Regulation of neutral ceramidase in glomerular mesangial cells

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Dekan:	Prof. Dr. W. Müller
Gutachter:	Prof. Dr. J. Pfeilschifter Prof. Dr. D. Steinhilber
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meinen Eltern

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Franzen R, Fabbro D, Aschrafi A, Pfeilschifter J, and Huwiler A
Nitric oxide induces degradation of the neutral ceramidase in rat renal mesangial cells and is counterregulated by protein kinase C.
J Biol Chem 2002 Nov 29, 277:46184-90

Franzen R, Pfeilschifter J, and Huwiler A
Nitric oxide induces neutral ceramidase degradation by the ubiquitin/proteasome complex in renal mesangial cell cultures.
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Contents

1 INTRODUCTION

1.1 Lipids and signal transduction

Lipids are integral structural components of cell membranes, which through their ability to form a bilayer produce a permeability barrier between extracellular and intracellular compartments, a function essential for cell survival. In addition, lipids are essential for signal transduction in response to agonist stimulation as their hydrolysis produces bioactive molecules known to trigger many downstream signalling cascades. The first evidence for such a signalling role came in the 1970s with the discovery of the phosphoinositide (PI) cycle. Subsequently, many studies have shown that a primary event following receptor activation is hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by PI-phospholipase C (PI-PLC), releasing the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG). IP_3 modulates intracellular calcium levels by controlling calcium channels at both the plasma membrane and endoplasmic reticulum [Berridge 1987, Putney & Ribeiro 2000] and DG binds to and activates protein kinase C (PKC) [Nishizuka 1995] which initiates a distinct and separate signalling cascade. Further studies have shown the production of many bioactive lipids generated by receptor-mediated hydrolysis of glycerophospholipids such as phosphatidic acid (PA) produced by a phospholipase D (PLD) acting on phosphatidylcholine (PC) [Exton 1997] or by DG kinases phosphorylating DG [Topham & Prescott 1999]. Arachidonic acid produced by the action of a phospholipase A_2 (PLA_2) is also recognised as an important signalling molecule as well as being the precursor of a diverse group of bioactive compounds, the eicosanoids [Piomelli 1993]. More recently, 3-phosphoinositides generated following growth factor and G protein-coupled receptor activation by the action of PI 3-kinases (PI3Ks) on inositol phospholipids have been recognised as important signalling lipids [Leevers *et al.* 1999]. One target of these lipid messengers is protein kinase B, an important cell survival mediator [Vanhaesebroeck & Alessi 2000]. In addition to glycerolipids, a second class of lipids – sphingolipids – are now known to act as a reservoir of signalling molecules [Okazaki *et al.* 1989, Hannun 1994, Huwiler *et al.* 2000]. Sphingolipids, of which there are more than 300, are found in all eukaryotic cells and are enriched in plasma membranes, Golgi membranes and lysosomes [Merrill *et al.* 1997, Huwiler *et al.* 2000]. In 1986, the sphingolipid derivative sphingosine was shown to inhibit PKC [Hannun *et al.* 1986], suggesting an important role in cell signalling.

1.1.1 Sphingolipid metabolism

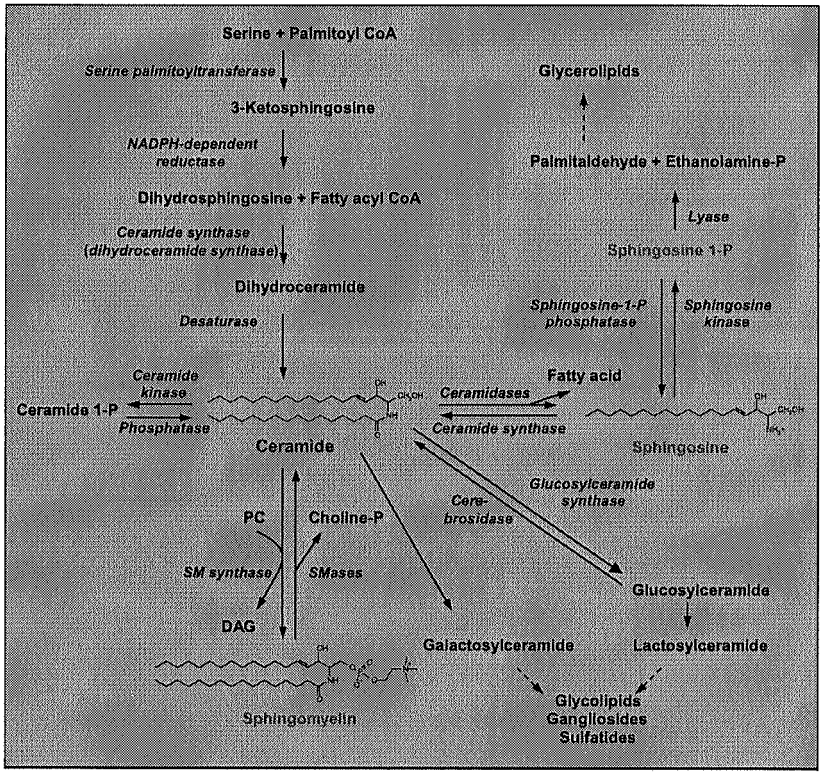


Figure 1: Sphingolipid metabolism

Sphingolipids are characterised by their sphingoid backbone. In mammalian cells, sphingosine is the most common sphingoid base, while in yeast and plant cells, phytosphingosine is more common (Fig. 2). Sphingolipid biosynthesis (Fig. 1) starts with the condensation of serine and palmitoyl-CoA forming 3-ketosphingosine which in turn undergoes reduction to dihydrosphingosine. A fatty acyl group is added by an amide linkage to form dihydroceramide, which is converted directly to ceramide, the precursor of all sphingolipids, by the introduction of a trans double bond between carbons 4 and 5 of the sphingoid base [Merrill & Jones 1990]. Different head groups may then be added to ceramide to form more complex sphingolipids, the simplest of which is ceramide-1-phosphate, formed by ceramide kinase. More complex head groups include β-glycosidically-linked glucose- or galactose-cerebrosides, the addition of a sulfate group

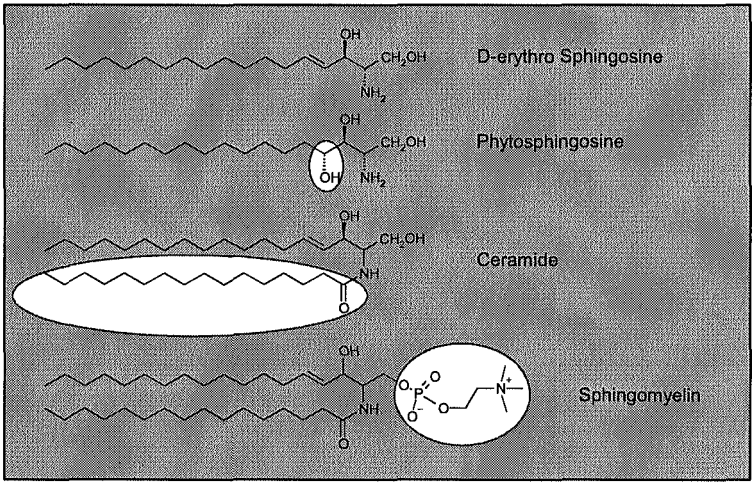


Figure 2: Chemical structure of selected sphingolipids

to galactosylceramide yields sulfatides and di-, tri- and tetra-glycosylceramides are known as glycosphingolipids. Gangliosides are a subclass of glycosphingolipids identified by the presence of sialic acid in the carbohydrate head group [Huwiler *et al.* 2000]. The addition of phosphorylcholine to ceramide, transferred from PC by sphingomyelin synthase, forms sphingomyelin [Merrill & Jones 1990]. Lyso-sphingolipids, N-deacylated derivatives such as 1-galactosyl-sphingosine, glucosyl-sphingosine, sphingosine-1-phosphate, sphingosine and lyso-sphingomyelin are also found. These sphingolipids are present at very low concentrations but may have important signalling effects either as second messengers or through their lytic and membrane-destabilising effects [Iwabuchi *et al.* 2000].

1.1.2 The sphingolipid network

The sphingolipid network is an ubiquitous signalling system that is conserved from yeast to humans [Ballou *et al.* 1996, Hannun 1996, Spiegel *et al.* 1996, Pena *et al.* 1997]. Ceramide, the central molecule in this network, serves as a second messenger for several cellular functions ranging from proliferation and differentiation to growth arrest and apoptosis (Fig. 3). The manifold nature of ceramide signalling is due to the fact that ceramide is linked to different downstream effectors involving distinct signalling pathways depending on cell type. This nature is further closely associated with activity of enzymes that convert ceramide into other metabolites.

The catabolic pathway for ceramide generation involves the action of sphingomyelinases which hydrolyse the phosphodiester bond of sphingomyelin producing ceramide and phosphocholine [Kolesnick 1991, Spiegel *et al.* 1996, Merrill *et al.* 1997]. There are

several isoforms of sphingomyelinase, distinguished by pH optima and therefore classified in acid, neutral and alkaline sphingomyelinases. Both neutral and acid sphingomyelinases are rapidly and transiently activated by diverse exogenous stimuli like interleukin-1β (IL 1β), tumour necrosis factor-α (TNFα), interferon γ (IFNγ), UV-light, radiation, heat shock, or oxidative stress, leading to an increase in ceramide levels in a time frame of seconds to minutes [Ballou *et al.* 1996, Hannun 1996, Spiegel *et al.* 1996, Pena *et al.* 1997]. Neutral sphingomyelinase also showed more prolonged activation [Hannun 1996]. However, until now it remains unclear which of the sphingomyelinases is responsible for the stress-induced production of ceramide.

In addition, ceramide can be synthesized de novo by condensation of serine and palmitoyl-CoA as the primary step. This pathway requires several hours to generate a detectable increase of ceramide [Bose *et al.* 1995] Once generated, ceramide accumulates or is converted into various metabolites (Fig. 3).

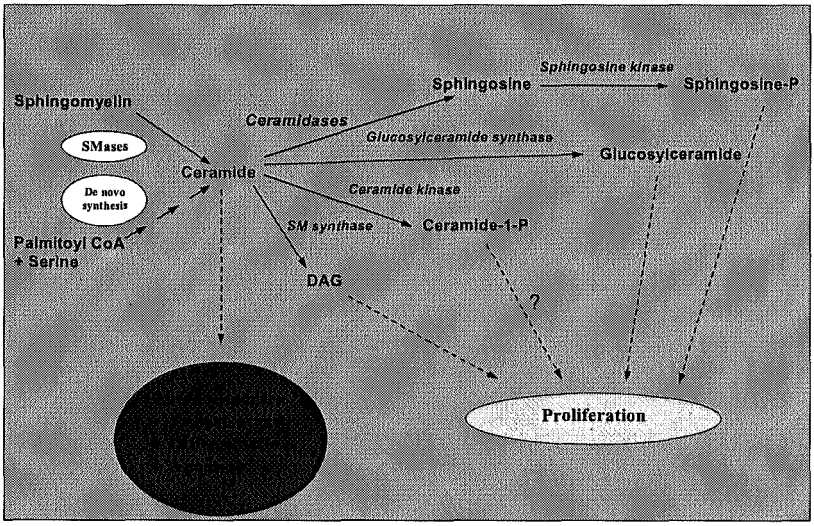


Figure 3: Sphingolipid biology

1.1.3 The role of ceramide in cell signalling

The observation that sphingolipids could act as bioactive molecules promoted interest in the role of sphingolipids and especially of ceramide in cellular responses. Due to this growing research ceramide has been found to be involved in cellular stress response including apoptosis, growth arrest and differentiation [Riboni *et al.* 1997, Perry & Hannun 1998, Levade & Jaffrézou 1999, Huwiler *et al.* 2000] whereas several ceramide metabolites are proposed to exert opposing biological effects such as proliferation

[Gomez-Munoz *et al.* 1997, Pyne & Pyne, 2000, Uchida *et al.* 2002] (Fig. 3). However, the mechanisms of action are still poorly defined.

1.1.3.1 Apoptosis

Programmed cell death, or apoptosis, is an essential process that regulates many aspects of normal and pathophysiological development and homeostasis in general [Hale *et al.* 1996, Nicotera *et al.* 1999]. Most eukaryotic cells have the ability to commit suicide by activating a suicide programme in case that they are no longer needed or are seriously damaged or infected. Failure or suppression of apoptosis may contribute to the development of severe diseases such as cancer and autoimmune diseases. On the other hand, uncontrolled overshooting apoptosis may aggravate the development and symptoms of diseases like neurodegenerative processes or HIV infection. It is believed that cellular sensors exist that register cell damage and can either initiate repair processes, or, if the injury is too severe, can induce apoptosis [Hale *et al.* 1996, Nicotera *et al.* 1999]. The major biochemical pathways include the activation of caspases and of mitochondria-associated death events leading to the release of cytochrome c.

A number of studies support the assumed role of ceramide in apoptosis. Several cytokines and environmental stress factors that initiate apoptosis, including TNFα, Fas ligand, ionising radiation, UV-light, heat shock, and oxidative stress, appeared to induce ceramide generation [Levade & Jaffrézou 1999, Huwiler *et al.* 2000], closely correlating with the induction of apoptosis.

Moreover, exogenous cell permeable ceramide, but not dihydroceramide (Fig. 2) mimicked the effect of endogenous ceramide to induce apoptosis.

Furthermore, it has been shown in several cell lines that exogenous ceramide led to an activation of caspase-3 [Mizushima *et al.* 1996, Kuo *et al.* 1997, Tepper *et al.* 1997, Anjum *et al.* 1998, Machleidt *et al.* 1998, Spinedi *et al.* 1998, Yoshimura *et al.* 1998, Lievremont *et al.* 1999, Turnbull *et al.* 1999] which was supported by studies with caspase-3 inhibitors [Gamen *et al.* 1996, Takeda *et al.* 1999], whereas ceramide was not able to activate caspase-1 or caspase-8 [Brenner *et al.* 1998, Tepper *et al.* 1999]. Thus, it was tempting to speculate that ceramide acts downstream of the early initiator caspases (e.g. caspase 8) but upstream of the executioner caspases (e.g. caspase 3). Moreover, caspase-3 was demonstrated to be an activator of neutral sphingomyelinase [Tokeda *et al.* 1999] leading to an amplification loop resulting in of high amounts of ceramide.

Studies using mutants of the cytoplasmic domain of the 55kDa TNF receptor showed that specific receptor domains were linked to different sphingomyelinases. These observations suggested that neutral and acid sphingomyelinases were activated through distinct molecular mechanisms. However, acid and neutral sphingomyelinases were both thought

to be involved in signalling leading to programmed cell death. The acid enzyme is activated through the death domain adaptor protein system leading to a stimulation of cathepsin D that has been shown to be involved in TNF-induced cell death [Heinrich *et al.* 1999], whereas neutral sphingomyelinase was connected with another receptor domain, termed neutral sphingomyelinase activation domain (NSD), by binding, factor associated with neutral sphingomyelinase (FAN), that functionally coupled this domain to neutral sphingomyelinase [Adam-Klages *et al.* 1996]. Depending on cell type and stimulus, both ceramide generating enzymes could either act concurrently or act separately in TNF-induced apoptotic signalling [Segui *et al.* 2001].

Another pathway involved in the regulation of apoptosis and survival that may be affected by ceramide is the PI-3 kinase/Akt/Bad pathway. This pathway results in phosphorylation of the Bcl-2 family member Bad and thereby protects cells from undergoing apoptosis. Ceramide has been shown to inhibit this pathway either by downregulating PI-3 kinase activity [Zundel & Giaccia 1998, Zundel *et al.* 2000] involving caveolin 1 recruitment to PI-3 kinase-associated receptor complex [Zundel *et al.* 2000] or by preventing Akt/PKB activation [Salinas *et al.* 2000, Hajduch *et al.* 2001, Stratford *et al.* 2001] either through activating ceramide-activated protein phosphatase (CAPP) [Salinas *et al.* 2000] or by phosphatase-independent events [Rölz *et al.* 2002].

It was further reported that ceramide may modulate cell death by dephosphorylation of Bcl-2 in HL-60 cells [Ruvolo *et al.* 1999] losing the protection that was normally sustained by phosphorylation.

In addition, ceramide may interact with the mitochondrial way of apoptosis by triggering the release of cytochrome c [Rosse *et al.* 1998, Ghafourifar *et al.* 1999]. Moreover, it has been shown that ceramide reduced the mitochondrial oxidative phosphorylation [Gudz *et al.* 1997] leading to a disruption of mitochondrial function [Arora *et al.* 1997].

1.1.3.2 Growth arrest

Growth and division of proliferating cells are strictly controlled processes that are regulated by a complex interplay of a variety of enzymes within the cell cycle. A prominent role in the regulation of the cell cycle is exerted by cyclins, cyclin-dependent kinases (Cdks) and Cdk inhibitors. During normal cell cycling in the G1 phase, an active complex of cyclin D and Cdk4/Cdk6 phosphorylates the retinoblastoma (Rb) protein which then releases a family of transcription factors called E2Fs that induce expression of various genes required for cell proliferation. The phosphorylated Rb protein is essential for a cell to enter the following S phase [Chen *et al.* 1989]. This sequential activation of cyclin D/Cdks/Rb/E2F is again regulated by the tumour suppressor p53 which induces

expression of the Cdk inhibitor $p21^{WAF1/CIP1}$ [El Deiry *et al.* 1993] and thereby prevents Rb phosphorylation and progression into the S phase.

Several studies proposed that ceramide may interact with different points of the cell cycle as shown by exogenously applied ceramides causing cell cycle arrest of the G1/S and the G2/M transition [Dbaibo *et al.* 1995, Rani *et al.* 1995, Alesse *et al.* 1998]. Hence, it has been speculated that Rb was dephosphorylated by ceramide and thereby the cell cycle could be stopped [Dbaibo *et al.* 1995, Alesse *et al.* 1998].

Besides, ceramide was able to induce $p21^{WAF1/CIP1}$ protein levels leading to prevention of cyclin-dependent kinase activation and Rb phosphorylation [Alesse *et al.* 1998, Oh *et al.* 1998] so that re-entry into the S phase was blocked.

1.1.3.3 Differentiation

Differentiation of pluripotent cells is a crucial step in embryogenesis and tissue development, especially the neuronal development. To investigate this kind of cell response, undifferentiated cells were very suitable because of their potential to undergo differentiation after a certain stimulus. For example, the promyelocytic leukemia cell line HL-60 could differentiate to either a macrophage-like cell (i.e., by vitamin D3 or phorbol ester) or a granulocytic cell (i.e., by retinoic acid or cAMP).

Ceramide has been shown to be able to mimic vitamin D3 or TNFα-induced monocytic differentiation [Okazaki *et al.* 1989]. Moreover, vitamin D3 itself represented an inducer of ceramide formation in HL-60 cells indicating that vitamin D3 may act as differentiator via ceramide or some of its downstream targets, respectively.

In other studies retinoic acid activated neutral sphingomyelinase in neuroblastoma cells leading to increased ceramide levels which were paralleled with differentiation of these cells detected by enhanced neurite outgrowth [Riboni *et al.* 1995]. This finding was supported by exogenously applied ceramide causing a comparable effect [Harel & Futerman 1993, Riboni *et al.* 1998].

1.1.4 Ceramidases

The regulating mechanisms that determine the intracellular ceramide level are still poorly investigated. Most studies have focused on the ceramide-generating enzymes, i.e. the acid and neutral sphingomyelinases. Based on activity measurements from cell extracts, activators of acid and / or neutral sphingomyelinases have been determined and include pro-inflammatory cytokines, growth factors and other environmental stress stimuli [Levade & Jaffrézou 1999, Huwiler *et al.* 2000]. However, sphingomyelinases only depict one side of the regulation of ceramide level. Therefore, it is equally important to understand the involvement of ceramide-degrading enzymes, the ceramidases.

Ceramidase is an enzyme that catalyses hydrolysis of the N-acyl linkage of ceramide to produce sphingosine, which subsequently can be phosphorylated to sphingosine-1-phosphate by sphingosine kinase [Merrill *et al.* 1997]. Sphingosine is not produced by de novo synthesis [Michel *et al.* 1997], and thus the activity of ceramidase is crucial not only for switching off the ceramide-induced signalling but also for generation of sphingosine and sphingosine-1-phosphate (Fig. 3). Ceramidases are classified into three categories: acid, neutral and alkaline enzymes depending on their pH optimum. However, this classification is not only based on the pH value of optimal enzyme function but also on their primary genetic structure. Furthermore, the intracellular distribution is also quite different, the acid enzymes are exclusively present in lysosomes [Koch *et al.* 1996], neutral enzymes in endosome-like organelles [Mitsutake *et al.* 2001], mitochondria [El Bawab *et al.* 2000] or at the plasma membrane [Mitsutake *et al.* 2001], and alkaline enzymes in the endoplasmatic reticulum and the Golgi apparatus [Mao *et al.* 2001], respectively. With regards to these differences, the particular forms of cermidases may account for specific physiological roles.

Furthermore, the acid and neutral ceramidases were found to be released by murine endothelial cells [Romiti *et al.* 2000a], although the characterisation of this secretory enzyme still needs to be performed.

1.1.4.1 Acid ceramidase

Acid ceramidase is thought to be a housekeeping enzyme to catabolise ceramide in lysosomes and thereby perform the final step of glycosphingolipid catabolism [Bernado *et al.* 1995]. Glycosphingolipids form cell type-specific patterns on the cell surface, which can change with cell growth, differentiation, viral transformation, or oncogenesis [Hakamori 1981]. Degradation of glycosphingolipids takes place in the acidic compartments of the cell, namely lysosomes [Sandhoff & Kolter 1997]. The lysosomal degradation of ceramide requires sphingolipid activator proteins (SAP) as cofactors in vivo, characterised as a group of small, heat-stable, enzymatically inactive glycoproteins [Fürst & Sandhoff 1992].

Acid ceramidase was purified from human urine [Bernado *et al.* 1995], and the cDNA encoding the enzyme was isolated from human [Koch *et al.* 1996] and mouse [Li *et al.* 1998] cDNA libraries. Regarding tissue specificity, acid ceramidase is ubiquitously expressed whereby kidney shows the highest activity followed by brain.

Human acid ceramidase is a heterodimeric protein consisting of an unglycosylated α subunit of 13 kDa and a N-glycosylated β subunit of 40 kDa. Both subunits arise from a single glycosylated precursor protein of about 55 kDa which is processed into the mature enzyme. Endoglycosidase F treatment results in a reduced molecular mass for the

precursor from 55 kDa to 40 kDa and the β subunit from 40 kDa to 29 kDa whereas the size of the α subunit remains unchanged [Koch *et al.* 1996].

A deficiency of the enzyme causes the so-called Farber disease (FD) which is an autosomal recessive disorder that manifests during the first few months after birth. In these patients ceramide accumulates in the lysosomes of most tissues [Moser 1995], leading to painful swelling of the joints and tendons, pulmonary insufficiency, and a shortened life-span. The clinical diagnosis of FD is usually confirmed by biochemical methods, including the determination of lysosomal ceramide accumulation and / or the deficiency of AC activity. To date, seven FD subtypes have been described with varying degrees of clinical outcome. Six of which are believed to be primarily caused by mutations in the acid ceramidase gene. Farber disease type 7 is the result of a complete lack of sphingolipid activator proteins (SAPs) due to a mutation in the initiation codon of the SAP precursor protein prosaposin and is also known as sphingolipid activator protein deficiency. This deficiency not only affects the degradation of ceramide by AC but also the degradation of other glycosphingolipis such as glucosylceramide and galactosylceramide by glucocerebrosidase and galactocerebrosidase, respectively [Moser 1995].

Murine acid ceramidase amino acid sequence is highly homologous to the human protein, with an overall amino acid identity of 90%. Five of the six human N-glycosylation sites were identical in the mouse, as well as the cleavage site that generates the human subunits [Li *et al.* 1998].

In vivo, acid ceramidase activity could be stimulated by IL-1β in rat hepatocytes [Nikolova-Karakashian *et al.* 1997] and this induction appeared to be tyrosine kinase-dependent, whereas TNFα was able to activate acid ceramidase in rat renal mesangial cells [Huwiler *et al.* 1999b].

1.1.4.2 Neutral ceramidase

Neutral ceramidase changes the balance of ceramide / sphingosine / sphingosine-1-phosphate in response to various stimuli including cytokines and growth factors [Coroneos *et al.* 1995, Nikolova-Karakashian *et al.* 1997, Huwiler *et al.* 1999b], and due to its localisation it is an attractive candidate for regulation of sphingolipid-mediated signalling. However, the biological function of the enzyme still needs to be investigated.

Molecular cloning of neutral ceramidases has been performed in rat [Mitsutake *et al.* 2001], mouse [Tani *et al.* 2000], human [El Bawab *et al.* 2000], bacteria [Okino *et al.* 1999], and drosophila melanogaster [Yoshimura *et al.* 2002]. The genetic information of the neutral ceramidase gene family was clearly distinguished from that of acid ceramidase. In contrast to the acid enzymes, the neutral ceramidases are composed of a single polypeptide of 70 - 110 kDa.

The rat enzyme was characterised as a 112 kDa membrane-bound ceramidase having nine putative N-glycosylation sites and one possible transmembrane domain. By treatment with tunicamycin, a known inhibitor of N-linked glycosylation, the protein was converted to an unglycosylated 87 kDa form collaterally with loss of activity. In addition, rat neutral ceramidase possesses eight putative casein kinase II and nine protein kinase C phosphorylation sites. Northern blot analysis indicated high expression of the rat enzyme in heart, brain and kidney which was paralleled with enzyme activity [Mitsutake *et al.* 2001].

The rat neutral ceramidase amino acid sequence was found to be homologous to the sequence found for neutral ceramidase isolated from mouse liver and from human brain. 92% and 76% identity were recognised, respectively. Therefore, by analysis of the protein sequence, the enzymes showed similar pattern of characteristic sites.

