PPARγ AS MOLECULAR TARGET OF EPITHELIAL FUNCTIONS IN THE GASTROINTESTINAL TRACT

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Meinen Eltern

Es ist nicht genug, zu wissen,
man muss auch anwenden;

Es ist nicht genug zu wollen,
man muss auch tun.

Johann Wolfgang von Goethe

(Wilhelm Meisters Wanderjahre)

I CONTENTS

1 Introduction

The major functions of the gastrointestinal tract are not only digestion, excretion and absorption but also recognition of food signals and transduction of these signals to various internal body systems. The intestine is unique in the body with regard to its constant exposure to an abundant commensal bacterial microbiota combined with regular if not frequent contact with pathogenic invaders, *e.g.* microbes and germs in contaminated foods. Although the normal host's relationship with resident luminal bacteria is often mutually beneficial, the host also requires protection against these microorganisms. Effective control of the invaders either in the intestinal lumen or after invasion into the mucosa is not only important for protecting the host from infections with enteric pathogens but also for maintaining intestinal structure and function. This protection is provided to a large extent by the mucosal immune system consisting of multiple specific and non-specific components. Thus, the human intestine forms an interface with the environment serving to both protect the body and absorb nutrients. The relationship between the mammalian host and its microbiotica is to a large extent symbiotic. Disturbance of the delicate balance can result in several gastrointestinal diseases, especially inflammatory bowel diseases (IBD) and colorectal cancer (CRC). The disruption of the epithelial lining elicits the secretion of potent inflammatory mediators, such as the activation of the nuclear transcription factor NFκB, altered expression of antimicrobial peptides (APs), and abnormalities in apoptotic functions, *e.g.* regulated by the caspase cascade. Moreover, there is strong evidence that these processes are under control of nuclear hormone receptors, in particular the peroxisome proliferator-activated receptor γ (PPARγ) and the vitamin D receptor (VDR).

Mesalazine and the dietary histone deacetylase (HDAC) inhibitors butyrate and sulforaphane (SFN) have attracted substantial interest as chemopreventive and anti-inflammatory agents, especially in the gastrointestinal tract. However, the exact mechanisms underlying their effects are not entirely understood. Therefore, the aim of this thesis was to further unravel the influence of the drugs on above mentioned pathways and molecules with regard to the potential involvement of the nuclear hormone receptors, especially PPARγ.

2 The gastrointestinal ecosystem

2.1 The intestinal epithelium and gastrointestinal diseases

2.1.1 The intestinal epithelium

Besides the absorption of nutrients and water, epithelial cells of the gastrointestinal mucosa forms an important mechanical barrier separating the host's internal milieu from the environment to keep microorganisms out [1, 2]. The strong physical barrier is achieved by several defence mechanisms both constitutive and inducible [3]. Innate immune defences produced by intestinal epithelial cells include mucins and a variety of antimicrobial compounds, such as lysozymes, cathelicidins and defensins [4, 5]. These molecules directly inhibit bacterial growth and protect the epithelium by preventing microbial adhesion and by retaining APs and secretory antibody close to the epithelial surface [4, 5]. Additionally, epithelial cells secrete several cytokines, monocyte chemoattractant proteins, granulocyte-macrophage colony-stimulating factors, the tumor necrosis factor α (TNFα) and the transforming growth factor β (TGF-β) [1]. Moreover, intestinal peristaltic movements of food and infectious agents are further examples of defensive mechanisms [3].

The intestinal epithelium has a well defined architecture. A number of invaginations, called the crypts of Lieberkühn are the functional proliferative units. In the small intestine these crypts are surrounded by numerous villi [1, 6]. This crypt-villi unit results in a greater surface area available for the absorption of nutrients. The epithelium is continuously and rapidly renewed by a process involving cell generation and migration from the stem cell population located at the bottom of the crypt [*Figure 1*] [1]. The cells then migrate upwards to the top of the villi where they preferentially die by apoptosis [1, 7]. The turnover time is considered to be 3-5 days. The plasticity of the system ensures that damaged and/or infected cells are dismissed and leave the body with the faeces, providing yet another defence mechanism against pathogenic bacteria [1].

Three of the four principal epithelial cell lineages arising from the multipotent stem cell differentiate during an upward migration from the crypt to an associated villus [1, 8]. They include columnar absorptive enterocytes, comprising 80% of all epithelial cells, mucus-producing goblet cells and enteroendocrine cells [1, 8]. Paneth cells represent the fourth lineage arising from the multipotent stem cell [8]. A variety of functions have been attributed to paneth cells, including modulation of the microbial flora through secretion of lysozyme and

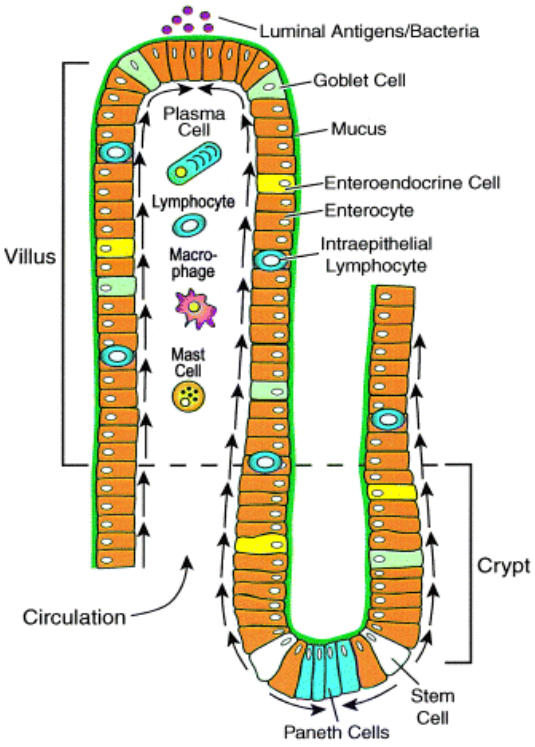

Figure 1: **The effector side of the gastrointestinal ecosystem**. The gastrointestinal ecosystem consists of a rapidly renewing epithelial stem cell population that differentiates into multiple intestinal epithelial cell types (goblet cells, enteroendocrine cells, enterocytes, Paneth cells and M cells). These epithelial cells serve as a barrier between the luminal contents (food antigens and resident microbiota) and the complex mucosal immune system. [adapted from [1]]

of APs [4]. A specialised type of epithelial cells is the M cell [1]. This cell line is an epithelial cell by all characteristics, but executes important functions in antigen presentation to the gut associated lymphoid tissue [1].

Hence, the intestinal epithelium is a complex ecosystem system generated by alliance of a rapidly renewing epithelial stem cell population and a number of defence processes [1]. Disturbance of the balance will lead to structural and functional changes, contributing to the pathogenesis of several gastrointestinal diseases. Unlike to normal colonic mucosa, however, inflamed colonic mucosa shows alterations *e.g.* in the defence mechanisms. Moreover, in IBD, like Crohn's disease (CD) and ulcerative colitis (UC), as well as in CRC enhanced secretion of pro-inflammatory cytokines and rapid epithelial cell turnover with increased cell proliferation and reduced cell death was observed, respectively [9, 10].

2.1.2 Gastrointestinal diseases

2.1.2.1 Inflammatory bowel disease

The inflammatory bowel diseases (IBD) UC and CD are inflammatory disorders of the gastrointestinal tract, which are characterised by chronic and spontaneously relapsing inflammation of the gut [9, 11]. Both diseases lead to long-term and sometimes irreversible impairment of gastrointestinal structure and function [9]. Although UC and CD share many clinical and pathological characteristics, they also have some markedly different features [9]. In UC, inflammation appears continuously and is limited to the colon and invariably involves the rectum [11]. Inflammation in UC only affects superficial layers of the mucosa with infiltration of granulocytes and lymphocytes with a frequent appearance of ulcerations and crypt abscesses [9]. In contrast, CD is a transmural inflammatory process that may affect any part of the gastrointestinal tract from the mouth to the anorectum. Moreover, the disease is not confined to the lining of the bowel, but affects all layers of the intestinal wall with dense infiltrations of macrophages and lymphocytes to form abscesses and fistulas, thereby sometimes causing bowel obstruction. The presence of granulomas is common [9, 11]. A further characteristic of CD is the presence of normal bowel segments between affected areas in contrast to the continuous inflammatory process seen in UC [9]. In some of the patients, IBD is associated with extra-intestinal manifestations involving the liver, skin, eyes and/or the joints [11]. UC and CD manifest in the young population, the onset often occurs during the early twenties [12]. Symptoms can include abdominal pain, diarrhoea, weight loss, loss of appetite, arthralgias, fever and fatigue and anemia [13, 14]. The symptoms and the possible complications of the diseases can impact significantly on patients' physical and systemic performance, leading to emotional anxiety, psychological distress and an overall reduction in quality of life [14].

Although the aetiology of IBD remain obscure, its pathogenesis is gradually being unravelled [11]. IBD is a result of an inappropriate and exaggerated mucosal immune response directed against constitutes of the mucosal microflora, normally present in the intestinal milieu [15, 16]. The interaction of environmental factors with genetic susceptibility leads to inflammation of the gut mucosa [15]. CD is associated with a Th1-type immune response with excessive production of TNFα, interferon-γ and interleukin-12 (IL-12) [9]. In contrast, UC results mainly of a Th2-type response, associated with increased production of

IL-4, IL-5 and IL-13 [17]. Thus, the consequent excessive production of inflammatory mediators and, in addition, reactive oxygen metabolites, eicosanoids and proteases, together with defective repair mechanisms, perpetuate the inflammatory reaction resulting in tissue damage [11]. A further area is the identification of specific genetic abnormalities, responsible for IBD. The most important finding is the detection of mutations in the gene encoding NOD2 on chromosome 16 occurring exclusively in patients with CD but not in patients with UC [9].

Besides the conventional medical treatment with corticosteroids and aminosalicylates, especially mesalazine, alternative immune-modulatory medication, such as methotrexate, and specific targeted cytokine therapies, *e.g.* infliximab, a monoclonal antibody to TNFα, are increasingly used in patients suffering from IBD [11]. Severe cases of IBD may require surgery, such as bowel resection, strictureplasty, or a temporary or permanent colostomy or ileostomy [11, 12].

Patients suffering from CD or UC have an increased risk for the development of CRC and the risk depends on disease duration and the extent of inflammation [18, 19]. The risk of CRC for patients with UC is estimated to 2% after 10 years, 8% after 20 years and 18% after 30 years of disease [19]. CD also increases the hazard of CRC. The relative risk with extensive colonic involvement appears to be similar to that of UC [19].

2.1.2.2 Colorectal cancer

Colorectal cancer (CRC) is the third most common malignant disease and the second most frequent cause of cancer-related death in the United States, with 145.290 new cases and 56.290 deaths anticipated in 2005 [20, 21]. Worldwide, CRC is the fourth most commonly diagnosed malignant disease, with an estimated 1.023.000 new cases and 529.000 deaths each year [21]. In developed countries, the lifetime incidence of CRC is 5% [21]. Both men and women are almost equally affected [20]. The disease is rare in persons below the age of 50 years, but thereafter increases with age [20]. The median age at diagnosis is slightly above 70 years. The mortality ratio of patients suffering from CRC can decrease substantially in the next years, if the available measures for prevention, *e.g.* colonoscopy, will be implemented effectively [20]. There are at least three major types of CRC, which includes sporadic, IBD-associated and hereditary CRC [*Table 1*] [19]. Only a small population of cases are attributed in the setting of the two main forms of hereditary cancer syndromes, the familial

- **Sporadic colorectal cancer (88-94 %)**

  Older age
  Male sex
  Cholecystectomy
  Ureterocolic anastomosis
  Hormonal factors: nulliparity, late age at first pregnancy, early menopause

  Environmental factors
  - Diet rich in meat and fat, and poor in fibre, folate, and calcium
  - Sendentary lifestyle
  - Diabetes mellitus
  - Obesity
  - Smoking
  - Previous irradiation
  - Occupational hazards (*e.g.*, asbestos exposure)
  - High alcohol intake

  Personal history of sporadic tumors
  - History of colorectal polyps
  - History of colorectal cancer (risk is 1.5-3 % for second such cancer in first 5 years)
  - History of small bowel, endometrial, breast, or ovarian cancer

  Familial colorectal cancer (20 %)
  First or second degree relatives with this cancer, criteria for hereditary colorectal cancer not fulfilled:
  - One affected first-degree relative increases risk 2.3-fold
  - Two or more affected first degree relatives increase risk 4.25-fold
  - Index case < 45 years increase risk 3.9-fold
  - Familial history of colorectal adenoma increases risk 2-fold

- **Colorectal cancer in inflammatory bowel disease (1-2 %)**
  - Ulcerative colitis
  - Crohn's colitis

- **Hereditary colorectal cancer (5-10 %)**
  - Polyposos-syndromes: familial adenomatous polyposis (FAD), Gardner's syndrome, Turcot's syndrome, attenuated adenomatous polyposis coli, flat adenoma syndrome
  - Hereditary non-polyposis colorectal cancer (HNPCC)
  - Hamartomatous polyposis syndromes (Peutz-Jeghers syndrome, juvenile polyposis syndrome, Crowden syndrome)

Table 1: **Classification of CRC, risk factors and causes.** [adapted from [19]]

adenomatous polyposis (FAP) and the hereditary nonpolyposis colorectal cancer (HNPCC), while the majority of CRC are considered sporadic [19]. The third type of CRC is the result of long-standing UC and CD of the colon. CRC accounts for approximately 15% of all deaths in patients with IBD [22].

Several factors have been suggested to be associated with a higher risk of CRC in patients with IBD. The magnitude of CRC risk increases *e.g.* with early age at IBD diagnosis, more extensive colitis, anatomic extent of disease and in patients having concomitant primary sclerosing cholangitis [22]. The most important risk factors for sporadic CRC are familial

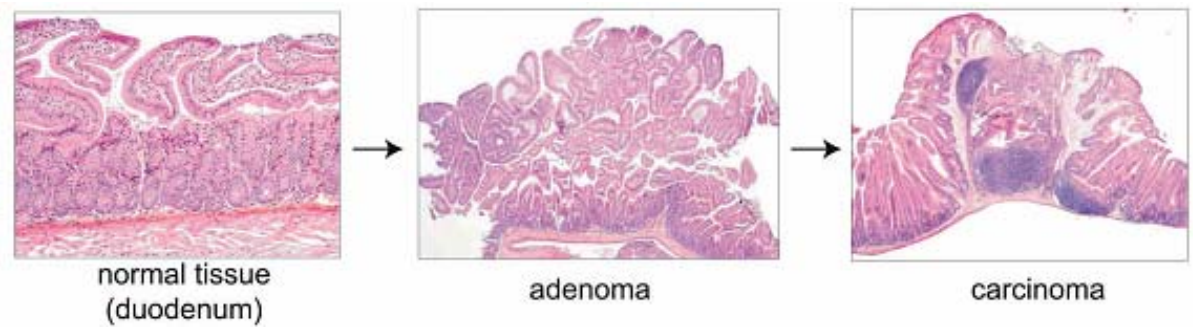

Figure 2: **Schematic overview of tumor progression in the digestive tract.** Haematoxylin/eosin stained murine tissue sections. From left to right: normal tissue from the duodenum, an adenomatous polyp, and a malignant, invasive adenocarcinoma. See text for further explanations. [depicted modified from [10]]

history, genetic mutations, epigenetic changes as well as lifestyle factors such as nutrition, obesity, inactivity and smoking [19]. Lifestyle-related risks offer a broad area for implementing primary preventive measures, which have not yet been adequately exhausted [19]. Several methods are available for secondary prevention *e.g.* fecal occult blood test and endoscopy [23]. Consistent encouragement of these possibilities for prevention could reduce incidence and mortality substantially and render colorectal tumors less frequent [23].

The intestinal epithelium with its distinct crypt-villus units is an intriguing model for several basic aspects of cellular behaviour, such as the control of cell number in a mammalian tissue [10]. The continuous process of self-renewal involves an intricate interplay between proliferation, controlled cell death, and cellular differentiation [10]. Imbalance of this tightly regulated system results in hyper-proliferation and de-differentiation, and can lead to drastic changes of the cell growth of the digestive tract, notably colorectal tumors [10]. The development of CRC from an adenoma to a carcinoma may take several decades [*Figure 2*] [10]. The process can be divided into three phases, tumor initiation, tumor promotion and tumor progression [24]. According to Hanahan and Weinberg, cancer then is the result of an accumulation of genetic alterations that allows growth of neoplastic cells with phenotypic characteristics *e.g.* self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and the ability to invade tissues and metastasis [25, 26].

The first phase of tumorigenesis, tumor initiation, involves multiple steps of genetic alterations, including the loss of function of tumor suppressor genes, and the activation of oncogenes [26]. Traditionally, colorectal carcinogenesis is explained by two pathways, the microsatellite instability (MSI) pathway also called the caretaker pathway, and the

chromosomal instability (CIN) pathway - the gatekeeper pathway [27]. The CIN pathway has originally been identified by Fearon and Vogelstein and is characterised by numeric and structural defects in chromosomes [28]. The authors proposed a multistep model of colorectal carcinogenesis, in which tumor suppressor genes, mainly the adenomatous polyposis coli (APC) gene, frequently called the "gatekeeper of CRC", mutated in 60-80% of the cases, and the oncogene K-ras with a mutation rate of 50% are involved [28]. Several studies indicate that many other tumor suppressor genes (*e.g.* DCC, DPC4/Smad4, p53) and oncogenes (*e.g.* *c-myc*, *c-neu*) contribute to this regulation [19]. Besides the mutation of oncogenes and tumor suppressor genes in the CIN pathway, the MSI pathway is characterised by mutations or epigenetic changes of genes that maintain genetic stability *e.g.* mismatch repair genes (MSH1, MSH2) [29]. In addition, tumor suppressor genes such as TGF-β-RII and BAX are mutated in this pathway; changes in K-ras, APC and p53 are rare [19, 27]. The CIN pathway is responsible for about 85% of the sporadic CRC, and is the mechanism leading to carcinogenesis in patients with FAP. On the other hand, the MSI pathway is responsible for 15% of sporadic CRC and also for the HNPCC [19]. Colorectal cancer of the CIN pathway is mainly located to the distal colon, whereas the MSI pathway can primarily be found in the proximal colon [27]. The major carcinogenic pathways that lead to sporadic CRC, namely CIN and MSI also occur in IBD-associated CRC [22]. However, recent progress in molecular biology has shown that colorectal carcinogenesis is not necessarily clearly divided into these two pathways, since the APC gene can act as a caretaker and mismatch repair gene [30]. Moreover, additional pathways exist, *e.g.* the serrated pathway, the distinct pathways for carcinogenesis of flat and depressed colorectal neoplasms and the pathway for the carcinogenesis in IBD [19]. Epigenetic mechanisms such as change in DNA methylation, loss of imprinting, and histone acetylation, as well as modifier genes, such as cyclooxygenase-2 (COX-2), PPARγ and VDR also seem to be involved in the genesis of CRC [19].

The second phase of tumorigenesis, tumor promotion, is characterized by the clonal expansion of initiated cells, owing to increased cell proliferation and/or reduced cell death [24]. Finally, invasion and metastasis, as well as an increase in tumor size, are characteristics of the third phase of tumorigenesis, the tumor progression [24]. The molecular pathway leading to IBD-associated CRC appears to differ from the well-known adenoma to CRC sequence as described above. These cancers seem to arise from either flat dysplastic tissue or dysplasia-associated lesions or masses [22]. An important model for IBD-associated carcinogenesis follows progression from an absence of dysplasia, to indefinite dysplasia, to

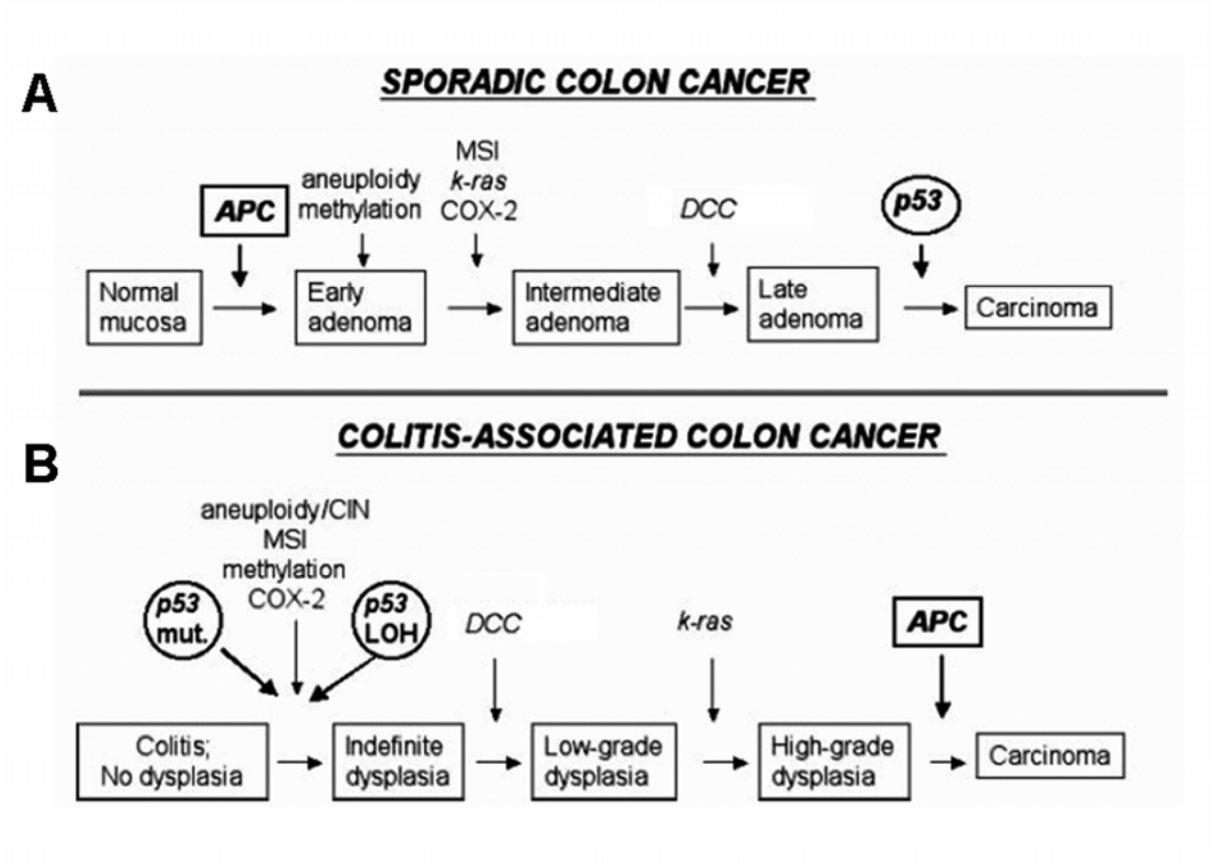

Figure 3: **Molecular pathogenesis of CRC.** (A) Sporadic CRC and (B) Colitis (ulcerative colitis, Crohn's coltis)-associated CRC. *Abbreviations:* COX, cyclooxygenase; CIN, chromasomal instability; MSI, microsatellite instability; mut., mutation; LOH, loss of heterozygosity; DCC, deleted in colorectal cancer; APC, adenomatous polyposis coli. [adapted from [18]]

low-grade dysplasia, on to high-grade dysplasia, and ultimately to invasive CRC [*Figure 3*] [18]. Molecular events distinguishing sporadic CRC and IBD-associated CRC can mainly be associated in frequency and timing of the alteration of tumor suppressor genes, especially of APC and p53 [*Figure 3*] [18]. For example, APC loss of function, considered to be a very common early event in sporadic CRC, is much less frequent and usually occurs late in the IBD-associated dysplasia carcinoma sequence [18]. Loss of p53 gene function plays an early instrumental role in IBD-associated CRC. p53 mutations are often detected in mucosa that is nondysplastic or only indefinite for dysplasia. In contrast, loss of p53 gene function occurs late in sporadic CRC [18].

2.2 Peroxisome proliferator-activated receptor γ (PPARγ) as a molecular target of epithelial functions in the intestinal tract

2.2.1 PPARγ structure and activation

Peroxisome proliferator-activated receptors (PPARs) are ligand-inducible transcription factors belonging to the nuclear hormone receptor superfamily, also including the receptors for estrogen, thyroid, glucocorticoid and vitamin D [31, 32]. The PPAR subfamily consists of distinct genes which code for three PPAR isoforms termed α, β and γ, sharing about 60-80% homology in their ligand binding (LBDs) and DNA binding domains (DBDs) [32]. Amongst the three subtypes, PPARγ has been the most intensively investigated receptor in the intestine [32]. Three PPARγ mRNA isoforms have been described in the human genome, *i.e.* PPAR-γ1, -γ2 and -γ3, differing at their 5'-end as a consequence of alternate promoter usage and splicing [32]. However, up to now the precise actions of these distinct PPARγ isoforms remain unclear [32].

As shown for most members of the nuclear hormone receptor superfamily, PPARγ has a characteristic modular structure consisting of several functional domains [*Figure 4*]: the NH_2-terminal A/B domain harbors a ligand-independent transcriptional activation function (AF-1) which can be regulated by phosphorylation [31]. The central C region contains the DBD, composed of two zinc fingers, which target the receptor to specific DNA sequences known as peroxisome proliferator response elements (PPREs) [31]. The hinge region D is important for coactivator docking [31]. The complex multifunctional region (E) encompasses the LBD and the ligand-dependent activation domain AF-2 [31]. The LBD contributes to ligand binding, dimerization, nuclear import signalling, interaction with the transcriptional machinery, and transcriptional activation/inhibition. In addition, it contains several helices and dimerization interfaces [31]. For the F domain on the COOH-terminal end of the receptor, no functional relevance has been described yet [33].

Upon activation in the cytoplasm, PPARγ changes its confirmation in the AF-2 domain to the active form, heterodimerizes with retinoid X receptors (RXR), and forms a complex that translocates to the nucleus [*Figure 5*] [32]. The heterodimer then binds to PPREs located within the promoter region of target genes consisting of a direct repetitition of the consensus AGGTCA half-site, thereby regulating gene expression [32].

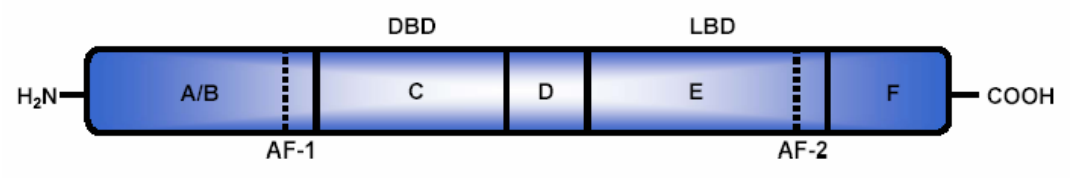

Figure 4: **Structural organisation of the nuclear hormone receptor PPARγ.** See text for explanation.

In addition to the heterodimer complex, several accessory proteins *i.e.* coactivators and corepressors can bind to the nuclear receptor PPARγ/RXRα in a ligand-dependent manner *e.g.* steroid receptor coactivator-1 (SCR-1) and CBP/p300 [32]. They impact the transcriptional process by either remodelling chromatin structure or acting as adapter molecules linking the nuclear receptor complex to the key transcriptional machinery [32]. Ligand binding to PPARγ appears to trigger conformational changes that permit its dissociation from corepressors and favours its association with coactivators [32]. The coactivator proteins either possess or recruit histone acetyltransferase (HAT) activity to the transcription start site [32]. There, acetylation of histone proteins alters chromatin structure, thus facilitating the binding of RNA polymerase II and initiating transcription [32]. This is often achieved through the recruitment of HDACs [32]. Besides to its stimulatory effect on gene transcription, PPARγ can suppress gene expression in a DNA binding-dependent manner through the recruitment of corepressors to unliganded PPARγ bonded to PPRE or in a DNA binding-independent manner by interfering with key transcription factors via physical interaction [32].

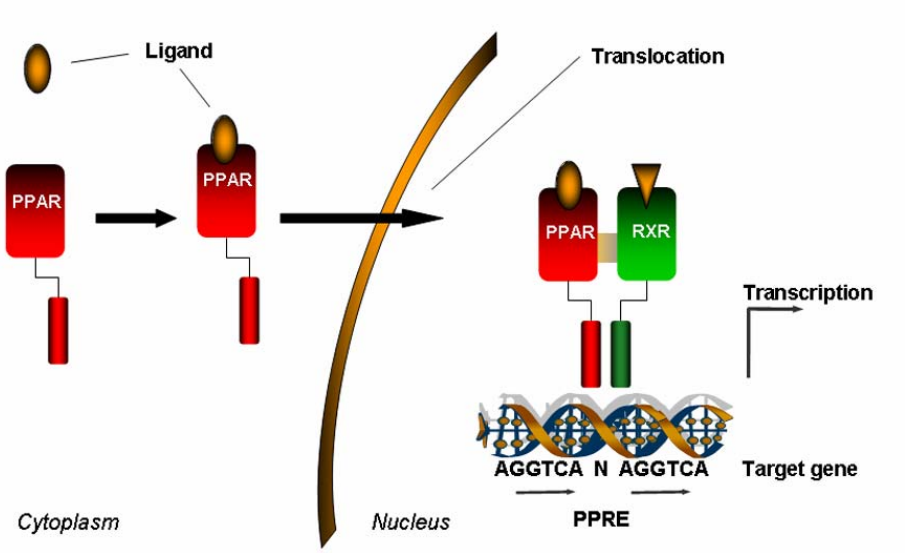

Figure 5: **Activation mechanism of PPARγ.** PPARγ may be activated by different natural and synthetic ligands allowing its heterodimerisation with RXR in the cytoplasm. The heterodimer complex is then translocated to the nucleus of the cell and binds to peroxisome proliferator response elements (PPREs) located within the promoter region of target genes consisting of a direct repetitition of the consensus AGGTCA half-site, thereby regulating gene expression of many biological processes.

2.2.2 Expression of PPARγ in humans

Originally described as a receptor expressed by adipose tissue where it plays a role in adipocyte differentiation and in the regulation of insulin responses, other tissues and cells are now known to express PPARγ [*Figure 6 A*] [34]. Amongst them, the colon is a major tissue expressing PPARγ in epithelial cells and to a lesser degree in macrophages and lymphocytes [34]. In both humans and rodents the expression of PPARγ has been reported to be higher in the distal parts of the colon compared to the more proximal parts and the small intestine [35]. Furthermore, human CRC cell lines, tumors and polyps derived from CRC often contain high levels of PPARγ [36]. Since expression is primarily localised to the postmitotic, differentiated epithelial cells facing the lumen, the expression pattern in the colon mucosa is consistent with the proposed role of PPARγ in differentiation [35]. Thus, in colon cells, the expression of the receptor has been shown to increase during the differentiation process [*Figure 6 B*] [36, 37].

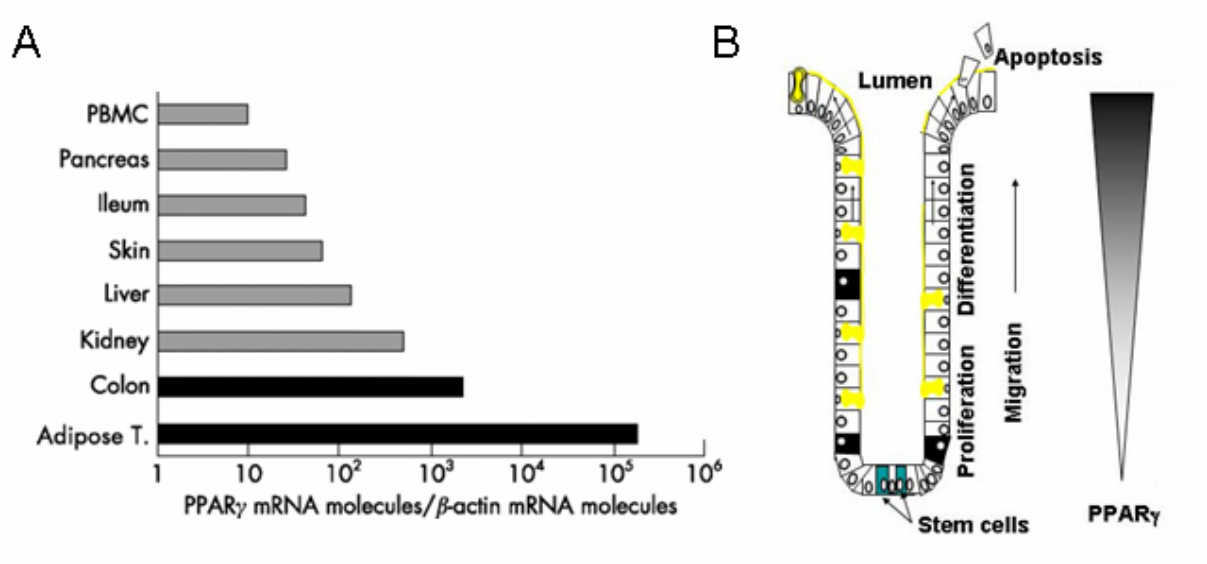

Figure 6: **Expression of PPARγ in different tissues and in the colonic crypt** (A) PPARγ mRNA expression in different tissues. PPARγ mRNA was quantified by reverse transcription-competitive polymerase chain reaction in different human organs and tissues. The main sources of PPARγ are adipose tissue and the colon. *Abbreviation:* PBMC, peripheral blood mononuclear cells. [adapted from [34]] (B) Expression of PPARγ in the colonic crypt. Stem cells are located at the bottom of the crypt and migrate up through zones of proliferation to their final destination as terminally differentiated cells on the top where they preferentially die by apoptosis. Expression of PPARγ increases upon differentiation of colon cells.

2.2.3 Natural and synthetic ligands of PPARγ

Several groups of endogenous synthetic and natural compounds are known to bind and to selectively activate PPARγ [*Figure 7*] [31, 34]. Thiazolidinediones (TZDs), a class of synthetic insulin-sensitizing agents useful in the therapy of non-insulin dependent diabetes mellitus (diabetes-type II) including troglitazone (Rezulin®, withdrawn from the market in the year 2000 due to an increased incidence of drug-induced hepatitis [38]), rosiglitazone (Avandia®) and pioglitazone (Actos®) were amongst the initially studied PPARγ agonists [31, 34, 39]. In addition, a variety of nonsteroidal anti-inflammatory drugs (NSAIDs) *e.g.* indomethacin, ibuprofen, mesalazine and several natural ligands have been described to activate PPARγ *in vitro* and *in vivo* [34, 40, 41]. Natural ligands include 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15-PGJ_2), 9-hydroxyoctadecadienoic acid (9-HODE), 13-hydroxyoctadecadienoic acid (13-HODE) and 8(*S*)-hydroxyeicosatetraenoic acid (8(*S*)-HETE), all derived from the metabolism of arachidonic acid as well as certain polyunsaturated fatty acids [31, 34]. Moreover, our group could demonstrate that also the

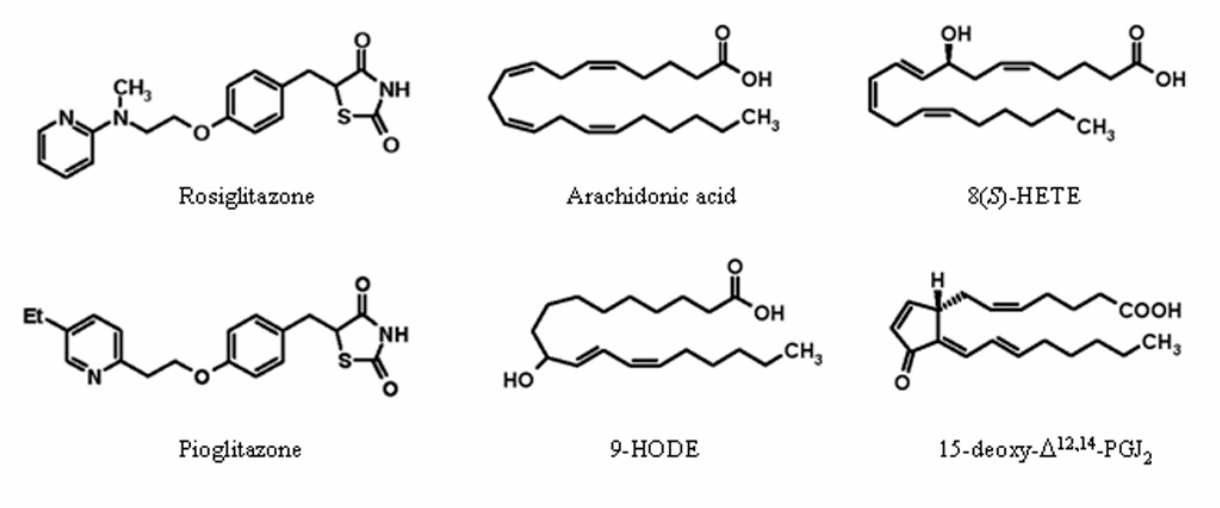

Figure 7: **Selection of natural and synthetic PPARγ ligands**

SCFA butyrate is able to up-regulate and to activate PPARγ [42, 43]. However, ligands are not the only way to modulate PPARγ activity, *e.g.* phosphorylation of specific serine structures by members of the mitogen-activated protein kinase (MAPK) family has been shown to activate PPARγ in a ligand-independent fashion [44].

2.2.4 Functions of PPARγ

Much attention has been addressed to the role of PPARγ in controlling the expression of a large number of regulatory genes in lipid metabolism and insulin sensitisation [31]. PPARγ is the master regulator of differentiation and energy storage by adipocytes [31, 45]. As adipose tissue is required for proper glucose homeostasis, it has been demonstrated that absence leads to severe insulin resistance [46]. Classical PPARγ agonists such as rosiglitazone not only induce adipose differentiation, but also improve glucose control [46]. Besides those fundamental mechanisms, a variety of pleiotropic functions for PPARγ affecting multiple fundamental pathways in the cell have been described [31]. PPARγ has been demonstrated to play important roles in cellular proliferation and differentiation, inflammation, apoptosis, immune-modulation and angiogenesis [31, 47]. Thus, PPARγ is part of a group of versatile and potent regulators of several cellular functions [47].

2.2.5 Role of PPARγ in colorectal cancer and inflammatory bowel disease

PPARγ is highly expressed in the colonic epithelium (see 2.2.2), indicating an important role of the receptor in the physiology of the human colon [32, 47, 48]. Moreover, PPARγ has been implicated in the pathogenesis of CRC and IBD by modulating a variety of cellular functions involved in the pathogenesis of both diseases [32, 49].

In the gastrointestinal tract, PPARγ is known to regulate cellular proliferation, and differentiation, induction of apoptosis and to modulate cell cycling [50, 51]. Moreover, reports demonstrate that PPARγ ligands lead to tumor suppression and inhibition of perineal metastasis in gastrointestinal cancer cells *in vivo* and *in vitro*. The growth of cultured human colon tumor cells and transplanted tumors in nude mice is inhibited by activators of PPARγ, including troglitazone, rosiglitazone and 15-PGJ_2 [52-55]. In addition, several reports not only describe the initiation of the caspase cascade by PPARγ ligands in a variety of cancer cells, but also an association between loss of function mutations and the development of CRC in humans, suggesting the involvement of the receptor in the regulation of apoptosis [56-58]. These observations raised the possibility that the somatic mutation of PPARγ can contribute to the carcinogenic process and that PPARγ can function as a tumor suppressor gene by inhibiting cell growth and inducing apoptosis [47]. However, the role of PPARγ in tumor formation is still uncertain and highly debated [32, 50]. Beyond the tumor-inhibiting effects, also tumor-promoting effects were reported for PPARγ agonists in models of CRC [50, 59]. These differences can, in part, be explained by PPARγ-independent effects of PPARγ agonists and by differences in the models that were used [50]. Since it is still unclear how PPARγ impacts on CRC, careful monitoring of patients receiving PPARγ agonists and additional basic research is indicated before recommendations on the use of PPARγ ligands in CRC can be made [32, 50].

Besides the role of PPARγ in the modulation of cell growth and cell proliferation, the involvement of the receptor in immune and inflammatory responses has been indicated [40, 49]. In particular, the participation of PPARγ in the pathogenesis of IBD has been established [40, 49]. The PPARγ agonists troglitazone and rosiglitazone dramatically reduced disease severity in several experimental colitis animal models compared with the placebo treated group, thereby, reducing mortality, intensity of macroscopic and histological lesions, as well as levels of biological markers of colonic inflammation [60]. Moreover PPARγ deficient mice exhibited an aggravation of inflammation in a trinitrobenzene sulfonic acid (TNBS) model

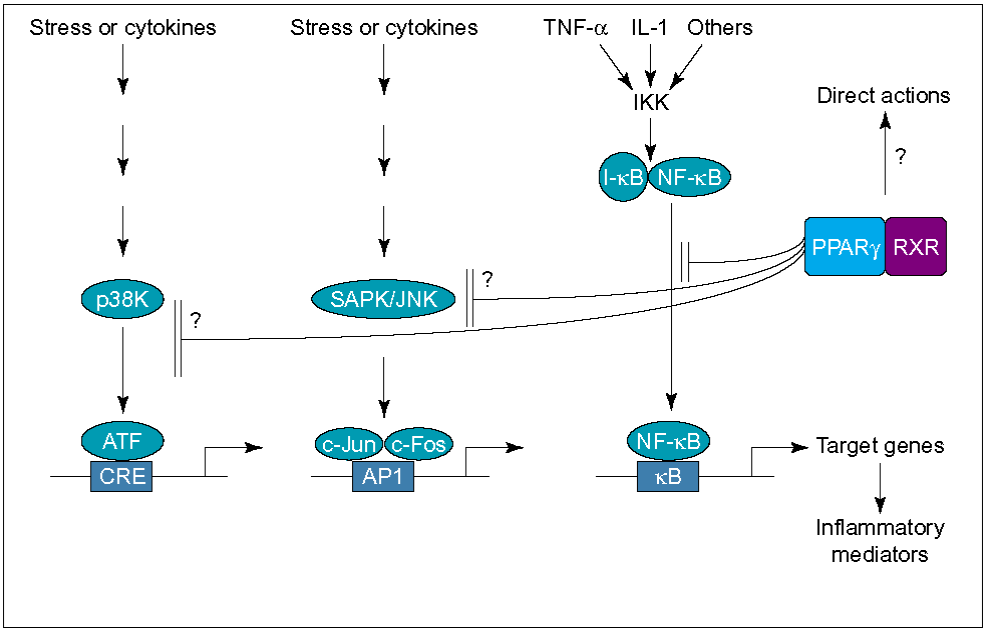

Figure 8: **Possible mechanisms of anti-inflammatory effects of PPARγ-associated pathways.** *Abbreviations:* AP1, activator protein 1; CRE, cAMP response element; IκB, inhibitor of NFκB; JNK, cJun N-terminal kinase; IL, interleukin; NFκB, nuclear factor κB; TNFα, tumor necrosis factor α. [adapted from [49]]

compared to wild-type mice [60]. However, decreased expression of PPARγ was observed in the colon of UC patients compared with patients suffering from CD and with the control group, both in the mucosa of healthy and inflamed colon [40]. All these observations indicate a role of PPARγ as an anti-inflammatory brake such that a decrease in PPARγ levels might cause the development or exacerbation of inflammation [49]. However, it is still unclear if either decreases in the endogenous ligands for PPARγ, which remain to be identified, or decreases in the levels of PPARγ itself might be associated with the pathogenesis of IBD [49]. Elucidating the molecular pathways involved in the interaction between the receptor and IBD, recently it was indicated that activation of PPARγ may interfere with a variety of signalling pathways regulating the expression of pro-inflammatory genes, such as those controlled by NFκB, signal transducers and activators of transcription *e.g.* signal transducers and activators of transcription (STATs), activating protein 1 (AP1), and the nuclear factor of activated T-cells [32, 34, 49]. Moreover, a network of interactions between NFκB and MAPK signalling has been established which is summarised in *Figure 8* [49]. Consequent to the inhibition of these pathways, PPARγ activators may reduce the production of inflammatory cytokines, chemokines, and cell-adhesion molecules, thereby limiting the recruitment of inflammatory cells [60].

2.3 The vitamin D receptor (VDR) as a molecular target of epithelial function in the intestinal tract

2.3.1 VDR structure and activation

The vitamin D receptor (VDR) also belongs to the nuclear steroid hormone receptor superfamily [61]. The amino acid sequence and the functional domains of VDR show a significant homology with other members of its superfamily, with highest similarity to the thyroid, PPARγ and RXR [61]. VDR is believed to be located in the nucleus prior to activation with the active form of vitamin D hormone, which then dissociates from the serum vitamin D binding protein, or other carrier proteins; enters the cell by diffusion and binds with the receptor [62]. Moreover, substantial amounts of unoccupied VDR have been detected in the cytoplasm [63]. VDR has a similar structural and functional organisation as described for PPARγ (see 2.2.1). In contrast to other nuclear receptors, VDR lacks a ligand-independent AF-1 motif in its aminoterminal A/B domain [64]. Ligand activation results in conformational changes of the receptor, exposure of surfaces for coactivating factor binding, and in the induction of heterodimerization usually with RXR [64]. The VDR-RXR heterodimer then binds with high affinity to the vitamin D response element (VDRE), present within the promoter region of vitamin D controlling genes [64]. These initial macromolecular interactions initiate a communication process that ultimately influences the rate of RNA polymerase II-directed transcription [65]. Moreover, calcitriol-dependent phosphorylation at distinct serine residues in the ligand/hinge domains of VDR has been reported and might also be involved in the regulation of DNA binding, ligand binding, nuclear localisation and gene transactivation [63].

VDR has been shown to interact with various compounds of the transcriptional machinery [65]. Several members of the steroid receptor coactivator gene family (*e.g.* SRC/p160), transcriptional intermediary factors (*e.g.* SRC-1), receptor associated coactivator integrator proteins (*e.g.* CBP/p300), the coactivator complex DRIP, corepressor proteins (*e.g.* SMRT) and a variety of other factors have emerged as central players in the communication process, connecting ligand-activated receptors to the preinitiation VDR complex [65]. The group of coactivator integrator proteins such as CBP/p300 can cooperate with VDR, coactivators, corepressors, and the preinitiation complex, thereby forming larger coactivator complexes [65]. Coactivators such as SRC-1 and PCAF and cointegrators like CBP/p300

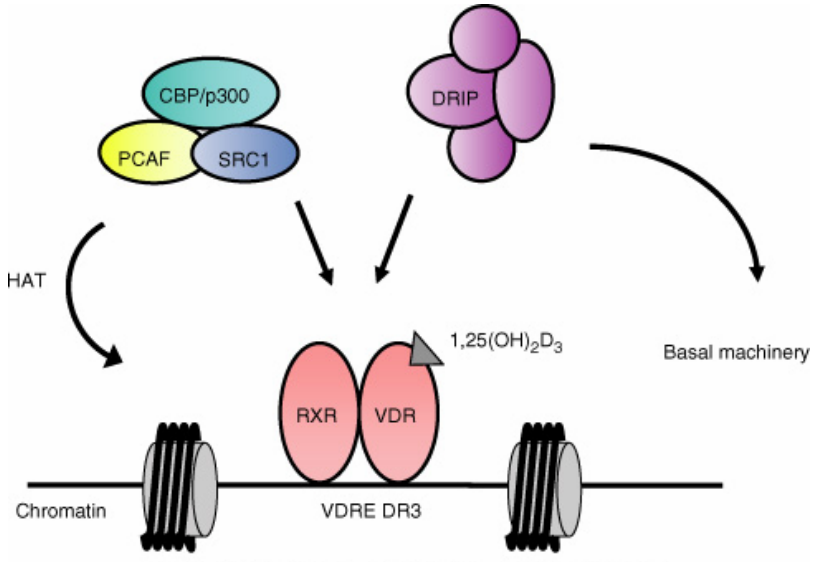

Figure 9: **Model for VDR-mediated gene regulation.** The VDR and RXR bind to the promoter of a target gene as a heterodimer in response to $1,25(OH)_2D_3$. Transcriptionally active complexes that include the SRC1, CBP/p300 HAT coactivator complex or the DRIP-Mediator complex or perhaps others are subsequently recruited to facilitate changes in chromatin architecture and to enhance RNA polymerase II entry. Both may be needed to modulate gene expression. [Figure taken from [66]]

contain intrinsic HAT activities forming a link between nuclear receptor signalling and chromatin organization [65]. Presumably, coactivator function is primarily the recruitment of CBP/p300 appearing to be the primary source of HAT activity [*Figure 9*] [67].

2.3.2 Ligands for VDR

The main ligand for VDR is the active form of vitamin D, the seco-steroid hormone 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$, calcitriol; VD_3) [61, 68]. VD_3 is obtained from dietary sources, mainly fish and meat, dietary supplements and, mostly, conversion of 7-dehydrocholesterol to colecalciferol by the action of solar UV-B radiation (280-320 nm) in the skin [61]. Colecalciferol is then hydroxylated in the liver to 25-hydroxyvitamin D_3 (25-hydroxycholecalciferol, califediol) by vitamin D_3 25-hydroxylase [61]. Subsequently, the intermediate is again hydroxylated to render 1α,25-dihydroxyvitamin, the most active metabolite of vitamin D, in the kidney and several cell types, by the actions of 25-hydroxyvitamin D_3 1α-hydroxylase [*Figure 10*] [61, 68]. Besides the active form of vitamin D and several vitamin D analoges like calcipotriol or tacalcitol, a variety of molecules

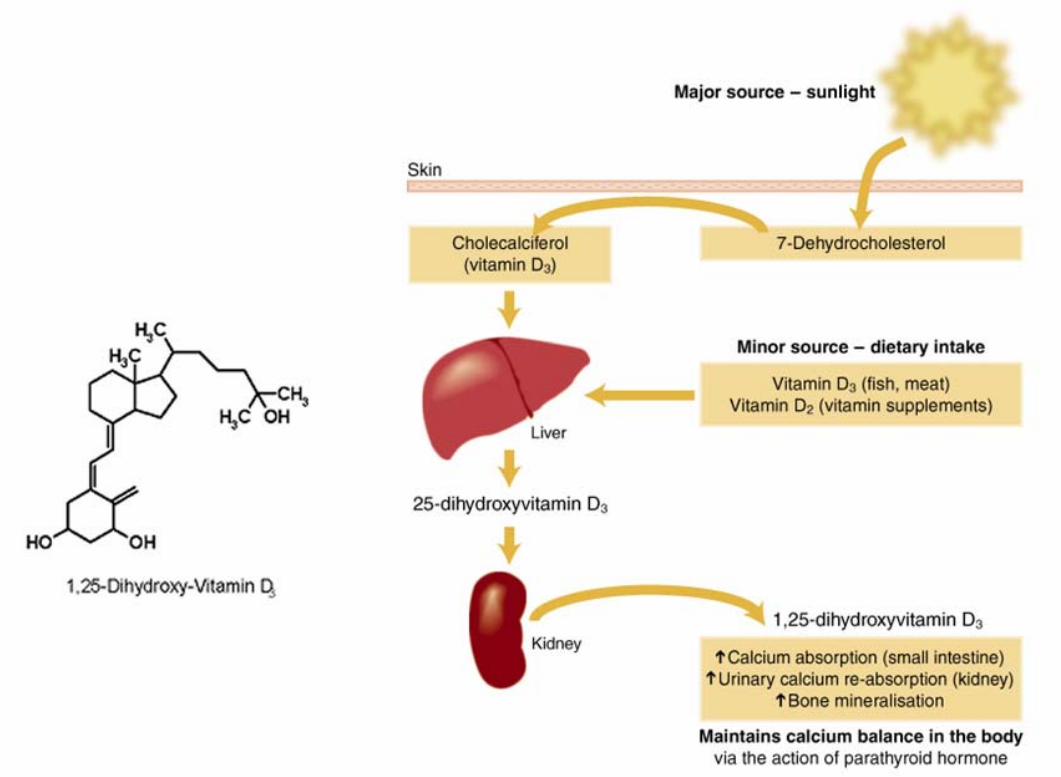

Figure 10: **Chemical structure, biosynthesis and actions of Vitamin D.** See text for further explanations. [adapted from [68]]

modulate VDR by mimicking many of the activities of VD_3 without a direct structural relationship to the molecule, *e.g.* LG190119 [64]. Recently, our group could demonstrate that also the SCFA butyrate is able to activate and to up-regulate the expression of VDR in CRC cells [69, 70].

Like other nuclear hormones, VD_3 is a lipophilic compound that easily passes biological membranes and enters the nucleus [71]. Most of the known biological effects of VD_3 are genomic effects, mediated through specific and high affinity binding to VDR [63, 67]. Moreover, rapid, non-genomic actions of VD_3 are known which have been suggested to function via putative membrane receptors [63, 67]. They have been reported to involve activation of Ca^{2+} channels, G-protein-coupled receptors (GPCR), and downstream protein kinase C (PKC) and MAPK pathways [63, 67, 72].

2.3.3 Tissue distribution of VDR

VDR was first discovered in chicken intestine, and subsequently in a wide variety of cells and tissues [62]. In human, VDR is not only found in the classic target organs such as the intestinal tract, kidney and bone, but also in many other epithelial and mesenchymal cells. Moreover, high levels of VDR have been reported in several types of malignant cells like leukemic and osteosarcoma, as well as in colon, breast, lung, and prostate carcinoma cells [62, 73]. In addition, VDR is expressed by cells of the immune system like monocytes and activated B and T lymphocytes [73].

Numerous studies have demonstrated variable levels of VDR expression in epithelial and human CRC cells [74]. VDR expression is low in normal colon tissue, mainly in differentiated luminal crypt cells [74]. In addition, in mice studies, VDR expression has been found to be higher in the proximal than in the distal colon [75]. Examination of VDR expression in the CRC cell line Caco-2 showed that the expression of the receptor was increased when the cells became confluent and differentiated in culture [76]. Subsequent studies corroborated that VDR is associated with a high degree of cell differentiation [72]. The comparison between human malignant colonic tissue and normal mucosa of the same patient revealed significant higher VDR expression in tumoral than in normal tissue [72]. However, VDR expression is only enhanced in low grade tumor tissue, whereas in advanced carcinomas, it diminishes or disappears completely, - suggesting that CRC cells express VDR as long as they retain a certain level of differentiation [72, 74].

2.3.4 Cellular functions of the VDR

Tissue responsiveness to VD_3 depends mainly on the expression levels of VDR and the hydroxylases regulating calcitriol synthesis and degradation [64]. Besides the major roles of VDR, mainly caused by VD_3, in calcium homeostasis and bone metabolisms, immune-modulatory functions as well as anti-inflammatory, -carcinogenic, -invasive, -angiogenic, and anti-metastatic activities have been well established [62, 77].

VD_3 and its analogues are potential therapeutics in diverse disease states of inflammation like rheumatoid arthritis, psoriatic arthritis, IBD; dermatological indications like psoriasis, photoaging and skin rejuvenation; osteoporosis; cancers like breast, prostate, CRC,

leukemia and myelodysplastic syndrome; and autoimmune diseases as multiple sclerosis, type I diabetes and systemic lupus erythematosus [64, 73]. However, the use of VD_3 in above mentioned indications as well as in the oral therapy for psoriasis and even in the topical therapy for severe psoriasis is hampered by its associated toxicity, namely hypercalcemia [64]. New VDR ligands have been synthesized which exhibit greater specificity by retaining desirable properties, but with reduced hypercalcemic potential .

The anti-cancer activity of VD_3, especially in colon cells, stems from the inhibition of proliferation and the induction of apoptosis and differentiation. This not only maintains normal growth of the colonic crypt, but potentially can prevent the progression into pre-malignancy [72]. The actions can operate in combination and the predominant effect varies from one to another cell [72]. The growth inhibitory actions of VD_3 have been observed in many human CRC cell lines and also in cultured primary human colon adenoma- and carcinoma-derived cells and xenograft models [72].

