Regulation of matrix-metalloproteinase 9 (MMP-9) in glomerular mesangial cells

Dissertation
zur Erlangung des Doktorgrades
der Naturwissenschaften

vorgelegt beim Fachbereich
Chemische und Pharmazeutische Wissenschaften
der Johann Wolfgang Goethe-Universität
in Frankfurt am Main

von
El-Sayed Akool
aus Ägypten

Frankfurt am Main 2003

vom Fachbereich Chemische und Pharmazeutische Wissenschaften

der Johann Wolfgang Goethe-Universität als Dissertation angenommen

Dekan: Prof. Dr. Schwalbe

Gutachter: Prof. Dr. J. Pfeilschifter
Prof. Dr. M. Schubert-Zsilavecz

Datum der Disputation: 31.03.2004

To my beloved parents

and

to my wife

whom I honor and cherish

To them I owe everything

The work outlined in this thesis is based on experimental studies published in the following articles:

Akool, El-S.,* Eberhardt, W.,* Rebhan, J., Frank, S., Beck, K.F., Franzen, R., Hamada, F.M., and Pfeilschifter, J. Inhibition of cytokine-induced matrix metalloproteinase 9 expression by peroxisome proliferator-activated receptor α agonists is indirect and due to a NO-mediated reduction of mRNA stability.
J. Biol. Chem. (2002), 277: 33518-33528.

* Both authors contributed equally.

Akool, El-S., Kleinert, H., Hamada, F.M.A., Abdelwahab, M.H., Forstermann, U., Pfeilschifter, J., and Eberhardt, W. Nitric oxide increases the decay of MMP-9 mRNA by inhibiting the expression of mRNA stabilizing factor HuR.
Mol. Cell. Biol. (2003), 23: 4901-4916.

Akool, El-S.,* Huwiler, A.,* Aschrafi, A., Hamada, F.M.A., Pfeilschifter, J., and Eberhardt, W. ATP potentiates IL-1ß-induced MMP-9 expression in mesangial cells via recruitment of the ELAV protein HuR.
J. Biol. Chem. [Epub ahead of print, Oct 2003].

* Both authors contributed equally.

Contents

1. Introduction

Matrix-Metalloproteinases : general aspects

Matrix metalloproteinases (MMPs) are a tightly regulated family of enzymes having a potential to degrade the extracellular matrix and basement membrane components [Nelson et al., 2000]. The first report of an enzyme from vertebrate (as opposed to bacterial) sources that was capable of attacking the triple helix of native type I collagen was published in 1962 by Jerome Groos and Charles Lapiere [Groos and Lapiere, 1962] . In this first report the authors could demonstrate that the enzyme activity, secreted by cultured tissue fragments of tail fin skin from resorbing tadpole tails in metamorphosis, was a true collagenase which was able to degrade collagen at 27 °C at neutral pH. Mechanistically, by these studies it was demonstrated that collagenase is able to cut the triple helix at a point one-quarter of the distance in from the C-terminal end and that the activity was metal dependent. Meanwhile, more than 20 endopeptidases have been classified as MMPs. Historically, this enzyme family was divided into collagenases, gelatinases, stromelysins and matrilysins [Egeblad and Werb, 2002] The MMPs have several common characteristics which are as follows [Powel et al., 1996]: 1. Members of the MMP family have a characteristic pattern of conserved domains within their structure. 2. Common substrates for all MMPs members are proteins which build up the extracellular matrix (ECM) and the basement membrane. 3. Their proteolytic activity is specifically inhibited by their endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). 4. Some MMPs are either secreted as inactive zymogen or transmembrane proenzymes that both require activation to exert their matrix degrading activities. 5. The active site contains a zinc ion and requires a second cofactor such as calcium. 6. The enzymatic activity of all MMPs is optimal in a physiological pH range. As the list of MMP substrates has increased, a sequential numbering system has been adopted and MMPs are meanwhile grouped according to their substrates as shown in table 1.

Enzyme	Descriptve name	Size (kDa) Latent/active	Collagen substrates	Additional substrates
MMP-1	Interstitial collagenase	55/45	I, II, III, VII, VIII, X	Aggrecan, Gelatin, MMP-2, MMP-9
MMP-2	Gelatinase A	72/66	I, II, III, IV, V, VII, X, XI	Aggrecan, Elastin, Fibronectin, Gelatin, Laminin, MMP-9, MMP-13
MMP-3	Stromelysin-1	57/45	II, III, IV, IX, X, XI	Aggrecan, Elastin, Fibronectin, Gelatin, Laminin, MMP-7, MMP-8, MMP-13
MMP-7	Matrilysin	28/19	IV, X	Aggrecan, Elastin, Fibronectin, Gelatin, Laminin, MMP-1, MMP-2, MMP-9
MMP-8	Neutrophil collagenase	75/58	I, II, III, V, VII,VIII,X	Aggrecan, Elastin, Fibronectin, Gelatin, Laminin
MMP-9	Gelatinase B	92/86	IV, V, VII, X, XIV	Aggrecan, Elastin, Fibronectin, Gelatin
MMP-10	Stromelysin-2	57/44	III, IV, V	Aggrecan, Elastin, Fibrpnectin, Gelatin, Laminin, MMP-1, MMP-8
MMP-11	Stromelysin-3	51/44		Aggrecan, Fibronectin, Laminin
MMP-12	Metalloelastase	54/45	IV	Elastin, Fibronectin, Gelatin, Laminin
MMP-13	Collagenase-3	60/48	I, II, III, IV	Aggrecan, Gelatin
MMP- 18	Xenopus collagenase	70/53		
MMP-19	RASI-1	54/45	IV	Fibronectin, Aggrican, Laminin, Gelatin
MMP-20	Enamelysin	54/22		Aggrecan, Amelogenin
MMP-23	CA-MMP			
MMP-26	Matrilysin-2	28/19	IV	Gelatin, fibrinocetin
MMP-28	Epilysin			
MMP-14	MT1-MMP	66/56	I ,II, III	Aggrecan, Elastin, Fibronectin, Gelatin, Laminin, MMP-2, MMP-13
MMP-15	MT2-MMP	72/60		Fibrionectin, laminin, gelatin, MMP-2
MMP-16	MT3-MMP	64/52		MMP-2
MMP-17	MT4-MMP	57/53		Fibrin, gelatin
MMP-24	MT5-MMP	-/62		
MMP-25	MT6-MMP		IV	Gelatin, Fibronectin, laminin

Table 1: The family of MMPs (adapted from Matrisian, 1990, Nelson et al., 2000 and Murphy et al., 2002)

The structural complexity of MMPs

Analysis of the human genome has enabled to get insight into the MMP gene family, a gallery of proteinases which are encoded by 24 distinct genes [Overall and Lopez-Otin, 2002]. The secreted types of MMPs contain a signal peptide necessary for secretion, a propeptide which keeps the enzyme in an inactive state and a catalytic domain with a zinc ion (Zn^{2+}) in its center (Figure 1). Moreover, most of the secreted types of MMPs at their carboxy terminal end contain a hemopexin domain (Figure 1) which is important for determining the substrate specificity and for the interaction with the TIMPs. A subset of MMPs, known as membrane-type MMPs (MT-MMPs), additionally contains a transmembrane domain. Unlike the other members of MMP family, MT-MMPs are not secreted but instead are inserted into the plasma membrane by a transmembrane segment or a glycosylphosphatidylinositol (GPI). There is good evidence that one of the main function of MT-MMPs is to localize and activate some of the secreted MMPs mainly the MMP-2 (gelatinase-A) [Nelson et al., 2000].

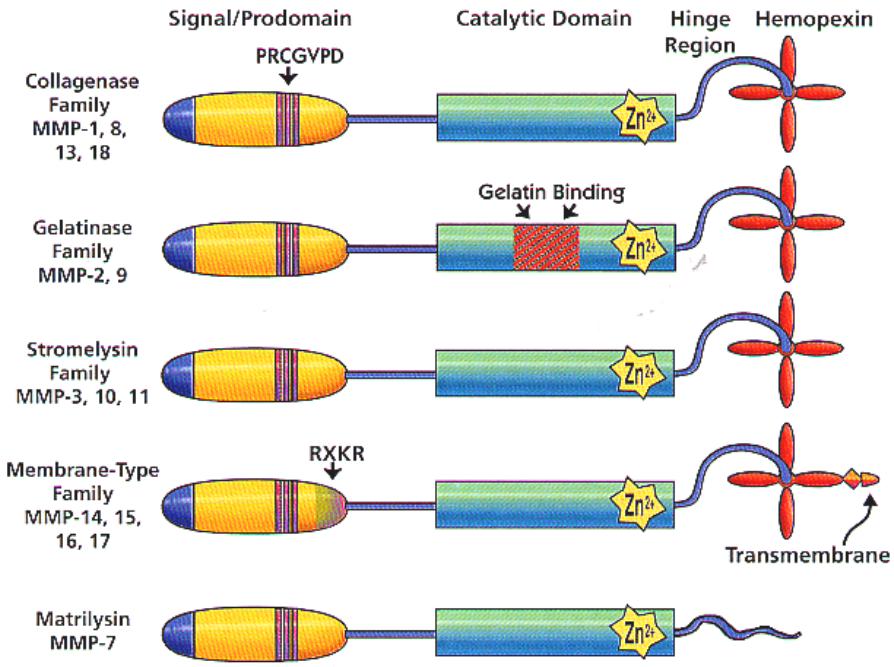

Figure 1. the domain structure of MMPs

Further complexity is derived by addition of other protein modules or smaller inserts. For example, the gelatinases A and B (MMP-2 and MMP-9) similarly have an additional fibronectin-like domain inserted in the middle of the catalytic domain [Sternlicht and Werb, 2001]. Unique to gelatinase B is a collagen V-like domain located between the catalytic domain and the hemopexin domain [Wilhelm et al., 1989].