The murine neutral ceramidase protein also had several putative post-translational phosphorylation motifs, one tyrosine specific kinase, nine casein kinase II, and ten protein kinase C phosphorylation sites. In addition, nine N-myristoylation sites were found in the sequence [Tani *et al.* 2000].

The human neutral ceramidase protein sequence revealed a similar pattern of potential post-translational modification sites such as N-myristoylation and N-glycosylation, protein kinase C, cAMP-dependent protein kinase, and casein kinase II motifs. The major difference concerns the localisation within the cell. The human sequence showed a potential mitochondrial targeting region and the protein was exclusively localised to mitochondria in HEK 293 and MCF7 cells when overexpressed as a fusion protein with green fluorescent protein (GFP) [El Bawab *et al.* 2000].

The molecular masses are 94 kDa for the murine and 90 kDa for the human enzyme.

Furthermore, neutral ceramidase was identified in Pseudomonas aeruginosa and Mycobacterium tuberculosis whereas sequence homology to neutral ceramidase was found in hypothetical proteins encoded in Arabidopsis thaliana and Dictyostelium discoideum [Okino *et al.* 1999].

Recently, a secretory neutral ceramidase of Drosophila melanogaster was cloned and characterised [Yoshimura *et al.* 2002]. Again, this ceramidase exhibited marked sequence identity to other neutral ceramidases: 33.2% for Pseudomonas aeruginosa, 28.6% for Mycobacterium tuberculosis, 44.5% for mouse, 44.7% for rat, and 43.5% for human, respectively. Typically this enzyme was secreted through the classical pathway involving the endoplasmatic reticulum and the Golgi compartment.

Neutral ceramidase has been shown to be regulated by cytokines [Nikolova-Karakashian *et al.* 1997, Huwiler *et al.* 1999b]. Furthermore, bacterial ceramidase was activated by anionic glycerophospholipids [Kita *et al.* 2002] indicating a possible involvement of

ceramide hydrolysis in atopic skin by the ceramidase of Pseudomonas aeruginosa. Although the etiologic factors in atopic dermatitis have yet to be fully elucidated, dry and barrier-disrupted skin is a distinctive feature of this disease which could be evoked by a decrease of ceramide in the stratum corneum [Imokawa *et al.* 1991]. In this context, Okino *et al.* [1998] suggested that the activity of bacterial ceramidases was related to the decrease of ceramide in atopic dermatitis.

1.1.4.3 Alkaline ceramidase

The 30 kDa alkaline ceramidase, so far now only cloned from yeast [Mao *et al.* 2000] and human [Mao *et al.* 2001], efficiently hydrolyses phytoceramide, which is resistant to hydrolysis by acid and neutral enzymes. Phytoceramide is characterised by introduction of a hydroxyl group in position 4 of the sphingoid base and is a constituent of complex sphingolipids in lower eukaryotes such as Saccharomyces cerevisiae. However, its existence has also been shown in many mammalian tissues.

The alkaline ceramidase did not share any sequence homology with the two other described types of ceramidases, although they catalyse identical cleavage reactions, however, with differences in substrate specificity, pH optimum, and cellular localisation. This enzyme had a pH optimum of 9.5, was activated by calcium and inhibited by zinc and sphingosine [Mao *et al.* 2001].

In addition, an alkaline membrane-associated ceramidase which was not further characterised has been found to be regulated by growth factors, apparently via a tyrosine kinase phosphorylation mechanism [Coroneos *et al.* 1995].

1.2 The mesangial cell

The glomerulus is built up by four cell types: endothelial cells outlining the glomerular capillaries, mesangial cells functioning as pericytes adjacent to the glomerular capillaries, visceral glomerular epithelial cells, also named podocytes, attached to the glomerular basement membrane, and parietal glomerular epithelial cells covering the inner surface of Bowman's capsule [Remuzzi & Bertami 1998]. Among these cell types glomerular mesangial cells are critically involved during various types of glomerular injury [Sterzel *et al.* 1992, Floege *et al.* 1994, Pfeilschifter 1994, Schlöndorff 1996]. As active part of the inflammatory response to glomerular injury, mesangial cells cross-communicate with the invading immune cells such as neutrophils or macrophages resulting in increased production of mediators and extracellular matrix and increased mesangial cell proliferation [Sterzel *et al.* 1992, Floege *et al.* 1994, Pfeilschifter 1994, Schlöndorff 1996]. Quiescent mesangial cells do not produce any pro-inflammatory mediator constitutively. However, regarding injurious mechanisms that are associated with the invasion of productive

immune cells, mesangial cells become self-competent to synthesize bioactive molecules like eicosanoids, nitric oxide, growth factors and inflammatory cytokines. This interplay of mediators may end in repair of damage, if properly controlled. Otherwise, the scenario leads to connective tissue accumulation and irreversible alteration in glomerular structure. These processes have been shown to impair glomerular filtration and may finally result in sclerosis and renal failure [Pfeilschifter *et al.* 1993, Pfeilschifter 1994].

Due to this critical involvement of mesangial cells in glomerular response to injury much effort has been made to analyse mesangial cell function in normal conditions as well as in glomerular diseases. To study the role of mesangial cells during progression of glomerular diseases, cell culture was extensively used over the last years.

1.3 Aim of this thesis

In the past years it has become clear that sphingolipids and in particular the central molecule ceramide act as key compounds in the regulation of cell homeostasis particularly with respect to cell differentiation, growth arrest and apoptosis [Riboni *et al.* 1997, Perry & Hannun 1998, Levade & Jaffrézou 1999, Huwiler *et al.* 2000]. Therefore, the enzymes of the sphingolipid network are important players of cell signalling in general.

This thesis focuses on the regulation of ceramidases which are important ceramide-metabolising enzymes, in renal mesangial cells. Among these I focused on the neutral enzyme which, due to its intracellular localisation, is considered to critically regulate ceramide action.

Particularly, I wanted to analyse the involvement of neutral ceramidase in cell signalling and its role in cellular stress response. Moreover, I assumed that ceramide metabolism may have important consequences for the balance between cell death and survival.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Acrylamide / bisacrylamide-solutions	Roth, Karlsruhe
Actinomycin D	Sigma Aldrich Fine Chemicals, Deisenhofen
Agar	Gibco Life Technologies, Karlsruhe
Agarose	Biozym, Oldendorf
Ammoniumpersulfate	Sigma Aldrich Fine Chemicals, Deisenhofen
Ampicillin	Sigma Aldrich Fine Chemicals, Deisenhofen
Angiotensin II	BACHEM, Biochemica, Heidelberg
Aprotinin	Roche Biochemicals, Mannheim
[γ-^{32}P]ATP	Amersham Pharmacia, Freiburg
Bovine serum albumin, fatty acid free	Sigma Aldrich Fine Chemicals, Deisenhofen
Bovine serum albumin, fraction V	Sigma Aldrich Fine Chemicals, Deisenhofen
5-Bromo-4-Chloro-3-indolyl-β-D-galactoside	Roth, Karlsruhe
Cell culture media	Gibco Life Technologies, Karlsruhe
[^{14}C]ceramide	ICN Biomedicals, Eschwege
CGP 41251	Novartis Pharma, Basel
clasto-Lactacystin β-Lactone	Calbiochem, Schwalbach
Coomassie-Brillant-Blue G250	Sigma Aldrich Fine Chemicals, Deisenhofen
[α-^{32}P]CTP	Amersham Pharmacia, Freiburg
Cycloheximide	Sigma Aldrich Fine Chemicals, Deisenhofen
DETA-NONOate	Alexis, Läufelingen, Switzerland
Diethylpyrocarbonate	Sigma Aldrich Fine Chemicals, Deisenhofen
Dimethylformamide	Roth, Karlsruhe
Dithiothreitol	Sigma Aldrich Fine Chemicals, Deisenhofen
Ethidium bromide	Sigma Aldrich Fine Chemicals, Deisenhofen
EDTA	Sigma Aldrich Fine Chemicals, Deisenhofen
EGTA	Sigma Aldrich Fine Chemicals, Deisenhofen
Fetal calf serum	Gibco Life Technologies, Karlsruhe
G 418	Gibco Life Technologies, Karlsruhe
Glycine	Merck, Darmstadt
Guanidinium thiocyanate	Sigma Aldrich Fine Chemicals, Deisenhofen
Hydroxylammoniumchloride	Merck, Darmstadt
Insulin-Transferrin-Sodium Selenite Supplement	Roche Biochemicals, Mannheim
Interleukin-1β	Novartis Pharma, Basel
Isopropylthiogalactopyranoside	Roth, Karlsruhe
Leupeptin	Roche Biochemicals, Mannheim
Lipofectamine	Gibco Life Technologies, Karlsruhe
L-N-monomethyl-arginine	Calbiochem, Schwalbach
β-Mercaptoethanol	Sigma Aldrich Fine Chemicals, Deisenhofen

[^{35}S]methionine- and [^{35}S]cysteine pro mix	Amersham Pharmacia, Freiburg
MitoTracker® Orange CMTMRos	MoBiTec, Göttingen
Molecular weight markers (DNA)	MBI Fermentas, St. Leon-Rot
Molecular weight markers (protein)	Amersham Pharmacia, Braunschweig
Myelin basic protein	Sigma Aldrich Fine Chemicals, Deisenhofen
Nucleotide triphosphates	PE Biosystems, Weiterstadt
Oligonucleotides	Roth, Karlsruhe; Gibco Life Technologies, Karlsruhe
[^{32}P]orthophosphate	Amersham Pharmacia, Freiburg
PDGF-BB	Hofmann La Roche, Basel
Pepstatin A	Roche Biochemicals, Mannheim
Peptone 140	Gibco Life Technologies, Karlsruhe
Phenol	Roth, Karlsruhe
Phenylmethylsulfonyl fluoride	Roche Biochemicals, Mannheim
Ponceau S	Serva, Heidelberg
Protein A-sepharose 4B CL	Amersham Pharmacia, Freiburg
Ro 31 8220	Calbiochem, Schwalbach
γ-S-ATP	Sigma Aldrich Fine Chemicals, Deisenhofen
[^{14}C]serine	Amersham Pharmacia, Freiburg
Skim milk (non fat)	Fluka, Deisenhofen
Sodiumlaurylsarcosyl	Serva, Heidelberg
Spermine	Calbiochem, Schwalbach
Spermine-NONOate	Alexis, Läufelingen, Switzerland
Sphingomyelin	Calbiochem, Schwalbach
[^{14}C]sphingomyelin	Amersham Pharmacia, Freiburg
Tetramethylethylendiamine	Sigma Aldrich Fine Chemicals, Deisenhofen
12-O-tetradecanoyl-phorbol-13-acetate	Calbiochem, Schwalbach
TNF-α	Knoll, Ludwigshafen
Triton X-100	Sigma Aldrich Fine Chemicals, Deisenhofen
Tween 20	Sigma Aldrich Fine Chemicals, Deisenhofen
U0126	Calbiochem, Schwalbach
Yeast extract	Gibco Life Technologies, Karlsruhe

Acetone, chloroform, ethanol, ether, methanol, isopropyl alcohol, acids and lyes were from the central store of the university hospital Frankfurt. All other, not special listed solvents and salts were supplied from Merck (Darmstadt), Roth (Karlsruhe) or Sigma Biochemicals (Deisenhofen).

2.1.2 Antibodies and antisera

Anti-acid ceramidase (rabbit, polyclonal)	kindly provided by K. Sandhoff, Bonn
Anti-β-actin (goat, monoclonal)	Santa Cruz Biotechnologies, Heidelberg
Anti-green fluorescent protein (mouse, monoclonal)	Roche Biochemicals, Mannheim
Anti-neutral ceramidase (rabbit, polyclonal)	Eurogentec, Belgium
Anti-ubiquitin (rabbit, polyclonal)	Calbiochem, Schwalbach
Anit-GM130 (mouse, monoclonal)	Transduction Laboratories, Kentucky, USA

Anti-goat IgG (horseradish-peroxidase coupled)	Amersham Pharmacia, Freiburg
Anti-mouse IgG (horseradish-peroxidase coupled)	Amersham Pharmacia, Freiburg
Anti-rabbit IgG (horseradish-peroxidase coupled)	Amersham Pharmacia, Freiburg
Anti-mouse IgG (FluoroLink™ Cy™3 labelled)	Amersham Pharmacia, Freiburg
Anti-rabbit IgG (Alexa™ 488)	Molecular Probes Europe, Leiden, Netherlands

2.1.3 Enzymes

Pfu-DNA polymerase	Stratagene, Heidelberg
Recombinant Protein Kinase C α, δ, ε, ζ	kindly provided by D. Fabbro, Novartis Pharma Inc., Basel
Restriction enzymes	MBI-Fermentas, St. Leon-Rot
Reverse transcriptase	MBI-Fermentas, St. Leon-Rot
RNase A	QIAGEN Inc., Hilden
T4-DNA ligase	Roche Biochemicals, Mannheim
Taq-DNA polymerase	MBI-Fermentas, St. Leon-Rot

2.1.4 Plasmids

pBluescript II KS (+)	Stratagene, Heidelberg
pAP3neo vector	kindly provided by M. Ito, Fukuoka
PEGFP-N1	Clontech Labotories, Heidelberg

2.1.5 Eukaryotic cell lines

HEK 293	kindly provided by H. Radeke, Frankfurt
mMC, C57/Bl6	preparation of primary cells from mouse kidney by A. Huwiler, Frankfurt
mMC, MAPKAPK-2 -/-	k.o. mice kindly provided by M. Gaestel, Halle, preparation of primary cells from mouse kidney by A. Huwiler, Frankfurt
rMC, B1	preparation of primary cells from rat kidney by J. Pfeilschifter, Frankfurt

2.1.6 Bacterial strains

E. coli XL-1 blue	Stratagene, Heidelberg

2.1.7 Buffers

PBS-buffer 10 fold	1.5 M NaCl, 30 mM KCl, 15 mM KH_2PO_4, 60 mM Na_2HPO_4
TAE-buffer 10 fold	400 mM Tris acetate, 10 mM EDTA
TBE-buffer 10 fold	900 mM Tris borate, 20 mM EDTA
SSC-buffer 20 fold	3 M NaCl, 300 mM Na_2Citrat

All buffers were prepared with highly purified water from a Milli-Q-system (Millipore).

2.2 Cell culture

All cell lines were cultured at 37°C in a humidified atmosphere containing 5% CO_2 (Heraeus BBD 6220 incubator). For subcultivation, cells were washed once in phosphate buffered saline (PBS), subsequently trypsinised (Trypsin, EDTA) and diluted in an appropriate ratio (1:3 for mouse mesangial cells, 1:4 for rat mesangial cells and 1:10 for HEK 293 cells). For long-term storage, the cells were treated as follows: after trypsinisation, cells were pelleted (5 min at 400 x g, Heraeus Megafuge 1.0, rotor 75750F), diluted in freezing medium (growth medium with 20% FCS supplemented with 10% DMSO) and stored in cryotubes (Nunc). The cryotubes were cooled down slowly and finally stored in liquid nitrogen.

2.2.1 Culture and stimulation of mesangial cells

Rat mesangial cells were grown in Roswell Park Memorial Institute 1640 medium (RPMI) supplemented with 10% FCS, the antibiotics penicillin (100 U/ml) / streptomycin (100 µg/ml), insulin (5 µg/ml), transferrin (5 µg/ml), sodium selenite (5 ng/ml) and 10 mM HEPES, whereas mouse mesangial cells were grown with 15% FCS, β-mercaptoethanol and one fold non-essential amino acid solution in addition.

For induction experiments, cells were grown to confluency and rendered quiescent by 24 h incubation in serum-free Dulbecco's Modified Eagle's Media (DMEM) including fatty acid-free BSA (0.1 mg/ml). Cells were then incubated for varying time periods in fresh DMEM including fatty acid-free BSA (0.1 mg/ml) containing the factors or reagents of interest.

2.2.2 Culture and transfection of HEK 293 cells

HEK 293 cells were grown in DMEM supplemented with 10% FCS and the antibiotics penicillin (100 U/ml) / streptomycin (100 µg/ml).

For transfection experiments $2x10^5$ cells / 3.5 cm dish were plated so that they were 50-80% confluent on the day of transfection. The DNA of interest was diluted into unsupplemented DMEM and incubated at room temperature for 15 minutes. At the same time the lipofectamine reagent was also diluted in DMEM without serum and antibiotics. Thereafter the DNA and lipofectamine were combined and incubated for 15 minutes at room temperature. While complexes were forming the complete medium on the cells was replaced with unsupplemented DMEM. Then the complexes were added and incubated at 37°C at 5% CO_2 for at least 3 hours. After this incubation complete medium was given to bring the final concentration of serum to that of normal growth medium. One day after transfection the medium containing the complexes was replaced with fresh complete medium. After reaching confluency the cells were either harvested or passaged into fresh

culture medium for stable transfection. For this purpose, two days after transfection the appropriate antibiotic was added to select for expression of the transfected antibiotic-resistance gene.

2.3 Bacterial culture

The E. coli strain XL1-blue (Stratagene) was used for amplification of plasmid DNA. The bacteria were grown in liquid LB (Lauria-Bertani) medium (1% bacto-tryptone w/v, 0.5% bacto-yeast-extract w/v and 1% NaCl w/v). For selection, the media contained ampicillin (50 µg/ml). Agar plates were generated with LB-ampicillin medium supplemented with agar (15 g/l). For long-term preservation of transformed bacteria (2.3.2), cells were mixed with 30% sterile glycerol v/v and stored at –80°C.

2.3.1 Competent bacteria

To yield high transformation efficiencies from plasmid DNA in bacteria, cells were chemically pretreated. For this purpose, 100 ml LB-medium was inoculated with 200 µl of an overnight bacterial culture and grown at 37°C until the suspension reached an optical density of 0.5 ($OD_{600\ nm}$). Bacterial cells were pelleted by centrifugation (15 min, 5000 x g, 4°C; Heraeus Megafuge 1.0, rotor 7570F) and subsequently resuspended in 100 ml of the following salt solution: 100 mM RbCl, 50 mM $MnCl_2$, 10 mM $CaCl_2$, 30 mM KOAc and 15% glycerol v/v. The suspension was mixed and incubated on ice for 45 min before recentrifugation at 15 min, 5000 x g, 4°C). The pellet was gently resuspended in 9 ml of a second salt solution (10 mM RbCl, 75 mM $CaCl_2$, 10 mM MOPS and 15% glycerol v/v) and incubated on ice for additional 15 min. Aliquots of competent bacteria were snap frozen in liquid nitrogen and stored at –80°C.

2.3.2 Transformation

200 µl of a competent bacteria suspension (2.3.1) were thawed on ice and 20 µl of the ligation reaction (2.4.7.2) were added. The bacteria / DNA mixture was incubated for 30 min followed by 3 min at 42°C. The bacteria were chilled on ice again for 2 min, before 200 µl of LB-medium was added. For initial expression of the plasmid encoded ampicillin resistance, bacteria were incubated for 45 min at 37°C with 180 rpm on a circular shaker (Unitron Infors AG, Bottmingen, Switzerland). Subsequently, 100 µl of this transformation solution was plated on ampicillin containing agar plates. To enable a blue/white screening for recombinant clones, the agar plate was supplemented with 100 µl X-Gal (2% w/v in dimethylformamide (DMF)) and 40 µl isopropylthiogalactopyranoside (IPTG) (0.1 M in distilled water). The plates were incubated overnight at 37°C.

2.4 Nucleic acid techniques

2.4.1 Preparation of plasmid DNA

Plasmids were routinely isolated from bacteria cultures using a modified protocol originally described by Birnboim and Doly [1979]. 3 ml of medium containing the appropriate antibiotic(s) were inoculated with a single bacterial colony from a selective agar plate and incubated overnight by vigorous shaking at 37°C. 1.5 ml of the cell-suspension were centrifuged for 2 min at 7000x g, and the medium was removed carefully by aspiration. The bacterial pellet was resuspended in 100 µl of solution I (50 mM glucose, 25 mM Tris/HCl pH 8.0, 10 mM EDTA). Subsequently, 200 µl of freshly prepared solution II (200 mM NaOH, 1% SDS w/v) were added to the dispersed bacteria, mixed five times by inverting the tubes and stored on ice for exactly five minutes. This step lyses the bacterial cells and denatures the DNA. The lysate was neutralised by 150 µl 3 M acidic potassium buffer pH 5.5 (solution III), and stored on ice for 10 min. The high salt concentration causes SDS to precipitate, and the denatured proteins, cellular debris and chromosomal DNA become trapped by salt-detergent complexes. Plasmid DNA, being smaller and covalently closed, renatures correctly and remains in solution. The tube was centrifuged for 10 min at 15,000 g and the supernatant containing the plasmids transferred to a fresh Eppendorf tube. A subsequent cleaning step using phenol / chloroform was performed optionally, as trace amount of phenol could disturb subsequent enzymatic reactions processing the plasmid DNA. Plasmid DNA was precipitated using 2 volumes of ethanol at room temperature and a centrifugation step for 10 min at 15,000 x g. The pellet was air dried for 10-15 min and the DNA was finally dissolved in 15 µl of distilled water.
High amounts of pure plasmid DNA (up to 100 µg) were prepared using the QIAGEN Plasmid Midi Kit as described by the manufacturer.

2.4.2 RNA isolation from cultured cells

RNA isolation was performed according to a protocol from Chomczynski and Sacchi [1987]. Cells were grown and stimulated as described above (2.2.1). Cells were washed twice with PBS and last traces of PBS were removed by a pipette tip attached to a vacuum line. Subsequently, cells were lysed with 400 µl of GSCN solution (50% guanidinium thiocyanate w/v, 0.5% sodium laurylsacrosyl w/v, 15 mM sodium citrate pH 7.0 and 0.7% β-mercaptoethanol v/v) per 10 cm-plate, scraped with a rubber policeman and the lysate was transferred into an Eppendorf tube. After addition of 40 µl 2M NaOAc pH 4.0, 400 µl acidic phenol (H_2O-saturated) and 120 µl chloroform, the samples were vortexed vigorously for 20 sec. The vortexed tubes were stored on ice, centrifuged (15,000 x g, 10 min) and the aqueous upper phase was transferred into a fresh tube. RNA was precipitated using 1 ml of isopropyl alcohol, and isolated by a single centrifugation

step (15,000 x g, 10 min). The RNA pellet was washed twice with absolute ethanol, air dried and dissolved in 20 µl diethylpyrocarbonate (DEPC) treated water. Following a 10 min incubation at 65°C, the amount of isolated RNA was quantified photometrically (2.4.3). 3 µg of the isolated RNA was controlled for integrity by agarose gel electrophoresis (2.4.4). Finally, RNA was stored at –20°C until use.

2.4.3 Quantification of nucleic acids

Concentrations of nucleic acids were determined photometrically using a wavelength of 260 nm (Gene Quant II, Amersham Pharmacia). An optical density (OD) of 1 corresponds to approximately 50 µg/ml double-stranded DNA or 40 µg/ml for single stranded DNA and RNA [Sambrook *et al.* 1989]. The ratio of the readings at 260 nm and 280 nm (OD_{260}/OD_{280}) provides an estimation of the purity of the nucleic acid preparation. Highly pure DNA or RNA are characterised by ratios between 1.8 and 2.0. Low amounts of DNA were estimated by agarose gel electrophoresis (2.4.4) in comparison with a known standard concentration.

2.4.4 Agarose gel electrophoresis

Nucleic acids were usually separated by gel electrophoresis using agarose gels. The gel concentration was dependent on the molecular weight of the analysed nucleic acids. Agarose (Biozym) was dissolved in 1x TAE gel electrophoresis buffer. Ethidium bromide was added to a final concentration of 500 ng/µl. Ethidium bromide binds to DNA or RNA by intercalation between the bases and thus enables an ultraviolet fluorescence illumination of nucleic acids. The DNA / RNA probes were diluted with loading buffer (6x loading buffer: 30% glycerol v/v, 0.25% bromophenolblue w/v, 0.25% xylenecyanole w/v, 60% 10x TAE buffer v/v) and transferred into the appropriate gel wells. Electrophoresis was performed in 1x TAE buffer with a voltage of 5-10 V/cm gel. DNA fragment sizes were estimated using molecular weight markers (MBI Fermentas).

2.4.5 DNA isolation from agarose gels

The use of the NucleoSpin-DNA-Extraction-kit (Machery & Nagel, Düren, Germany) enables a pure extraction of DNA fragments directly from agarose gels. The system is based on a silica matrix, which binds single and double-stranded DNA. The DNA fragments of interest were cut from the gel with a razor blade and further processed according to the instructions of the manufacturer.

2.4.6 Reverse transcriptase polymerase chain reaction

2.4.6.1 Reverse transcription

The enzyme reverse transcriptase synthesises a complementary DNA strand using RNA as a template. This enzymatic activity provides access to the generation of cDNA. In general, eukaryotic mRNAs are characterised by a series of adenine nucleotides at the 3´-end, the so called poly-(A) tail. Through hybridisation with oligo-(dT) primers, these poly-(A) sites are ideal start points for the reverse transcriptase enzyme. Additionally, random hexamers (50 ng/µl) were used as internal enzyme start sites.

Reverse transcriptase reaction:
- RNA 5µg
- oligo-(dT) primer 0.5µg
- 1x reverse transcriptase buffer
- dNTP-Mix 2 mM
- RNase inhibitor 20 U
- reverse transcriptase 40 U

2.4.6.2 Polymerase chain reaction (PCR)

This methods enables the *in vitro* amplification of DNA fragments without time consuming cloning and identification steps [Mullis & Faloona, 1987]. The method is based on the availability of heat-stable DNA polymerases which allow multiple denaturing of template DNA, annealing of driver sequences (primer) and synthesis of DNA by amplification steps within one tube.