2.3.5 Role of VDR in colorectal cancer and inflammatory inflammatory bowel disease

One of the earliest observations suggesting that VD_3 may play a role in CRC prevention and treatment was that people, who lived at northern latitudes and therefore received less solar radiation, had an increased risk for dying due to this disease [77]. Moreover it was shown that low serum levels of VD_3 correlated with a higher risk of developing CRC [77]. In addition, studies in wild-type mice have demonstrated that a high-fat diet, with low levels of VD_3, increases the risk of CRC. In long-term studies, these mice showed hyperproliferation in colonic epithelial cells [77]. When the diet was supplemented with calcium and VD_3 these effects were suppressed, suggesting that neoplasia could be prevented by increasing dietary calcium and VD_3 [77]. To examine the direct functional role of VDR in CRC, VDR deficient mice have been further analyzed. These mice display hyperproliferation and increased DNA damage, mainly in the colon [75]. Although they do not have high rates of spontaneous CRC, the results implicate the action of VD_3 in the prevention of hyperproliferation and oxidative DNA damage, at least in the distal colon [75]. Moreover, several studies have demonstrated that polymorphisms of the VDR gene may influence the development of CRC [78, 79].

Besides the participation of VDR in inhibition of the development of CRC, the critical role of the receptor in the control of IBD has also been established [80, 81]. There is

mounting evidence for a link between VD_3 availability either from sunshine or diet and the prevalence of IBD [81]. In several experimental models, VD_3 deficiency has been linked to IBD, *e.g.* absence of VD_3 has been shown to exacerbate chronic IBD in IL-10 KO mice [82, 83]. Treatment of these mice with VD_3 resulted in the suppression of IBD symptoms [82]. Moreover, VDR-deficiency in mice resulted in an acceleration of the progress of severe IBD [84]. In addition, polymorphisms of the VDR gene have been implicated in susceptibility for CD in patient studies [85]. Direct effects on T cells and innate immune cells and the suppression of TNFα are mechanisms underlying the efficacy of VD_3 *in vivo* [86]. In the gut it is likely that the targets of VD_3 will include epithelial cells, innate as well as acquired immune cells [81]. Furthermore, the modulation of the NFκB pathway by VD_3 has been postulated [87, 88].

2.4 Drugs with promising characteristics to treat colorectal cancer and inflammatory bowel disease

2.4.1 Mesalazine

5-Aminosalicylic acid (5-ASA, 5-AS, 5-aminosalicylate, 5-aminosalicylic acid), including its derivates sulfasalazine, olsalazine and balsalazine are the most commonly described anti-inflammatory drugs in patients with IBD which have been used extensively in the treatment of IBD for more than 50 years [89]. Moreover, a series of epidemiological investigations and preliminary clinical trials have affirmatively suggested that regular intake of oral mesalazine may have anti-neoplastic and potentially prophylactic properties reducing the occurrence of CRC in patients with IBD, in particular UC and colonic CD [90]. Furthermore, MSI and CIN as well as dysplasia were found to be reduced after long-term mesalazine therapy [91, 92].

Oral administration of simple 5-ASA tablets or capsules is ineffective because, although absorption is poor, the surface area and transit time through the small bowel is not sufficient for most of the drug to be absorbed whilst passing through the small bowel [93]. As a result, therapeutic concentrations are not reached in the distal gut [93]. The development of pH-dependent, delayed-release formulations of 5-aminosalicylic acid, olsalazine and balsalazine not only abolishes the toxicity associated with the sulfapyridine part of sulfasalazine but also delivers 5-ASA in therapeutic concentrations to the large bowel [93]. The effectiveness of these drugs depends on the splitting of the diazo-bond of the molecule by

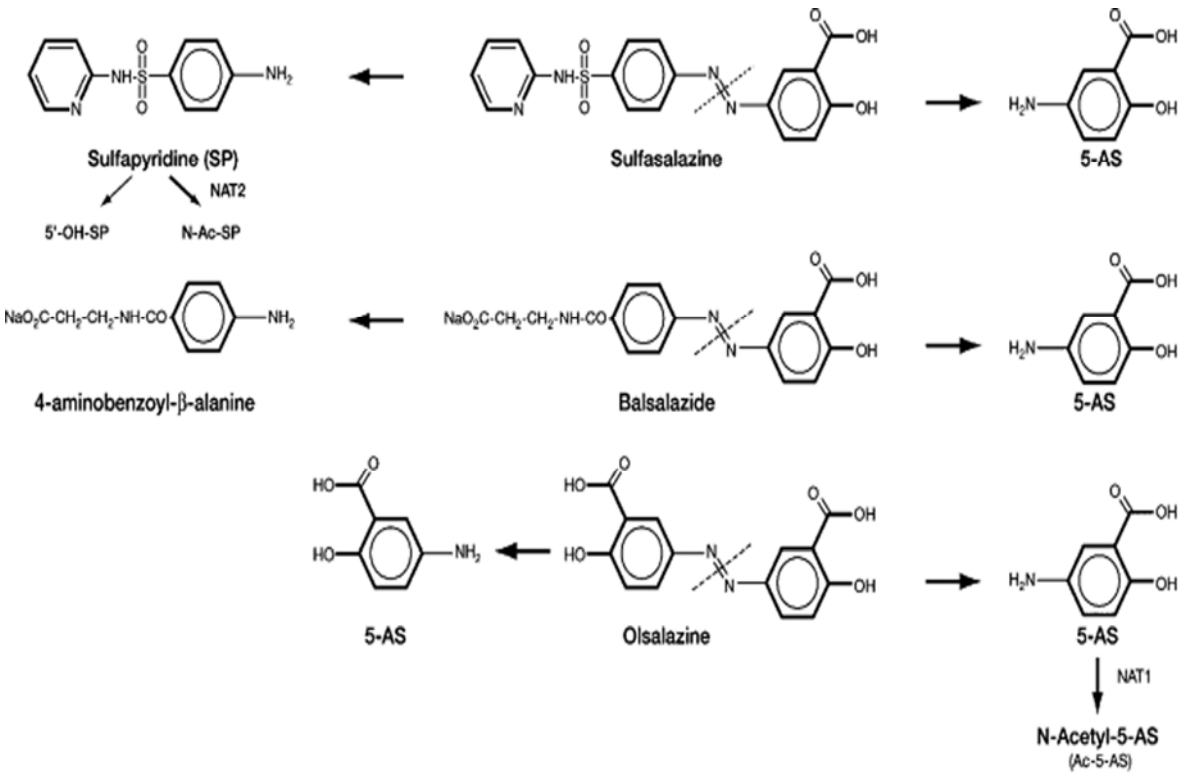

Figure 11: **Chemical structures of 5-aminosalicylates (5-AS).** See text for further explanations. [adapted from [93]]

the action of bacteria in the large bowel, releasing the pharmacologically active moiety, 5-AS [*Figure 11*] [93].

Although mesalazine has been identified as a candidate chemopreventive agent in CRC prophylaxis and in the treatment of IBD caused by its anti-proliferative and pro-apoptotic effects, the precise mechanisms of action are not fully understood [94]. The widespread mechanisms leading to its actions include the inhibition of inflammatory cascades and reactions involved in cell growth and proliferation, such as the COX pathways (COX-1 and COX-2) which regulate cell proliferation through the formation of prostaglandins; the lipoxygenase pathway; direct DNA stabilizing effects; the transcription factor NFκB, responsible for the subsequent expression of pro-inflammatory molecules; MAPKs and the Bcl-2 family, as well as the activation of apoptotic processes via caspase-3 signalling [94]. In addition, recently Rousseaux *et al.* demonstrated that the PPARγ receptor is the key receptor for mesalazine [34, 95]. The drug increased the expression of the receptor, promoted its translocation from the cytoplasm to the nucleus and induced a modification of its conformation permitting the recruitment of coactivators and the activation of a PPRE driven gene, thereby affecting the anti-inflammatory actions of the drug [34, 93, 95]. Activation of

PPARγ may also have anti-tumorogenic effects, which manifests as anti-proliferative, pro-differentiation, and pro-apoptotic activities, inhibition of the formation of aberrant crypts foci and inhibition of the development of colon tumors [93]. Therefore it is possible that mesalazine also acts directly as a PPARγ ligand to suppress tumor formation, rather than directly by suppressing inflammation.

2.4.2 Dietary Histone Deacetylase inhibitors

2.4.2.1 Butyrate

Butyrate (butyric acid) [*Figure 12*], a SCFA and normal constituent of the colonic luminal content, is formed by anaerobic bacterial fermentation of undigested carbohydrates and dietary fibre in the colon, where it is present at millimolar concentrations [96]. Besides its role as the major energy source for the colonocyte, there is increasing evidence that butyrate also acts as a critical factor in the physiological turnover of the colonic epithelium [96, 97]. Although butyrate increases proliferation of normal colonic epithelium, it decreases proliferation of neoplastic colonocytes *in vitro* and *in vivo* (*butyrate paradox*) [96]. In addition, roles of butyrate have been established for cell differentiation, morphology, motility, as well as induction of cell cycle arrest and apoptosis, through interaction with the caspase signalling cascade [96, 98, 99]. Moreover, butyrate exerts immune-modulatory effects and anti-inflammatory properties in the gastrointestinal tract, *i.e.* several studies have demonstrated that butyrate stimulates the release of the APs cathelicidin and β-defensin-2 and influences cytokine-activated gene expression in colonic epithelial cells via inhibition of NFκB signalling [100-104].

Although human data are still inconsistent, at least in rodents, evidence has been provided that alimentary fibres exert a protective role with regard to colorectal carcinogenesis [105, 106]. The benefits of a high intake of dietary fibre can be attributed to their fermentation in the colonic lumen [96]. In addition, deficiencies in luminal butyrate production have been linked to chronic bowel inflammation, such as diversion colitis and pseudomembranous colitis [96, 107, 108]. Moreover, a series of experimental results corroborates the role of butyrate in the pathogenesis of UC [96]. It has been postulated that UC is due to either exogenous or endogenous starvational factors limiting fatty acid oxidation in colonocytes; the latter is maybe associated with a mucosal defect [96].

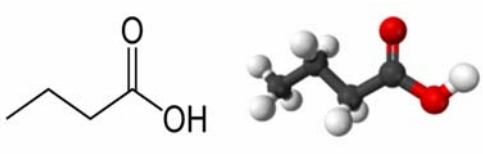

Figure 12: **Chemical structure of butyric acid**

Mechanisms of butyrate affecting the colonic epithelium are mainly relied due to its function as a HDAC inhibitor, opening the chromatin structure of the DNA, thereby possibly enhancing the transcriptional activity of several genes [*Figure 13*] [96, 109]. Moreover, butyrate has been revealed both to phosphorylate and to methylate DNA-histone complexes, to influence the expression of various oncogenes (*c-myc, c-myb, c-ras*), and to reduce urokinase activity [96]. Additionally, several studies have demonstrated that butyrate up-regulates and activates the nuclear hormone receptors, PPARγ and VDR, in colonic epithelial cells, indicating the possible involvement of both receptors in butyrate-mediated actions on the colonic epithelium [42, 69, 70].

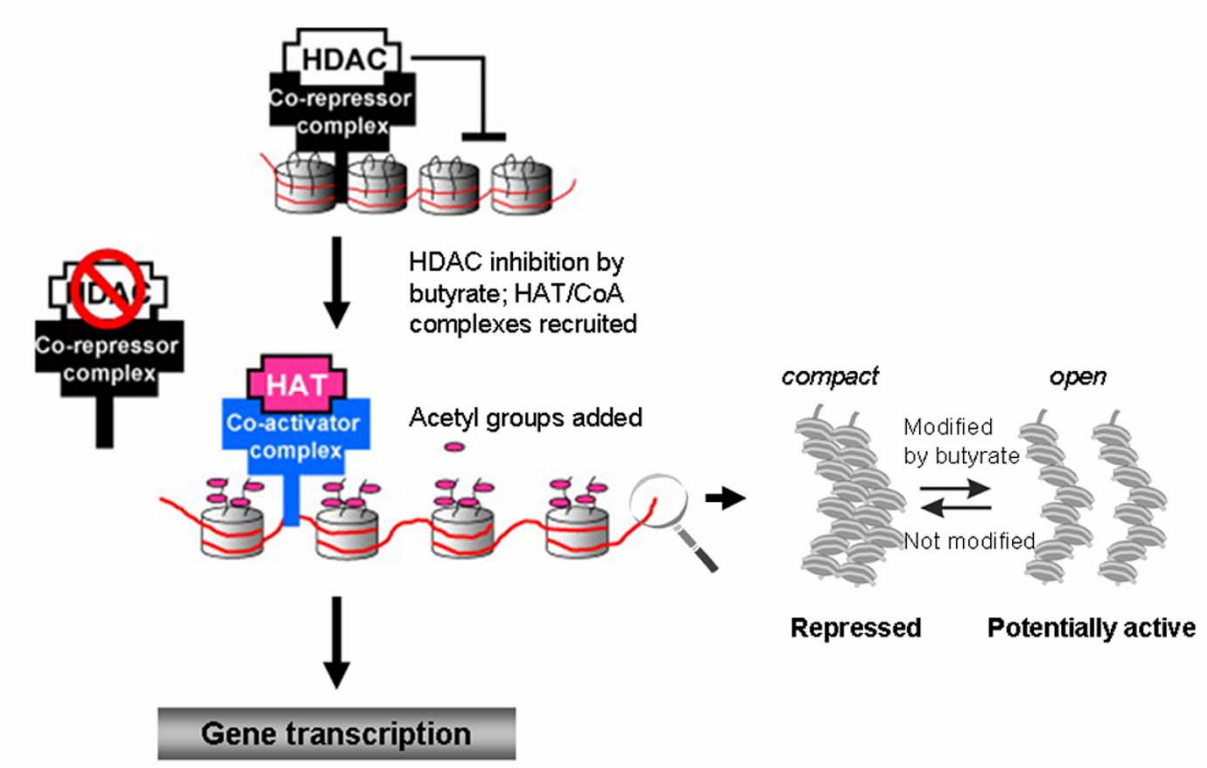

Figure 13: **Possible working mechanism for dietary HDAC inhibitors like butyrate.** HDAC/co-repressor complexes maintain a tightly restricted chromatin configuration, which limits access of transcription factors to DNA, and the repression of genes. HDAC inhibition by dietary agents enables histone acetyltransferase/coactivator (HAT/CoA) complexes to add acetyl groups to histone tails, loosening DNA/chromatin interactions, and allowing access of transcription factors to the promoters of genes. [depicted modified from [109]]

2.4.2.2 Sulforaphane

Sulforaphane (SFN, [1-isothiocyanato-4-(methyl-sulfinyl)butane]) is one of the most promising dietary chemopreventive agents and is present at high concentrations in some cruciferous vegetables, especially broccoli, broccoli sprouts, cauliflower and cabbage (*Brassica oleracea*) [98, 110, 111]. SFN is an isothiocyanate derived from hydrolysis of the glucosinolate glucoraphanin [*Figure 14*] [110]. The hydrolytic reaction is catalyzed by the endogenous myrosinase which is related by disruption of the plant during harvesting, processing, or chewing [110, 111]. The mercapturic acid pathway then concerts SFN sequentially to the metabolites SFN-glutathione, SFN-cysteine, SFN-N-acetylcysteine [112].

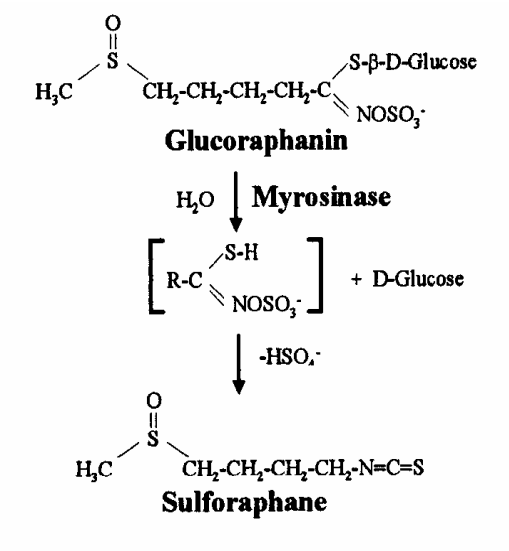

Figure 14: **Enzymatic conversion of glucoraphanin to sulforaphane.** [adapted from [110]]

SFN has received a great deal of attention because of its ability to simultaneously modulate multiple targets involved in cellular protection, including DNA protection by inducing carcinogen-metabolising enzymes of Phase II and by blocking the action of mutagens [111]. Moreover, in human CRC cells, SFN has been reported to be involved in the inhibition of cell proliferation, tumor progression, and neoangiogenesis as well as in the initiation of cell growth arrest and apoptosis [98, 111]. In addition, several studies indicate that SFN also exhibits immune-modulatory and anti-inflammatory capacities by interfering with the actions of the transcription factor NFκB, modulating the production of pro- and anti-inflammatory cytokines, and by interacting with the COX system [113, 114]. The exact molecular mechanisms behind these regulations are not entirely clear. The chemopreventive effects of SFN, similar to butyrate (see 2.4.1), may be achieved mainly through its function as a HDAC inhibitor by altering histone structures including acetylation, methylation, phosphorylation, ubiquination and biotinylation [109, 112]. This results in chromatin relaxation and increased transcription of target genes [109, 112]. A recent study demonstrated that direct application of SFN parent compound and SFN-glutathione to nuclear extracts *in vitro* did not effect on HDAC activity, whereas SFN-cysteine and SFN-N-acetylcysteine attenuated HDAC activity significantly, indicating that incubation with cells is necessary to allow for metabolism [112].

2.5 Targets for nuclear hormone receptors involved in inflammation and cell cycling

In the following chapter, basics of the caspase cascade, of APs and of NFκB signalling are described. Within the scope of this thesis, the interaction of butyrate, mesalazine and SFN with these signalling pathways and molecules was scrutinized. Moreover, the putative role of the nuclear hormone receptors PPARγ and VDR involved in these regulatory mechanisms was elucidated.

2.5.1 The caspase cascade

Apoptosis is a physiological suicide program that is critical for the development of healthy tissues [115]. Dysregulation of cell death pathways leading to increased cellular growth and decreased death occurs *e.g.* in autoimmune and immunodeficiency diseases, reperfusion injury after ischemic episodes, in neurodegenerative disorders and in several types of cancer, in particular CRC [115]. Although over the past years major advances have increased the understanding of the molecular mechanisms triggering apoptosis, the regulation of apoptotic cell death has yet not been fully uncovered. Major pathways of apoptosis include the caspase-, proteasome- and the autophagic-induced cell death, which frequently occur in parallel or in cross-talk [115-117]. Among these, the caspase cascade pathway is the best characterized one [*Figure 15*]. One key endpoint represents caspase-3, a member of the cysteine aspartyl-specific protease (caspase) family, specifically involved in the initiation and execution of the apoptotic program [115]. Caspase-3 is a downstream effector caspase which is activated by upstream caspases, predominantly caspase-8 and -9. Activation of caspase-3 then leads to cleavage of cellular substrates, *e.g.* poly(ADP-ribose) polymerase (PARP), DNA fragmentation factor (DFF) and disassembling of the cell [115]. Two pathways operate as sensors for caspase-3 activation and can stimulate the cellular death program of apoptosis. The extrinsic or death receptor-mediated pathway involves members of the tumor-necrosis-factor receptor (TNFR) superfamily and is engaged in response to cytokines, especially of the TNF family, and extracellular signals, leading to activation of caspase-8 [115]. The intrinsic or mitochondrial pathway is activated in response to intracellular signals and cytotoxic insults at the level of mitochondria [115]. After activation by an apoptotic stimulus, mitochondria

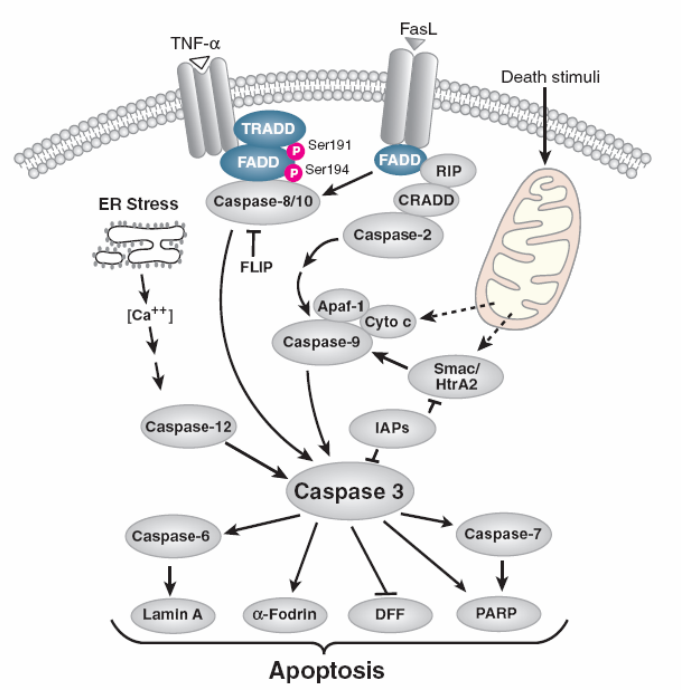

Figure 15: **The caspase cascade.** Activation of the caspase cascade can be suppressed by the inhibitor of apoptosis proteins (IAPs) Survivin and Xiap. See text for further explanations. [adapted from [118]]

releases *cytochrome c* into the cytosol, where it binds to the apoptotic protease activating factor-I (Apaf-1) to form the apoptosome. At the apoptosome, the initiator caspase-9 is then activated [115]. However, cross-talk between the death receptor-mediated pathway and the mitochondrial pathway has been described [115]. Besides the prototypic caspase-dependent apoptosis pathways, there are also molecular less-well defined cell death pathways that do not require caspase activation. These pathways share some, but not all, the characteristics of apoptotic classical pathways [115].

The balance between apoptotic and anti-apoptotic proteins plays an important role in determining the response of cancer cells to chemotherapeutic agents [119]. Various proteins regulate the apoptotic process at different levels, *e.g.* the FLICE-inhibitory protein (FLIP) has been shown to interfere with the initiation of apoptosis directly at the level of death receptors [115]. Moreover, death initiated at the mitochondrial level is regulated by the members of the Bcl-2 family which can be divided into anti-apoptotic and pro-apoptotic proteins [115]. The family of the inhibitor of apoptosis proteins (IAP), *i.e.* Survivin and X linked inhibitor of apoptosis (Xiap), is a recently identified, novel category of apoptosis-regulatory proteins [120]. IAPs can inhibit the activation of caspases by both the extrinsic and intrinsic pathway

[120]. IAPs may thereby set the threshold for apoptosis-activation and play a key role in the regulation of apoptotic cell death.

2.5.2 Antimicrobial peptides

2.5.2.1 Fundamentals

Antimicrobial peptides (APs) are endogenous antibiotic effector molecules of the innate immune system [121, 122]. In mammals, they are mainly expressed in the epithelia and in blood cells. In the past few years, different lines of investigations have implicated a central role of APs in gastrointestinal infections and inflammatory diseases [122, 123]. APs are small polypeptides of less than 50 amino acid residues, with the ability to directly kill *Gram-positive* and *Gram-negative* bacteria as well as fungi and viruses [124]. In addition, these peptides modify the local inflammatory response and activate mechanisms of cellular and adaptive immunity [125]. The activity spectrum is unique for every peptide and single amino acid substitutions can affect the activity [126]. The net charge of nearly all APs is positive, and therefore they are often designated cationic APs. The tertiary structures of the peptides are diverse. However, they have an amphiphilic character in common [127].

The two main families of APs expressed in mammals are defensins and cathelicidins [124]. The defensins are characterised by three disulphide bonds, while the cathelicidin family is characterised by a conserved prepro-region [128, 129]. The defensins are subdivided into α, β and γ defensins. Four human β-defensin (HBD 1-4) peptides have been characterised. They are mainly expressed in epithelial cells [130]. In addition, 28 β-defensin genes have been identified in the human genome [131]. On the other hand, only one human member of the cathelicidin family has been identified, namely LL-37 [132]. The name hCAP18 is often used when referring to its proform [132]. The APs known to be produced by the colonic epithelium are LL-37 and the β-defensins HBD-1 and HBD-2, contributing to the regulation of the intestinal balance [121]. Both LL-37 and HBD-1 are constitutively expressed, while HBD-2 is induced during inflammation by pro-inflammatory stimuli [133]. There is increasing evidence, that the expression of LL-37 and HBD-2 in the colon is modulated by SCFAs [100, 104].

2.5.2.2 Mechanisms of antimicrobial activity

APs are mainly membrane active and lyse the target by disrupting the integrity of the membrane. They exhibit many of the desirable features of a novel antibiotic class [134]. In particular, they have a broad spectrum of activity, kill bacteria rapidly, are unaffected by classical antibiotic resistance mutations, do not easily select antibiotic resistant variants, show synergy with classical antibiotics and neutralize endotoxin [135].

Several models explaining the mode of action of APs have been proposed, of which the barrel-stave pore, toroidal pore, and the carpet model are well established [*Figure 16*] [127]. In the barrel-stave pore model the peptides transverse the membrane with the hydrophobic surfaces directed towards the lipid membrane and the hydrophilic surfaces inwards forming a pore allowing water and electrolyte leakage [127]. The killing mechanism of the toroidal pore model is similar to the barrel-stave pore model. In this model, the peptides are suggested to aggregate and induce the lipid monolayers to bend continuously through the pore, causing the lipid head groups to be directed towards the water core [127]. In the third mechanism, the carpet model, bacterial membrane surface is covered with APs after an electrostatic interaction, and the membrane is disrupted in a detergent-like manner [127].

Caused by their positive charge, APs are more attracted to the negatively charged membranes of bacteria than to the neutral mammalian membranes [136]. This difference in the membrane charge is due to a higher level of acidic phospholipids in the bacterial membranes, while the eukaryotic membranes have more cholesterol and bipolar phospholipids facing the extracellular room [136]. Moreover, these acidic phospholipids are positioned at the cytoplasmatic side [136]. Besides the initiation of transmembrane pores, there is increasing evidence that APs have other intracellular targets regulating microbial killing. Proposed mechanisms include the alteration of cytoplasmatic membrane septure formation as well as the inhibition of cell-wall biosynthesis, nucleic-acid synthesis, protein synthesis or enzymatic activity [127]. Moreover, non-membrane external targets such as autolysis and phospholipases are activated by APs [127].

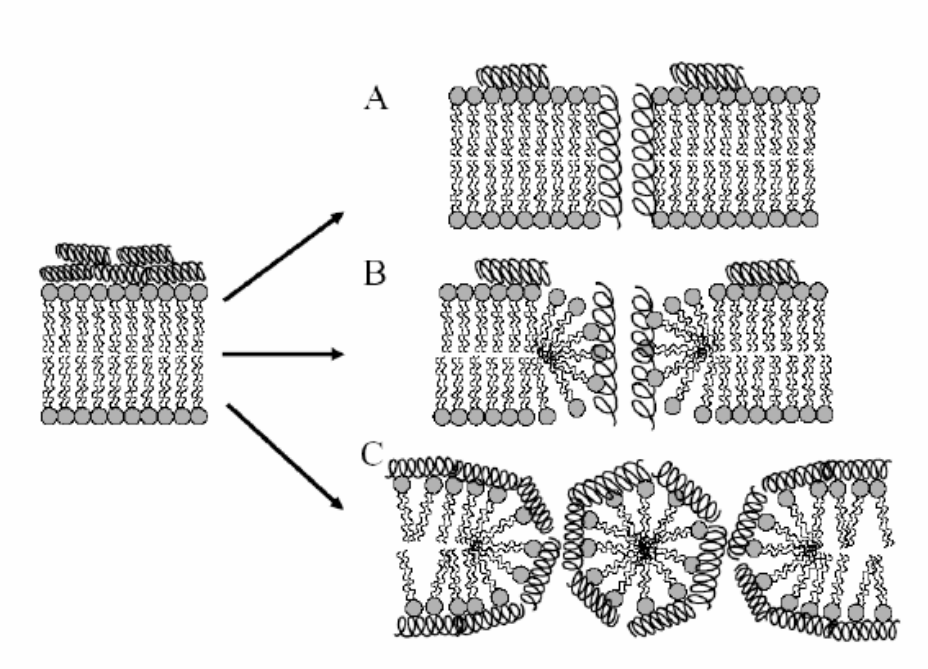

Figure 16: **Mechanisms of action of APs.** (A) Barrel-stave pore model of AP-induced killing. (B) Toroidal pore of AP-induced killing model. (C) Carpet model of AP-induced killing. [modified from [127]]

2.5.2.3 Additional functions of antimicrobial peptides

Aside from being able to directly kill or inhibit growth of microorganisms, APs exert multifunctional roles in the immune system. They have been shown to chemo-attract cells of both the innate and adaptive immune system, thereby forming a link between these two systems [137]. APs exhibit immune-modulatory properties, such as modulating differentiation and enhancing the endocytic capacity of dendritic cells, up-regulating the expression of chemokines in epithelial cells and macrophages, neutralising lipopolysaccharides (LPS) bioactivity, thereby protecting against endotoxic shock, increasing phagocytosis, inducing mast cell degradation, regulating the complement system, stimulating angiogenesis and playing part in the re-epithelisation in wounds [121, 138-140]. Moreover, they seem to be involved in anti-tumor and mitogen activity, or they may act as signalling molecules [141].

2.5.3 The nuclear factor NFκB

NFκB is an ubiquitously expressed family of transcription factors holding a central role in the inducible expression of a high number of genes involved in inflammation, host defence, cell survival, proliferation and thus also tumorigenesis [24, 142]. The transcription factor is triggered by pro-inflammatory stimuli and genotoxic stress, including cytokines such as TNFα and IL-1, bacterial cell-wall components such as LPS, viruses, and DNA-damaging agents [24]. Increased NFκB activation is not only associated with IBD, but also with CRC [142]. CRC cell lines, as well as nuclei of stromal macrophages in sporadic adenomatous polyps, were found to have elevated NFκB activity [24, 142]. NFκB inhibitors might, therefore be useful in treating both human diseases [142]. The NFκB family consists of five different proteins (p50 (NFκB1), p52 (NFκB2), p65 (RelA), RelB and c-Rel) which tend to dimerize [24, 142]. The majority of NFκB dimers is composed of the p65 and the p50 or p52 subunits [24, 142]. In resting conditions, NFκB is sequestered in the cytoplasm of unstimulated cells and must be translocated into the nucleus to function [24, 142]. The subcellular location of NFκB is controlled by a family of inhibitory proteins called IκBs [24, 142]. One of the most important regulators of mammalian NFκB is IκBα. When IκBα binds to the p50/p65 heterodimer complex, it maintains the heterodimer in the cytoplasm and prevents activation of NFκB target gene transcription [*Figure 17*] [24, 142]. In response to cell stimulation, IκBα kinase (IKK) is activated and phosphorylates IκBα on two serine residues leading to polyubiquitination and 26S proteasome-mediated degradation [24, 142]. Degradation of IκBα allows a rapid and transient translocation of NFκB to the nucleus, where it activates transcription from a wide variety of promoters, including that of its own inhibitor IκBα. The newly synthesized IκBα enters the nucleus and removes NFκB from its DNA-binding sites and transports it back to the cytoplasm, thereby terminating NFκB dependent transcription [24, 142]. In addition, there is accumulating evidence that the activity of NFκB is regulated by direct modification of NFκB proteins through phosphorylation and possible acetylation *e.g.* protein kinase A (PKA)-mediated phosphorylation of p65 has been shown to be important for its transcriptional activity [143].

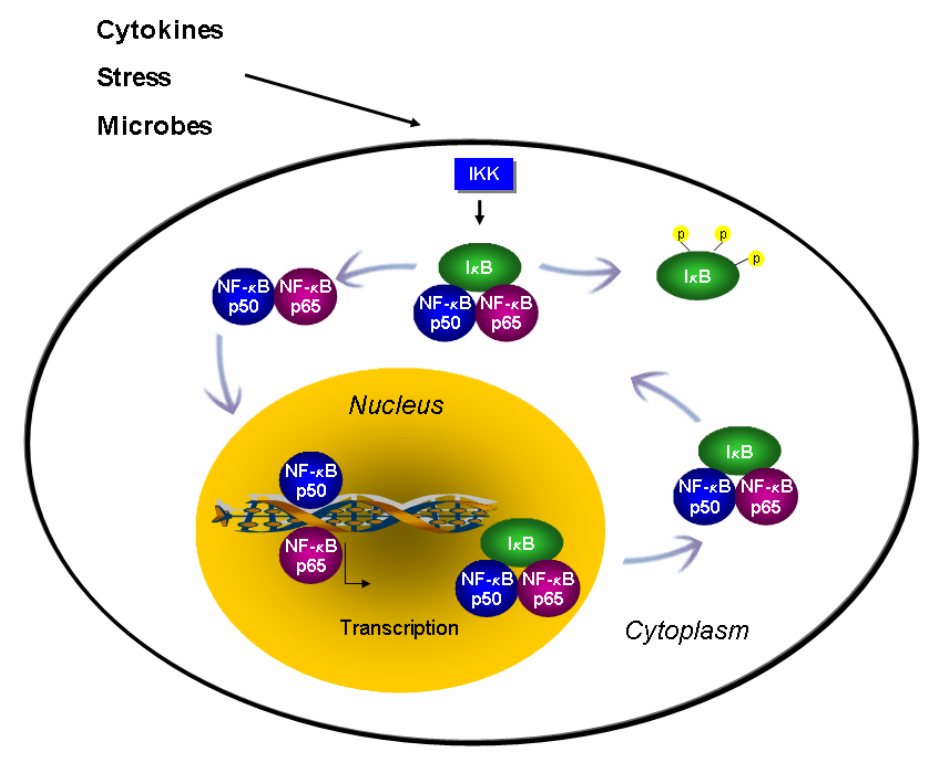

Figure 17: **NFκB activation.** Various stimuli induce transcription of NFκB target genes by facilitating nuclear import of NFκB and binding to cognate DNA sites. In unstimulated cells, p50/p65 is inhibited by IκB.

3 Aim of the present investigations

Due to increasing relevance of the nuclear hormone receptor PPARγ in the therapy of CRC and IBD, the major purpose of the studies presented in this thesis has been to reveal roles for the receptor in the pro-apoptotic, immune-modulatory and anti-inflammatory actions caused by butyrate, mesalazine and SFN in colonic epithelium cells. The specific aims have been:

Paper I: Butyrate is known to exert pro-apoptotic effects in CRC cells by inducing apoptosis via caspase-3 signalling [144, 145]. However, little is known with respect to the underlying events participating in this regulation. There is increasing evidence supporting the role of PPARγ as a key player in the induction of cellular apoptosis. PPARγ does not only control the expression of genes involved in differentiation, but also modulates cell cycling [50, 51]. In addition, recent reports indicate that the pro-apoptotic action of PPARγ is dependent on caspase-3 activation [56, 58]. Besides revealing that the expression of PPARγ is under control of butyrate, our group could demonstrate that the receptor is also involved in butyrate-induced growth inhibition of CRC cells [42, 146]. **On the basis of the current findings, this study addressed the potential role of PPARγ in the regulation of caspase-3 activation caused by butyrate in Caco-2 cells. The second aim was to point out the involvement of the mitogen-activated protein kinase kinase/extracellular-regulated kinase (MEK-ERK) signalling pathway in butyrate-mediated up-regulation of PPARγ expression and caspase-3 activity.**

Paper II: Mesalazine has been demonstrated to reduce the development of CRC in patients suffering from IBD by decreasing cell proliferation and inducing apoptosis [90, 147]. However the mechanisms of these chemopreventive actions are not yet completely elucidated. Moreover, mesalazine has been recently identified to modulate the activity and expression of PPARγ *in vitro* and *in vivo*, thereby exerting its anti-inflammatory effects in the colon [40, 93]. **Based on the observations in Paper I, the present study was addressed to investigate the possible role of PPARγ in mesalazine-induced pro-apoptotic effects in CRC cells along the caspase**

signalling pathway. Moreover, the involvement of PPARγ in the alteration of cell cycle distribution and cell growth in response to mesalazine was investigated.

Paper III and Paper IV: The APs human β-defensin-2 (HBD-2) and cathelicidin hCAP18 (LL-37) are molecules of the innate immune system located at epithelial surfaces in particular at the surface of the gastrointestinal mucosa [124, 128]. APs influence microbial growth and inflammation and may play a role in IBD, characterized by breakdown of colon epithelial barrier function [148]. The capacity of butyrate to induce HBD-2 and LL-37 in CRC cells has been demonstrated in several studies [101, 104]. However the underlying molecular mechanisms are rarely understood. **The intention of this study was to determine the role of several pathways, *e.g.* MEK-ERK signalling, and of the two nuclear hormone receptors PPARγ and VDR in butyrate-mediated induction of both APs in CRC cells. Moreover, in Paper IV, the influence of the dietary HDAC inhibitor SFN on HBD-2 expression and signalling was investigated.**

Paper V: High levels of circulating and mucosal pro-inflammatory cytokines resulting from increased NFκB activity have been reported in the intestinal mucosa of patients suffering from IBD [149-151]. Modulating NFκB signalling may therefore be an interesting target to suppress inflammation and the development of cancer in IBD. Several studies have demonstrated that butyrate antagonises inducible NFκB signalling in colonocytes [103, 152, 153]. However, comprehensive knowledge by which butyrate interacts with the NFκB pathway is still lacking. There is increasing evidence that PPARγ and VDR are under control of butyrate implying that the receptors may participate in butyrate-mediated suppression of NFκB activation [42, 69, 70]. **The aim of this study was to elucidate the putative role of both nuclear receptors in butyrate-mediated inhibition of inducible NFκB activation. Moreover, the involvement of the receptors in basal NFκB signalling was studied.**

4 Methods

The following table lists the methods used in this thesis and indicates in which paper the respective method is described:

Method	*Paper*
Assay for cell proliferation	II
Beta-defensin-2 ELISA	IV
Caspase-3 activity assay	I, II
Caspase-8 and -9 activity assay	I
Cytoplasmatic and nuclear protein extraction	I, II, V
Determination of cell count	II
Determination of cytotoxicity	I-V
Flow cytometry analysis	II
Immunofluorescence assay	III, IV
mRNA isolation	I, III, IV
NFκB p65/NFκB p50 transcription factor assay	V
PPARγ transactivation assay	II, V
PPARγ transfection assay	I-V
SDS–polyacrylamide gel electrophoresis and immunoblot analysis	I, II, IV, V
Semiquantitative RT-PCR	I, III, IV
Total protein extraction	II, IV

The following CRC cell lines have been used:

Cell line	*Paper*
Caco-2 (COX-1 non-expressing)	I-IV
HT-29 (mutant $p53^{R273H}$, COX-2 non-expressing)	II-V
HCT-116 (hMLH1 mutant, WT p53)	II
SW480 (mutant p53/Arg^{273}His)	IV
Transfected dominant-negative PPARγ and empty-vector Caco-2 cells	I-III
Transfected dominant-negative PPARγ and empty-vector HT-29 cells	II, IV, V

5 Summary of the Results

Paper I: **PPARγ is a key target of butyrate-induced caspase-3 activation in the colorectal cancer cell line Caco-2**; Schwab M, Reynders V, Ulrich S, Zahn N, Stein J, Schröder O; *Apoptosis.* 2006 Oct; 11(10):1801-11

Paper II: **PPARγ is involved in mesalazine-mediated induction of apoptosis and inhibition of cell growth in colon cancer cells**; Schwab M, Reynders V, Loitsch S, Shastri Y, Steinhilber D, Schröder O, Stein J; *Manuscript submitted to Clin Cancer Res.*

Paper III: **Role of nuclear hormone receptors in butyrate-mediated up-regulation of the antimicrobial peptide cathelicidin in epithelial colorectal cells**; Schwab M, Reynders V, Shastri Y, Loitsch S, Stein J, Schröder O; *Mol Immunol*. 2007 Mar; 44(8):2107-14

Paper IV: **The dietary histone deacetylase inhibitor sulforaphane induces human beta defensin-2 in intestinal epithelial cells**; Schwab M, Loitsch S, Reynders V, Steinhilber D, Stein J, Schröder O*; Manuscript submitted to Mol Immunol.*

Paper V: **Involvement of different nuclear hormone receptors in butyrate-mediated inhibition of inducible NFκB signalling**; Schwab M, Reynders V, Loitsch S, Steinhilber D, Stein J, Schröder O; *Mol Immunol.* 2007 Jul; 44(15):3625-32

5.1 Paper I: PPARγ is a key target of butyrate-induced caspase-3 activation in the colorectal cancer cell line Caco-2

Background and rationale: Butyrate belongs to a promising new class of chemopreventive agents in CRC. Several studies have demonstrated that butyrate exerts pro-apoptotic effects by inducing apoptosis via activation of caspase-3 [144, 145]. However, the mechanisms by which butyrate modulates the caspase cascade are not fully understood. Recently our group not only elucidated the capacity of butyrate to up-regulate PPARγ, but also demonstrated the involvement of the receptor in butyrate-mediated inhibition of cell growth in the CRC cell line Caco-2 [42, 146]. Moreover, several studies indicate that PPARγ agonists like troglitazone induce apoptosis via activating caspase-3 [56, 154]. The objective of the study was to investigate a possible role of PPARγ in butyrate-induced caspase-3 activity along the caspase cascade in Caco-2 cells. In this context, the involvement of the MEK-ERK signalling pathway in butyrate-mediated elevation of PPARγ expression and caspase-3 activity was studied.

Results: Challenge of Caco-2 wild-type cells with butyrate (3 mM) resulted in a time-dependent increase of PPARγ mRNA expression. Initial effects were observed after 24 h and were boosted up to 1.7-fold after 48 h of treatment. Up-regulation of PPARγ mRNA was followed by transient changes on the protein level. PPARγ protein expression was elevated up to 1.5-fold after 48 h of treatment. Moreover, increased caspase-3 activity (2.2-fold) was observed after stimulation of cells with butyrate for 24 h.

To elucidate the role of MEK-ERK signalling in butyrate-induced caspase-3 activity and PPARγ expression, the effect of butyrate on p38 MAPK / phospho-p38 MAPK and ERK1/2 / phospho-ERK1/2 levels in Caco-2 wild-type cells was examined. Stimulation of cells with butyrate (3 mM) resulted in increased expression of phospho-p38 MAPK (1.6-fold) after 24 h, while p38 MAPK was not affected. Moreover, the expression of ERK1/2 and phospho-ERK1/2 was unchanged in response to butyrate treatment. In order to determine the involvement of any MAPK in butyrate-induced PPARγ expression and caspase-3 activation, specific MAPK inhibitors, the p38 MAPK inhibitor SB203580 and the ERK1/2 inhibitor PD98059 were employed. Pre-incubation of Caco-2 wild-type cells with the p38 MAPK inhibitor SB203580 (20 μM) for 12 h, followed by stimulation of cells with butyrate for

additional 48 h, resulted in a significant inhibition of both butyrate-induced up-regulation of PPARγ mRNA as well as protein expression. In contrast, the combination of butyrate and the ERK1/2 inhibitor PD98059 (40 μM) did not influence butyrate-mediated up-regulation of PPARγ expression at any time. To corroborate the findings of a signalling link between p38 MAPK and PPARγ, Caco-2 cells were treated with arsenite (50 μM), a direct stimulator of p38 MAPK [155]. Arsenite treatment resulted in a time-dependent augmentation of PPARγ protein expression (6 h: 1.4-fold, 24 h: 2.4-fold), indicating the participation of p38 MAPK in butyrate-mediated up-regulation of PPARγ expression. Similar to the increase of PPARγ, the involvement of both MAPK signalling pathways in butyrate-mediated activation of caspase-3 activity was examined. Incubation with the p38 MAPK inhibitor resulted in a significant inhibition of elevated caspase-3 activity caused by butyrate (−63%). Again, the specific ERK1/2 inhibitor PD98059 had no impact on the induction of caspase-3 activity caused by butyrate.

Revealing the transduction pathway leading to increased caspase-3 activity in response to butyrate, caspase-8 and -9 activities as well as the expression of the inhibitor of apoptosis proteins Xiap and Survivin were further examined. Both caspase-8 (1.3-fold) and -9 (1.8-fold) activities were increased after incubation of cells with butyrate for 24 h, while the expression of Xiap (−59%) and Survivin (−68%) was reduced simultaneously. In order to identify a possible role of PPARγ in butyrate-mediated apoptosis, above scrutinized pro- and anti-apoptotic markers were also analysed in Caco-2 cells transfected with a dominant-negative PPARγ mutant to inhibit wild-type receptor action. For this purpose, at first, PPAR*γ* receptor activity in Caco-2 wild-type, empty-vector and dominant-negative PPAR*γ* mutant cells was evaluated by determining the expression of cytokeratin 20, described to be a specific target gene of PPAR*γ* activity in CRC cells [37]. A time-dependent increase of cytokeratin 20 caused by butyrate could be observed in wild-type and empty-vector cells (2.0-fold, 48 h). As expected, cytokeratin 20 was not expressed in both unstimulated and even butyrate challenged dominant-negative PPAR*γ* mutant cells, indicating not only that butyrate is able to increase PPARγ receptor activity but also demonstrating the functional successful transfection of the cell system. In dominant-negative PPARγ Caco-2 cells, butyrate-mediated increased caspase-3, -8 and -9 activities were almost completely abolished compared to wild-type and empty-vector cells after 24 h, and down-regulation of Xiap and Survivin expression was significantly reduced.

Except for the western blot for p38 / phospho-p38 MAPK and ERK1/2 / phospho-ERK1/2 expression (Figure 1 in Paper I) and transfection of dominant-negative PPARγ and empty-vector cells, all experiments and results of Paper I were performed by the author of this thesis. Above mentioned blots were performed by Nadine Zahn. Transfected cells were kindly provided by Sandra Ulrich. Moreover, interpretation and presentation of findings, including graphic display of results as well as writing of the scientific manuscript was performed by the author of this thesis.

5.2 Paper II: PPARγ is a key target of mesalazine-mediated inhibition of cell growth and induction of apoptosis in colon cancer cells

Background and rationale: Recent data identified PPARγ as a target of mesalazine's anti-inflammatory effects on the colon [34, 40]. Moreover, mesalazine has been recognized as a candidate chemopreventive agent in CRC prophylaxis for its pro-apoptotic effects [93, 156]. However, the precise mechanisms of these actions are not fully understood. In analogy to Paper I, the aims of this study were to investigate the participation of PPARγ in mesalazine's anti-carcinogenic actions in colonocytes along the caspase signalling pathway. In addition, the role of the receptor in mesalazine-mediated growth-inhibitory abilities was investigated.

Results: Caco-2, HT-29 and HCT-116 cells were treated with increasing concentrations of mesalazine (10-50 mM) for 24, 48 and 72 h. Mesalazine provoked a significant time- and dose-dependent decrease in both cell growth and proliferation for all time points at doses of 10 mM and higher. To establish whether mesalazine also leads to changes in cell cycle progression, HT-29 and HCT-116 cells were cultured in presence of the drug (40 mM) for 24 and 48 h and cell cycle distribution was analysed by flow cytometry. All cell lines showed changes in the cell cycle profile within 48 h, with an apparent increase in the number of cells in the G0/G1 phase.

To establish whether mesalazine affects PPARγ signalling, PPARγ protein expression and activity was studied. Stimulation of HT-29 cells with mesalazine (30-50 mM) led to a time-dependent up-regulation of PPARγ protein expression. In analogy to Paper I, here, mesalazine increased the expression of cytokeratin 20 up to 1.4-fold in Caco-2 wild-type and empty-vector cells, while in dominant-negative PPARγ mutant cells the expression of

cytokeratin 20 was not affected. Comparable effects were obtained for direct determination of PPARγ transcriptional activity in HT-29 cells.

In order to investigate the role of PPARγ in mesalazine-induced inhibition of cell proliferation and changes in cell cycle distribution, the effect of the drug was studied in dominant-negative PPARγ Caco-2 and HT-29 mutant CRC cells. Mesalazine (50 mM) treatment led to a significant decrease in cell proliferation in both wild-type and empty-vector cells after 72 h of treatment. In the dominant-negative mutant cell lines, the decrease in cell proliferation was partially neutralized compared to wild-type and empty-vector cells. In dominant-negative PPARγ HT-29 cells, change in cell cycle distribution in response to mesalazine (40 mM) was partially reversed. Nevertheless, statistical significant effects compared to the wild-type were not reached.

To study further molecular mechanisms responsible for mesalazine's growth-inhibitory effects, expressions of the oncoprotein *c-myc* and of the tumor suppressor gene PTEN after incubation with the drug were determined. *c-myc* is known to modulate a variety of biological activities including cellular proliferation and cell growth [157]. After stimulation of HT-29 cells with mesalazine (40 mM) for 4 h, the expression of *c-myc* was decreased in a PPARγ-independent mechanism. On the other hand, treatment of HT-29 wild-type and empty-vector cells with mesalazine (40 mM) up to 8 h provoked a significant increase in the expression of the tumor suppressor gene PTEN, well-established to decrease cell proliferation through cell-cycle arrest in the G0/G1 phase and to induce apoptosis via the caspase cascade [158, 159]. In dominant-negative PPARγ HT-29 cells, expression of PTEN in response to mesalazine was unchanged, suggesting that the tumor suppressor gene takes part in mesalazine-mediated PPARγ-dependent growth-inhibitory and pro-apoptotic actions.

To unravel whether growth inhibition and changes in cell cycle distribution in response to mesalazine were accompanied by a modification of the caspase cascade, the pro-apoptotic markers PARP, caspase-8, caspase-9 as well as the anti-apoptotic proteins Survivin and Xiap were examined by western blot analysis in Caco-2 cells. Direct caspase-3 activity was determined by ELISA. Mesalazine (30 mM) increased the expression of activated PARP (2.2-fold), caspase-3 (1.4-fold) and caspase-8 (1.5-fold) after incubation of cells for 48 h, whereas the expression of Xiap (−32%) and Survivin (−22%) was decreased simultaneously. In contrast, no influence on the expression of activated caspase-9 could be observed. Similar results were obtained in the CRC cell line HT-29. In order to scrutinize any involvement of PPARγ, experiments were performed in dominant-negative PPARγ Caco-2 and HT-29 cells. In both cell lines, mesalazine-mediated increased expressions of activated PARP, caspase-3 as

well as the down-regulation of Survivin and Xiap were almost reversed. Moreover, cleavage of caspase-8 could not be observed in dominant-negative Caco-2 cells in response to mesalazine treatment.

All experiments and results, published in this manuscript, were performed by the author of this thesis. Transfected cells were provided by Stefan Loitsch. Moreover, interpretation and presentation of findings, including graphic display of results as well as writing of the scientific manuscript was performed by the author of this thesis.

5.3 Additional results to Paper II: Enhanced anti-proliferative and pro-apoptotic effects of mesalazine and butyrate in the colorectal cancer cell line Caco-2

Except for determining the role of PPARγ in mesalazine-mediated anti-carcinogenic actions [Paper II], it was further examined whether mesalazine and butyrate exert synergistic effects in regard to growth inhibition and apoptosis in Caco-2 cells:

Mesalazine and butyrate act additive on the inhibition of cell count and cell proliferation

Cells were challenged with mesalazine (30 mM), butyrate (3 mM) or the combination of both substances for 24, 48 or 72 h. Both mesalazine, butyrate and their combination resulted in a time-dependent decrease of cell count and proliferation. *Figure 18* demonstrates the anti-proliferative effect of Caco-2 cells in response to mesalazine (−19%, $p < 0.001$) and butyrate (−32%, $p < 0.001$) after 72 h. Co-incubation of both substances had an additive effect on the inhibition of cell proliferation (−48%, $p < 0.001$). Similar effects could be observed after 24 and 48 h of treatment and the same tendency was seen for cell counts.

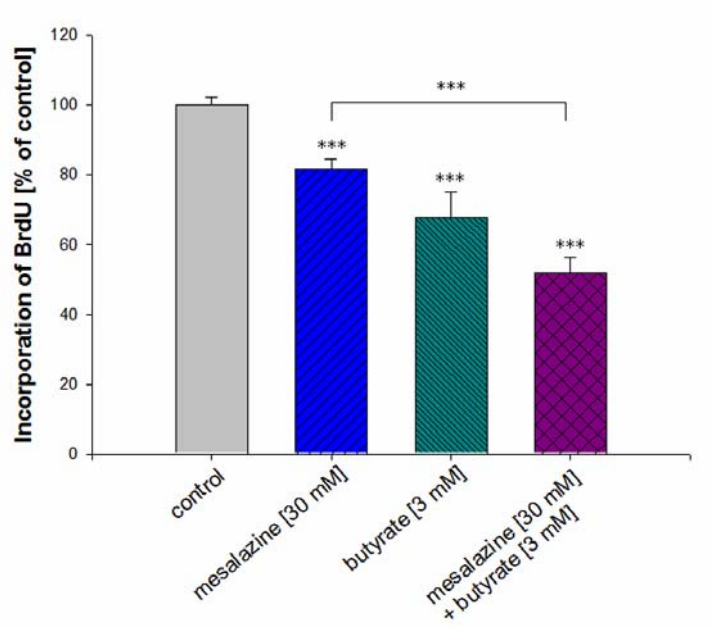

Figure 18: **Additional results to Paper II.** Cell proliferation measurement for Caco-2 wild-type cells after incubation with mesalazine (30 mM), butyrate (3 mM) or co-incubation of both substances for 72 h compared to control level. n = 8, ***$p < 0.001$. Bromodeoxyuridine (BrdU) test and statistics was performed as described in Paper II.

Additive effect on cleavage of PARP and caspase-8 caused by co-incubation of mesalazine and butyrate

Challenge of Caco-2 cells with mesalazine (30 mM) and butyrate (3 mM) resulted in an increase of cleaved PARP (*Figure 19* A, butyrate: 7.9-fold, mesalazine: 3.2-fold) after 48 h. Co-incubation of both substances led to an additive effect and up-regulated cleaved PARP protein levels up to 10.5-fold [*Figure 19* A]. To further unravel the responsible elements in the caspase pathway leading to increased cleavage of PARP, cleavage of caspase-8 and -9 were examined [*Figure 19* B, C]. As shown in *Figure 19* C, incubation with mesalazine (30 mM) for 48 h led to elevated cleavage of caspase-8 (1.5-fold of control, $p < 0.01$). In contrast, no cleavage of caspase-9 was observed [*Figure 19* B]. Co-incubation of mesalazine with butyrate increased cleavage of caspase-8 (3.2-fold, $p < 0.001$, *Figure 19* C) and also induced cleavage of caspase-9 [*Figure 19* B].

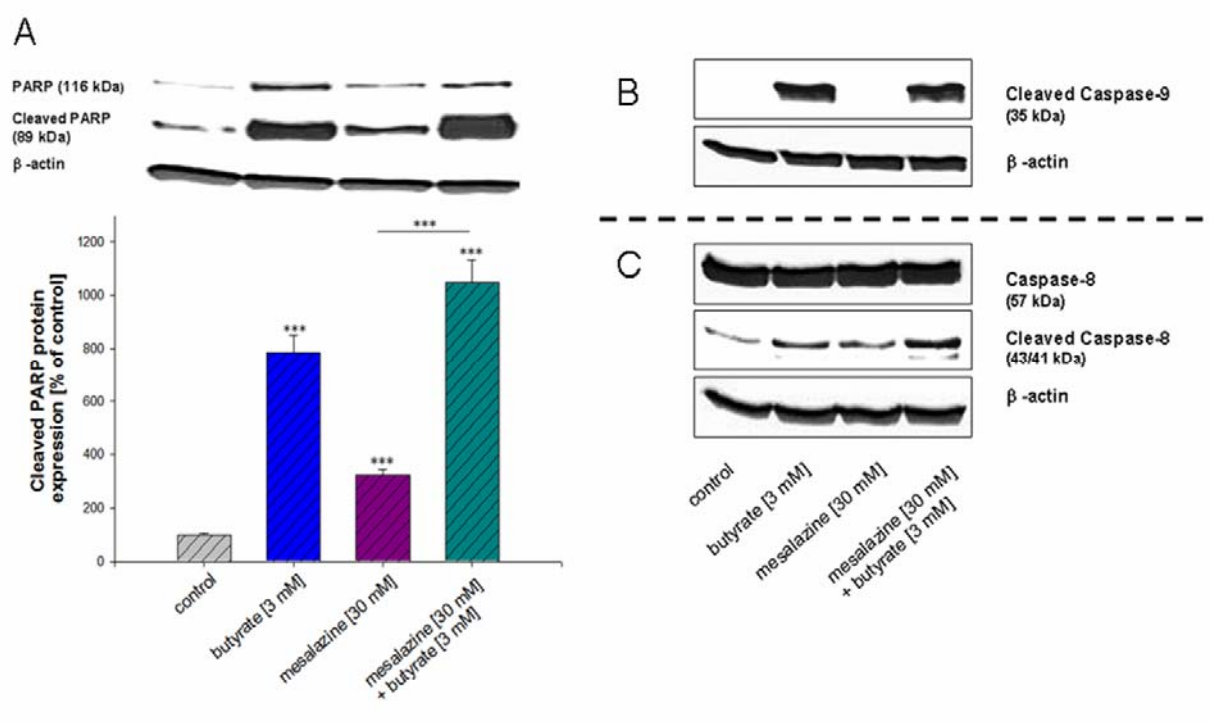

Figure 19: **Additional results to Paper II.** (A) Effect of mesalazine (30 mM), butyrate (3 mM) or the combination of both drugs on cleaved PARP protein expression after 48 h of treatment in Caco-2 wild-type cells. The band at 89 kDa corresponds to the cleaved PARP protein. Densitometric data are corrected for β-actin levels. ***$p < 0.001$ (B,C) Western Blot for cleaved caspase-9 (B) and cleaved caspase-8 (C) after treatment with mesalazine (30 mM), butyrate (3 mM) or the co-incubation of mesalazine with butyrate in Caco-2 wild-type cells for 48 h. Western-blotting and statistics was performed as described in Paper II.