Regulation of MMPs

Generally, the proteolytic activity of MMPs is regulated mainly at three levels involving, transcription, proenzyme activation and inhibition by the TIMPs (Figure 2). Further mechanisms by which the abundance of MMPs can be modulated involve the regulation of MMP mRNA stability, changes in the translational efficiency, enzyme compartmentalization, enzyme secretion and the cell-surface recruitment (Figure 2). All MMPs are synthesized as latent zymogens (pro-MMPs) and are kept inactive due to the interaction between a cysteine-sulphohydryl group present in the propeptide domain and the central Zn^{+2} of the enzyme's catalytical domain [Egeblad and Werb, 2002]. Most of the MMPs are activated extracellularly by other active MMPs or serine proteinases whereas MMP-11, MMP-28 and MMP-14 (MT1-MMP) can be activated intracellularly by the action of furin-like serine proteinases [Sternlicht and Werb, 2001]. For example, MMP-2 underlies a complex activation pathway which involves active MMP-14 (MT1-MMP) and TIMP-2 which binds to pro-MMP-2 at its C-terminus. Secondly, the activity of MMPs is tightly controlled by the action of endogenous inhibitors. In addition to the already mentioned TIMPs, an other important inhibitor of MMPs is α-2 macroglobulin which acts via binding to the MMP scavenger receptors thereby generating an inactive MMP-macroglobulin complex. A further inhibitor of MMPs are thrombospondin-1 and –2 which by binding to pro-gelatinases directly can inhibit their activation. Finally, a recently found protein called RECK (reversion-inducing cysteine-rich protein with kazal motifs) was characterized as the first known membrane-bound MMP

inhibitor [Egeblad and Werb, 2002].The transcriptional mechanisms regulating MMP expression are quite complex [Fini et al., 1998; Westermarck & Kahari, 1999]. The expression of most MMPs under normal physiologic conditions is low in tissue but is induced under conditions where remodeling of ECM is required [Westermack and Kahari,1999]. The expression of MMPs is regulated at the transcriptional level and induced by various signals, such as cytokines, growth factors and oncogene products [Kheradmand et al., 1998]. Similarily, the signal transduction pathways that mediate the activity of MMP transcriptional activators are also diverse. These include, for example, the mitogen activated protein kinase (MAPK) pathways including, the p38- MAPK, the jun-N-terminal kinase (JNK) and the extracellular signal-regulated kinase (ERK) pathways [Westermarck & Kahari, 1999; Simon et al., 1998; Johansson et al., 2000; Eberhardt et al., 2000b]. All of these signal transduction pathways however, converge at the activation of only a few transcriptional factors which finally control MMP expression which actions however account for the variable inducibility of MMPs by several agents. Some of the common transcription factors involved in MMP expression include the ETS family of oncoproteins, which bind to their PEA3 sites that are present in most of the MMP gene promoters[Fini et al., 1998], nuclear factor kappaB (NF-κB) which is necessary for expression of MMP-1,3,9,13 and 14 genes [Bond et al., 1999; Han et al., 2001] and the binding-site for transcription factor activated protein-1 (AP-1) which also is present in the promoter region of most inducible MMP genes [Pendas et al., 1997].The DNA-binding and transactivation capacity of AP-1 and ETS transcription factors are activated by phosphorylation by MAPKs and the balance between the distincit MAPK pathways is thought to regulate cell growth, differentiation, survival and cell death [Westermack and Kahari, 1999]. A further important group of transcription factors for regulating the MMP gene expression are the signal transducers and activators of transcription (STATs), which mainly mediate the effects of interferons (INFs) but also of many other cytokines [Overall and Lopez-Otin, 2002].

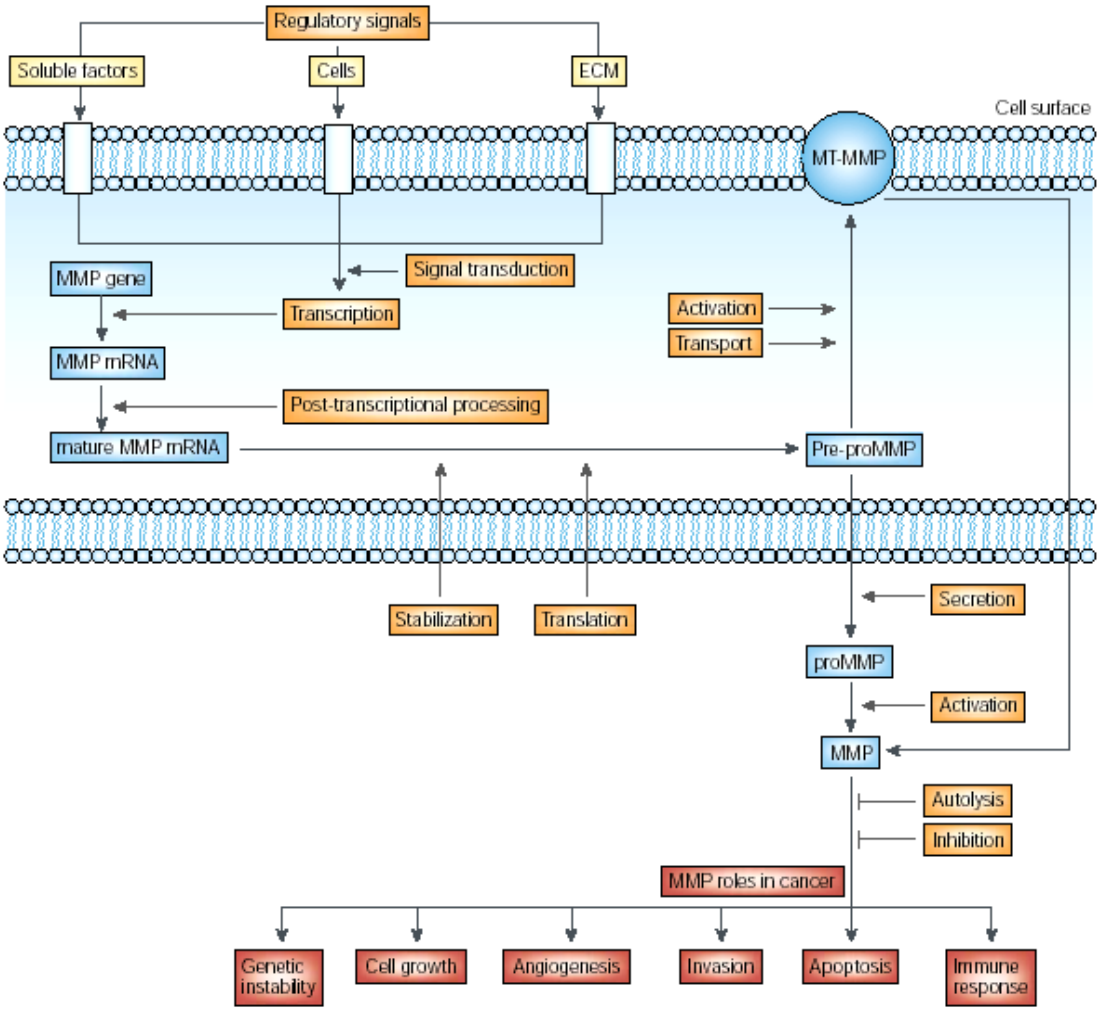

Figure 2. regulation of MMPs (Overall & Lopez-Otin, 2002)