The reaction was performed in a thermocycler (GeneAmp 2400 or 9600, *PE* Biosystems) with the following sequences for PCR: 94°C for 5 min (1 cycle), and 94°C for 1 min, 52°C for 1.5 min and 72°C for 1 min (with variable numbers of cycles) and final extension at 72°C for 7 min. The number of cycles were: 30 for murine neutral ceramidase, 35 for rat neutral ceramidase and 25 for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Sequences of the primers for analysis of mRNA: mouse neutral ceramidase: forward: TTC AAT TCG GGA CTT CAG TGG; reverse: CAA GAA TGT TGG GTG ACA CG; rat neutral ceramidase: forward: TGA AGA CGT GTA AAG CCG C; reverse: TGC GAT AAC GAC AGT CAT ATC C; GAPDH: forward: AAT GCA TCC TGC ACC ACC AA; reverse: GTC ATT GAG AGC AAT GCC AGC. PCR products (length: 793 bp for mouse neutral ceramidase, 377 bp for rat neutral ceramidase and 470 bp for GAPDH) were run on a 1.5% agarose gel containing 0.5 µg/ml ethidium bromide. Identity of amplicons were confirmed by sequencing using a 310 Genetic Analyser (Perkin Elmer Corp.) The samples were stored at 4°C and analysed by agarose gel electrophoresis (2.4.4).

Polymerase chain reaction:	2 µl	cDNA (2.4.6.1)
	10 µl	5x taq-polymerase buffer
	5 µl	dNTP-Mix 2 mM
	1 µl	forward primer 50 µM
	1 µl	reverse primer 50 µM
	1 µl	taq-polymerase 1 U
	ad 50 µl	H_2O

2.4.7 Manipulation of DNA

2.4.7.1 Restriction

Type II endonucleases isolated from bacteria specifically bind palindromic sequences with a subsequent cleavage of the DNA molecule at their recognition site. This process generates either blunt end fragments or overhanging cohesive ends, which allow the generation of recombinant DNA by enzymatic ligation. The standard approach for DNA digestion is subsequently listed. After incubation at the appropriate temperature, DNA cleavage was checked by agarose gel electrophoresis (2.4.4).

DNA restriction:	10 µl	DNA 500 ng
	2 µl	10x restriction buffer
	0.2 µl	restriction enzyme 3 U
	ad 20 µl	H_2O

2.4.7.2 Ligation

Generation of covalent phosphodiester bonds between the 5´-phosphate and the 3´-OH of DNA fragments is catalysed by T4-DNA ligase. The ligation reaction was performed with restricted or PCR amplified DNA. The DNA was separated in a low-melting agarose gel, and the DNA fragment of interest was subsequently cut out. The gel piece was melted at 68°C and added to the ligation reaction. The mixture was incubated for at least 5 h at room temperature. Afterwards, an aliquot of this reaction was transformed into competent bacteria as described in section 2.3.2.

DNA ligation:	1 µl	vector DNA (500 ng)
	10 µl	melted gel / DNA fragment
	4 µl	10x ligase buffer
	1 µl	T4-DNA ligase 1 U
	ad 40 µl	H_2O

2.4.8 DNA sequencing

DNA sequencing was performed using the ABI-Prism 310 Genetic Analyser (*PE* Biosystems) based on the dideoxynucleotide chain termination method [Sanger *et al.* 1977]. In the termination labeling mix, the four dideoxy terminators (ddNTPs) were tagged

with different fluorescent dyes. This technique allows the simultaneous sequencing of all four reactions (A, C, G, T) in one reaction tube. The probes were separated electrophoretically using a micro capillary. As each dye terminator emits light at a different wavelength when excited by laser light, all four colors corresponding to the four nucleotides can be detected and distinguished within a single run. Raw data were evaluated by the Abi Prism sequencing analysis software on a Power G3 Macintosh computer. The sequencing reaction, as listed below, was performed in a thermocycler (GeneAmp 2400, *PE* Biosystems) with 25 cycles of the following temperature steps: 96°C for 10 sec, 55°C for 5 sec, 60°C for 2 min. For the detection process, probes were prepared as described by the manufacturer.

DNA sequencing:
1 µl DNA (250 ng plasmid / 50 ng PCR derived)
2 µl sequencing premix
1 µl primer 10 µM
ad 10 µl H_2O

2.4.9 Northern blot analysis

Total RNA was isolated using guanidinium isothiocyanate solution as described (2.4.2). 25 µg of RNA was separated by electrophoresis on 1% agarose formaldehyd gels. RNA was transferred to a nylon membrane by vacuum blotting for 2 h at 55 mbar and cross-linked by UV light. Blots were hybridised with a 540 bp RT-PCR product (forward primer: CCA GTG GGT GAA CAT GAC AG; reverse primer: GAT GTA TGC AGA CAG GGT GT) of the rat neutral ceramidase, and a 1206 bp RT-PCR product (forward primer: GGG GTA CCT GGG AAG ATG GGG GGC CAA AGT CTT CTC; reverse primer: GAC TAC TGC TCA CCA GCC TAT ACA AG) for the acid ceramidase, which were labeled with α-[^{32}P]-dCTP using the Multiprime DNA Labeling system (Amersham Pharmacia Biotech). Hybridisation was carried out at 42°C for 16 h, and the membranes were exposed on a Phosphorimager (Fuji). To correct for variations in RNA amounts, blots were finally rehybridised with α.–[^{32}P]-labeled GAPDH cDNA probe.

2.5 Protein Techniques

2.5.1 Preparation of lysates

Confluent mesangial cells in 60 mm-diameter dishes were stimulated for the indicated time periods in DMEM containing 0.1 mg/ml of fatty acid-free BSA. To stop the reaction, the medium was removed and the cells washed with ice-cold PBS. Cells were then scraped directly into lysis buffer (50 mM Hepes pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 10% glycerol v/v, 1% Triton X-100 v/v, 20 mM β-glycerophosphate, 50 mM sodium fluoride, 1 mM Na_3VO_4, 10 µg/ml leupeptin, 10 µg/ml

aprotinin, 1 µM pepstatin A, 1 mM phenylmethylsulfonyl fluoride (PMSF)) and homogenised by ten passes through a 26-gauge needle fitted to a 1 ml syringe. The homogenate was centrifuged for 10 min at 15,000 x g and the supernatant taken for protein determination (2.5.2). Aliquoted samples were stored at –80°C until use.

2.5.2 Quantification of proteins

The amount of protein in cellular and tissue lysates was determined using the Bio-Rad protein assay (Bradford method). 10 µl of the samples diluted in 790 µl of distilled water were combined with 200 µl Bio-Rad 5x dye solution, vortexed and pipetted into appropriate wells of a 96-well plate. BSA concentrations between 3 - 48 µg/ml were used to generate a standard curve. After 10 min of incubation, the optical density was measured at a wavelength of 595 nm using a microplate reader (Bio Rad). The absorption values were calculated using the Microplate Manager 4.0 software (Bio Rad).

2.5.3 Trichloracetic acid (TCA) precipitation

This method was used to concentrate proteins from a defined volume of cell culture supernatants for Western blot analysis. 70% trichloroacetic acid (TCA) w/v were added to protein extracts in lysis buffer to yield a final concentration of 7%, mixed and incubated for 30 min on ice. TCA-precipitated proteins were pelleted by centrifugation (15,000 x g, 30 min, 4°C). The protein pellet was washed in 200 µl of ice-cold acetone, centrifuged for 5 min at 14,000 g and finally resuspended in 1x SDS buffer (10 mM Tris/HCl pH 7.4, 1 mM EDTA, 181 mM DTT, 13% SDS w/v, 13.25% glycerol v/v, 1.0% bromphenolblue m/v). After neutralisation (1 µl of 1 M Tris/HCl pH 8.5 per 50 µl), the samples were ready to use for SDS-PAGE (2.5.6).

2.5.4 Immunoprecipitation

Samples of 1 ml volume, containing the indicated amount of proteins, 5% fetal calf serum v/v and 1.5 mM iodoacetamide in lysis buffer, were incubated overnight at 4°C with a polyclonal antiserum against the neutral ceramidase (2.5.7) at a dilution of 1:100. Then 100 µl of a 50% slurry of protein A sepharose 4B-CL in PBS was added and the mixture was rotated for 1 h at room temperature. After centrifugation for 5 min at 3,000 g immunocomplexes were washed 3 times with a low salt buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% Triton X100 v/v, 2 mM EDTA, 2 mM EGTA, 0.1% SDS w/v), 3 times with a high salt buffer (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.2% Triton X100 v/v, 2 mM EDTA, 2 mM EGTA, 0.1% SDS w/v) and once with 10 mM Tris. Pellets were either boiled for 5 min in Laemmli dissociation buffer and subjected to SDS-PAGE (SDS-

polyacrylamide-gel-electrophoresis) or used for in vitro phosphorylation experiments (2.5.9).

2.5.5 Metabolic labelling

Confluent mesangial cells in 100 mm-diameter dishes were washed with PBS and incubated in methionine-free MEM in the absence or presence of the stimulators for the indicated time periods. For the last 4 h of incubation a mix [^{35}S]methionine and [^{35}S]cysteine was added (140 µCi/plate). After labelling, cells were washed twice with ice-cold PBS and scraped directly into 1 ml of lysis buffer and homogenised. The homogenate was centrifuged for 10 min at 14,000 g and 5 µl of the supernatant were measured in a β-counter. Samples of 1 ml volume, containing 250 x 10^6 cpm of labelled proteins were taken for immunoprecipitation (2.5.4) and following SDS-PAGE. After fixing in 25% isopropyl alcohol v/v, 10% acetic acid v/v, the gels were dried and exposed on a Phosphoimager (Fuji). Labelled bands corresponding to neutral ceramidase were analysed.

2.5.6 Western blot analysis

The Western blot technique represents a sensitive method to detect specific polypeptides within a complex mixture of proteins. Proteins are separated electrophoretically and transferred to a membrane, which is subsequently incubated with antibodies specific for the protein of interest. Finally, the bound antibody is recognised by a second anti-immunoglobulin antibody that is coupled to horseradish peroxidase which was visualised with enhanced chemiluminescence (ECL) system (Amersham) according to the company's specifications.

2.5.6.1 SDS gel electrophoresis

Electrophoretic separation of proteins was carried out in the discontinuous buffer system for SDS polyacrylamide gels as originally described by Laemmli [1970]. 100 µg of total protein were dissolved in 1x SDS buffer. After heating for 5 min at 95°C, samples were loaded on the gel. Subsequently, the gel was run at 80 V for a period of 2-3 hours.

2.5.6.2 Protein transfer to nitrocellulose membrane

After gel electrophoresis, proteins were transferred onto a nitrocellulose membrane by semi-dry electroblotting (Trans-Blot SD, Bio Rad). Prior to use, membrane and filterpapers (Whatman 3MM) were damped in blotting buffer (25 mM Tris, 190 mM glycin, 20% methanol v/v) as two appropriate pieces of filter. After soaking in blotting buffer, one filter was positioned on the anode side of the transfer apparatus. The nitrocellulose membrane

was placed directly on the Whatman 3MM paper. The SDS gel containing the separated proteins was taken off the glasplates, rinsed shortly in blotting buffer, and placed on the top of the membrane. Finally, the gel was covered with one additional, blotting buffer-soaked Whatman 3MM filter. Air bubbles were squeezed out by a roller apparatus. The upper electrode (cathode) was positioned on the top of the stack and a voltage of 11 volts was applied. Transfer of proteins was carried out at room temperature and terminated after 60 min. After blotting, the membrane was checked by Ponceau S (0.2% Ponceau-S w/v, 3% TCA w/v) staining for correct electrophoretic transfer and equal loading.

2.5.6.3 Immunodetection

The nitrocellulose membrane was rinsed with distilled water. Non-specific binding sites were blocked by shaking the membrane in blocking buffer (50 mM Tris/HCl pH 7.4, 200mM NaCl, 0.2% Triton X-100 w/v, 3% BSA fraction V w/v, 10% horse serum v/v) for 1 h at room temperature or overnight at 4°C. The membrane was subsequently exposed to antibodies (diluted in blocking buffer in a range of 1:500 to 1:2,000) specific for the protein of interest. The incubation time varied between 1 h at room temperature, or 15 h at 4°C depending on the antiserum. The blot was washed three times for 15 min in 1x TNTX (50mM Tris/HCl pH 7.4, 200mM NaCl, 0.2% Triton X-100 v/v) and incubated with a specific secondary antibody coupled to horseradish peroxidase (diluted in blocking buffer in a range of 1:5,000 to 1:20,000) for 1 h at room temperature. For visualisation of the bands, the enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia) was used according to the instructions of the manufacturer. The membrane was exposed to a Hyperfilm MP (Amersham Pharmacia) and developed (Hyperprocessor, Amersham Pharmacia). Developed films were scanned (GS 700 Imaging Densitometer, Bio Rad) and analysed using the Molecular Analyst software from Bio Rad.

2.5.7 Generation and characterisation of a neutral ceramidase antibody

A synthetic peptide (ENHKDSGNHWFSTC) based on the N-terminal sequence of the murine neutral ceramidase (GenBank™/EBI Data Bank accession number AB037111) was synthesised, coupled to keyhole limpet hemocyanin, and used to immunise rabbits. For characterisation of the antibody, lysates of IL-1β-stimulated (8 h) rat mesangial cells were separated on a MonoQ column coupled to a BioLogic FPLC® system. The cell lysate was loaded into 25 mM Tris (pH 7.4) and eluted with a linear gradient of 1 M NaCl in 25 mM Tris (pH 7.4) at a flow rate of 2 ml/min. The eluted fractions were analysed by Western blotting (2.5.6) and neutral ceramidase activity assay (2.6.1.1).

2.5.8 In vivo phosphorylation

Confluent mesangial cells in 100 mm-diameter dishes were washed twice with PBS and incubated for 48 h in DMEM containing fatty acid free 0.1 mg BSA/ml. The cells were washed three times with phosphate-free MEM in order to remove all phosphate. Afterwards the cells were metabolically labelled for 4 h with [^{32}P]orthophosphate (0.5 mCi/plate). After labelling, cells were stimulated at 37°C for the indicated time periods with various agents. To stop the reaction, the medium was removed and the cells were washed twice with ice-cold buffer containing 20mM Tris/HCl pH 7.5 / 150 mM NaCl. Cells were then scraped directly into 1.0 ml of ice-cold lysis buffer and homogenised by ten passes through a 26-gauche needle fitted to a 1 ml syringe. The homogenate was centrifuged for 15 min at 15,000 x g and 5 µl of the supernatant was taken for measurement in a β-counter.

The supernatants (containing 2.5 x 10^6 cpm of labelled proteins) were taken for immunoprecipitation (2.5.4) and following SDS-PAGE as described above (2.5.6.1). After fixing in 25% isopropyl alcohol v/v, 10% acetic acid v/v, the gels were dried and exposed to an Imaging System (Fuji). Phosphorylated bands corresponding to neutral ceramidase were analysed.

2.5.9 In vitro phosphorylation

Immunocomplexes of interest were tested for direct phosphorylation by using recombinant PKC isoenzymes in the presence or absence of calcium. The beads were incubated with 1 µg of the partially purified enzymes in a total volume of 40 µl containing 20 mM Tris/HCl pH 7.4, 10 mM $MgCl_2$, 100 µM Na_2ATP, 8 µCi [γ-^{32}P]ATP, 1µg phosphatidylserine, 0.1 µg diolein, 1 µM TPA and either 100 µM $CaCl_2$ or 100 µM EDTA for 15 min at 32°C. To show PKC isoenzymes' activities 5 µg of MBP being a well known substrate for PKC [Geiges *et al.* 1997] were included. Thereafter SDS buffer was added to stop the reaction and the samples were separated on SDS-PAGE (8% acrylamide for neutral ceramidase and 13% acrylamide for MBP). Phosphorylated bands corresponding to neutral ceramidase and MBP, respectively, were analysed on an Imaging system (Fuji).

2.5.10 Trypsin digestion

[^{32}P]phosphorylated neutral ceramidase that was affinity-purified on an anti-ceramidase sepharose column was incubated with or without trypsin (100µg/ml) in a final volume of 50µl for 3h at 37°C. Thereafter, undigested samples were separated on a Tris-glycine SDS-PAGE (7% acrylamide gel) and trypsin-digested samples were separated on a Tris-tricine SDS-PAGE (10-20% acrylamide gel). Phosphorylated bands were analysed on an Imaging system.

2.6 Measurement of cell parameters

2.6.1 Enzyme activity

2.6.1.1 Acid and neutral ceramidase activity

Confluent mesangial cells in 60mm dishes were stimulated as described above (2.2.1) and homogenised in lysis buffer containing 50 mM sodium acetate pH 4.5, 0.5% Triton X-100 v/v, 5 mM $MgCl_2$,1 mM EDTA, 5 mM D-galactonic acid γ–lactone for the acid ceramidase, and 50 mM Tris pH 7.5, 0.5% Triton X-100 v/v, 5 mM $MgCl_2$,1 mM EDTA, 5 mM D-galactonic acid γ–lactone for the neutral ceramidase. Cell homogenates were centrifuged for 10 min at 15,000 x g, and the supernatant was taken for an in vitro assay. 100 µg of protein in a total volume of 100 µl were incubated for 20 h at 37 °C with 20 nCi of [^{14}C]ceramide. Thereafter, the reaction was stopped by the addition of 200 µl of water, and lipid extraction was performed by addition of 2 ml of chloroform / methanol (2:1, v/v). The lower phase was concentrated and lipids were resolved by TLC (thin layer chromatography) using chloroform / methanol / ammonia (90:20:0.5, v/v) as a solvent. Spots corresponding to ceramide (Rf =0.8) and sphingosine (Rf =0.25) were analysed and quantified using a Phosphoimager (Fuji).

2.6.1.2 Acid and neutral sphingomyelinase activity

For the sphingomyelinase activity cells were homogenised in lysis buffer containing 50 mM sodium acetate pH 4.5, 0.5% Triton X-100 v/v, 5 mM $MgCl_2$, 1 mM EDTA for the acidic sphingomyelinase, and 50 mM Tris pH 7.4, 0.5% Triton X-100 v/v, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM dithiothreitol for the neutral sphingomyelinase. Cell homogenates were centrifuged for 10 min at 15,000 x g, and the supernatant was taken for an in vitro assay. 100 µg of protein in a total volume of 100 µl were incubated for 30 min at 37 °C with 20 nCi of [^{14}C]sphingomyelin. Thereafter, the reaction was stopped by the addition of 200 µl of water and 2 ml of chloroform / methanol (2:1, v/v) and vortexed for 10 sec. After phase separation, the radioactivity in the upper phase containing the phosphocholine was counted in a β-counter.

2.6.2 Ceramide formation

Confluent mesangial cells in 30mm-diameter dishes were labelled for 24 h with [^{14}C]serine (0.2 µCi/ml) and stimulated as indicated. Lipids were extracted as follows: the stimulation was stopped by withdrawing the stimulating medium and incubating the cells with 1.0 ml ice-cold methanol for 1 h at 4°C. Thereafter, cells were scraped with a rubber policeman and the lysate was transferred into a test tube. 1.6 ml of salt solution (0.74% KCl w/v, 0.04% $CaCl_2$ w/v, 0.034% $MgCl_2$ w/v) and 2.0 ml chloroform were added followed by vigorous shaking which resulted in the lipids solved in the lower chloroform phase. This

separated lipid phase was transferred into a centrifuge tube. This extraction step was repeated by adding 35 µl HCl / 2.0 ml chloroform and a final extraction with 1.0 ml chloroform alone. The united chloroform phases were subsequently concentrated in a speedvac vacuum concentrator. Ceramide was resolved by sequential one-dimensional TLC using chloroform / methanol / ammonia (65:35:7,5, v/v) followed by chloroform / methanol / acetic acid (9:1:1, v/v). Spots corresponding to ceramide were analysed and quantified using a Phosphoimager (Fuji).

Alternatively, ceramide was quantified by LC/MS/MS spectrometry. The LC unit consisted of a Jasco DG 1580-53 degasser, a Jasco LG-1580-02 ternary gradient unit, a Jasco PU-1585 pump and a Jasco AS 1550 autosampler (Gross-Umstadt,Germany). The mass spectrometer consisted of a PE Sciex API 3000 triple quadrupol mass spectrometer (Applied Biosystems Langen, Germany) equipped with a turbo ion spray interface. Nitrogen at high purity was supplied by a Whatman nitrogen generator (Whatman GmbH, Goettingen, Germany).

Chromatographic separation of extracted samples was performed in isocratic mode with a Nucleosil C18 column (30×2.0 mm I.D., 5 µm particle size and 100 Å pore size, Macherey-Nagel, Dueren, Germany). The mobile phase consisted of methanol containing 2 mM ammonium acetate. The flow rate was set at 0.1 ml/min. The injection volume was 10 µl and the running time was 4 min. The turbo ion spray interface operated in the positive ion mode at 5200 V and 200°C and was supplied by an auxiliary gas flow of 4500 ml/min. The nebuliser gas was set at 1.23 l/min (setting 10), the curtain gas flow was set at 1.08 l/min (setting 9) and the collision gas at 3.7×10^{-6} HPa ($3.02 * 10^{15}$ molecules/cm^2) (setting 4) . Nitrogen was used for all gases. C(16)-ceramide standards and cellular lipid extracts were resuspended in 1000 µl of a 5 mM ammonium acetate/methanol buffer just prior to MS analysis. Standards were analysed at concentrations ranging from 25 nM to 10 µM.

Quantification was performed in multiple reaction monitoring (MRM) (dwell time 200ms) of the protonated precursor ion and related product ions. The mass transition used for quantification was m/z 538.4→264.2 (collision energy 33eV). The mass transitions used as qualifier were m/z 538.4→82.1 (collision energy 77eV) and 538.4→252.1 (collision energy 39eV). The analytical data were processed by Analyst software (version 1.1).

2.6.3 Apoptosis

Apoptotic cell death is characterised by membrane blebbing, chromatin condensation, and the activation of endonucleases which cleave the double-stranded DNA at the most accessible internucleosomal linker regions, finally generating mono- and oligonucleosomes. As DNA degradation occurs several hours before plasma membrane

disassembly, these mono- and oligonucleosomes are enriched in the cytoplasm of apoptotic cells. The ELISA uses the determination of these soluble DNA / histone complexes as readout for cellular apoptosis (Cell Death Detection ELISA, Roche Biochemicals).

2.7 Confocal microscopy

Sequential images of the same cell were collected at 1-5 min intervals using LaserSharp software through a Bio-Rad MRC 1024 confocal scan head mounted on a microscope (Axioskop FS, Carl Zeiss, Jena, Germany) with a 603 planapochromat lens. Excitation at 488 nm was provided by a krypton-argon gas laser with a 522/32 emission filter for green fluorescence.

For mitochondrial-specific staining cells were incubated with prewarmed medium containing MitoTracker® Orange CMTMRos (250nM) for 30 minutes as suggested by the manufacturer. After replacing the loading medium labelled cells were viewed by confocal microscopy as described above.

For Golgi-specific staining with Anit-GM130 and for visualising uncoupled neutral ceramidase, respectively, cells were washed with PBS and fixed in methanol containing 0.02% EGTA (w/v) at -20°C for at least 1 h. Thereafter, cells were rinsed with PBS and incubated with 1mg/ml BSA in PBS (PBS/BSA) for 1 h to block nonspecific binding sites. Cells were then incubated with a monoclonal GM130-specific antibody (1:250) or neutral ceramidase antibody (1:200) for 1 h. The cells were rinsed with PBS/BSA, incubated for 1 h with FluoroLink™ Cy™3 labelled goat anti-mouse IgG (1:2000) or Alexa™ 488 goat anti-rabbit IgG (1:2000) and rinsed with PBS. The labelled cells were viewed by confocal microscopy using a 63x fold magnification objetive.

2.8 Statistical analysis

Statistical analysis was performed by one way analysis of variance (ANOVA). For multiple comparisons with the same control group, the limit of significance was divided by the number of comparisons according to Bonferroni.

3 RESULTS

3.1 Interleukin 1β induces chronic activation and de-novo synthesis of neutral ceramidase in renal mesangial cells

Introduction

Inflammatory diseases of the renal glomerulus are accompanied by enhanced formation of the cytokine interleukin-1 β (IL-1 β). The primary source is the activated macrophage, but IL-1β is also released by many other cell types after exposure to an inflammatory environment. Soluble IL-1 β is the predominant form in biological fluids, and it binds to specific receptors in target tissues. IL-1 is an exemplary cytokine that is particularly important in the systemic response to inflammation. It synergises with tumour necrosis factor-α (TNF-α) for many of its actions, and its synthesis is stimulated, in turn, by TNF-α. Furthermore, it is implicated in the pathogenesis of diseases such as rheumatoid arthritis, inflammatory bowel disease, septic shock, and several autoimmune reactions.

In this part of the thesis, the effect of the IL-1β on the neutral sphingomyelinase and neutral ceramidase activities was investigated.

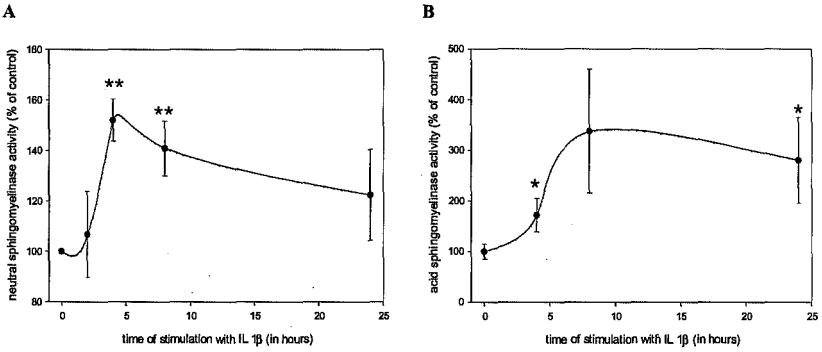
Results

IL-1β stimulates chronic activation of neutral sphingomyelinase

Previously, it has been shown that IL-1β causes a rapid (within minutes) and transient activation of neutral sphingomyelinase activity in rat mesangial cells, which leads to increased ceramide formation [Huwiler *et al.* 1996, Kaszkin *et al.* 1998]. Now, these studies have been extended and it has been found that prolonged treatment of mesangial cells with IL-1 β resulted in a delayed second peak of neutral sphingomyelinase activation that was first detectable after 2 h of stimulation and reached a maximum after 4 h (Fig. 4A). The acid sphingomyelinase also showed a time-dependent delayed activation after IL-1 β treatment (Fig. 4B).