Synergistic effect of mesalazine and butyrate on the attenuation of the inhibitor of apoptosis protein Survivin

To specify the molecular mechanism of mesalazine-induced apoptosis, changes in Xiap [*Figure 20 A*] and Survivin [*Figure 20 B*] were examined. Levels of both proteins were reduced in Caco-2 wild-type cells in response to mesalazine (50 and 30 mM) and butyrate (3 mM). The decrease in Survivin [*Figure 20 B*] was even more pronounced after co-incubation with butyrate, whereas no cumulative effect on the down-regulation of Xiap was observed [*Figure 20 A*].

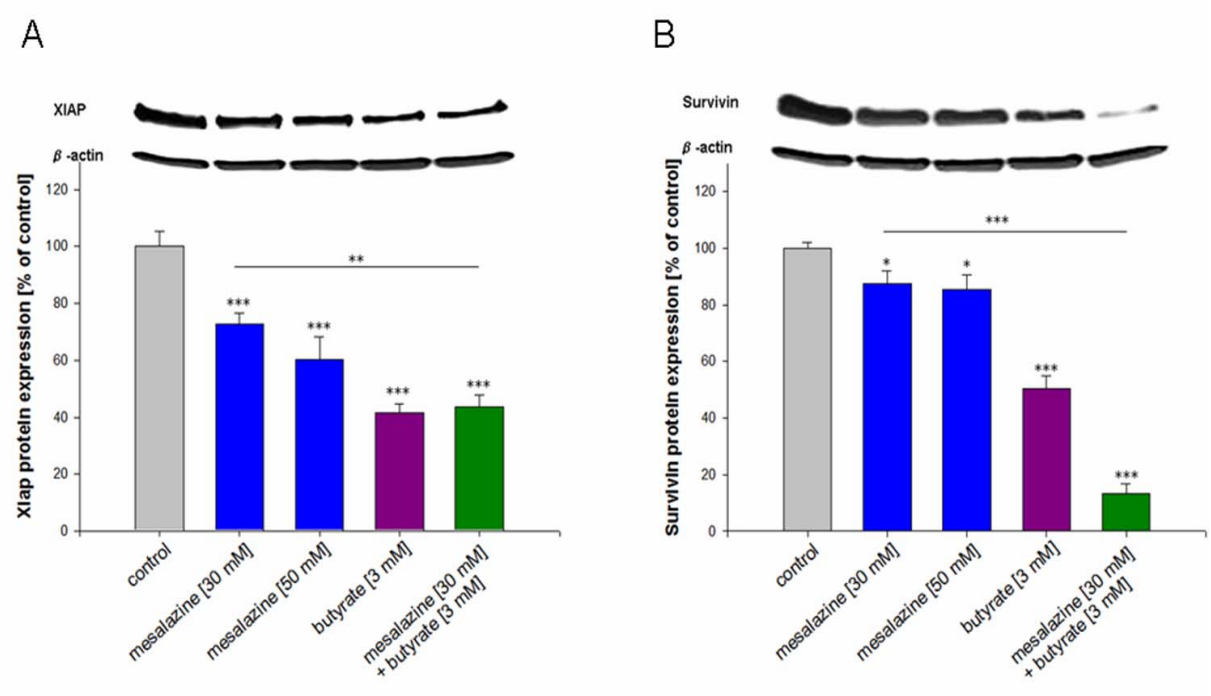

Figure 20: **Additional results to Paper II.** Xiap (A) and Survivin (B) expression in response to mesalazine (30 mM), butyrate (3 mM) or the combination of both drugs in Caco-2 wild-type cells after 48 h of treatment. One representative western blot of three independent experiments is shown. Densitometric data are corrected for β-actin levels. ***$p < 0.001$, **$p < 0.01$, **$p < 0.05$. Western-blotting and statistics was performed as described in Paper II.

All experiments and results, in this additional part of Paper II, were performed by the author of this thesis. Moreover, interpretation and presentation of findings, including graphic display of results was performed by the author of this thesis.

5.4 Paper III: Role of nuclear hormone receptors in butyrate-mediated up-regulation of the antimicrobial peptide cathelicidin in epithelial colorectal cells

Paper IV: The dietary histone deacetylase inhibitor sulforaphane induces human beta defensin-2 in intestinal epithelial cells

Background and rationale: APs like human β-defensin-2 (HBD-2) and cathelicidin hCAP18 (LL-37) play an important role in the non-specific immune system protecting the intestinal mucosa against bacterial invasion [124, 128]. Recent studies have demonstrated that butyrate induces HBD-2 and LL-37 in colonic epithelial cells [101, 104]. However the precise underlying molecular mechanisms have not yet been elucidated. The objective of this study was to investigate several pathways, *e.g.* the MEK-ERK signalling pathway, and the two nuclear hormone receptors PPARγ and VDR, involved in the up-regulation of both peptides in response to butyrate [Paper III + IV]. In addition, the influence of the dietary HDAC inhibitor SFN on HBD-2 expression and signalling was studied [Paper IV].

Results: Stimulation of the colorectal carcinoma cell lines Caco-2 and HT-29 with increasing concentrations of butyrate (1-5 mM) up to 48 h, resulted in a time-dependent up-regulation of both LL-37 and HBD-2 mRNA expression compared to control level [Paper III + IV]. Similar results were observed after incubation of colonocytes with SFN (1-20 μM) [Paper IV + *Figure 21*].

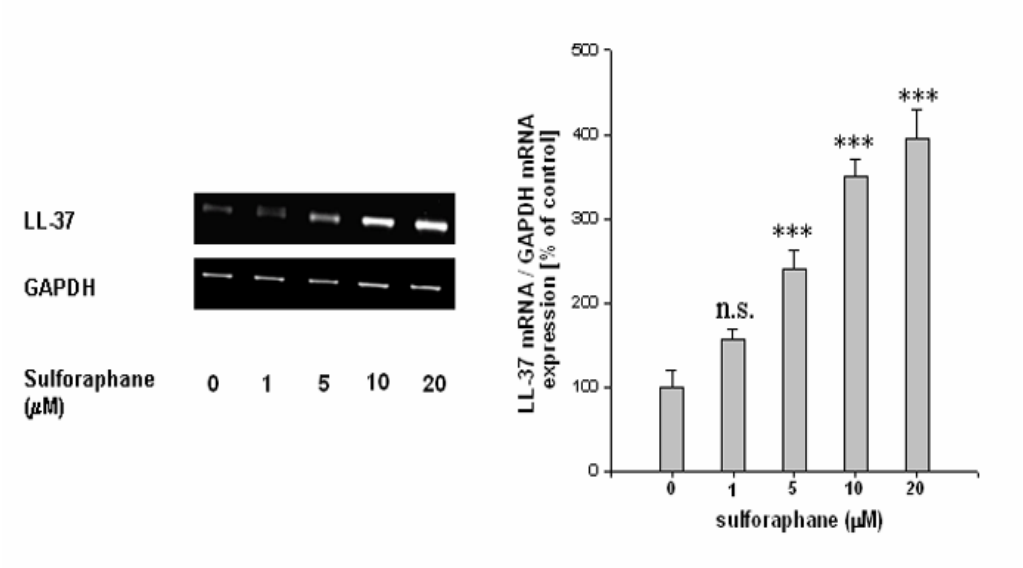

Figure 21: **Additional results to Paper IV.** Dose-dependent effect of sulforaphane (1-20 μM) on LL-37 mRNA expression in HT-29 cells after 24 h of treatment. LL-37 mRNA expression was measured by semi-quantitative RT-PCR with the fluorescent dye Pico Green. All values for mRNA levels are normalized to corresponding mRNA amounts for Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). One representative gel of three independent experiments is shown. Induction of LL-37 mRNA is displayed as relative percentage to solvent treated control cells. ***$p < 0.001$, n.s. = not significant. Semi-quantitative RT-PCR and statistics was performed as described in Paper III.

Increased expression of HBD-2 mRNA was accompanied by a comparable up-regulation on the protein level as measured by ELISA. HBD-2 protein levels were elevated by butyrate (3 mM) up to 2.6-fold and by SFN (20 μM) up to 2.0-fold after 48 h of exposure with the substances [Paper IV]. To further analyse the subcellular localisation of HBD-2 and LL-37, Caco-2 cells were treated with butyrate (3 mM) up to 48 h and APs were detected via immunofluorescence analysis. Both control and butyrate-treated cells showed intense HBD-2 and LL-37 staining mainly in the cytosol. Moreover, both peptides were increased in response to butyrate stimulation for 48 h [Paper III + IV].

To determine the potential role of the nuclear hormone receptors PPARγ and VDR in butyrate- and SFN-mediated up-regulation of LL-37 and HBD-2 mRNA expression, induction of mRNA levels were analysed in PPARγ dominant-negative cells to inhibit wild-type PPARγ function and by use of the specific VDR inhibitor ZK191732, respectively. In dominant-negative PPARγ mutant Caco-2 cells, an equal time-dependent increase of LL-37 mRNA compared to wild-type cells was observed after stimulation with butyrate (2-3 mM) [Paper III]. Similarly, equivalent amount of augmented HBD-2 mRNA in dominant-negative PPARγ

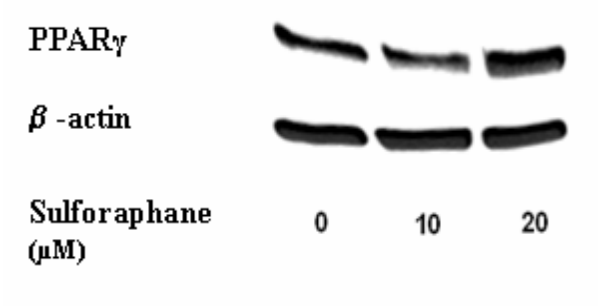

Figure 22: **Additional results to Paper IV.** Western blot for PPARγ expression after treatment of Caco-2 cells with SFN (10-20 μM) for 24 h. One representative blot of three independent experiments is shown. Western-blotting and statistics was performed as described in Paper I.

and wild-type HT-29 cells in response to butyrate (3 mM) or SFN (10 μM) was obtained [Paper IV]. In contrast, pre-incubation of Caco-2 cells with the VDR antagonist ZK191732 (10 μM) for 5 h followed by stimulation with butyrate (3 mM) or SFN (10 μM) for 24 h, significantly reduced the induction of the APs on mRNA level, indicating the essential role of VDR in the signalling pathway.

Up-regulation of VDR and PPARγ expression in colonocytes in response to butyrate has been demonstrated recently [42, 69, 70]. To elucidate whether the receptors are also targets of SFN signalling, protein expression of VDR and PPARγ in response to this HDAC inhibitor was also determined in colonoytes. Incubation of HT-29 and Caco-2 cells with SFN (10-20 μM) for 24 h resulted in a dose-dependent augmentation of VDR protein expression in both cell lines [Paper IV]. Moreover PPARγ protein expression was increased by SFN as determined in Caco-2 cells [*Figure 22*].

In order to establish the involvement of MEK-ERK signalling in LL-37 and HBD-2 mRNA, the p38 MAPK inhibitor SB203580 (20 μM) and the ERK1/2 inhibitor PD98059 (40 μM) were employed. Pre-treatment of Caco-2 cells for 5 h with the two specific MEK-ERK inhibitors prior to stimulation with butyrate (3 mM) up to 24 h diminished the up-regulation of LL-37 mRNA expression with the strongest effects seen by inhibiting the p38 MAPK trail [Paper III]. Moreover, the induction of LL-37 mRNA in response to butyrate was counteracted by the application of the transforming growth factor TGF-β1 inhibitor SB431542 (10 μM) [Paper III]. However, different results were obtained by inhibition of MEK-ERK signalling pathways in SFN-mediated induction of HBD-2 mRNA expression for this point in time. In this *in vitro* model, pre-treatment with the ERK1/2 inhibitor PD98059

reduced the up-regulation of HBD-2 mRNA expression [Paper IV] in response to SFN after 24 h. In contrast, co-incubation with the p38 MAPK inhibitor SB203580 did not influence SFN-induced HBD-2 levels [Paper IV]. These data are in accordance to the findings on the phosphorylation pattern. Exposure of Caco-2 cells with SFN (10-20 μM) resulted in a rapid expression of phospho-ERK1/2 after 8 h, while the amount of the ERK1/2 protein was not affected. The expression of phospho-p38 MAPK and p38 MAPK proteins were unchanged by SFN in Caco-2 cells [Paper IV].

All experiments and results, published in the manuscripts, were performed by the author of this thesis. Transfected cells were provided by Stefan Loitsch. Moreover, interpretation and presentation of findings, including graphic display of results as well as writing of the scientific manuscripts was performed by the author of this thesis.

5.5 Paper V: Involvement of different nuclear hormone receptors in butyrate-mediated inhibition of inducible NFκB signalling

Background and rationale: NFκB plays a major role in the control of immune and inflammatory responses [160]. Recently, it has been demonstrated that butyrate suppresses inducible NFκB activation in CRC cells [103, 152, 153]. However, the underlying molecular mechanisms are yet not fully understood. Ligands for PPARγ and VDR receptor have been shown to interfere with the activity of NFκB and thus to influence the ability of colonocytes to express immunmodulatory cytokines [42, 69, 70]. The purpose of this study was to examine a possible role of PPARγ and VDR in butyrate-mediated inhibition of NFκB activation induced by TNFα and LPS. Besides, the involvement of both receptors in basal NFκB signalling was explored.

Results: Treatment of HT-29 wild-type cells with butyrate (4 mM) for 24 h reduced basal p50 dimer NFκB DNA binding activity in the nucleus (−45%). In contrast, basal p65 dimer activity was not affected. Incubation of cells with TNFα for 30 min (100 ng/mL) led to a strong increase of both NFκB DNA binding activities (p50: 1.7-fold, p65: 4.9-fold). A comparable up-regulation of NFκB DNA binding activity was observed after stimulation with LPS (10 ng/mL) for 30 min (p50: 1.5-fold, p65: 3.0-fold). However, TNFα- and LPS-

mediated increased activities of NFκB were significantly attenuated in response to butyrate (TNFα: p50: −54%; p65: −52%; LPS: p50: −63%; p65: −49%). Cytosolic expression of the NFκB inhibitor protein IκBα was reduced by butyrate (−60%) and TNFα (−37%) but not by LPS. Moreover, combined treatment of butyrate and TNFα enhanced the decrease of IκBα (−83%).

To elucidate the potential involvement of PPARγ and VDR in the activation of NFκB in resting HT-29 and also after treatment with butyrate, NFκB activity was studied by application of the VDR inhibitor ZK191732 (10 μM), the PPARγ antagonist GW9662 (5 μM) or in transfected PPARγ dominant-negative cells to inhibit PPARγ wild-type action. To scrutinize the functional effective transfection of the cell system, at first, PPARγ receptor activity was determined in HT-29 wild-type, empty-vector and dominant-negative PPARγ mutant cells via a transcriptional factor assay. Similar to the investigations on the expression of cytokeratin 20 in Caco-2 cells in Paper I, butyrate (4 mM) provoked a direct up-regulation of PPARγ activity in both HT-29 wild-type and empty-vector cells. In the dominant-negative PPARγ mutant cell line, PPARγ activity was unchanged in response to butyrate. Stimulation of HT-29 wild-type cells with ZK191732 (p50: 1.6-fold, p65: 1.8-fold) alone or in combination with butyrate (p50: 1.6-fold, p65: 1.8-fold) led to an increase of basal NFκB DNA binding activity, while the expression of IκBα was attenuated simultaneously. On the contrary, treatment of cells with the PPARγ antagonist GW9662 did neither affect basal NFκB activity nor the expression of IκBα. Co-incubation of GW9662 did also not influence butyrate's actions on NFκB activity and IκBα expression. Comparable results were obtained by the use of dominant-negative PPARγ mutant cells.

In order to investigate the putative role of PPARγ and VDR in butyrate-mediated (4 mM) suppression of inducible p50 and p65 activity caused by LPS (10 ng/mL) or TNFα (100 ng/mL), experiments were performed in presence of the VDR inhibitor ZK191732 (10 μM) or in the PPARγ mutant HT-29 cell line. Pre-treatment with ZK191732 in presence of TNFα almost reversed the inhibitory effect caused by butyrate on both p50 and p65 NFκB dimer activities, while the effects on NFκB activation in dominant-negative PPARγ mutant HT-29 cells were comparable to the wild type. Reversed effects were obtained after stimulation of cells with LPS. Hence, inhibition of VDR did not affect butyrate-mediated suppression of LPS-induced NFκB activity, while inhibition of LPS-induced NFκB activity caused by butyrate was almost abolished in the PPARγ mutant cell line. Thus, butyrate-mediated inhibition of NFκB activation via LPS seems to be modulated by PPARγ, while VDR is

involved in the suppression of TNFα-induced NFκB activity caused by butyrate. Moreover, stimulation of HT-29 wild-type cells with LPS led to a time-dependent up-regulation of PPARγ expression, confirming the role of the receptor in LPS signalling. Regarding IκBα expression, comparable down-regulation of the protein in response to butyrate (4 mM), TNFα (100 ng/mL) or their combination was observed in all three cell lines. In addition, the decrease in IκBα caused by co-treatment of TNFα and butyrate was reduced in response to ZK191732.

All experiments and results, published in this manuscript, were performed by the author of this thesis. Transfected cells were provided by Stefan Loitsch. Moreover, interpretation and presentation of findings, including graphic display of results as well as writing of the scientific manuscript was performed by the author of this thesis.

6 Discussion

6.1 Importance of PPARγ in mesalazine's and butyrate's pro-apoptotic abilities

Apoptosis is a key component in the development and maintenance of tissues within multicellular organisms, providing a tightly regulated and selective mechanism for the deletion of superfluous, infected, mutated or aged cells. Dysregulation of apoptosis contributes to a variety of pathologic conditions, including cancer [161]. As tumor cells show a disturbed equilibrium between proliferation and apoptosis, conventional cancer therapies take advantage of this apoptotic mechanism by employing ionizing radiation or chemotherapeutic drugs to damage DNA and to induce selective apoptosis of rapidly growing cells [115]. Apoptosis can be initiated either through the death receptor or the mitochondrial pathway (see 2.5.1) [115]. Caspases that cleave cellular substrates leading to characteristic biochemical and morphological changes are activated in both pathways [115]. Caspases are a family of aspartyl specific cysteine proteases. They exist within the cell as inactive pro-caspases or zymogenes. The pro-domain is usually removed during the process of activation. Their initiation in turn triggers other caspases, resulting in a sequence of events instructing the cell to undergo programmed death [115].

The functional role of the nuclear hormone receptor PPARγ contributing to apoptosis and especially to the involvement in CRC has been implicated at several levels (see 2.2.5). There is increasing evidence that the receptor can be modulated by mesalazine and the dietary HDAC inhibitor butyrate *e.g.* by increasing its expression [42, 95]. Moreover, mesalazine was recently shown to be a new functional synthetic ligand for PPARγ in colonic epithelial cells [95]. In a variety of *in vivo* studies it has been indicated that long-term use of mesalazine in patients suffering from IBD may significantly reduce the risk for the development of CRC by decreasing cell proliferation and inducing apoptosis via the caspase cascade [147, 162, 163]. Similarly, the protective effect of butyrate reducing the hazard for CRC in the long-term therapy of UC has been indicated *in vivo* [164]. However, detailed molecular knowledge in this regulation is still missing. Therefore, in the first part of this thesis the involvement of PPARγ in the anti-carcinogenic abilities of mesalazine [Paper II] and butyrate [Paper I] was enlightened.

To elucidate the participation of PPARγ in the pro-apoptotic properties of both drugs, a dominant-negative PPARγ cell system was developed [Paper I + II]. Therefore, colonocytes were transfected with a mutant receptor to inhibit wild-type PPARγ action. In this mutant the highly conserved hydrophobic and charged residues (Leu468 and Glu471) in helix 12 of the LBD are mutated to alanine. As a consequence, this cell mutant retains PPARγ ligand and DNA binding, but exhibits markedly reduced transactivation due to impaired coactivator recruitment [165]. To assure the functional successful transfection of this *in vitro* system, cells were challenged with mesalazine or butyrate, and PPARγ activity was determined by the expression of cytokeratin 20 or transcription factor assay, respectively [Paper I + II]. Cytokeratin 20 has been described to be a specific target gene of PPARγ activation in CRC cells [37]. As expected in wild-type and empty-vector cells, the activity of the receptor was increased, while in the dominant-negative PPARγ cell lines, up-regulation of PPARγ activity in response to both substances was suppressed. In accordance with recent studies, augmentation of PPARγ protein expression in wild-type CRC cells caused by butyrate or mesalazine was shown [42, 95]. Moreover, increased transcriptional PPARγ activity in response to mesalazine has been demonstrated by Rousseaux *et al.* [95].

To reveal the influence of butyrate [Paper I] and mesalazine [Paper II] along the caspase cascade in this *in vitro* model, several characteristic signalling molecules were examined. Both substances led to a significant increase of activated caspase-3 and caspase-8 by decreasing the expression of the IAPs Survivin and Xiap simultaneously [Paper I + II] [166]. Survivin and Xiap have been well-characterized in mammalians and are known to inhibit caspases of the death receptor and of the mitochondrial pathway [161] (see 2.5.1). However, butyrate increased the cleavage of caspase-9 [Paper I], while activation of the enzyme was not affected by mesalazine [Paper II]. In addition, exaggerated expression of cleaved PARP in response to mesalazine was observed [Paper II]. Cleavage of PARP facilitates cellular disassembly and apoptosis, and cleavage products are commonly used markers for activation of the effector caspase-3 [167, 168]. Regarding the interference of mesalazine with the caspase cascade [Paper II], the findings of the experiments are in line with previous studies demonstrating increased cleavage of PARP and caspase-3 activity after mesalazine treatment in HT-29 and colo205 cells, respectively [169, 170]. The increase of levels in cleaved caspase-8 by mesalazine in the present study indicates that the death receptor pathway plays part in caspase-3 activation. In addition, both caspase-dependent and caspase-independent mechanisms have been reported for mesalazine's apoptotic processes,

demonstrating that the drug activates multiple cell death signalling pathways, *e.g.* caspase-9, Bcl-2, intestinal sphingomyelinase and the induction of intracellular peroxides [94, 169]. The initiated mechanism of cell death in response to the drug seems to be cell type specific. Accordingly, a similar down-regulation of IAPs as obtained for mesalazine in this study, has been demonstrated for the NSAID sulindac and also for PPARγ ligands *e.g.* troglitazone and pioglitazone in colorectal carcinoma cells [171-173]. Moreover, increased activation of caspase-3 by butyrate has also been demonstrated in a variety of malignant cancer cell lines including CRC cells [144, 145]. Up-regulation of caspase-8 and -9 activity in response to the drug [Paper I] indicates that both, the death receptor, and the mitochondrial trail share in caspase-3 activation. This finding is confirmed by previous studies [144, 174]. A comparable down-regulation of Survivin and Xiap by butyrate for this *in vitro* setting has recently been shown in human glioma and neuroblastoma cells [175, 176].

To establish the involvement of MEK-ERK signalling in butyrate-mediated induction of caspase-3 activity and PPARγ expression, specific inhibitors against p38 MAPK and ERK1/2 were applied [Paper I] [*Figure 23*]. p38 MAPK belongs to a group of protein serine/threonine kinases which has been demonstrated to play an essential role in the regulation of several cellular processes including controlling inflammation, growth, apoptosis and differentiation [177-179]. p38 MAPK can be activated in response to inflammatory cytokines, but also by hormones, ligands for GPCR, cell stress and heat shock [178]. ERK1/2 signalling has been implicated as a key regulator of cell survival and proliferation [179]. ERKs are mainly activated by growth factors, cytokines, virus infection, transforming agents, and also by activators of GPCR [178]. The data of this study demonstrate that inhibition of p38 MAPK counteracts both the induction of caspase-3 activity and the up-regulation of PPARγ caused by butyrate [Paper I]. In contrast, blocking of ERK1/2 did not affect both processes [Paper I]. Moreover, butyrate increased the expression of phospho-p38 MAPK, while ERK1/2 activity was not affected [Paper I]. In addition, stimulation of cells with the direct p38 MAPK stimulator arsenite resulted in an augmentation of PPARγ protein expression [Paper I], demonstrating that p38 MAPK plays a key role in PPARγ signalling. Nevertheless, butyrate-mediated apoptotic effects were not completely reversed by the p38 MAPK inhibitor SB203580 [Paper I], indicating that transduction pathways other than p38 MAPK take part in the activation of caspase-3 by butyrate. Indeed, expression of bak, a member of the bcl2-gene family which has been shown to be involved in the regulation of

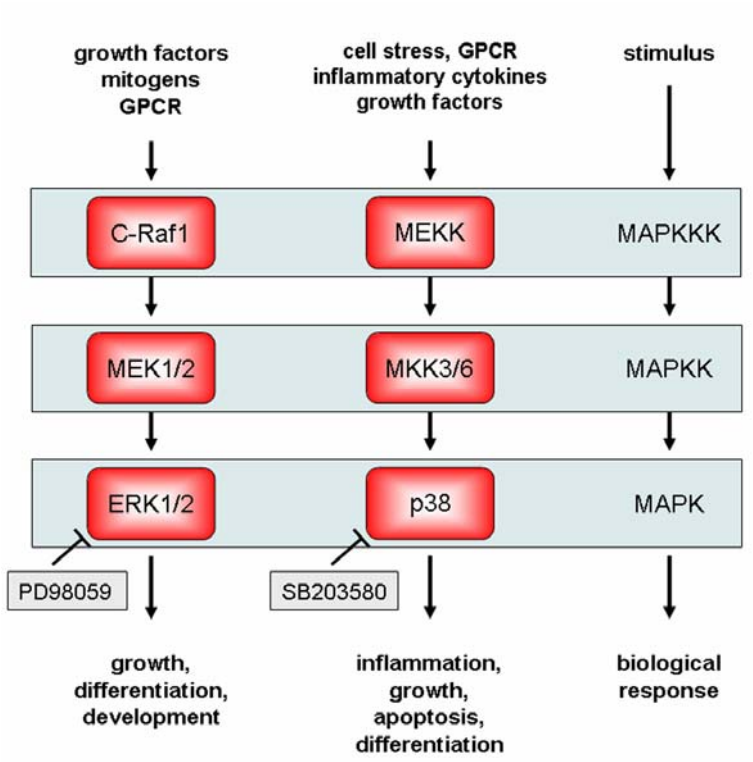

Figure 23: **MAPK phosphorylay system.** *Abbreviation:* GPCR, G-protein-coupled receptor. MAPKs are regulated by an up-stream relay of three protein kinases, *i.e.* MAPKKK – MAPKK – MAPK, which phosphorylate and subsequently activate another. See text for further explanations.

apoptotic signalling, was found to be significantly increased in butyrate-stimulated Caco-2 cells [180]. Bak was also shown to be a potent activator of the caspase cascade [181]. However, since butyrate is known to affect the expression of several genes potentially involved in the regulation of programmed cell death [180], other targets such as cJun N-terminal kinase (JNK) [182, 183] and PKC-δ [184] may also be involved in butyrate-induced stimulation of caspase-3 and in the regulation of PPARγ expression in Caco-2. In addition, butyrate has been shown to activate p38 MAPK in several cancer cell lines including Caco-2 [155, 182, 184]. Moreover, activation of p38 MAPK is an important molecular signalling event in butyrate-mediated activation of the caspase cascade [182, 184]. Summarising experiments hitherto, a signal transduction pathway leading to increased cell death via p38 MAPK - PPARγ - caspase-3 in response to butyrate was unveiled [Paper I], which is depicted in *Figure 24*. The following investigations in dominant-negative PPARγ cells in this study provide further evidence for this proposed model.

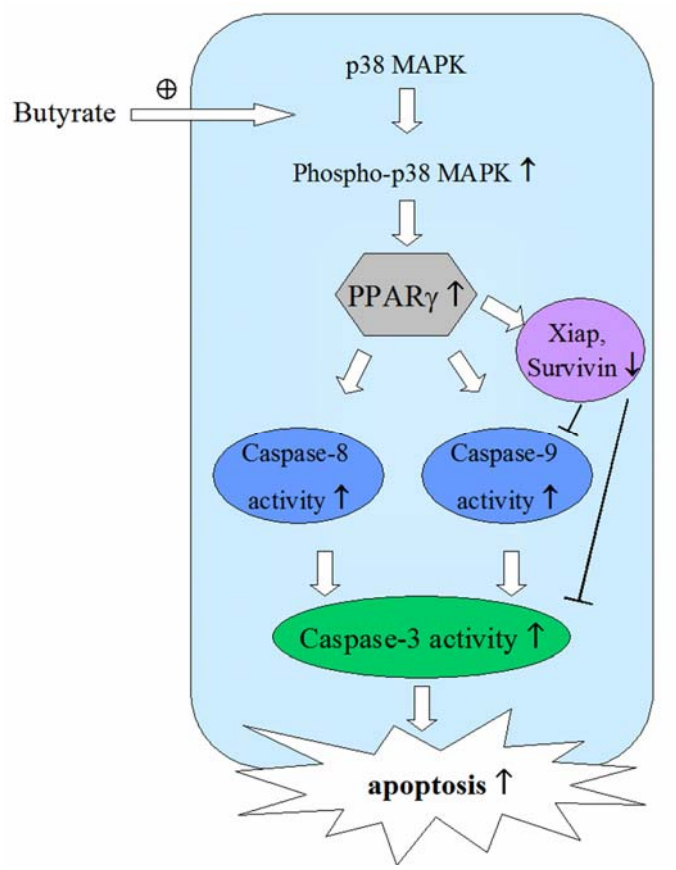

Figure 24: **Model of butyrate-induced cell death.** Butyrate activates p38 MAPK, which in turn up regulates PPAR*γ* expression and receptor activity. PPAR*γ* decreases the expression of Survivin and Xiap and activates caspase-8 and -9 leading to increased caspase-3 activity, eventually resulting in cell death.

Challenge of CRC cells with mesalazine resulted in a reduction of cell growth and proliferation and in the accumulation of cells in the G0/G1-phase of the cell cycle [Paper II]. Several *in vivo* and *in vitro* studies have pointed out the anti-proliferative features of mesalazine in colon carcinoma cell lines [163, 169, 170, 185]. A number of NSAIDs like indomethacin and sulindac, but also the PPARγ agonists rosiglitazone and pioglitazone have been demonstrated to induce a G1 arrest in CRC cell [154, 186-188]. These observations differ from further studies, illustrating a S- or G2-Phase arrest in response to mesalazine, whereas the discrepancy compared to other NSAIDs was not explained [170, 185].

To unravel further mechanisms contributing to mesalazine's growth-inhibitory abilities in colonocytes, expressions of the oncoprotein *c-myc* and of the tumor suppressor gene PTEN were determined [Paper II]. *c-myc* is overexpressed in nearly 70% of CRC. Moreover, dysplasia of colonocytes in UC is also associated with increased expression of the oncoprotein [55, 157]. *c-myc* is known to modulate a broad range of biological activities

including cellular proliferation and cell growth [55, 157]. Deregulated *c-myc* has been shown to increase apoptosis, genomic instability and to block differentiation [55, 157]. In colonocytes including HT-29, it was recently shown that mesalazine decreased the expression of *c-myc* [157]. These data are in accordance with the findings in the present study [Paper II], reflecting the participation of the oncoprotein in the growth-inhibitory effects caused by the drug. On the other hand, PTEN is a tumor suppressor gene involved in the regulation of cell survival signalling through the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) pathway [189]. PI3K/Akt signalling has been shown to be required for an extremely diverse array of cellular activities mainly involved in cell growth, proliferation, apoptosis and survival mechanisms [189, 190]. Activated Akt protects cell from apoptotic death by inactivating compounds of the cell-death machinery such as pro-caspases [189]. PTEN exercises its role as a tumor suppressor by antagonising the PI3K/Akt pathway [189]. Stimulation of HT-29 wild-type cells with mesalazine in this *in vitro* setting increased the expression of PTEN [Paper II]. Augmented expression of PTEN in CRC cells, *e.g.* by NSAIDs, has been demonstrated to decrease cell proliferation through cell-cycle arrest in the G0/G1 phase and to activate the caspase cascade [159, 190, 191], which is in line with the findings obtained in this study.

In the dominant-negative PPARγ cell system, mesalazine- and butyrate-mediated effects of all investigated apoptotic markers of the caspase cascade were annihilated [Paper I + II]. Moreover, the anti-proliferative effects caused by mesalazine were partially reduced and the up-regulation of PTEN in response of the drug was reversed [Paper II]. Altogether these results suggest the participation of PPARγ in mesalazine- [Paper II] and butyrate- [Paper I] mediated anti-carcionogenic effects. In contrast, a PPARγ-independent decrease of *c-myc* in response to mesalazine was obtained [Paper II]. Mesalazine's anti-carcinogenic effects obtained in this study are summarized in *Figure 25*. With regard to the interaction of cell growth and PPARγ, in several studies it could be demonstrated that agonists for the receptor like 15-PGJ_2, rosiglitazone and troglitazone exhibit inhibitory effects on the proliferation of CRC cell lines [154, 172, 188]. This growth inhibitory effect was partially reversed by use of the PPARγ inhibitor GW9662 [172, 188], supporting the findings for the partial reduction of cellular proliferation caused by mesalazine in the dominant-negative PPARγ cell system in this study [Paper II]. However, incomplete inhibition of cell proliferation indicates that other mechanisms besides PPARγ must be involved in this regulation [172]. Recently it was demonstrated that mesalazine also interferes with the colonic cell growth by disrupting epidermal growth factor signalling via enhanced SH-phosphatase-2 [192]. Moreover, this study demonstrates a PPARγ-independent down-regulation of the oncprotein *c-myc*, known to

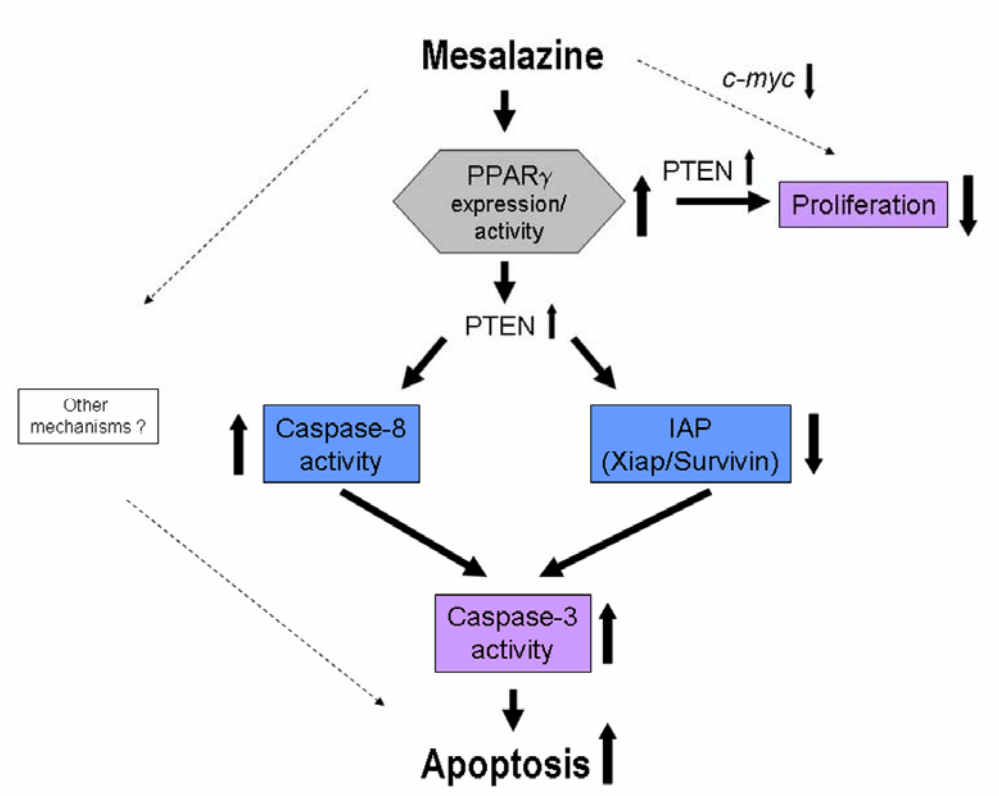

Figure 25: **Possible molecular mechanism of mesalazine-induced apoptosis and inhibition of cell proliferation in colorectal carcinoma cells.** See text for further explanations.

modulate cell growth [Paper II]. This finding is supported by previous studies. Although PPARγ agonists have been demonstrated to inhibit the expression of the oncoprotein [55, 193], the effects seem to be PPARγ-independent because no PPRE sequences have been found in its promoter [193]. Thus, in this study, *c-myc* seems to contribute to the modulation of the PPARγ-independent growth-inhibitory effects caused by mesalazine.

Several observations propose an interaction between PTEN and PPARγ. Except for the ability of PPARγ agonists like rosiglitazone to increase the expression of PTEN, corroborated by inhibitor and antisense experiments, PPARγ has also been demonstrated to bind two PPREs in the genomic sequence upstream of PTEN [194-196], which is in accordance with the present investigations. The PPARγ-dependent increase of PTEN caused by mesalazine in these experiments does not only indicate that the tumor suppressor gene contributes to the growth-inhibitory activities of the drug, but also may trigger its pro-apoptotic actions.

Increased caspase-3 activity resulting in apoptosis has been shown by several PPARγ ligands like 15-PGJ_2 and troglitazone in a variety of cell types including CRC cells [56, 58, 154]. With regard to the action by which PPARγ controls caspase signalling, the molecular

mechanisms are not fully enlightened. It may be speculated that PPARγ activates the caspase pathway via modulation of the IAPs *e.g.* via a spermidine/spermidine N^1-acetyltransferase (SSAT) dependent mechanism [197, 198]. Indeed, several NSAIDs have been demonstrated to induce the transcription of SSAT via activation of PPARγ and to inhibit the expression of IAPs [173, 198]. Other factors known to modulate the expression of the IAPs such as JNK, epithelial growth factor (EGF) and STAT-3 may also be involved [166, 199-201]. Only recently, a direct interaction of PPARγ with the death receptor signalling pathway was disclosed, demonstrating the ability of PPARγ ligands to down-regulate FLIP, an apoptosis-suppressing protein that blocks early events in death receptor signalling pathway [202]. However, decreased expressions of Survivin and Xiap by the PPARγ ligands troglitazone and pioglitazone were unveiled as important apoptotic-inducing mechanisms [172, 203].

Conclusions: These data further elucidated the molecular events involved in butyrate- [Paper I] and mesalazine- [Paper II] mediated apoptosis in CRC cells, clearly demonstrating the key role of PPARγ in this sequential process. Upon activation by p38 MAPK in response to butyrate, PPARγ triggers the regulation of several pro- and anti-apoptotic markers finally followed by activation of caspase-3 eventually resulting in cell death [Paper I, *Figure 24*]. In contrast, mesalazine-mediated apoptosis appears to be merely modulated by the caspase-8 signalling pathway and the IAPs [Paper II, *Figure 25*]. Moreover, the data of this thesis provide evidence that mesalazine acts via PPARγ-dependent and -independent mechanisms to induce growth-inhibitory effects and apoptosis in colonocytes which may in part be triggered by the modulation of the oncoprotein *c-myc* and the tumor suppressor gene PTEN, respectively [Paper II, *Figure 25*].

6.2 Crucial role of VDR in HDAC-mediated induction of the anti-microbial peptides HBD-2 and LL-37

The innate immune system modulates a number of biological processes so as to properly defend against pathogens [204]. The APs LL-37 and HBD-2 are secreted by a variety of epithelial cells, belonging to the innate immune system and are potential factors contributing to infection control [124]. In the gastrointestinal tract, these peptides regulate the composition and number of colonizing microbes and protect the host from food-borne and water-borne

pathogenesis [123]. In many cases they are effective against pathogens which are resistant to conventional antibiotics [205]. Hence, APs have been proposed as natural templates for the design of novel antimicrobial drugs [205].

Aberrances in the expression of AP expression have not only been observed in IBD but also during infections caused by pathogens including several *Helicobacter* strains, *Shigella*, *Salmonella* und *Campylobacter jejuni* [124, 206, 207]. The site-specific disruption of the critical balance between epithelial APs and luminal bacteria might explain adherent bacteria to the mucosa, as well as different disease locations, such as found in colonic CD compared with UC [123]. In the pathology of UC, epithelial expression of HBD-2 and LL-37 is enhanced [123]. When stratified according to disease activity, the induction is clearly restricted to the inflamed mucosa [208]. However, the difference in the expression of HBD-2 between non-inflamed mucosa in UC and healthy controls is not significant [208]. In contrast, a slight but significant induction of LL-37 in non-inflamed UC was observed [123]. Regarding colonic CD, the state of innate immune affairs is dramatically different [123]. There is a broad lack or at least attenuation of HBD-2 and LL-37 response during severe inflammation [123]. A model of antimicrobial defence of APs in the normal mucosa and in colonic CD is demonstrated in *Figure 26* [123]. The expression of HBD-2 and LL-37 shows a strong degree of correlation, especially in case of inflammation [123]. This defective regulation of HBD-2 could *inter alia* be explained by a genetic predisposition through diminished gene copy numbers in the HBD-2 gene cluster. In contrast, the gene copy number of HBD-2 is normal in UC, allowing full transcriptional response [209]. Elucidating the underlying molecular mechanisms involved in the complex regulation and biology of these APs could open new therapeutic avenues [210].

Cross-talk between nuclear hormone receptors, such as VDR and PPARγ, and the innate immune system may influence multiple biological functions during an immune response [204]. Moreover, the function of nuclear hormone receptors in innate immune responses and inflammation of the gastrointestinal tract has been extensively established [81, 204, 211]. The dietary SCFA butyrate is able to modulate the activity and expression of the nuclear hormone receptors PPARγ and VDR and can also interact with the innate immune system by regulating the expression of APs [70, 100, 104, 212, 213]. In several *in vivo* studies of IBD and experimental *Shigelloses* butyrate has been demonstrated to decrease inflammation [212, 214], probably caused by an induction of APs. The aim of this study was therefore to investigate a possible modulation of AP expression in response to the dietary HDAC inhibitors butyrate and SFN by the nuclear hormone receptors PPARγ and VDR.

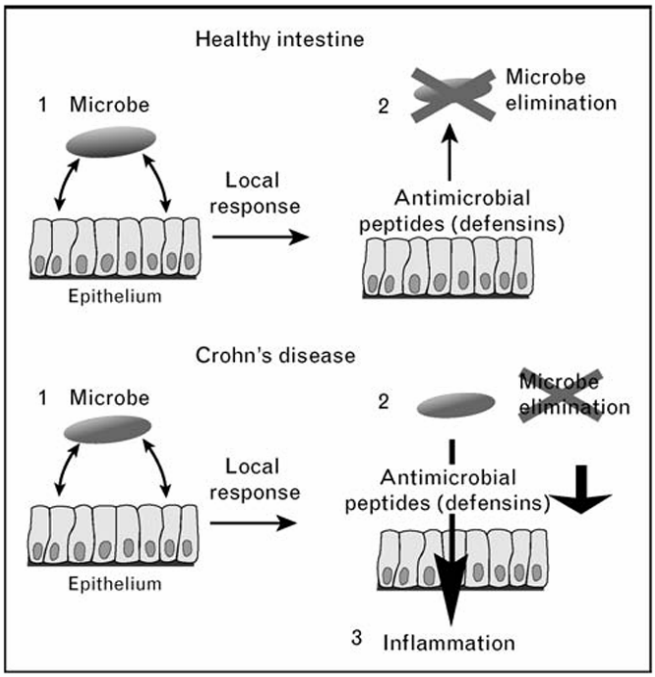

Figure 26: **Proposed model for the role of APs in IBD, especially colonic CD.** In the healthy gut, the microbes can not invade the mucosa because of an effective antimicrobial barrier. In CD of the colon and small intestine (lower panel), different components of this antimicrobial barrier are disturbed and bacteria can invade the mucosa. According to this hypothesis, a bacterial invasion due to AP deficiency is the primary cause for a secondary inflammation. [adapted from [123]]

Stimulation of several CRC cell lines with butyrate in this *in vitro* study led to a time- and dose-dependent up-regulation of HBD-2 and LL-37 mRNA expression, reflecting a common mechanism in CRC cells [Paper III + IV]. The results are in line with the observations found in a variety of epithelial cell types including colon epithelial cells, gastrointestinal cells and lung cells [100, 101, 104, 215]. Moreover, the data provide evidence that the CRC cell lines used in this study express basal levels of LL-37 and HBD-2 mRNA which is in accordance with the findings in HT-29 and Caco-2 cells [100, 216], but differs from the observations in the lung epithelial cell line EBD-1 for LL-37 mRNA expression and from the human gastric epithelial cell line AGS for HBD-2 [217]. Moreover, protein expression of both APs in response to the HDAC inhibitors was increased as detected by immunostaining [Paper III + IV] and ELISA [Paper IV], respectively. In conformity with previous studies [100, 104], both peptides were expressed in the cytoplasm of the cells. Moreover, the induction of HBD-2 on mRNA and protein level in response to SFN has been demonstrated in this thesis for the first time [Paper IV].

To elucidate the putative participation of PPARγ and VDR in butyrate and SFN-mediated induction of LL-37 and HBD-2 mRNA, experiments were performed in CRC cells transfected with a dominant-negative PPARγ mutant to inhibit wild-type PPARγ action or by application of the specific VDR antagonist ZK191732, respectively [Paper III + IV]. Challenge of cells with ZK191732 reversed the expression of the APs in response to both HDAC inhibitors [Paper III + IV]. In contrast, in the dominant-negative PPARγ cell lines, the induction of the APs was not affected [Paper III + IV]. The findings for the role of VDR in butyrate-mediated induction of the APs are supported by the presence of a consensus VDRE in the promoters of the human HBD-2 and LL-37 genes [218]. Moreover, induction of LL-37 by VD_3 has been observed in cell lines from various tissue types, *e.g.* several carcinoma cell-types of the tongue, lung cancer, CRC, hematopoetic cell lines, primary tissues, bone marrow cells and in keratinocytes [104, 219, 220]. Likewise, increased HBD-2 mRNA in response to VD_3 has been shown in human cell lines and primary cultures [218]. In addition, the up-regulation of VDR protein expression after challenge of cells with butyrate [69, 70] and SFN [Paper IV] refers to the involvement of the receptor in the signalling pathway of APs. On the other hand, the PPARγ agonist troglitazone was unable to induce the expression of LL-37 in U937 cells [219], indicating that PPARγ doesn't contribute to the signal transduction pathway of the AP.

The MEK-ERK signalling pathway controls a huge variety of physiological processes [178]. In general, these enzymes are regulated by an up-stream relay of three protein kinases, *i.e.* MAPKKK – MAPKK – MAPK, which phosphorylate and subsequently activate another [178] [see 6.1; *Figure 23*]. Since p38 MAPK and ERK1/2 have already been indicated in HDAC-mediated functions on cellular physiology [155, 221], their role in butyrate- and SFN-mediated induction of LL-37 and HBD-2 was determined [Paper III + IV]. Pre-treatment with two MEK-ERK inhibitors diminished the induction of LL-37 mRNA expression caused by butyrate with strongest effects observed for the p38 MAPK inhibitor SB203580 [Paper III]. Conversely, inhibition of the ERK1/2 pathway by PD98059 in this study prevented the induction of HBD-2 mRNA caused by SFN and butyrate, while blocking of the p38 trail had no impact on this regulatory process [Paper IV]. The participation of MEK-ERK signalling in butyrate-mediated up-regulation of LL-37 [Paper III] found in these experiments is in accordance with data in human lung epithelial cells, demonstrating reduced LL-37 mRNA after pre-incubation with inhibitors for the ERK1/2, JNK and p38 MAPK signalling pathway [215]. Moreover, inhibition of p38 MAPK in human keratinocytes led to decreased LL-37 gene expression, whereas JNK and ERK1/2 pathways were not involved [222]. Conflicting

results have been obtained in SW620 cells. In this cell line, inhibition of the ERK1/2 pathway suppressed butyrate-mediated LL-37 mRNA expression, while blocking the p38 MAPK trail boosted the expression of the gene [101]. Regarding the influence of the MEK-ERK signalling pathway on the induction of HBD-2 caused by SFN [Paper IV], the data from this study are in line with middle ear epithelial cell transcriptional activation of the HBD-2 gene caused by IL-1α which is mediated through an Raf-MEK1/2-ERK1/2 signalling pathway [223]. Different results were obtained in the lung epithelial cell line A549, in which IL-1β-induced up-regulation of HBD-2 was partly attenuated by inhibiting the p38 MAPK but not by the ERK1/2 signalling pathway [224]. Taken together, all these different observations indicate a cell specific and stimulus dependent kinase pathway leading to the induction of both APs. However, the importance of the MEK-ERK signalling pathway in this regulation was underscored by the present findings. The results in this study are further supported by the phosphorylation pattern of MEK-ERK signalling obtained after treatment with butyrate [Paper I] and SFN [Paper IV]. In this *in vitro* setting, butyrate was shown to increase the expression of phospho-p38 MAPK, while phosphorylation of ERK1/2 was not affected [Paper I]. Reversed effects were obtained after stimulation of cells with SFN [Paper IV]. Increased phosphorylation of p38 MAPK by butyrate has been demonstrated in several studies [155, 213]. Moreover, SFN was shown to increase phospho-ERK1/2 in various cell lines including Caco-2 cells, while the amount of ERK1/2 protein was not affected. SFN treatment had no impact on p38 MAPK activation [225, 226].

The transforming growth factor TGF-β1 is a multifunctional peptide that controls cellular proliferation, differentiation, migration, embryonic development, wound healing and other functions in diverse cell types [227, 228]. During active inflammation of IBD, TGF-β1 was found to be increased in the affected mucosa [227]. To further elucidate the participation of the peptide in butyrate-mediated induction of LL-37, cells were pre-treated with a specific inhibitor against the TGF-β1 receptor kinase. Stimulation with the inhibitor prior to butyrate treatment diminished the up-regulation of LL-37 mRNA [Paper III]. Previous investigations reporting the necessity of TGF-β1 in butyrate-induced Caco-2 cell differentiation [229] as well as the increase of LL-37 expression in spontaneously differentiating Caco-2 cells [100] are supporting these findings of this study. Moreover, in keratinocytes a TGF-β1-dependent induction of LL-37 mRNA in response to VD_3 has been demonstrated, also reflecting the requirement for VDR in the signalling pathway of the AP. This effect was counteracted by application of the VDR antagonist ZK159222 [230]. Furthermore, direct cross-talk between VDR and TGF-β1 has been reported [231].

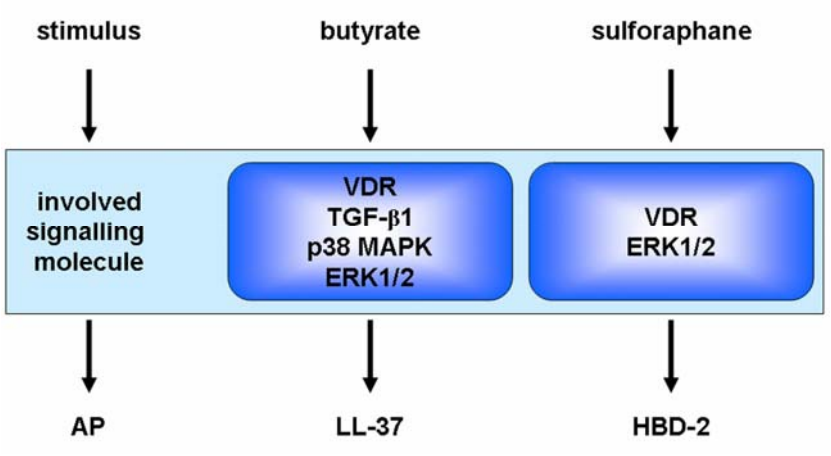

Figure 27: **Established signalling molecules in HDAC-mediated induction of APs.** See text for further explanations.

Conclusion: These findings provide evidence for the involvement of the nuclear hormone receptor VDR in butyrate and SFN-mediated induction of the APs LL-37 and HBD-2 in colonocytes [*Figure 27*]. Moreover, the participation of MEK-ERK signalling in the expression of both peptides was revealed. On the contrary, the participation of PPARγ in this regulatory process could be debarred.

6.3 Stimuli-dependent participation of PPARγ and VDR in butyrate-mediated suppression of cytokine-induced NFκB activation

Most NFκB target genes have been intensively studied for their participation in immune and inflammatory responses. However, the transcription factor also regulates cell proliferation, apoptosis and cell migration [142]. A causal connection between inflammation and cancer has been assumed for a long time. Nevertheless, the mechanistic link between inflammation and tumorigenesis is not well understood [142]. Since NFκB becomes activated in response to a variety of inflammatory stimuli and environmental stressors and since the constitutive activation of the transcription factor has been associated with cancer of several types, NFκB might be a missing link between the two processes [142, 232-234]. Moreover, NFκB mediates the production of many factors playing an active role in the pathology of IBD and in inflammation-driven tumor progression [234]. High levels of circulating and mucosal pro-inflammatory cytokines resulting from increased activity of NFκB have been reported in the

intestinal mucosa of patients with IBD, especially UC and CD [149, 150]. Hence, targeting NFκB may be a possibility to suppress inflammation and also the development of cancer in IBD. Current studies have demonstrated that the SCFA butyrate suppresses NFκB signalling in resting and cytokine stimulated epithelial cells [103, 152, 153]. Moreover, it was shown that the nuclear hormone receptors VDR and PPARγ can inhibit NFκB activity, representing an important link between the endocrine and immune systems [87, 235]. However, activation and increased expression of PPARγ and VDR in response to butyrate has been demonstrated recently [42, 69, 70, 213]. Based on these observations, the involvement of both receptors in the modulation of butyrate-mediated suppression of NFκB signalling as well as in basal NFκB activation in the CRC cell line HT-29 was scrutinized [Paper V].