The biological roles of the MMPs

MMPs are important regulators of many developmental and physiological events. Because MMPs can degrade ECM molecules, their main function has been presumed to be remodeling of the ECM. Since ECM remodeling is a critical process of tissue growth and morphogenesis, MMPs are thought to play important roles during embryonic development. Moreover, ECM remodeling is important for proliferation of cells and angiogenesis since for both processes

the breakdown of ECM seems to be a prerequisite. Beyond their classical connective tissue remodeling functions, increasing experimental evidence has been given, demonstrating that MMPs are key-players in the precise regulation of many bioactive molecules, mainly by their proteolytic and processing activity [Overall and Lopez-Otin, 2002]. For example, MMPs can generate growth-promoting signals as demonstrated by the decreased cell proliferation rates of tumor cells injected into MMP-9 deficient mice when compared to wild-type mice [Coussens et al., 2000; Bergers et al., 2000]. Mechanistically, MMPs can promote cell proliferation by increasing the release of cell membrane-bound precursors of some growth factors, most importantly the transforming growth factor α (TGF-α) [Peschon et al., 1998] and the insulin-like growth factor (IGF) [Manes et al., 1997; 1999]. In contrast, by liberating transforming growth factor β (TGFß) from the latent TGFß complex some MMPs can additionally inhibit cancer cell growth, thus demonstrating the diversity of MMPs in regulation of cell growth [Egeblad and Werb, 2002]. A further biological role of MMPs is their contribution to regulation of apoptosis. In this context, overexpression of MMP-3,-7,-9 and –11 has been demonstrated to cause an increase in apoptotic cell death in mammary epithelial cells [Alexander et al., 1996; Witty et al., 1995]. One main mechanism by which MMPs can regulate apoptosis is the release of membrane-bound FAS ligand (FASL) which by binding to its cognate transmembrane death receptor FAS can activate the receptor tyrosine kinase thereby inducing apoptotic cell death [Powel et al., 1999; Mitsiades et al., 2001]. However, release of FASL in some cell types can also lead to a reduction of apoptosis demonstrating the strong cell-type specificity of MMP-related cell death [Powel et al., 1999]. A further pivotal function of MMPs in the carcinogenic process is the regulation of angiogenesis which plays a povital role in the sprouting of newly formed vessels during tumor growth. One of the most convincing trials proving the contribution of MMPs in tumor angiogenesis is the use of synthetic MMP inhibitors which are able to significantly reduce tumor angiogenesis in different animal experiments [Rodriguez-Manzaneque et al., 2001; Oh

et al., 2001]. One of the mechanisms by which MMPs are thought to regulate angiogenesis is the release of pro-angiogenic factors such as vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF) [Bergers et al., 2000]. Mainly the contribution of MMP-9 and -14 was demonstrated by the use of MMP-9 and MMP-14-deficient mice, which both showed impaired angiogenesis during development [Vu et al., 1998; Zhou et al., 2000]. In addition to angiogenesis, MMPs play an important role in metastasis as demonstrated by promoting the invasion of certain cell lines through collagen-containing matrices when MMP-2, -3, -13 and –14 were overexpressed [Ahonen et al., 1998; Kim et al., 2000]. Experimental evidence for functional roles of MMPs in the metastatic processes has furthermore been derived from *in vitro* invasion assays as well as from *in vivio* xenograft models [Egeblad and Werb, 2002]. The latter is a method of transplantation of human tumor cells into immune-deficient mice strains [Egeblad and Werb, 2002]. By these assays, it could be demonstrated that the number of metastatic colonies formed in the lungs of these mice was significantly reduced in the MMP-2 and MMP-9 null mice when compared with the wild-type mice thus demonstrating that both gelatinases play a critical role for the migration of metastatic cells [Itoh et al., 1998; 1999]. Finally, MMPs are involved in the escape of tumor cells from the attacks of immune responses by different mechanisms. MMP-9 e.g., can cleave the α receptor of interleukin-2 (IL-2Rα) and thereby suppress the proliferation of T-lymphocytes which strongly depends on IL-2 dependent signaling pathways [Sheu et al., 2001]. Furthermore, MMPs can activate TGF-ß [Yu and Stamenkovic, 2000] an important inhibitor of the T-lymphocyte response against tumors [Gorelik and Flavell, 2001]. In addition, MMPs can affect several chemokines resulting in both increased or reduced chemotaxis of leukocytes. MMP-9 has been shown to cleave the neutrophil chemoattractant CXCL8 and its murine homolog, thereby increasing its activity [Egeblad and Werb, 2002]. In contrast, MMP-2 can cleave CCL7 producing an inactive cleaved fragment which becomes an antagonist of the receptors [McQuibban et al., 2000].

MMPs in renal glomerular diseases

The tight balance between ECM synthesis and degradation is thought to be a main prerequisite for maintaining the structural and functional integrity of the glomerulus [Arthur, 1998; Lenz et al., 2000]. Thus, changes in MMP expression or activity will directly translate into altered ECM turnover, which may lead to glomerular scarring and a decline in renal function. Many forms of glomerular diseases are characterized by a change in cellularity, which in turn may affect ECM composition and turnover. A main mechanism how MMPs can influence ECM turnover is the release of certain growth factors from their inactive precursors as mentioned on page 7. A regulation of growth factor activity by MMPs has been observed by use of MMP-9-deficient mice, which exhibit abnormal growth plate vascularization and ossification [Vu et al., 1998]. Furthermore, binding proteins for insulin-like growth factor 1 have been identified as substrates of MMP [Fowlkes et al., 1995]. Especially, diseases which are characterized by an altered matrix composition, such as Alport disease, the increased susceptibility of the basement membrane to the degradation by MMPs has been postulated to cause glomerular damage [Kalluri et al., 1997]. These findings illustrate that MMPs are involved at several levels of ECM turnover, thereby keeping a tight balance between ECM synthesis and degradation necessary for normal physiological function of the glomerulus. The role of MMPs in the development of glomerular damage has been studied in a variety of experimental conditions. In general terms, a downregulation of MMPs is often associated with the progression of noninflammatory diseases such as hypertensive glomerulosclerosis [Singhal et al., 1996; Trachtman et al., 1996], cyclosporin nephrotoxicity [Duymelinck et al., 1998] and diabetic nephropathy [Abrass et al., 1998; Schnaper and Robson, 1992]. In contrast to noninflammatory diseases, the findings are quite different in inflammatory glomerular diseases. It has been reported that the increased levels of MMPs are functionally associated with the progression of disease and paralleled by an increased influx of inflammatory cells

[Nakamura et al., 1995]. Therefore, the overall amount and duration of MMPs activity may critically determine the structural glomerular damage as demonstrated by elevated levels of MMPs in various forms of glomerulonephritis [Steinmann-Niggli et al., 1998; Akiyama et al., 1997; Koide et al., 1996]. In addition, it has been reported that the inhibition of MMPs significantly can attenuate the formation of glomerular lesions observed during glomerulonephritis demonstrating a beneficial role of MMP inhibitors in the treatment of acute glomerulonephritis [Steinmann-Niggli et al., 1998].

In summary, an overall reduction of MMP level within the glomerulus seems to critically determine the progression and degree of glomerulosclerosis since downregulation of MMPs increases the matrix accumulation which consequently causes a decrease of the filtration capacity. In contrast, upregulation of MMPs in many cases is associated with the generation of glomerulonephritis. Therefore, future therapeutic strategies by selectively targeting single member of the MMP family and/or their endogenous inhibitors may have beneficial effects in preventing progressive as well as inflammatory renal diseases.

The mesangial cell as an important regulator of inflammatory processes within the kidney

The functional unit of the kidney, which represents the main excretory organ of mammalians is the nephron. Each human kidney contains about 1.0-1.5 million nephrons, that of rats approximately 30,000 to 34,000 (Brenner, 1996). Essential components of the nephron are the renal glomerulus with the adjacent vas afferens and vas efferens, the proximal and the distal convoluted tuble and the loop of Henle. The glomerulus itself builts up a functional unit of the kidney which enables the production of a highly concentrated plasma ultrafiltrate. The functionality of a glomerulus is guaranteed by the action of at least four different cell-types which each of them perform special functions.

1. *Glomerular endothelial cells* which form the initial barrier to the passage of blood constituents from the lumen of the capillaries to the Bowman space (Brenner, 1996). Due to a negatively charged surface endothelial cell contribute to the charge-selective properties of the capillary wall.

2. *Visceral epithelial cells* also called podocytes, characterized by long cytoplasmatic processes which come into direct contact with the glomerular basement membrane. There is also some evidence that visceral epithelial cells are responsible for the synthesis and maintenance of the glomerular basement membrane.

3. *Parietal glomerular epithelial cells*, which built up the main component of the Bowman's capsule.

4. *Glomerular mesangial cells* which together with surrounding matrix material and resident monocytes-macrophages constitute the mesangium. The mesangium is localized inbetween the capillary loops of the glomerulus and represents a separate component, which is mainly involved in the regulation of glomerular circulation and filtration (Brenner, 1996). Morphologically the mesangial cell is characterized by its irregular shape, its dense nucleus and the existence of elongated processes which can extend around the capillary lumen. Mesangial cells have also been characterized as modified smooth muscle cells as they possess an extensive array of contractile cytoskeletal elements as for example actomyosin, microtubles and intermediate filaments all of which can exert mechanical traction on the glomerular basement membrane in response to vasoactive agents such as angiotensin II, vasopressin, cAMP-and cGMP elevating hormones (Pfeilschifter, 1989). Therefore it has been concluded that the contractile state of mesangial cells finally regulates the glomerular filtration rate by a direct alteration of the filtration surface of glomerular capillaries. However, mesangial cells not only control glomerular filtration but are also involved in the responses to local injury, including cell proliferation, basement membrane remodeling and production of inflammatory mediators (Schlondorff, 1987). Experimental data and findings from human

studies have suggested that mainly the enhanced degradation of ECM accompanying cell lysis in response to acute inflammatory processes determines the extent of glomerular damage [Fogo, 2001]. In contrast, excessive matrix deposition seems as a paradigm of interstitial fibrosis and glomerulosclerosis which count for the most common complications of the many renal diseases. Due to this critical involvement of MCs in glomerular response to injury much effort has been made to analyse mesangial cell function in normal condition as well as in glomerular diseases. To study the role of MCs during progression of glomerular diseases, MC culture is extensivly used as a powerful tool to investigate the main signaling pathways underlying MC responses.