When the level of ceramide was measured by tandem mass spectrometry after IL-1β stimulation, no increase was observed up to 24 h after stimulation (Fig. 5), thus pointing toward additional compensatory mechanisms that regulate the ceramide content of the cell. In contrast, 1 h of stimulation with a bacterial sphingomyelinase led to an 8-10-fold increase in ceramide levels (Fig. 5).

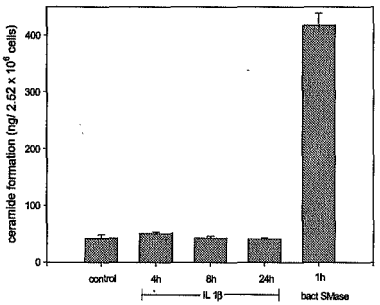
Figure 4



Time course of IL-1β-induced neutral (A) and acid (B) sphingomyelinase activities in rat mesangial cells.

Confluent rat mesangial cells were stimulated for the indicated time periods with IL-1β (2 nM). Thereafter, cell lysates containing 100 µg of protein were taken for in vitro neutral (A) or acid (B) sphingomyelinase activity assay as described under 2.6.6.2. The [^{14}C]phosphocholine generated was extracted and counted in a β-counter. Results are expressed as a percent of control values and are means ± S.D. (n = 3-4). *$p < 0.05$; **$p < 0.01$ (statistically significant difference compared with the unstimulated control).

Figure 5



Time course of IL-1β-induced ceramide formation in rat mesangial cells.

Cells were stimulated for the indicated time periods with vehicle (control), IL-1β (2 nM), or bacterial sphingomyelinase (bact SMase; 0.1 milliunit/ml). Thereafter, lipids were extracted, and ceramide was analysed by liquid chromatography-tandem mass spectrometry as described under 2.6.7. Results are means of two independent experiments performed in duplicate (n=4).

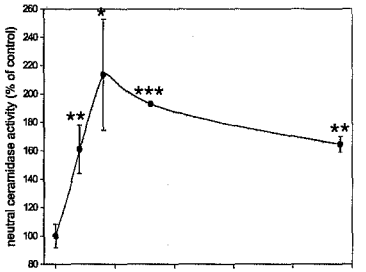
IL-1β stimulates chronic activation of a neutral ceramidase

To investigate the effect of IL-1β on the ceramide-degrading enzymes, rat mesangial cells were stimulated for different time periods with the cytokine, and ceramidase activity was measured. As shown in Fig. 6 (A and B), IL-1β caused a chronic activation of acid and neutral ceramidases, with maximal stimulation occurring 4 h after cytokine exposure and subsequently declining for the neutral enzyme (Fig. 6A). This decline is due to the induction of the inducible nitric oxide synthase (iNOS) by IL-1β followed by the production of high concentrations of nitric oxide which in turn trigger proteolytic degradation of neutral ceramidase and loss of neutral ceramidase activity (chapter 3.2) [Franzen *et al.* 2002a]. This notion is supported by co-stimulation of mesangial cells with the nitric oxide synthase

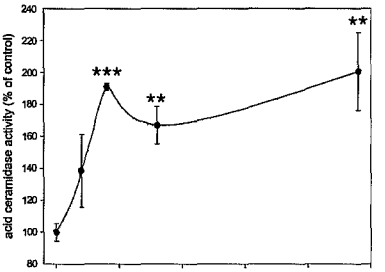
(NOS)-inhibitor N^G-monomethyl-L-arginine (L-NMMA) which prevents the decline of neutral ceramidase activity (Fig. 6C).

Figure 6

A



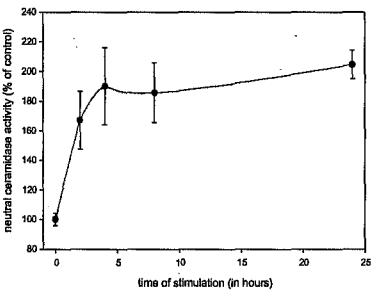
B



Time course of IL-1β-induced neutral (A) and acid (B) ceramidase activities in rat mesangial cells.

Quiescent rat mesangial cells were stimulated for the indicated time periods with IL-1β (2 nM). Thereafter, cell lysates containing 100 µg of protein were taken for in vitro neutral (A) or acid (B) ceramidase activity assay as described under 2.6.6.1. [^{14}C]sphingosine generated was separated by thin layer chromatography and evaluated on a Fuji phosphoimager. Results are expressed as a percent of control values and are means ± S.D. (n = 3-4). Neutral ceramidase activity in control cells was 23 ± 3.4 pmol/mg/h. Acid ceramidase activity in control cells was 450 ± 32.4 pmol/mg/h. *p < 0.05; **p < 0.01; ***p < 0.001 (statistically significant difference compared with the unstimulated control).

C



Time course of neutral ceramidase activity in rat mesangial cells after co-stimulation with IL-1β and L-NMMA.

Quiescent rat mesangial cells were stimulated for the indicated time periods with IL-1β (2 nM) plus L-NMMA (2 mM). Thereafter, cell lysates containing 100 µg of protein were taken for in vitro neutral ceramidase activity assay as described under 2.6.6.1. [^{14}C]sphingosine generated was separated by thin layer chromatography and evaluated on a Fuji phosphoimager. Results are expressed as a percent of control values and are means ± S.D. (n = 3-4).

IL-1β-stimulation leads to neutral ceramidase mRNA and protein up-regulation

To test whether the increase in neutral ceramidase activity is due to an increased amount of neutral ceramidase protein, Western blot analysis was performed using a polyclonal antiserum raised against a peptide comprising the N-terminus of the murine neutral ceramidase. The antiserum recognised a double band of ~110-120 kDa. This size is in agreement with the recently described size of rat kidney neutral ceramidase [Mitsutake *et al.* 2001]. To determine whether the recognised protein does indeed show neutral ceramidase activity, cell lysates from IL-1β-stimulated mesangial cells were separated on

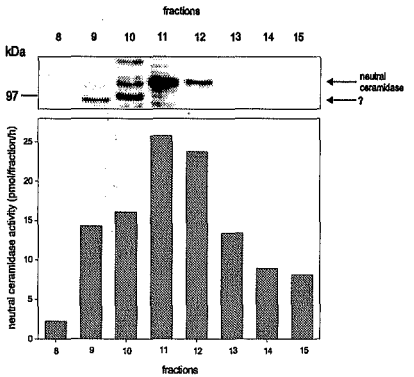
a MonoQ column, and fractions were analysed by Western blotting (Fig. 7, upper panel) and for neutral ceramidase activity (lower panel). Earlier fractions (fractions 9 and 10) showed an ~94-kDa protein of still unknown identity that was recognised by the neutral ceramidase antibody. Fractions 11 and 12 showed exclusive expression of a 110-120-kDa protein, the predicted size of rat neutral ceramidase [Mitsutake *et al.* 2001]. The neutral ceramidase activity was highest in fractions 11 and 12, which also showed the highest protein amounts, thus suggesting that this band is indeed a neutral ceramidase. Furthermore, we investigated whether the antibody could immunoprecipitate a fully active enzyme. As shown in Fig. 8, Western blot analysis of the supernatant after immunoprecipitation of neutral ceramidase revealed a disappearance of the protein that was dependent on the antibody dilution used (Fig. 8, upper panel). Preimmune serum did not deplete the protein from the supernatant. In parallel, a reduction of neutral ceramidase activity was observed in the supernatant (Fig. 8, lower panel). Consistent with a depletion of the enzyme in the supernatant, an increased amount of enzyme was observed in the immunoprecipitates by Western blotting (Fig. 9). However, no increased neutral ceramidase activity was recovered in the immunoprecipitates (data not shown). These data suggest that binding of the antibody to its antigen leads to neutralisation of the enzyme activity.

Stimulation of mesangial cells with IL-1β led to a marked and time-dependent up-regulation of the neutral ceramidase protein (Fig. 10A). In contrast, the acid ceramidase protein, which runs at 55 kDa as a holoenzyme under non reducing conditions [Bernardo *et al.* 1995], was not significantly changed (Fig. 10B).

It was further investigated whether the up-regulation of neutral ceramidase is due to increased de novo synthesis. For this purpose, mesangial cells were stimulated with IL-1β for different time periods, and [^{35}S]methionine and [^{35}S]cysteine were included in the culture medium for the last 4 h of stimulation. Thereafter, the cells were lysed, and the neutral ceramidase was immunoprecipitated and analysed by SDS-PAGE. Fig. 5 shows that IL-1β triggered increased de novo synthesis of the neutral ceramidase. A similar increase was also observed with another pro-inflammatory cytokine, TNF-α (Fig. 11). In contrast, the degradation of the neutral ceramidase was not affected by IL-1β treatment (data not shown) as analysed by pulse-chase experiments. In a next step, it was tested whether there is also an induction of the mRNA coding for the neutral ceramidase. Based on the mouse sequence of neutral ceramidase, mouse primers were selected and used to obtain a cDNA for the rat sequence. Using this partial sequence of the rat neutral ceramidase, new primers were generated and used to perform reverse transcriptase-PCR of IL-1β-stimulated rat mesangial cells. IL-1β stimulation indeed led to a clear induction of the neutral ceramidase mRNA level (Fig. 12). A maximal induction was obtained after 4 h

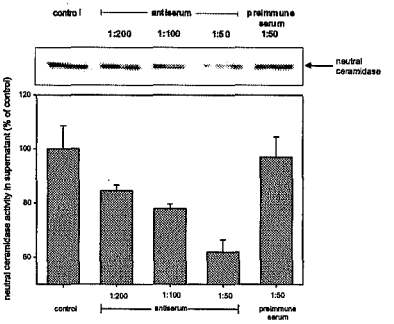
of stimulation and slightly decreased over the next 20 h. A similar induction was obtained in mouse mesangial cells using mouse primers (data not shown). Furthermore, the induction of neutral ceramidase by IL-1β was confirmed by Northern blot analysis (Fig. 13). Interestingly, two transcripts were detected for the neutral ceramidase at 3.5 kilobases, which were both induced by IL-1β (Fig. 13, upper panel). In contrast, the acid ceramidase mRNA was not significantly altered by IL-1β stimulation (Fig. 13, lower panel).

Figure 7



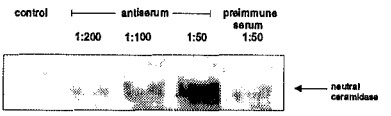
Cell lysate (200 µg of protein) of IL-1β-stimulated (8 h) mesangial cells was loaded onto a MonoQ column, and fractions were collected by elution with a linear gradient of NaCl as described under 2.5.7. Half of the fractions were concentrated and subjected to SDS-PAGE, and Western blot analysis using the anti-neutral ceramidase antibody at a dilution of 1:500 was performed (upper panel). In parallel, fractions were taken for neutral ceramidase activity measurement (lower panel).

Figure 8



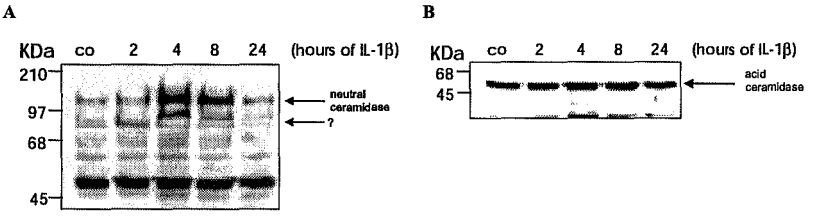
Lysates were subjected to immunoprecipitation using either the anti-neutral ceramidase antibody at the indicated dilutions or the preimmune serum at 1:50. Thereafter, aliquots of the supernatant were taken for Western blot analysis using the anti-neutral ceramidase antibody at a dilution of 1:500 (upper panel), or neutral ceramidase activity was measured (lower panel).

Figure 9



Immunoprecipitates were subjected to SDS-PAGE, and Western blot analysis was performed using the anti-neutral ceramidase antibody at a dilution of 1:500.

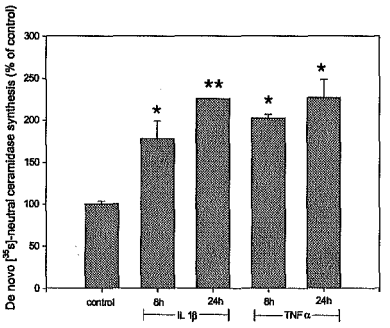
Figure 10



IL-1β treatment enhances neutral ceramidase protein levels in mesangial cells.

Quiescent mesangial cells were stimulated for the indicated time periods with 2 nM IL-1β. Thereafter, cell lysates were prepared, and 100 µg of protein were subjected to SDS-PAGE (7 and 10% acrylamide gels for the neutral and acid ceramidases, respectively) and transferred to nitrocellulose membrane. Western blot analysis was performed using an anti-neutral ceramidase antiserum at a dilution of 1:500 (A) or an anti-acid ceramidase antiserum at a dilution of 1:2000 (B). Bands were visualised by the ECL methods. The blot is representative of three independent experiments showing similar results.

Figure 11



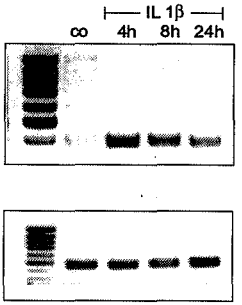
IL-1β stimulates de novo synthesis of neutral ceramidase in mesangial cells.

Confluent rat mesangial cells were stimulated for the indicated time periods with vehicle (control), IL-1β (2 nM), or TNF-α (2 nM). During the last 4 h of stimulation, a mixture of [^{35}S]methionine and [^{35}S]cysteine was added. Thereafter, cell lysates were prepared, and the neutral ceramidase was immunoprecipitated as described under 2.5.4. Immunoprecipitates were separated by SDS-PAGE, and the radioactive amount of neutral ceramidase was evaluated on a Fuji phosphoimager. The data are expressed as a percent of control stimulation and are the means of two independent experiments performed in duplicate (n=4). *$p < 0.05$; **$p < 0.01$ (statistically significant difference compared with the unstimulated control).

It was further investigated whether the up-regulation of neutral ceramidase is due to increased de novo synthesis. For this purpose, mesangial cells were stimulated with IL-1β for different time periods, and [^{35}S]methionine and [^{35}S]cysteine were included in the culture medium for the last 4 h of stimulation. Thereafter, the cells were lysed, and the neutral ceramidase was immunoprecipitated and analysed by SDS-PAGE. Fig. 5 shows that IL-1β triggered increased de novo synthesis of the neutral ceramidase. A similar increase was also observed with another pro-inflammatory cytokine, TNF-α (Fig. 11). In contrast, the degradation of the neutral ceramidase was not affected by IL-1β treatment (data not shown) as analysed by pulse-chase experiments. In a next step, it was tested whether there is also an induction of the mRNA coding for the neutral ceramidase. Based on the mouse sequence of neutral ceramidase, mouse primers were selected and used to obtain a cDNA for the rat sequence. Using this partial sequence of the rat neutral

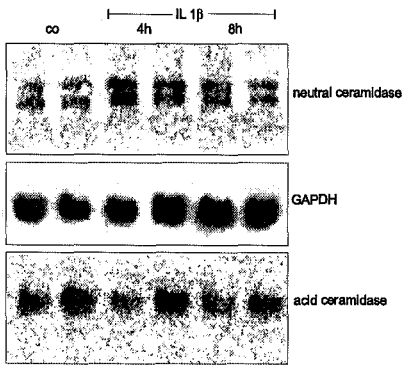
ceramidase, new primers were generated and used to perform reverse transcriptase-PCR of IL-1β-stimulated rat mesangial cells. IL-1β stimulation indeed led to a clear induction of the neutral ceramidase mRNA level (Fig. 12). A maximal induction was obtained after 4 h of stimulation and slightly decreased over the next 20 h. A similar induction was obtained in mouse mesangial cells using mouse primers (data not shown). Furthermore, the induction of neutral ceramidase by IL-1β was confirmed by Northern blot analysis (Fig. 13). Interestingly, two transcripts were detected for the neutral ceramidase at 3.5 kilobases, which were both induced by IL-1β (Fig. 13, upper panel). In contrast, the acid ceramidase mRNA was not significantly altered by IL-1β stimulation (Fig. 13, lower panel).

Figure 12



Quiescent rat mesangial cells were stimulated for the indicated time periods with IL-1 (2 nM). Thereafter, RNA was prepared, and reverse transcriptase-PCR of the neutral ceramidase (upper panel) and GAPDH (lower panel) was performed as described under 2.4.6. The data are representative of three independent experiments giving similar results.

Figure 13



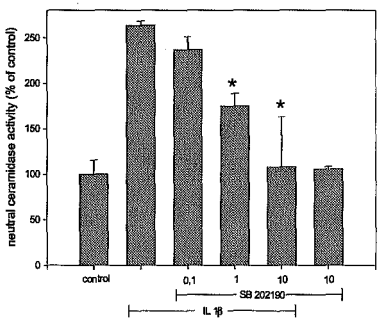
Quiescent mesangial cells were stimulated for 4 and 8 h with IL-1 (2 nM). Thereafter, RNA was prepared, separated on an agarose gel, and transferred to a nylon membrane; and Northern blot analysis was performed as described under 2.4.9 using a probe for the rat neutral ceramidase (upper panel), the rat acid ceramidase (lower panel), or GAPDH (middle panel). The data show two independent stimulation experiments.

IL-1β-induced up-regulation of neutral ceramidase involves the p38-MAPK pathway, but not MAPKAPK-2

To further elucidate mechanistically the pathway by which IL-1β increases neutral ceramidase activity, inhibitors against the different MAPK cascades were tested, i.e. the classical ERK and the stress-activated protein kinase p38 MAPK, since these MAPKs are known to play an important role in activating transcription factors and subsequently gene transcription and are targeted by rather specific low molecular mass inhibitors.

SB 202190, which is a quite selective inhibitor of p38 MAPK [Cuenda *et al.* 1995], caused a dose-dependent decrease in IL-1β-induced neutral ceramidase activity (Fig. 14) as well as in protein induction (Fig. 15). SB 202190 alone had no effect on ceramidase activity or protein levels (Fig. 14 and 15). Consequently, it was found that co-treatment of IL-1β with SB 202190, which blocks neutral ceramidase activity, but leaves the IL-1β-induced persistent sphingomyelinase activation unaffected, resulted in increased formation of ceramide (Fig. 16). In parallel, an enhanced rate of apoptosis was seen under co-treatment conditions (data not shown). SB 202190 had no effect on IL-1β-stimulated neutral or acid sphingomyelinase or acid ceramidase activities (data not shown). In contrast to SB 202190, U0126, which inhibits the MAPK kinase MEK, and Ro 318220, which potently blocks protein kinase C isoenzymes, were ineffective in blocking neutral ceramidase activity (data not shown).

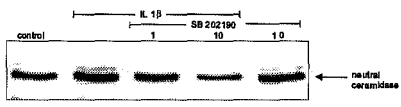
Figure 14



Effect of the p38 MAPK inhibitor SB 202190 on IL-1β-induced neutral ceramidase activity

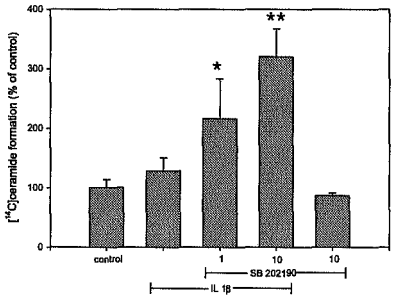
Quiescent mesangial cells were stimulated for 8 h with vehicle (control), IL-1β (2 nM) in the presence of the indicated micromolar concentrations of SB 202190, or SB 202190 alone. Thereafter, neutral ceramidase activity was detected as described under 2.6.1.1. Neutral ceramidase activity in control cells was 18.6 ± 2.8 pmol/mg/h. Results are expressed as a percent of control values and are means ± S.D. (n = 3-4). *p < 0.05; (statistically significant difference compared with the IL-1β-stimulated control).

Figure 15



Quiescent mesangial cells were stimulated for 8 h with vehicle (control), IL-1β (2 nM) in the presence of the indicated micromolar concentrations of SB 202190, or SB 202190 alone. Thereafter, protein levels were detected as described under 2.5.6.

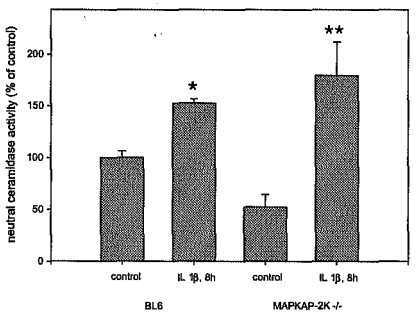
Figure 16



[^{14}C]serine-labelled cells were stimulated for 24 h with IL-1β (2 nM) in the presence of the indicated micromolar concentrations of SB 202190 or SB 202190 alone. Thereafter, lipids were extracted, and [^{14}C]ceramide was analysed as described under 2.6.2. Results are expressed as a percent of control values and are means ± S.D. (n = 3-4). *p < 0.05; **p < 0.01 (statistically significant difference compared with the IL-1β-stimulated control).

As MAPKAPK-2 is a downstream substrate of p38 MAPK, which can phosphorylate various transcription factors and thereby regulate gene transcription [Rouse *et al.* 1994, Tan *et al.* 1996, Kotlyarov *et al.* 1999], it was further investigated whether MAPKAPK-2 is involved in IL-1β-mediated neutral ceramidase activation. For this purpose, mesangial cells from MAPKAPK-2 knockout mice [Kotlyarov *et al.* 1999] and corresponding control mice were isolated and stimulated with IL-1β. As shown in Fig. 17, IL-1β-induced neutral ceramidase activity was not abolished in these mice, thus suggesting that MAPKAPK-2 does not mediate p38 MAPK-triggered neutral ceramidase induction.

Figure 17



IL-1β-stimulated neutral ceramidase activity in mesangial cells from MAPKAPK-2-deficient mice.

Quiescent mouse mesangial cells from control C57/BL6 mice (BL6) or MAPKAPK-2 -/- mice (MAPKAP-2K -/-) were stimulated for 8 h with IL-1β (2 nM). Thereafter, neutral ceramidase activity was measured as described under 2.6.1.1. Results are expressed as a percent of control values and are means ± S.D. (n = 6). Neutral ceramidase activity in control C57/BL6 cells was 38.4 ± 2.3 pmol/mg/h, and the activity in control MAPKAPK-2/ cells was 20.1 ± 4.5 pmol/mg/h. *p < 0.05; **p < 0.01 (statistically significant difference compared with the corresponding unstimulated controls).

Discussion

In this part of the thesis, it has been shown that IL-1β evokes a biphasic activation of neutral sphingomyelinase activity in renal mesangial cells. As previously reported, a first peak of activation occurs after minutes of IL-1β stimulation, leading to increased ceramide levels in mesangial cells [Huwiler *et al.* 1996, Kaszkin *et al.* 1998]. Now, these studies have been extended and it was reported on a second increase in neutral sphingomyelinase activity occurring after hours of IL-1β treatment. Surprisingly, this late

phase of neutral sphingomyelinase activation is not paralleled by an increase in ceramide levels in the cells, thus arguing for additional counter-regulatory mechanisms that maintain a normal ceramide level. Obvious candidates are ceramide-degrading enzymes such as the ceramidases, which deacylate ceramide to form sphingosine. Indeed, activity assays for neutral and acid ceramidases revealed that these enzymes are activated in a delayed fashion by IL-1β after hours of stimulation. This increase in activity is due to transcriptional and translational activation of the gene, as both the mRNA level (Fig. 12 and 13) and the de novo protein synthesis (Fig. 11) of the neutral ceramidase were up-regulated. The consequence of this dual action on the ceramide-generating and -degrading enzymes is a stable level of ceramide, which even tends to decrease over prolonged time periods of stimulation. Similar results regarding a balanced regulation of neutral ceramidase and neutral sphingomyelinase activities and ceramide levels in mesangial cells were also observed for TNF-α [Huwiler *et al.* 1999b].

These findings are consistent with the data of Nikolova-Karakashian *et al.* [1997], who found that, in rat hepatocytes, IL-1β also chronically increases neutral ceramidase activity and fails to accumulate ceramide in the cells. Additionally, these authors found that vanadate, a tyrosine phosphatase inhibitor, dramatically enhances IL-1β-induced neutral ceramidase activity, whereas the nonspecific tyrosine kinase inhibitor genistein partially inhibits it. Whether this is due to phosphorylation and subsequent changes in enzyme activity or changes in the expression level of the enzyme were not addressed.

Furthermore, Coroneos *et al.* [1995] reported that platelet-derived growth factor is a potent activator of ceramidase activity in rat mesangial cells, whereas cytokines such as IL-1β and TNF-α are ineffective in activating ceramidase after 1 h of stimulation. These data do not contrast with our results, as 1 h of IL-1β stimulation was not sufficient to increase the neutral ceramidase activity, and at least 2-4 h of stimulation were required to see significant stimulation of enzyme activity (Fig. 6A).