There are two main key receptor families that can activate NFκB, Toll-Like Receptors (TLRs) and TNFRs [*Figure 28*]. TLRs recognize LPS, a cell wall component of *Gram-negative* bacteria. TNFRs are triggered by their ligand TNFα [236-238]. Each of the different groups of receptors activate NFκB utilizing a specific signalling cascade [238]. Thus, the differences assigned to NFκB functions can be ascribed to specific cell surface receptors recognizing a variety of unrelated ligands [238]. Stimulation of HT-29 cells with TNFα or LPS increased the expression of both p50 and p65 NFκB dimer DNA binding activities, while this activation was attenuated in response to butyrate [Paper V]. These findings are in line with numerous investigations in colonocytes, murine macrophages and adenocarconoma cells, indicating the efficacy of butyrate to attenuate increased NFκB signalling [103, 152, 153, 239]. Furthermore, the data in this study provide evidence that only the p50 but not p65 basal NFκB DNA binding activity was reduced in response to butyrate [Paper V]. The study of Inan *et al.* demonstrated by using supershift analysis that the constitutive complex of NFκB in HT-29 cells is the p50 dimer, although p65 NFκB activation was observed upon cytokine stimulation [103]. Exposure to butyrate lead to a selective reduction of p50 NFκB DNA binding activity as observed in the present experiments [103]. Cytosolic levels of IκBα investigated in this thesis were decreased after stimulation with butyrate, TNFα but not in response to LPS [Paper V]. However, conflicting results have been obtained in previous studies. In regard to stimulation with TNFα, Inan *et al.* could not detect any change in the expression of IκBα after exposure of the cytokine and even not in the presence or absence of butyrate up to 1 h [103]. In further experiments, the same research group demonstrated reduced levels of IκBα in TNFα treated cells caused by butyrate, which is in accordance with the present experiments [103]. In contrast, Yin *et al.* established that IκBα was completely

degradated in response to TNFα after 15 min in HT-29 cells, whereas its degradation was dampened in butyrate-conditioned cells [153]. However, after 30 min the protective effect of butyrate on IκBα was completely reversed. The data obtained for exposure of cells to LPS in this study differ from previous studies. Stimulation of human intestinal microvascular endothelial cells with LPS decreased the expression of IκBα, while the degradation was not affected by pre-treatment with butyrate [107]. In contrast, in THP-1 cells a decrease in IκBα expression only 4 h after stimulation with LPS was obtained [108]. In presence of butyrate this effect was abolished. Altogether, these findings refer to a cell type, concentration and time point specific mechanism in the expression of IκBα. Nevertheless, it is unlikely that the prevention of IκBα degradation is the only mechanism influencing NFκB activity. Other mechanisms or other members of the IκB family may also be responsible for the inhibitory effects of butyrate on NFκB actions.

To elucidate a possible role for PPARγ and VDR in basal NFκB activity and after challenge of cells with butyrate, experiments were performed in HT-29 cells transfected with a dominant-negative PPARγ mutant to inhibit wild-type PPARγ action or by use of the specific PPARγ receptor antagonist GW9662 or the VDR antagonist ZK191732, respectively [Paper V]. Challenge of cells with ZK191732 or in combination with butyrate increased both p50 and p65 basal NFκB dimer activities, while reducing the expression of IκBα simultaneously. However, the PPARγ antagonist GW9662 did not affect butyrate-mediated actions on basal NFκB activity and IκBα expression. Basal NFκB activities were not influenced either. Comparable results for these observations were obtained in dominant-negative PPARγ mutant HT-29 vector cells. To further scrutinize any involvement of the receptors in butyrate-induced suppression of cytokine-mediated activation of NFκB, experiments were additionally performed in the presence of the VDR inhibitor ZK191732 or in the PPARγ mutant HT-29 cell line. Co-incubation of cells with the VDR antagonist ZK191732 prior to stimulation of cells with TNFα abolished the inhibitory effect of butyrate on NFκB activity, while in this *in vitro* model inhibition of PPARγ signalling did not affect the suppressive activity of butyrate. In contrast, the participation of PPARγ but not of VDR could be observed after stimulation of cells with LPS. Thus, these findings refer to the involvement of different signalling pathways responsible for the reduction of inducible NFκB activity caused by butyrate. Butyrate-mediated suppression of inducible NFκB activity in response to LPS seems to be modulated via PPARγ and therefore via TLR signalling.

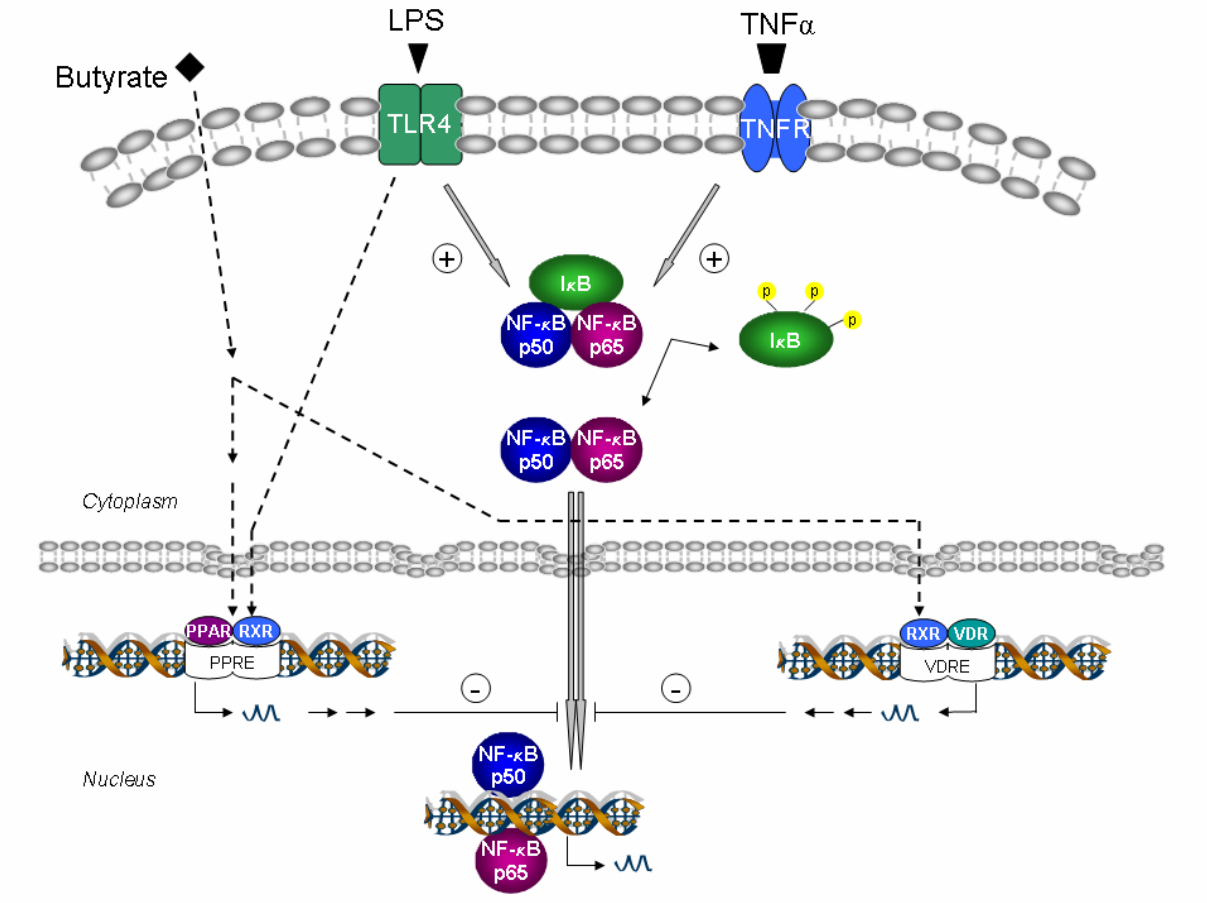

Figure 28: **Proposed model of butyrate-mediated inhibition of inducible NFκB activation dependent on the stimulated signalling pathway caused by TNFα or LPS.** *Abbreviation:* NFκB, nuclear factor κB; PPAR, peroxisome proliferators-activated receptor; PPRE, peroxisome proliferator response element; RXR, retinoid X receptor; TLR4, toll-like receptor 4; TNFR, tumor-necrosis-factor receptor; VDR, vitamin D receptor, VDRE, vitamin D response element. See text for further explanations.

Conversely, VDR affects the TNFR signalling pathway leading to increased NFκB activation. A proposed model of this interaction is depicted in *Figure 28*.

There is little consensus about the precise molecular mechanisms affected by nuclear hormone receptors to inhibit NFκB activity. Different mechanisms for this cross-talk, influencing the activity of the transcription factor have been indicated [235]. Proposed mechanisms can be divided generally into two categories, nuclear and cytoplasmatic models. Modes of nuclear models include *e.g.* physical interaction between NFκB and nuclear hormone receptors, interference with the basal transcription machinery and cofactor-based activation. Cytoplasmatic interaction is mainly induced by cytoplasmatic sequestration of NFκB and the interference of the transcription factor with other signal transduction pathways [235]. However, the dynamic nature of the protein shuttling events make it likely that such

strict boundaries do not exist in the real setting. Multi-targeting mechanisms have rather been considered [235]. Several mechanisms have been reported by which PPARγ can inhibit the actions of NFκB activity. The transcription factor can not only physically interact with the NFκB subunits p65, subunit p50, or both, but can also inhibit degradation of the inhibitory protein IκB [240, 241]. In addition, up-regulation of IκB levels and a frequent interaction of PPARγ with cyclic AMP response element binding protein (CREB), a coactivator interacting with p65 have been described [240, 241]. A precise interaction between NFκB and the VDR receptor has not been elucidated yet. However, integration of p65 in the VDR transcription complex has been indicated [242].

Revealing the participation of PPARγ in butyrate-mediated inhibition of TLR-mediated NFκB signalling, the following observations found in literature support the data of the present study. In a rat model of acute UC caused by TNBS, the PPARγ agonist rosiglitazone has been shown to modulate inflammation by reducing increased levels of NFκB [243]. In addition, the ability of LPS to up-regulate PPARγ levels has been demonstrated *in vitro* and *in vivo* [34, 40, 244] which is in accordance with the data of this thesis. Moreover, the involvement of TLR4 in PPARγ signalling caused by LPS has been shown by a weak expression of PPARγ in the colon of mice with non-functional TLR4 due to a naturally occurring mutation within the TLR4 gene [34, 40]. These data were further supported by the transfection of Caco-2 cells with the constitutive active form of TLR4 leading to increased induction of PPARγ expression [40]. In general, the up-regulation of PPARγ has been ultimately considered as a negative feedback resulting in attenuation of the pro-infammatory NFκB signalling pathway induced by LPS [244, 245].

The discovery for the participation of VDR in basal and in butyrate-mediated inhibition of TNFα-stimulated NFκB activity by the present study, is underpinned by several *in vitro* and *in vivo* studies, demonstrating cross-talk between VDR and NFκB signalling. In a variety of cell types it was shown that the active metabolite of vitamin D, VD_3 counteracts NFκB activation [246-249]. This suppressive effect of VD_3 on NFκB activation therefore appears to be a general effect that occurs in various cell types and immune processes [249]. In peripheral blood mononuclear cells of healthy and patients with CD, the vitamin D analogue TX 527 not only decreased basal and TNFα-induced activation of NFκB, but also increased IκBα protein levels, even in association with TNFα [87]. In a mouse model lacking the VDR receptor, a reduction of IκBα was observed [88]. The degradation of IκBα induced by TNFα was inhibited by VD_3 in normal but not in VDR lacking cells [88]. In human keratinocytes

VD_3 is able to reduce NFκB DNA binding activity by increasing IκBα protein levels [247]. Moreover, in murine macrophages, VD_3 up-regulates IκBα levels by increasing mRNA stability and decreasing IκBα phosphorylation. The increase in IκBα levels reduces nuclear translocation of NFκB and thereby downgrades its activity [249]. Finally, in mouse embryonic fibroblasts lacking the VDR receptor, basal IκBα protein levels were markedly decreased probably caused by the lack of VDR-mediated stabilization of IκBα by VD_3. However, degradation of IκBα and its phosphorylation were not altered. Moreover, these mutants appear to be more pro-inflammatory due to the increased intrinsic NFκB activity [250].

Conclusion: The data presented in this paper provided evidence for the participation of the nuclear hormone receptors PPARγ and VDR in butyrate-mediated inhibition of inducible NFκB activation dependent on stimulated signalling pathway caused by TNFα or LPS [*Figure 28*]. In addition, the inhibitory role of VDR in basal NFκB signalling was demonstrated.

7 Summary - Achievement of this work and outlook

Disruption of the complex gastrointestinal ecosystem between the resident microflora and the colonic epithelial cells has been associated with increased inflammation and altered cell growth. Possible endpoints of this disturbance are IBD and CRC. The data presented in this thesis, entitled *"PPARγ as molecular target of epithelial functions in the gastrointestinal tract"*, shed further light on the underlying molecular mechanisms contributing to the well ordered homeostasis of this gastrointestinal ecosystem. Except for elucidating important roles for mesalazine and the dietary HDAC inhibitors butyrate and SFN in a) the modulation of cellular growth, b) the induction of APs, and c) the control of NFκB signalling in CRC cells, the involvement of the nuclear hormone receptors PPARγ und VDR as "gatekeepers" in these intricate regulatory mechanisms were established. Future work will be engaged in analysing whether these *in vitro* findings are also physiologically relevant in regard to prevention and therapy of gastrointestinal diseases.

Within the scope of this work, in Paper I and II it could be demonstrated that butyrate and mesalazine act via PPARγ to induce their anti-proliferative and pro-apoptotic actions along the caspase signalling pathway. Activation of the intrinsic and extrinsic signalling trail and the down-regulation of anti-apoptotic proteins are responsible for increased caspase-3 activity caused by butyrate. In contrast, mesalazine merely activates this cascade via the extrinsic trail and the IAPs. Moreover, a signal transduction pathway leading to increased cell death via p38 MAPK - PPARγ - caspase-3 in response to butyrate was unveiled. In addition, there is strong evidence that mesalazine-mediated pro-apoptotic and growth-inhibitory abilities are controlled by PPARγ-dependent and -independent mechanisms which appear to be triggered at least in part by the modulation of the tumor suppressor gene PTEN and the oncoprotein *c-myc*, respectively.

In Paper III and IV the induction of the APs HBD-2 and LL-37 in response to the dietary HDAC inhibitors butyrate and SFN was pinpointed. Regarding the molecular events of this regulation, the data presented in this thesis provide strong evidence for the involvement of VDR in HBD-2- and LL-37-induced gene expression, while the participation of PPARγ was excluded. Moreover, the role for p38 MAPK and TGF-β1 in the up-regulation of LL-37 caused by butyrate was established. In contrast, SFN-mediated induction of HBD-2 is modulated via ERK1/2 signalling.

The findings in Paper V clearly refer to the involvement of the nuclear hormone receptors PPARγ and VDR in butyrate-mediated suppression of inducible NFκB activation dependent on the stimulated signalling pathway caused by LPS or TNFα. Moreover, an inhibitory role for VDR in the regulation of basal NFκB activation was revealed. On the contrary, a modulating role for PPARγ on basal NFκB could be debarred.

Altogether the data presented in this thesis not only provide new insights in the understanding of the fundamental gastrointestinal physiology regulated by nuclear hormone receptors, but also may offer opportunities for the development of potential drug targets and therapeutic strategies in the treatment of IBD and CRC.

8 Zusammenfassung

Schlüsselworte: Peroxisome Proliferator-Activated Rezeptor (PPARγ), Vitamin D Rezeptor (VDR), nukleäre Hormonrezeptoren, kolorektales Karzinom, gastrointestinale Zellregulierung, Butyrat, Mesalazin, Sulforaphan, Caspase-Kaskade, antimikrobielle Peptide, Cathelicidin, Beta-Defensin-2, NFκB-Signaltransduktionsweg

Die Beteiligung der nukleären Hormonrezeptoren PPARγ und VDR an der intestinalen Zellregulierung ist in zahlreichen *in vivo* und *in vitro* Studien hinreichend belegt. Ferner konnte der Einfluss der beiden Rezeptoren an der Pathogenese des kolorektalen Karzinoms sowie der chronisch entzündlichen Darmerkrankungen (CED), Morbus Crohn und Colitis ulcerosa, aufgezeigt werden. In einer Reihe von Zellkultur-, Tier- und Humanstudien wurde außerdem eine Verminderung des Tumorwachstums sowie eine Remission von CED durch Modulierung der PPARγ- und VDR-Rezeptoraktivitäten beobachtet.

Die Mukosa des Gastrointestinaltraktes befindet sich in einem komplexen dynamischen Gleichgewicht. Schon kleinste Störungen dieses epithelialen Systems können zu einer veränderten Expression von antimikrobiell wirkenden Peptiden sowie zur Aktivierung des Transkriptionsfaktors NFκB verbunden mit der Freisetzung zahlreicher Entzündungsparameter führen. Diese Faktoren begünstigen die Entstehung gastrointestinaler Erkrankungen. In der gesunden Darmschleimhaut stehen außerdem proliferierende Zellen und deren geregelter Zelltod (Apoptose) in einem Fließgleichgewicht. Der Zelltod wird dabei klassischerweise durch die Caspase-Kaskade reguliert. Bei der Tumorentwicklung geht diese Kontrolle durch Hemmung der Apoptose verloren; hieraus resultiert ein unkontrolliertes Zellwachstum. Die nukleären Hormonrezeptoren PPARγ und VDR tragen vermutlich zur Regulation all der hier aufgeführten Prozesse bei.

Mesalazin sowie die natürlich vorkommenden Histondeacetylase (HDAC)-Inhibitoren Butyrat und Sulforaphan (SFN) haben in der letzten Zeit aufgrund ihrer chemopräventiven und anti-inflammatorischen Wirkungen in Hinblick auf die Prävention des kolorektalen Karzinoms und von CED zunehmend an Bedeutung gewonnen. Im Rahmen dieser Arbeit sollte eine potentielle Wirkung dieser Substanzen auf die oben aufgeführten Signaltransduktionswege und Moleküle sowie eine mögliche Beteiligung der nukleären Rezeptoren aufgezeigt werden. Dazu wurde die Arbeit in 3 Teile gegliedert:

Zahlreiche Studien belegen dass sowohl Butyrat als auch Mesalazin pro-apoptotische und anti-proliferative Eigenschaften aufweisen. Diese werden vorwiegend durch die Aktivierung der Caspase-Kaskade mittels gesteigerter Caspase-3-Aktivität und Zellzyklushemmung eingeleitet. Außerdem ist bekannt, dass beide Substanzen in die PPARγ-Signaltransduktion involviert sind. Im ersten Teil dieser Arbeit wurde der Einfluss von Butyrat und Mesalazin auf die Caspase-Kaskade sowie weitere Regulationsvorgänge in diesem Zusammenhang untersucht [Paper I, II]. Dazu wurden die Aktivitäten bzw. die Proteinexpressionen von charakteristischen Signalmolekülen dieser Kaskade in kolorektalen Karzinomzellen mittels Western-Blot-Analyse und ELISA ermittelt. Sowohl Mesalazin [Paper II] als auch Butyrat [Paper I] steigerten die Aktivität der Effektor-Caspase-3, was zu einem apoptotischen Zelltod über sekundäre Zielproteine führt. Darüber hinaus verursachte die Mesalazin-Behandlung eine gesteigerte Aktivität der Poly(ADP-Ribose)-Polymerase (PARP), einem indirekten Marker für aktive Caspase-3 [Paper II]. Um den Einfluss sowohl des extrinsischen als auch des intrinsischen Signalweges zu untersuchen, welche in einer verstärkten Caspase-3-Aktivität resultieren, wurde die Aktivierung der jeweiligen Schlüsselenzyme dieser Signalwege, Caspase-8 und -9, nach Behandlung der Zellen mit Butyrat [Paper I] bzw. Mesalazin [Paper II] bestimmt. Darüber hinaus wurde die Wirkung dieser Substanzen auf die inhibitorischen Apoptoseproteine (IAP) Survivin und Xiap ermittelt [Paper I, II]. Mesalazin [Paper II] und Butyrat [Paper I] führten koinzident zu einem Anstieg von aktivierter Caspase-8 bei gleichzeitiger Hemmung der beiden IAP. Die Caspase-9 Aktivität wurde hingegen lediglich durch Butyrat gesteigert [Paper I]. Mesalazin führte außerdem zu einer zeit- und dosisabhängigen Reduktion der Zellzahl und -proliferation, einem Zellzyklusarrest mit einer Akkumulation der Zellen in der G0/G1-Phase, einer Expressionsminderung von c-Myc, sowie in einer gesteigerten Proteinexpression des Tumor-Suppressor-Gens PTEN [Paper II]. Durch Co-Inkubation mit Butyrat wurden synergistische Effekte auf die durch Mesalazin-vermittelten anti-proliferativen Wirkungen sowie auf sämtliche apoptotischen Marker erzielt. Lediglich die Expression von Xiap blieb unverändert [zusätzliche Daten].

Um die Beteiligung des MEK-ERK-Signalweges an der durch Butyrat gesteigerten PPARγ-Expression sowie Caspase-3-Aktivität zu ermitteln, wurden Caco-2 Zellen mit der Kombination aus Butyrat und des p38-MAPK Inhibitors SB20350 bzw. des ERK1/2-Inhibitors PD98059 stimuliert [Paper I]. In Gegenwart des p38-MAPK-Inhibitors SB203580

blieben die durch Butyrat vermittelten Effekte nahezu vollständig aus, hingegen wurde durch Inhibierung des ERK1/2-Transduktionsweges keine antagonisierende Wirkung beobachtet. Butyrat steigerte außerdem die p38-MAPK-Aktivität, nicht jedoch die Aktivität von ERK1/2 [Paper I]. Die Behandlung der Zellen mit dem direkten p38-MAPK-Induktor Arsenit induzierte zudem zeitabhängig die PPARγ-Expression. Diese Beobachtungen lassen auf eine Beteiligung von p38-MAPK an der durch Butyrat induzieren PPARγ- und Caspase-3-Aktivität schließen [Paper I].

In weiteren Experimenten wurden sämtliche apoptotische Marker zudem in transfizierten kolorektalen Karzinomzellen mit unterdrückter PPARγ-Funktion untersucht, um eine potentielle Beteiligung von PPARγ an den durch Butyrat [Paper I] und Mesalazin [Paper II] vermittelten pro-apoptotischen und anti-proliferativen Wirkungen zu ermitteln. Zunächst wurde durch Bestimmung der PPARγ-Aktivität nach Butyrat- [Paper I + V] und Mesalazin-Behandlung [Paper II] mittels Detektion der Expression von Cytokeratin-20, einem indirekten Marker für PPARγ-Aktivität, bzw. mittels Transkriptionsfaktorassays ein funktionsfähiges Zellsystem sichergestellt. Sowohl Butyrat [Paper I + V] als auch Mesalazin [Paper II] steigerten die Aktivität des PPARγ-Rezeptors in Wildtypzellen, indessen blieb, wie erwartet, eine gesteigerte Aktivität in dem dominant-negativ PPARγ-transfizierten Zellsystem aus. Butyrat [Paper I] und Mesalazin [Paper II] induzierten außerdem die PPARγ-Expression in Wildtypzellen. Alle Wirkungen auf die untersuchten apoptotischen Marker der Caspase-Kaskade waren unter Stimulation von dominant-negativ PPARγ-transfizierten Zellen mit Butyrat [Paper I] sowie Mesalazin [Paper II] nicht mehr nachweisbar. Lediglich die Expressionsminderung von c-Myc blieb unverändert. Ebenso wurden die durch Mesalazin-vermittelten anti-proliferativen Effekte partiell aufgehoben; eine durch Mesalazin-verursachte Expressionssteigerung von PTEN blieb aus [Paper II].

Zusammenfassend belegen diese Daten erstmals die Bedeutung von PPARγ als zentrales zwischengeschaltetes Signalmolekül an den durch Butyrat und Mesalazin induzierten pro-apoptotischen Wirkungen entlang der Caspase-Kaskade. Zudem konnte ein durch Butyrat-vermittelter p38-MAPK-PPARγ-Caspase-3 Signaltransduktionsweg etabliert werden. Ferner wurden PPARγ-abhängige und -unabhänige Regulierungsmechanismen an den durch Mesalazin-induzierten pro-apoptotischen und anti-proliferativen Wirkungen nachgewiesen. Diese Vorgänge werden u.a. über die modulierende Funktion von Mesalazin auf das Tumor-Suppressor-Gene PTEN bzw. auf das Onkoprotein c-Myc vermittelt.

Im zweiten Teil dieser Arbeit wurde der Einfluss der HDAC-Inhibitoren Butyrat und SFN auf die antimikrobiell wirkenden Proteine Cathelicidin (LL-37) [Paper III] und β-Defensin-2 (HBD-2) [Paper IV] sowie die zugrunde liegenden molekularen Regulationsvorgänge untersucht. Antimikrobielle Peptide sind Bestandteil des angeborenen Immunsystems, die zur Aufrechterhaltung der schützenden Mukosabarriere im Rahmen infektiöser Prozesse beitragen. Mittels semiquantitativer RT-PCR konnte eine zeit- und dosisabhängige Induktion der LL-37- [Paper III + zusätzliche Daten] und HBD-2-mRNA-Expression [Paper IV] nach Inkubation kolorektaler Karzinomzellen mit Butyrat bzw. SFN beobachtet werden. Parallel zur Steigerung der Expression von HBD-2-mRNA induzierten Butyrat und SFN auch die HBD-2-Proteinexpression [Paper IV]. Mithilfe einer Immunofluoreszenz-Analyse konnte außerdem eine überwiegende Lokalisation von HBD-2 [Paper IV] und LL-37 [Paper III] im Zytosol ermittelt werden.

Um den Einfluss der nukleären Rezeptoren PPARγ und VDR auf die Induktion der mRNA-Expression der antimikrobiellen Peptide durch HDAC-Inhibitoren zu untersuchen, erfolgte die Bestimmung der Expression unter dem Einfluss des VDR-Antagonisten ZK191732, sowie in PPARγ dominant-negativ transfizierten HT-29 Zellen [Paper III, IV]. Die durch Butyrat- und SFN-induzierten LL-37- bzw. HBD-2-mRNA-Level wurden jeweils durch die Co-Inkubation mit dem VDR-Antagonisten partiell aufgehoben [Paper III, IV]. Die Behandlung von PPARγ dominant-negativen Zellen übte hingegen keinen Einfluss auf die Induktion von LL-37- [Paper III] bzw. HBD-2-mRNA [Paper IV] im Vergleich mit dem Wildtyp aus. Ferner deutet die unter SFN-Behandlung gesteigerte VDR-Expression auf eine Beteiligung des Rezeptors in der durch SFN induzierten Signalkaskade hin [Paper IV]. Eine vergleichbare Induktion der VDR-Expression nach Butyrat-Behandlung ist in der Literatur hinreichend belegt. Zudem steigerte SFN die Proteinexpression von PPARγ [zusätzliche Daten].

Darüber hinaus wurde die Beteiligung des MEK-ERK-Signalweges an der induzierbaren LL-37- und HBD-2-mRNA-Expression bestimmt [Paper III, IV]. Hierzu wurden die Zellen mit spezifischen Inhibitoren gegen p38-MAPK (SB203580) bzw. ERK1/2 (PD98059) vorinkubiert und anschließend mit Butyrat bzw. SFN behandelt. Durch Co-Inkubation mit dem p38-MAPK-Inhibitor wurde die durch Butyrat induzierte LL-37-mRNA nahezu vollständig aufgehoben [Paper III]. Co-Stimulierung von Butyrat mit dem ERK1/2-Inhibitor übte hingegen nur eine partielle Reduktion auf die Expression von LL-37-mRNA aus [Paper III]. Umgekehrte Effekte der Inhibitoren waren nach SFN-Behandlung auf die

Expression von HBD-2-mRNA zu beobachten [Paper IV]. SFN induzierte zudem die Aktivierung von ERK1/2; hingegen wurde die p38-MAPK-Aktivität nicht beeinflusst [Paper IV]. Durch Blockierung der TGF-β1-Rezeptorkinase mittels des spezifischen Antagonisten SB431542 wurde die durch Butyrat induzierte LL-37-mRNA-Expression teilweise aufgehoben [Paper III].

Allgemein weisen diese Daten darauf hin, dass eine durch die natürlichen HDAC-Inhibitoren Butyrat und SFN induzierbare Expression der antimikrobiellen Peptide LL-37 und HBD-2 von einer Vielzahl von Signalkaskaden und Rezeptoren reguliert wird. In diesem Regulationsprozess spielen sowohl VDR als auch der MEK-ERK-Signaltransduktionsweg eine bedeutende Rolle. Im Gegensatz dazu kann eine Beteiligung des PPARγ-Rezeptors ausgeschlossen werden.

Die supprimierende Wirkung von Butyrat auf die induzierbare NFκB-Aktivität ist hinreichend belegt. Ferner konnte eine Modulierung der NFκB-Aktivität durch nukleäre Rezeptoren aufgezeigt werden. Im letzten Teil der Arbeit wurde daher eine mögliche Beteiligung der beiden nukleären Rezeptoren PPARγ und VDR am basalen NFκB-Signaltransduktionsweg sowie an der Butyrat-vermittelten Hemmung von TNFα- bzw. LPS-induzierter NFκB-Aktivität untersucht [Paper V].

Mittels Transkriptionsfaktorassays konnte nach Stimulierung von HT-29 Zellen mit TNFα sowie mit LPS eine erhöhte p50- und p65-NFκB-Dimer-Aktivität gemessen werden. Die Prä-Inkubation mit Butyrat führte zu einer signifikanten Hemmung der basalen p50- sowie der Zytokin-induzierten p50- und p65-NFκB-Aktivität. Im Gegensatz dazu konnte keine hemmende Wirkung auf die basale p65-Untereinheit nach Butyrat-Behandlung beobachtet werden. Die cytosolische Expression des NFκB-Inhibitors IκBα wurde nach Inkubation der Zellen mit TNFα und Butyrat erniedrigt, jedoch nicht nach LPS-Stimulation. Um eine potentielle Beteiligung von PPARγ und VDR an der supprimierenden Wirkung von Butyrat auf die basale sowie Zytokin-vermittelte NFκB-Aktivität zu bestimmen, wurden die Experimente in transfizierten HT-29 Zellen mit unterdrückter PPARγ-Funktion sowie unter dem Einfluss von rezeptorspezifischen VDR- (ZK191732) und PPARγ-Antagonisten (GW9662) durchgeführt. Die Co-Inkubation mit ZK191732 nach Inkubation mit TNFα hob die supprimierende Wirkung von Butyrat auf die NFκB-Aktivität partiell auf; eine regulatorische Beteiligung von PPARγ konnte in diesem Modell dagegen ausgeschlossen werden. Im Gegensatz dazu wurde nach Behandlung mit LPS eine Beteiligung von PPARγ,

nicht jedoch von VDR beobachtet. Die basale NFκB-Aktivität wurde durch Inkubation des VDR-Antagonisten bei gleichzeitiger Reduktion von IκBα im Zytoplasma signifikant erhöht; der PPARγ-Inhibitor GW9662 übte hingegen keinen Einfluss auf die basale NFκB-Aktivität und die IκBα- Expression aus. Die unterschiedlichen Effekte der supprimierenden Wirkung von Butyrat auf die durch TNFα- oder LPS-induzierbare NFκB-Aktivität lassen sich durch die Beteiligung von unterschiedlichen Signaltransduktionskaskaden erklären. TNFα aktiviert NFκB dabei vornehmlich über den TNF-Repeptor, hingegen interagiert LPS mit dem Toll-Like-Rezeptor-Signaltransduktionsweg. Eine erhöhte PPARγ-Expression nach Behandlung der Zellen mit LPS deutet ferner auf PPARγ als Signalmolekül im Toll-Like-Rezeptor-Signaltransduktionsweg hin.

Zusammenfassend belegen diese Daten erstmals die Bedeutung von PPARγ und VDR als zwischengeschaltete Signalmoleküle der inhibitorischen Wirkung von Butyrat auf die NFκB-Aktivität in Abhängigkeit von dessen Triggerung durch pro-inflammatorische Stimuli. Darüber hinaus konnte eine Beteiligung von VDR an der basalen NFκB-Aktivität aufgezeigt werden.

Mit den vorliegenden Resultaten in kolorekatalen Tumorzellen wird das Spektrum der intestinalen Zellregulierung durch die modulierende Funktion von Mesalazin, Butyrat und SFN auf die Caspase-Kaskade, auf antimikrobielle Peptide sowie auf den NFκB-Signaltransduktionsweg erweitert. Ferner konnte eine Beteiligung der nukleären Hormonrezeptoren PPARγ und VDR an den genannten Signaltransduktionsprozessen aufgezeigt werden. Die vorliegende Arbeit unterstreicht somit nicht nur die allgemeine zellbiologische Bedeutung der nukleären Hormonrezeptoren PPARγ und VDR an gastrointestinalen Regulierungsvorgängen, sondern impliziert auch deren Potential zur Entwicklung neuer therapeutischer Ansätze in der Behandlung des kolorektalen Karzinoms und von CED. Die prinzipielle Relevanz der gefundenen Daten muss jedoch durch weitere grundlagenwissenschaftliche und intensive klinische Forschungsbemühungen untersucht werden.

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10 Appendix

10.1 Abbreviations

5-ASA	5-Aminosalicylic acid, 5-AS, 5-aminosalicylate, 5-aminosalicylic acid
15-PGJ$_2$	15-deoxy-$\Delta^{12,14}$-prostaglandin J_2
AF	activation function
AP	antimicrobial peptide
AP1	activating protein 1
Apaf-1	apoptotic protease activating factor-I
APC	adenomatous polyposis coli
BAX	Bcl-2 associated X protein
BrdU	bromodeoxyuridine
Butyrate	butyric acid
Caspase	cysteine aspartyl-specific protease
CD	crohn's disease
CIN	chromosomal instability
COX	cyclooxygenase
CRC	colorectal cancer
CRE	cAMP response element
CREB	cyclic AMP response element binding protein
DBD	DNA binding domain
DCC	deleted in colorectal cancer
DFF	DNA fragmentation factor
EGF	epithelial growth factor
ERK	extracellular signal-regulated kinase
FAP	familial adenomatous polyposis
FLIP	FLICE-inhibitory protein
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GPCR	G-protein-coupled receptor
HAT	histone acetylase
HBD	human beta defensin

HDAC	histone deacetylase
HETE	hydroxyeicosatetraenoic acid
HNPCC	hereditary nonpolyposis colorectal cancer
HODE	hydroxyoctadecadienoic acid
IAP	inhibitor of apoptosis protein
IBD	inflammatory bowel disease
IKK	IκB kinase
IL	Interleukin
IκB	inhibitor of NFκB
JNK	cJun N-terminal kinase
LBD	ligand binding domain
LOH	loss of heterozygosity
LPS	lipopolysaccharides
MAPK	mitogen-activated protein kinase
MEK-ERK	mitogen-activated protein kinase kinase/extracellular-regulated kinase
MSI	microsatellite instability
NFκB	nuclear factor κB
NSAID	nonsteroidal anti-inflammatory drug
PARP	poly(ADP-ribose) polymerase
PBMC	peripheral blood mononuclear cells
PI3K/Akt	phosphatidylinositol 3-kinase/Akt
PKA	protein kinase A
PKC	protein kinase C
PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome proliferator response element
RXR	retinoid X receptor
SCFA	short chain fatty acid
SCR	steroid receptor coactivator
SFN	sulforaphane
Smad	small mothers against decapentaplegic homologue (Drosophila)
SSAT	spermidine/spermidine N1-acetyltransferase
STAT	signal transducer and activator of transcription
TGF	transforming growth factor

TLR	toll-like receptor
TNBS	trinitrobenzene sulfonic acid
TNF	tumor necrosis factor
TNFR	tumor-necrosis-factor receptor
TZD	thiazolidinedione
UC	ulcerative colitis
VD_3	1α,25-dihydroxyvitamin D_3
VDR	vitamin D receptor
VDRE	vitamin D response element
Xiap	X linked inhibitor of apoptosis

10.2 List of figures and tables

11 Curriculum Vitae

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Schwab M, Reynders V, Loitsch S, Steinhilber D, Stein J, Schröder O; “Involvement of different nuclear hormone receptors in butyrate-mediated inhibition of inducible NFκB signalling”; Mol Immunol. 2007 Jul;44(15):3625-32

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Schwab M, Schröder O, Zahn N, Stein J; “Butyrat induziert die PPARγ-Expression über P38 MAPK“; 60. Jahrestagung der Deutschen Gesellschaft für Verdauungs- und Stoffwechselkrankheiten mit Sektion für Gastroenterologische Endoskopie und Deutsche Gesellschaft für Viszeralchirurgie; Köln; September 14-17; 2005. Abstract published in: Z Gastroenterol 2005; 43: 806

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Schwab M, Schröder O, Stein J; “Stimuli-dependent participation of PPARγ and VDR in butyrate-mediated suppression of NFκB activation”; Jahrestagung 2007 der Deutschen Pharmazeutischen Gesellschaft (DPhG); Erlangen; October 10-13; 2007. Abstract published in: Abstractband DPhG Jahrestagung 2007: 116

Schwab M, Schröder O, Stein J, “Sulforaphane and butyrate are direct inducers of human HBD-2 expression in colonocytes”; Jahrestagung 2007 der Deutschen Pharmazeutischen Gesellschaft (DPhG); Erlangen; October 10-13; 2007. Abstract published in: Abstractband DPhG Jahrestagung 2007: 116

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Schwab M, Zahn N, Stein J, Schröder O; “PPARγ is a key target of butyrate-induced caspase-3 activation in the colorectal cancer cell line Caco-2”; 14th United European Gastroenterology Week "UEGW 2006"; Berlin; October 21-25; 2006. Abstract published in: Gut; Suppl Nov; 55: A76

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12 ZAFES-Zertificat

13 Eidesstattliche Versicherung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Arbeit selbständig angefertigt und mich anderer Hilfsmittel als der in ihr angegebenen nicht bedient habe, insbesondere, dass aus Schriften Entlehnungen, soweit sie in der Dissertation nicht ausdrücklich als solche mit Angabe der betreffenden Schrift bezeichnet sind, nicht stattgefunden haben.

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Markus Schwab

Erklärung

Ich erkläre hiermit, dass ich mich bisher keiner Doktorprüfung unterzogen habe.

Frankfurt am Main, den _____

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Die vorliegende Dissertation entstand im Gastrolabor der Medizinischen Klinik I der Johann Wolfgang Goethe-Universität Frankfurt am Main unter Anleitung von Prof. Dr. Dr. Jürgen Stein.
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15 Publications (I-V)

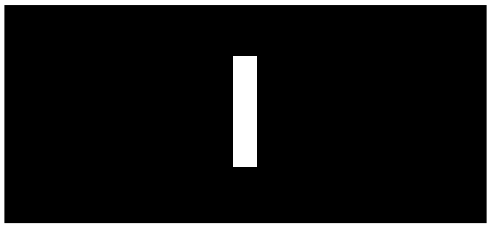
Paper I: **PPARγ is a key target of butyrate-induced caspase-3 activation in the colorectal cancer cell line Caco-2**; Schwab M, Reynders V, Ulrich S, Zahn N, Stein J, Schröder O; *Apoptosis.* 2006 Oct; 11(10):1801-11

Paper II: **PPARγ is involved in mesalazine-mediated induction of apoptosis and inhibition of cell growth in colon cancer cells**; Schwab M, Reynders V, Loitsch S, Shastri Y, Steinhilber D, Schröder O, Stein J; *Manuscript submitted to Clin Cancer Res.*

Paper III: **Role of nuclear hormone receptors in butyrate-mediated up-regulation of the antimicrobial peptide cathelicidin in epithelial colorectal cells**; Schwab M, Reynders V, Shastri Y, Loitsch S, Stein J, Schröder O; *Mol Immunol*. 2007 Mar; 44(8):2107-14

Paper IV: **The dietary histone deacetylase inhibitor sulforaphane induces human beta defensin-2 in intestinal epithelial cells**; Schwab M, Loitsch S, Reynders V, Steinhilber D, Stein J, Schröder O*; Manuscript submitted to Mol Immunol.*

Paper V: **Involvement of different nuclear hormone receptors in butyrate-mediated inhibition of inducible NFκB signalling**; Schwab M, Reynders V, Loitsch S, Steinhilber D, Stein J, Schröder O; *Mol Immunol.* 2007 Jul; 44(15):3625-32



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PPARγ is a key target of butyrate-induced caspase-3 activation in the colorectal cancer cell line Caco-2

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Published online: 21 August 2006

Abstract *Background:* Butyrate, a potent histone deacetylase inhibitor, belongs to a promising new class of antineoplastic agents with the capacity to induce apoptosis of cancer cells. However, the underlying mechanisms of action have yet not been elucidated. *Aim:* To further investigate the molecular events involved in butyrate-induced caspase-3 activation in Caco-2 wild-type, empty-vector and dominant-negative PPARγ mutant cells along the signalling pathway. In this context, the involvement and up-regulation of PPARγ was examined. *Results:* Stimulation of cells with butyrate resulted in increased expression of PPARγ mRNA, protein, and activity as well as phospho-p38 MAPK protein expression and caspase-3 activity. Arsenite, a direct stimulator of p38 MAPK, also led to an increased PPARγ expression, thereby mimicking the effects of butyrate. In contrast, butyrate-mediated up-regulation of PPARγ was counteracted by co-incubation with the p38 MAPK inhibitor SB203580. Treatment of cells with butyrate resulted in both increased caspase-8 and -9 activity and reduced expression of XIAP and survivin. However, butyrate-mediated effects on these apoptosis-regulatory proteins leading to caspase-3 activation were almost completely abolished in Caco-2 dominant-negative PPARγ mutant cells. *Conclusions:* Our data clearly unveil PPARγ as a key target in the butyrate-induced signalling cascade leading to apoptosis via caspase-3 in Caco-2 cells.

Keywords Apoptosis · Butyrate · Caspase-3 · p38 MAPK · PPARγ

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Abbreviations

DFF	DNA Fragmentation Factor
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
ECACC	European Collection of cell cultures
EGF	Epithelial growth factor
ERK	Extracellular signal-regulated kinase
FCS	Foetal calf serum
FLIP	FLICE-inhibitory protein
HDAC	Histone deacetylase
IAP	Inhibitor of apoptosis protein
JNK	cJun N-terminal kinase
LDH	Lactate dehydrogenase
MAPK	Mitogen-activated protein kinase
PARP	Poly(ADP-ribose) Polymerase
PBS	Phosphate buffered saline
PKC-δ	Protein kinase C-δ
PPARγ	Peroxisome proliferator-activated receptor γ
SCFA	Short chain fatty acid
STAT3	Signal transducer and activator of transcription-3
TBS-T	Tris-buffered saline containing 0.05% Tween 20
TNF	Tumor necrosis factor
XIAP	X linked inhibitor of apoptosis

Introduction

Although human data are still inconsistent, at least in rodents evidence has been provided that alimentary fibres exert a protective role with regard to colorectal carcinogenesis [1]. The benefits of a high intake of dietary fibre can be attributed to their fermentation in the colonic lumen to short chain fatty acids (SCFA), including butyrate. Besides its role as the major energy source for the colonocyte [2], there is increasing

evidence that butyrate also acts as a critical factor in the physiological turnover of the colonic epithelium [3]. Roles for butyrate have been established for cell proliferation [4], differentiation [5], morphology [6], motility [7], as well as induction of cell cycle arrest [8] and apoptosis [9]. Therefore butyrate may be considered as a chemopreventive agent in colorectal cancer [10, 11].

Although over the past years major advances have increased our understanding of the molecular mechanisms that trigger apoptosis, the regulation of apoptotic cell death has yet not been fully uncovered. Major pathways in butyrate-induced apoptosis include the caspase- [12, 13], proteasome- [14] and the autophagic-induced [13] cell death, which frequently occur in parallel or in cross talk [13, 14]. Among these, the caspase cascade pathway is the best characterized one in butyrate-mediated apoptosis. One key endpoint represents caspase-3, a member of a cysteine protease family, specifically involved in the initiation and execution of the apoptotic programme [12, 13]. Activation of p38 Mitogen-activated protein kinase (MAPK) seems to be a critical upstream event in butyrate-induced caspase-3 activity [15]. Moreover, caspase-3 is a downstream effector caspase that is activated by upstream caspases, predominantly caspase-8 and -9. Activation of caspase-3 then leads to cleavage of cellular substrates e.g. Poly(ADP-ribose) Polymerase (PARP) [16], DNA Fragmentation Factor (DFF) [17] and disassembling of the cell [18]. Two pathways operate as sensors for caspase-3 activation and can stimulate the cellular death program of apoptosis. The extrinsic or receptor-mediated pathway involves members of the Tumor necrosis factor (TNF) receptor superfamily and is engaged in response to cytokines and extracellular signals leading to activation of caspase-8 [19]. The intrinsic pathway is activated in response to intracellular signals and cytotoxic insults and is mediated by members of the Bcl-2 family at the level of mitochondria resulting in activation of caspase-9 [20].

The family of inhibitor of apoptosis proteins (IAP) is a recently identified, novel category of apoptosis-regulatory proteins. IAPs can inhibit the activation of caspases by both the extrinsic and intrinsic pathway. IAPs are of particular interest as they are up-regulated in many cancer cells and are decreased by several histone deacetylase (HDAC) inhibitors [21].

Previously, we have uncovered for the first time the decisive role of the peroxisome proliferator-activated receptor γ (PPARγ) in butyrate-induced growth inhibition of colon cancer cells [22, 23]. There is also a body of evidence indicating that PPARγ may interact with both p38 MAPK and caspase-3 [15, 24], which makes this nuclear receptor an intriguing target in butyrate-mediated apoptosis. On the basis of these current findings, our study addressed the potential role of PPARγ in the regulation of apoptosis by butyrate in the colorectal adenocarcinoma cell line Caco-2.

Materials and methods

Cell culture

The human colorectal cancer cell line Caco-2 was obtained from the European Collection of cell cultures (ECACC). Cells were cultured in a humidified incubator at 37°C in an atmosphere of 95% air and 5% CO_2. Caco-2 cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% foetal calf serum (FCS), 1% non-essential amino acids, 1% sodium pyruvate and 1% penicillin/streptomycin. Medium of the dominant-negative PPARγ mutant and empty-vector Caco-2 cells was supplied with 400 μg/ml Geneticin 418 sulphate (G418, Gibco-BRL, Eggenstein, Germany). Cells were regularly screened for mycoplasma contamination using the VenorGem Mycoplasma detection kit (Minerva Biolabs, Berlin, Germany).

For experiments, cells were seeded in plastic cell culture wells and were cultivated in DMEM at 80% confluency. Medium was then removed and replaced by a medium containing either the solvent or butyrate (3 mmol/l), arsenite (50 μmol/l), p38 MAPK inhibitor SB203580 (20 μmol/l), extracellular signal-regulated kinase (ERK) 1/2 inhibitor PD98059 (40 μmol/l) or the combination of butyrate and one of the inhibitors. Butyrate (Merck-Schuchardt, Hohenbrunn, Germany) and arsenite (Fluka, Sigma Aldrich-Chemie, Steinheim, Germany) were solubilized in phosphate buffered saline (PBS) and added to the medium (final maximum concentration of PBS in medium was 0.1% (v/v)). PD98059 and SB203580 (Calbiochem, Schwalbach/Taunus, Germany) were dissolved in dimethyl sulfoxide (DMSO, Fluka, Sigma-Aldrich-Chemie, Steinheim, Germany, final maximum concentration of DMSO in medium was 0.1% (v/v)). The medium was changed every day. Cells were then harvested at the times indicated in the figure legends.

Transfection assay

The following plasmids were used for transfection: pcDNA3 (Invitrogen), as an empty-vector for control transfection and the plasmid pcDNA3-PPAR$\gamma_{L468A/E471A}$, a dominant-negative PPARγ double-mutant, that was kindly provided by VK Chatterjee (Department of Medicine, University of Cambridge, Addenbrooke's Hospital, Cambridge, UK) [25]. These constructs were transfected into subconfluent Caco-2 cells with lipofectamine 2000 (Invitrogen) in serum-free conditions. After 6 h, the cells were supplied with fresh medium containing 10% FCS. 24 h later, the cells were supplied with medium containing G418 (400 μg/ml) and culture medium supplemented with G418 was replaced twice a week. G418-resistant colonies were collected and used for further analysis.

Cytotoxicity

Cytotoxicity was excluded by lactate dehydrogenase (LDH) release assay using a commercial kit (LDH kit, Roche, Mannheim, Germany).

Cellular extraction

Caco-2 cells were stimulated with indicated substances at 80% confluency. Cellular extraction was performed using the Active Motif Nuclear cell extraction kit according to the manufacturer's instructions (Active Motif Nuclear extract kit, Rixensart, Belgium). Protein content was determined via Bio-Rad colorimetric assay according to the method of Bradford (Bio-Rad Laboratories, Munich, Germany).

SDS-polyacrylamide gel electrophoresis and immunoblot analysis

Equal amounts of total protein lysates were separated on a 10% SDS-polyacrylamide gel for PPARγ and cytokeratin 20 protein and on a 12.5% SDS-polyacrylamide gel for p38 MAPK, phospho-p38 MAPK, XIAP and survivin protein, respectively. Proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Subsequently, membranes were blocked overnight at 4°C with 3% (w/v) nonfat dried milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T). Membranes were then incubated for one hour with 1:2000 dilutions of human PPARγ-antibody (Calbiochem, La Jolla, CA), phospho-p38 MAPK-, XIAP- and survivin-antibody (all from Cell signaling, Beverly, MA) or with a 1:500 dilution of human cytokeratin 20-antibody (Santa Cruz Biotechnologies, Santa Cruz, CA), respectively. After washing, the blots were incubated for half an hour with corresponding horseradish peroxidase-conjugated antibodys (all from Santa Cruz Biotechnologies, Santa Cruz, CA, dilution 1:2000) in 0.05% TBS-T and 3% (w/v) nonfat dried milk. The washing steps were repeated, and subsequently enhanced chemoluminescence detection was performed according to the manufacturer's instructions (ECL, Amersham Pharmacia Biotech, Buckinghamshire, UK) on Hyperfilm-MP (Amersham International plc, Buckinghamshire, UK). Blots were then reprobed with β-actin antibody (Santa Cruz Biotechnologies, Santa Cruz, CA). For quantitative analysis, bands were detected by scanning densitometry, using a Desaga CabUVIS scanner and Desaga ProViDoc software (Desaga, Wiesloch, Germany).

Semiquantitative RT-PCR

Cells were cultivated in 6-well plates and were treated at 80% confluency with butyrate, any inhibitor or one of the combinations butyrate + SB203580 or butyrate + PD98059 for 24 and 48 h, respectively. Total RNA was isolated from cells by RNA isolation reagent (TRIR, Abgene, UK), followed by phenol extraction and ethanol precipitation. Reverse transcription of total RNA was performed using Superscript II RNase H reverse transcriptase (Invitrogen, Karlsruhe, Germany) and random hexanucleotide primers (Applied Biosystems, Weiterstadt, Germany). PCR was conducted using the following sense and antisense primers, respectively (Biospring, Frankfurt, Germany): PPARγ-forward: 5′-atg-ggt-gaa-act-ctg-gga-gat-tct-cct-a-3′; PPARγ-reverse: 5′-ggt-cat-ttc-ttg-tga-tat-gtt-tgc-aga-cag-t-3′; thermal cycling conditions: 30 cycles, denaturation at 94°C for 30 s, annealing at 55°C for 60 s, and extension at 72°C for 60 s. Cycles were selected as optimal amplification conditions to produce a long-linear relationship between the amount of each mRNA and the intensity of the PCR product. The PCR reaction contained 0.2 mmol/l dNTPs (Eurogentec, Seraing, Belgium), 1 μmol/l primers, 0.3 μl Taq DNA Polymerase (1.5 U) and 1.5 mmol/l $MgCl_2$ per 30 μl reaction. PCR products were separated electrophoretically using a 2% agarose gel containing ethidium bromide and visualized by UV illumination. For semi-quantitative analysis of amplified PCR products, the fluorescent dye PicoGreen (Molecular Probes, Eugene, OR) was used according to the manufacturer's instructions. In brief, two microliters of amplified DNA in 100 μl TE buffer were mixed with an equal volume of diluted PicoGreen reagent (1:200, v/v in TE buffer). Samples were incubated for 5 min at room temperature protected from light in a microtiter plate. The fluorescence was measured ($\lambda_{ex} = 485$ nm; $\lambda_{em} = 538$ nm) in the fluorescence microplate reader Tecan SpectraFluor PLUS (Tecan GmbH Crailsheim, Germany). The standard curve for the quantitative analysis was obtained with λ DNA standard in TE buffer and was linear from 1 to 200 ng/well.

Measurement of caspase-3 activity

The EnzCheck caspase-3 assay kit #2 (Molecular Probes, Leiden, The Netherlands) was used according to the manufacturers instructions. Caco-2 cells were grown to 80% confluency and then stimulated with indicated substances. In inhibitor experiments, cells were pretreated with the inhibitors for 12 h, followed by challenge of cells with butyrate for 24 h. Cells were trypsinized and counted. For each sample 10^6 cells were pelleted and stored at -80°C until further preparation. Pellets were then thawed on ice and resuspended in lysis buffer. After complete lysis of the cells, the particulate material was sedimented by centrifugation at $2000 \times g$ for 5 min. Protein concentration of the supernatant was measured by Bio-Rad colorimetric assay and equal amounts of protein were diluted in lysis buffer for each reaction. The normalized supernatant was incubated with the Z-DEVD-R110 substrate for 30 min. Fluorescence was measured

($\lambda_{ex} = 492$ nm; $\lambda_{em} = 535$ nm) with the fluorescence microplate reader Tecan SpectraFluor PLUS.

Measurement of caspase-8 and -9 activity

The FLICE/Caspase-8 Colorimetric Assay Kit (BioVision Mountain View, CA) and the caspase-9 Colorimetric Assay Kit (Calbiochem, La Jolla, CA) were used to determine enzymatic activity of caspase-8 or -9 class of proteases in apoptotic cells by colorimetric reaction. The three cancer cell lines Caco-2 wild-type, Caco-2 dominant-negative PPARγ, and Caco-2 empty-vector were treated with butyrate (3 mmol/l) for 24 h. After trypsinisation, cells (5×10^6/sample) were collected in PBS and pelleted. 100 μg of total protein was loaded into each well of a 96-well flat bottom microplate and adjusted to 50 μl with dilution buffer. Subsequently, 50 μl of 2 $\times$ reaction buffer containing 10 mmol/l of dithiothreitol was added. After adding 5 μl of caspase colorimetric substrate, the plate was incubated at 37°C for 2 h. Caspase-8 and -9 activity was determined using IETD-pNA and LEHD-pNA as substrate, respectively. Optical density was determined by a microplate reader ($\lambda_{ex} = 405$ nm). Results were expressed as the percent of control.

Statistics

If not otherwise stated, data are expressed as means $\pm$ SEM from three independent experiments performed in duplicates. Student's *t* test was used for statistical comparison (SigmaStat, SPSS, Chicago, IL). *P* value <0.05 was considered to be significant.

Results

Phospho-p38 MAPK is involved in butyrate-induced caspase-3 activation

To establish a possible role of MAPKs in butyrate-induced apoptosis in colonic epithelial cells, we first examined the effect of butyrate on p38 MAPK and ERK1/2 expression in the Caco-2 cell line. As shown in Fig. 1, incubation of cells with butyrate at a concentration of 3 mmol/l for 24 h significantly increased phospho-38 MAPK as compared to control. In contrast, expression of phospho-ERK1/2 was not affected by treatment of the Caco-2 cell line with butyrate (data not shown).

In order to unveil the involvement of any MAPK in butyrate-induced apoptosis, we employed specific MAPK inhibitors, the p38 MAPK inhibitor SB203580 and the ERK1/2 inhibitor PD98059, and investigated their effects on butyrate-induced caspase-3 activation. Incubation of Caco-2 cell with butyrate (3 mmol/l) for 24 h significantly increased caspase-3 activity up to $+215 \pm 45\%$ compared to control level ($p < 0.01$) as determined by cleavage of Z-DEVD-R110. In contrast, pretreatment of the Caco-2 cell line with the p38 MAPK inhibitor SB203580 (20 μmol/l) for 12 h, followed by treatment of cells with butyrate for additional 24 h, resulted in a significant inhibition of butyrate-induced caspase-3 activity ($-63 \pm 10\%$, $p < 0.05$). The specific ERK1/2 inhibitor PD98059 (40 μmol/l) had no impact on the induction of caspase-3 activity caused by butyrate (data not shown).