Aim of the work

Progressive glomerulosclerosis leading to end-stage renal failure is a complication of a variety of diseases, such as diabetes mellitus, glomerulonephritis, focal sclerosis, chronic transplant rejection [Abrass et al., 1988; Schnaper & Robson, 1992; Jeong et al., 1996]. Although many studies have tried to elucidate the pathogenesis, the exact underlying mechanisms are still not completely understood. While increased synthesis of ECM certainly plays an important role, recent studies have focused on the degradative systems [Lenz et al., 2000]. The major physiologic regulators of ECM degradation in the glomerulus are matrix metalloproteinases (MMPs) [Woessner, 1991]. As described before, the tight regulation of most of these proteases is accomplished at different levels. The present work focuses on the regulation of MMP-9 which is one of the most prominent MMPs induced under inflammatory conditions within the renal mesangium. This work aimed to elucidate:

(i) the possible modulation of MMP-9 by peroxisome proliferator-activated receptors (PPAR) since each ligand by binding to its specific intracellular receptor can mediate a broad spectrum of physiological responses including lipid metabolism, glucose homeostasis,

inflammatory and antiinflammatory responses, cell cycle progression, cell differentiation and for this work, most importantly, extracellular matrix remodeling.

(ii) The molecular mechanisms by which NO can inhibit the expression of MMP-9 as described by Eberhardt et al., (2000a) since MC under inflammatory conditions similar to MMP-9 highly express the inducible type of NO synthase (iNOS) thereby producing high amounts of NO [Pfeilschifter and Schwarzenbach, 1990; Pfeilschifter et al., 1992]. The high output levels of NO may contribute to the excessive matrix accumulation [Cattel et al., 1993; Cook et al., 1994] triggering the sclerotic processes within the glomerulus in response to inflammatory injury [Johnson et al., 1994]. Furthermore, it has been reported that the adminstration of specific iNOS inhibitors can reduce the intensity of inflammation in various forms of glomerulonephritis. Therefore, iNOS inhibitors may be of certain value in the future therapy of inflammatory diseases of the kidney [Noris and Remuzzi. 1999].

(iii) The modulatory effects of extracellular ATP on MMP-9 expression since nucleotides can mediate a variety of cell responses in MC including cell growth, inhibition of apoptosis, and proliferation [Schulze-Lohoff et al., 1998; Huwiler and Pfeilschifter, 1994]. Since both processes can be linked with excessive degradation of ECM, I tested whether extracellular nucleotides may influence the expression of MMP-9 in MC.

2. Materials and Methods

I. Materials

A. Chemicals :

Acrylamide solution (30% acrylamide with 0.8% bisacrylamide)	Roth, Karlsruhe
Actinomycin D	Sigma Aldrich Fine chemicals
Agar	Gibco Life Technologies, Karlsruhe
Agarose	Biozym, Oldendorf
Ammoniumpersulfate	Sigma Aldrich Fine Chemicals
Ampicillin	Sigma Aldrich Fine Chemicals
Aprotinin	Roch Biochemicals, Mannheim
Bovine serum albumin, fatty acid free	Sigma Aldrich Fine Chemicals
Cell culture media	Gibco Life Technologies
Chemicals for luciferase reporter assay	Promega, Heidelberg
Chemicals for sequence analysis (BigDye terminator premix, template supression reagent)	PE Biosystems, Weiterstadt
Cycloheximide	Sigma Aldrich Fine chemicals
DETA-NONOate	Alexis, Grunberg
Diethylpyrocarbonate	Sigma Aldrich Fine chemicals
Dithiothreitol	Sigma Aldrich Fine chemicals
ECL reagent	Amersham Pharmacia, Freiburg
Effectine transfection reagent	QIAGEN inc.,Hilden
Ethidium bromide	Sigma Aldrich Fine chemicals
EDTA	Sigma Aldrich Fine chemicals
EGTA	Sigma Aldrich Fine chemicals
Fetal calf serum	Gibco Life Technologies, Karlsruhe
Glycine	Merk, Darmstadt
Griess reagent	Merk, Darmstadt
Insulin (source: Bovine pancreas & activity: 28.1 IU/mg)	Roch Biochemicals
IL-1ß (recombinant human IL-ß produced in E.coli with activity 5-500 units/ml)	Cellconcept, Germany
Leupeptin	Roch Biochemicals
N^G-monomethyl-L-arginine	Alexis

Ly-17,1883	Alexis
Molecular weight markers (DNA)	MBI Fermentas, St. Leon-Rot
Molecular Weight markers (protein)	Amersham Pharmacia
Nucleotide triphosphates	PE Biosystems, Weiterstadt
Pepstatin A	Roch Biochemicals, Mannheim
Peptone 140	Gibco Life Technologies
Phenylmethylsulfonyl fluoride	Roch Biochemicals
Ponceau S	erva, Heidelberg
Radiolabeled nucleotide [α-^{32}P]ATP(6000 ci/mmol)	Amersham, Freiburg
Radiolabeled nucleotide [α-^{32}P]CTP(3000 ci/mmol)	Amersham, Freiburg
Radiolabeled nucleotide [α-^{32}P]UTP(3000 ci/mmol)	Amersham, Freiburg
Rediprime DNA labelling Kit	Amersham, Freiburg
SNAP	Alexis, Grunberg
SDS	Applichem, Darmstadt
Tetramethylethylenediamine	Sigma Aldrich Fine chemicals
TritonX-100	Sigma Aldrich Fine chemicals
TRIzol reagent	Gibco Life Technologies
Tween 20	Sigma Aldrich Fine chemicals
Wy-14,643	Alexis

All nucleotides were purchased from Sigma Aldrich Fine chemicals.

B. Antibodies and antisera :

Anti-MMP-9 (Rabbit polyclonal)	Chemicon, Hofheim, Germany
Anti-HuR (mouse monoclonal)	Santa Cruz Biotechnology
Anti-HuB (goat polyclonal)	Santa Cruz Biotechnology
Anti-HuC (goat polyclonal)	Santa Cruz Biotechnology
Anti-HuD (goat polyclonal)	Santa Cruz Biotechnology
Anti-NF-κß p65 (Rabbit polyclonal)	Santa Cruz Biotechnology
Anti.NF-κß p50 (Rabbit polyclonal)	Santa Cruz Biotechnology
Anti-PPAR-α (Rabbit polyclonal)	Affinity Bioreagent, Inc
Anti-ß-actin (goat, monoclonal)	Santa Cruz Biotechnology
Anti-α-actin (mouse monoclonal)	Sigma Aldrich Fine chemicals
Anti-goat IgG (horseradish-peroxidase coupled)	Amersham Pharmacia
Anti-mouse IgG (horseradish-peroxidase coupled)	Amersham Pharmacia

Anti-iNOS : It was generated as previously described [Kunz et al., 1994] by cloning a cDNA-fragment that represent 196 amino acids of the iNOS N-terminus into the expression vector pDS56 and expressed in E.coli strain M15.The His_6-tagged polypeptide was purified using the xpress-kit (Invitrogen, Groningen, Netherlands). Female new zealand rabbits were immunized and the antisera were tested in Western blots. A prominent iNOS band at the expressed size of 130 kDa was obtained when the protein was analyzed from MC treated with IL-ß.