Again, the short-term activation of neutral/alkaline ceramidase by platelet-derived growth factor observed by Coroneos *et al.* [1995] was suggested to involve tyrosine kinases since the platelet-derived growth factor-induced activation was completely inhibited by genistein. Taken together, these different results make it tempting to speculate that the neutral ceramidase is regulated by two different mechanisms: (i) a rapid post-translational regulation by phosphorylation/dephosphorylation reactions, which is further supported by the presence of various putative protein kinase phosphorylation sites in the sequence of the neutral ceramidase, and (ii) a long-term regulation by gene transcription, as documented for the first time in this study.

Furthermore, the data reveal that p38 MAPK is critically involved in the up-regulation of IL-1β-induced neutral ceramidase activity. As previously reported, IL-1β indeed potently

activates the p38 MAPK pathway in mesangial cells [Guan *et al.* 1997]. p38 MAPK has been attributed an important role in transcription of many genes [Obata *et al.* 2000, Ono & Han 2000] due to its ability to phosphorylate and activate various transcription factors, including activating transcription factor-2, myocyte enhancer factor-2C, and CHOP/GADD153, which is a member of the CAAT/enhancer-binding protein family of transcription factors. Furthermore, p38 MAPK can phosphorylate and activate MAPKAPK-2, which in turn can phosphorylate transcription factors, including cAMP response element-binding protein and activating transcription factor 1 [Tan *et al.* 1996], and thereby activate gene transcription.

Using mesangial cells from MAPKAPK-2 knockout mice [Kotlyarov *et al.* 1999], the involvement of MAPKAPK-2 in the cytokine-induced up-regulation of neutral ceramidase can, however, be excluded. The exact pathway by which p38 MAPK up-regulates the neutral ceramidase protein and activity is presently under investigation.

This data further suggest an inverse correlation between neutral ceramidase activity and the rate of apoptosis. Whenever increased neutral ceramidase activity is observed, such as after IL-1β treatment, no DNA fragmentation can be detected [Huwiler *et al.* 1999b]. However, when neutral ceramidase activity is blocked by co-incubation with the p38 MAPK inhibitor SB 202190, ceramide levels (Fig. 16) and also DNA fragmentation (data not shown) are increased. Moreover, when neutral ceramidase activity drops, such as after nitric oxide donor treatment, an increased rate of DNA fragmentation is observed [Huwiler *et al.* 1999b]. However, a causative role of increased ceramide levels in cell death induction is controversial and not yet settled. Some studies have shown that ceramide activates different caspases and thereby feeds the signal into the apoptotic pathway [Mizushima *et al.* 1996, Kuo *et al.* 1997, Tepper *et al.* 1997, Machleidt *et al.* 1998]. Other reports have documented that ceramide can inhibit the survival signal resulting from the phosphatidylinositol 3-kinase cascade [Zhou et al. 1998, Zundel & Giaccia 1998], and it may well be that turning off a survival signal will finally direct a cell toward apoptosis.

Further evidence for an involvement of ceramidases in cell survival has recently been forwarded by Strelow *et al.* [2000]. These authors showed that overexpression of the acid ceramidase in fibroblast L292 cells leads to reduced TNF-α-induced apoptosis. In contrast, Tohyama *et al.* [1999] and Segui *et al.* [2000] reported that fibroblasts [Tohyama *et al.* 1999] and lymphoid cells [Segui *et al.* 2000] from patients suffering from Farber's disease, which results in a deficiency in acid ceramidase activity, do not show enhanced rates of apoptosis compared with healthy controls.

Another interesting observation of this study is the appearance of a double band in Northern blot analysis (Fig. 13) as well as Western blot analysis (Fig. 10A). It is very

tempting to speculate that these two bands represent different splice variants or isoforms of the neutral ceramidase. However, additional work is needed to unambiguously define the identity of both bands. In any case, the pathways controlling ceramide levels in cells exposed to stressful stimuli such as inflammatory cytokines seem to exert a stringent control on both the ceramide-generating enzymes as well as the ceramide-metabolising enzymes, thus arguing for the relevance of ceramide and the products derived thereof for cellular functions.

3.2 Nitric oxide induces degradation of the neutral ceramidase in rat renal mesangial cells and is counterregulated by protein kinase C.

Introduction

In recent years, nitric oxide (NO), has become established as a diffusible universal messenger mediating cell-cell communication throughout the body. Excessive and uncontrolled production of NO is associated with severe diseases like septic shock, stroke, neurodegeneration, diabetes mellitus, arthritis, and other forms of acute and chronic inflammation [Nathan 1992, Knowles & Moncada 1994, Kröncke *et al.* 1995, Pfeilschifter 1995]. NO-induced apoptosis has been described for a variety of cell types but it seems to be a matter of concentration whether NO acts pro- or anti-apoptotic [Brüne *et al.* 1998, Beck *et al.* 1999]. The early and rapid mechanisms of NO signalling depend primarily on post-translational modifications of pre-existing cellular proteins such as guanylate cyclase. However, the late phases that are required to accommodate the microenvironmental changes are mediated by alterations in gene expression [Pfeilschifter *et al.* 2001a, Pfeilschifter *et al.* 2001b].

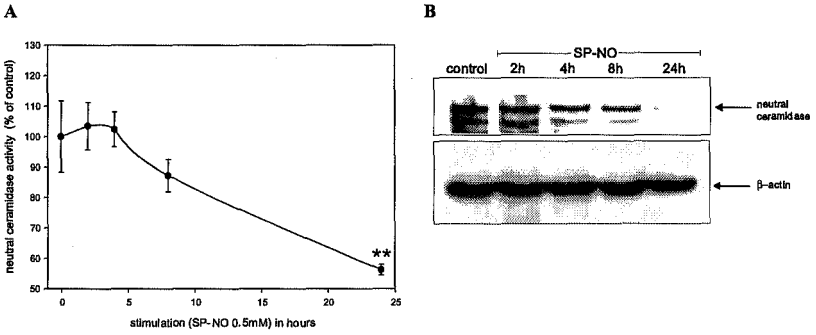
Previously, it was shown that glomerular cells exposed to NO donors respond with a drastic increase in ceramide formation [Huwiler *et al.* 1999a, Huwiler *et al.* 1999b]. This was due to a dual action of NO, on one side by activation of sphingomyelinases, and on the other side by inhibition of ceramidase activities. In this part, the mechanism by which NO reduces neutral ceramidase activity in mesangial cells was investigated.

Results

Nitric oxide stimulation leads to a decrease in neutral ceramidase activity and protein level.

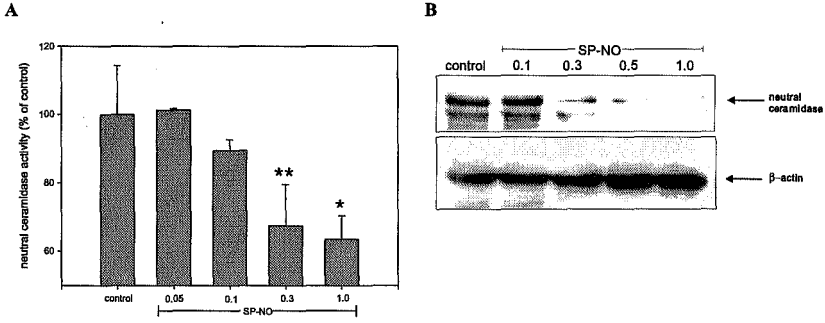
Recently, it has been demonstrated that high levels of nitric oxide caused an increased ceramide formation with subsequent apoptosis of mesangial cells which was due to enhanced sphingomyelinase activity and reduced ceramidase activity [Huwiler *et al.* 1999b]. These studies were now extended and the mechanism by which NO inhibits ceramidase activity was investigated. Stimulation of renal mesangial cells with the nitric oxide donor spermine-NO leads to a delayed and time-dependent reduction of neutral ceramidase activity (Fig. 18A) with a first significant effect after 8 h of treatment. This reduction of activity is paralleled by a decrease of neutral ceramidase protein amount as shown by a Western blot analysis (Fig. 18B). The effect on neutral ceramidase activity (Fig. 19A), and protein level (Fig. 19B) occurs in a concentration-dependent manner. A

Figure 18



Time-dependent effect of spermine-NO on neutral ceramidase activity (A) and protein (B) in rat mesangial cells.
Confluent rat mesangial cells were stimulated for the indicated time periods with spermine-NO (SP-NO; 0.5mM). Thereafter, cell lysates containing 100µg of protein were taken for a neutral ceramidase activity assay (A) or SDS-PAGE (7% acrylamide gel) and subsequent Western blot analysis (B) as described in chapters 2.5.6. and 2.6.1.1. The generated [^{14}C]sphingosine was separated on thin layer chromatography and evaluated on an Imaging System (Fuji). Results in A are expressed as % of control values and are means ±S.D. (n=3-4). Neutral ceramidase activity in control cells was 21.5 ± 2.5 pmol/mg/h. **p<0.01 statistically significant difference compared to the unstimulated control. Data in B are representative of at least 3 independent experiments giving similar results.

Figure 19

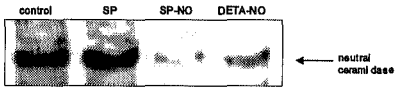


Concentration-dependent effect of spermine-NO on neutral ceramidase activity and protein level in rat mesangial cells.
Quiescent rat mesangial cells were stimulated for 24h with the indicated concentrations of spermine-NO (SP-NO; in mM) (A and B). Thereafter, cell lysates containing 100µg of protein were taken for a neutral ceramidase activity assay (A) or SDS-PAGE (7% acrylamide gel) and subsequent Western blot analysis (B) as described in chapters 2.5.6 and 2.6.1.1. The generated [^{14}C]sphingosine was separated on thin layer chromatography and evaluated on an Imaging System (Fuji). Data in A are expressed as % of control values and are means ±S.D. (n=3-4). Neutral ceramidase activity in control cells was 20.8 ± 3.0 pmol/mg/h. *p<0.05, **p<0.01, statistically significant difference compared to the unstimulated control. Data in B are representative of 3 independent experiments giving similar results.

maximal reduction to approximately 60% of control level is obtained with 0.3 mM of spermine-NO which is not further reduced by increasing the concentration of the NO donor. Interestingly, the protein level of neutral ceramidase is almost completely reduced at 0.3 mM of NO. The total amount of protein was not changed upon NO-treatment as

shown by staining for the housekeeping enzyme β-actin (Fig. 18B and 19B, lower panels). To verify that the observed effect of spermine-NO is indeed mediated by released NO and not by an unspecific effect, spermine was used as a control substance. As seen in Fig. 20, spermine has no effect on neutral ceramidase protein levels nor on activity (data not shown). Furthermore, Deta-NO, another NO donor which possesses a much longer half-life than spermine-NO, shows the same effect on ceramidase protein degradation (Fig. 20)

Figure 20



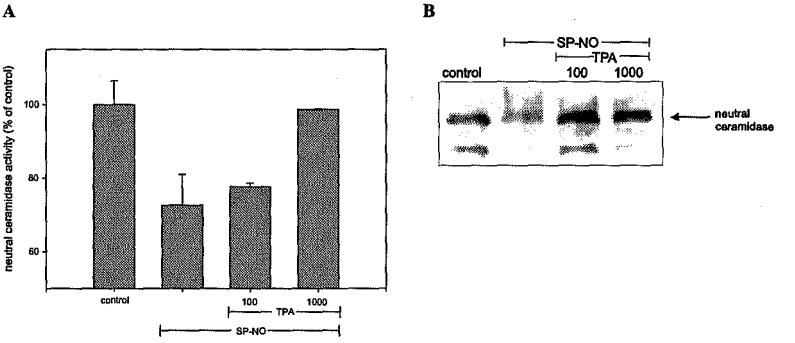
Quiescent rat mesangial cells were stimulated for 24h with the indicated concentrations of spermine (SP; 0.5mM), spermine-NO (SP-NO; 0.5mM) or DETA-NO (0.5mM). Thereafter, cell lysates containing 100μg of protein were taken for SDS-PAGE (7% acrylamide gel) and subsequent Western blot analysis as described under 2.5.6.

Activation of PKC prevents NO-induced down-regulation of neutral ceramidase activity and protein.

When mesangial cells were exposed to NO donors in the presence of phorbol esters which directly activate PKC, both ceramide accumulation and DNA fragmentation were completely blocked [Huwiler *et al.* 1999b]. Therefore, the effect of PKC on the NO-induced down-regulation of neutral ceramidase was investigated. Treatment of mesangial cells with spermine-NO in the presence of 12-O-tetradecanoylphorbol-13-actetate (TPA) dose-dependently reversed the inhibitory action of NO on neutral ceramidase activity (Fig. 21A). In parallel, the protein level of neutral ceramidase also recovered in the presence of TPA (Fig. 21B). Neither short-term nor long-term stimulation with TPA alone had any effect on neutral ceramidase activity or protein level (data not shown).

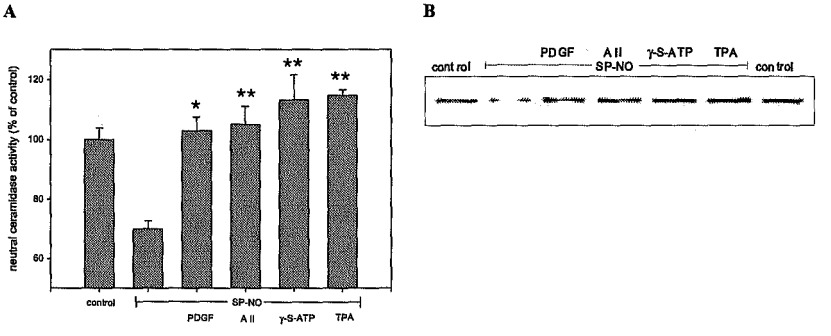
Furthermore, physiological activators of PKC, like platelet-derived growth factor BB (PDGF-BB), angiotensin II and the stable ATP analog γ-S-ATP, which all evoke phosphoinositide hydrolysis and generation of 1,2-diacylglycerol, the endogenous activator of PKC [Pfeilschifter 1986, Pfeilschifter 1990, Pfeilschifter & Hosang 1991], were tested. All these substances mimicked the effect of TPA and reversed the NO-mediated inhibition of neutral ceramidase activity (Fig. 22A), as well as neutral ceramidase protein level (Fig. 22B). Consistent with the observed increase of neutral ceramidase protein by all PKC activators, a decrease of NO-induced ceramide formation was found by these substances in intact mesangial cells when performing mass spectrometry of extracted lipids (Fig. 23).

Figure 21



Effect of protein kinase C on NO-reduced neutral ceramidase activity (A) and neutral ceramidase protein (B) in rat mesangial cells. Confluent rat mesangial cells were stimulated for 24 h with vehicle (control) or spermine-NO (SP-NO; 1.0mM) in the absence or presence of the indicated concentrations of the phorbol ester TPA (in nM). Thereafter, cell lysates containing 100µg of protein were taken for a neutral ceramidase activity assay (A) or SDS-PAGE (7% acrylamide gel) and subsequent Western blot analysis (B) as described in chapter 2.5.6 and 2.6.1.1. The generated [^{14}C]sphingosine was separated on thin layer chromatography and evaluated on an Imaging System (Fuji). Results in A are expressed as % of control values and are means ±S.D. (n=3-4). Neutral ceramidase activity in control cells was 24.5 ± 2.1 pmol/mg/h. Data in B are representative of 2 independent experiments giving similar results.

Figure 22



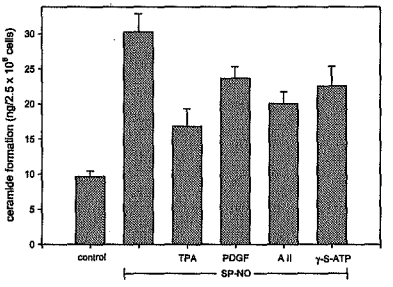
Effect of TPA, PDGF, angiotensin II and γ-S-ATP on NO-reduced neutral ceramidase activity (A) and protein level (B)

Quiescent rat mesangial cells were stimulated for 24h with either vehicle (control) or spermine-NO (SP-NO; 1.0mM) in the absence or presence of TPA (1.0µM), PDGF-BB (3.0ng/ml), angiotensin II (100nM) or γ-S-ATP (100µM) as indicated.

(A) Cell lysates containing 100µg of protein were taken for a neutral ceramidase activity assay as described in chapter 2.6.1.1. The generated [^{14}C]sphingosine was separated on thin layer chromatography and evaluated on an Imaging System (Fuji). Results are expressed as % of control values and are means ±S.D. (n=4). *p<0.05, **p<0.01, statistically significant difference compared to the NO-stimulated control. Neutral ceramidase activity in control cells was 27.3 ± 1.0 pmol/mg/h.

(B) Cell lysates containing 100µg of protein were subjected to SDS-PAGE (7% acrylamide gel) and transferred to nitrocellulose membrane. Western blot analysis was performed using an anti-neutral ceramidase antiserum at a dilution of 1:500. Data are representative of 2 independent experiments giving similar results.

Figure 23



Effect of TPA, PDGF, angiotensin II and γ-S-ATP on NO-induced ceramide formation.

Quiescent rat mesangial cells were stimulated for 24h with either vehicle (control) or spermine-NO (SP-NO; 1.0mM) in the absence or presence of TPA (1.0μM), PDGF-BB (3.0ng/ml), angiotensin II (100nM) or γ-S-ATP (100μM) as indicated. Lipids were extracted and ceramide was analysed by LC/MS/MS spectrometry as described under 2.6.2. Results are means ± S.D. (n=4). *p<0.05 statistically significant difference compared to the NO-stimulated control.

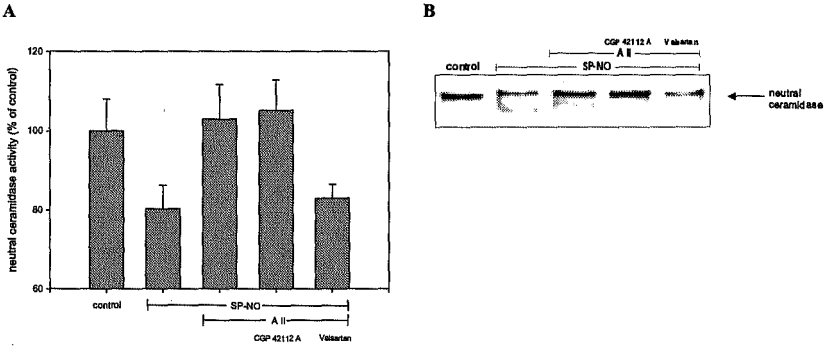
The effect of angiotensin II was further characterised and was found to involve the AT_1-receptor. The protective effect of angiotensin II reversing the NO-mediated inhibition of neutral ceramidase activity (Fig. 22A), as well as neutral ceramidase protein level (Fig. 22B) was abrogated by co-stimulation with valsartan (Fig. 24), a specific inhibitor of the AT_1-receptor-subtype [Criscione *et al.* 1993, Timmermans *et al.* 1993]. In contrast, the specific AT_2-receptor-agonist CGP 42112A [Hines *et al.* 2001] delivers no further activation compared to the reversion of neutral ceramidase activity and protein by angiotensin II alone (Fig. 24). To definitely exclude that the protective effect is not mediated by the AT_2-receptor a specific AT_2-receptor antagonist like PD 123319 [Blankley *et al.* 1991] has to be evaluated. In this context, it is important to note that mesangial cells exclusively express the AT_1 receptor [Pfeilschifter 1989, Pfeilschifter *et al.* 1990].

NO promotes degradation of neutral ceramidase.

To investigate how NO down-regulates the protein amount of neutral ceramidase, the protein synthesis inhibitor cycloheximide [Ch'ih *et al.* 1979] was used. As seen in Fig. 25, the presence of cycloheximide does not abrogate the NO-mediated reduction of neutral ceramide protein nor does it affect the reversal by the PKC activators TPA, angiotensin II or γ-S-ATP, thus suggesting that NO modulates the degradation of neutral ceramidase rather than its de-novo synthesis.

Furthermore, the involvement of caspases in mediating NO-induced degradation of neutral ceramidase was investigated. For this purpose, the broad caspase family inhibitor Z-VAD-FMK as well as the specific caspase-3 inhibitor Z-DEVD-FMK were tested. However, none of these two inhibitors is able to block NO-induced enzyme degradation at the recommended concentration of 2μM (data not shown). In contrast, the highly selective proteasome inhibitor lactacystin inhibited the NO-induced degradation of neutral ceramidase (Fig. 26).

Figure 24



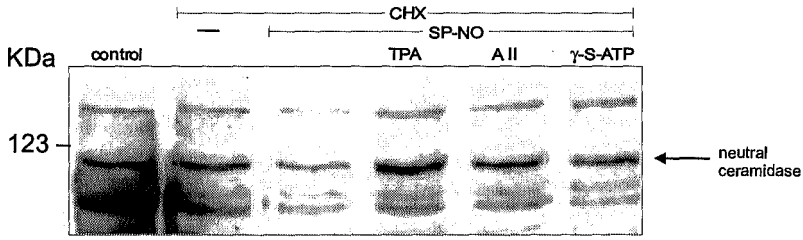
Effect of angiotensin II, CGP 42112A and valsartan on NO-reduced neutral ceramidase activity (A) and protein level (B)

Quiescent rat mesangial cells were stimulated for 24h with either vehicle (control) or spermine-NO (SP-NO; 1.0mM) in the absence or presence of angiotensin II (AII, 100nM), CGP 42112A (10μM), or valsartan (10μM) as indicated.

(A) Cell lysates containing 100μg of protein were taken for a neutral ceramidase activity assay as described in chapter 2.6.1.1. The generated [^{14}C]sphingosine was separated on thin layer chromatography and evaluated on an Imaging System (Fuji). Results are expressed as % of control values and are means ±S.D. (n=4). Neutral ceramidase activity in control cells was 27.3 ± 1.0 pmol/mg/h.

(B) Cell lysates containing 100μg of protein were subjected to SDS-PAGE (7% acrylamide gel) and transferred to nitrocellulose membrane. Western blot analysis was performed using an anti-neutral ceramidase antiserum at a dilution of 1:500. Data are representative of 2 independent experiments giving similar results.

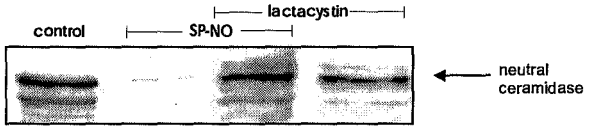
Figure 25



Effect of cycloheximide on NO-reduced neutral ceramidase protein level in mesangial cells.

Quiescent mesangial cells were stimulated for 24h in the absence or presence of cycloheximide (CHX; 10μM) with either vehicle (-) or spermine-NO (SP-NO; 1.0mM) in the absence or presence of TPA (1.0μM), angiotensin II (AII, 100nM) or γ-S-ATP (100μM). Thereafter, cell lysates containing 100μg of protein were subjected to SDS-PAGE (7% acrylamide gel) and transferred to nitrocellulose membrane. Western blot analysis was performed using an anti-neutral ceramidase antiserum at a dilution of 1:500. Data are representative of 2 independent experiments giving similar results.

Figure 26



Effect of lactacystin on NO-reduced neutral ceramidase protein level in mesangial cells.
Cells were stimulated for 24h in the absence or presence of lactacystin (20μM; pretreated for 2 h) with either vehicle (control) or spermine-NO (SP-NO; 1.0mM). Thereafter, cell lysates containing 100μg of protein were subjected to SDS-PAGE (7% acrylamide gel) and transferred to nitrocellulose membrane. Western blot analysis was performed using an anti-neutral ceramidase antiserum at a dilution of 1:500. Data are representative of 3 independent experiments giving similar results.

PKC activation induces phosphorylation of neutral ceramidase.

To study whether PKC exerts its effect on neutral ceramidase via phosphorylation of the enzyme either directly or indirectly we performed in vivo phosphorylation assays. Mesangial cells were labelled with ^{32}P-orthophosphate before stimulation. Thereafter, neutral ceramidase was immunoprecipitated and incorporated ^{32}P was analysed. As seen in Fig. 27, stimulation of cells for 10 min with TPA causes a dose-dependent increase of phosphorylated neutral ceramidase. Similarly, PDGF-BB (Fig. 27) and angiotensin II (Fig. 28) also induce phosphorylation of the enzyme. The TPA-induced phosphorylation is inhibited by the potent PKC inhibitor Ro 318220 [Wilkinson *et al.* 1993] but not by CGP 41251 [Meyer *et al.* 1989], an inhibitor that preferentially blocks Ca^{2+}-dependent PKC isoenzymes (Fig. 29) [Ochsner *et al.* 1993, Marte *et al.* 1994].

Moreover, it was investigated whether PKC directly phosphorylates the neutral ceramidase in an in-vitro system. Neutral ceramidase was enriched by immunoprecipitating the enzyme from unstimulated mesangial cell lysates, and was then incubated with recombinant PKC of the four subtypes that have been identified in mesangial cells, i.e. PKC-α, –δ, –ε and –ζ [Huwiler *et al.* 1991, 1992, 1993].

Figure 27

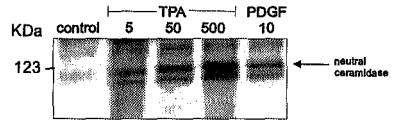
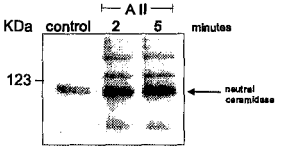
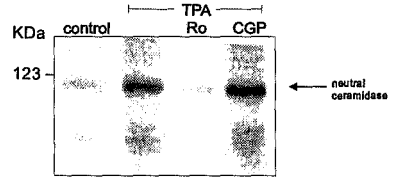


Figure 28



Effect of PKC activators on phosphorylation of neutral ceramidase in intact mesangial cells.
^{32}P-labelled mesangial cells were stimulated for 15 min with either vehicle (control) or the indicated concentrations of TPA (Figure 27; in nM) and PDGF-BB (Figure 27; in ng/ml), or for the indicated time periods with angiotensin II (A II) (Figure 28; 100nM). Cells were then lysed and neutral ceramidase was immunoprecipitated with a polyclonal antibody at a dilution of 1:100, as described in the materials and methods section. The immunoprecipitates were separated by SDS-PAGE (7% acrylamide gels) and visualised on an Imaging System (Fuji). Data are representative of 3 independent experiments giving similar results.