PPARγ plays a pivotal role in p38 MAPK-mediated caspase-3 activation by butyrate

Stimulation of the colorectal carcinoma cell line Caco-2 with butyrate (3 mmol/l) resulted in a time-dependent increase of PPARγ mRNA. Significant effects were first observed after 24 h (data not shown) and reached its maximum at 48 h of treatment with a 1.7-fold increase in PPARγ mRNA. Whereas the combination of butyrate and the ERK1/2 inhibitor PD98059 did not influence butyrate-induced PPARγ mRNA expression at any time, co-incubation of butyrate with the p38 MAPK inhibitor SB203580 (20 μmol/l) significantly reversed the butyrate-induced up-regulation of PPARγ mRNA expression at 48 h of treatment (Fig. 2(A)). Up-regulation of PPARγ mRNA was followed by transient changes on the protein level. Indeed, after 48 h of treatment, butyrate significantly augmented PPARγ protein expression ($+45 \pm 14\%$ of control, $p < 0.05$, Fig. 2(B)). Again, incubation with the p38 MAPK inhibitor completely abolished the induction of PPARγ protein expression by butyrate, whereas the ERK1/2 inhibitor PD98059 had no impact on PPARγ expression levels.

To corroborate our findings of a signalling link between p38 MAPK and PPARγ, Caco-2 cells were treated with arsenite (50 μmol/l), a known direct stimulator of p38 MAPK. As shown in Fig. 3, arsenite augmented PPARγ protein expression in a time-dependent manner, already beginning at 6 h of stimulation ($+44 \pm 18\%$ of control, $p < 0.05$) with a maximum of $+135 \pm 10\%$ vs. control at 24 h of incubation, indicating that p38 MAPK is involved in butyrate-induced up-regulation of PPARγ expression.

In order to confirm the pivotal role of PPARγ in butyrate-mediated apoptosis, activation of caspase-3 by butyrate was also analysed in Caco-2 cells transfected with a dominant-negative PPARγ mutant to inhibit wild-type receptor action. As shown in Fig. 4, no significant effects on caspase-3 activity could be detected in PPARγ dominant-negative Caco-2 cells treated with butyrate (3 mmol/l) for 24 h as compared with both Caco-2 wild-type and Caco-2 empty-vector cells.

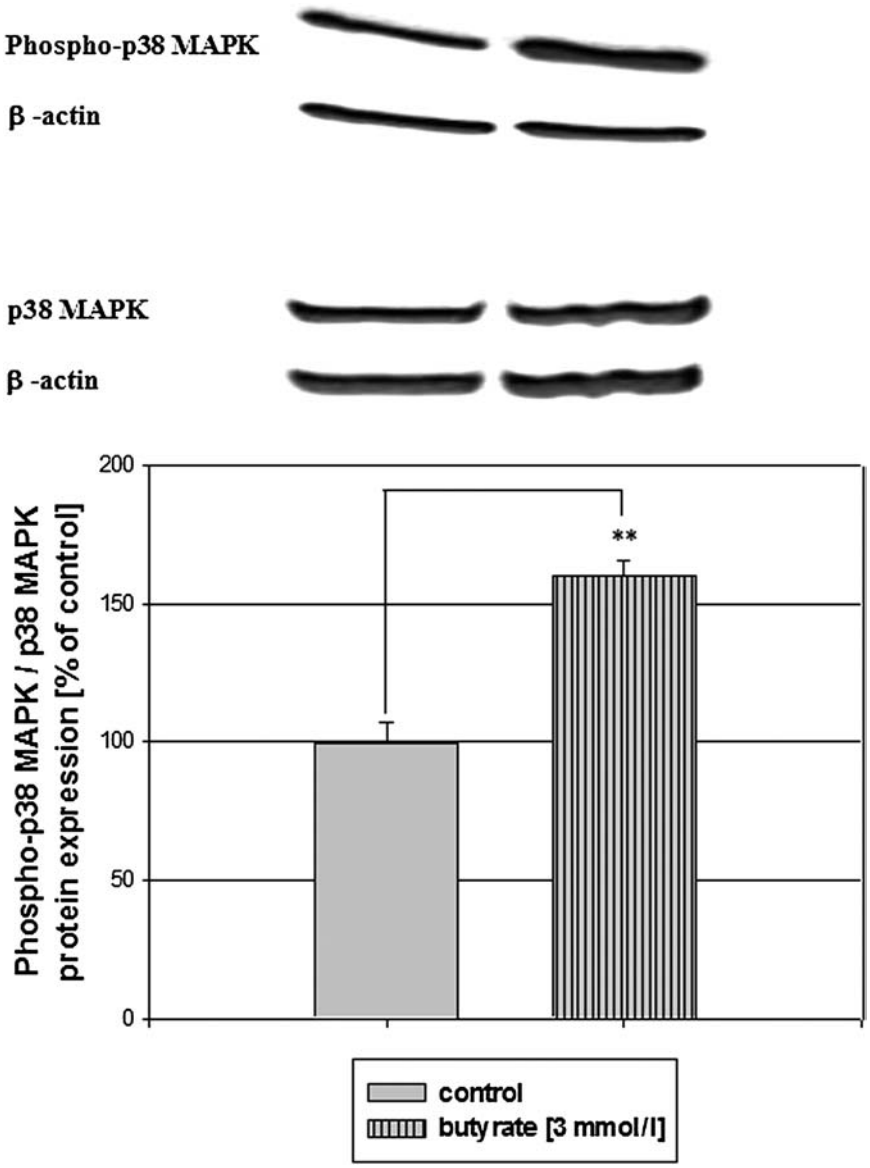

Fig. 1 Effect of butyrate (3 mmol/l) on p38 MAPK and phospho-p38 MAPK protein expression in Caco-2 cells after 24 h. One representative gel of three independent experiments is shown. Quantitative data are corrected for β-actin levels. $^{**}p < 0.01$

PPARγ is also involved in caspase-8 and caspase-9 activation and controls the expression of the inhibitor of apoptosis proteins XIAP and survivin after butyrate treatment

To investigate the fine tuning in the caspase pathway leading to elevated caspase-3 activity after butyrate treatment, the activities of caspase-8 and -9 were examined. Incubation with butyrate (3 mmol/l) resulted in a significant increase in caspase-8 ($\sim$2.3-fold, $p < 0.01$) and caspase-9 ($\sim$1.7-fold, $p < 0.01$) activity after 24 h in both, Caco-2 wild-type and Caco-2 empty-vector cells. In contrast, up-regulation of caspase-8 ($\sim$1.2-fold, n.s.) and caspase-9 ($\sim$1.2-fold, n.s.) activity in response to butyrate was found to be almost reversed in PPARγ dominant-negative Caco-2 cells.

To specify the molecular mechanism of butyrate-induced apoptosis, changes in IAPs, X linked inhibitor of apoptosis (XIAP) and survivin, were further examined (Fig. 5(A) and (B)). In Caco-2 wild-type cells, levels of survivin and XIAP were reduced to $-68 \pm 8\%$ ($p < 0.001$) and $-59 \pm 10\%$ ($p < 0.01$) of control in response to butyrate (3 mmol/l) for 24 h, respectively. Similar effects were obtained in Caco-2 empty-vector cells. In contrast, butyrate-mediated down-regulation of both IAP family members was partially neutralized when incubated in Caco-2 dominant-negative PPARγ mutant cells.

To evaluate receptor activity of PPARγ in Caco-2 wild-type and dominant-negative PPARγ mutant Caco-2 cells, we determined the expression of cytokeratin 20 which has been described to be a specific target gene of PPARγ activity in colorectal cancer cells [26]. A time-dependent increase

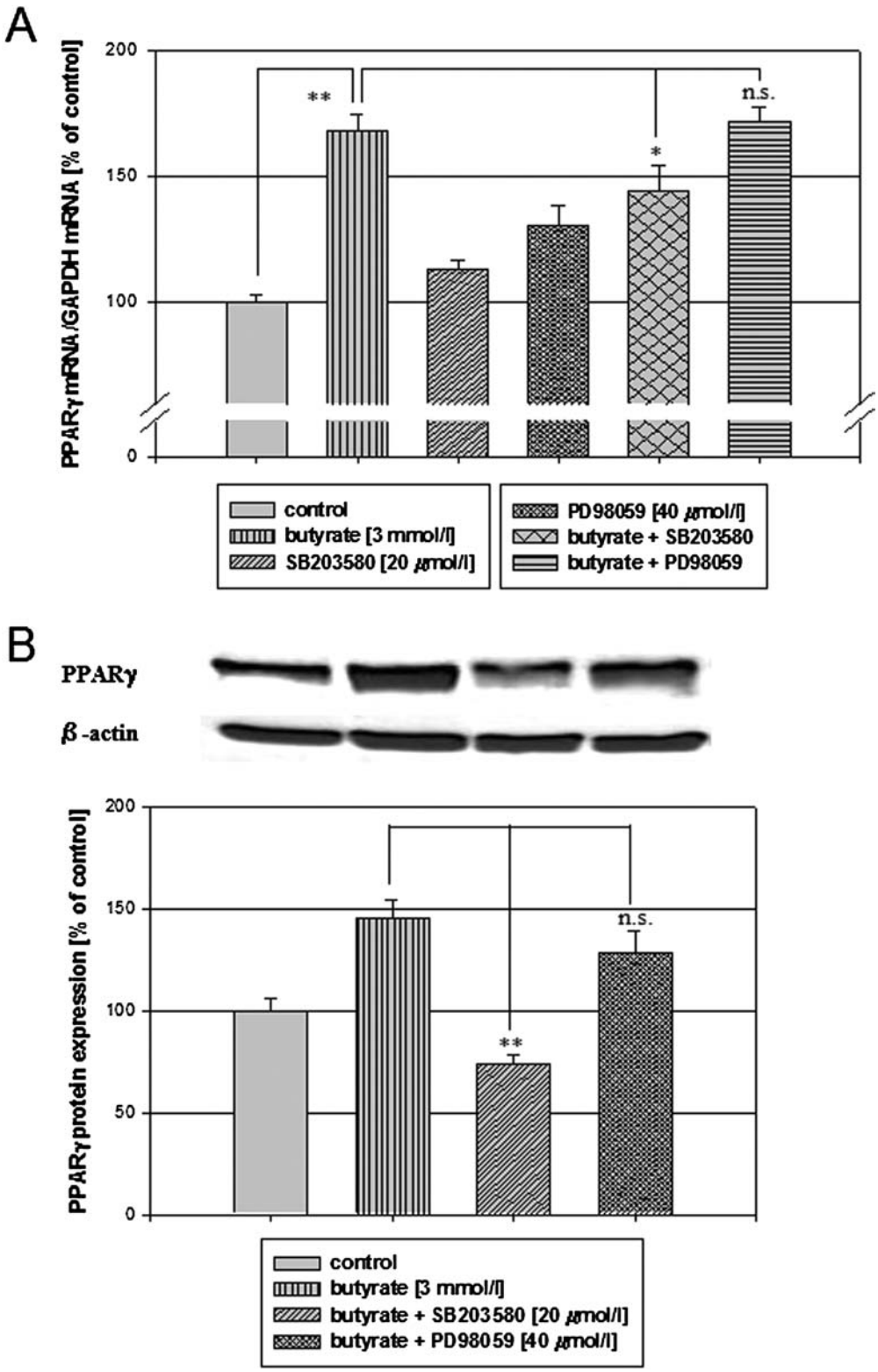

Fig. 2 (A) PPARγ mRNA-expression in Caco-2 cells after treatment with butyrate (3 mmol/l), p38 MAPK inhibitor SB203580 (20 μmol/l), ERK1/2 inhibitor PD98059 (40 μmol/l) and the combined treatment of butyrate and one of the inhibitors for 48 h. Semiquantitative analysis of PCR products was performed using Pico Green. All values for mRNA levels are normalized to corresponding mRNA amount of the housekeeping gene GAPDH. $^{*}p < 0.05$, $^{**}p < 0.01$, n.s. = not significant (B) Effect of the specific inhibitors SB203580 (20 μmol/l) and PD98059 (40 μmol/l), respectively, on butyrate-induced PPARγ protein expression after 48 h of treatment. The band at 60 kDa corresponds to the PPARγ protein. One representative gel of three independent experiments is shown. Quantitative data are corrected for β-actin levels. $^{**}p < 0.01$, n.s. = not significant

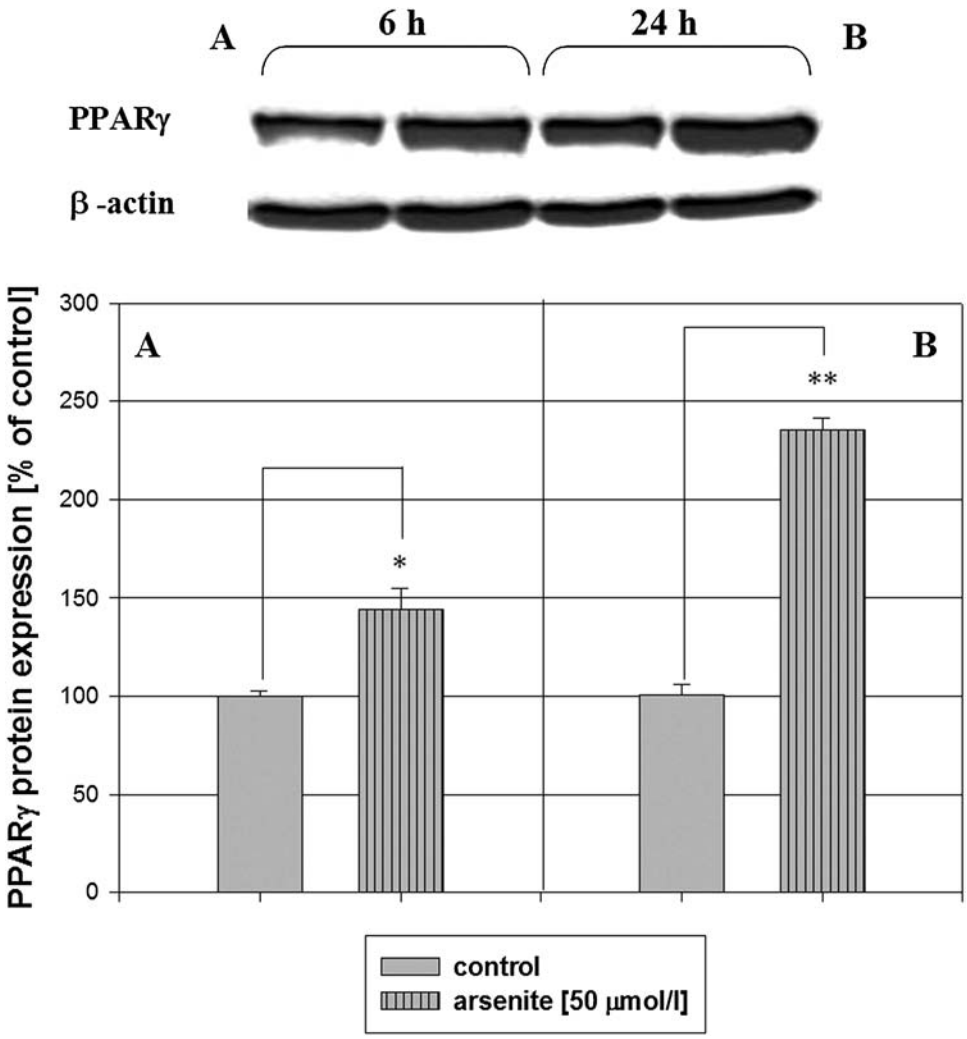

Fig. 3 Time-dependent effect of the chemical p38 MAPK stimulator arsenite (50 μmol/l) on PPARγ protein expression after 6 h (A) and 24 h (B) of treatment. One representative gel of three independent experiments is shown. Quantitative data are corrected for β-actin levels. $^{*}p < 0.05$, $^{**}p < 0.01$

of cytokeratin 20 could be observed after butyrate incubation (3 mmol/l) in Caco-2 wild-type cells. Up-regulation of protein was already detected after 24 h and reached its maximum to approximately 2.0-fold as compared with control after 48 h ($p < 0.01$, Fig. 5(C)). As expected, cytokeratin 20 was not expressed in both unstimulated and butyrate-challenged Caco-2 dominant-negative PPARγ mutant cells (Fig. 5(C)).

Discussion

The HDAC inhibitor butyrate exerts potent anti-tumor effects by inhibition of proliferation, induction of differentiation, and stimulation of apoptosis [27]. Revealing the transduction pathway of butyrate-mediated apoptotic cell death is not only important to understand fundamental biological processes, but also may be useful for developing pharmacological tools for treating cancers with apoptotic defects. Among the different apoptotic pathways involved in butyrate-mediated apoptosis, activation of the caspase cascade appears to play a protruding role. Indeed, induction of caspase-3, a downstream key member of the caspase pathway, by butyrate has been demonstrated in a variety of malignant cancer cell lines including colorectal cancer cells [28–30]. However, little is known with respect to the underlying upstream events participating in the regulation of caspase-3 by butyrate.

p38 MAPK belongs to a group of protein serine/threonine kinases which has been demonstrated to play an essential role in the regulation of several cellular processes including apoptosis [31–34]. Moreover, butyrate has been shown to activate p38 MAPK in several cancer cell lines including Caco-2 [15, 32, 35, 36]. Recent data indicate that activation of p38 MAPK is an important molecular signalling event in butyrate-mediated activation of the caspase cascade [15, 32, 37]. Activation of p38 MAPK by butyrate in the human colorectal adenoma cell line AA/C1 resulted in activation of caspase-3 [15]. Furthermore, butyrate-mediated activation of p38 MAPK appeared to be signalled via

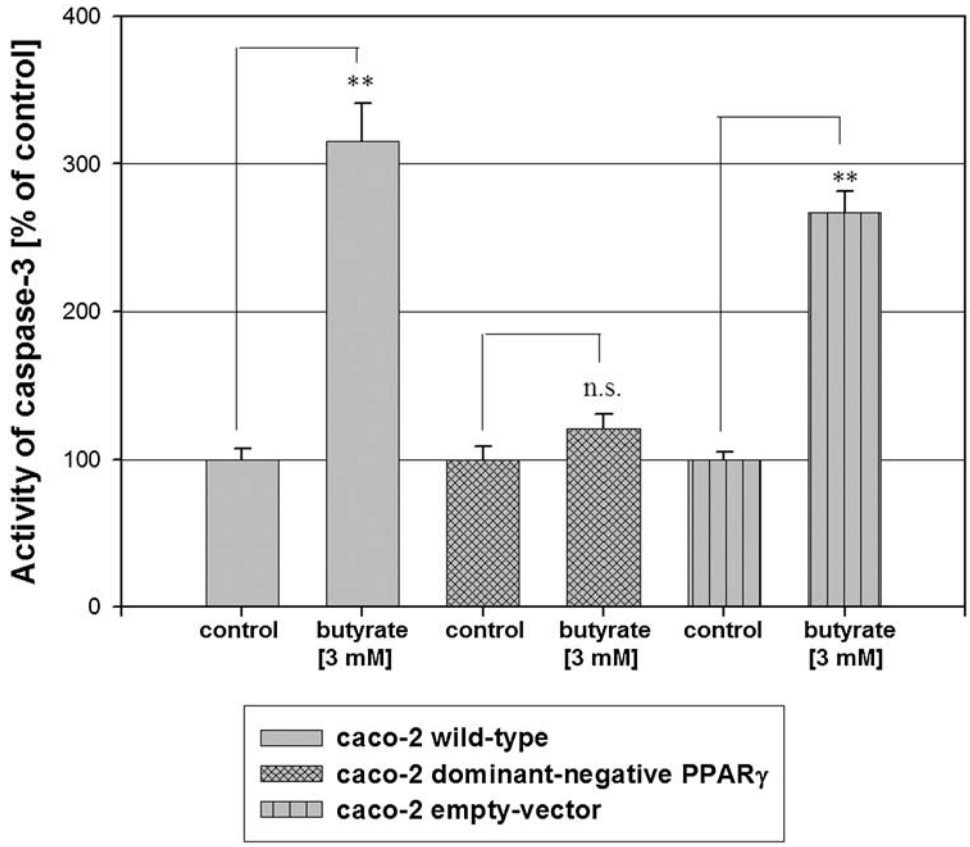

Fig. 4 Caspase-3 activity of Caco-2 wild-type, Caco-2 dominant-negative PPARγ, and Caco-2 empty-vector cells 24 h after incubation with butyrate (3 mmol/l) compared to control. $^{**}p < 0.01$, n.s. = not significant

protein kinase C-δ (PKC-δ) [15]. Our data corroborate these findings by demonstrating that butyrate-induced activation of caspase-3 was significantly reduced by the specific p38 MAPK inhibitor SB203580. Nevertheless, butyrate-mediated apoptotic effects were not completely reversed by the p38 MAPK inhibitor SB203580 indicating that transduction pathways other than p38 MAPK are involved in activation of caspase-3 by butyrate at least in Caco-2. Indeed, expression of bak, a member of the bcl2-gene family which has been shown to be involved in the regulation of apoptotic signalling was found to be significantly increased in butyrate-stimulated Caco-2 cells [38]. Moreover, bak expression does not only correlate positively with apoptosis in intestinal epithelium [39] but also has been demonstrated to be a potent activator of the caspase cascade [40]. However, since butyrate is known to affect the expression of several genes potentially involved in the regulation of programmed cell death [38], other targets such as cJun N-terminal kinase (JNK) [32, 41] and PKC-δ [15] may also be involved in butyrate-induced stimulation of caspase-3 in Caco-2.

There is increasing evidence supporting the role of PPARγ, a ligand-activated transcription factor belonging to the nuclear hormone receptor superfamily, as a key player in the induction of cellular apoptosis. PPARγ does not only control the expression of genes involved in differentiation, but also modulates cell cycling [42, 43]. Indeed, recent reports indicate that the pro-apoptotic action of PPARγ is dependent on caspase-3 activation. Thus, the PPARγ agonists 15-deoxy-delta^{12,14}-prostaglandin J_2 and troglitazone were shown to induce apoptosis via elevated activity of caspase-3 in human neuroblastoma [44] and HepG2 [45] cells, respectively. Finally, an association between loss-of-function mutations of PPARγ and the development of colorectal cancer in humans [46] also suggests the involvement of this receptor in the regulation of apoptosis.

Previous studies have shown that expression of PPARγ mRNA and protein [22, 23, 47] is under the control of butyrate implying that PPARγ may participate in butyrate-induced caspase-3 apoptotic signalling. To prove such a possible role for PPARγ, butyrate-mediated pro-apoptotic properties were investigated in Caco-2 cells, transfected with a mutant receptor to inhibit wild-type PPARγ action. In this mutant the highly conserved hydrophobic and charged residues (Leu468 and Glu471) in helix 12 of the ligand binding domain are mutated to alanine. As a consequence, this cell mutant indeed retains PPARγ ligand and DNA binding, but exhibits markedly reduced transactivation due to impaired coactivator recruitment [25]. Indeed, butyrate-mediated activation of caspase-3 was almost completely abolished in these mutant cells indicating that PPARγ plays a critical role in signalling the pro-apoptotic effects of butyrate. Furthermore, our data suggest that PPARγ

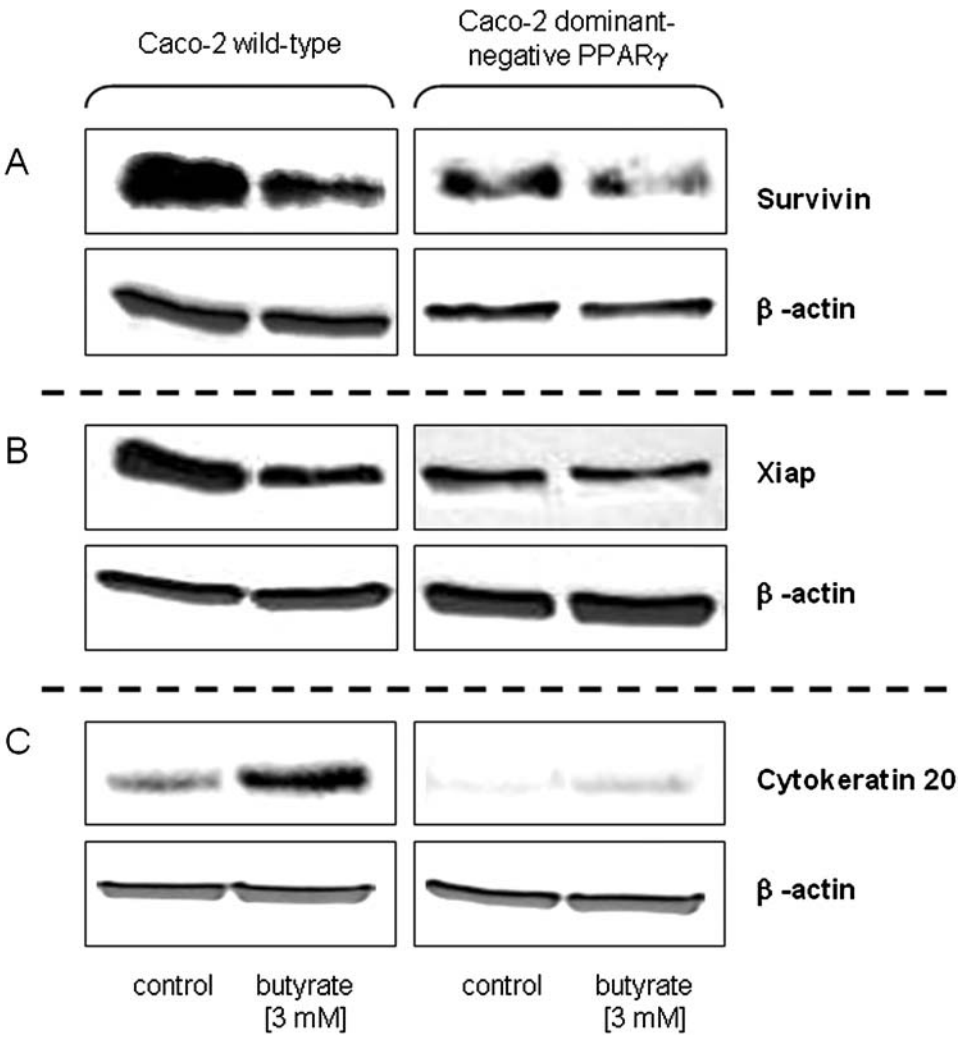

Fig. 5 Western Blot of survivin (A) and XIAP (B) in Caco-2 wild-type and dominant-negative PPARγ cells after incubation with butyrate (3 mmol/l) for 24 h. One representative gel of three independent experiments is shown. Quantitative data are corrected for β-actin levels. (C) Induction of cytokeratin 20 in Caco-2 wild-type and dominant-negative PPARγ cells after treatment with butyrate (3 mmol/l) for 48 h. A representative immunoblot of three independent experiments is shown

acts downstream of p38 MAPK as demonstrated by both up-regulation of PPARγ protein by the direct p38 MAPK stimulator arsenite and down-regulation of this nuclear receptor on the mRNA and protein level in presence of the p38 MAPK inhibitor SB203580.

In addition to the contribution of PPARγ in butyrate-mediated Caco-2 cell apoptosis, our data provide further insight in the molecular events leading to activation of caspase-3 as depicted in Fig. 6. The increase in caspase-8 and -9 activity by butyrate indicates that both the extrinsic (caspase-8) and the intrinsic (caspase-9) caspase pathway share in caspase-3 activation. This finding is supported by recent studies illustrating that both caspase-8 and -9 lead to increased caspase-3 activity after butyrate incubation [29, 48]. Moreover, in our *in vitro* model butyrate also significantly decreased protein levels of survivin and XIAP, two proteins known to potently block caspase-3 and -9, thereby inhibiting apoptosis. Accordingly, a similar down-regulatory effect on survivin and XIAP by butyrate has been previously shown in human glioma cells [49]. Again, butyrate-mediated effects on both pro-apoptotic and anti-apoptotic events were vigorously diminished in PPARγ dominant-negative Caco-2 cells. The underlying mechanism(s) by which PPARγ controls the caspase signalling pathway are yet not fully enlightened. It might be speculated that PPARγ activates both the intrinsic and extrinsic caspase pathway via modulation of the IAPs. However, incomplete abolishment in PPARγ dominant-negative Caco-2 cells suggests that other factors known to modulate the expression of the IAPs such as JNK, epithelial growth factor (EGF) and activator of transcription-3 (STAT3) [50–53] might also be involved. A direct interaction of PPARγ and the death receptor signalling pathway was disclosed only recently. Kim et al. demonstrated that PPARγ ligands were able to down-regulate the FLICE-inhibitory protein (FLIP), an apoptosis-suppressing protein that blocks early events in death receptor signalling pathway [54]. Accordingly, reduced levels of FLIP after butyrate treatment were detected in human colon cancer [55] and pancreatic cancer cells [48], allowing apoptosis to occur via activation of caspase-8.

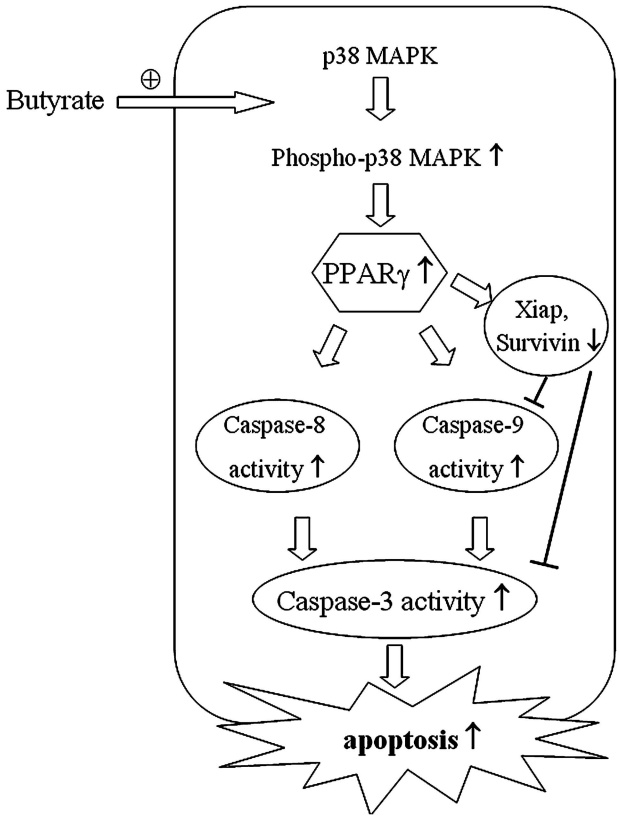

Fig. 6 Model of butyrate-induced cell death: Butyrate activates p38 MAPK, which in turn up-regulates PPARγ expression and receptor activity. PPARγ decreases the expression of survivin and XIAP and activates caspase-8 and -9 leading to increased caspase-3 activity, eventually resulting in cell death

Conclusion

Our data shed further light in the underlying mechanisms of butyrate-mediated apoptosis via caspase-3 in colorectal cancer cells. Following the activation of p38 MAPK by phosphorylation, up-regulation of PPARγ receives a key role in mediating death receptor signalling by decreasing the expression of survivin and XIAP and increasing the activity of caspase-8 and -9 finally leading to increased caspase-3 activity, eventually resulting in cell death. Further studies are warranted to elucidate the complex mechanisms underlying butyrate-mediated apoptosis.

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II

PPARγ is involved in mesalazine-mediated induction of apoptosis and inhibition of cell growth in colon cancer cells

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Running title

Mesalazine induces apoptosis via PPARγ

Keywords

Colorectal cancer, apoptosis, mesalazine, caspase, PPARγ

Abstract

Purpose: Mesalazine has been identified as a candidate chemopreventive agent in colon cancer prophylaxis because of its pro-apoptotic and anti-proliferative effects. However, the precise mechanisms of action are not entirely understood. The aim of our study was to investigate the involvement of PPARγ in mesalazine's anti-carcinogenic actions in colorectal cancer cells.

Experimental Design: The effects of mesalazine on cell cycle distribution, cell count, proliferation and caspase-mediated apoptosis were examined in Caco-2, HT-29 and HCT-116 cells used as wild-type, dominant-negative PPARγ mutant and empty-vector cultures. We focused on caspase-3 activity, cleavage of PARP, caspase-8, and caspase-9, as well as on expression of survivin, Xiap, PTEN and c-Myc. Techniques employed included transfection assays, immunoblotting, flow cytometry analysis, colorimetric and fluorometric assays.

Results: Mesalazine provoked a time- and dose-dependent decrease in both cell growth and proliferation. Growth inhibition was accompanied by a G1/G0 arrest, a significant increase in PTEN, caspase-3 activity, cleavage of PARP and caspase-8; while the expression of Xiap, survivin and c-Myc was decreased simultaneously. Cleavage of caspase-9 was not observed. Moreover, PPARγ expression and activity were elevated. The growth-inhibitory effect of mesalazine was partially reduced in dominant-negative PPARγ mutant cells, while the expression of c-Myc was not affected. Mesalazine-mediated increased caspase-3 activity, the expression of PTEN, cleavage of PARP and caspase-8 as well as reduced levels of survivin and Xiap were completely abolished in the PPARγ mutant cell lines.

Conclusions: This study clearly demonstrates that mesalazine-mediated pro-apoptotic and anti-proliferative actions are regulated via PPARγ-dependent and -independent pathways in colonocytes.

Introduction

Colorectal cancer (CRC) is one of the most fearsome complications of inflammatory bowel diseases (IBD) mainly ulcerative colitis (UC) but also Crohn's colitis (1, 2). CRC risk increases significantly with extent and duration of the disease (1). Main strategy for CRC prevention in chronic UC is currently based on identification of neoplasia by surveillance colonoscopy, but there is great interest in the possibility of primary chemoprevention (3). A series of epidemiological investigations and preliminary clinical trials have affirmatively suggested that regular intake of oral mesalazine (5-ASA, 5-aminosalicylate) may have anti-neoplastic and potentially prophylactic properties reducing the occurrence of CRC in patients with IBD, in particular UC (4, 5). Furthermore, chromosomal and microsatellite instability as well as dysplasia was found to be reduced after long-term mesalazine therapy (6-8).

The molecular mechanisms responsible for mesalazine's chemopreventive effects are not entirely understood. The widespread mechanisms leading to its anti-carcinogenic actions include the inhibition of inflammatory cascades such as the cyclooxygenase pathway (COX-1 and COX-2) which regulates cell proliferation through formation of prostaglandins (9). Moreover, recent studies suggest that 5-ASA may reduce the cancer risk by mechanisms other than simply controlling inflammation, such as increasing apoptosis, decreasing cellular proliferation and by activating the Peroxisome-Proliferator-Activated Receptor γ (PPARγ) (10, 11).

In a rodent CRC model, mesalazine inhibits tumor growth and reduces the number of aberrant crypt foci while in patients with sporadic polyps or cancer of the large bowel, mesalazine induces apoptosis and decreases proliferation in the colorectal mucosa (12). In addition, induction of apoptosis through activation of caspase-3 in colon cancer cells has also

been indicated (13). However, the precise pathway of activating the caspase cascade is still unknown.

Rousseaux *et al.* demonstrated that PPARγ is a target of mesalazine (11, 14). The drug increased PPARγ expression, promoted its translocation from the cytoplasm to the nucleus and induced a modification of its conformation permitting the recruitment of coactivators and the activation of a PPARγ response element driven gene (11, 14, 15). PPARγ is a transcription factor belonging to the nuclear hormone receptor superfamily (11). The receptor is highly expressed in the colonic epithelium and can not only be activated by natural ligands such as eicosanoids and fatty acids like butyrate, but also by synthetic ligands such as thiazolidinediones (16, 17). In the gastrointestinal tract, PPARγ is known to regulate cellular proliferation, differentiation, and to induce apoptosis (18, 19). Moreover, reports demonstrate that PPARγ ligands lead to tumor suppression (20). The growth of cultured human colon tumor cells and of transplanted tumors in nude mice is inhibited by activators of PPARγ, including troglitazone, rosiglitazone and 15-deoxy-delta^{12,14}-prostaglandin J_2 (19, 20).

Recently, the participation of PPARγ, both in cell growth inhibition and in the regulation of several pro- and anti-apoptotic markers of the caspase signalling pathway was demonstrated by our group in CRC cells (21, 22). Based on these observations, the present study was addressed to elucidate the putative role of PPARγ in mesalazine-mediated anti-proliferative and pro-apoptotic effects.

Materials and methods

Cell culture

The human CRC cell lines Caco-2, HT-29 and HCT-116 were obtained from the European Collection of cell cultures (ECACC). Cells were cultured in a humidified incubator at 37 °C in an atmosphere of 95% air and 5% CO_2. Caco-2 cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% foetal calf serum (FCS), 1% non-essential amino acids, 1% sodium pyruvate and 1% penicillin/streptomycin. HT-29 and HCT-116 cells were grown in McCoy's 5A Medium, supplemented with 10% FCS and 1% penicillin/streptomycin. Medium of the dominant-negative PPARγ mutant and empty-vector Caco-2 and HT-29 cells was supplied with 400 µg/ml Geneticin 418 sulphate (G418, Gibco-BRL, Eggenstein, Germany). Cells were regularly screened for mycoplasma contamination using the VenorGem Mycoplasma detection kit (Minerva Biolabs, Berlin, Germany).

For experiments, cells were seeded in plastic cell culture wells and were cultivated in DMEM until 80% confluency was reached. Medium was then removed and replaced by a medium containing mesalazine (10-50 mM). Mesalazine (5-aminosalicylate, 5-ASA, Sigma, Deisenhofen, Germany) was dissolved as a 100 mM stock solution in culture medium. The pH of the drug solution was adjusted to 7.0 with NaOH and afterwards, the solution was sterile filtrated. All experiments using mesalazine were protected from light. The medium was changed every day. Cells were then harvested at the times indicated in the figure legends.

Transfection assay

The following plasmids were used for transfection: pcDNA3 (Invitrogen, Karlsruhe, Germany), as an empty-vector for control transfection and the plasmid pcDNA3-

PPAR$\gamma_{L468A/E471A}$, a dominant-negative PPARγ double-mutant, that was kindly provided by VK Chatterjee (Department of Medicine, University of Cambridge, Addenbrooke's Hospital, Cambridge, United Kingdom) (23). These constructs were transfected into subconfluent Caco-2 and HT-29 cells with lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) in serum-free conditions. After 6 h, the cells were supplied with fresh medium containing 10% FCS. 24 h later, the cells were supplied with medium containing G418 (400 µg/mL) and culture medium supplemented with G418 was replaced twice a week. G418- resistant colonies were collected and used for further analysis.

Cytotoxicity

Cytotoxicity of mesalazine in concentrations used in our experiments was excluded by lactate dehydrogenase (LDH) release assay using a commercial kit (LDH kit, Roche, Mannheim, Germany).

Cell counts

Cells were suspended and cultured on 96-well dishes at a density of 10^4/well (0.28 cm^2). 24 h after plating, cells were incubated for 24–72 h with mesalazine. At given time points following treatment, cell numbers were assessed by crystal violet staining. Medium was removed from the plates and cells were fixed with 5% formaldehyde for 5 min. After washing with PBS, cells were stained with 0.5% crystal violet for 10 min, washed again with PBS and unstained with 33% acetic acid. Absorption, which correlates with the cell number, was measured at λ_{ex}= 620 nm.

Assay for cell proliferation

The effect of mesalazine on DNA synthesis of cells was assessed using a cell proliferation ELISA kit (Roche Diagnostics, Tokyo, Japan). This assay is a colorimetric immunoassay for quantification of cell proliferation based on the measurement of bromodeoxyuridine (BrdU) incorporation during DNA synthesis, and is a nonradioactive alternative to the [^{3}H]-thymidine incorporation assay. Incorporated BrdU was measured colorimetrically.

Caspase-3 activity assay

Caco-2 and HT-29 cells were stimulated with mesalazine at 80% confluency. Fluorometric immunosorbent enzyme assay (FIENA, Roche, Mannheim, Germany) was used according to the manufacturers instructions. Protein concentration was analysed and samples were normalized in lysis buffer to equal protein concentrations.

PPARγ transactivation assay

PPARγ activity was assayed using an ELISA-based transactivation TransAM® PPARγ kit (Active Motif, Rixensart, Belgium) following the manufacturer's protocol. The PPARγ TransAM® kit contains a 96-well plate with immobilized oligonucleotides containing a peroxisome proliferator response element (5'-AACTAGGTCAAAGGTCA-3').

Flow Cytometry Analysis

Cells were starved for 72 h and then treated with mesalazine (40 mM) up to 48 h. Cells were harvested by trypsinization, fixed with 80% ethanol and kept at -20°C. Cells were then centrifuged for 10 min at 500 × *g* and resuspended in PBS containing 0.25% Triton-X100 for 5 min. After washing with PBS, cells were stained with PBS containing propidium iodide (20 µg/ml; Sigma Chemicals, Deisenhofen, Germany) and RNAse A (2 mg/ml; Sigma

Chemicals, Deisenhofen, Germany) at room temperature for 30 minutes. Subsequently, DNA content was measured by flow cytometry (FACSCalibur, Becton Dickinson, Heidelberg, Germany) and cell cycle distribution was calculated using Cell Quest Software (Becton Dickinson Technology, Mountain View, California, USA).

Protein extraction

Caco-2 and HT-29 cells were stimulated with mesalazine at 80% confluency. Cells were washed three times with ice-cold PBS and incubated with cell lysis buffer (Cell signaling, Beverly, Massachusetts, USA) containing multiple protease inhibitors (Complete, Roche, Mannheim, Germany) for 20 min at 4 °C. Protein extracts were obtained after sonication of cell lysates (2 × 5 s) and centrifugation at 10.000 rpm at 4 °C (10 min). Protein content was determined via Bio-Rad colorimetric assay according to the method of Bradford (Bio-Rad Laboratories, Munich, Germany).

SDS–polyacrylamide gel electrophoresis and immunoblot analysis

Equal amounts of total protein lysates were separated on a 15% SDS-polyacrylamide gel for PARP, Caspase-8, -9 and on a 12.5% SDS-polyacrylamide gel for Xiap, survivin, PPARγ, PTEN, c-Myc and cytokeratin 20, respectively. Proteins were transferred onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Subsequently, membranes were blocked for one hour with 5% (w/v) nonfat dried milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T). Membranes were then incubated overnight with 1:1000 dilutions of PPARγ-, c-Myc (both from Calbiochem, La Jolla, CA), PTEN-, PARP-, Caspase-8-, Caspase-9-, Xiap- and survivin- antibody (all from Cell signaling, Beverly, Massachusetts, USA) or with a 1:500 dilution of human cytokeratin 20- antibody (Santa

Cruz Biotechnologies, Santa Cruz, California, USA) in 0.05% TBS-T and 5% (w/v) nonfat dried milk, respectively. After washing, the blots were incubated for half an hour with corresponding horseradish peroxidase-conjugated antibodys (all from Santa Cruz Biotechnologies, Santa Cruz, California, USA, dilution 1:2000) in 0.05% TBS-T and 5% (w/v) nonfat dried milk. The washing steps were repeated, and subsequently enhanced chemoluminescence detection was performed according to the manufacturer's instructions (ECL, Amersham pharmacia biotech, Buckinghamshire, UK) on Hyperfilm-MP (Amersham International plc, Buckinghamshire, UK). Blots were then reprobed with β-actin antibody (Santa Cruz Biotechnologies, Santa Cruz, California, USA). For quantitative analysis, bands were detected by scanning densitometry, using a Desaga CabUVIS scanner and Desaga ProVilDoc software (Desaga, Wiesloch, Germany).

Statistics

All statistical analyses were performed using GraphPad Prism 4.01 (San Diego, California, USA). Analysis of variance (ANOVA) was performed when more than two groups were compared and, when significant ($P < 0.05$), multiple comparisons were performed with the Newman-Keuls test. If not otherwise stated, data are expressed as means ± SD from three independent experiments. P value < 0.05 was considered to be significant.

Results

Mesalazine reduces cell count, inhibits cell proliferation and affects cell cycle progression of colorectal cancer cells

First, we evaluated the effect of mesalazine on cell number and proliferation of Caco-2, HT-29 and HCT-116 cells. Cells were treated with increasing concentrations of mesalazine (10-50 mM) for 24, 48 and 72 h. As shown in Fig. 1 A, treatment of CRC cells with mesalazine for 48 h significantly reduced the cell count in a dose-dependent manner. Similar effects were obtained for 24 and 72 h and for the inhibition of cell proliferation, measured by the BrdU test (data not shown). To establish whether the reduction in cell growth and proliferation after mesalazine treatment of colon cancer cells was due to changes in cell cycle progression, HT-29 and HCT-116 cells were cultured in presence of the drug (40 mM) for 24 and 48 h, and cell cycle distribution was analysed by flow cytometry (Fig. 1 B). Both cell lines showed changes in the cell cycle profile within 48 h, with an apparent accumulation of cells in the G0/G1 phase.

Mesalazine up-regulates the expression and activity of PPARγ

As depicted in Fig. 2 A, stimulation of HT-29 cells with mesalazine (30-50 mM) for 48 h led to a concentration-dependent up-regulation of PPARγ protein expression. To evaluate receptor activity of PPARγ in HT-29 wild-type, dominant-negative PPARγ and empty-vector HT-29 cells, PPARγ activation was determined via a transcriptional factor assay (Fig. 2 B). Treatment of both HT-29 wild-type and empty-vector cells with mesalazine (30 mM) for 48 h increased the activity PPARγ. As expected, PPARγ activity was not affected in the dominant-negative PPARγ cell line. Similarly, treatment of Caco-2 wild-type and empty-vector cells with mesalazine (30 mM) provoked +1.4-fold increase of cytokeratin 20

expression (vs. control, $p < 0.01$, 48 h), a specific target gene of PPARγ activity in CRC cells (data not shown) (24). In contrast, the induction of cytokeratin 20 could not be observed in dominant-negative PPARγ mutant Caco-2 cells (data not shown).

PPARγ is partially involved in mesalazine-induced inhibition of cell proliferation

In order to elucidate a potential role for PPARγ in mesalazine-induced inhibition of cell proliferation and changes in cell cycle distribution, the effect of the drug was studied in dominant-negative PPARγ colon cancer cells. Mesalazine (50 mM) treatment led to a significant decrease in cell proliferation in both HT-29 wild-type (-50%, $p < 0.001$) and empty-vector cells (-49%, $p < 0.001$) after 72 h of treatment (Fig. 3 A). In contrast, in the dominant-negative PPARγ cell line, the decrease in cell proliferation was partially reversed (-34%, $p < 0.001$, vs. wild-type and empty-vector). Similar effects were obtained in the cell line Caco-2 (data no shown). In dominant-negative PPARγ HT-29 cells, change in cell cycle distribution in response to mesalazine (40 mM) was partially neutralised (data no shown). Nevertheless, statistical significant effects compared to the wild-type were not reached.

Mesalazine down-regulates the expression of c-Myc in a PPARγ-independent mechanism

To study further molecular mechanisms responsible for mesalazine's growth-inhibitory effects, expression of the oncoprotein c-Myc after incubation with the drug was determined. c-Myc is known to modulate a broad range of biological activities including cell proliferation and growth (25). Stimulation of HT-29 wild-type, dominant-negative PPARγ and empty-vector (data not shown) cells with mesalazine (40 mM) for 4 h decreased the expression of c-Myc, indicating that PPARγ does not contribute to the down-regulation of the oncoprotein in response to the drug (Fig. 3 B).

PPARγ is involved in mesalazine-mediated up-regulation of the tumor suppressor gene PTEN

The tumor suppressor gene PTEN modulates several cellular functions, including cell survival signalling (26). In particular, it is well established that PTEN decreases cell proliferation through cell-cycle arrest in the G0/G1 phase and induces apoptosis via the caspase-cascade (27, 28). Treatment of HT-29 wild-type and empty-vector (data not shown) cells with mesalazine (40 mM) up to 8 results in a significant increase of PTEN protein expression (Fig. 3 C). In dominant-negative PPARγ HT-29 cells, the effect of mesalazine on the expression of PTEN was annihilated (Fig. 3 C).

Mesalazine-induced up-regulation of caspase-3 activity by the caspase-8 signalling pathway occurs via PPARγ

Challenge of both, Caco-2 wild-type and empty-vector cells, with mesalazine (30 mM) resulted in increased cleavage of the nuclear poly (ADP-ribose) polymerase (PARP) protein after 48 h (+2.2-fold, $p < 0.001$), a marker for caspase-3 activity (Fig. 4 A). In contrast, up-regulation of cleaved PARP protein expression in response to mesalazine (30 mM) was found to be attenuated in dominant-negative PPARγ mutant Caco-2 cells. Analogue effects could be observed in HT-29 wild-type, dominant-negative PPARγ and empty-vector cells after mesalazine treatment (data not shown). Direct determination of caspase-3 activity with a fluorometric immunosorbent enzyme assay (Fig. 4 B) showed a similar pattern of results as compared to the cleavage of PARP (Fig. 4 A). Incubation with mesalazine resulted in a significant increase in caspase-3 activity after 48 h both, in Caco-2 wild-type and empty vector cells (+1.4-fold, $p < 0.001$). In contrast, up-regulation of caspase-3 activity in

response to mesalazine was found to be almost reversed in PPARγ dominant-negative Caco-2 cells. Comparable results were also obtained in the cell line HT-29 (data not shown).

To further unravel the responsible elements in the caspase pathway leading to increased caspase-3 activity after mesalazine treatment, cleavage of caspase-8 and -9 were examined. Incubation with mesalazine (30 mM) for 48 h led to elevated cleavage of caspase-8 (+1.5-fold, $p < 0.01$) in Caco-2 wild-type and empty-vector cells. In contrast, no cleavage of caspase-9 in response to mesalazine could be observed. In dominant-negative PPARγ Caco-2 cells, mesalazine-mediated (30 mM) cleavage of caspase-8 was also reversed.

PPARγ controls the expression of inhibitor of apoptosis proteins after mesalazine treatment

To specify the molecular mechanism of mesalazine-induced apoptosis, changes in protein levels of the inhibitor of apoptosis proteins (IAP), survivin (Fig. 5) and X linked inhibitor of apoptosis (Xiap) (Fig. 6) were examined. Levels of survivin (5 A) and Xiap (6 A) were reduced in HT-29 wild-type and empty-vector cells in response to mesalazine after 48 h. In contrast, mesalazine-mediated down-regulation of both IAP family members was completely neutralized when incubated in HT-29 dominant-negative PPARγ mutant cells. A similar pattern of results was obtained in the cell line Caco-2 (5 B, 6 B).

Discussion

The increased risk for the development of CRC in patients suffering from UC and Crohn's colitis remains a significant problem in the long-term management of IBD (1). One of the first candidate chemopreventive drugs that has specific relevance in IBD patients seems to be mesalazine, an anti-inflammatory drug which has been used extensively in the treatment of IBD for more than 50 years (29). Several retrospective studies have suggested that long-term use of 5-ASA in IBD patients may significantly reduce the risk for the development of CRC by decreasing cell proliferation and inducing apoptosis (4, 9). However, despite comprehensive clinical and experimental experience with the drug, the mechanisms leading to its pro-apoptotic actions are largely unknown. Unravelling the signalling pathways behind these effects could deliver insights into mesalazine's mode of action in colon cancer prevention.

There is increasing evidence supporting the role of PPARγ as a regulator of proliferation and a modulator of cell growth (21, 30). Recent studies demonstrate that activators of PPARγ suppress the growth response of colon cancer cells (19, 20). Thus, inhibition of cell growth is partly due to the induction of apoptosis upon PPARγ activation. In addition, several reports describe not only an induction of caspase-3 by PPARγ ligands in a variety of cancer cells (31, 32), but also an association between loss of function mutations of PPARγ and the development of CRC (33). These observations raised the possibility that somatic mutations of PPARγ contribute to the carcinogenic process and the receptor functions as a tumor suppressor gene by inhibiting cell growth and inducing apoptosis.

Recent data showed that PPARγ was the major functional receptor mediating the common mesalazine activities in IBD and have corroborated involvement of the receptor in controlling intestinal inflammation (14). Mesalazine has been identified to act as an agonist

of PPARγ and was shown to up-regulate the expression and activity of the receptor in HT-29 cells (14), which is in accordance to the findings in the present study. Moreover, PPARγ has also been proposed to mediate the anti-proliferative and pro-apoptotic effects of mesalazine (10). In the present study, we shed light on PPARγ as a possible key target in mesalazine-mediated anti-carcinogenic abilities. For that purpose, mesalazine-induced anti-proliferative effects and pro-apoptotic actions along the caspase signalling cascade were examined in colonocytes transfected with a dominant-negative PPARγ vector to inhibit wild-type PPARγ receptor action (23). The therapeutic effect of 5-ASA depends more on the direct contact of the molecule with the colonic epithelium than to tissue concentration in the colon (14). It has been demonstrated that stool concentrations in patients conventionally treated with 5-ASA are in the median order of 30 mM, ranging from 10 to 100 mM (14). These concentrations have been demonstrated *in vivo* to modulate several cellular functions including inhibition of cell proliferation (13, 14). Hence, the concentrations used in the present study are clinically and biological relevant.

In our *in vitro* model, mesalazine decreased cell proliferation of colonocytes in a time- and dose-dependent manner. These anti-proliferative abilities were partially reversed in the PPARγ dominant-negative cell lines. However, the inhibition was not complete, indicating that other mechanisms besides PPARγ are involved in mesalazine's anti-proliferative effects. The anti-proliferative actions of mesalazine on colon carcinoma cell lines are in line with several *in vivo* and *in vitro* studies (9, 13, 34, 35). In addition, PPARγ agonists like rosiglitazone and troglitazone have been shown to decrease proliferation of colon cancer cell lines, merely mediated in part by a PPARγ-dependent mechanism (36, 37). Moreover, our data clearly demonstrate that mesalazine induced a G0/G1 arrest in colon cancer cells which is in line with the robust G1 arrest of other non-steroidal anti-inflammatory drugs (NSAIDs) *e.g.* indomethacin, sulindac and direct PPARγ agonists *e.g.*

rosiglitazone (38-40). Our results differ from the observations of former studies illustrating a S- or G2-Phase arrest in response to mesalazine, however, the authors could not reasonably explain the discrepancy compared to other NSAIDs (13, 35).

To unravel further mechanisms contributing to mesalazine's growth-inhibitory abilities in colonocytes, expressions of the oncoprotein c-Myc and of the tumor suppressor gene PTEN were determined. c-Myc is overexpressed in nearly 70% of CRC. Moreover, dysplasia of colonocytes in UC is also associated with increased expression of the oncoprotein (19, 25). c-Myc is known to modulate a broad range of biological activities including cellular proliferation and cell growth (19, 25). Deregulated c-Myc has been shown to increase apoptosis, genomic instability and to block differentiation (19, 25). In colonocytes including HT-29, it was recently shown that mesalazine decreased the expression of c-Myc (25). Although several PPARγ agonists inhibit the expression of the oncoprotein (19, 41), the effects seem to be PPARγ-independent because no peroxisome proliferator response element (PPRE) has been found in its promoter (41). Therefore, it is not surprising that a PPARγ-independent down-regulation of c-Myc in response to mesalazine was obtained in our *in vitro* setting which seems to contribute to the modulation of the PPARγ-independent growth-inhibitory effects caused by the drug.

PTEN is a tumor suppressor gene involved in the regulation of cell survival signalling through the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) pathway (42). PI3K/Akt signalling has been shown to be required for an extremely diverse array of cellular activities mainly involved in cell growth, proliferation apoptosis and survival mechanisms (42, 43). Activated Akt protects cell from apoptotic death by inactivating compounds of the cell-death machinery such as procaspases (42). PTEN exercises its role as a tumor suppressor by antagonising the PI3K/Akt pathway (42). In CRC cells, enforced expression of PTEN, *e.g.* by NSAIDs, has been demonstrated to decrease cell proliferation through cell-

cycle arrest in the G0/G1 phase and to activate the caspase cascade (28, 43, 44). Stimulation of HT-29 wild-type cells with mesalazine in our *in vitro* model increased the expression of PTEN. In contrast, in the dominant-negative PPARγ cell line, the effect of mesalazine on the expression of PTEN was reversed. Besides the ability of PPARγ agonists to up-regulate the expression of PTEN, confirmed by inhibitor and antisense experiments, two PPREs in the genomic sequence upstream of the tumor suppressor gene have been found (26, 45), supporting the findings of our investigations. The PPARγ-dependent increase of PTEN caused by mesalazine in our experiments not only indicates that the tumor suppressor gene contributes to the growth-inhibitory activities of the drug, but also may trigger its pro-apoptotic actions.