C. Enzymes :

Pfu-DNA polymerase (5 units/ml)	Stratagene, Heidelberg
Restriction enzymes	MBI Fermentas
BamH I (Source: *Bacillus amyloliquefciens* H & activity: 10,000 units/ml)	
EcoRV (Source: *Escherichia coli* & activity: 10,000 units/ml)	
Hind III (Source: *Haemophilus influenzae* & activity: 10,000 units/ml)	
Xba I (Source: *Xanthomonas badrii* & activity: 10,000 units/ml)	
Reverse transcriptase	MBI Fermentas
(source: Avian Myeloplastosis virus & activity: 10,000-100,000 units/ml)	
T4-DNA ligase	Roch Biochemicals
(Source: T4-infected E-coli & activity: 500-6,000 units/ml)	
T4-PNK	MBI Fermentas
(source : E-coli strain that carries cloned T4 polynucleotide kinase gene)	
Taq-DNA polymerase	MBI Fermentas
(Source: Thermus aquaticus & activity: 2.5 units/100µl)	
Dpn-I	MBI Fermentas
(Source: Diplococcus pneumoniae & activity: 10,000 units/ml)	

D. Membranes :

PVDF blotting membrane	Millipore, Eschborn
Nylon blotting membranes	Life science, Schleicher and Schull, Dassei

E. Buffers and Solutions :

PBS buffer (pH 7.4)	9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150 mM NaCl
Denaturation buffer	63.3 % formamide, 13.3 % 10 x MOPS, 23.4 % Formaldehyde (37 %)
10 x MOPS buffer	0.4 M Morpholinopropanesulfonic acid, 0.1 M

	sodium acetate-$3H_2O$,10 mM EDTA-Na_2-$2H_2O$
20 x SSC buffer	3 M sodium chloride, 0.3 M sodium citrate dihydrate
Prehybridization buffer	50 ml deionized formamide,25 ml 20 x SSC, 5 ml 100 x Denhardt's, 5 ml 20 % SDS, 15 ml H_2O, 10 gm Dextran sulfate .
Wash buffer–1	2 x SSC, 0.1 % SDS
Wash buffer-2	0.2 x SSC, 1 % SDS
Lysis buffer	20 mM Tris-HCl (pH 8.0), 2 mM EDTA (pH 8) 137mM NaCl, 10% glycerin, 1% Triton x-100, 0.2mM PMSF, 0.5 mM DDT, 5 mg/ml Leupeptin
staining solution for RNA (pH 5.2)	0.5 M sodium acetate, 0.02 % Methylene blue
10 x PAGE (pH 8.3)	3 % Tris-HCl, 14.4% glycine, 1% SDS
Towbin-transfere buffer	25 mM Tris base, 192 mM glycine (pH 8.3), 20 % Methanol
TBS solution	10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 % BSA
TBST solution	TBS plus 0.05 % Tween 20
Blocking solution	2 % BSA
2 x sample buffer	4 % SDS, 0.005 % Bromophenol blue, 20 % glycerol, 0.5 M Tris-HCl (pH6.8)
6 x loading buffer	30% glycerol, 0.25% bromophenol blue, 0.25% xylenecyanol and 60% 10x TAE buffer.
Developing buffer	0.2 M NaCl, 5 mM $CaCl_2$, 0.02 % Brij 35, 0 mM Tris-HCl (pH 7.6)
Staining soln for zymogen	30 % Methanol, 10 % glacial acetic acid, 0.5 % Coomassie G 250
Destain solution	30 % Methanol, 10 % glacial acetic acid
50 x TAE	1.6 M Tris-base, 0.8 M sodium acetate-3 H_2O, 40 mM EDTA-di-sodium-$2H_2O$
10 x TBE buffer	1M Tris base, 1 M boric acid, 0.25 M EDTA (pH 8.2)
Buffer A(hypotonic buffer)	10 mM HEPES (pH 7.9), 10 mM KCl,

	0.1mM EDTA, 0.1 mM EGTA, 0.5 mM PMSF*, 0.1 mM DDT*, 50 mg/ml Leupeptin* *ingredients were added immediately before use .
Buffer C (Nuclear extract buffer)	20 mM HEPES (PH 7.9), 25 % glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 0.5 mM PMSF*, 1 mM DTT*, 50 mg/ml Leupeptin* * ingredients were added immediately before use.
3 x Binding buffer	12 % Ficoll, 60 mM HEPES (pH 7.9), 150 mM KCl, 3 mM EDTA, 3 mM DTT, 0.75 mg/ml BSA
Buffer-1 (for DNA preparation)	50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 100 µg/ml Rnase A
Buffer-2 (for DNA preparation)	200 mM NaOH, 1 % SDS (W/V)
Buffer-3(for DNA preparation)	3.0 M Potassium acetate (pH 5.5)
Buffer N2(equilibration buffer)	100 mM Tris/H_3PO_4, 15 % Ethanol, 900 mM KCl (pH6.3), 0.15 % Triton X-100
Buffer N3 (washing buffer)	100 mM Tris/H_3PO_4, 15 % Ethanol, 1150 mM KCl (pH6.3)
Buffer N5 (elution buffer)	100 mm Tris/H_3PO_4, 15 % ethanol, 100 mM KCl (pH 8.5)
Depc-H_2O	1ml Diethylpyrocarbonate,1000 ml dd H_2O
Fix soln	100 ml isopropanol, 100 ml Acetic acid, 800 ml dd H_2O
Luria Bertani (LB) media	For one liter, 10 gm Tryptone, 5 gm yeast extract,10gm NaCl

II. Methods

1. Cell culture :

Rat glomerular MC were cultured by a limited dilution procedure as described previousely (Pfeilschifter & Vosbeck, 1991). MC were grown at 37°C and 5% CO_2 and cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 2mM glutamate, 5ng/ml insulin, 100

U/ml Penicillin and 100ug/ml Streptomycin. To obtain quiescent cells, MC were maintained in serum-free Dulbecco's modified Egale 's medium (DMEM) supplemented with 0.1mg/ml of fatty acid-free bovine serum albumin for 24 hours before cytokine treatment. For passaging, the cells were washed with PBS and incubated with trypsin-EDTA solution for 2 minutes at 37°C. After cells have dislodged from the surface, culture medium was added and cells were diluted and transferred to new culture dishes. MC were used between passage 8 and 19. For the experiments, 3.0-5.0X10^6 cells per 10 cm culture dish were used . The determination of cell numbers was done by the use of a Neubauer chamber. The amount of dead cells was determined by tryban blue exclusion. Viability of MC was not altered under the conditions used for the experiments described as determined by lactate dehydrogenase measurement using a cytotoxicity detection kit. For determination of the gelatinolytic activity of cellular supernatants, 1.0-1.5 X 10^6 MCs were grown on six-well plates and the experiments were performed in triplicates .

2. Manipulation of DNA

A. Molecular cloning of PCR products :

To get a permenent source of any foreign DNA it is often desired to clone PCR products into appropriate cloning vectors.

Restriction

restriction endonucleases isolated from bacteria specifically bind palindromic sequences with a subsequent cleavage of the DNA molecule at their specific recognition sites. This process can generate either blunt end fragments or overhanging cohesive ends, which allow the generation of recominent DNA by appropriate enzymatic ligation. Blunt end PCR products were produced by using a Pfu DNA polymerase for the polymerase chain reaction since this enzyme removes single stranded ends. The cloning vector bluescript KS was linearized by digestion with the restriction endonuclease EcoRV which produces blunt ends. Generally, 10

µg of the TOPO cloning vector were incubated with 10 Units of EcoRV and 2 µl of the appropriate 10 x restriction endonuclease buffer in a total volume of 20 µl for at least 2 hours at 37°C. Both PCR products and cloning vector were separated on a 1% agarose gel in 1 x TBE-buffer. The bands which correspond to the expected DNA were excised and extracted from the gel using the QIA quick gel extraction kit from Qiagen (Hilden, Germany). The elution of this column results in a final volume of 30 µl containing the purified PCR product.

Ligation

To create recombinant plasmid DNA, 2 µl of the cloning vector and 15 µl of eluted PCR product, 2 µl of 10x T4 DNA ligase buffer and 1 µl of T4 DNA ligase enzyme were mixed and incubated overnight at 15°C.

B. Introduction of plasmid DNA into *E*.coli (Transformation) :

Competent bacterial cells

To yield high transformation efficiencies, competent cells were prepared as followed. One single colony of E. coli was inoculated in 5 ml LB medium and incubated overnight at 37°C with shaking. 2 ml of the culture were inoculated into 200 ml LB medium and let grown at 37°C until the suspension reached an optical density of 0.5 (OD 600 nm). The bacterial culture was pelleted by centrifugation at 1600 x g and the pellet subsequently resuspended in 10 ml of ice-cold $CaCl_2$ solution. The resuspended cells were kept on ice for 30 minutes and centrifuged again 5 minutes at 1100 x g at 4°C. The supernatant was discarded and the pellet was resuspended in 2 ml ice-cold $CaCl_2$ solution. 250 µl aliquots were dispensed into prechilled polypropylene tubes and frozen immediately at –70°C.

Transformation

For transformation, approximately 10 µl of ligation reaction (10 ng DNA) were mixed gently with 100 µl of competent cells and placed on ice for 30 minutes. The cells were heat shocked at 42°C for 45 seconds. 500 µl of LB-medium was added and the tubes were incubated at

37C° for 1 hour with shaking. 50 µl of the transformation culture was plated on LB/ampicillin plates and incubated overnight at 37C°.

C. Isolation of plasmid DNA

Isolation of small quantities of plasmid DNA (miniprep) :

Isolation of small quantities of plasmid DNA was performed by alkaline lysis as described previously (Birnboim and Doly 1979). 2 ml sterile LB/ampicillin medium was inoculated with a single bacterial colony from a freshly streaked LB/ampicillin plate and let grown at 37°C with shaking overnight. 1 ml of bacterial cells were spined 1 minute at maximum speed (13,000 rpm) and the supernatant was removed. The pellet was resuspended in 100 µl of buffer 1 and 200 µl of buffer 2 ,150 µl of buffer 3 were added and mixed. The cells were centrifugated 15 minutes with maximum speed at 4°C to pellet the cell debris and chromosomal DNA. The supernatant was transferred to a fresh tube and mixed with 800 µl of isopropanol to precipitate nucleic acids. The tubes were spined 15 minutes with maximum speed at 4°C to pellet the plasmid DNA. The supernatant was removed and the pellet was washed with 100 µl 96 % ethanol (by centrifugation for 10 minutes at full speed), air dried and resuspended in 30-50 µl H_2O.