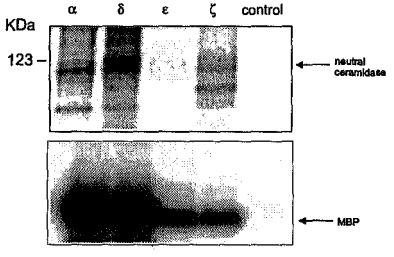
Figure 29



Effect of PKC inhibitors on TPA-induced phosphorylation of neutral ceramidase in mesangial cells.

^{32}P-labelled mesangial cells were preincubated for 20 min with either Ro 318220 (1.0μM) or CGP 41251 (1.0μM) before stimulation with TPA (100nM) for 10 min. Cells were then lysed and neutral ceramidase was immunoprecipitated with a polyclonal antibody at a dilution of 1:100, as described in the materials and methods section. The immunoprecipitates were separated by SDS-PAGE (7% acrylamide gels) and visualised on an Imaging System (Fuji). Data are representative of 2 independent experiments giving similar results.

Figure 30



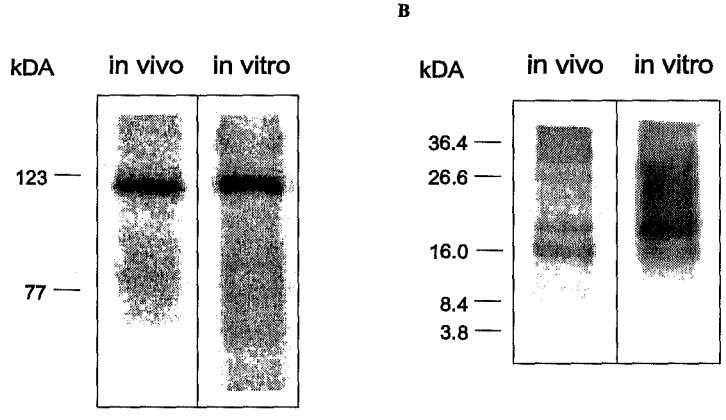
Effect of recombinant PKC isoenzymes on neutral ceramidase phosphorylation in vitro.

1μg of recombinant PKC-α, -δ, -ε, and -ζ isoenzymes or buffer (control) were incubated for 15 min with neutral ceramidase that was immunoprecipitated from unstimulated mesangial cells (upper panel) or with myelin basic protein (lower panel), as described in the materials and methods section. Thereafter, samples were separated on SDS-PAGE (7% acrylamide gel for neutral ceramidase and 13% for MBP) and analysed on an Imaging System (Fuji). Data are representative of 2 independent experiments giving similar results.

As seen in Fig. 30, upper panel, PKC-δ is the most efficient isoform to phosphorylate neutral ceramidase, which appears as a double band at approximately 120 kDa. PKC-α and PKC-ζ induce only minor phosphorylation of the neutral ceramidase. In contrast, PKC-ε has no effect.

To verify that all PKC isoenzymes used are active enzymes, phosphorylation of myelin basic protein (MBP), which is a well accepted substrate for PKC isoenzymes [Geiges *et al.* 1997] is shown (Fig. 30, lower panel). It was further investigated whether the phosphorylation of neutral ceramidase by PKC in vitro occurs at the same phosphorylation sites as in intact cells. For this the phosphorylation pattern of in vivo phosphorylated neutral ceramidase was compared to in vitro phosphorylated ceramidase after trypsin digestion. As seen in Fig. 31A, [^{32}P]phosphorylated ceramidase from in vivo and in vitro assays runs at 120 kDa on a 7% Tris-glycine gel system. Upon trypsin digestion, enzyme preparations are both fragmented into an identical pattern of polypeptides. Using a 10-20% gradient Tris-tricine gel system to allow a better separation of small polypeptides, two radioactive fragments between 15-20 kDa can be detected (Fig. 31B).

Figure 31



Phosphopeptide mapping of trypsin-digested phosphorylated neutral ceramidase.

Neutral ceramidase, either purified from mesangial cells and subjected to *in vitro* phosporylation by PKC-δ (A, right lane) or affinity-purified from *in vivo*-P_i-labelled TPA-stimulated mesangial cells (A, left lane), was digested with trypsin as described in the materials and methods section. Undigested control samples were separated on a Tris-glycine SDS-PAGE (7% acrylamide gel) (A) whereas trypsin-digested samples were separated on a Tris-tricine SDS-PAGE (10-20% gradient) (B). Gels were analysed on an Imaging system (Fuji) for radioactive fragments.

Discussion

Mesangial cells have been shown to respond to high amounts of NO with an increased generation of the sphingolipid molecule ceramide, which involves a dual mechanism, the activation of sphingomyelinases and the concomitant inhibition of ceramidase activities [Huwiler *et al.* 1999b].

In this part of the study, the mechanism by which NO reduces neutral ceramidase activity was investigated. The data clearly show that the NO-mediated reduction of neutral ceramidase activity is paralleled by a decrease of the ceramidase protein level. This suggests that either the de-novo synthesis of neutral ceramidase is blocked by NO or that NO induces degradation of the enzyme. The experiments performed in the presence of the protein synthesis inhibitor cycloheximide, which does not affect the NO-triggered down-regulation nor the basal level of neutral ceramidase (Fig. 25) rather argues for an increased degradation of the enzyme. This notion is further substantiated by the observation that a specific proteasome inhibitor blocked NO-induced ceramidase degradation, whereas several caspase inhibitors were without effect. Thus ceramidase is targeted by the ubiquitin proteasome machinery in mesangial cells. Interestingly, the NO-mediated degradation of ceramidase can be reversed by PKC activating agents, like the phorbol ester TPA, but also the physiologically important ligands PDGF-BB, angiotensin II and ATP. The mechanisms by which PKC prevents degradation is still unclear but it is

tempting to speculate that phosphorylation events on neutral ceramidase play an important role.

Phosphorylation is a well known mechanism for regulating protein stability. Thus many proteins are subjected to phosphorylation and as a consequence are degraded more rapidly, like the inhibitor of κb (IκB) [Steffan *et al.* 1995], the transcription factor RelB, which is a member of the Rel/NF-κB family of transcription factors [Marienfeld *et al.* 2001] or the tumor suppressor protein p53 [Chernov *et al.* 2001]. On the contrary, phosphorylation may also stabilise certain proteins like the epidermal growth factor receptor [Bao *et al.* 2000], the growth arrest and DNA-damage-inducible gene Gadd45 [Leung *et al.* 2001], or the human proto-oncogene ets-2 [Fujiwara *et al.* 1988], and thereby lead to their accumulation. In the case of neutral ceramidase, it seems that phosphorylation of the enzyme by PKC directly or indirectly by alteration of another kinase or phosphatase activity, leads to reduced protein degradation. Our data further suggest, that in mesangial cells, the PKC-δ isoenzyme is the most likely candidate for a direct stabilising effect. This finding is corroborated by the fact that (i) only the broad spectrum PKC inhibitor Ro 31-8220, but not the inhibitor of the Ca^{2+}-dependent PKC isoenzymes, CGP 41251, is able to block TPA-stimulated phosphorylation of neutral ceramidase, and that (ii) recombinant PKC-δ is the most effective isoform to phosphorylate neutral ceramidase in an in vitro system. In accordance with our data, Leung *et al.* [2001] reported that PKC-δ plays an important role in protein stabilisation of Gadd45 in A431 cells. Mechanistically, they showed that epidermal growth factor, by activating PKC-δ, decreased ubiquitination of Gadd45, an effect that was blocked by the PKC-δ selective inhibitor rottlerin. It is tempting to speculate that PKC-δ also decreases ubiquitination of neutral ceramidase and thus stabilises the enzyme in mesangial cells.

By sequence analysis of neutral ceramidase, nine putative PKC and several casein kinase II consensus phosphorylation sites can be found in the primary sequence [Mitsutake *et al.* 2001]. However, site directed mutagenesis studies of these phosphorylation sites will be required in order to identify which of these putative PKC phosphorylation sites, if any, are indeed relevant for ceramidase stability.

Remarkably, phosphorylation of neutral ceramidase per se, either by PKC or another kinase, seems not to be sufficient for activation, since short-term stimulation with either TPA, PDGF-BB or angiotensin II did not change the enzyme's activity (data not shown). This contrasts to a report by Coroneos *et al.* [1995], who found that PDGF activated an alkaline ceramidase in mesangial cells. This activation was under the regulation of a tyrosine phosphatase because activity increased in the presence of vanadate. Whether this alkaline ceramidase is identical to the neutral ceramidase investigated in this study is not clear, since no biochemical characterisation of the alkaline enzyme has been reported.

Moreover, when we added vanadate to mesangial cells, it had no effect on neutral ceramidase activity (data not shown).

One obvious cellular consequence of neutral ceramidase stabilisation by PKC is a more efficient elimination of ceramide which in turn may protect the cell from the proapoptotic action of this lipid mediator. There is increasing evidence that PKC can act as a cytoprotective enzyme preventing stress-induced programmed cell death (apoptosis) in various cell types, including mesangial cells [Huwiler *et al.* 1999b, Meinhardt *et al.* 2000]. Furthermore, Jun *et al.* [1999] described that overexpression of PKC-βII, -δ and -η isoenzymes protected RAW 264.7 macrophages from NO-induced apoptosis by blocking NO-activating effects on c-Jun N-terminal kinase/stress-activated protein kinase and p38 kinase. Moreover, overexpression of PKC-α in 32D myeloid progenitor cells leads to an increased activation of the protein kinase B/Akt which promotes cell survival [Li *et al.* 1999]. Obviously, there is a cell type-specific involvement of PKC isoenzymes in cell protection. In all, it is tempting to speculate that especially PKC-δ is a prime candidate for exerting an anti-apoptotic effect in mesangial cells via stabilising neutral ceramidase. This fits to previous findings and hypothesis that PKC-δ mediates mitogen-activated protein kinase activation in mesangial cells and promotes proliferation in response to extracellular nucleotides [Huwiler & Pfeilschifter 1994].

In this context it is worth noting that ceramide is able to directly bind to PKC-δ in mesangial cells [Huwiler *et al.* 1998]. Such a binding leads to a decreased autophosphorylation of PKC-δ probably reflecting an inhibitory effect on the enzyme's activity. This may constitute an intriguing positive feedback loop, with increased ceramide levels causing inhibition of PKC-δ , which in turn leads to an increased degradation of neutral ceramidase and amplified ceramide accumulation. Powerful negatively acting regulatory pathways are required to terminate amplification loops such as the one suggested here. Noteworthy, ceramide also binds to and triggers PKC-α-mediated feedback inhibition of cytokine-induced ceramide formation [Huwiler *et al.* 1998, Kaszkin *et al.* 1998, Pfeilschifter & Huwiler 2000]. Can these seeming disparate results be reconciled? It is suggesting that ceramide acts as a negative feedback regulator to control its own synthesis in response to cytokine stimulation via PKC-α activation, but as soon as a critical threshold of activation is reached and high amounts of NO are produced by a delayed cytokine-induced expression of the inducible NO-synthase, ceramide now functions as a positive feedback modulator, to amplify its production via ceramidase destabilisation due to inhibition of PKC-δ. This may operate in a switch like mechanism [Pfeilschifter *et al.* 2002] and initiate programmed cell death which is a common phenomenon in acute inflammation as well as resolution of disease [Savill & Johnson 1995].

Taken together the results of this chapter provide evidence that NO blocks neutral ceramidase activity by inducing degradation of the enzyme and that this effect can be reversed by activating PKC, especially the δ–isoform. The neutral ceramidase may thus represent a novel attractive target to interfere with cellular stress response and to modulate programmed cell death which is a typical feature of many inflammatory diseases.

3.3 Nitric oxide induces neutral ceramidase degradation by the ubiquitin/proteasome complex in renal mesangial cells.

Introduction

The ubiquitin/proteasome system is the major pathway of selective protein degradation in eukaryotic cells. Degradation of a protein via this system occurs via three distinct and successive steps. Initially, the target proteins are conjugated to the polypeptide ubiquitin; in the second step, the ubiquitin-conjugated proteins are recognised by the 26S proteasome, a large, multicatalytic protease, and in a last step degraded. The ubiquitin system targets normal short-lived proteins and misfolded or otherwise abnormal proteins for degradation. Although the components of the ubiquitin system are cytosolic, the known targets of the system comprise not only soluble cytosolic and nuclear proteins, but also membrane proteins and even luminal endoplasmic reticulum (ER) proteins. Some misfolded ER proteins were shown to be retransported to the cytosol, ubiquitinated, and then degraded by the proteasome [Plemper & Wolf 1999].

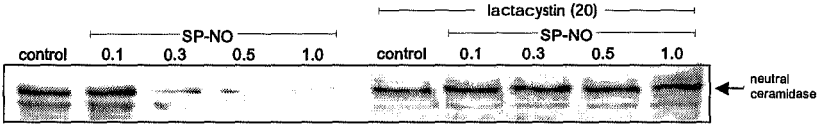
Results

To test whether the degradation of neutral ceramidase induced by nitric oxide is mediated through the ubiquitin/proteasome system, lactacystin, a highly selective and cell permeable inhibitor of the ubiquitine/proteasome complex, was applied. Analysing neutral ceramidase protein levels by immunodetection of mesangial cell lysates reveals that the NO donor spermine-NO leads to a concentration-dependent down-regulation of ceramidase protein (chapter 3.2) [Franzen *et al.* 2002a]. This depletion is completely prevented by pretreatment of renal mesangial cells with lactacystin (Fig. 32A) in a concentration-dependent manner (Fig. 32B), thus confirming and extending our previous observations (Fig. 19, Fig. 26). To fully block the NO-mediated effect a concentration of 10µM of lactacystin is required. In parallel, neutral ceramidase activity measurements reveal that the NO-mediated reduction of activity is also reverted by lactacystine (Fig. 33). A similar effect is obtained with clasto-lactacystin β-lactone [Dick *et al.* 1997], which is the active metabolite of the natural product lactacystin (data not shown). Consistent with the observed increase of ceramidase protein and activity in lactacystin-treated mesangial cells, a decrease of NO-induced ceramide formation was found in the cells as analysed by mass spectrometry of extracted lipids (Fig. 34).

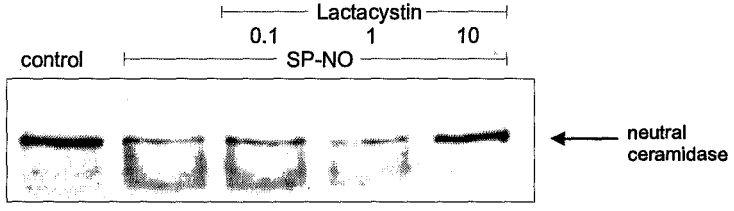
To further confirm the degradation of ceramidase by the ubiquitin/proteasome complex in response to nitric oxide, a potential ubiquitination of the enzyme in response to NO was investigated. For this purpose cells were pretreated with lactacystin in order to block

Figure 32

A



B

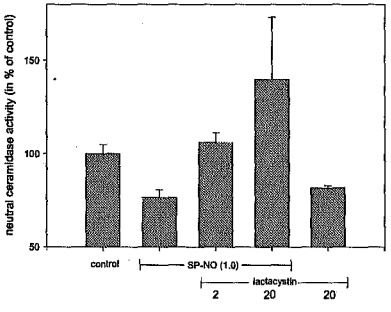


Effect of lactacystin on NO-reduced neutral ceramidase protein levels.

(A): Quiescent rat mesangial cells were stimulated for 24h with either vehicle (control) or spermine-NO (in mM) in the absence or presence of lactacystin (20μM; 2h pretreated).

(B): Quiescent cells were pretreated for 2 h with the indicated concentrations of lactacystin (in μM) before stimulation with spermine-NO (0.5mM) in the absence or presence of the indicated concentrations of lactacystin for 24 h. Thereafter, cell lysates containing 100μg of protein were subjected to SDS-PAGE (7% acrylamide gel) and transfered to nitrocellulose membrane. Western blot analysis was performed using an anti-neutral ceramidase antiserum at a dilution of 1:500. Data are representative of 2 independent experiments giving similar results

Figure 33



Effect of lactacystin on NO-reduced neutral ceramidase activity.

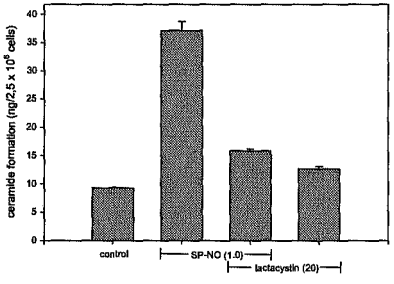
Quiescent rat mesangial cells were stimulated for 24h with either vehicle (control) or spermine-NO (SP-NO; 1.0mM) in the absence or presence of the indicated concentrations of lactacystin for 24h as indicated.

Cell lysates containing 100μg of protein were taken for a neutral ceramidase activity assay as described in chapter 2.6.1.1. The generated [^{14}C]sphingosine was separated on thin layer chromatography and evaluated on an Imaging System (Fuji). Results are expressed as % of control values and are means ±S.D. (n=4). Neutral ceramidase activity in control cells was 24.0 ± 1.5 pmol/mg/h.

degradation and thereafter stimulated for various time periods with spermine-NO. As shown in Fig. 35A, immunprecipitated ceramidase shows a time-dependent increase of ubiquitination when Western blot analysis was performed using an anti-ubiquitin antibody. Maximal ubiquitination occurs 30-120 minutes after treatment with spermine-NO. Similar data were obtained when cell extracts were subjected to immunoprecipitation with an anti-

ubiquitin antibody followed by Western blot detection using the anti-ceramidase antibody (Fig. 35B).

Figure 34



Effect of lactacystin on NO-induced ceramide formation in mesangial cells.
Quiescent cells were pretreated for 2 h with lactacystin (20 μM) before stimulation with spermine-NO (1.0mM) in the absence or presence of the indicated concentrations of lactacystin for 24 h. Thereafter, lipids were extracted and ceramide was analysed by LC/MS/MS spectrometry as described under 2.6.2. Results are means ± S.D. (n=4).

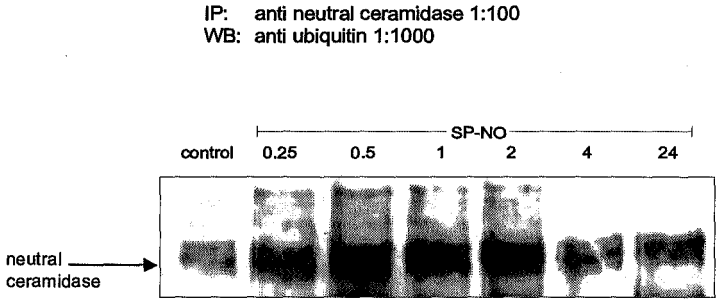
Discussion

The availability of the signalling molecule ceramide is fine tuned by the rate of generation involving sphingomyelinases and de-novo synthesis and the rate of degradation catalysed by ceramidases [Huwiler *et al.* 2000]. Whereas, sphingomyelinases have attracted interest due to their immediate regulation by an increasing list of stimuli like vitamin D_3, UV light, γ-irradiation, Fas ligand or doxorubicin, more recently, the neutral ceramidase has turned out to be a key enzyme in the regulation of intracellular free ceramide concentration particularly in response to the stress mediator NO and NO-inducing cytokines [Nikolova-Karakashian *et al.* 1997, Huwiler *et al.* 1999a, Huwiler *et al.* 1999b, Huwiler *et al.* 2001]. In terms of neutral ceramidase regulation, NO was found to inhibit neutral ceramidase activity in mesangial cells [Huwiler *et al.* 1999b]. This effect is due to down-regulation of the protein by increased degradation [Franzen *et al.* 2002a]. In this study, it is show that the degradation of neutral ceramidase by NO depends on the ubiquitin/proteasome pathway. Activation of the proteasome is a well known, fundamental mechanism for protein turnover, cell cycle control and signal transduction [Hershko & Ciechander 1998]. Conceptually, proteolysis by the ubiquitin/proteasome system is considered to comprise three steps: identification of the protein to be degraded, marking of that protein by attachment of ubiquitin to lysine residues, and delivering it to the proteasome, a multienzyme protease complex specific for multiubiquitinated substrates [Pickart 2001] that will degrade it and recycle ubiquitin. How NO is mechanistically activating the proteasome system is presently unknown. In principle, NO can mediate, especially under inflammatory conditions when also large amounts of reactive oxygen species are generated, protein nitration, nitrosation and oxidation. In addition, it can form nitrosyl complexes with metalloproteins [Pfeilschifter *et al.* 2001a].

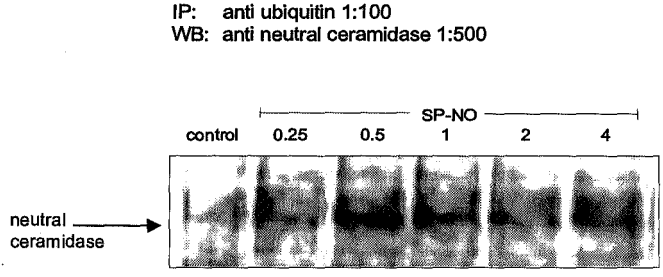
It could be speculated that one ubiquitinating enzyme or a component of the 26 S proteasome complex is modified at a critical site by NO. Alternatively, it is possible

Figure 35

A



B



NO induces ubiquitination of neutral ceramidase in mesangial cells.

Quiescent mesangial cells were pretreated for 2 h with 20μM lactacystin and then left untreated (control) or treated with 1mM spermine-NO (SP-NO) for the indicated time periods (in hours). Thereafter, cells were lysed and processed for immunoprecipitation by using either a polyclonal antibody against neutral ceramidase followed by Western blot analysis with an anti-ubiquitin antibody (A) or immunoprecipitated with the anti-ubiquitin antibody followed by Western blot analysis with neutral ceramidase antibody (B). Data are representative of 2 independent experiments giving similar results.

that NO stimulation leads to nitration or nitrosation of neutral ceramidase itself, which subsequently may facilitate faster degradation of the enzyme by the ubiquitine/proteasome system. In this context, it is interesting to note that Souza *et al.* [2000] reported an increased degradation of nitrated proteins by the proteasome and thus guarantees the removal of nitrated proteins in vivo. Furthermore, others have shown that oxidative stress leading to the generation of peroxynitrite, increases the degradation of various proteins, including aconitase by the proteasome pathway [Grune *et al.* 1998]. Peroxynitrite is thought to cause nitration of target proteins at tyrosine residues [Pfeilschifter *et al.* 2001b].

A proposed activation of the proteasome by NO, as suggested by our data from renal mesangial cells and other studies in K562 leukemia cells and pheochromocytoma PC 12 cells, is contrasted by the finding that in the mouse macrophage cell line RAW 264.7 NO inhibited the proteasome [Glockzin *et al.* 1999]. That study showed that lactacystin mimicked the effect of NO to induce accumulation of the tumor suppressor p53. Furthermore, NO was shown to trigger deubiquitination of p53.

However, it is worth noting that RAW 264.7 macrophages and renal mesangial cells respond differentially to NO stimulation most likely due to their different capacities to handle reactive nitrogen and oxygen species determined by differences in their glutathione redox system [Sandau *et al.* 1998, Pautz *et al.* 2002, Sumbayev *et al.* 2002]. Thus macrophages and endothelial cells are very sensitive to endogenous NO production and readily undergo apoptosis after stimulation with tumour necrosis factor α (TNF-α) [Manderscheid *et al.* 2001], whereas mesangial cells are completely resistant to TNFα stimulation, although endogenous NO production takes place. This mesangial cell protection correlates with high levels of reduced glutathione and high levels of inhibitor of apoptosis (IAP) protein levels [Manderscheid *et al.* 2001].

By sequence analysis of neutral ceramidase, several lysine residues as necessary targets for ubiquitin attachment can be found in the primary sequence [Mitsutake *et al.* 2001]. Further obvious sequence characteristics like a destruction box (D box) composed of the sequence R-X-X-L-X-X-X-X-N found in the N-terminus of proteins [Glotzner *et al.* 1991] or a KEN box composed of the sequence K-E-N-X-X-X-N/D located in the N or C terminus of the proteins [Pfleger & Kirschner 2000] which are targets for one special class of ubiquitin ligase, the anaphase promoting complex are missing. However, several other ubiquitin ligase complexes have been identified.

Further studies are required to characterise the involved ligase complex in more detail.

3.4 PKC-dependent translocation of neutral ceramidase to the nuclear membrane

Introduction

The green fluorescent protein (GFP) isolated from the Pacific jellyfish, Aequoria victoria [Morin & Hastings 1971] was been shown to transduce, by energy transfer, the blue chemiluminescence of another protein, aequorin, into green fluorescent light [Ward & Cormier 1979]. The molecular cloning of GFP cDNA [Prasher *et al.* 1992] and the demonstration by Chalfie *et al.* that GFP can be expressed as a functional transgene [Chalfie *et al.* 1994] have opened exciting new avenues of investigation in cell, developmental and molecular biology. GFP has been used as a fluorescent reporter molecule in the localisation of membrane receptors, cytoplasmic proteins, and secretory proteins. In the present study, we report on the development of a green fluorescent protein conjugated neutral ceramidase (GFP-NCER) to study neutral ceramidase translocation in response to stimulation with PKC activators like the phorbol ester TPA.