In addition to the contribution of PPARγ in mesalazine-induced growth inhibition, our data provides insight into the molecular mechanisms leading to activation of the caspase cascade, demonstrated directly by increased caspase-3 activity and indirectly by augmented levels of cleaved PARP. Our findings are supported by former studies demonstrating increased cleavage of PARP and caspase-3 activity after mesalazine treatment in HT-29 and colo205 cells, respectively (13, 34). Caspase-3 can be activated via two signalling pathways, the extrinsic pathway and the intrinsic trail, initiated by caspase-8 or caspase-9 signalling, respectively (46). The increase in levels of cleaved caspase-8 by mesalazine in the present study indicates that the extrinsic signalling pathway takes part in caspase-3 activation. Both caspase-dependent and caspase-independent mechanisms have been reported for mesalazine's apoptotic processes, indicating that the drug activates multiple cell death signalling pathways, *e.g.* caspase-9, Bcl-2, intestinal sphingomyelinase and the induction of intracellular peroxides (34, 47). Moreover, in our *in vitro* model mesalazine significantly decreased the protein levels of survivin and Xiap, two anti-apoptotic proteins known to potently block caspase-3, thereby inhibiting apoptosis (46). Accordingly, a similar down-

regulation of IAPs has not only been demonstrated for sulindac, but also for PPARγ ligands *e.g.* pioglitazone in colorectal carcinoma cells (36, 48). Mesalazine-mediated effects on both pro-apoptotic and anti-apoptotic markers were almost reversed in PPARγ dominant-negative cells. Taken together, these results indicate the involvement of PPARγ in mesalazine-mediated apoptosis via activating the caspase cascade. The underlying mechanism by which PPARγ controls the caspase signalling pathways are not fully explained. It may be speculated that the receptor regulates the cascade via modulation of the IAPs by a spermidine/spermidine N^1-acetyltransferase-dependent mechanism (49, 50).

In conclusion, this study provides evidence for the involvement of PPARγ-dependent and -independent mechanisms, responsible for mesalazine's pro-apoptotic and anti-proliferative abilities which appear to be triggered at least in part by the modulation of PTEN and c-Myc, respectively. In addition, activation of caspase-8 and down-regulation of Xiap and survivin contribute to elevated caspase-3 activity caused by mesalazine. Revealing this transduction pathway is not only important to understand the fundamental biological processes, but also may provide new opportunities in the chemoprevention of IBD.

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Competing Interests

The authors of this study have no conflict of interest or any financial disclosures to make.

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Abbreviations

BrdU, Bromodeoxyuridine

COX, Cyclooxygenase

CRC, Colorectal cancer

DMEM, Dulbecco's modified Eagle's medium

ECACC, European Collection of cell cultures

FCS, Foetal calf serum

IAP, Inhibitor of apoptosis protein

IBD, Inflammatory bowel disease

NSAID, Non-steroidal anti-inflammatory drug

PARP, Poly(ADP-ribose) Polymerase

PBS, Phosphate buffered saline

PI3K/Akt, Phosphatidylinositol 3-kinase/Akt

PPARγ, Peroxisome proliferator-activated receptor γ

PPRE, Peroxisome proliferator response element

TBS-T, Tris-buffered saline containing 0.05% Tween 20

TNF, Tumor necrosis factor

UC, Ulcerative colitis

Xiap, X linked inhibitor of apoptosis

Figure Legends

Fig. 1: (A) Cell counts of Caco-2, HT-29 and HCT-116 cells after incubation with increasing concentrations of mesalazine (10-50 mM) for 48 compared to control. n = 8, p value < 0.001 (vs. control) was obtained for all concentrations and all cell lines. (B) Cell cycle distribution in mesalazine-treated cells. HT-29 and HCT-116 cells were treated with mesalazine (40 mM) up to 48 h. After staining with propidium iodide, the cellular DNA content was visualized by flow cytometry, and the cell cycle distribution was analysed.

Fig. 2: (A) Western blot for PPARγ expression after treatment of HT-29 wild-type cells with mesalazine (30-50 mM) for 48 h. One representative blot of three independent experiments is shown. (B) Effect of mesalazine (30 mM) on PPARγ transcriptional activity in HT-29 wild-type, PPARγ dominant-negative and empty-vector cells after 48 h of stimulation. ***p < 0.001, n.s. = not significant.

Fig. 3: (A) Cell proliferation measurement of HT-29 wild-type, dominant-negative PPARγ and empty-vector cells after incubation with mesalazine (50 mM) for 72 h compared to control. n = 8, ***p < 0.001, n.s. = not significant (B) Western blot for the expression of c-Myc after treatment of HT-29 cells with mesalazine (30 mM) for 4 h. One representative blot of three independent experiments is shown. (C) Western blot for the expression of the tumor suppressor PTEN in HT-29 wild-type and dominant-negative PPARγ cells in response to mesalazine (40 mM) up to 8 h. One representative blot of three independent experiments is shown.

Fig. 4: (A) Effect of mesalazine (30 mM) on cleaved PARP protein expression after 48 h of treatment in Caco-2 cells used as wild-type, dominant-negative PPARγ mutant and empty-vector cultures. One representative western blot of three independent experiments is shown. The band at 89 kDa corresponds to the cleaved PARP protein. Densitometric data are corrected for β-actin levels. ***$p < 0.001$, n.s. = not significant (B) Caspase-3 activity in Caco-2 wild-type, dominant-negative PPARγ mutant and empty-vector cells measured via fluorometric immunosorbent enzyme assay after stimulation with mesalazine (30 mM) for 48 h. n = 6, ***$p < 0.001$, n.s. = not significant

Fig. 5: (A) Effect of mesalazine (30 mM) on the expression of survivin in HT-29 wild-type, dominant-negative PPARγ mutant and empty-vector cells after incubation for 48 h. One representative western blot of three independent experiments is shown. Quantitative data are normalized for β-actin levels. *$p < 0.05$, n.s. = not significant (B) Effect of mesalazine (30 mM) on the expression of survivin in Caco-2 wild-type, dominant-negative PPARγ mutant and empty-vector cells after incubation for 48 h. One representative western blot of three independent experiments is shown. Densitometric data are corrected for β-actin levels. ***$p < 0.001$, n.s. = not significant

Fig. 6: (A) Effect of mesalazine (30 mM) on the expression of Xiap in HT-29 wild-type, dominant-negative PPARγ mutant and empty-vector cells after incubation for 48 h. One representative western blot of three independent experiments is shown. Densitometric data are corrected for β-actin levels. ***$p < 0.001$, n.s. = not significant (B) Effect of mesalazine (30 mM) on the expression of Xiap in Caco-2 wild-type, dominant-negative PPARγ mutant and empty-vector cells after incubation for 48 h. One representative western blot of three

independent experiments is shown. Densitometric data are corrected for β-actin levels. ***$p < 0.001$, n.s. = not significant

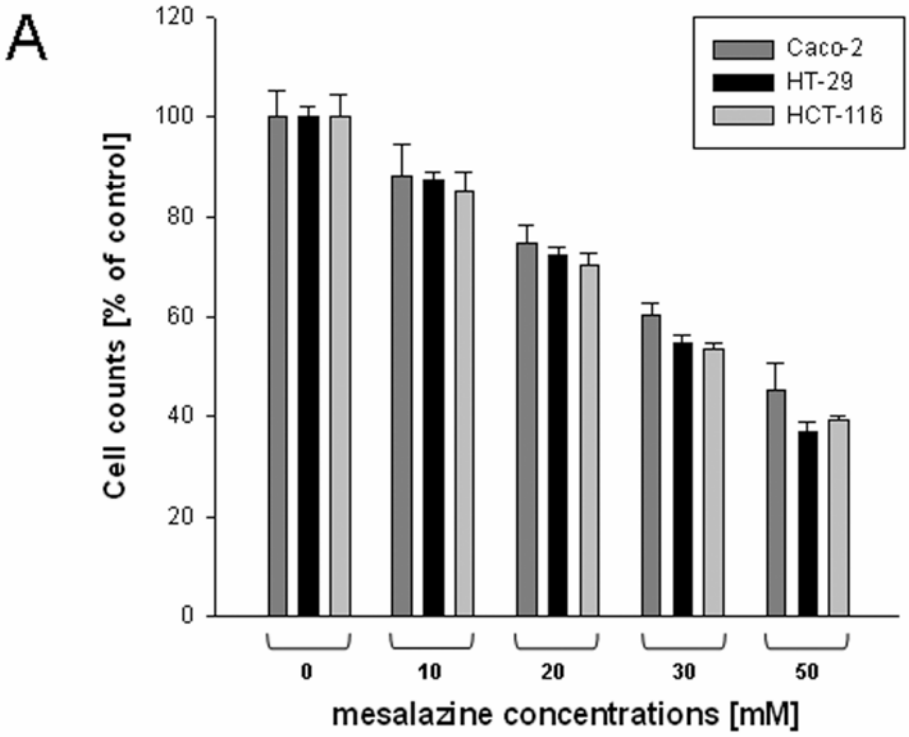

A
120
100
80
60
40
20
0
Cell counts [% of control]
Caco-2
HT-29
HCT-116
0
10
20
30
50
mesalazine concentrations [mM]

Figure 1

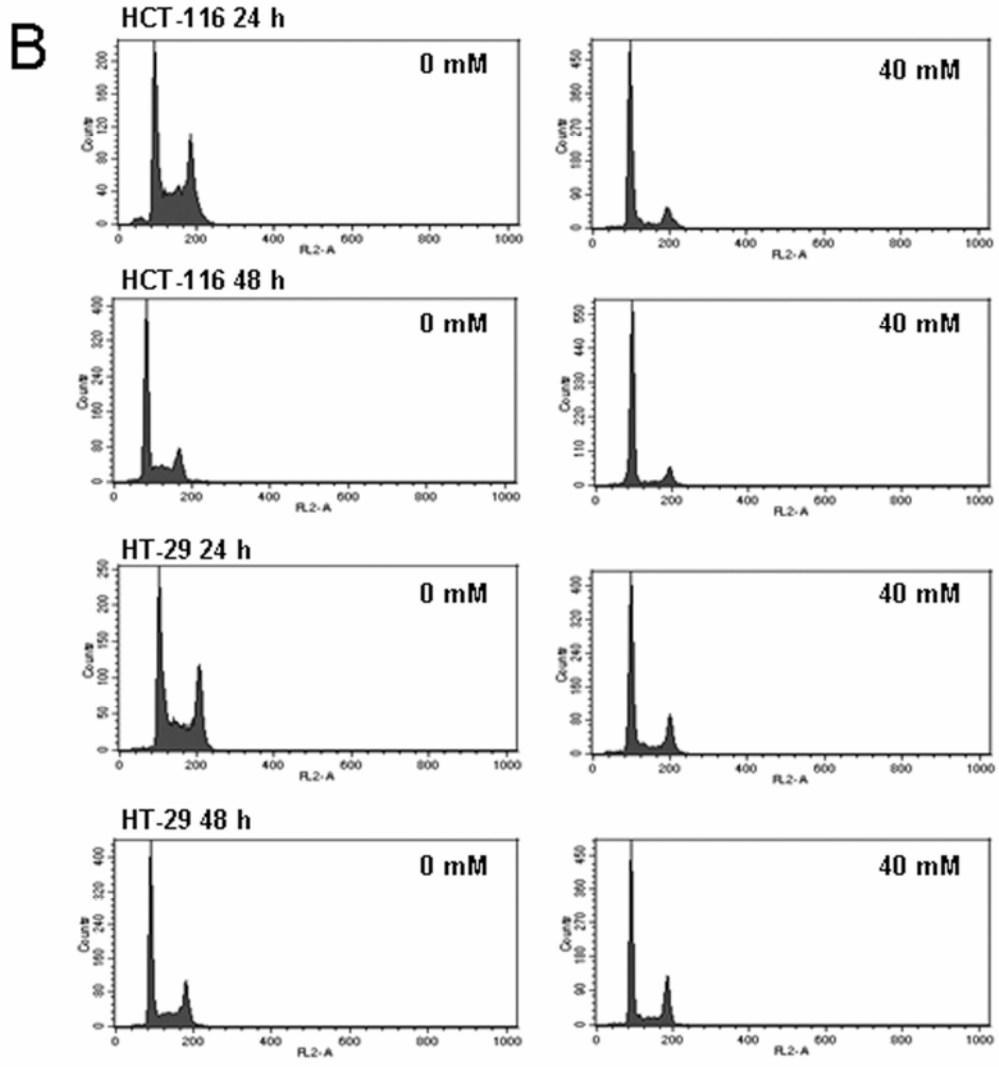

B
HCT-116 24 h
0 mM
40 mM
HCT-116 48 h
0 mM
40 mM
HT-29 24 h
0 mM
40 mM
HT-29 48 h
0 mM
40 mM
Counts
FL2-A

Figure 2

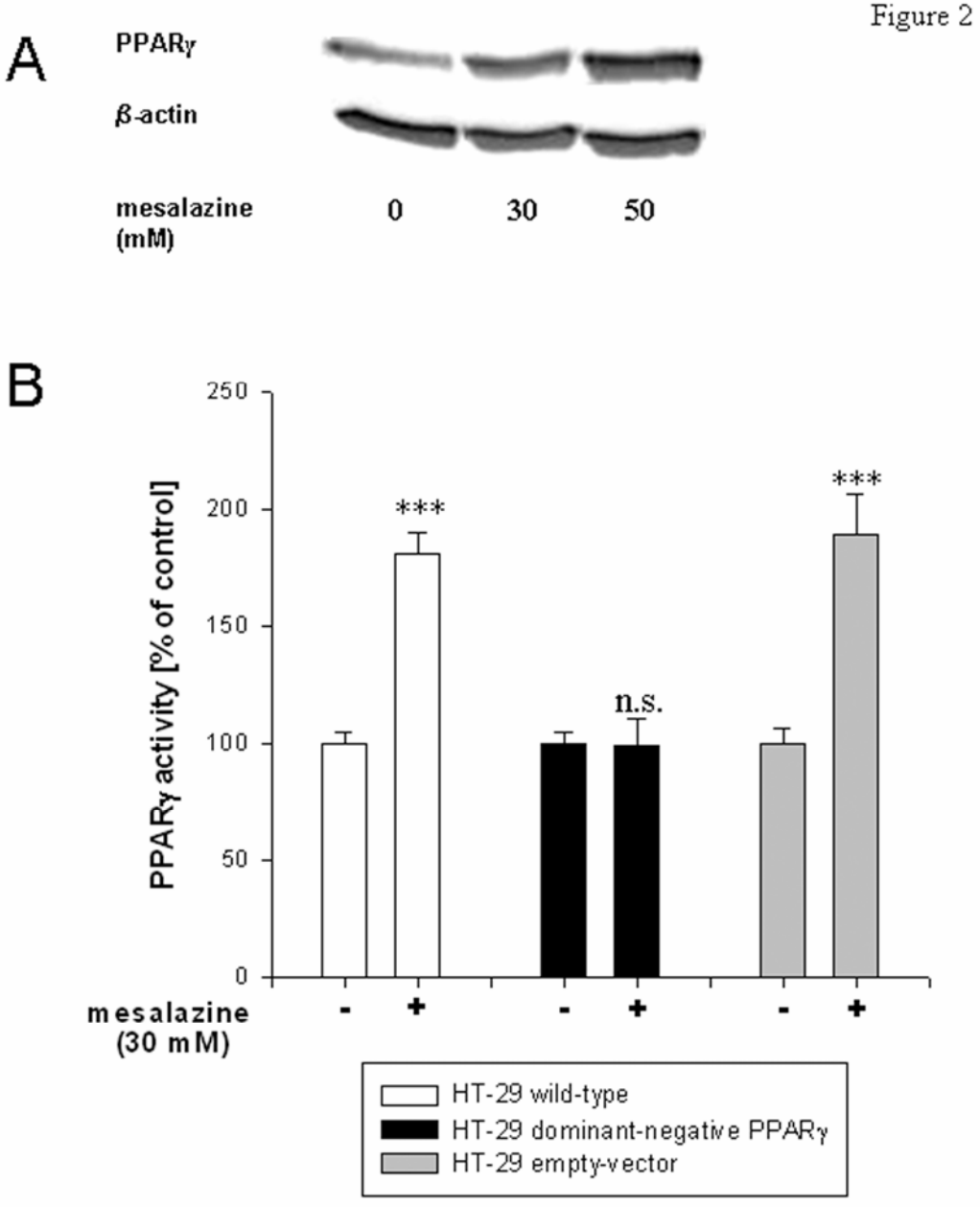

A
PPARγ
β-actin
mesalazine (mM)
0
30
50
B
PPARγ activity [% of control]
250
200
150
100
50
0
***
n.s.
***
mesalazine (30 mM)
-
+
-
+
-
+
HT-29 wild-type
HT-29 dominant-negative PPARγ
HT-29 empty-vector

Figure 3

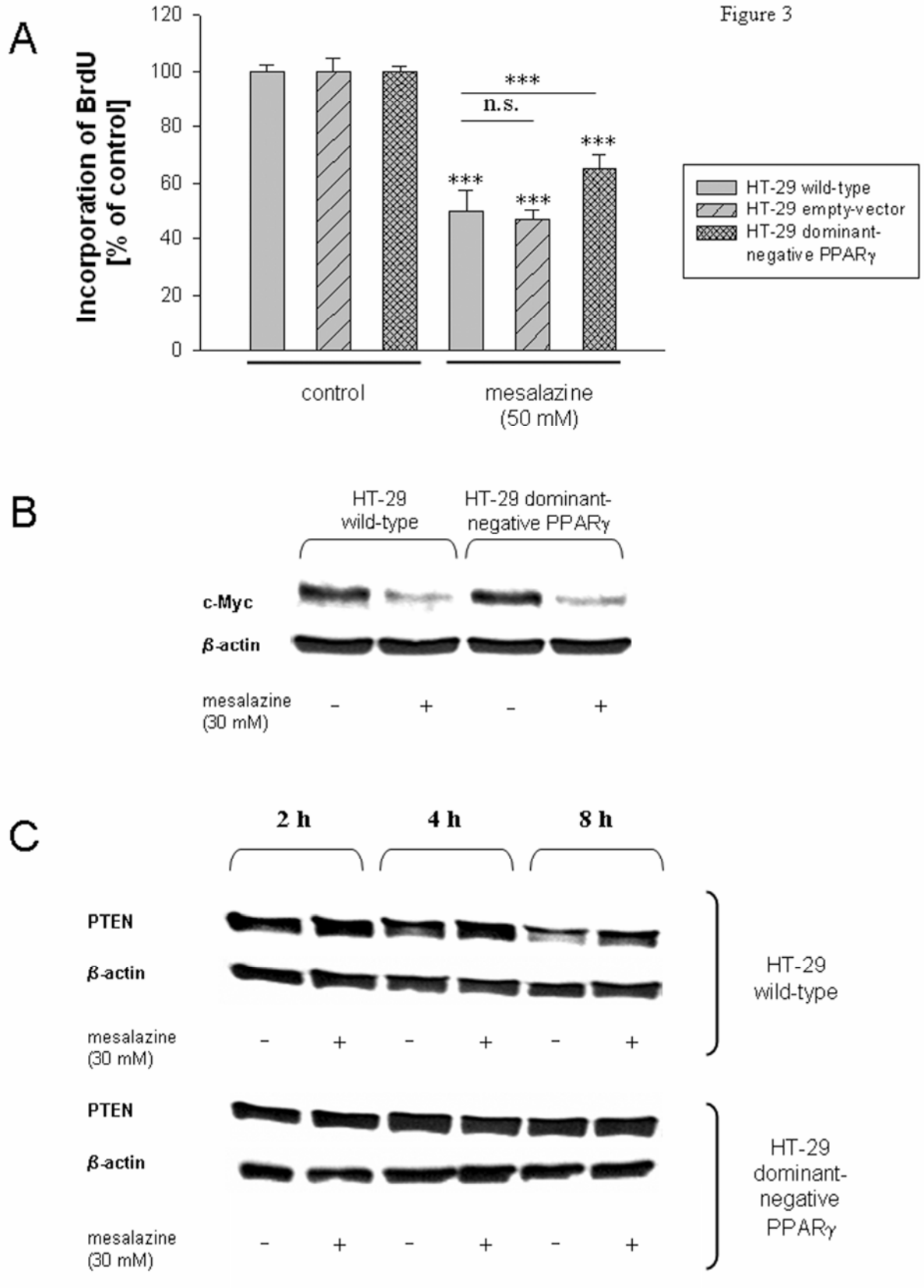
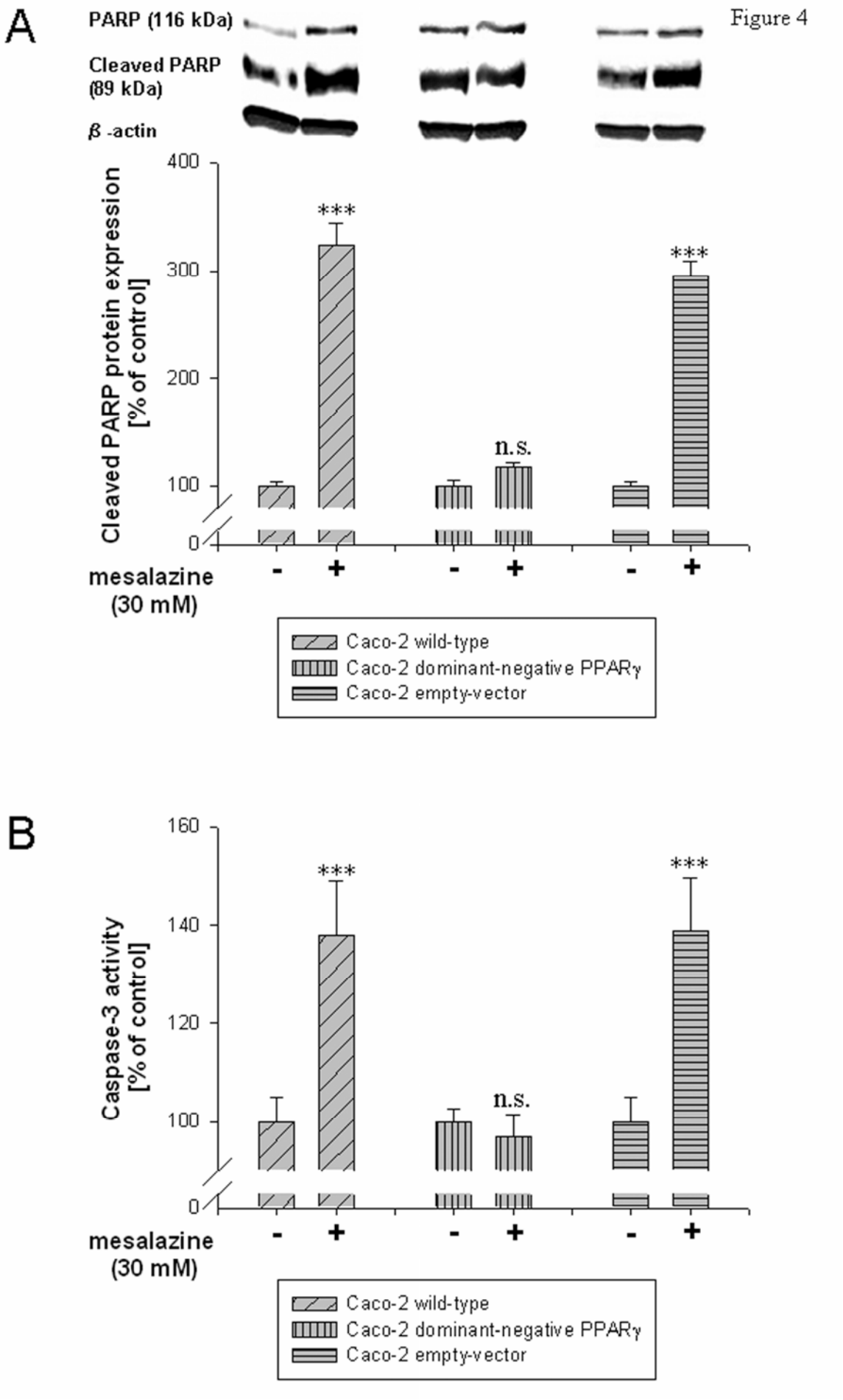
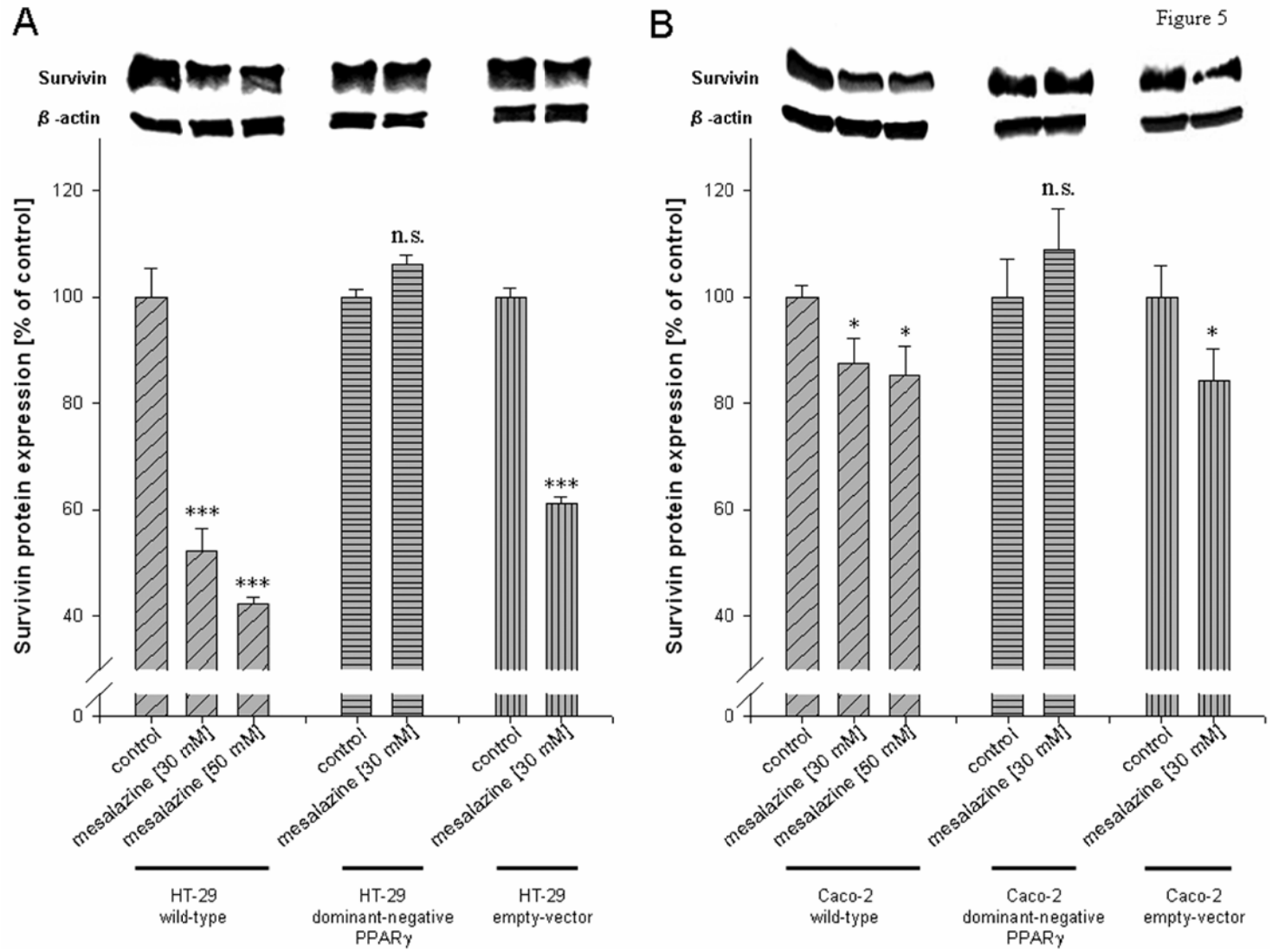
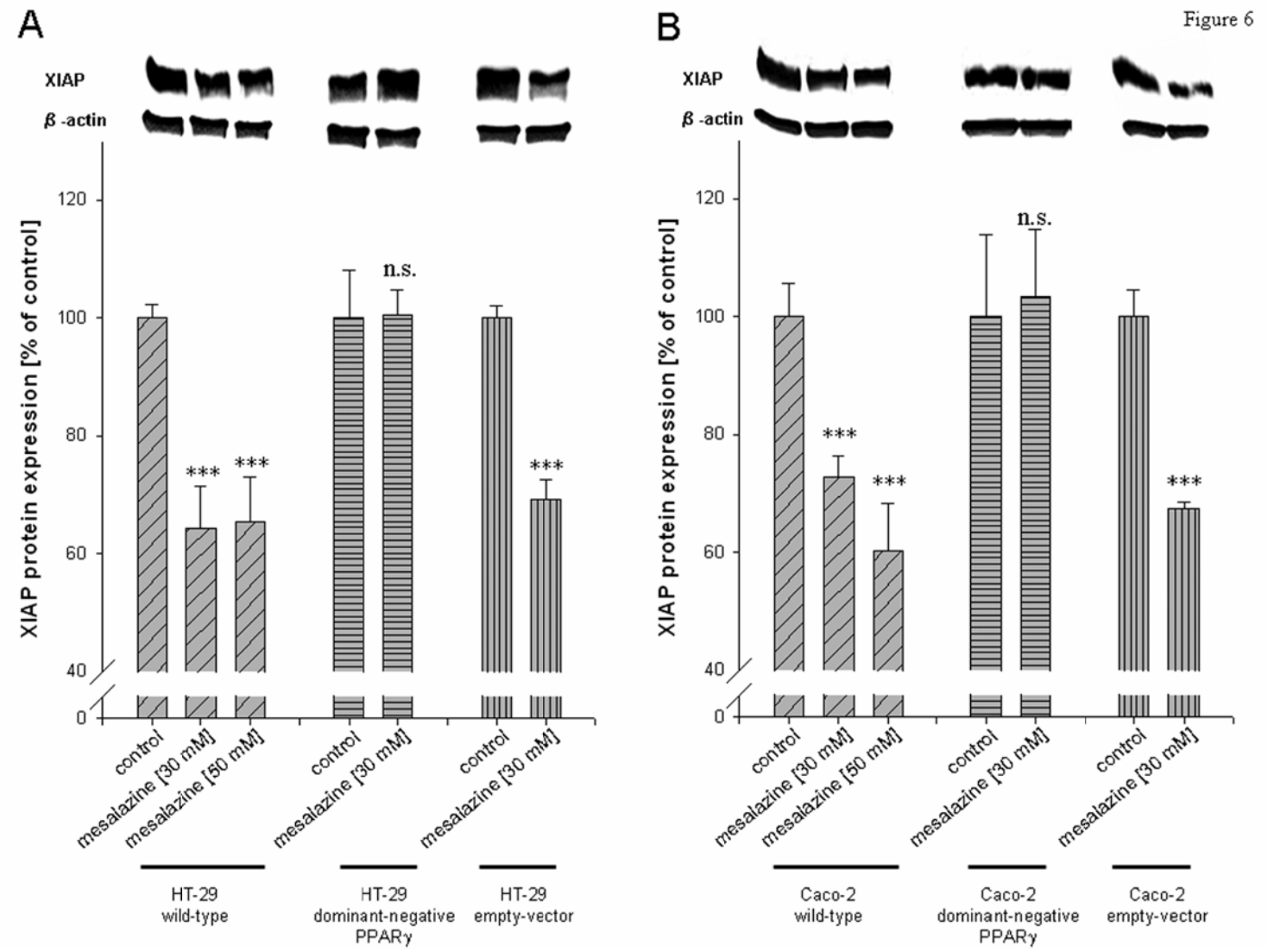
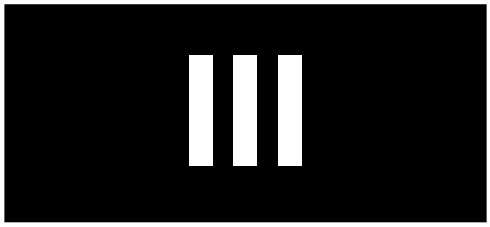

A
Incorporation of BrdU
[% of control]
120
100
80
60
40
20
0
***
n.s.
***
***
***
control
mesalazine
(50 mM)
HT-29 wild-type
HT-29 empty-vector
HT-29 dominant-negative PPARγ
B
HT-29
wild-type
HT-29 dominant-negative PPARγ
c-Myc
β-actin
mesalazine
(30 mM)
- + - +
C
2 h
4 h
8 h
PTEN
β-actin
mesalazine
(30 mM)
- + - + - +
HT-29
wild-type
PTEN
β-actin
mesalazine
(30 mM)
- + - + - +
HT-29
dominant-negative
PPARγ

Figure 4


A
PARP (116 kDa)
Cleaved PARP (89 kDa)
β -actin
Cleaved PARP protein expression [% of control]
400
300
200
100
0
***
***
n.s.
mesalazine (30 mM)
-
+
-
+
-
+
Caco-2 wild-type
Caco-2 dominant-negative PPARγ
Caco-2 empty-vector
B
Caspase-3 activity [% of control]
160
140
120
100
0
***
***
n.s.
mesalazine (30 mM)
-
+
-
+
-
+
Caco-2 wild-type
Caco-2 dominant-negative PPARγ
Caco-2 empty-vector


A
Survivin
β -actin
Survivin protein expression [% of control]
120
100
80
60
40
0
***
***
n.s.
***
control
mesalazine [30 mM]
mesalazine [50 mM]
control
mesalazine [30 mM]
control
mesalazine [30 mM]
HT-29
wild-type
HT-29
dominant-negative
PPARγ
HT-29
empty-vector
B
Survivin
β -actin
Survivin protein expression [% of control]
120
100
80
60
40
0
*
*
n.s.
*
control
mesalazine [30 mM]
mesalazine [50 mM]
control
mesalazine [30 mM]
control
mesalazine [30 mM]
Caco-2
wild-type
Caco-2
dominant-negative
PPARγ
Caco-2
empty-vector


A
XIAP
β -actin
XIAP protein expression [% of control]
120
100
80
60
40
0
***
***
n.s.
***
control
mesalazine [30 mM]
mesalazine [50 mM]
control
mesalazine [30 mM]
control
mesalazine [30 mM]
HT-29
wild-type
HT-29
dominant-negative
PPARγ
HT-29
empty-vector
B
XIAP
β -actin
XIAP protein expression [% of control]
120
100
80
60
40
0
***
***
n.s.
***
control
mesalazine [30 mM]
mesalazine [50 mM]
control
mesalazine [30 mM]
control
mesalazine [30 mM]
Caco-2
wild-type
Caco-2
dominant-negative
PPARγ
Caco-2
empty-vector



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Role of nuclear hormone receptors in butyrate-mediated up-regulation of the antimicrobial peptide cathelicidin in epithelial colorectal cells

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Abstract

Background and aims: The human cathelicidin (LL-37) is one of the major antimicrobial peptides of the non-specific innate immune system in the intestinal tract. Altered expression has been associated with gastrointestinal disease. Recent studies demonstrated that butyrate induces LL-37 mRNA in colonic epithelial cells, however the underlying molecular mechanisms have not been elucidated. The objective of this study was to investigate the regulatory pathways involved in butyrate-induced up-regulation of LL-37.

Methods and results: Treatment of Caco-2 and HT-29 cells with butyrate led to a time-dependent up-regulation of LL-37 mRNA expression as determined by semi-quantitative RT-PCR. Up-regulation of LL-37 mRNA by butyrate was subsequently followed by an increase in LL-37 protein expression as observed by immunofluorescence. Co-incubation of butyrate with a VDR, p38 MAPK, ERK 1/2 and TGF-β1 receptor kinase inhibitor all reduced butyrate-mediated LL-37 mRNA up-regulation. In contrast, transfection of Caco-2 cells with a dominant-negative PPARγ mutant vector did not affect butyrate-mediated up-regulation of LL-37 mRNA.

Conclusion: Our results clearly demonstrate that butyrate-mediated up-regulation of LL-37 is influenced by several signalling pathways and receptors including MAPKs as well as VDR and TGF-β1, but not by PPARγ. These data may provide new opportunities in the treatment of gastrointestinal diseases.

Keywords: Butyrate; Cathelicidin; Colon; Innate immunity; LL-37; MAPK; PPARγ; TGF-β1; VDR

1. Introduction

Antimicrobial peptides, such as cathelicidins, are active defence molecules in the innate immune system. Several studies confirm their importance at epithelial surfaces as immediate barrier effectors in preventing gastrointestinal infections (O'Neil et al., 1999; Schauber et al., 2006; Schmid et al., 2004).

Cathelicidins constitute a family of cationic precursor proteins with a well-conserved cathelin pro-region, followed by a highly variable C terminal antimicrobial domain (Bals, 2000; Eckmann, 2005). They are widespread in nature and found in many mammalian species (Sorensen, 2005). The only human cathelicidin gives rise to LL-37, a 37-amino acid mature antimicrobial peptide, after cleavage from the cathelin propart (De Smet and Contreras, 2005). LL-37 is present in neutrophils, lymphocytes and in epithelial cells of the testis, skin, respiratory and the gastrointestinal tract (Sorensen, 2005). In the colon, the protein is produced constitutively in differentiated surface and upper crypt epithelial cells. Its expression in these cells coincides with epithelial cell differentiation and is not up-regulated in response to inflammatory markers, which contrasts with the finding in keratinocytes (Hase et al., 2002). So far, the molecular mechanisms regulating cathelicidin expression remain largely unknown. Besides its antimicrobial activity,

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindol; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; ECACC, European collection of cell cultures; ERK, extracellular signal-regulated kinase; FCS, foetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IBD, Inflammatory bowel disease; JNK, cJun N-terminal kinase; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; PBS, phosphate buffered saline; PPARγ, peroxisome proliferator-activated receptor γ; SCFA, short chain fatty acid; TGF-β1, transforming growth factor β1; VDR, Vitamin D receptor; VDRE, Vitamin D response element

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cathelicidin exerts a variety of other functions such as chemoattraction of immune cells, degranulation of mast cells, stimulation of wound vascularisation and induction of antitumoral effects (De Smet and Contreras, 2005). Moreover, LL-37 binds and neutralizes lipopolysaccharide bioactivity and protects against endotoxic shock (De Smet and Contreras, 2005).

Altered levels of cathelicidin expression are observed in inflammatory bowel disease (IBD) and Shigellosis, indicating its important role in gastrointestinal host defence (Raqib et al., 2006; Schauber et al., 2006; Zasloff, 2006a). In IBD, increased levels of the antimicrobial peptide are measured in patients with ulcerative colitis but not in patients with Crohn's disease (Schauber et al., 2006). A triggering factor however could not be identified. In contrast, cathelicidin expression is suppressed in *Shigella* infections. Oral treatment with butyrate however, stimulated the release of the active peptide and promoted elimination of *Shigella* in a rabbit experimental model (Raqib et al., 2006).

Butyrate, a short chain fatty acid (SCFA) and normal constituent of the colonic luminal content, is formed by bacterial fermentation of undigested dietary fibre in the colon (Wachtershauser and Stein, 2000). Besides its role as the major energy source for the colonocyte (Wachtershauser and Stein, 2000), it exerts profound effects on the colonic physiology by influencing colonocyte proliferation and differentiation, gut motility and mucosal inflammation (Klurfeld, 1999). Moreover, recent studies demonstrated an immune modulating role for butyrate by inducing cathelicidin in human gastrointestinal cells (Hase et al., 2002; Schauber et al., 2003, 2004, 2006). Although the requirement of the mitogen-activated protein kinase (MAPK) pathway in this up-regulation has recently been indicated (Schauber et al., 2003; Zasloff, 2006b), the intracellular signalling pathways are mainly unknown.

In the present study we demonstrate for the first time the involvement of several signalling pathways and receptors like p38 MAPK, extracellular signal-regulated kinase 1/2 (ERK), Vitamin D receptor (VDR) and transforming growth factor β1 (TGF-β1) in butyrate-mediated induction of LL-37. On the contrary, the participation of the peroxisome proliferator-activated receptor γ (PPARγ) in this regulatory process could be excluded.

2. Materials and methods

2.1. Cell culture

Both human colorectal cancer cell lines Caco-2 and HT-29 were obtained from the European collection of cell cultures (ECACC). Cells were cultured in a humidified incubator at 37 °C in an atmosphere of 95% air and 5% CO_2. Caco-2 cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% foetal calf serum (FCS), 1% non-essential amino acids, 1% sodium pyruvate and 1% penicillin/streptomycin. HT-29 cells were grown in McCoy's 5A Medium, supplemented with 10% FCS and 1% penicillin/streptomycin. Medium of the dominant-negative PPARγ mutant and empty-vector Caco-2 cells was supplied with 400 μg/ml Geneticin 418 sulphate (G418, Gibco-BRL, Eggenstein, Germany). Cells were regularly screened for mycoplasma contamination using the VenorGem Mycoplasma detection kit (Minerva Biolabs, Berlin, Germany).

For experiments, cells were seeded in plastic cell culture wells and cultivated in DMEM and McCoy's 5A Medium, respectively, until 80% confluency was reached. Medium was then removed and replaced by medium containing either the solvent, butyrate (2 and 3 mM), or one of the combinations of butyrate (3 mM) with the Vitamin D receptor inhibitor ZK191732 (10 μM), the p38 MAPK inhibitor SB203580 (20 μM), the ERK1/2 inhibitor PD98059 (40 μM), or the TGF-β1 receptor kinase inhibitor SB431542 (10 μM), respectively. In these experiments, cells were pre-treated with the inhibitors for 5 h, followed by challenge of cells with butyrate for up to 48 h. Butyrate (Merck-Schuchardt, Hohenbrunn, Germany) was solubilized in phosphate buffered saline (PBS) and added to the medium. SB431542 (Sigma, Saint Louis, Missouri, USA) was dissolved in ethanol (Merck, Darmstadt, Germany), PD98059, SB203580 (both Calbiochem, Schwalbach/Taunus, Germany) and ZK191732 (supplied by the Department of Medicinal Chemistry at Schering AG, Berlin, Germany) were dissolved in dimethyl sulfoxide (DMSO, Fluka, Sigma–Aldrich–Chemie, Steinheim, Germany). Final maximal concentration of solvents in medium was kept below 0.1% (v/v). The medium was changed every day. Cells were then harvested at the times indicated in the figure legends.

2.2. Transfection assay

The following plasmids were used for transfection: pcDNA3 (Invitrogen, Karlsruhe, Germany), as an empty-vector for control transfection and the plasmid pcDNA3-PPARγ$_{L468A/E471A}$, a dominant-negative PPARγ double-mutant, that was kindly provided by VK Chatterjee (Department of Medicine, University of Cambridge, Addenbrooke's Hospital, Cambridge, United Kingdom) (Gurnell et al., 2000). These constructs were transfected into subconfluent Caco-2 cells with lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) in serum-free conditions. After 6 h, the cells were supplied with fresh medium containing 10% FCS. 24 h later, the cells were supplied with medium containing G418 (400 μg/ml) and culture medium supplemented with G418 was replaced twice a week. G418-resistant colonies were collected and used for further analysis.

2.3. Cytotoxicity

Cytotoxicity was excluded by lactate dehydrogenase (LDH) release assay using a commercial kit (LDH kit, Roche, Mannheim, Germany).

2.4. Immunofluorescence assay

Caco-2 cells were grown on glass chamber slides (Lab-Tek, Nunc, Rochester, New York, USA) and allowed to reach confluency. They were then treated with butyrate (3 mM) for 24 and 48 h. Cells were fixed in ice-cold ethanol (95%) for 10 min and permeabilized by addition of 0.1% Triton X-100

in PBS for 10 min. After washing, unspecific binding of antibodies was blocked by incubating cells in 8% horse serum in PBS containing 0.1% Tween-20 for 1 h at room temperature. LL-37 antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA) was diluted 1:100 in blocking buffer. After 1.5 h of incubation, cells were washed three times with PBS before secondary antibody (cy3 conjugated rabbit-anti-goat, Sigma, Saint Louis, Missouri, USA) was added for 45 min. Following washing and air-drying, the cells were embedded in 4′,6-Diamidino-2-phenylindol (DAPI) mounting medium (Vector Laboratories, Burlingame, California, USA) and evaluated by immunofluorescence microscopy (Eclipse E 600, Nikon, Tokyo, Japan) with a digital camera DX 20H (Kappa, Monrovia, California, USA).

2.5. mRNA isolation

Cells were cultivated in six-well plates and were treated at 80% confluency with butyrate, or the combination of butyrate and one of the inhibitors for 24 and 48 h, respectively. Total RNA was isolated from cells by RNA isolation reagent (TRIR, Abgene, United Kingdom), followed by phenol extraction and ethanol precipitation.

2.6. Semi-quantitative RT-PCR

RT-PCR was conducted with the Gene Amp RNA PCR kit (Applied Biosystems, Branchburg, New Jersey, USA) according to the manufacturer's protocol, starting from 1 μg total RNA. Amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as control. From the results of preliminary experiments, 20 PCR cycles for GAPDH and 35 cycles for LL-37 were selected as optimal amplification conditions to produce a log-linear relationship between the amount of each mRNA and intensity of PCR product. The PCR reaction contained 0.2 mM dNTPs (Invitrogen Corporation, Carlsbad, California, USA), 0.05 U μl^{-1} AmpliTaqGold DNA polymerase (Applied Biosystems, Branchburg, New Jersey, USA), 1.5 mM Mg_2Cl (Applied Biosystems, Branchburg, New Jersey, USA) and either 0.2 μmol/L primers of LL-37 or GAPDH, respectively (Biospring, Frankfurt, Germany). PCR cycling conditions for LL-37 were: initial denaturation at 94 °C for 1 min followed by annealing at 53 °C for 1 min, extension at 72 °C for 2 min and a final extension at 72 °C for 7 min after the last cycle. The respective annealing temperature for GAPDH was 45 °C. Primers for amplification were as follows: LL-37 sense 5′-cggtcctcggatgctaacctcta-3′, LL-37 antisense 5′-cctgggtacaagattccgcaaa-3′; GAPDH sense 5′-gcaccgtcaaggctgagaac-3′, GAPDH antisense 5′-ccaccaccctgttgctgtag-3′. The expected sizes of LL-37 and GAPDH were 357 and 803 base pairs, respectively. Aliquots of the PCR mixtures (10 μL) were analyzed by electrophoresis using a 1.5% agarose gel containing ethidium bromide and visualized by UV illumination.

For semi-quantitative analysis of amplified PCR products, the fluorescent dye PicoGreen (Molecular Probes, Eugene, Oregon, USA) was used according to the manufacturer's instructions. In brief, 2 μl of amplified DNA in 100 μl TE buffer were mixed with an equal volume of diluted PicoGreen reagent (1:200, v/v, in TE buffer). Samples were incubated for 5 min at room temperature and protected from light in a microtiter plate. The fluorescence was measured (λ_{ex} = 485 nm; λ_{em} = 538 nm) in the fluorescence microplate reader Tecan SpectraFluor PLUS. A λ DNA linear standard curve was applied for each experiment.

2.7. Statistics

All statistical analyses were performed using GraphPad Prism 4.01 (San Diego, California, USA). Analysis of variance (ANOVA) was performed when more than two groups were compared and, when significant, multiple comparisons were performed with the Tukey's test. If not otherwise stated, data are expressed as means ± S.D. from three independent experiments. A p value < 0.05 was considered to be significant.

3. Results

3.1. Butyrate up-regulates LL-37 mRNA in colorectal cancer cells in a time-dependent manner

Challenge of the colorectal carcinoma cell line Caco-2 with butyrate (3 mM) resulted in a time-dependent increase of LL-37 mRNA compared to control (Fig. 1) (24 h: +149% versus control, $p < 0.001$; 48 h: +489% versus control, $p < 0.001$). Similar results were observed in the HT-29 cell line. In these cells, LL-37 mRNA expression was augmented up to 338% ($p < 0.001$) after 24 h and even up to 770% ($p < 0.001$) after 48 h of treatment.

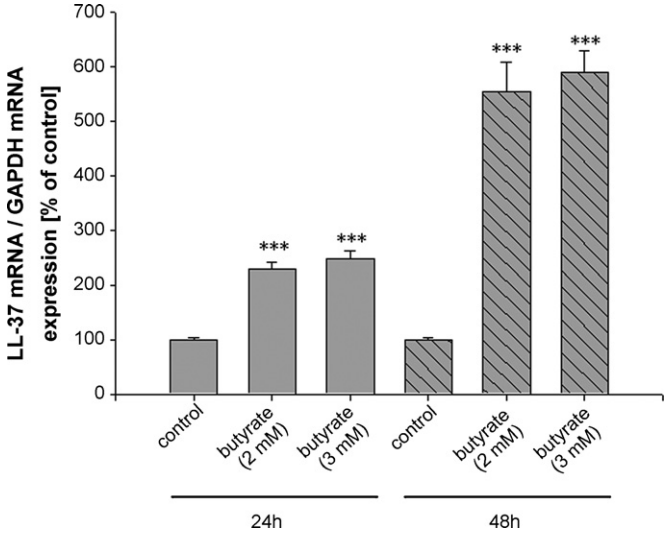

Fig. 1. Time-dependent effect of butyrate on LL-37 mRNA expression in Caco-2 cells after 24 and 48 h of treatment. LL-37 mRNA was performed by semiquantitative RT-PCR with the fluorescent dye Pico Green. All values for mRNA levels are normalized to corresponding mRNA amounts for GAPDH. Induction of LL-37 mRNA is displayed as relative percentage to solvent treated control cells. $^{***}p < 0.001$.

3.2. LL-37 protein exhibits a predominant cytoplasmatic localisation in Caco-2 cells

The subcellular localisation of LL-37 protein in Caco-2 cells was determined via immunofluorescence. Caco-2 cells were treated with butyrate (3 mM) for 24 and 48 h (Fig. 2). Microscopy analysis revealed that the natural antibiotic is predominantly expressed in the cytosol of Caco-2 cells, both in treated and control cells. Moreover, butyrate challenge led to increased staining of the cytoplasm, which is in line with the results obtained at the mRNA level.

3.3. The Vitamin D receptor is involved in butyrate-induced LL-37 mRNA expression

To determine a possible role of the VDR in butyrate-induced LL-37 mRNA expression, Caco-2 cells were treated with butyrate (3 mM) in presence of the VDR antagonist ZK 191732 for 24 h (Fig. 3A) and 48 h (Fig. 3B). The antagonistic characteristics of the inhibitor have been described previously by our group (Gaschott et al., 2001). Application of ZK 191732 significantly reversed the LL-37 induction already after 24 h (−54%, $p < 0.05$) of incubation and an even more pronounced effect was obtained at 48 h of treatment (−75%, $p < 0.001$).

3.4. MAPK and TGF-β1 play a pivotal role in butyrate-mediated LL-37 mRNA up-regulation

In addition, Caco-2 cells were incubated with butyrate (3 mM) in presence of the p38 MAPK, ERK1/2 or TGF-β1 receptor kinase inhibitor to investigate the involvement of TGF-β1 and intracellular signalling pathways in butyrate-regulated LL-37 mRNA expression.

Combined treatments of butyrate with the p38 MAPK inhibitor SB203580, the ERK1/2 inhibitor PD98059, and the TGF-β1 receptor kinase inhibitor SB431542 all reduced the induction of LL-37 mRNA expression caused by butyrate after 24 h of treatment (Fig. 4). Largest effects were obtained for co-incubation of butyrate with the p38 MAPK inhibitor SB203580.

3.5. PPARγ is not involved in butyrate-mediated LL-37 mRNA up-regulation

Butyrate is known to induce the expression of the transcription factor PPARγ. To investigate whether PPARγ plays a role in the up-regulation of LL-37 mRNA induced by butyrate, expression of LL-37 mRNA was examined in Caco-2 cells transfected with a dominant-negative PPARγ mutant (Fig. 5). This mutant still exhibits PPARγ DNA binding activity, but lacks transcriptional activation capacity (Gurnell et al., 2000). In this mutant

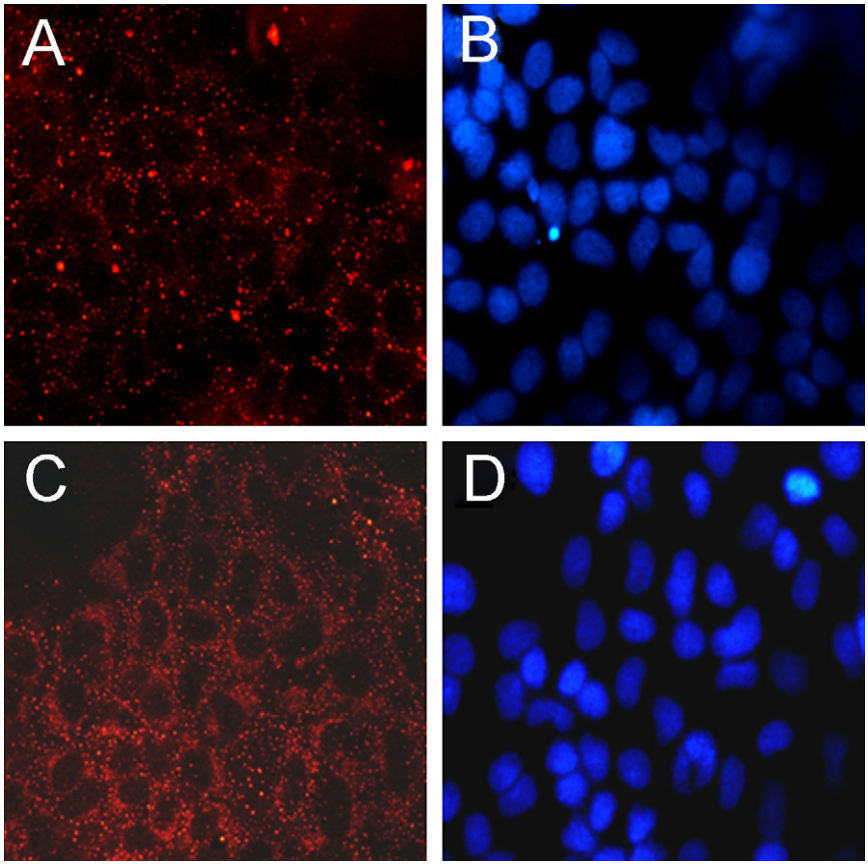

Fig. 2. Representative immunofluorescence pictures for LL-37 protein in Caco-2 cells. Confluent Caco-2 cells were challenged with butyrate (3 mM) for 48 h (C) or were grown in culture medium as control (A). (B and D) Nuclear counterstaining with DAPI mounting medium for each of the adjacent panels. Magnification, 400×.