Isolation of large quantities of plasmid DNA (maxiprep) :

Isolation of large scale of plasmid DNA was also performed by alkaline lysis. The QIAGEN maxi kit was used, which uses an anion exchange resin as an additional purification step. The bacterial cells (100 µl) were let grown in 100 ml LB/ampicillin medium at 37°C overnight with vigorous shaking. The bacterial cells were harvested by centrifugation at 4,000 rpm for 10 minutes at 4°C. The pellet of bacterial cells were resuspended in buffer 1 (12 ml), Subsequently buffer 2 (12 ml) was added and immediately mixed gently and incubated at room temp for 5 minutes. Buffer 3 (12 ml) was added and immediately mixed gently. The suspension was incubated on ice for 5 minutes before being centrifugated at high speed

(14,000rpm) for 40 minutes at 4°C. The supernatant carefully removed from the white precipitate and loaded onto a NUCLEOBOND AX cartilag previously equilibrated with buffer N2 (6 ml). The cartilage washed twice with buffer N3. The plasmid DNA was eluted with buffer N5 (15 ml) and precipitated by use of isopropanol (11 ml). Finally, the precipitated DNA was washed with 70 % ethanol and subsequently air-dried at room temperature before the DNA was dissolved in H_2O or TE-buffer.

3. Sequence analysis :

The 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. For sequencing, 250 ng DNA, 10 pmol primer and 2 µl BigDyeTerminator premix (PE Biosystems) were diluted to 10 µl with douple distilled H_2O and cycled in a thermocycler (GeneAmp 2400, PE Biosystems) under the following conditions: 25 cycles (denaturation:96°C, 10 seconds; annealing: 55°C, 5 seconds; elongation: 60°C, 4 minutes). The DNA was isolated by sodium acetate and precipitated using 1/10 volume of 96% ethanol. The DNA pellet was resuspended in 25 µl template suppression reagent (TSR) and denatured at 95°C for 2 minutes. This sequencing reaction was subjected to the Genetic Analyser.

4. Reverse transcriptase polymerase chain reaction (RT-PCR)

A. Reverse transcription (generation of cDNA from total RNA) :

The enzyme reverse transcriptase synthesizes a complementary DNA strand using RNA as a template. 1 µg of total RNA isolated from cultured MC in 12.5 µl DEPC-treated water and 1 µl of 20 µM oligo(dt) primer were added to a 0.2 ml PCR tube, heated to 72°C for 2 minutes and snap cooled on ice. The following components were added to the tube :

4 µl 5x reaction mixture

1 µl dNTP mix (2mM) [dATP, dCTP, dGTP, dTTT]

0.5 µl Rnase inhibitor (20 Units)

1 µl MMLV reverse transcriptase (40 Units)

The content of the tube was mixed by pipetting up/down and the reaction was incubated at 42°C for 1 hour. Subsequently the enzyme was heat inactivated by heating the reaction mixture at 95°C for 5 minutes and diluted to a final volume of 100 µl by adding 80 µl of DEPC-treated water. This reaction product is subsequently used as " RT-product".

B. Polymerase chain reaction (PCR) : The polymerase chain reaction is used to amplify a sequence of DNA using a pair of oligonucleotide primers each complementary to one end of the DNA target sequence. These are extended towards each other by a thermostable DNA polymerase in a reaction cycle of three steps: denaturation, primer annealing and polymerisation. In a PCR tube the following components were mixed :

2 µl of " RT product "

10 µl 5x Taq-polymerase buffer

5 µl d NTP-mix (2mM) [dATP, dCTP, dGTP, dTTT]

1 µl forward primer 50 µM

1 µl reverse primer 50 µM

1 µl Taq-polymerase 1 Units

The PCR reaction was performed in a thermocycler (Gene Amp 2400 or 9600, PE Biosystems). The PCR products were run on 1 % agarose gels containing 0.5µg/µl ethidium bromide to analyze nucleic acids. Usually the identification of PCR products was confirmed by sequencing analysis using a 310 genetic analyser (Perkin Elmer Corp) as described on page 22.

5. Quantification of nucleic acids

Concentration of nucleic acids were determined photometrically using a wavelength of 260 nm (Gene Quant, Amersham Pharmacia). An optical density (OD) of 1 corresponds to approximately 50 µg/ml double-stranded DNA or 40 µg/ml for RNA (Sambrook et al; 1989).

6. 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 analyzed nuleic acids. Agarose was dissolved in 1x TAE gel electophoresis buffer by boiling in a microwave. The solution was let to cool down with moderate stirring to avoid air bubbles. Ethidium bromide was added to a final concentration of 500 ng/µl. Ethidium bromide binds to DNA by intercalation between the bases and thus enables visualization upon ultraviolet fluorescence illumination of nucleic acids. The DNA probes were diluted with loading buffer (6x loading buffer) 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 base pair markers.

7. DNA isolation from agarose gels

The "NucleoSpin-DNA-Extraction-kit" was used to get 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.

8. cDNA clones and Plasmids :

A cDNA insert of 0.7 kb for rat 92 kDa type IV collagenase was generated by reverse transcription from mRNA of MC stimulated with IL-ß using internal primers from the complete sequence of rat mRNA (deposited in the Genebank/EMBL Database under accession No. U 36476).

The following primers were used :

5`-CTTAGATCATTCTTCAGTGCC-3`

5`-GATCCACCTTCTGAGACTTCA-3`

The blunt-ended polymerase chain reaction fragment was cloned into EcoRV cloning site of pBluescript-II ks to generate PKs-MMP-9 rat . A cDNA insert for HuR was a generous gift from Dr. Henry Furneaux (Memorial Sloan Kettering Cancer center, New York,USA). It was generated as described (Ma et al.,1996). A cDNA insert for rat TIMP-1 was generated using internal primers of a partial coding sequence of TIMP-1 mRNA (accession No. L29512). The primers were 5′-CAG ACG GCG TTC TGC AAC TCG (sense) and 5′-AGA CCC AAG GGA TTG CCA GGT-3′ (antisense). PCR fragments was then cloned into EcoRV-cut pBluescript-II KS+ to generate "pKS-TIMP-1 rat". A cDNA for rat TIMP-2 was generated using internal primers of coding sequence of rat TIMP-2 mRNA (accession no AJ 409332). The following primers were used: 5′-CGG GAA TGA CAT CTA TGG CAA CC-3′(sense) and 5′-AAA GCT GGA CCA GCC TCG ATG TC-3′(antisense).

A GAPDH cDNA clone was generated using internal primers of coding sequence of rat GAPDH mRNA (accession no. NM 017008). A cDNA insert from mouse 18S rRNA was from Ambion (Austin, TX, USA).

9. Isolation of total RNA and Northern blot analysis

A. Isolation of total RNA : Total cellular RNA was extracted from MCs using Trizol reagent. MCs that were cultured in 10 cm dishes were rinsed with PBS and 1ml of Trizol reagent was added. MC were scraped by rubber policeman and mixed several times by pipetting up/down and transferred to RNase-free 1.5 ml Epp. tubes. 0.2 ml chloroform was added, mixed by vortexing for 10 seconds and the tubes were kept at room temperature for 5 minutes and centrifugated at 13,000 rpm for 15 minutes at 4°C. The upper aqueous phase was transferred to a clean tubes and precipitated with isopropanol by centrifugation at 13000 rpm for 30 minutes at 4°C. The pellet was washed with 70% Ethanol-Depc water and air dried by incubation at 37°C untile the white pellet become opaque. The pellet was redissolved in 50-80 µl of Depc-treaed water according to the size of pellet. The RNA was quantified photometrically using a wavelength of 260 nm (GeneQuant, Amersham pharmacia, Freiburg, Germany).

B. Northern blot analysis (Transfer of total RNA by N.b.)

For 150 ml gel solution : 1.5 gm agarose (final 1%) and 131 ml H_2O were boiled in a microwave oven in a 200 ml Erlenmeyer flask and let to cool down with moderate stirring (using fish) to avoid air bubbles and then : 15 ml 10x MOPS ,4ml 37% formaldehyde were added. The prepared solution (gel solution) was poured into gel-tray under the hood and let to dry for 30 minutes .