Results

Characterisation of neutral ceramidase–GFP (NCER-GFP) fusion protein

In order to investigate the cellular localisation of neutral ceramidase in living cells, a plasmid was constructed encoding GFP-tagged neutral ceramidase fusion protein. The cDNA construct of GFP-tagged neutral ceramidase was confirmed by sequencing. Transient transfection experiments with the human embryonic kidney cell line 293 (HEK 293) lead to overexpression of the fusion protein which was confirmed by Western blot analysis of cell extracts as seen in Fig. 35A. NCER–GFP was recognised as a double band at 140 kDa corresponding well to the calculated mass of the cDNA complex and to previous Western blot results of neutral ceramidase which also detected two bands (chapter 3.1). Furthermore, lysates containing overexpressed NCER–GFP protein displayed more than 10.000-fold increased ceramidase activity compared to vector control (Fig. 35B) indicating that GFP-tagged neutral ceramidase was a functionally active fusion protein.

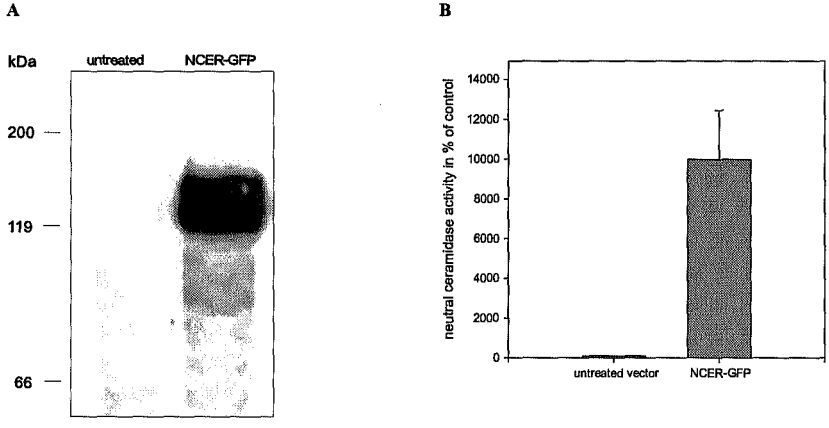
Localisation of neutral ceramidase-GFP

HEK 293 cells were transiently transfected for 24h with neutral ceramidase-GFP or GFP alone, respectively, and thereafter viewed by confocal microscopy. As seen in Fig. 36, unstimulated cells showed a staining of the cytoplasm with a characteristic accumulation in a perinuclear region. HEK 293 cells expressing only the GFP protein show fluorescence throughout the cell including the nucleus (data not shown). To identify the perinuclear region in which NCER-GFP accumulated, colocalisation studies were performed using MitoTracker®

Orange CMTMRos as useful tool to stain mitochondria and an antibody directed against GM130 as a specific Golgi complex marker.

Overlapping images demonstrate that NCER-GFP was not localised in mitochondria but the perinuclear region was stained by NCER-GFP as well as by the GM130 antibody indicating a possible partial localisation of neutral ceramidase in the Golgi complex (Fig. 37A and 37B).

Figure 35



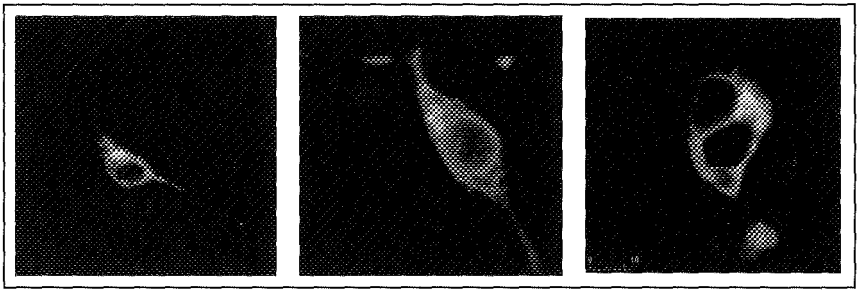
Expression (A) and activity (B) of neutral ceramidase-GFP fusion protein in HEK293 cells.

HEK 293 cells were transfected with pEGFP-N1 vector containing neutral ceramidase-GFP or with empty vector as described in chapter 2.2.2. Thereafter, cell lysates containing 100µg of protein were taken for SDS-PAGE (7% acrylamide gel) and subsequent Western blot analysis using a specific anti-GFP antibody at a dilution of 1:1000 (A) or a neutral ceramidase activity assay (B) as described in chapters 2.5.6. and 2.6.1.1. The generated [^{14}C]sphingosine was separated on thin layer chromatography and evaluated on an Imaging System (Fuji). Data in A are representative of al least 2 independent experiments giving similar results. Results in B are expressed as % of control values and are means ±S.D. (n=3-4).

PKC dependent translocation of neutral ceramidase-GFP

In a next step, transient transfected HEK 293 cells by NCER-GFP were stimulated with the PKC activator TPA followed by confocal microscopy (5 minute intervals). Upon TPA stimulation, GFP-tagged neutral ceramidase translocated to the nuclear membrane. This was first detectable after 10 minutes by using 25nM TPA (data not shown) and reached a maximum after 45 minutes (Fig. 38).

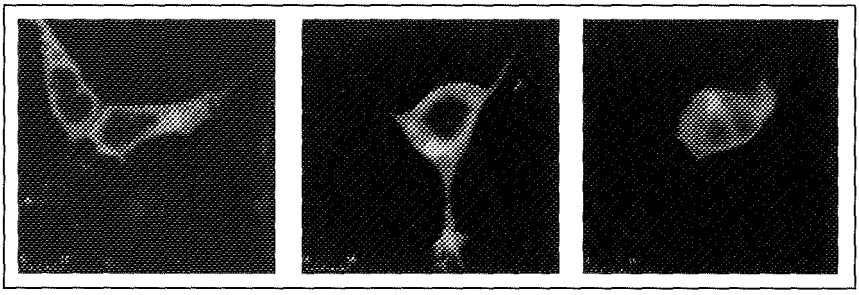
Figure 36



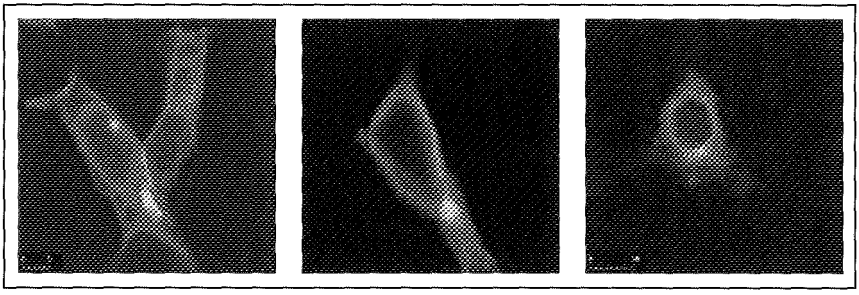
Localisation of neutral ceramidase-GFP fusion protein in HEK 293 cells.
HEK 293 cells were transfected with pEGFP-N1 vector containing neutral ceramidase-GFP or with empty vector as described in chapter 2.2.2. Thereafter, cells were viewed by confocal microscopy as described under 2.7.

Figure 38

control



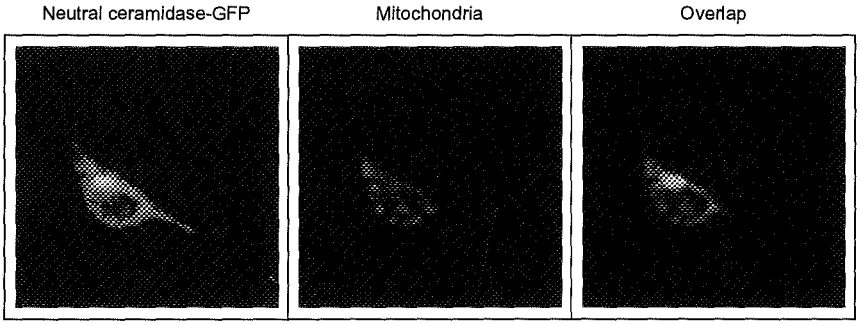
TPA 25nM, 45min



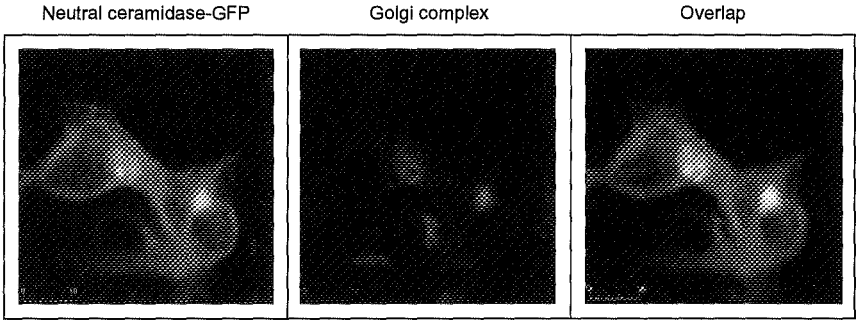
Translocation of neutral ceramidase-GFP fusion protein by stimulation of PKC in HEK 293 cells.
HEK 293 cells were transfected with pEGFP-N1 vector containing neutral ceramidase-GFP as described in chapter 2.2.2. Thereafter, cells were stimulated with vehicle (control) or 25nM TPA and viewed by confocal microscopy every 5 minutes up to one hour stimulation as described under 2.7.

Figure 37

A



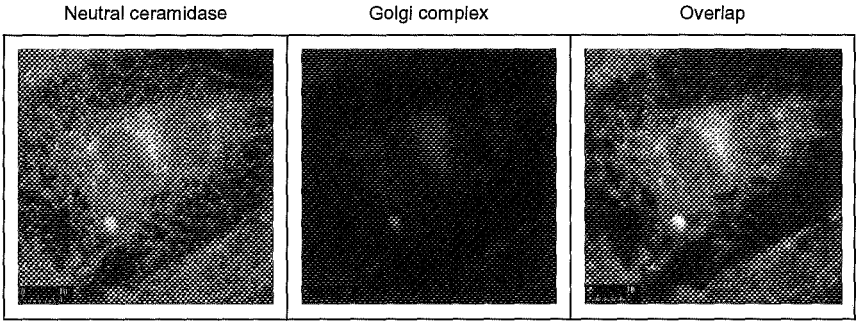
B



Localisation of neutral ceramidase-GFP fusion protein in HEK 293 cells.

HEK 293 cells were transfected with pEGFP-N1 vector containing neutral ceramidase-GFP as described in chapter 2.2.2. Thereafter, cells were treated either with MitoTracker® Orange CMTMRos (250nM) or fixed (2.7) and afterwards incubated with GM130 antibody. Finally, living and fixed cells were viewed by confocal microscopy as described under 2.7.

C



- Localisation of neutral ceramidase in rat mesangial cells.

Mesangial cells cells fixed and incubated with a mix of neutral ceramidase antibody (2.5.7) and GM130 antibody as described in chapter 2.7. Fixed cells were viewed by confocal microscopy as described under 2.7.

Discussion

In this part of the thesis the cellular localisation of neutral ceramidase was investigated using green fluorescent protein (GFP) as fusion protein which is a well established model to examine cellular distribution and translocation of proteins.

Unstimulated HEK 293 cells reveal after transient transfection experiments that neutral ceramidase is preferentially localised in the cytoplasm. A similar cytoplasmic localisation is also seen in rat mesangial cells. PKC activation leads to a striking accumulation of neutral ceramidase at the nuclear membrane.

A recent study showed that neutral as well as acid ceramidase activities are actively secreted by murine endothelial cells, RAW 264.7 murine macrophages and IMR-90 human fibroblasts. However, no further biochemical identification of the secreted enzymes was provided [Romiti *et al.* 2000a]. The exact distribution of neutral ceramidase remained unclear. Furthermore, Romiti *et al.* [2000a] reported that the release of ceramidases is inhibited by brefeldin A, a known inhibitor of secretion from the Golgi network [Orci *et al.* 1991, Rosa *et al.* 1992]. The release of neutral ceramidase was further potentiated by bradykinin and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) strongly indicating the implication of PKC activation in secretion of neutral ceramidase. The present study, however, did not show neutral ceramidase translocation to the plasma membrane but rather to the nuclear membrane in HEK 293 cells after PKC activation (Fig. 38) pointing towards a cell type specific regulation of neutral ceramidase trafficking. It remains to be shown whether neutral ceramidase is also secreted by TPA treatment of HEK 293 and rat mesangial cells.

Interestingly, acid sphingomyelinase was also shown to be secreted, either constitutively [Hurwitz *et al.* 1994, Schissel *et al.* 1998] or after platelet activation [Romiti *et al.* 2000b] or cytokine exposure [Marathe *et al.* 1998] suggesting agonist-regulated mechanisms. The concomitant release of sphingomyelinases and ceramidases combined with recent findings of PKC-dependent activation, translocation and secretion of sphingosine kinase [Johnson *et al.* 2002] suggest that, besides sphingolipid action as second messenger in signalling cascades, additional important roles as autocrine/paracrine signalling molecules. This is further corroborated by the existence of sphingosine-1-phosphate receptors on the surface of endothelial cells and their potential to evoke biological activity like differentiation, migration and mitogenesis [Hla 2001, Mandala *et al.* 2002].

Nevertheless, the findings of this study support also the important role of sphingolipids as signalling molecules inside the cell. In view of the reported lack of increase in neutral ceramidase activity upon TPA stimulation [Franzen *et al.* 2002a], it is tempting to speculate that phosphorylation of neutral ceramidase and subsequent translocation to the nuclear membrane protects the enzyme from degradation triggered by nitric oxide [Franzen *et al.* 2002a]. Several studies reported on nuclear localisation of a neutral sphingomyelinase

[Alessenko & Chatterjee 1995, Jaffrézou *et al.* 2001, Mizutani *et al.* 2001] thus giving hints for an important role of the nuclear region in sphingolipid-mediated signalling and thereby reinforcing the putative function of neutral ceramidase translocation to the nuclear membrane as shown in this chapter.

In summary, this part has shown that neutral ceramidase is localised in the cytoplasm of unstimulated HEK 293 and rat mesangial cells. Upon TPA stimulation a translocation to the nuclear membrane is found which may point to an additional functional role of neutral ceramidase at the cell nucleus.

4 SUMMARIZING DISCUSSION

4.1 Regulation of ceramidases

Among the different pathways regulating ceramide formation this thesis focused on ceramide degrading enzymes: the ceramidases. This class of enzymes regulated the hydrolysis of ceramide to sphingosine which is a substrate for the sphingosine kinase yielding sphingosine-1-phosphate, a potent mitogen for various cells.

In a first part of the study the effect of the pro-inflammatory cytokine IL-1β was investigated. IL-1β leads to activation of neutral ceramidase activity which is due to transcriptional and translational upregulation [Franzen *et al.* 2001]. Mechanistically this upregulation of neutral ceramidase by IL-1β involves the stress-activated protein kinase p38-MAPK, since a specific inhibitor of this kinase efficiently blocked the protein upregulation as well as activity. Consistently, increased intracellular ceramide levels are measured upon IL-1β stimulation in the presence of SB 202190 which leads to an increased rate of apoptosis. Among the different downstream targets of p38 MAPK, MAPKAP-K-2 could be excluded as a mediator of the described neutral ceramidase activation by using mesangial cells from MAPKAP-K-2 knockout mice [Kotlyarov *et al.* 1999].

Furthermore, the results clearly show that the stimulation with IL-1β delivered a maximal activation of the neutral ceramidase after 4 to 5 hours of stimulation which thereafter declines again (Fig. 6A). This delayed regulation is due to the expression of the inducible nitric oxide synthase (iNOS) by IL-1β followed by the production of high concentrations of nitric oxide which in turn trigger proteolytic degradation of neutral ceramidase as shown by co-stimulation with the nitric oxide synthase (NOS)-inhibitor L-NMMA which prevents the decline of neutral ceramidase activity (Fig. 6C).

Molecular cloning and sequence analysis has revealed the existence of three distinct NOS isotypes that are either constitutively expressed in endothelial cells (eNOS) and neurones (nNOS) or are inducible (iNOS) by endotoxin and by inflammatory cytokines such as IL-1 and TNFα in macrophages and many other cell types [Moncada & Palmer 1991; Nathan & Xie 1994]. For example, IL-1β is a strong stimulus for iNOS transcription in rat mesangial cells [Pfeilschifter & Schwarzenbach 1990, Kunz *et al.* 1994]. iNOS requires a delay of 6–8 h before the onset of NO production but, once induced, this enzyme is active for hours to days and produces NO in 1000-fold larger quantities than the constitutive enzymes eNOS and nNOS. At low concentrations, NO stimulates guanylate cyclase activity and triggers the formation of cyclic GMP (cGMP), an important messenger mediating physiological functions of NO such as vascular homeostasis. Higher concentrations of NO

produced by iNOS interact with thiol groups or transition metal-containing proteins and can alter protein function or initiate gene expression to protect cells. There is a continuous shift at even higher concentrations of NO towards cell damage or apoptosis, with other factors in the microenvironment of a cell critically influencing the final outcome [Brüne *et al.* 1998].

This activation of iNOS by IL-1β in rat mesangial cells is well compatible with the second part of this thesis dealing with the down-regulation of neutral ceramidase by nitric oxide [Franzen *et al.* 2002a]. Exposure to high concentrations of nitric oxide leads to a significant decrease of neutral ceramidase activity and protein in mesangial cells [Huwiler *et al.* 1999b, Franzen *et al.* 2002a] which could, at least partially, explain the observed counter-regulation after long-term stimulation with IL-1β.

NO was found to increase ceramide levels in the cell by a dual mechanism: on the one hand it stimulates the ceramide producing enzymes acid and neutral sphingomyelinases, thus leading to an increased ceramide formation, and on the other hand NO inhibits the ceramide degrading enzymes, the acidic and neutral ceramidases, and thereby results in an amplified increase in ceramide steady-state levels [Huwiler *et al.* 1999b]. By contrast, pro-inflammatory cytokines like TNFα and IL-1β, besides activating the sphingomyelinases cause a potent activation of the ceramidases, which fully compensates for the increased generation of ceramide [Huwiler *et al.* 1999b, Franzen *et al.* 2001]. This may also explain why mesangial cells undergo apoptosis when NO is delivered by exogenous sources but are resistant to endogenously produced NO after cytokine-induced inducible NOS expression [Mühl *et al.* 1996, Pfeilschifter & Huwiler 1996, Nitsch *et al.* 1997, Franzen *et al.* 2001]. The NO-mediated inhibition of ceramide-degrading enzymes was further investigated and found to be due to degradation of neutral ceramidase protein [Franzen *et al.* 2002a]. Mechanistically, this degradation is mediated by the ubiquitin/proteasome complex in renal mesangial cells as shown in the third part of this studies [Franzen *et al.* 2002b].

The NO-induced degradation of neutral ceramidase can be reversed by PKC activating agents, like the phorbol ester TPA, but also the physiologically important ligands PDGF-BB, angiotensin II and ATP. As found in in vitro and in vivo phosphorylation experiments PKC-δ is able to phosphorylate neutral ceramidase and appears to be the prime candidate for exerting the anti-apoptotic action in mesangial cells by stabilising neutral ceramidase. It is well established that activation of protein kinase C, either directly or indirectly, could regulate the level of proteins by posttranslational mechanisms. One possible way of action may be phosphorylation of components of the ubiquitin/proteasome machinery. In the case of growth arrest and DNA-damage-inducible gene (Gadd45) the deubiquitination is PKC-δ dependent [Leung *et al.* 2001] which offers an obvious link to the degradation of

neutral ceramidase. Therefore it is tempting to speculate on two different mechanisms by which PKC activation could be connected with neutral ceramidase stability and subsequent improved elimination of ceramide which in turn may protect the cell from apoptosis: (i) direct phosphorylation of neutral ceramidase leading to changes in conformation and enzymatic accessibility, its cellular transport and subsequent localisation, and (ii) phosphorylation of enzymes of the ubiquitin/proteasome pathway causing altered ubiquitination either by inhibition of ubiquitin-conjugating enzymes or by activation of deubiquitinating enzymes.

Furthermore, cytokine-induced activation of neutral SMase was inhibited by stimulation of PKC by TPA with subsequently reduced formation of ceramide [Kaszkin *et al.* 1998], indicating that PKC acts at least at two different levels to prevent ceramide accumulation and subsequent apoptosis, i.e. at the level of sphingomyelinase, and at the level of ceramidase.

In the last part of the thesis the localisation of neutral ceramidase was investigated using green fluorescent protein as tool to visualise proteins of interest inside the cell. Referring to transfection experiments with GFP-tagged neutral ceramidase and subsequent staining of cellular compartments neutral ceramidase seemed to be preferentially located in the cytoplasm of unstimulated cells. This distribution changed upon PKC activation by TPA leading to a striking accumulation of neutral ceramidase at the nuclear membrane.

4.2 Clinical relevance

With regard to the known properties of the different components of the sphingolipid network in tissue physiology and pathophysiology, several strategies to get useful therapeutic approaches for a variety of diseases have been developed. Elevation of cellular ceramide is being used for therapies aiming to arrest cell growth or to promote apoptosis. Conversely, agents that reduce ceramide or raise sphingosine-1-phosphate tend to attenuate apoptosis and support proliferation.

It was shown that ceramide-coated balloon catheters significantly reduced neointimal hyperplasia induced by balloon angioplasty in rabbit carotid arteries in vivo. This ceramide treatment decreased the number of vascular smooth muscle cells entering the cell cycle without inducing apoptosis [Charles *et al.* 2000] pointing out the benefit of cell-permeable ceramide as novel therapy for reducing neointimal hyperplasia after balloon angioplasty.

Secondly, exogenously applied ceramide augmented paclitaxel-induced apoptosis in human Tu138 head and neck squamous carcinoma cell line in vitro when added in combination with paclitaxel [Mehta *et al.* 2000] indicating effectiveness of ceramide in cancer therapy. This was corroborated by the study of Selzner *et al.* [2001] demonstrating that human colon cancer showed a more than 50% decrease in the cellular content of

ceramide when compared with healthy colon mucosa. Application of ceramide analogues and ceramidase inhibitors induced rapid cell death through activation of various proapoptotic molecules, such as caspases and release of cytochrome c. Ceramidase inhibition increases the ceramide content of tumour cells, resulting in maximum activation of the apoptotic cascade. Importantly, normal cells were completely resistant to inhibitors of ceramidases. Treatment of nude mice with B13, the most potent ceramidase inhibitor, completely prevented tumour growth using two different aggressive human colon cancer cell lines. Therefore, B13 and related analogues of ceramide and inhibitors of ceramidases offer a promissing therapeutic strategy with selective toxicity towards malignant but not normal cells [Selzner *et al.* 2001]. Moreover, safingol, a sphingosine kinase inhibitor that prevents formation of sphingosine-1-phosphate from degraded ceramide, combined with fenretinide, which was reported to increase ceramide generation via the de novo pathway [Maurer *et al.* 2000, Wang *et al.* 2001], synergistically enhanced cytotoxicity in various tumour cell lines [Maurer *et al.* 2000]. Hence, safingol was already used in a phase I clinical trial without dose-limiting toxicity [Schwartz *et al.* 1997].

On the other hand, blocking ceramide formation or manipulation of sphingosine-1-phosphate levels, respectively, may also be of therapeutic utility and protect from side effects of cancer treatment. For example, genetic inactivation of acid sphingomyelinase (A-Smase) prevented microvasculature endothelial cells of the small intestine from apoptotic damage [Paris *et al.* 2001]. Furthermore, it was shown that female A-Smase knock out mice suppressed the normal apoptotic deletion of fetal oocytes, leading to neonatal ovarian hyperplasia [Morita *et al.* 2000]. Ex vivo, oocytes lacking the gene for acid sphingomyelinase or wild-type oocytes treated with sphingosine-1-phosphate resisted physiological apoptosis and apoptosis induced by anti-cancer therapy. Moreover, radiation-induced oocyte loss in adult wild-type female mice, the event that drives premature ovarian failure and infertility in female cancer patients, was completely prevented by in vivo therapy with sphingosine-1-phosphate [Morita *et al.* 2001, Spiegel & Kolesnick 2002]. Thus, the sphingomyelin pathway and especially sphingosine-1-phosphate represent a new target to preserve ovarian function upon cancer therapy.

Additionally, sphingosine-1-phosphate and structural analogues like FTY720 revealed immunosuppressive qualities through altered lymphocyte trafficking mediated by activation of sphingosine-1-phosphate receptors [Brinkmann *et al.* 2001, Mandala et al. 2002]. Both species were high-affinity agonists of at least four of the five S1P receptors. These agonists produce lymphopenia in blood and thoracic duct lymph by sequestration of lymphocytes in lymph nodes. S1P receptor agonists induced emptying of lymphoid sinuses by retention of lymphocytes and inhibition of outlet into lymph. Thus, inhibition of lymphocyte recirculation by activation of S1P receptors may result in therapeutically useful

immunosuppression [Mandala *et al.* 2002] which has already been applied successfully for FTY720 in clinical trials in renal transplant patients [Brinkmann et al. 2001].

Taken together, the sphingolipid network provides several compounds and enzymes to be targeted in a pharmacological reasonable way. Especially the ceramidases, as "power button" in the regulation of ceramide and sphingosine-1-phosphate being key opponents in the balance of cell death and survival, provide the opportunity to interfere in pathophysiology in general, particularly with regard to cancer therapy.

Concerning the results of this thesis, it could be demonstrated that several ways of interference with the regulation of ceramidases do exist. Physiological or pathophysiological stimuli like the pro-inflammatory cytokine IL-1β and nitric oxide were shown to alter ceramidase activity in mesangial cells. IL-1β led to an increase of ceramidase activity [Franzen *et al.* 2001] whereas nitric oxide was able to decrease ceramidase activity through the ubiquitin/proteasome pathway [Franzen *et al.* 2002a, 2002b]. Both events subsequently impact on cellular biology.

In summary, the neutral ceramidase represents an attractive target enzyme for novel therapeutic approaches in the treatment of inflammatory diseases and tumour growth.