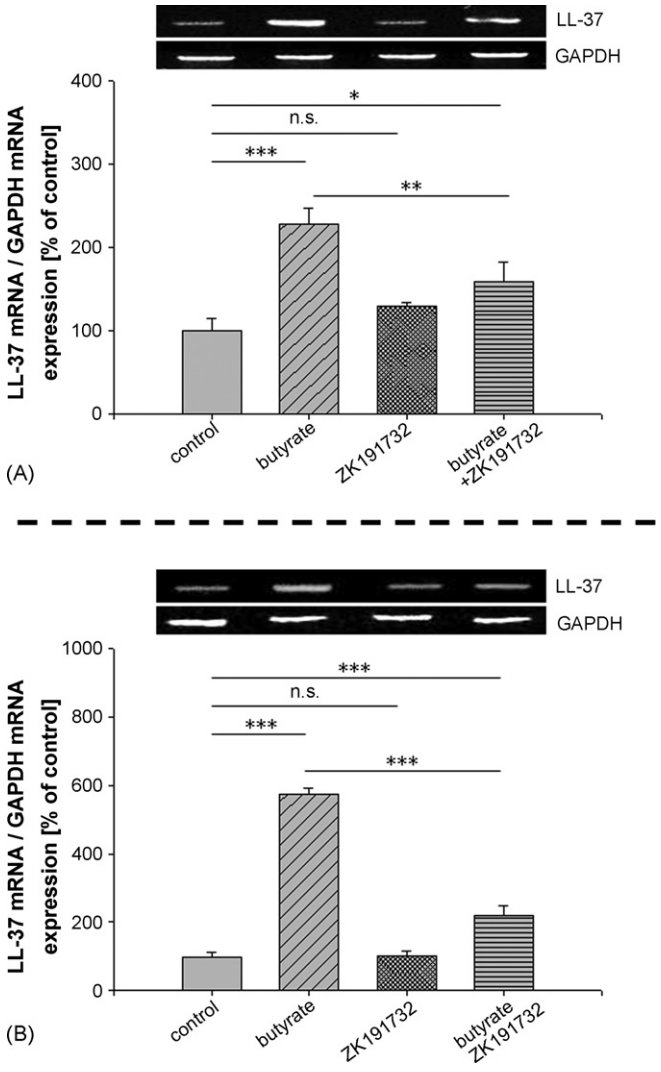

Fig. 3. LL-37 mRNA expression in Caco-2 cells. Medium was supplemented with (1) the solvent, or (2) butyrate (3 mM), or (3) the VDR inhibitor ZK191732 (10 μM), or (4) the combination of butyrate and ZK191732 for 24 (A) and 48 h (B). Semiquantitative analysis of PCR products were performed using Pico Green. All values for mRNA levels are normalized to corresponding mRNA amounts of the housekeeping gene GAPDH. One representative gel of three independent experiments is shown. Induction of LL-37 mRNA is displayed as relative percentage to solvent treated control cells. $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$; n.s.: not significant.

cell line, no statistical difference compared to wild-type Caco-2 cells in butyrate-induced (3 mM) LL-37 mRNA up-regulation could be observed.

4. Discussion

Cathelicidin is an important natural peptide antibiotic that actively contributes to the host bactericidal defence system at mucosal surfaces. Changes in cathelicidin expression have been associated with gastrointestinal disease. In IBD differential expression of human cathelicidin was observed. While inflamed and non-inflamed colon mucosa in ulcerative colitis patients showed increased expression of cathelicidin mRNA, in Crohn's disease no induction compared to non-inflamed healthy

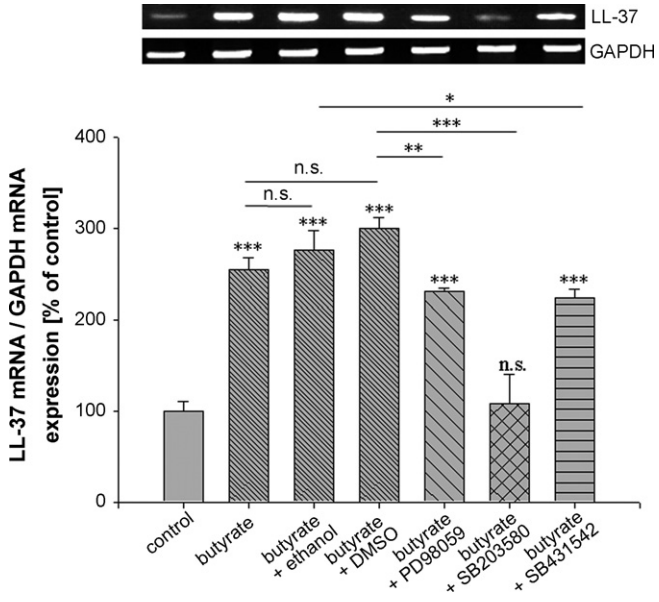

Fig. 4. Influence of the p38 MAPK inhibitor SB203580 (20 μM), the ERK1/2 inhibitor PD98059 (40 μM) and the TGF-β1 inhibitor SB431542 (10 μM) on butyrate-induced (3 mM) LL-37 mRNA expression in Caco-2 cells after 24 h of treatment. LL-37 expression was analysed by semiquantitative RT-PCR with the fluorescent dye Pico Green. All values for mRNA levels are normalized to corresponding mRNA amounts of GAPDH. One representative gel of three independent experiments is shown. Induction of LL-37 mRNA is displayed as relative percentage to solvent treated control cells. $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$; n.s.: not significant.

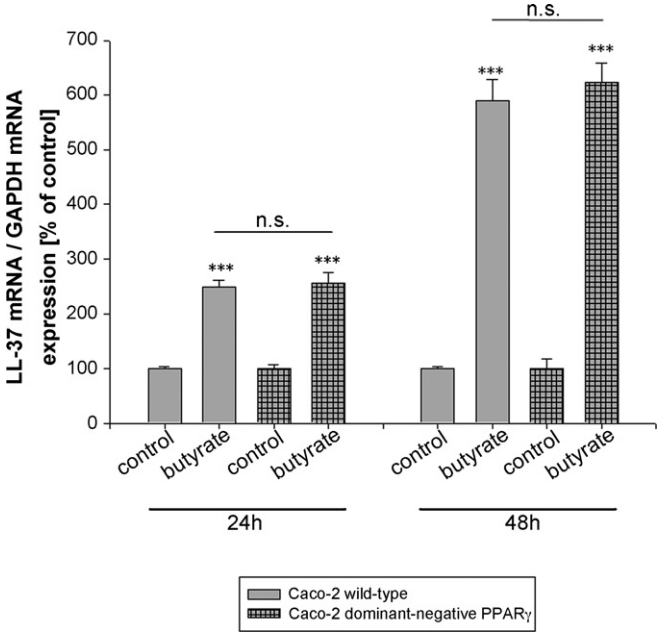

Fig. 5. Time-dependent effect of butyrate (3 mM) on LL-37 mRNA expression in wild-type and PPARγ mutant Caco-2 cells after 24 and 48 h of treatment. LL-37 mRNA was performed by semiquantitative RT-PCR with the fluorescent dye Pico Green. All values for mRNA levels are normalized to corresponding mRNA amounts for GAPDH. Induction of LL-37 mRNA is displayed as relative percentage to solvent treated control cells. $^{***}p < 0.001$; n.s.: not significant.

colon mucosa was seen (Schauber et al., 2006; Zasloff, 2006b). Furthermore, previous studies found that cathelicidin is down-regulated in the rectal epithelium of patients during Shigellosis and that butyrate up-regulates the expression of LL-37 in colonic epithelial cells *in vitro* and decreases severity of inflammation in experimental Shigellosis (Raqib et al., 2006). Comprehensive knowledge of cathelicidin regulation and expression is still lacking. The SCFA butyrate is known to up-regulate LL-37 in a variety of human colon cells (Hase et al., 2002; Schauber et al., 2003, 2004). The aim of the present study was to shed light on the regulatory pathways by which butyrate induces LL-37. In this context, the role of the VDR, PPARγ as well as the ERK-MAPK pathways and TGF-β1 were examined.

Stimulation of both Caco-2 and HT-29 cells with butyrate led to a time-dependent increase of LL-37 mRNA and protein. These findings are in line with the study of Schauber et al. (2003), who reported a similar LL-37 expression pattern in several colon cells including HT-29. Furthermore, our data provide evidence that both colorectal cancer cell lines express basal levels of LL-37, which differs from lung epithelial EBC-1 cells (Kida et al., 2006).

Butyrate affects the expression of a diverse array of target genes potentially involved in the regulation of cell death (Ruemmele et al., 1999) and immune-modulation of the gastrointestinal tract and exhibits thereby anti-inflammatory properties (Schauber et al., 2006). Mechanisms that have been proposed for butyrate-induced effects include hyperacetylation of histones due to inhibition of histone deacetylase, leading to chromatin relaxation and increased transcriptional activity and both phosphorylation and methylation of DNA-histone complexes (Wachtershauser and Stein, 2000).

PPARγ and VDR are target genes for butyrate and are up-regulated by this substance (Gaschott and Stein, 2003; Wachtershauser et al., 2000). Both are ligand-activated transcription factors that belong to the nuclear hormone receptor family and participate in a variety of immunological processes (Tirona and Kim, 2005). VDR is widely expressed in epithelial tissues, cells of the immune system and several cancer cell lines including the colon cancer cell line Caco-2 (Giuliano et al., 1991; Segaert and Bouillon, 1998). PPARγ is activated by natural ligands such as fatty acids and eicosanoids and is highly expressed in colonic epithelium, indicating an important role of the receptor in the physiology of the human colon (Desvergne and Wahli, 1999). All these characteristics make both receptors potential targets in butyrate-mediated LL-37 up-regulation. We therefore examined butyrate-induced LL-37 gene expression through application of the VDR inhibitor ZK191732 and a Caco-2 PPARγ mutant cell line to inhibit wild-type action. Our data indeed demonstrate that VDR activity, but not PPARγ activity, is required for the butyrate-induced LL-37 expression. The presence of a consensus vitamin D response element (VDRE) in the promoter of the human cathelicidin gene supports our findings (Wang et al., 2004). Furthermore, LL-37 mRNA levels were up-regulated after treatment with 1,25-dihydroxyvitamin D_3 and its analogues in several human cell types (Gombart et al., 2005; Wang et al., 2004). This mechanism seems to be conserved in primates but not in other mammals as suggested by the absence of the VDRE in murine and canine cathelicidin promotors (Gombart et al., 2005).

In addition, the participation of MAPKs in LL-37 regulation was scrutinized. MAPKs, such as ERK1/2 and p38 MAPK, comprise a group of protein serine/threonine kinases that are able to modulate the activity of a variety of transcription factors (Narayanan et al., 2004; Qi et al., 2004). They are activated in response to a variety of stimuli through dual phosphorylation at conserved threonine and tyrosine residues (Seger and Krebs, 1995). The p38 MAPK signalling transduction pathway has been demonstrated to play an essential role in the regulation of several cellular processes including, cell differentiation (Nagata et al., 1998), cell growth, apoptosis, as well as inflammation (Nebreda and Porras, 2000). Increased phosphorylation of p38 MAPK by butyrate in different cell types has been demonstrated by several groups including our own (Daniel et al., 2004; Schwab et al., 2006; Witt et al., 2000). Furthermore, we have recently shown that p38 MAPK is involved in the up-regulation of the VDR after butyrate treatment (Daniel et al., 2004), suggesting a possible role for this signalling pathway also in the regulation of LL-37 expression. Indeed, pre-treatment with two MAPK-ERK inhibitors diminished the induction of cathelicidin by butyrate, the strongest effect thereby observed for the p38 MAPK inhibitor SB203580, underscoring the importance of the p38 MAPK signalling pathway in the induction process of cathelicidin in Caco-2 cells. In accordance to our findings, butyrate-induced transactivation of the cathelicidin promotor in a human lung epithelial cell line was reported to be blocked after pre-incubation with inhibitors for the ERK 1/2, JNK and p38 MAPK signalling pathway (Kida et al., 2006). However, conflicting results have been obtained in other cell types. In SW620 cells, inhibition of the ERK1/2 pathway suppressed butyrate-mediated LL-37 mRNA expression while blocking the p38 MAPK trail supported the expression of the gene (Schauber et al., 2003). Moreover, inhibition of p38 MAPK in human keratinocytes led to decreased cathelicidin gene expression, whereas cJun N-terminal kinase (JNK) and ERK 1/2 pathways were not involved (Sayama et al., 2005). Altogether, these findings suggest the involvement of cell specific kinase pathways in the induction of LL-37 by butyrate.

Transforming growth factors, including TGF-β1, are key regulatory peptides that modulate mucosal cell populations critical to IBD (Babyatsky et al., 1996). During active inflammation of IBD, TGF-β1 was found to be increased in the affected mucosa modulating epithelial cell restitution and functional features of cells within the lamina propria (Babyatsky et al., 1996). In addition, TGF-β1 expression was reported to be enhanced in wound healing and this growth factor response ceased after regeneration of the injured tissue, when the physical barrier protecting against microbial infections was re-established (Sorensen et al., 2003). We therefore reasoned that growth factors are logical candidates as inducers of antimicrobial peptides. Our data demonstrate that pre-treatment of Caco-2 cells with a TGF-β1 receptor kinase inhibitor diminished LL-37 up-regulation caused by butyrate. These findings are supported by former studies reporting the necessity of TGF-β1 in butyrate-induced Caco-2 cell differen-

tiation (Schroder et al., 1999) and the increase of LL-37 expression in spontaneously differentiating Caco-2 cells (Hase et al., 2002).

In summary, the data presented here give new insights into the regulation of the antimicrobial cathelicidin in the colon cancer cell line Caco-2. Our results clearly demonstrate that butyrate-induced up-regulation of LL-37 in Caco-2 cells is influenced by several signalling pathways and receptors including MAPKs as well as VDR and TGF-β1. In contrast, PPARγ does not participate in this cascade. Revealing this transduction pathway is not only important to understand fundamental biological processes, but also may provide new opportunities in the treatment of gastrointestinal diseases.

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IV

The dietary histone deacetylase inhibitor sulforaphane induces human beta defensin-2 in intestinal epithelial cells

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Abstract

Background and aims: Antimicrobial peptides like human β-defensin-2 (HBD-2) play an important role in the innate immune system protecting the intestinal mucosa against bacterial invasion. The dietary histone deacetylase (HDAC) inhibitors sulforaphane (SFN) and butyrate have received a great deal of attention because of their ability to simultaneously modulate multiple cellular targets involved in cellular protection. In this study the influence of SFN and butyrate on HBD-2 expression as well as molecular pathways involved in the HBD-2 regulatory process were scrutinized.

Methods and Results: Treatment of Caco-2, HT-29 and SW480 cells with SFN led to a time- and dose-dependent up-regulation of HBD-2 mRNA expression as determined by semi-quantitative RT-PCR. Moreover, HBD-2 protein was increased in response to SFN, measured by ELISA. Immunofluorescence analysis revealed that the protein is localised in the cytosol. Co-incubation of SFN with a vitamin D receptor (VDR) antagonist and a ERK1/2 inhibitor reduced HBD-2 mRNA up-regulation. In contrast, transfection of cells with a dominant-negative peroxisome proliferator-activated receptor γ (PPARγ) mutant vector to inhibit PPARγ wild-type action and inhibition of p38 MAPK signalling did not affect SFN-mediated up-regulation of HBD-2 mRNA. Similar results for all experiments were obtained after butyrate stimulation. Moreover, SFN induced the expression of VDR and phosphorylated ERK1/2 but did not affect p38 MAPK activation.

Conclusion: The data clearly demonstrate for the first time that the dietary HDAC inhibitor SFN is able to induce antimicrobial peptides in colonocytes. In this process HBD-2 expression is regulated via VDR and the MEK/ERK signalling pathway.

Keywords

β-defensin-2, butyrate, innate immunity, sulforaphane, vitamin D receptor

Introduction

The gastrointestinal tract is constantly in contact with bulk of commensal microorganisms, residing in the colon and distal small intestine. Although the presence of this native flora in general is mutually beneficial, the host also requires protection against these microorganisms. The role of antimicrobial peptides of the defensin family mediating protective responses has been established (Ganz, 2003; Sansonetti, 2004; Wehkamp and Stange, 2006; Zasloff, 2002). Disturbances in their expression in the intestinal tract were shown to be linked to inflammatory bowel diseases (IBD) (Wehkamp et al., 2005b; Wehkamp et al., 2005c; Wehkamp et al., 2005d; Zasloff, 2006).

Defensins are small cationic peptides with a broad spectrum of antimicrobial activity having characteristic pairs of intramolecular disulfide bonds, a beta-sheet structure and a mass of 3-5 kDa (Bevins et al., 1999; Eckmann, 2004). They are classified as α- and β-defensins based on the position of three disulfide bridges (Eckmann, 2005). The β-defensins are ubiquitously expressed throughout the gastrointestinal tract, including the colon (Sansonetti, 2004). Among the β-defensins, HBD-1 is constitutively expressed (O'Neil et al., 1999; Wehkamp et al., 2003), whereas HBD-2 is inducible in response to infection, pro-inflammatory mediators such as IL-1β, TNF-α, and probiotic bacteria (De Smet and Contreras, 2005; Wehkamp et al., 2004). Permeabilization of target membranes is the crucial step in defensin-mediated antimicrobial activity and cytotoxicity (Ganz, 2003). The positively charged defensin molecules are inserted into the bacterial membranes under the influence of cell-generated transmembrane potentials and local electrostatic fields, resulting in the cessation of RNA, DNA, and protein synthesis in bacteria (Ganz, 2003; Wehkamp et al., 2005a). Besides their direct antimicrobial effects, defensins exert further functions related to host defence such as the induction of histamine release by mast cells and chemoattraction of various cells of the immune system including neutrophils and T cells (Befus et al., 1999; De

Smet and Contreras, 2005). Moreover, they seem to be involved in carcinogenesis (Bullard et al., 2008; Markeeva et al., 2005; Papo and Shai, 2005).

The dietary histone deacetylase (HDAC) inhibitor sulforaphane (SFN) is one of the most biologically active phytochemicals in the human diet and is present at high concentrations in some cruciferous vegetables, especially broccoli (*Brassica oleracea*) (Fimognari and Hrelia, 2007). SFN has received a great deal of attention because of its ability to simultaneously modulate multiple cellular targets involved in cellular protection (Myzak and Dashwood, 2006; Myzak et al., 2006). SFN is regarded as a highly promising dietary preventative agent because of its capacity to induce apoptosis and to inhibit cell proliferation in various tumour cells, including colorectal cancer (Fimognari and Hrelia, 2007; Myzak and Dashwood, 2006). In addition, several studies indicate that SFN exhibits immune-modulatory capacities by interfering with the actions of the pro-inflammatory transcription factor NFκB and by the modulation of the production of pro- and anti-inflammatory cytokines (Bertl et al., 2006; Heiss et al., 2001; Thejass and Kuttan, 2007). Recent studies including our own report that HDAC inhibitors like butyrate and Trichostatin A induce the expression of antimicrobial peptides in several colorectal cancer cells (Hase et al., 2002; Schauber et al., 2006; Schauber et al., 2004; Schwab et al., 2007b). In analogy, the aim of this work was to explore a possible role for SFN in the induction of HBD-2 expression as well as to scrutinize molecular pathways involved in this regulatory process.

Materials and methods

Cell culture

The human colorectal cancer cell lines Caco-2, HT-29 and SW480 were obtained from the European Collection of cell cultures (ECACC). Cells were cultured in a humidified incubator at 37 °C in an atmosphere of 95% air and 5% CO_2. Caco-2 and SW480 cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% foetal calf serum (FCS), 1% non-essential amino acids, 1% sodium pyruvate and 1% penicillin/streptomycin. HT-29 cells were grown in McCoy's 5A Medium, supplemented with 10% FCS and 1% penicillin/streptomycin. Medium of the dominant-negative PPARγ mutant and empty-vector HT-29 cells was supplied with 400 µg/ml Geneticin 418 sulphate (G418, Gibco-BRL, Eggenstein, Germany). Cells were regularly screened for mycoplasma contamination using the VenorGem Mycoplasma detection kit (Minerva Biolabs, Berlin, Germany).

For experiments, cells were seeded in plastic cell culture wells and were cultivated in DMEM and McCoy's 5A Medium, respectively until, 80% confluency was reached. Medium was then removed and replaced by medium containing either the solvent, SFN (1-20 µM), butyrate (1-5 mM) or one of the combinations of SFN (10 µM) or butyrate (3 mM) with the vitamin D receptor inhibitor ZK191732 (10 µM), the p38 MAPK inhibitor SB203580 (20 µM) or the ERK1/2 inhibitor PD98059 (40 µM), respectively. In these experiments, cells were pre-treated with the inhibitors for 5 h, followed by challenge of cells with SFN or butyrate for up to 48 h. Sodium butyrate (Merck-Schuchardt, Hohenbrunn, Germany) was dissolved in phosphate buffered saline (PBS), SFN (Axxora, San Diego, California, USA) solubilised in aqua ad iniectabilia (DeltaSelect, Pfufflingen, Germany). PD98059 and SB203580 (both Calbiochem, Schwalbach/Taunus, Germany) was dissolved in dimethyl sulfoxide (DMSO, Fluka, Sigma-Aldrich-Chemie, Steinheim, Germany), ZK191732 was (supplied by the Department of Medicinal Chemistry at Schering AG, Berlin, Germany)

dissolved in ethanol. The maximal concentration of solvents in each medium was kept below 0.1% [v/v]. The medium was changed every day. Cells were then harvested at the times indicated in the figure legends. Concentrations of SFN and butyrate in our experiments correspond to appropriate physiologic concentrations (Jakubikova et al., 2005; Traka et al., 2005; Wachtershauser and Stein, 2000).

Transfection assay

The following plasmids were used for transfection: pcDNA3 (Invitrogen, Karlsruhe, Germany), as an empty-vector for control transfection and the plasmid pcDNA3-PPARγ$_{L468A/E471A}$, a dominant-negative PPARγ double-mutant, that was kindly provided by VK Chatterjee (Department of Medicine, University of Cambridge, Addenbrooke's Hospital, Cambridge, United Kingdom) (Gurnell et al., 2000). These constructs were transfected into subconfluent HT-29 cells with lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) in serum-free conditions. After 6 h, the cells were supplied with fresh medium containing 10% FCS. 24 h later, the cells were supplied with medium containing G418 (400 µg/ml) and culture medium supplemented with G418 was replaced twice a week. G418-resistant colonies were collected and used for further analysis. The functional successful transfection of the dominant-negative PPARγ cell system was demonstrated recently (Schwab et al., 2007a; Schwab et al., 2006).

Cytotoxicity

Cytotoxicity was excluded by lactate dehydrogenase (LDH) release assay using a commercial kit (LDH kit, Roche, Mannheim, Germany).

mRNA isolation

Cells were cultivated in 6-well plates and were treated at 80% confluency with SFN (20 μM), butyrate (3 mM), or the combination of SFN or butyrate with one of the inhibitors for 24 and 48 h, respectively. Total RNA was isolated from cells by RNA isolation reagent (TRIR, Abgene, United Kingdom), followed by phenol extraction and ethanol precipitation.

Semi-quantitative RT-PCR

RT-PCR was conducted with the Gene Amp RNA PCR kit (Applied Biosystems, Branchburg, New Jersey, USA) according to the manufacturer's protocol, starting from 1 μg total RNA. All RNA samples were subjected to DNase-treatment during the RT-step to remove traces of genomic DNA (Shrimp Nuclease, Abgene, United Kingdom). Amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as control. From the results of preliminary experiments, 20 PCR cycles for GAPDH and 35 cycles for HBD-2 were selected as optimal amplification conditions to produce a log-linear relationship between the amount of each mRNA and intensity of PCR product. The PCR reaction contained 0.2 mM dNTPs (Invitrogen Corporation, Carlsbad, California, USA), 0.05 U × μl^{-1} AmpliTaqGold DNA polymerase (Applied Biosystems, Branchburg, New Jersey, USA), 1.5 mM Mg_2Cl (Applied Biosystems, Branchburg, New Jersey, USA) and either 0.2 μmol/L primers of HBD-2 and GAPDH, respectively (Biospring, Frankfurt, Germany). PCR conditions for HBD-2 were: initial denaturation at 94°C for 1 min followed by annealing at 58°C for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 7 min after the last cycle. The respective annealing temperature for GAPDH was 45°C. Primers for amplification were as follows: HBD-2 sense 5'-ggtggtataggcgatcctgtt-3', HBD-2 antisense 5'-agggcaaaagactggatgaca-3'; GAPDH sense 5'-gcaccgtcaaggctgagaac-3', GAPDH antisense 5'-ccaccaccctgttgctgtag-3'. The expected sizes of HBD-2 and GAPDH were 66 and 803 base pairs, respectively. Aliquots of the PCR mixtures (10 μL) were analyzed by electrophoresis using a 1.5% agarose gel

containing ethidium bromide. Gels were placed on a UV transilluminator and digitalized by DocuGel V-System (Scananalytics). For semi-quantitative analysis of amplified PCR products, the fluorescent dye PicoGreen (Molecular Probes, Eugene, Oregon, USA) was used according to the manufacturer's instructions. In brief, two microliters of amplified DNA in 100 µl TE buffer were mixed with an equal volume of diluted PicoGreen reagent (1:200, v/v in TE buffer). Samples were incubated for 5 min at room temperature and protected from light in a microtiter plate. The fluorescence was measured (λ_{ex}= 485 nm; λ_{em} = 538 nm) in the fluorescence microplate reader Tecan SpectraFluor PLUS. A λ DNA linear standard curve was applied for each experiment.

Immunofluorescence Assay

Caco-2 cells were grown on glass chamber slides (Lab-Tek, Nunc, Rochester, New York, USA) and allowed to reach 80% confluency. Cells were fixed in ice-cold ethanol (95%) for 10 min and permeabilized by addition of 0,1% Triton X-100 in PBS for 10 min. After washing, unspecific binding of antibodies was blocked by incubating cells in 8% horse serum in PBS containing 0,1% Tween-20 for 1 h at room temperature. HBD-2 antibody (Immundiagnostik, Bensheim, Germany) was diluted 1:25 in blocking buffer. After 1.5 h of incubation, cells were washed three times with PBS before secondary antibody (cy3 conjugated rabbit-anti-goat, Sigma, Saint Louis, Missouri, USA) was added for 1 h. Following washing and air-drying, the cells were embedded in 4',6-Diamidino-2-phenylindol (DAPI) mounting medium (Vector Laboratories, Burlingame, California, USA) and evaluated by immunofluorescence microscopy (Eclipse E 600, Nikon, Tokyo, Japan) with a digital camera DX 20H (Kappa, Monrovia, California, USA).

β-defensin-2 ELISA

Caco-2 cells were grown in 6-well plates and were treated at 80% confluency with SFN or butyrate for 24 and 48 h, respectively. Cells were washed three times with ice-cold PBS and incubated with cell lysis buffer (Cell signaling, Beverly, Massachusetts, USA) containing multiple protease inhibitors (Complete, Roche, Mannheim, Germany) for 5 min at 4 °C. Protein extracts were obtained after sonication of cell lysates (2 × 5 s) and centrifugation at 13.000 rpm at 4 °C (10 min). Protein content was determined via a colorimetric assay according to the method of Bradford (Bio-Rad Laboratories, Munich, Germany). Cell lysates were diluted 1:3 with PBS containing 0,1% bovine serum albumin. 100 µL were used for each reaction. HBD-2 ELISA Kit (Immundiagnostik, Benzheim, Germany) was then used according to the manufacturers instructions. Protein concentration of HBD-2 was analysed and samples were normalized to equal protein concentrations.

SDS–polyacrylamide gel electrophoresis and immunoblot analysis

Caco-2 and HT-29 cells were stimulated with SFN at 80% confluency. Cells were than harvested and protein content was determined via Bio-Rad colorimetric assay (see β-defensin-2 ELISA). Equal amounts of total protein lysates were separated on a 10% SDS-polyacrylamide gel for VDR and on a 12.5% SDS-polyacrylamide gel for ERK1/2, phospho-ERK1/2, p38 MAPK and phospho-p38 MAPK. Proteins were transferred onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Subsequently, membranes were blocked for one hour with 5% (w/v) nonfat dried milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T). Membranes were then incubated overnight with a 1:1000 dilution of VDR-, ERK1/2-, phospho-ERK1/2-antibody (all from Santa Cruz Biotechnologies, Santa Cruz, California, USA) or with p38 MAPK- and phospho-p38 MAPK-antibody (both from Cell signalling, Beverly, Massachusetts, USA) in 0.05% TBS-T and 5% (w/v) nonfat dried milk, respectively. After washing, the blots were incubated for half an hour with corresponding

horseradish peroxidase-conjugated antibody (Vector Laboratories, Burlingame, California, USA, dilution 1:2000) in 0.05% TBS-T and 5% (w/v) nonfat dried milk. The washing steps were repeated, and subsequently enhanced chemoluminescence detection was performed according to the manufacturer's instructions (ECL, Amersham pharmacia biotech, Buckinghamshire, UK) on Hyperfilm-MP (Amersham International plc, Buckinghamshire, UK). Blots were then reprobed with β-actin antibody (Sigma, Saint Louis, Missouri, USA). For quantitative analysis, bands were detected by scanning densitometry, using a Desaga CabUVIS scanner and Desaga ProVilDoc software (Desaga, Wiesloch, Germany).

Statistics

All statistical analyses were performed using GraphPad Prism 4.01 (San Diego, California, USA). Analysis of variance (ANOVA) was performed when more than two groups were compared and, when significant ($P < 0.05$), multiple comparisons were performed with the Tukey's test. Data are expressed as means ± SD from three independent experiments. P value < 0.05 was considered to be significant.

Results

Sulforaphane is a direct inducer of HBD-2 mRNA expression in colonocytes

Challenge of HT-29 cells with increasing concentrations of SFN (1-20 μM) provoked a dose-dependent up-regulation of HBD-2 mRNA after 24 h (Fig. 1) and 48 h of treatment (data not shown). Significant effects were seen at concentrations of ≥ 5 μM. Increased expression of the HBD-2 gene in response to SFN (20 μM) was also obtained in the colon cancer cell lines Caco-2 and SW480 after 24 h (Fig. 1), reflecting a common mechanism in colorectal cancer cells. In analogy, stimulation of HT-29 cells with increasing concentrations of the dietary HDAC inhibitor butyrate (1-5 mM) also resulted in a dose-dependent up-regulation of HBD-2

mRNA compared to control cells (Fig. 1) after 24 h. A similar up-regulation was seen in Caco-2 cells (data not shown).

Sulforaphane elevates HBD-2 protein expression in colonocytes

Up-regulation of HBD-2 mRNA was accompanied by a similar increase in protein level as measured by ELISA. In Caco-2 cells, a time-dependent increase of HBD-2 protein levels was observed after exposure with SFN (20 μM) or butyrate (3 mM) (Fig. 2 A: SFN: 24 h: +62%, $p < 0.05$; 48 h: +104%, $p < 0.001$ / butyrate: 24 h: +47%, $p < 0.05$; 48 h: +161%, $p < 0.001$). Moreover, the subcellular localisation of HBD-2 protein in Caco-2 cells was determined via immunofluorescence analysis (Fig. 2 B). Caco-2 cells were treated with butyrate (3 mM) up to 48 h. Both control and butyrate-treated cells showed strong HBD-2 staining predominantly in the cytosol. Moreover, butyrate challenge for 48 h led to an increased expression of the HBD-2 protein, which is in line with the results obtained by ELISA. Similar results were obtained after challenge of cells with SFN used in the concentration of 20 μM (data not shown).

The vitamin D receptor (VDR) but not the peroxisome proliferator-activated receptor γ (PPAR γ) is involved in sulforaphane-induced β-defensin-2 mRNA expression

The nuclear receptors vitamin D receptor (VDR) and peroxisome proliferator-activated receptor γ (PPARγ) are potent regulators of immune responses. To evaluate the involvement of PPARγ in SFN-induced HBD-2 mRNA expression, HT-29 cells transfected with a dominant-negative PPARγ mutant to inhibit wild-type receptor action were used (Fig. 3). In these mutant cells, an equal time-dependent increase of HBD-2 compared to wild-type and empty-vector (data not shown) cells was observed after challenge of SFN, indicating that PPARγ activity is not required for HBD-2 regulation. Similar effects were obtained after

challenge of cells with butyrate (data not shown). Moreover, wild-type Caco-2 cells were treated with SFN (10 μM) in presence of the VDR antagonist ZK191732. Inhibition of the VDR significantly antagonized the SFN-induced HBD-2 mRNA after 24 h of incubation, demonstrating its pivotal role in SFN-induced HBD-2 up-regulation (-71% vs. control, $p < 0.001$, Fig. 4 A). A similar pattern for HBD-2 mRNA levels was observed after treatment of Caco-2 cells with ZK191732 and butyrate (data not shown). To unveil whether VDR is a target of the SFN signalling pathway, colonocytes were stimulated with SFN (10-20 μM) for 24 h. As depicted in Fig. 4 B, SFN increased VDR protein expression in HT-29 cells. A similar pattern was observed for the colorectal cancer cell line Caco-2 (Fig. 4 B).

The mitogen-activated protein kinase kinase/extracellular-regulated kinase (MEK/ERK) signalling pathway plays a role in sulforaphane-induced HBD-2 mRNA expression

To determine the involvement of intracellular signal transduction pathways such as p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK1/2) in SFN-mediated HBD-2 expression, Caco-2 cells were pre-incubated for 5 h with specific inhibitors prior to stimulation with SFN (10 μM). Combined treatment of SFN with the ERK1/2 inhibitor PD98059 reduced the induction of HBD-2 mRNA expression after 24 h (Fig. 5 A). On the contrary, co-incubation with the p38 MAPK inhibitor SB203580 did not affect elevated HBD-2 mRNA expression caused by SFN for this point in time (Fig. 5 A). Similar effects were obtained after challenge of cells with butyrate (data not shown). Moreover, exposure of Caco-2 cells with SFN (10-20 μM) resulted in a rapid phosphorylation of ERK1/2 after 8 h, while the amount of total ERK1/2 protein was not affected. The expression of phospho-p38 MAPK and p38 MAPK proteins were unchanged by SFN in Caco-2 cells (Fig. 5 B).

Discussion

The discovery of antimicrobial peptides such as defensins has extended the knowledge of unspecific defence mechanisms. A deficiency in the antimicrobial defence system of defensins may be a reasonable and plausible explanation for the breakdown of barrier functions with increased invasion of infectious pathogens, leading to inflammation or deleterious immune responses. Aberrances in defensin expression have not only been observed in inflammatory bowel diseases (Wehkamp et al., 2005a; Zasloff, 2006), but also in cystic fibrosis where the inactivation of the defensins due to high salt conentrations has been linked to recurrent bronchopulmonary infections (Goldman et al., 1997). In contrast to ulcerative colitis and ileal Crohn's disease, colonic Crohn's disease is characterized by an impaired induction of the epithelial HBD-2 (Fellermann et al., 2006; Wehkamp et al., 2002; Wehkamp et al., 2005b). This defective regulation could be explained by the lower HBD-2 gene copy number in the HBD-2 locus in colonic Crohn's disease (Fellermann et al., 2006). A normal HBD-2 copy number distribution was seen in ileal Crohn's disease and also in ulcerative colitis (Fellermann et al., 2006). Eventually, the lack of HBD-2 induction may contribute to a defective antimicrobial barrier leading to chronic inflammation in Crohn's disease affecting the large bowel (Fellermann et al., 2006). Detailed knowledge of HBD-2 expression and regulation is still lacking. The aim of this study was to determine a possible role for the dietary HDAC inhibitor SFN in the regulation of the HBD-2. In this context, the involvement of the nuclear receptors VDR and PPARγ as well as MEK-ERK pathways were examined. Equal experiments were accomplished with the dietary HDAC inhibitor butyrate.

Accumulating evidence suggests that SFN is a highly promising dietary preventive agent, due to its ability to exert anti-inflammatory effects via multiple mechanisms of action (Dashwood et al., 2006; Thejass and Kuttan, 2007). The anti-inflammatory effects of SFN and butyrate may be achieved via different pathways: The agents can affect the expression of a

diverse array of genes potentially involved in immune modulation through their ability to inhibit HDAC activity via histone hyperacetylation (Dashwood et al., 2006). This results in chromatin relaxation and increased transcription of target genes (Dashwood et al., 2006). SFN and butyrate are able to block TNFα- and LPS-induced NFκB translocation resulting in reduced expression of pro-inflammatory cytokines (Heiss et al., 2001; Place et al., 2005; Thejass and Kuttan, 2007). Moreover, the involvement of HDAC inhibitors like butyrate and Trichostatin A in the modulation of the intestinal antimicrobial peptides make the proteins possible targets for SFN's actions (Schauber et al., 2006; Schauber et al., 2003; Schwab et al., 2007b). The present study demonstrated for the first time that the dietary HDAC inhibitor SFN is a direct inducer of HBD-2 mRNA and protein in colonocytes. Similar results were obtained after butyrate treatment. Immunofluorescence experiments showed that the peptide exhibits a primarily cytoplasmatic localisation.

PPARγ and VDR are highly expressed in the colonic epithelium indicating that both receptors are important agents in the physiology of the human colon (Desvergne and Wahli, 1999; Nagpal et al., 2005; Schwab et al., 2006). The receptors partly mediate their anti-inflammatory actions through negative interference with pro-inflammatory transcription factors such as NFκB (Delerive et al., 2001; Schwab et al., 2007a; Sun et al., 2006). Moreover, there is great influx of information that PPARγ ligands may influence the inflammatory response in IBD and colon cancer (Auwerx, 2002; Bull, 2003). Similarly, the active form of vitamin D, 1,25-dihydroxyvitamin D_3, was shown to inhibit the development of various autoimmune diseases, including IBD (Cantorna et al., 2004). In addition, a VDR gene polymorphism has been associated with Crohn's disease susceptibility (Simmons et al., 2000). All these features implicate a possible involvement of both receptors in SFN-mediated HBD-2 regulation. Using the VDR inhibitor ZK191732 and a Caco-2 dominant-negative PPARγ mutant cell line in our experiments, we demonstrated that VDR activity, but not PPARγ

activity, is required for HBD-2 expression induced by SFN or butyrate. The properties of the VDR inhibitor and the functional successful transfection of the dominant-negative PPARγ mutant cell system has described recently (Schwab et al., 2007a). Our findings are supported by the presence of a consensus vitamin D response element (VDRE) in the promoter of the human HBD-2 gene (Wang et al., 2004). Accordingly, stimulation of several human cell types with 1,25-dihydroxyvitamin D_3 resulted in increased expression levels of HBD-2 mRNA (Wang et al., 2004). Moreover, our *in vitro* model demonstrates for the first time the up-regulation of VDR after SFN stimulation in colonocytes. A similar increase of the receptor was observed after butyrate treatment (Gaschott and Stein, 2003; Gaschott et al., 2001).

SFN stimulation may activate different MAPKs in colorectal cancer cells (Jakubikova et al., 2005; Keum et al., 2006; Shen et al., 2006). Kinase pathways act as signal sorters and conduct a variety of upstream signals to the nucleus of the eukaryotic cell, where transcription of specific target genes will be affected (Seger and Krebs, 1995). To gain insight into the regulatory pathways by which SFN augments HBD-2 expression in Caco-2 cells, our *in vitro* model focused on two major MAPKs: p38 MAPK and ERK1/2. These MAPKs are involved in a large variety of cellular activities, including cell survival, proliferation and inflammatory responses (Ballif and Blenis, 2001; Chang and Karin, 2001; Nebreda and Porras, 2000; Seger and Krebs, 1995). Increased phosphorylation of ERK1/2 by SFN in various cell lines including human colon adenocarcinoma Caco-2 cells has been demonstrated by several groups, although the amount of ERK1/2 protein was not affected. In contrast, SFN treatment had no impact on p38 MAPK activation (Jakubikova et al., 2005; Keum et al., 2006). These observations are confirmed by our experiments. Moreover, inhibition of the ERK1/2 pathway in this study prevented SFN-mediated induction of HBD-2 mRNA expression in Caco-2 cells, while blocking of the p38 MAPK trail did not affect HBD-2 levels for this point in time. Our data are in accordance with the observations in middle ear epithelial cells, demonstrating

transcriptional activation of the HBD-2 gene caused by IL-1α which is mediated through an Raf-MEK1/2-ERK1/2 signalling pathway (Moon et al., 2002). Conflicting results were obtained in the lung epithelial cell line A549, in which IL-1β-induced up-regulation of HBD-2 was partly attenuated by inhibiting the p38 MAPK but not by the ERK1/2 signalling pathway (Jang et al., 2004). These different observations indicate a cell specific and stimulus dependent kinase pathway leading to induction of HBD-2. However, all these studies underscore the importance of the MAPK signalling pathways in innate immunity through the regulation of HBD-2 levels.

Recapitulating our present findings, we could demonstrate for the first time that SFN induces the expression of the antimicrobial peptide HBD-2 on mRNA and protein level in colorectal cancer cells. Furthermore, we revealed that SFN-mediated induction of HBD-2 is modulated via the VDR and the MEK/ERK signalling pathway. Similar effects were obtained after butyrate treatment. These data support the potential usefulness of dietary HDAC inhibitors in the therapy of colonic Crohn's disease. Further *in vivo* studies are required to confirmly establish the relevance of these findings.

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Abbreviations

DAPI, 4',6-Diamidino-2-phenylindol

DMEM, Dulbecco's modified Eagle's medium

DMSO, Dimethyl sulfoxide

ECACC, European Collection of cell cultures

ERK, Extracellular signal-regulated kinase

FCS, Foetal calf serum

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase

HBD-2, human β-defensin-2

HDAC, Histone deacetylase

IBD, Inflammatory bowel disease

JNK, cJun N-terminal kinase

LDH, Lactate dehydrogenase

MAPK, Mitogen-activated protein kinase

MEK/ERK, Mitogen-activated protein kinase kinase/extracellular-regulated kinase

PBS, Phosphate buffered saline

PPARγ, Peroxisome proliferator-activated receptor γ

SCFA, Short chain fatty acid

SFN, Sulforaphane

VDR, Vitamin D receptor

VDRE, Vitamin D response element

Figure Legends

Fig. 1: Dose-dependent effect of sulforaphane (1-20 μM) and butyrate (1-5 mM) on β-defensin-2 mRNA expression in HT-29 cells after 24 h of treatment. Moreover the induction of β-defensin-2 mRNA expression in Caco-2 and SW480 cells in response to sulforaphane (20 μM) was demonstrated. β-defensin-2 mRNA expression was measured by semi-quantitative RT-PCR with the fluorescent dye Pico Green. All values for mRNA levels are normalized to corresponding mRNA amounts for GAPDH. One representative gel of three independent experiments is shown. Induction of β-defensin-2 mRNA is displayed as relative percentage to solvent treated control cells. ***$p < 0.001$, *$p < 0.05$, n.s. = not significant

Fig. 2: (A) β-defensin-2 protein expression in Caco-2 cells measured via ELISA: Cells were starved for 72 h and then stimulated with sulforaphane (10-20 μM) or butyrate (3 mM) for 24 and 48 h. Proteins were harvested and β-defensin-2 was measured by ELISA. The concentration range of all experiments was ng/ml. Induction of β-defensin-2 protein is displayed as fold induction relative to solvent treated control cells. ***$p < 0.001$, *$p < 0.05$ (B) Representative immunofluorescence assays for β-defensin-2 in Caco-2 cells: Confluent cells were challenged with butyrate (3 mM) for 48 h (III) or grown in culture medium as controls (I). (II, IV): Nuclear counterstaining with 4',6-Diamidino-2-phenylindol (DAPI) mounting medium for each of the adjacent panels. Magnification, x 400.

Fig. 3: Time-dependent effect of sulforaphane (10 μM) on β-defensin-2 mRNA expression in wild-type and PPARγ mutant HT-29 cells after 24 and 48 h of treatment. β-defensin-2 mRNA was measured by semi-quantitative RT-PCR with the fluorescent dye Pico Green. All values for mRNA levels are normalized to corresponding mRNA amounts for GAPDH.

Induction of β-defensin-2 mRNA is displayed as relative percentage to solvent treated control cells. n.s. = not significant

Fig. 4: (A) β-defensin-2 mRNA expression in Caco-2 cells. Medium was supplemented with the solvent, sulforaphane (10 μM), the VDR inhibitor ZK191732 (10 μM) or the combination of sulforaphane and ZK191732 for 24 h. Semi-quantitative analysis of PCR products was performed using Pico Green. All values for mRNA levels are normalized to corresponding mRNA amounts of the housekeeping gene GAPDH. Induction of β-defensin-2 mRNA is displayed as relative percentage to solvent treated control cells. ***$p < 0.001$, *$p < 0.05$, n.s. = not significant (B) Western blot for VDR expression after treatment of HT-29 and Caco-2 cells with SFN (10-20 μM) for 24 h. One representative blot of three independent experiments is shown.

Fig. 5: (A) Influence of the p38 MAPK inhibitor SB203580 (20 μM) and the ERK1/2 inhibitor PD98059 (40 μM) on sulforaphane-induced (10 μM) β-defensin-2 mRNA expression after 24 h of treatment in Caco-2 cells. β-defensin-2 expression was analysed by semi-quantitative RT-PCR with the fluorescent dye Pico Green. All values for mRNA levels are normalized to corresponding mRNA amounts of GAPDH. One representative gel of three independent experiments is shown. Induction of β-defensin-2 mRNA is displayed as relative percentage to solvent treated control cells. ***$p < 0.001$, n.s. = not significant (B) Western blot for ERK1/2, phospho-ERK1/2, p38 MAPK and phospho-p38 MAPK expression after treatment of Caco-2 cells with SFN (10-20 μM) for 8 h. One representative blot of three independent experiments is shown.

Figure 1

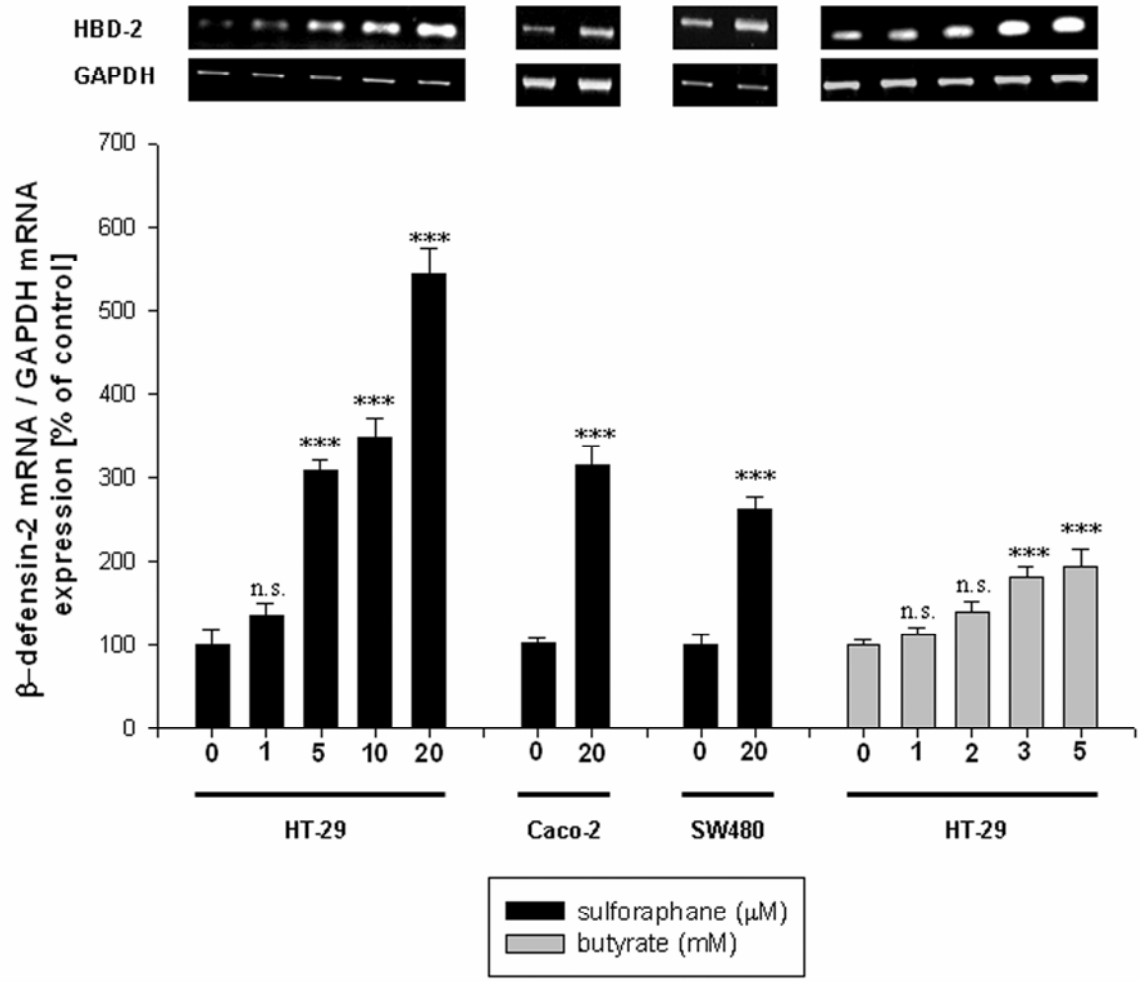
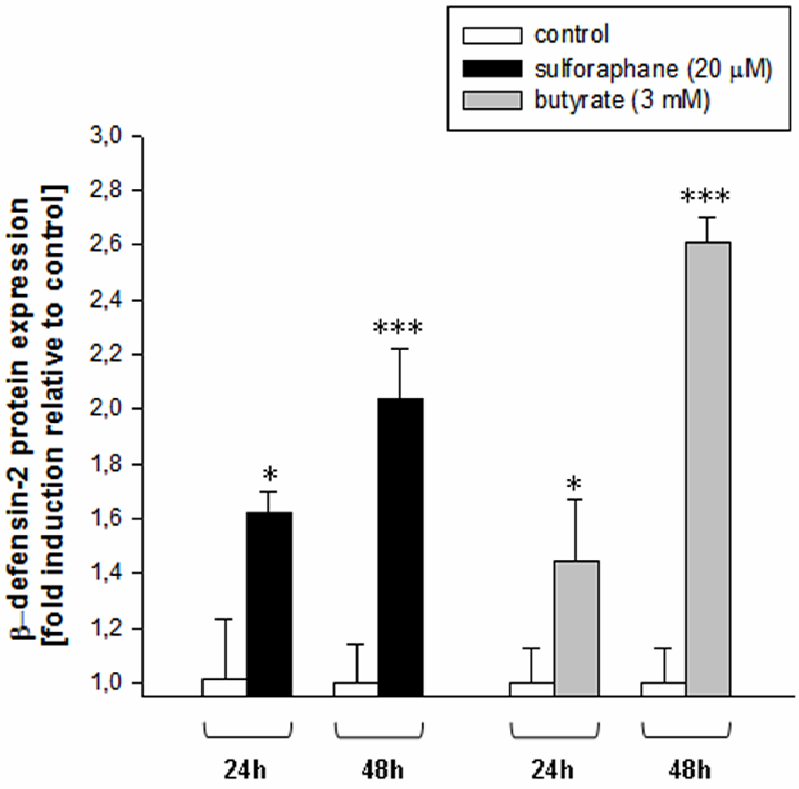

HBD-2
GAPDH
β-defensin-2 mRNA / GAPDH mRNA expression [% of control]
700
600
500
400
300
200
100
0
n.s.
***
0 1 5 10 20
0 20
0 20
0 1 2 3 5
HT-29
Caco-2
SW480
HT-29
sulforaphane (μM)
butyrate (mM)

Figure 2

A


control
sulforaphane (20 μM)
butyrate (3 mM)
β–defensin-2 protein expression
[fold induction relative to control]
3,0
2,8
2,6
2,4
2,2
2,0
1,8
1,6
1,4
1,2
1,0
*
***
*
***
24h
48h
24h
48h

B

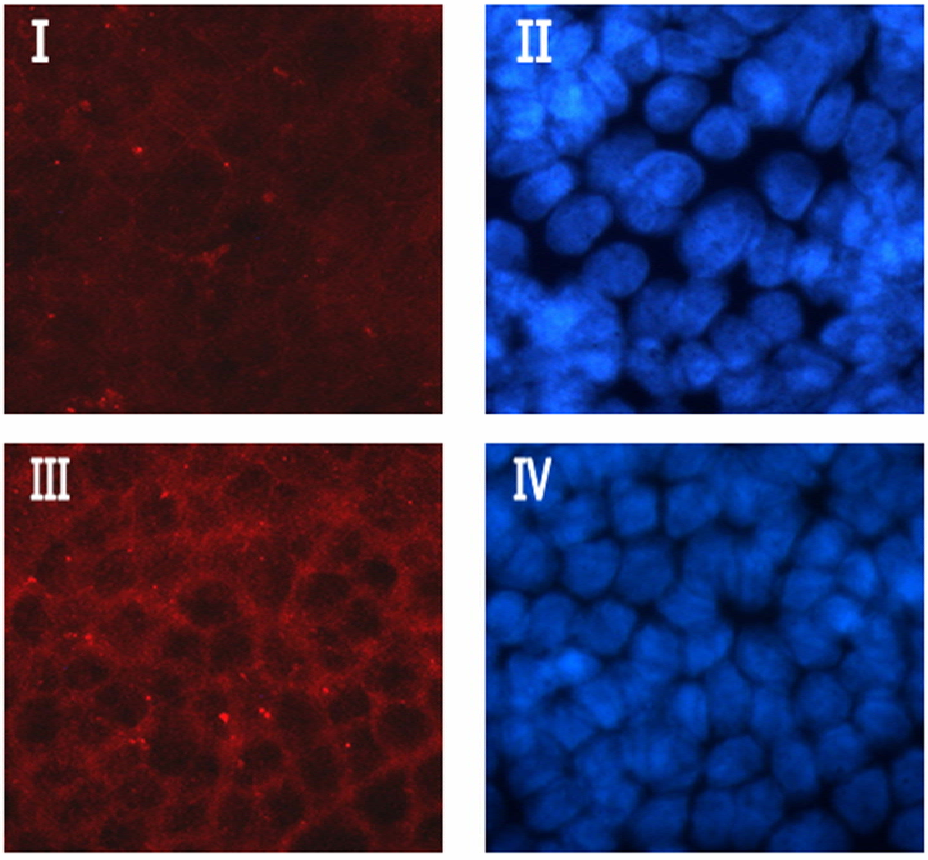

I
II
III
IV

Figure 3

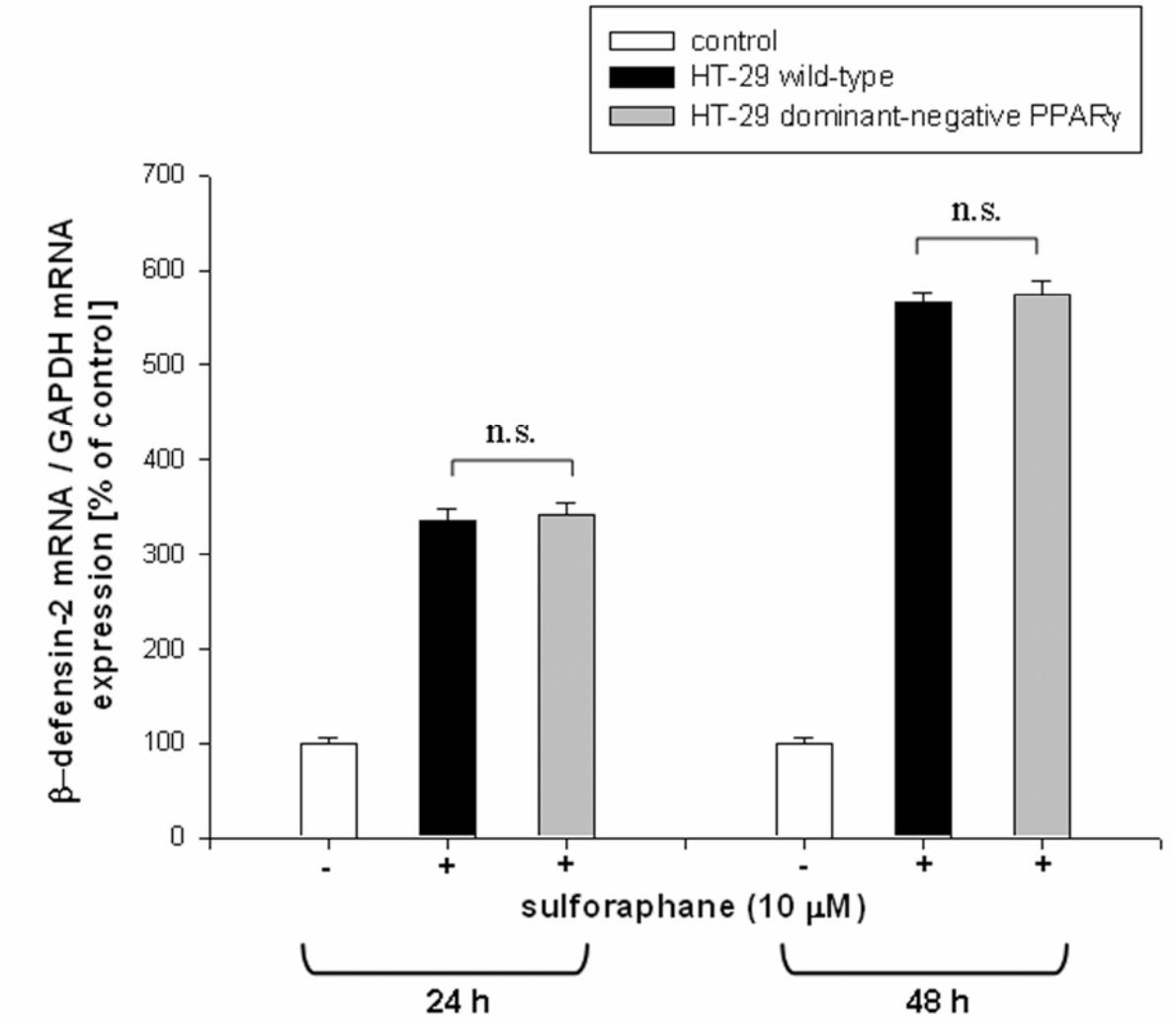

control
HT-29 wild-type
HT-29 dominant-negative PPARγ
β-defensin-2 mRNA / GAPDH mRNA expression [% of control]
700
600
500
400
300
200
100
0
n.s.
n.s.
-
+
+
-
+
+
sulforaphane (10 µM)
24 h
48 h