RNA samples were prepared as followed : 20µg RNA were adjusted to a total volume of 15 µl with Depc-treated H_2O and were mixed with equal volume (15 µl) of 2x denaturation

buffer (prepared freshly). The denaturating mixture was subsequently incupated at 65°C for 15 minutes and mixed with 6µl of a 6x RNA loading dye. The RNA samples were loaded on the gel and run in 1x MOPS at 80 V for approximately 4 hours . The RNA was then transferred from the gel to a nylon membrane by upward capillary transfer with 20 x SSC overnight and afterwards fixed by UV cross-linking. The membrane was stained with methylene blue solution to check for equal loading of RNA and destained with washing buffer 2 .The membrane was placed in a hybridization tube with 10 ml hybridization buffer and incubated in a hybridization oven with rotation for at least 3 hours at 42°C . Labeling of the cDNA probe by random priming using the (rediprime) DNA labelling system. 25 ng of the DNA were denatured for 5 minutes at 95°C before being added to the ready to use reaction tube (rediprime) and mixed with 5µl [α-^{32}P] dCTP (3000 Ci/mmol). The rediprime reaction was performed at 37°C for 30 minutes. A sephadex- Nick column was used to separate the DNA from unincorporated ^{32}P-labeled nucleotides. 200µl of ssDNA (10mg/ml) was added to the labeling reaction and subsequently denatured at 95°C for 5 minutes. The radioactive probe was added to the hybridization tube and hybridization was continued with rotation overnight at 42°C. The membrane was washed with washing buffer 1 for 20 minutes at 42°C three times and one time with washing buffer 2 for 30 minutes at 65°C and exposured overnight on a phosphoimaging screen. Specific signals were quantitated using an automated detector system BAS1500 from Fujifilm (Raytest, Straubenhardt, Germany).

10. Isolation of total cellular Proteins and Western blot analysis

A. Isolation of proteins :

MCs (approximately 3.0-5.0X10^6) cultured in 10 cm dishes were rinsed with PBS, scraped into 1ml of lysis buffer (with protease inhibitors) and left on ice for 15 minutes. The extracts were sonicated three times (10 seconds) and centrifuged at 13,000 rpm for 10 minutes.

Aliquots of SN (total protein) were collected, freezed in liquid nitrogen and stored at –80°C. The protein concentration was determined by micro-protein assay (Bio-Rad laboratories, Zurich, Switzerland).

B. Quantification of proteins

The proteins were quantified by using MicroBCAprotein assay reagent kit. TheMicroBCAprotein reagent is a highly sensitive reagent for the quantitative colorimetric determination of total protein in dilute aqueous solution. This unique reagent system utilizes bicinchoninic acid (BCA) as the detection reagent for Cu^{+}, which is formed when Cu^{2+} is reduced by protein in an alkaline environment (Smith et al., 1985). The purple colored reaction product is formed by the chelation of two molecules of BCA with one cuprous ion (Cu^{+}). This water soluble complex exhibits a strong absorbance at 540 nm that is linear with increasing protein concentrations. A fresh set of protein standards were prepared by diluting the 2mg/ml BSA stock standard. BSA concentrations between10-70 µg/ml were used to generate a standard curve. 100 µl of each standard, unknown samples (1 µl of each sample diluted in 100 µl H_2O) were pipetted into a 96 well plate and 100 µl of H_2O were used as blank. 100 µl of the working reagent (25 parts of reagent A, 24 parts of reagent B and 1 part of reagent C) were added to each well. The plate was covered and incubated at room temperature for 1 hour. The optical density was measured at a wavelength of 540 nm using a microplate reader (Bio Rad). The absorbition values were calculated using microplate manager 4.0 software (Bio Rad).

C. Western blot analysis :

i. SDS gel electrophoresis

Proteins were subjected to SDS-Polyacrylamide gel electrophoresis as originally described by Laemmli (1970). In general a total of 50-150µg of total protein from each sample was mixed with an equal volume of 2x electrophoresis sample buffer and incubated at 95°C for 10

minutes for denaturation. The samples were loaded on the gel and the gel was run at 20 mA for a period of 2-3 hours.

ii. Protein transfer to nitrocellulose membrane

After gel electrophoresis, the proteins were transfered onto a nitrocellulose (PVDF) membrane by semi-dry electroblotting. Prior to use , the membrane had to be activated with isopropanol, The SDS gel containing the separated proteins and filter papers (Whatman 3MM) were damped in blotting buffer (Towbin transfer buffer). The gel was placed on top of the membrane. Finally, the gel was covered with one additional blotting buffer-soaked Whatman 3MM filter. The transfer was performed at 70 mA (constant) per minigel for 70 minutes. After blotting, the membrane was checked by Ponceau S staining for correct electrophoresis transfer and equal loading.

iii. Immunodetection

Non-specific binding sites on the nitrocellulose membrane were blocked by shaking the membrane in 2% BSA overnight at 4°C. The membrane was incubated with the primary antibody (at the appropriate concentration as suggested by the supplier) diluted in 1x TBST-buffer for 1 hour at room temperature and washed 4 times for 10 minutes each with 1x TBST-buffer. Afterwards the membrane was incubated for 30 minutes with the appropriate secondary antibody (coupled to horseradish peroxidase) and subsequently washed 4 times for 10 minutes with 1x TBST-buffer. For detection of the immunoreactive protein, the membrane was incubated in 2 ml ECL reagent and exposed for 1-5 minutes to X-ray film (Bio-Max film: Kodak). X-ray films were scanned by the GS 700 Imaging Densitometer (BioRad) and analyzed using the molecular Analyst software.

11. Isolation of nuclear extracts and Electromobility Shift Assay

A. Isolation of nuclear extracts from cultured MC :

MC were cultured in 10 cm dishes and rinsed with cold PBS. Before being harvested for nuclear extracts 1ml of ice-cold PBS/EDTA was added and incubated for 5 minutes on ice before cells were scraped with a rubber policeman. The cells were pelleted by centrifugation at 6000 rpm for 2 minutes at 4°C and SN were removed completely before the pellet was resuspended in 300µl of ice-cold buffer A and incubated on ice for 15 minutes. 20µl of NP-40 was added, the samples vortexed vigorously for 10 seconds and centrifugated at 13,000 rpm for 1 minute. The SN (cytoplasmic fraction) was collected , frozen in liquid nitrogen and stored at –80°C. The nuclear pellets were dissolved in 70 µl of ice cold buffer C and vigorously rocked on an Eppendorf shaker for 20 minutes with full speed at 4°C. Finally samples were centrifuged at 13,000 rpm for 10 minutes at 4°C and the supernatants were collected as nuclear extracts. The protein concentration was determined by microprotein assay as described on page 28.

B. Electrophoretic Mobility Shift Assay (EMSA) :

EMSA is the method for identification and investigation of DNA-protein interaction.

i.Gel preparation

For 30 ml gel solution :	28.8	ml	H_2O
	1.8	ml	10 x TBE
	5.4	ml	Acrylamid gel (30%)
	30	µl	TEMED
	400	µl	10 x APS

ii. Endlabeling of double-stranded DNA with T4 Kinase :

Approximately 2µl (20 pmol) of double stranded oligonucleotide was diluted with 10µl of distilled water, heated at 95°C for 2 minutes and quickly chilled on ice. 2µl of 10x PNK-

buffer, (20 units) of PNK, 5µl of [γ-^{32}P] dATP (6000 Ci/mmol) were added and kinase reaction run at 37°C for 1 hour. To allow a correct renaturation of single strand DNA the sample was left to cool overnight. Nick-column was used to separate the labeled oligonucleotide from unincorporated label. 2µl approximately of the probe was used per binding reaction .

iii. The binding reaction : The binding reaction was assembled by combining the following :

Radiolabeled probe DNA (1.0-0.5ng)

3 x binding buffer

DNA-binding protein (5µg of nuclear extract)

The final reaction volume was adjusted to 15-20µl with water or buffer C and incubated on ice for 30 minutes. For supershift analysis the antibodies were preincubated overnight at 4°C before addition of the labeled oligonucleotide.

iv. Electrophoresis of protein-DNA complexes :

Gel running : 4µl of 6x loading dye were added to each sample (binding reaction). The samples were loaded into the appropriate well of the gel using a Hamilton glass syringe and the gel was run at 200V (constant voltage) in 1x TBE buffer. The glass plates and side spacers were removed from the gel. The EMSA-gel was fixed with Fix solution for 30 minutes at room temperature. The gel was dried and for autoradiography subsequently exposed to an intensifying screen on a phosphoimager.

12. Nitrite analysis

Nitrite contents of the cellular supernatants were measured as a readout for NOS activity. Confluent MC (1.0-1.5 $\times$ 10^6 cells) on six-well plates were incubated in DMEM without FCS and stimulated with or without agents for the indicated time periods. After the incubation

nitrite, the stable end product of NO, was measured in the culture medium with the Griess method (Green et al., 1982) using a ready to use Griess reagent. 100 µl of culture media were mixed with 100µl of Griess reagent. The absorbance was measured at 540 nm and nitrite concentration was determined using a calibration curve with sodium nitrite standards.