5 SUMMARY

During the past several years, ceramide has emerged as an important second messenger triggering cell responses including proliferation, differentiation, growth arrest and apoptosis. This thesis has focused on the regulation of neutral ceramidase which critically determines, in concert with ceramide generating sphingomyelinases, the intracellular ceramide levels.

In the first part it is reported that besides a rapid and transient increase in neutral sphingomyelinase activity a second delayed peak of activation occurs after hours of IL-1β treatment. This second phase of activation is first detectable after 2 h of treatment, and steadily increases over the next two hours reaching maximal values after 4 h. In parallel, a pronounced increase in neutral ceramidase activity is observed, which accounts for a constant or even decreased level of ceramide after long-term IL-1β treatment, despite continuous sphingomyelinase activation.

The increase in neutral ceramidase activity is due to expressional up-regulation, as detected by an increase in mRNA level and enhanced *de novo* protein synthesis. The increase of neutral ceramidase protein levels and activity can be blocked dose-dependently by the p38- mitogen-activated protein kinase (p38-MAPK) inhibitor, SB 202190, whereas the classical MAPK pathway inhibitor U0126, and the PKC inhibitor Ro 31-8220 were ineffective. Moreover, co-treatment of cells for 24 h with IL-1β and SB 202190 leads to an increase in ceramide formation. Interestingly, IL-1β-stimulated neutral ceramidase activation is not reduced in mesangial cells isolated from mice deficient in MAPK-activated protein kinase 2 (MAPKAPK-2), which is one possible downstream substrate of the p38-MAPK, thus suggesting that the p38-MAPK-mediated induction of neutral ceramidase occurs independently of MAPKAPK-2.

The results suggest a biphasic regulation of sphingomyelin hydrolysis in cytokine-treated mesangial cells with a delayed de novo synthesis of neutral ceramidase counteracting sphingomyelinase activity and apoptosis. Neutral ceramidase may thus represent a novel cytoprotective enzyme for mesangial cells exposed to inflammatory stress conditions.

In a second part, the effect of NO on neutral ceramidase was studied. Ceramide levels are strongly increased in a delayed fashion by stimulation of renal mesangial cells with NO. This effect is due to a dual action of NO, comprising an activation of sphingomyelinases and an inhibition of ceramidase activity. The inhibition of neutral ceramidase activity correlates with the decrease of neutral ceramidase protein. A complete loss of neutral ceramidase protein is obtained after 24h of NO stimulation. Moreover, the NO-induced degradation is reversed by the protein kinase C (PKC) activator, 12-O-

tetradecanoylphorbol-13-acetate (TPA), but also by the physiological PKC activators platelet-derived growth factor-BB (PDGF-BB), angiotensin II and ATP, resulting in a normalisation of neutral ceramidase protein as well as activity.

In vivo phosphorylation studies using $^{32}P_i$-labelled mesangial cells, reveal that TPA, PDGF-BB, angiotensin II and ATP trigger an increased phosphorylation of the neutral ceramidase, which is blocked by the broad-spectrum PKC inhibitor Ro-31 8220, but not by CGP 41251, which has a preferential action on Ca^{2+}-dependent PKC isoforms, thus suggesting the involvement of a Ca^{2+}-independent PKC isoenzyme. In vitro phosphorylation assays using recombinant PKC isoenzymes and neutral ceramidase immunoprecipitated from unstimulated mesangial cells, show that particularly the PKC-δ isoform, and to a lesser extent the PKC-α isoform, are efficient in directly phosphorylating neutral ceramidase.

The data show that NO is able to induce degradation of neutral ceramidase thereby promoting accumulation of ceramide in the cell. This effect is reversed by PKC activation, most probably by the PKC-δ isoenzyme, which may directly phosphorylate and thereby, prevent neutral ceramidase degradation.

In the third chapter it is demonstrated that the NO-triggered degradation of neutral ceramidase involves activation of the ubiquitin/proteasome complex. The specific proteasome inhibitor, lactacystin, completely reverses the NO-induced degradation of ceramidase protein and neutral ceramidase activity. As a consequence, the cellular amount of ceramide, which drastically increases by NO stimulation, is reduced in the presence of lactacystin. Furthermore, ubiquitinated neutral ceramidase accumulates after NO stimulation. The data clearly show that the ubiquitin/proteasome complex is an important determinant of neutral ceramidase activity and thereby regulates the availability of ceramide.

In a last part, the cellular localisation of neutral ceramidase was investigated using green fluorescent protein (GFP) as fusion protein to examine cellular distribution and translocation of neutral ceramidase. Unstimulated HEK 293 cells reveal after transient transfection experiments that neutral ceramidase is preferentially localized in the cytoplasm. PKC activation led to an accumulation of neutral ceramidase at the nuclear membrane.

In summary, this work demonstrates that the neutral ceramidase is a fine regulated protein that plays a critical role in regulating intracellular ceramide levels and thereby the cell's fate to undergo apoptosis or survive. Regulation of neutral ceramidase can be achieved on all levels, i.e. on the mRNA level, the protein level or posttranslationally by phosphorylation and subcellular translocation.

Future work will reveal whether neutral ceramidase can serve as a therapeutic target in the development of novel antiinflammatory and anti-tumour drugs.

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7 SUPPLEMENT

7.1 Abbreviations

AC	acid ceramidase
AP-1	activator protein 1
A-SMase	acid sphingomyelinase
AT II	Angiotensin II
ATF2	activator of transcription 2
ATP	adenosine triphosphate
BSA	bovine serume albumin
CAPP	ceramide-activated protein phosphatase
cdk	cyclin dependent kinase
cGMP	cyclic guanosine monophosphate
DG	diacylglycerol
DMEM	Dulbecco's Modified Eagles Media
ECL	enhanced chemiluminescence
eNOS	endothelial nitric oxide synthase
ER	endoplasmatic reticulum
ERK	extracellular signal regulated kinase
FAN	factor associated with neutral sphingomyelinase
FCS	fetal calf serume
FD	farber disease
GADD	growth arrest and DNA-damage
GFP	green fluorescent protein
HEK	human embryonic kidney
IFN γ	interferon γ
IL 1β	interleukin 1β
iNOS	inducible nitric oxide synthase
IP_3	inositol-1,4,5-trisphosphate
IκB	inhibitor of κB
JNK	c-Jun-N-terminal kinase
LB	Lauria-Bertani
LDL	low density lipoprotein
MAPK	mitogen activated protein kinase
MAPKAPK	mitogen activated protein kinase activated protein kinase
MBP	myelin basic protein
MEF2C	mouse embryonic factor 2C
MRM	mutiple reaction monitoring
MS	mass spectrometry
MSK	mitogen- and stress-activated kinase
NCER	neutral ceramidase
NF	nuclear factor
nNOS	neuronal nitric oxide synthase

NO	nitric oxide
NSD	neutral sphingomyelinase activation domain
N-SMase	neutral sphingomyelinase
OD	optical density
PA	phosphatidic acid
PAGE	polyacrylamid gel electrophoresis
PBS	phosphate buffered saline
PC	phosphocholine
PDGF	platelet-derived growth factor
PI	phosphoinositide
PI3K	phosphoinositide-3-kinase
PIP_2	phosphatidyl-4,5-biphosphate
PKB	protein kinase B
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
Rb	retinoblastoma
RPMI	Roswell Park Memorial Institute
S1P	sphingosine-1-phosphate
SAP	sphingolipid activator protein
SAPK	stress activated protein kinase
SM	sphingomyelin
TLC	thin layer chromatography
TNFα	tumour necrosis factor α
TPA	12-O-tetradecanoylphorbol-13-acetate

7.2 Publications

7.2.1 Journal publications

Manderscheid M, Messmer UK, **Franzen R**, and Pfeilschifter J:
Regulation of inhibitor of apoptosis expression by nitric oxide and cytokines: relation to apoptosis induction in rat mesangial cells and raw 264.7 macrophages.
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J Biol Chem 277:33518-28 (2002)

Franzen R, Fabbro D, Aschrafi A, Pfeilschifter J, and Huwiler A:
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J Biol Chem 277:46184-90 (2002)

Franzen R, Pfeilschifter J, and Huwiler A:
Nitric oxide induces neutral ceramidase degradation by the ubiquitin/proteasome complex in renal mesangial cell cultures.
FEBS Lett 532:441-444 (2002)

7.2.2 Poster presentations

Franzen R, Pautz A, Pfeilschifter J, and Huwiler A
Interleukin-1β induces chronic activation of neutral sphingomyelinase and neutral ceramidase in renal mesangial cells.
42. Spring meeting of DGPT, Mainz, 2001, N-S Arch Pharmacol 363: 39 Suppl.

Franzen R, Aschrafi A, Pautz A, Bräutigam L, Geislinger G, Pfeilschifter J, and Huwiler A
Interleukin-1β induces chronic activation and *de novo* synthesis of neutral ceramidase in renal mesangial cells.
Signal transduction meeting, Luxembourg, 2002, abstract booklet

Franzen R, Pfeilschifter J, and Huwiler A
Interleukin-1β-induced up-regulation of neutral ceramidase involves the p38 mitogen-activated protein kinase pathway in mesangial cells.
43. Spring meeting of DGPT, Mainz, 2002, N-S Arch Pharmacol 365: 27 Suppl.

Franzen R, Aschrafi A, Pfeilschifter J, and Huwiler A
Nitric oxide induces degradation of the neutral ceramidase in rat renal mesangial cells and is counterregulated by protein kinase C.
8. NO-Forum of German Speaking Countries, Frankfurt, 2002, abstract booklet

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7.4 Deutsche Zusammenfassung

7.4.1 Einführung

Dem deutschen Arzt Tudichum gelang, in der zweiten Hälfte des 19. Jahrhunderts bei fraktionierenden Kristallisationen von alkoholischen Hirnextrakten Verbindungen zu isolieren, die neben Zucker und Fettsäuren eine organische Base enthielten. Da diese Base ihm rätselhaft erschien, gab er dieser Substanz in Anlehnung an die griechische Sage von der Sphinx den Namen Sphingosin.

Nach dieser Entdeckung haben Sphingolipide einen großen Aufschwung erlebt, da sich herausstellte, dass Sphingolipide neben ihren Funktionen als Membranbausteine wichtige Eigenschaften als Signalmoleküle innerhalb der Zelle offenbaren. Ausgangspunkt für diese Entwicklung war die Beobachtung, dass die Proteinkinase C (PKC), ein Signalmolekül, welches durch den Lipid-Botenstoff Diacylglycerin (DAG) aktiviert wird, durch Sphingosin gehemmt wird.

Ceramid, eine zentrale Verbindung innerhalb der Klasse der Sphingolipide, wird durch verschiedene Sphingomyelinasen freigesetzt und zusätzlich auch im Zuge einer de novo Synthese von Sphingolipiden, bzw. beim lysosomalen Abbau glykosilierter Sphingolipide produziert. Da Sphingomyelin jedoch in großen Mengen in der Plasmamembran vorkommt, und nur ein Schritt zur Bildung von Ceramid nötig ist, geht man davon aus, dass vor allem die Sphingomyelinasen für eine schnelle Ceramid-Freisetzung im Rahmen von Signaltransduktions-Prozessen von Bedeutung sind. Die Signalmoleküle Sphingosin und Sphingosin-1-Phosphat (S1P) werden aus Ceramid durch Ceramidasen gebildet und scheinen im Gegensatz zu Ceramid, das vorwiegend zum Stop des Zell-Zyklus und zur Apoptose führt, vor allem an den Prozessen der Zell-Aktivierung wie Mitogenese und Proliferation beteiligt zu sein.

Ceramid als zentrales Signalmolekül wurde umfangreich charakterisiert. Nach Stimulation von Zellen mit entzündlichen Zytokinen wie Interleukin 1β (IL-1β), Tumornekrosefaktor α (TNFα) und Interferon γ, oder Stressfaktoren wie UV-Strahlung, Röntgenstrahlung, Hitzeschock oder oxidativem Stress kommt es zur Aktivierung von sauren und neutralen Sphingomyelinasen, die Sphingomyelin in Zellmembranen zu Ceramid spalten. Die Regulation von Sphingomyelinasen liefern aber nur einen Teilaspekt, die Bildung von Ceramid, für das molekulare Verständnis des Ceramid-Stoffwechsels in der Zelle. Einen ebenso wichtigen Beitrag liefern die den Abbau regulierenden Enzyme, die Ceramidasen.

Ceramidasen sind Enzyme, welche in der Lage sind, Ceramide in Sphingosin und freie Fettsäure zu spalten. Sphingosin kann dann in der Folge zu Sphingosin-1-phosphat phosphoryliert werden. Sphingosin-1-phosphat ist als ein Mitogen und damit Förderer von Wachstum in verschiedenen Zelltypen beschrieben.

Sphingosin entsteht nicht über de novo Synthese, so dass die Ceramidase-Aktivität nicht nur für den Abbau von Ceramid wichtig ist, sondern auch für das Entstehen relevanter Sphingosin- und Sphingosin-1-phosphat-Spiegel verantwortlich ist. Entsprechend der assoziierten Zellantworten auf Ceramide bzw. Sphingosin-1-phosphat stellen die Ceramidasen Schlüsselenzyme in der zellulären Regulation der Balance zwischen programmiertem Zelltod und Überleben dar.

Ceramidasen werden abhängig von ihrem pH-Optimum in drei Klassen unterteilt: man unterscheidet saure, neutrale und alkalische Ceramidasen. Diese Einteilung basiert jedoch nicht ausschließlich auf dem entsprechenden pH-Optimum, sondern gründet sich zusätzlich auf Unterschiede in der genetischen Information.

Bis heute ist über die Regulation und die Beteiligung der Ceramidasen an der zellulären Signalübertragung nur sehr wenig bekannt.

Ziel dieser Arbeit ist es, diese Rolle der Ceramidasen und im Besonderen der neutralen Ceramidase in der zellulären Signalübertragung zu erforschen, vornehmlich die Rolle bei zellulärem Stress und Entzündungen. Weiterhin gilt es, die Effekte auf die Elimination von Ceramid und die Produktion von Spingosin-1-phosphat zu erforschen, um damit die Konsequenzen für das Gleichgewicht zwischen programmiertem Zelltod auf der einen Seite und Wachstum und Überleben auf der anderen Seite aufzudecken.

7.4.2 Ergebnisse

Der Lipid-Botenstoff Ceramid wird gebildet durch saure und neutrale Sphingomyelinasen und abgebaut durch saure, neutrale und alkalische Ceramidasen. Kurzzeit-Stimulationen von Mesangiumzellen mit dem pro-entzündlichen Zytokin Interleukin 1β (IL-1β) führen zu einem schnellen und vorübergehenden Anstieg der neutralen Sphingomyelinase-Aktivität. Im ersten Teil dieser Arbeit wird über einen zweiten verzögerten Anstieg der Sphingomyelinase-Aktivität innerhalb von Stunden nach Gabe von IL-1β berichtet. Parallel dazu wird ein Anstieg der Ceramidase-Aktivitäten beobachtet, was dazu führt, dass die Ceramid-Spiegel innerhalb der Zelle nach Langzeit-Stimulation mit IL-1β relativ konstant bleiben.

Der Anstieg der neutralen Ceramidase-Aktivität erfolgt aufgrund einer expressionalen Hochregulation, welche sich sowohl durch erhöhte entsprechende Ribonukleinsäure-Spiegel als auch durch eine verstärkte Neusynthese des Proteins zeigen. Der beobachtete Anstieg von neutraler Ceramidase kann unter Costimulation mit dem p38-Mitogen-aktivierten-Protein-Kinase (MAPK)-Inhibitor SB 202190 dosisabhängig blockiert werden. Dazu passend führt diese Costimulation zu einem Anstieg der Ceramid-Formation. Mesangiumzellen, die von Mäusen isoliert wurden, denen das Gen für die MAPK-aktivierte-Protein-Kinase-2 fehlt, reagieren vergleichbar auf die beschriebenen

Stimuli, was darauf schließen lässt, das die MAPK-aktivierte-Protein-Kinase-2 als mögliches Target der p38-MAPK nicht in der Aktivierung der neutralen Ceramidase durch IL-1β involviert ist.

Daher sprechen die Resultate für eine biphasische Regulation der Sphingomyelin-Spaltung und eine verzögerte Ceramidase-Aktivierung nach einer Stimulation mit IL-1β.

Ceramid-Spiegel werden stark erhöht durch eine Stimulation von Mesangiumzellen mit dem physiologischen Botenstoff Stickstoffmonoxid (NO). Dieser Effekt ist durch eine Aktivierung der Sphingomyelinase-Aktivität und eine Hemmung der Ceramidase-Aktivität durch NO bedingt. In dem zweiten Teil dieser Arbeit wird beschrieben, dass diese Regulation der neutralen Ceramidase mit einer Reduzierung der Proteinmenge der neutralen Ceramidase einhergeht. Diese NO-vermittelte Reduzierung der Aktivität und der Proteinspiegel wird aufgehoben durch eine zeitgleiche Stimulation mit Stoffen, die zu einer Aktivierung der Proteinkinase C (PKC) führen, wie zum Beispiel Phorbolester oder die bekannten physiologischen Stimuli wie Angiotensin II und Adenosintriphosphat.

Phosphorylierungs-Experimente ergaben, dass durch diese PKC-Aktivatoren die neutrale Ceramidase direkt, wahrscheinlich durch eines der in Mesangiumzellen bekannten Enzyme aus der PKC-Familie, phosphoryliert wird. Weitere Studien mit selektiven PKC-Inhibitoren und rekombinaten PKC-Isoformen lassen den Schluss zu, dass wahrscheinlich die Isoform PKC-δ das entscheidende Enzym ist, welches die neutrale Ceramidase durch direkte Phosphorylierung davor schützt, durch NO abgebaut zu werden. Damit ergibt sich eine Möglichkeit, aktiv in die Stabilität der neutralen Ceramidase und infolgedessen auch in die Ceramid-Spiegel der Zelle einzugreifen.

Das dritte Kapitel dieser Arbeit beschreibt, dass der im zweiten Teil angeführte NO-vermittelte Abbau der neutralen Ceramidase durch eine Aktivierung des Ubiquitin/Proteasomen-Systems bedingt ist. Durch den spezifischen Proteasomen-Inhibitor Lactacystin kann der Abbau der neutralen Ceramidase vollständig wieder aufgehoben werden. Dazu passend wird gezeigt, dass der zelluläre Ceramid-Spiegel, der nach NO-Stimulation dramatisch erhöht ist, durch eine zusätzliche Stimulation mit Lactacystin wieder reduziert wird. Durch diesen Mechanismus ergibt sich ein weitere Möglichkeit, in den Ceramid-Stoffwechsel regulierend einzugreifen.

Der letzte Teil der Experimente befasst sich mit der zellulären Lokalisation der neutralen Ceramidase. Durch Herstellung eines Fusionsproteins bestehend aus der neutralen Ceramidase und einem grün fluoreszierenden Protein (GFP), welches speziell für derartige Lokalisationsexperimente verwendet wird, wird in Transfektions-Experimenten demonstriert, dass die neutrale Ceramidase in weiten Teilen des Zytoplasma vorkommt. Eine Doppelfärbung mit verschiedenen organelltypischen Antikörpern lässt darauf schließen, dass die neutrale Ceramidase im Zytoplasma vorhanden ist. Durch PKC-

Aktivierung kommt es zu einer Translokation der neutralen Ceramidase an die Kernmembran. Die Bedeutung dieser Translokation ist noch ungewiss.

7.4.3 Diskussion

Im Zuge der breiten Dokumentation, die Ceramid in vielen verschiedenen Zelltypen als pro-apoptotische Verbindung beschreibt, wächst mehr und mehr auch das Interesse an den am Ceramid-Stoffwechsel beteiligten Enzymen.

Wie schon ausgeführt, legt diese Arbeit den Schwerpunkt auf die Regulation der Klasse der Ceramidasen und damit auf die Schlüsselenzyme der Ceramid-Spaltung.

Die beschriebenen Regulationen, insbesondere der neutralen Ceramidase, durch pro-inflammatorische Zytokine wie Interleukin 1β oder Tumornekrosefaktor α und Stickstoffmonoxid zeigen einen möglichen Zusammenhang zwischen pathologischen Prozessen und dem Sphingolipid-Stoffwechsel.

Die Störung der Apoptose, des programmierten Zelltodes, als ein wichtiges zelluläres Instrument, um Proliferation zu begrenzen, ist bei der Pathogenese vieler Krankheiten, insbesondere bei der Krebsentstehung beteiligt.

Durch eine mögliche Regulation der Ceramid-Spiegel innerhalb der Zelle kann so gezielt auf apoptotische Prozesse Einfluss genommen werden, beziehungsweise diese können ausgelöst werden. Eine Ceramid-Abbau durch Ceramidasen hat zudem eine Erhöhung der Folgeprodukte Sphingosin und Sphingosin-1-phosphat zur Folge. Besonders Sphingosin-1-phosphat ist in vielen Zellsystemen als Mitogen und Wachstums-Förderer beschrieben worden. Zudem wird es nicht auf dem de novo Syntheseweg produziert, was die Bedeutung der Ceramidasen als Enzym, welches für die Produktion von Sphingosin bzw. Sphingosin-1-phosphat mit verantwortlich ist, noch weiter erhöht. Damit stellen die Ceramidasen als Enzyme, die das Ceramid/Sphingosin/Sphingosin-1-phosphat-Gleichgewicht maßgeblich beeinflussen, wichtige potentielle Ziele für eine Arzneistofftherapie dar. Durch Hemmung oder Abbau der Ceramidasen kann es zu einer Ceramid-Akkumulation kommen, während eine Induktion oder Aktivierung zu einer Verringerung der Ceramid-Spiegel und einer Erhöhung der Sphingosin- und Sphingosin-1-phosphat-Spiegel führt.

Einige klinische Studien beschreiben schon Erfolge einer Therapie, die in die Ceramidase-Regulation bzw. in das Ceramid/Sphingosin-1-phosphat-Gleichgewicht eingreifen.

Humane Kolon-Karzinom-Zellen zeigen eine 50%ige Abnahme des zellulären Ceramid-Gehaltes verglichen mit gesunder Darm-Mucosa. Die Behandlung mit einem potenten Hemmstoff der Ceramidase (B13) erhöht hier den Ceramid-Spiegel in den Tumorzellen, was zu einer maximalen Aktivierung der apoptotischen Kaskade führt, während gesunde

Zellen nicht wesentlich beeinflusst werden. Damit stellt diese Ceramidase-Inhibition eine vielversprechende Strategie dar, Tumorzellen selektiv zu eliminieren.
Weiterhin wird gezeigt, dass von außen appliziertes Ceramid die Wirkungen einer bestehenden Krebs-Therapie noch zu erhöhen vermag.
Auch ein Therapieansatz zur Prävention einer erhöhten Sphingosin-1-phosphat-Bildung mit einem Inhibitor der Sphingosin-1-kinase kombiniert mit einer Förderung der Ceramid de novo Synthese zeigt synergistische Effekte bezüglich der Zelltoxizität. Die beschriebene Therapie mit Safingol (Sphingosin-Kinase-Inhibitor) und Fenretinide (erhöht die Ceramid de novo Synthese) wird bereits in klinischen Phase II Studien bei verschiedenen Krebsformen getestet.

Bezogen auf die Ergebnisse dieser Arbeit ergeben sich vielfältige Möglichkeiten in die Regulation der neutralen Ceramidase einzugreifen. Es konnte gezeigt werden, dass die neutrale Ceramidase sowohl auf Ebene der Genexpression (transkriptionelle Regulation) als auch auf Ebene der Proteinexpression (translationale Regulation) reguliert wird. Weiterhin zeigen sich verschiedene post-translationale Regulationsmechanismen wie Phosphorylierung, Ubiquitinierung oder Translokation. All diese Mechanismen tragen dazu bei, die Ceramid-Spiegel innerhalb der Zelle zu regulieren, und damit das weitere Schicksal der Zelle zu bestimmen.
Zukünftige Arbeiten werden zeigen, ob die neutrale Ceramidase als ein mögliches Target für die Entwicklung neuer Therapie-Strategien im Kampf gegen Entzündung und Krebs erfolgreich angegangen werden kann.

7.5 Curriculum vitae

PERSÖNLICHE ANGABEN

Name:	Rochus Franzen
Geburtsdatum:	23. Oktober 1973
Geburtsort:	Meschede
Anschrift:	Hauptstraße 61, 59889 Eslohe Rotlintstraße 69, 60389 Frankfurt am Main

SCHULBILDUNG

1980 – 1984	Kath. Grundschule in Eslohe
1984 – 1992	Gymnasium der Benediktiner in Meschede

WEHRDIENST

Juli 1992 - Juni 1993	Wolfhagen / Hofgeismar

STUDIUM UND PROMOTION

Wintersemester 1993/94	Immatrikulation an der Universität zu Köln, Studiengang: „Medizin“
Sommersemester 1994	Immatrikulation an der Johann Wolfgang Goethe Universität Frankfurt am Main, Studiengang: „Pharmazie“
September 1996	Erstes Staatsexamen
Oktober 1998	Zweites Staatsexamen
November 1998 – Oktober 1999	Praktisches Jahr (STADA Arzneimittel AG, Bad Vilbel / Bock-Apotheke, Frankfurt am Main)
November 1999	Drittes Staatsexamen
Dezember 1999	Approbation als Apotheker
Dezember 1999 – November 2002	Experimentelle Arbeiten der Dissertation am Institut für Allgemeine Pharmakologie und Toxikologie (Direktor: Prof. Dr. J. Pfeilschifter) des Klinikums der Johann Wolfgang Goethe-Universität Frankfurt am Main unter Betreuung von PD Dr. A. Huwiler. Thema der Dissertation: „Regulation of neutral ceramidase in glomerular mesangial cells“

MITGLIEDSCHAFTEN IN BERUFSVERBÄNDEN

Deutsche Gesellschaft für experimentelle und klinische Pharmakologie und Toxikologie

Deutsche Pharmazeutische Gesellschaft