Figure 4

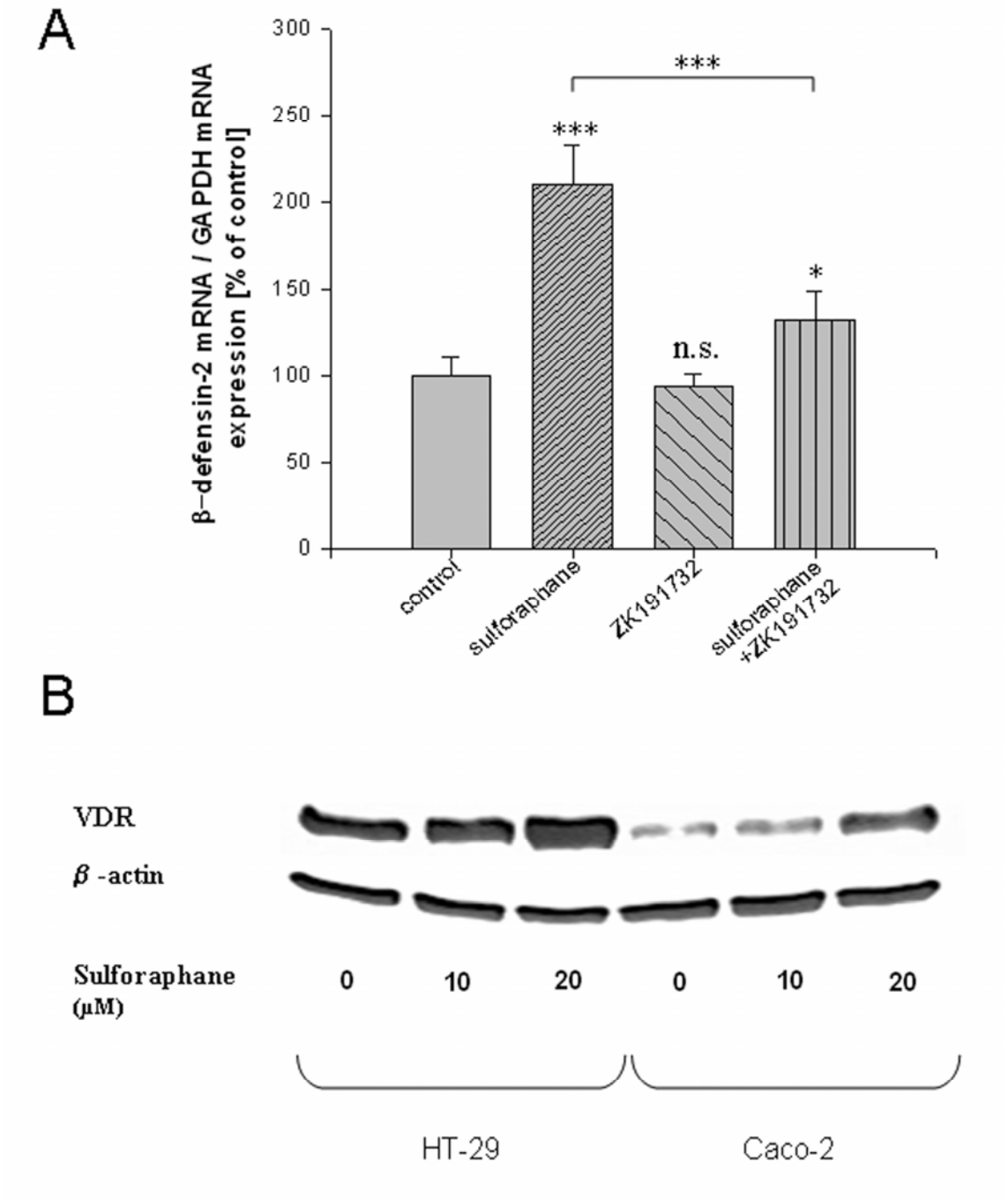
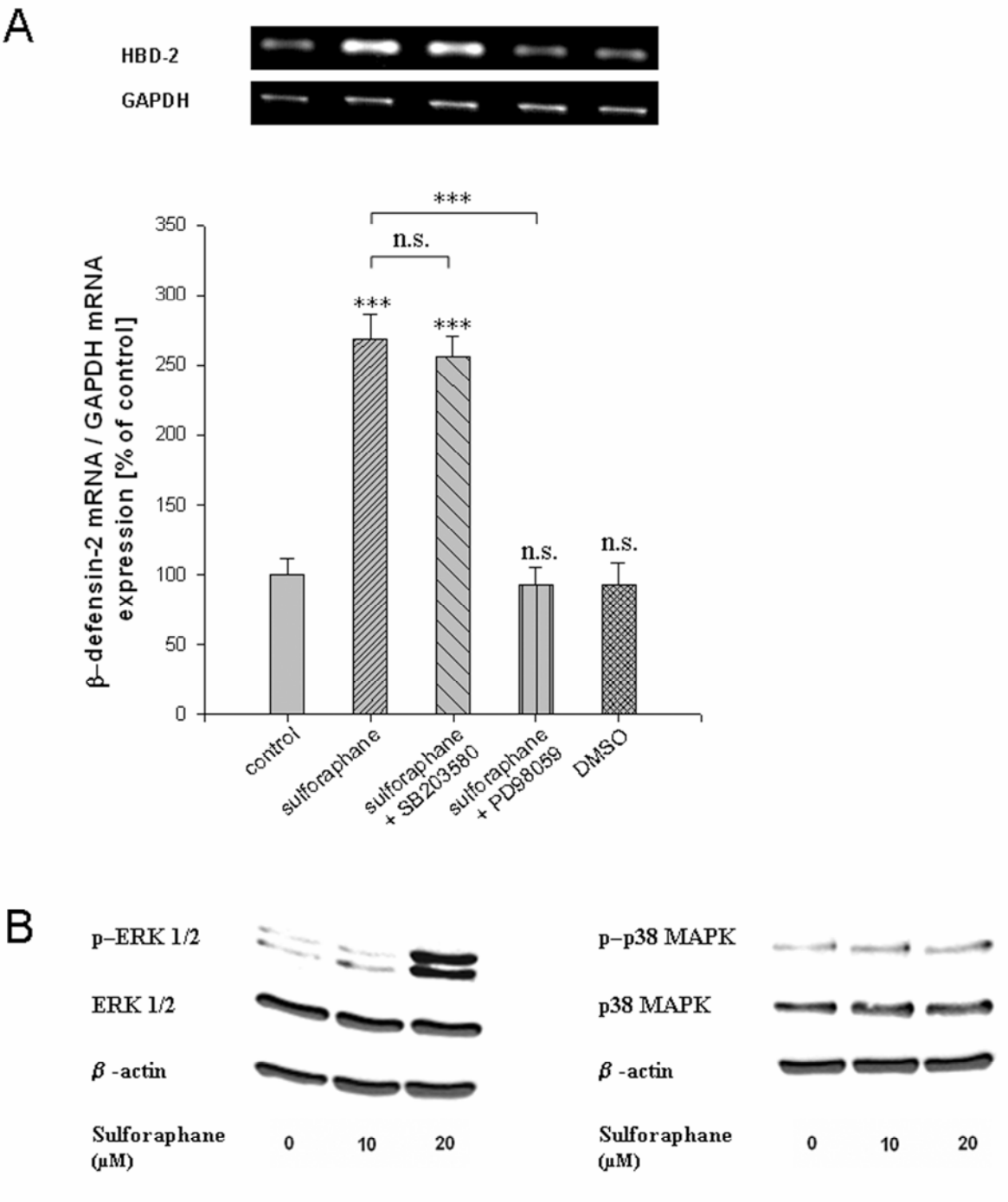

A
β-defensin-2 mRNA / GAPDH mRNA expression [% of control]
300
250
200
150
100
50
0
***
***
n.s.
*
control
sulforaphane
ZK191732
sulforaphane +ZK191732
B
VDR
β -actin
Sulforaphane (μM)
0
10
20
0
10
20
HT-29
Caco-2

Figure 5


A
HBD-2
GAPDH
β–defensin-2 mRNA / GAPDH mRNA expression [% of control]
350
300
250
200
150
100
50
0
***
n.s.
***
***
n.s.
n.s.
control
sulforaphane
sulforaphane + SB203580
sulforaphane + PD98059
DMSO
B
p–ERK 1/2
ERK 1/2
β -actin
Sulforaphane (μM)
0
10
20
p–p38 MAPK
p38 MAPK
β -actin
Sulforaphane (μM)
0
10
20

V

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Involvement of different nuclear hormone receptors in butyrate-mediated inhibition of inducible NFκB signalling

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Abstract

Background: NFκB plays a major role in the control of immune responses and inflammation. Recently, butyrate has not only been demonstrated to suppress NFκB activation in colorectal cancer cells, but also to modulate the activity and expression of the Peroxisome-Proliferator-Activated-Receptor γ (PPARγ) and the vitamin D receptor (VDR). Therefore, we investigated a putative involvement of both receptors in butyrate-mediated inhibition of inducible NFκB signalling.

Results: Treatment of HT-29 cells with butyrate attenuated basal p50 as well as TNFα- and LPS-induced p50 and p65 NFκB dimer activity in the nucleus as measured by transcription factor assay. Cytosolic expression of IκBα protein was reduced by butyrate, and TNFα but not by LPS. Challenge of cells with the VDR antagonist ZK191732 up-regulated basal NFκB activity by decreasing IκBα simultaneously, while basal signalling was not influenced by the PPARγ inhibitor GW9662. Pre-treatment with ZK191732 reduced the inhibitory effect of butyrate on NFκB activation caused by TNFα whereas no activation was noted in transfected dominant-negative PPARγ mutant vector cells. Adversely, the inhibitory effect of butyrate on NFκB activity induced by LPS was almost reversed in dominant-negative PPARγ mutant cells while pre-incubation of ZK191732 did not affect butyrate-mediated attenuation of LPS-induced NFκB signalling.

Conclusion: These findings provide evidence for the involvement of the nuclear hormone receptors PPARγ and VDR in butyrate-mediated inhibition of inducible NFκB activation dependent on the stimulated signalling pathway. Moreover, VDR appears to play an inhibitory role in the regulation of basal NFκB signalling.

Keywords: Butyrate; Inflammation; HT-29; LPS; NFκB; PPARγ; TNFα; VDR

1. Introduction

The short chain fatty acid (SCFA) butyrate results from anaerobic fermentation of undigested carbohydrates and dietary fiber in the colon (Wachtershauser and Stein, 2000). Butyrate regulates many epithelial cell activities in the colon including the induction of differentiation, inhibition of cell proliferation and the initiation of apoptosis (Klurfeld, 1999; Wachtershauser and Stein, 2000). Moreover, butyrate exerts immune-modulatory effects and anti-inflammatory properties by stimulating the release of antimicrobial peptides and by influencing cytokine-activated gene expression in colonic epithelial cells (Inan et al., 2000; Schauber et al., 2004; Schwab et al., 2007). Thus, deficiencies in luminal butyrate produc-

Abbreviations: CD, Crohn's disease; CREB, cyclic AMP response element binding protein; DMEM, dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; ECACC, European collection of cell cultures; FCS, foetal calf serum; IBD, inflammatory bowel disease; IKK, IκB kinase; LDH, lactate dehydrogenase; LPS, lipopolysaccharides; NFκB, nuclear factor-κB; PBS, phosphate buffered saline; PPARγ, peroxisome proliferator-activated receptor γ; SCFA, short chain fatty acid; TBS-T, tris-buffered saline containing 0.05% Tween 20; TLR, Toll-like Receptor; TNFR, Tumor-Necrosis-Factor Receptor; VD_3, 1,25-dihydroxyvitamin D3; VDR, vitamin D receptor

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tion have been linked to chronic bowel inflammation (Ogawa et al., 2003; Segain et al., 2000). There is substantial evidence that butyrate exerts some of its anti-inflammatory effects through suppression of nuclear factor-κB (NFκB) activity (Inan et al., 2000; Luhrs et al., 2002; Yin et al., 2001). However, the regulatory mechanisms are far from being understood.

NFκB is an ubiquitously expressed family of transcription factors controlling the expression of numerous genes involved in inflammatory and immune responses and cellular proliferation (Ben-Neriah, 2002; Ghosh and Karin, 2002; Karin and Ben-Neriah, 2000). NFκB rapidly enhances the transcription of various inflammatory cytokines, adhesion molecules, and chemokines (Baeuerle and Baltimore, 1996; Siebenlist et al., 1994). The NFκB family consists of five different proteins (p50 (NFκB1), p52 (NFκB2), p65 (RelA), RelB, and c-Rel) which tend to dimerize (Baeuerle and Baltimore, 1996; Siebenlist et al., 1994). The majority of NFκB dimers are composed of the p65 and the p50 or p52 subunits (Baeuerle and Baltimore, 1996; Siebenlist et al., 1994). In resting cells, NFκB is sequestered in the cytoplasm by association with inhibitory proteins called IκBs (Baeuerle, 1998). In response to cellular stimulation, by *e.g.* inflammatory cytokines, IκB kinase (IKK) is activated and phosphorylates IκB on two serine residues (Ben-Neriah, 2002; Quivy and Van Lint, 2004). IκB is then ubiquitinated and degraded by the proteasome, freeing NFκB to be translocated into the nucleus and to exert its functions as transcription factor (Ben-Neriah, 2002; Quivy and Van Lint, 2004; Schutze et al., 1992).

Previously, we have demonstrated that the nuclear hormone receptors Peroxisome-Proliferator-Activated-Receptor γ (PPARγ) and the vitamin D receptor (VDR), transcription factors with anti-inflammatory capacities, are up-regulated and activated by butyrate (Gaschott and Stein, 2003; Gaschott et al., 2001; Schwab et al., 2006; Wachtershauser et al., 2000). PPARγ and VDR are highly expressed in the colonic epithelium indicating that both receptors are important agents in the physiology of the human colon (Desvergne and Wahli, 1999; Nagpal et al., 2005). Ligands for both receptors have been shown to interfere with the activity of NFκB and to influence the ability of colonocytes to express immune-modulatory cytokines (Segain et al., 2000; Sun et al., 2006). Our aim in this study was to examine the role of PPARγ and VDR in butyrate-mediated inhibition of NFκB activity induced by TNFα and LPS. Moreover, the involvement of both receptors for basal NFκB signalling was explored.

2. Materials and methods

2.1. Cell culture

The human colorectal cancer cell line HT-29 was obtained from the European Collection of cell cultures (ECACC). Cells were cultured in a humidified incubator at 37 °C in an atmosphere of 95% air and 5% CO_2. HT-29 cells were grown in McCoy's 5A Medium, supplemented with 10% foetal calf serum (FCS) and 1% penicillin/streptomycin. Medium of the dominant-negative PPARγ mutant and empty-vector HT-29 cells was supplied with 400 μg/mL Geneticin 418 sulfate (G418, Gibco-BRL, Eggenstein, Germany). Cells were regularly screened for mycoplasma contamination using the VenorGem Mycoplasma detection kit (Minerva Biolabs, Berlin, Germany).

For experiments, cells were seeded in plastic cell culture wells and cultivated in McCoy's 5A Medium until 80% confluency was reached. Medium was then removed and replaced by medium containing either the solvent, butyrate (4 mM), TNFα (100 ng/mL), LPS (10 μg/mL), the vitamin D receptor antagonist ZK191732 (10 μM), the PPARγ antagonist GW9662 (5 μM) or one of the combinations of butyrate (4 mM) with TNFα, LPS, ZK191732, GW9662, TNFα + ZK191732 or LPS + ZK191732, respectively.

In the experiments, cells were pre-incubated with the inhibitors for 2 h, followed by challenge of cells with butyrate for up to 24 h and then stimulated with TNFα or LPS for 30 min. Butyrate (Merck-Schuchardt, Hohenbrunn, Germany) was solubilized in phosphate buffered saline (PBS) and added to the medium. TNFα (Cell Concepts, Umkirch, Germany) and LPS (lipopolysaccharides from *Escherichia coli* 055:B5, Sigma–Aldrich Chemie, Steinheim, Germany) were dissolved in aqua ad injectabilia (DeltaSelect, Pfufflingen, Germany), GW9662 (Sigma, Saint Louis, Missouri, USA) was dissolved in dimethyl sulfoxide (DMSO, Fluka, Sigma–Aldrich Chemie, Steinheim, Germany) and ZK191732 (supplied by the Department of Medicinal Chemistry at Schering AG, Berlin, Germany) was solubilized in ethanol (Merck, Darmstadt, Germany). Final maximal concentration of solvents in medium was kept below 0.1% (v/v). Solvent controls did not differ from the control group. The medium was changed every day. Cells were then harvested at the times indicated in the figure legends.

2.2. Transfection assay

The following plasmids were used for transfection: pcDNA3 (Invitrogen, Karlsruhe, Germany), as an empty-vector for control transfection and the plasmid pcDNA3-PPARγ$_{L468A/E471A}$, a dominant-negative PPARγ double-mutant, that was kindly provided by VK Chatterjee (Department of Medicine, University of Cambridge, Addenbrooke's Hospital, Cambridge, United Kingdom) (Gurnell et al., 2000). These constructs were transfected into subconfluent HT-29 cells with lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) in serum-free conditions. After 6 h, the cells were supplied with fresh medium containing 10% FCS. 24 h later, the cells were supplied with medium containing G418 (400 μg/mL) and culture medium supplemented with G418 was replaced twice a week. G418-resistant colonies were collected and used for further analysis.

2.3. Cytotoxicity

Cytotoxicity was excluded by lactate dehydrogenase (LDH) release assay using a commercial kit (LDH kit, Roche, Mannheim, Germany).

2.4. Cytoplasmatic and nuclear protein extraction

HT-29 cells were stimulated with indicated substances at 80% confluency. Cytoplasmatic and nuclear extraction was performed using the Active Motif Nuclear cell extraction kit (Rixensart, Belgium) according to the manufacturer's instructions. Protein content was determined via Bio-Rad colorimetric assay according to the method of Bradford (Bio-Rad Laboratories, Munich, Germany).

2.5. NFκB p65/NFκB p50 transcription factor assay

NFκB activity was determined with the TransAM® transcription factor assay kit (Active Motif, Rixensart, Belgium) following the manufacturer's protocol. In brief, 5 μg of nuclear extract was added to each well of a 96-well plate precoated with NFκB consensus binding oligonucleotide (5′-GGGACTTTCC-3′). After 1 h of incubation with smooth agitation, wells were washed three times with washing buffer and then incubated with p50 or p65 antibody (dilution 1:1000) for 1 h at 20 °C, respectively. After three successive washings, the wells were incubated for 1 h with diluted horseradish peroxidase-conjugated antibody (dilution 1:1000) followed by addition of 100 μL of developing solution. After 5 min of incubation, the reaction was blocked by adding 100 μL of stop solution reagent. Optical density was determined by a spectrophotometer at 450 nm with a reference wavelength of 655 nm.

2.6. PPARγ transactivation assay

PPARγ activity was assayed using an ELISA-based transactivation TransAM® PPARγ kit (Active Motif, Rixensart, Belgium) following the manufacturer's protocol (see NFκB transcription factor assay). The PPARγ TransAM® kit contains a 96-well plate with immobilized oligonucleotides containing a peroxisome proliferator response element (5′-AACTAGGTCAAAGGTCA-3′).

2.7. SDS-polyacrylamide gel electrophoresis and immunoblot analysis

Equal amounts of cytoplasmatic protein lysates were separated on a 12.5% SDS-polyacrylamide gel for IκBα and PPARγ, respectively. Proteins were transferred onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Subsequently, membranes were blocked for 1 h with 5% (w/v) nonfat dried milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T). Membranes were then incubated overnight with a 1:500 dilution of IκBα-antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) or a 1:2000 dilution of PPARγ-antibody (Calbiochem, La Jolla, CA) in 0.05% TBS-T and 5% (w/v) nonfat dried milk, respectively. After washing, the blots were incubated for half an hour with corresponding horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA, dilution 1:2000) in 0.05% TBS-T and 5% (w/v) nonfat dried milk. The washing steps were repeated, and subsequently enhanced chemoluminescence detection was performed according to the manufacturer's instructions (ECL, Amersham Pharmacia Biotech, Buckinghamshire, UK) on Hyperfilm-MP (Amersham International plc, Buckinghamshire, UK). Blots were then reprobed with β-actin antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA). For quantitative analysis, bands were detected by scanning densitometry, using a Desaga CabUVIS scanner and Desaga ProVilDoc software (Desaga, Wiesloch, Germany).

2.8. Statistics

All statistical analyses were performed using GraphPad Prism 4.01 (San Diego, CA, USA). Analysis of variance (ANOVA) was performed when more than two groups were compared and, when significant, multiple comparisons were performed with the Tukey's test. If not otherwise stated, data are expressed as means ± S.D. from three independent experiments. A p value < 0.05 was considered to be significant.

3. Results

3.1. Butyrate reduces the basal, TNFα- and LPS-induced nuclear activation of NFκB

Challenge of HT-29 wild-type cells with butyrate (4 mM) for 24 h resulted in a reduction of basal p50 dimer NFκB DNA binding activity in the nucleus (Fig. 1) (−45% versus control, $p < 0.001$). In contrast, basal p65 NFκB DNA binding activity was not affected in response to butyrate (Fig. 2).

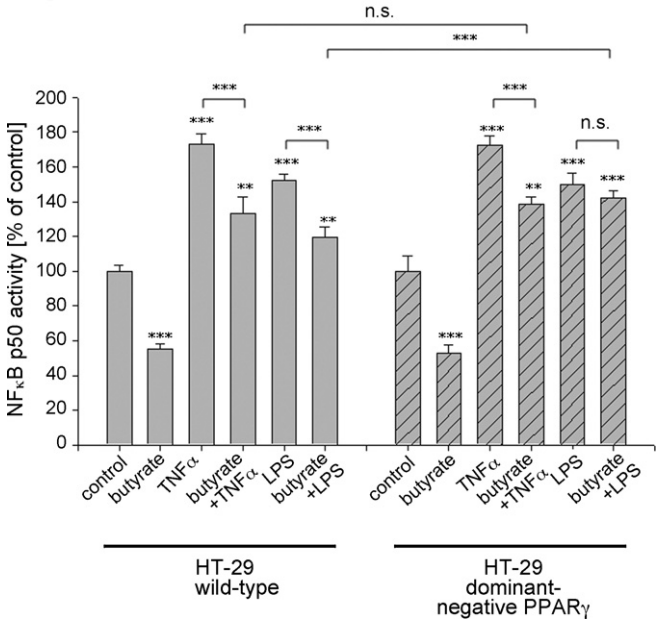

Fig. 1. Influence of butyrate (4 mM), TNFα (100 ng/mL), LPS (10 μg/mL) or one of the combinations of butyrate with TNFα or LPS on NFκB p50 DNA binding activity in HT-29 wild-type and PPARγ dominant-negative vector cells. Cells were pre-treated with butyrate for 24 h followed by TNFα or LPS stimulation for 30 min. Nuclear extracts were prepared and NFκB activity was analysed with the TransAM® transcription factor assay kit. ***$p < 0.001$, **$p < 0.01$, n.s. = not significant.

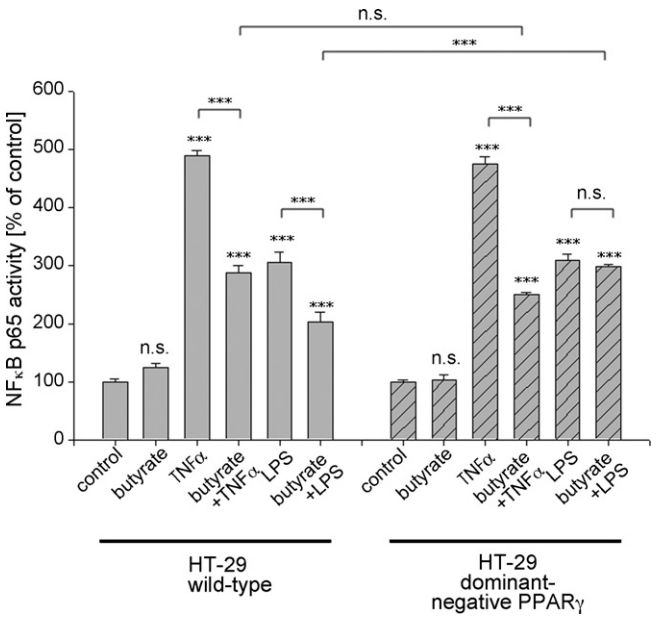

Fig. 2. Influence of butyrate (4 mM), TNFα (100 ng/mL), LPS (10 μg/mL) or one of the combinations of butyrate with TNFα or LPS on NFκB p65 DNA binding activity in HT-29 wild-type and PPARγ dominant-negative vector cells. Cells were pre-treated with butyrate for 24 h followed by TNFα or LPS stimulation for 30 min. Nuclear extracts were prepared and NFκB activity was analysed with the TransAM® transcription factor assay kit. ***$p < 0.001$, n.s. = not significant.

Moreover, stimulation of HT-29 wild-type cells with TNFα (100 ng/mL) or LPS (10 μg/mL) led to a strong increase of both p50 (TNFα: +73%, $p < 0.001$; LPS: +52%, $p < 0.001$) and p65 NFκB DNA binding activities (TNFα: +389%, $p < 0.001$; LPS: +205%, $p < 0.001$) (Figs. 1 and 2). Co-incubation with butyrate (4 mM) significantly attenuated these effects (TNFα: p50: −54%, $p < 0.001$; p65: −52%, $p < 0.001$; LPS: p50: −63%, $p < 0.001$; p65: −49%, $p < 0.001$) (Figs. 1 and 2).

3.2. Butyrate and TNFα decrease IκBα levels in the cytosolic fraction

Cytosolic expression of IκBα was reduced in response to TNFα (100 ng/mL; −37%, $p < 0.001$) stimulation for 30 min and was also decreased after butyrate (4 mM; −60%, $p < 0.001$) incubation for 24 h (Fig. 3). Co-incubation of butyrate and TNFα enhanced the decrease of IκBα (−83%, $p < 0.001$; Fig. 3). Stimulation of cells with LPS (10 μg/mL) for 30 min did either not affect cytosolic IκBα expression nor IκBα levels caused by butyrate (data not shown).

3.3. PPARγ is involved in butyrate-mediated inhibition of LPS-induced NFκB activity

To investigate whether PPARγ plays a role in the inhibition of NFκB activity caused by butyrate, NFκB p50 and p65 activity was examined in HT-29 cells transfected with a dominant-negative PPARγ mutant (Figs. 1 and 2). In this mutant the highly conserved hydrophobic and charged residues (Leu468 and Glu471) in helix 12 of the ligand binding domain are mutated to alanine. As a consequence, this cell mutant indeed retains

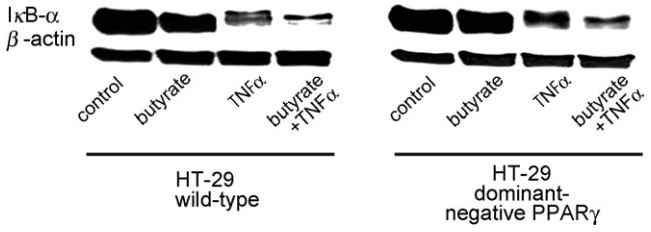

Fig. 3. Effect of butyrate (4 mM), TNFα (100 ng/mL) or the combination of both substances on IκBα protein expression in HT-29 wild-type and PPARγ dominant-negative vector cells. Cells were pre-treated with butyrate for 24 h followed by TNFα stimulation for 30 min. Cytoplasmic extracts were then prepared and analysed by western blotting. One representative blot of three independent experiments is shown. Quantitative data are normalized for β-actin levels. IκBα protein expression after stimulation with the substances was comparable in all cell types.

PPARγ ligand and DNA binding, but exhibits markedly reduced transactivation due to impaired coactivator recruitment (Gurnell et al., 2000). In the mutant cell line, basal and inducible NFκB transcriptional activation was similar compared to wild-type and empty-vector HT-29 cells after stimulation with butyrate (4 mM), TNFα (100 ng/mL) or their combination. Even the down-regulation of IκBα levels was comparable in all three cell lines (Fig. 3). The findings for basal NFκB activity were confirmed by co-incubation of butyrate (4 mM) with the irreversible PPARγ antagonist GW9662 (5 μM). Administration of both substances did not reverse the effect of butyrate on both basal NFκB p50 dimer (Fig. 4A) and p65 dimer activity (Fig. 4B). Similarly, the decrease of IκBα was not affected by co-treatment of GW9662 and butyrate compared to single stimulation with butyrate (Fig. 4C). In contrast, butyrate-mediated inhibition of LPS-induced (10 μg/mL) NFκB activity was almost reversed in dominant-negative PPARγ mutant cells (Figs. 1 and 2).

To evaluate receptor activity of PPARγ in HT-29 wild-type and dominant-negative PPARγ mutant Caco-2 cells, PPARγ activation was determined via a transcriptional factor assay (Fig. 5). Treatment of both HT-29 wild-type and empty-vector cells with butyrate increased PPARγ activity up to +215% (versus control, $p < 0.001$). As expected, PPARγ activity was not increased in butyrate-challenged HT-29 dominant-negative PPARγ mutant cells. In addition, co-incubation of butyrate with the PPARγ antagonist GW9662 reversed the increase in PPARγ activity caused by butyrate (Fig. 5).

3.4. LPS up-regulates the expression of PPARγ

To unveil whether PPARγ is a target of the LPS signalling pathway, HT-29 cells were stimulated with LPS (10 μg/mL) for several time points. As depicted in Fig. 6, LPS increased PPARγ protein expression in a time-dependent manner, already beginning at 0.5 h of stimulation.

3.5. VDR plays a role in butyrate-mediated inhibition of TNFα-induced and basal NFκB signalling activity

Challenge of HT-29 cells with the VDR antagonist ZK191732 for 24 h increased both, basal p50 (+58%, $p < 0.01$) and p65

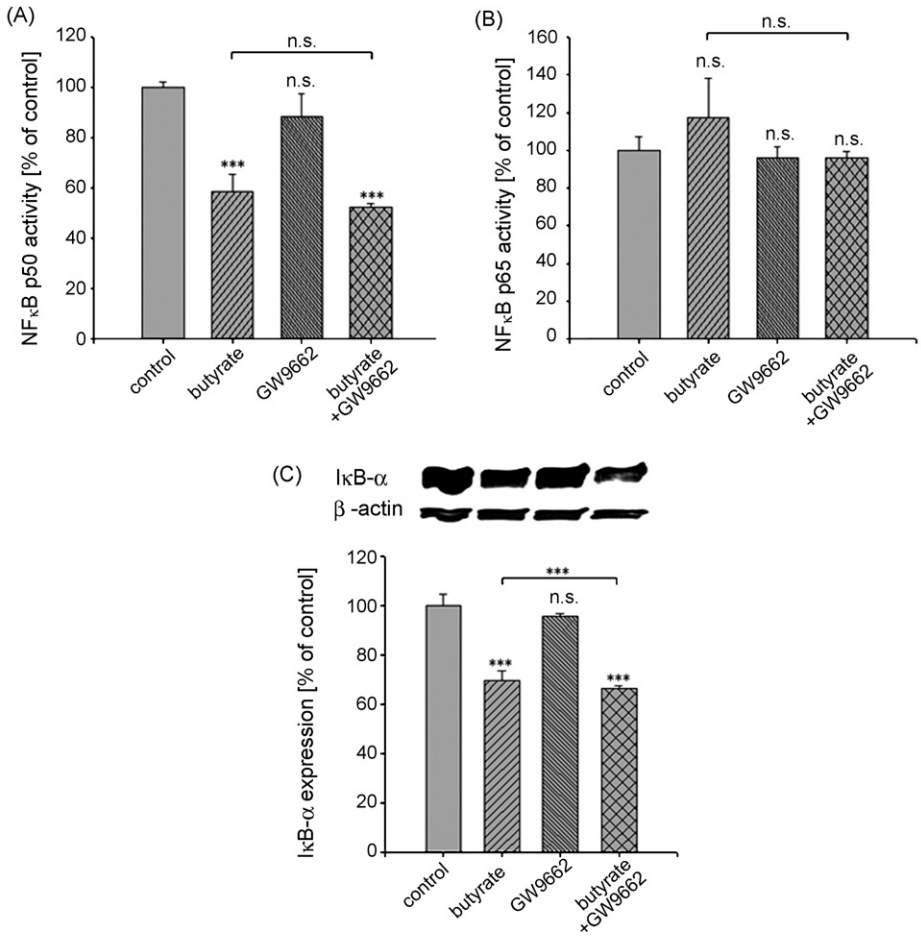

Fig. 4. NFκB p50 (A) and p65 (B) dimer DNA binding activity in HT-29 wild-type cells after stimulation with butyrate (4 mM), the PPARγ antagonist GW9662 (5 μM) or the combination of both substances. Cells were pre-treated with GW9662 for 2 h followed by butyrate stimulation for 24 h. Nuclear extracts were then prepared and NFκB activation was analysed with TransAM® transcription factor assay kit. ***$p < 0.001$, n.s. = not significant. (C) Western blot for IκBα levels after treatment with butyrate (4 mM), the PPARγ antagonist GW9662 (5 μM) or the combination of butyrate and GW9662 in HT-29 wild-type cells. Cells were pre-treated with GW9662 for 2 h followed by butyrate stimulation for 24 h. One representative blot of three independent experiments is shown. ***$p < 0.001$, n.s. = not significant.

dimer (+78%, $p < 0.001$) NFκB DNA binding activity (Fig. 7A and B) while reducing the expression of IκBα (Fig. 7C). The decrease in IκBα caused by combined treatment of TNFα and butyrate was attenuated in response to ZK191732 (Fig. 7C). Moreover, co-incubation of ZK191732 in presence of TNFα almost abolished the inhibitory effect of butyrate on NFκB DNA binding activity (Fig. 7A and B). In contrast, butyrate-mediated inhibition of LPS-induced (10 μg/mL) NFκB activity was not affected by co-stimulation with ZK191732 (Fig. 7A and B).

4. Discussion

Much of the diversity attributed to NFκB functions can be ascribed to specific cell surface receptors recognizing a variety of unrelated ligands (Orange and Geha, 2003). Two main key receptor families that can activate NFκB include the Toll-like Receptors (TRL) which recognize LPS, a cell wall component of Gram-Negative bacteria and Tumor-Necrosis-Factor Receptors (TNFR) which are triggered by their ligand TNFα (Akira and Takeda, 2004; Cario and Podolsky, 2005; Orange and Geha, 2003). Each of the different groups of receptors activates NFκB utilizing a specific signalling cascade (Orange and Geha, 2003). High levels of circulating and mucosal pro-inflammatory cytokines resulting from increased NFκB activity have been reported in the intestinal mucosa of patients with IBD, especially ulcerative colitis and Crohn's disease (CD) (Brynskov et al., 1994; Di Sabatino et al., 2005; Sartor, 1997). NFκB may therefore be an interesting clinical target to suppress inflammation and also the development of cancer in IBD. Several studies have demonstrated that the SCFA butyrate is able to modulate NFκB signalling in epithelial cells (Inan et al., 2000; Luhrs et al., 2002; Yin et al., 2001). However, comprehensive knowledge by which butyrate interacts with the NFκB signalling pathway is still lacking. Our study demonstrates for the first time that PPARγ plays part in butyrate-mediated inhibition of LPS-induced NFκB activation. Moreover, the VDR seems to modulate both the expression of basal NFκB signalling as well as attenuation of TNFα-induced NFκB activation.

In resting cells, butyrate has been shown to inhibit p50 but not p65 NFκB DNA binding activity (Inan et al., 2000). In addition, recent studies provide evidence that butyrate is able to attenuate both p50 and p65 NFκB dimer activity induced by TNFα

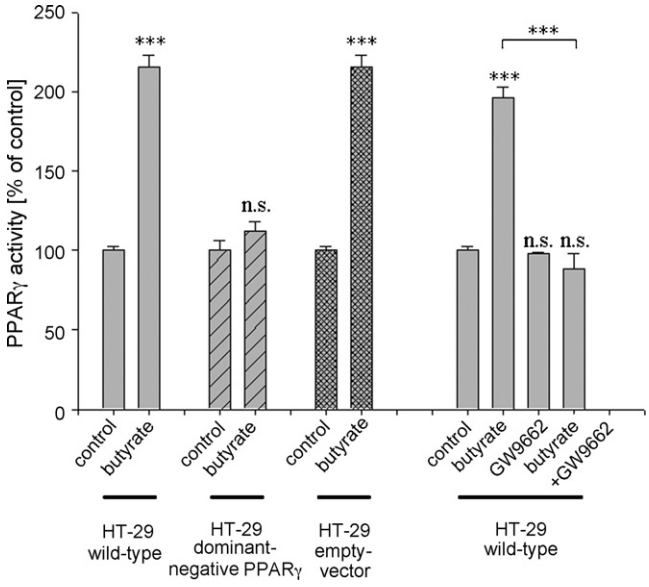

Fig. 5. Effect of butyrate (4 mM) on PPARγ DNA binding activity in HT-29 wild-type, PPARγ dominant-negative and empty-vector cells. In addition, the influence of butyrate (4 mM), the PPARγ antagonist GW9662 (5 μM) or the combination of butyrate and GW9662 on PPARγ activity in HT-29 wild-type cells was measured. Cells were pre-treated with GW9662 for 2 h followed by butyrate stimulation for 24 h. Nuclear extracts were prepared and PPARγ binding activity was determined with the TransAM® transcription factor assay kit. ***$p < 0.001$, n.s. = not significant. PPARγ binding activity was similar for HT-29 wild-type and empty-vector cells after stimulation and was suppressed using dominant-negative PPARγ cells or GW9662.

or LPS in several cell lines including HT-29 cells (Chakravortty et al., 2000; Inan et al., 2000; Luhrs et al., 2002; Segain et al., 2000; Yin et al., 2001). Our data corroborate these findings. Moreover, TNFα and LPS were reported to provoke a time-dependent decrease of IκBα, an inhibitor protein for NFκB activity, which was prevented by co-treatment with butyrate (Place et al., 2005; Segain et al., 2000; Yin et al., 2001). A decrease in IκBα expression was also observed after TNFα stimulation in our experiments. However, this reduction was even stronger after butyrate pre-treatment. Comparable results were reported in the study of Inan et al. (2000) suggesting that, for this specific point in time, the inhibitory effect of butyrate on NFκB activity is not achieved via the expression of IκBα. Nevertheless, other mechanisms or other members of the IκB family may be also responsible for the inhibitory effects of butyrate on NFκB actions.

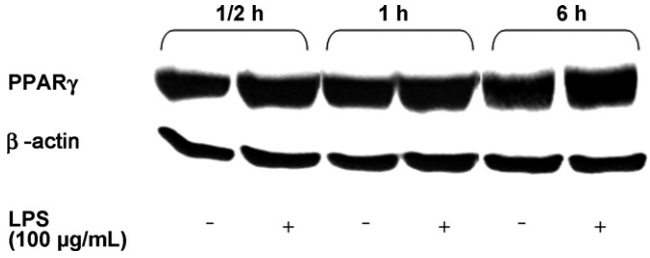

Fig. 6. Western blot for PPARγ expression after treatment of HT-29 wild-type cells with LPS (10 μg/mL) for 0.5, 1 and 6 h. One representative blot of three independent experiments is shown.

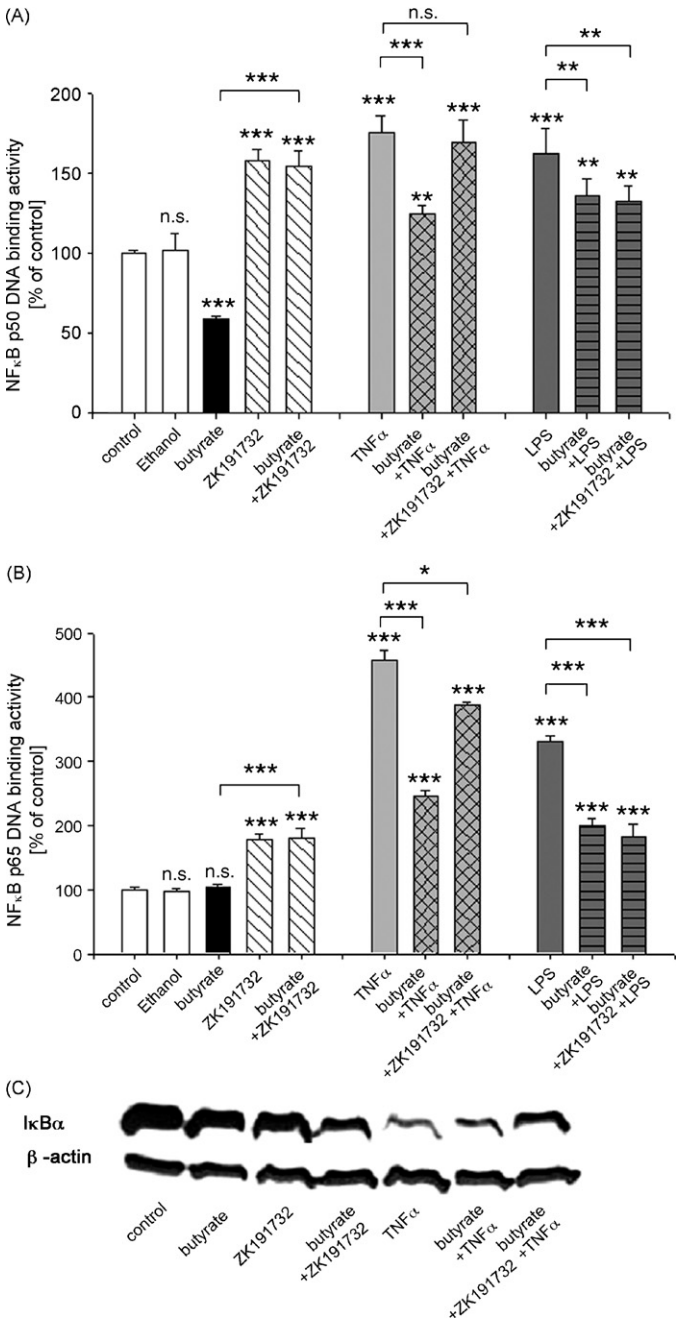

Fig. 7. NFκB p50 (A) and p65 (B) dimer DNA binding activity in HT-29 wild-type cells after stimulation with butyrate (4 mM), the VDR antagonist ZK191732 (10 μM), TNFα (100 ng/mL), LPS (10 μg/mL), butyrate + ZK191732, butyrate + TNFα, butyrate + LPS, or one of the combinations of butyrate + ZK191732 with TNFα or LPS, respectively. Cells were pre-treated with ZK191732 for 2 h followed by butyrate stimulation for 24 h. Cells were then stimulated with TNFα or LPS for 30 min. Nuclear extracts were prepared and NFκB activation was analysed with TransAM® transcription factor assay kit. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, n.s. = not significant. (C) Western blot for IκBα expression after treatment with butyrate (4 mM), the VDR antagonist ZK191732 (10 μM), the combination of butyrate and ZK191732, TNFα (100 ng/mL) or the combination of all three substances. Cells were pre-treated with ZK191732 for 2 h followed by butyrate stimulation for 24 h. Cells were then stimulated with TNFα for 30 min. One representative blot of three independent experiments is shown.

There is increasing evidence that the expression and activity of PPARγ and VDR are under the control of butyrate implying that the receptors may participate in butyrate-mediated suppression of NFκB activation (Gaschott and Stein, 2003; Gaschott et al., 2001; Schwab et al., 2006; Wachtershauser et al., 2000). PPARγ and VDR are both ligand-activated transcription factors that belong to the nuclear hormone receptor family and participate in a variety of immune processes (Tirona and Kim, 2005). VDR is widely expressed in epithelial tissues, cells of the immune system and several cancer cell lines including colorectal cancer cells (Giuliano et al., 1991; Segaert and Bouillon, 1998). PPARγ is activated by natural ligands such as fatty acids and eicosanoids and is highly expressed in colonic epithelium, indicating an important role of the receptor in the physiology of the human colon (Desvergne and Wahli, 1999). All these characteristics make both receptors potential targets in butyrate-mediated inhibition of NFκB signalling. To scrutinize a possible involvement of the receptors for the activation of NFκB in resting HT-29 cells and also after stimulation with butyrate, NFκB activity was examined after application of the VDR inhibitor ZK191732 or the PPARγ antagonist GW9662. Indeed, treatment of cells with ZK191732 alone or in combination with butyrate increased both p50 and p65 basal NFκB dimer activities while reducing the expression IκBα simultaneously. In contrast, challenge of cells with the PPARγ antagonist GW9662 did neither affect basal NFκB activity, nor the expression of IκBα. Similarly, co-stimulation of GW9662 did not alter the effect of butyrate on NFκB activity and IκBα expression. Comparable results were obtained in dominant-negative PPARγ mutant HT-29 vector cells.

In addition, the role of both receptors in butyrate-mediated inhibition of inducible NFκB activity was scrutinized. HT-29 cells were stimulated with TNFα or LPS, substances leading to increased NFκB activity via two different pathways, *i.e.* TNFR and TRL signalling, respectively. To reveal the involvement of the receptors in both pathways, experiments were performed in presence of the VDR inhibitor ZK191732 or in the PPARγ mutant HT-29 cell line. In our *in vitro* model, pre-treatment with ZK191732 in presence of TNFα almost reversed the inhibitory effect caused by butyrate on both p50 and p65 NFκB dimer activities while the effects on NFκB activity in HT-29 PPARγ mutant cells were comparable to wild-type cells. These results indicate that the VDR seems to play part in TNFR signalling pathway leading to increased NFκB activation. Previous works reporting direct modulation of NFκB activity through the active metabolite of vitamin D, 1,25-dihydroxyvitamin D_3 (VD_3) support our findings for the involvement of the VDR in basal and in butyrate-mediated inhibition of TNFα-stimulated NFκB activity. In human lymphocytes, VD_3 suppressed NFκB p50 and its precursor proteins p105 and p65 (Yu et al., 1995). Furthermore, VD_3 has been shown to decrease the DNA binding capacity of NFκB in human fibrinoblasts (Harant et al., 1998). In peripheral blood mononuclear cells of healthy controls and patients with CD, the vitamin D analogue TX 527 not only decreased basal and TNFα-induced activation of NFκB, but also increased IκBα protein levels, even in association with TNFα (Stio et al., 2007). In addition, in a mouse model lacking the VDR receptor a reduction of IκBα was observed (Sun et al., 2006). The degradation of IκBα induced by TNFα was inhibited by VD_3 in normal but not in VDR lacking cells (Sun et al., 2006). Revealing NFκB activation via the TLR signalling pathway our experiments demonstrate that butyrate-mediated inhibition of LPS-induced NFκB activity was almost completely abolished in HT-29 PPARγ mutant cells while inhibition of the VDR did not affect LPS-induced inhibition of NFκB activity caused by butyrate. Thus, butyrate-mediated inhibition of NFκB activation via the TLR seems to be modulated by PPARγ. Several mechanisms have been reported by which PPARγ can inhibit the actions of NFκB activity. The transcription factor can not only physically interact with the NFκB subunits p65, subunit p50, or both, but also inhibit degradation of the inhibitory protein IκB (Daynes and Jones, 2002; Dubuquoy et al., 2002). In addition, up-regulation of IκB levels and an increased interaction of PPARγ with cyclic AMP response element binding protein (CREB), a coactivator interacting with p65 have been described (Daynes and Jones, 2002; Dubuquoy et al., 2002). Moreover, the present study demonstrates that PPARγ protein expression is increased after stimulation with LPS. Our finding is supported by other *in vitro* and *in vivo* studies demonstrating the ability of LPS to elevate PPARγ levels through TLR4 signalling (Dubuquoy et al., 2003, 2006; Eun et al., 2006). This up-regulation may ultimately lead to attenuation of the pro-inflammatory NFκB signalling pathway induced by LPS (Dubuquoy et al., 2003; Eun et al., 2006).

In conclusion the data presented here demonstrate for the first time that the nuclear hormone receptors PPARγ and VDR play part in butyrate-mediated inhibition of inducible NFκB activation dependent on the stimulated signalling pathway. Moreover, VDR appears to play an inhibitory role in the regulation of basal NFκB signalling. Our results thereby complete the puzzle of several *in vivo* studies demonstrating that the anti-inflammatory actions of both nuclear hormone receptors are exhibited via interfering with NFκB signalling (Di Sabatino et al., 2005; Kaplan et al., 2005; Lee et al., 2006; Stio et al., 2007; Sun et al., 2006). Elucidating the NFκB pathway will add value to the understanding of the regulation of inflammatory processes. This in turn, may provide opportunities for the development of new preventive and therapeutic strategies in the treatment of IBD.

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PPARγ als molekulares Target epithelialer Funktionen im Intestinaltrakt

Schlüsselworte: Peroxisome Proliferator-Activated Rezeptor (PPARγ), Vitamin D Rezeptor (VDR), nukleäre Hormonrezeptoren, kolorektales Karzinom, gastrointestinale Zellregulierung, Butyrat, Mesalazin, Sulforaphan, Caspase-Kaskade, antimikrobielle Peptide, Cathelicidin, Beta-Defensin-2, NFκB-Signaltransduktionsweg

Die Mukosa des Gastrointestinaltraktes befindet sich in einem komplexen dynamischen Gleichgewicht. Schon kleinste Störungen dieses epithelialen Systems können zu einer veränderten Expression von antimikrobiell wirkenden Peptiden, zur Initiierung von unkontrolliertem Zellwachstum sowie zur Aktivierung des Transkriptionsfaktors NFκB verbunden mit der Freisetzung zahlreicher Entzündungsparameter führen. Diese Faktoren begünstigen die Entstehung gastrointestinaler Erkrankungen wie des kolorektalen Karzinoms und chronisch entzündlicher Darmerkrankungen.

Die vorliegende Arbeit mit dem Titel „PPARγ als molekulares Target epithelialer Funktionen im Intestinaltrakt“ hatte das Ziel, das Verständnis molekularer Mechanismen, die zur Aufrechterhaltung der gastrointestinalen Homöostase beitragen, zu vertiefen. Neben der Entschlüsselung wichtiger Funktionen von Mesalazin sowie der natürlich vorkommenden Histondeacetylase-Inhibitoren Butyrat und Sulforaphan in a) der Regulierung des Zellwachstums, in b) der Induktion der antimikrobiellen Peptide Beta-Defensin-2 und Cathelicidin, sowie in c) der Modulation des NFκB-Signaltransduktionsweges in kolorektalen Tumorzellen, konnte die Beteiligung der nukleären Hormonrezeptoren PPARγ und VDR als sog. „Gatekeepers“ in diesen komplexen Regulierungsvorgängen aufgezeigt werden.

Im Rahmen dieser Arbeit wurde erstmals die Bedeutung von PPARγ als zentrales zwischengeschaltetes Signalmolekül an den durch Butyrat- und Mesalazin-vermittelten pro-apoptotischen Effekten entlang der Caspase-Kaskade in Kolonozyten nachgewiesen. Die durch Butyrat-induzierte Caspase-3 Aktivität resultierte dabei aus der Initiierung sowohl des intrinsischen als auch des extrinsischen Signaltransduktionsweges bei simultaner Expressionshemmung der beiden inhibitorischen Apoptoseproteine Survivin und Xiap. Im Gegensatz dazu aktivierte Mesalazin diese Kaskade lediglich über den extrinsischen Weg. Zudem konnte ein durch Butyrat-vermittelter p38-MAPK-PPARγ-Caspase-3-Signalweg

etabliert werden. Im vorliegenden *in vitro* Zellsystem wurden ferner PPARγ-abhängige und -unabhängige Regulierungsmechanismen an den durch Mesalazin-induzierten pro-apoptotischen und anti-proliferativen Wirkungen nachgewiesen. Diese Vorgänge werden u.a. über die modulierende Funktion von Mesalazin auf das Tumor-Suppressor-Gene PTEN bzw. auf das Onkoprotein c-Myc vermittelt.

Des Weiteren konnte aufgezeigt werden, dass die durch Butyrat- und Sulforaphan-induzierte Expressionssteigerung der antimikrobiellen Peptide Beta-Defensin-2 und Cathelicidin von einer Vielzahl von Signalkaskaden und Rezeptoren reguliert wird. In diesem Induktionsprozess konnte erstmalig die essentielle Bedeutung des VDR aufgezeigt werden. Eine Beteilung von PPARγ wurde hingegen ausgeschlossen. Zudem wird die durch Butyrat-gesteigerte Cathelicidin-Expression vorwiegend über den p38-MAPK-Signalweg sowie über TGF-β1 reguliert. Der ERK1/2-Signalweg ist hingegen in die durch Sulforaphan-induzierte Expressionssteigerung von Beta-Defensin-2 involviert.

Im Rahmen dieser Arbeit konnte außerdem erstmals die Bedeutung von PPARγ und VDR als zwischengeschaltete Signalmoleküle der supprimierenden Wirkung von Butyrat auf die NFκB-Aktivität in Abhängigkeit von dessen Triggerung durch die pro-inflammatorischen Stimuli LPS und TNFα aufgezeigt werden. Darüber hinaus wurde eine regulatorische Funktion des VDR auf die basale NFκB-Aktivität nachgewiesen. Eine modulierende Wirkung von PPARγ auf die basale NFκB-Aktivität konnte hingegen nicht beobachtet werden.

Mit den vorliegenden Resultaten in kolorekatalen Tumorzellen wird das Spektrum der intestinalen Zellregulierung durch die modulierende Funktion von Mesalazin, Butyrat und Sulforaphan auf die Caspase-Kaskade, auf antimikrobielle Peptide sowie auf den NFκB-Signaltransduktionsweg erweitert. Ferner konnte eine Beteiligung der nukleären Hormonrezeptoren PPARγ und VDR an den genannten Signaltransduktionsprozessen aufgezeigt werden. Die vorliegende Arbeit unterstreicht somit nicht nur die allgemeine zellbiologische Bedeutung der beiden nukleären Hormonrezeptoren PPARγ und VDR an gastrointestinalen Regulierungsvorgängen, sondern impliziert auch deren Potential zur Entwicklung neuer therapeutischer Ansätze in der Behandlung des kolorektalen Karzinoms und von chronisch entzündlichen Darmerkrankungen. Die prinzipielle Relevanz der gefundenen Daten muss jedoch durch weitere grundlagenwissenschaftliche und intensive klinische Forschungsbemühungen untersucht werden.