13(A). SDS-PAGE Zymography

The gelatinolytic content of cellular supernatants was assessed by SDS-PAGE Zymography containing 0.1% gelatin as substrate. To ensure that the supernatants were derived from equal cell numbers, for each experimental condition, cell counts were obtained separately. In general 10-20µl of culture media were loaded on gels directly after the addition of 2 x sample buffer. After elecrophoresis, the denatured proteins were renatured by exchanging SDS with 2.5% Triton X-100 in two 15-minutes incubations. The gels were subsequently incubated overnight at 37°C in developing buffer. At the end of incubation, the gels were stained with staining solution. Destaining was performed using destaining solution for 30 minutes at room temperature and the proteins with gelatinolytic activity were visualized as area of lytic activity on an otherwise blue gel. Migration properties of proteins were determined by comparison with that of a prestained, full-range rainbow protein markers. Photographs of the gels were scanned by an imaging densitometer system from Bio-Rad laboratories (München, Germany)

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13(B). Cell-free incubation experiments :

By this assay, the direct effects of an exogenously given agent on MMP-9 can be monitored quickly. In general, supernatants (100µl) from MC treated for 24 hours with IL-ß was incubated with the agent to be tested for several hours at room temperature. As a positive control the same volume of supernatant was incubated with the indicated concentration of

APMA for the same time. At the end of the incubation, samples were mixed with 2x sample buffer and directly subjected to SDS-PAGE Zymography as described on page 32.

14. Isolation of cytoplasmic extracts and RNA-EMSA

A. Isolation of cytoplasmic extracts : Cytoplasmic extracts were prepared from rat MC by lysis in a hypotonic extraction buffer as described before (Page 29). Nuclei were removed by centrifugation and the supernatants used as cytoplasmic fractions. To achieve RNase-free conditions all buffers used were prepared with DEPC-treated water.

B. RNA Electromobility Shift Assay (EMSA) and (supershift analysis) : Radiolabeling of single stranded RNA oligonucleotides was performed by Kinase reaction using T4 polynucleotide kinase. Similar to the labeling protocol used for double-stranded DNA (page 30), after kinase reaction the labeled oligonucleotides were separated by Nick-Sephadex columns. The radiolabeled RNA oligonucleotides (approximately 30,000 cpm/reaction) were mixed with 6 µg of cytoplasmic extracts and incubated at room temperature for 15 minutes in the reaction buffer (containing 10 mM HEPES pH 7.6, 3mM $MgCl_2$, 40mM KCl, 2mM DTT, 5% glycerol and 0.5% Nonidet P-40). To reduce non-specific binding total yeast RNA (200 ng/ml final concentration) was added. The total volume of each reaction was 10 µl. RNA-protein complexes were separated in 6% non-denaturing polyacrylamide gels and run in 1x TBE buffer. The characterization of RNA-bound complexes was performed by supershift analysis. It was done by addition of supershift antibodies 15 minutes after addition of the radioactive labeled RNA oligo and incubated for a further 15 minutes at room temperature before the analysis was proceded as described before (page 31).

15. Transient transfection and reporter gene assay

i. Generation of reporter plasmids

A. Generation of PGL-MMP-9 plasmid (Cloning of rat MMP-9 promoter) :

The 5`-flanking region of the rat MMP-9 gene was cloned utilizing the Genome walker kit using internal (upstream) and external (downstream) primers from the rat MMP-9 cDNA (accession No. U36476) as follows :

MMP-9 internal primer: 5`-AGGGGCAGCAAAGCTGTAGCCTAG-3` .

MMP-9 external primer: 5`-TTTCAGGTCTCGGGGGAAGACCACATA-3`

A 1.8 kb fragment from a *EcoRV* cut library was isolated by PCR under stringent conditions. The fragment was subsequently subcloned into pBluescript-KS and sequenced using the automated sequence analyser ABI 310 (PE Applied Biosystems, Weiterstadt, Germany). The sequence has been deposited in the GenBank/EMBL databases (accession No. A7438266). Subsequently, the 1.8 kb fragment of MMP-9 promoter was subcloned into PGL-III Basic vector coding for beetle luciferase (Promega, Mannheim, Germany) using Xba I and Xho I restriction sites.

B. Generation of PGL-MMP-9-ΔPPRE-1 : Introduction of a double point mutation into a putative distal PPRE- like site (GT to CA) to generate PGL-MMP-9-ΔPPRE-1 was done, using the following (forward) primer: 5`- ATGGAGACTCAAGCACACCTATGTGT-3`(corresponding to a region from –1763 to –1783).

C. Generation of PGL-MMP-9-ΔPPRE-2 : Introduction of a double point mutation into second proximal laying PPRE-like binding sites to generate PGL-MMP-9-ΔPPRE-2 was done using the following (forward) primer : 5`-TCCCATCCAGCACACCCCGAGGCTTA-3`(corresponding to a region from –896 to –871). All mutant constructs were generated using the Quick Change Site-Directed Mutagenesis Kit.

**D. Generation of 3`-UTR-MMP-9 PGL3-MMP-9 :** The PGL3-MMP-9 encompassing a 1.3 kb fragment of the 5`-flanking region of the rat MMP-9 gene was cloned as described on page 34. The plasmid 3`-UTR-MMP-9 PGL3-MMP-9, 1.3 kb was generated by cloning a 670 bp fragment from the 3`-UTR of rat MMP-9 mRNA into the PGL3-MMP-9(Δ1.3kb) promoter plasmid. The 3`-UTR sequence of the rat MMP-9 gene was generated by PCR from reverse transcriptase products using the *Xba I*-flanked (underlined) forward primer 5`-TATTCTAGACCAACCTTTACCAGCTACTCGAA-3` and *Bam HI* flanked (underlined ) reverse primer : 5`-TATGGATCCATTCATTTATTTAAAAA-AGAGTGT-3`, corresponding to a region from 2315-2338 and 2954-2977 of the rat MMP-9 cDNA (GenBank, accession no U24441). The PCR products subsequently were digested with *XbaI* and *Bam HI* restriction enzymes and cloned into the *BamHI/XbaI* cut PGL3-MMP-9(Δ1.3) plasmid thereby allowing a forced insertion of the 3`-UTR of rat MMP-9 mRNA at the 3`-end of the luc+coding region of PGL3- MMP-9(Δ1.3).

**E. Generation of the 3`UTR-MMP-9 PGL3 control :** Also the *BamHI/XbaI* cut PCR fragment was cloned into an empty PGL3 control vector to generate 3`-UTR-MMP-9 PGL3 control. This vector was in parallel used in luciferase assays to test the influence of the 3`-UTR of MMP-9 on the stability of luciferase mRNA under the control of a none-MMP-9 related promoter sequence.

F. Generation of triple-point mutation within 3'-UTR-MMP-9 : Introduction of triple-point mutation into ARE-sites (ATTTA to ACCCA) to generate ΔARE mutations were performed using the following (sense) primer:

5´-TACCGGCCCTTTTACCCATTATGTATGTGG-3´ (corresponding to a region from 2494 to 2523) to generate "3´-UTR-ΔARE1 pGL3-MMP-9 (1.3kb)"

5´-TTCACACACATGTACCCAACCTATAGAATG-3´ (corresponding to a region from 2529 to 2558) to generate "3´-UTR-ΔARE2 pGL3-MMP-9 (1.3kb) "

5´-TTAGGGACAGAGGAACCCATTGGATGTTGG-3´ (corresponding to a region from 2735 to 2764) to generate "3´-UTR-ΔARE4 pGL3-MMP-9 (1.3kb) ".

All mutants were generated by use of the Quick Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The plasmid "3´-UTR-ΔARE1-2-4 pGL3-MMP-9 (1.3kb)" in which all three AREs have been mutated was generated using a construct bearing double mutated AREs ("3´-UTR-ΔARE1-2 pGL3-MMP-9 (1.3kb)" which then served as a template.

ii. Transient transfection of rat glomerular MC

Transient transfection of MC on 6-well plates was performed using the Effectene transfection reagent. Effectene represents a new class of lipid-based transfection reagent that spontaneously forms micelle structures. One day before transfection approximately 400,000 cells per well were seeded in 6-well plates. Optimally, Cells should have reached 40-80% confluence on the day of transfection. The cells were washed with PBS before 1.6 ml of medium (RPMI, 10% FCS) was added. Per well, 0.4 µg of firefly luciferase reporter vector DNA and 0.1 µg of renilla luciferase control reporter vector were mixed with the DNA condensation buffer (EC) and 3.2 µl of enhancer. The DNA mixture was vortexed for 1 sec and incubated at room temperature for 5 minutes. 10 µl of Effectene transfection reagent were added to the DNA mixture and the tube was vortexed for 10 seconds and incubated at room temperature for 10 minutes to allow complex formation. Per well, 600 µl of serum-free medium was added to the transfection complex and mixed by pipetting up/down. Finally, the transfection complex was added drop-wise onto the cells. The plates were swirled gently and incubated overnight at 37°C / 5% CO_2. The cells were washed with PBS and new medium with stimulating reagents was added for 24 hours.

iii. Reporter gene assay : To study reporter gene expression, the dual-luciferase reporter assay system from Promega was used. Luciferase catalyzes a bioluminescent reaction when mixed with a substrate, a flash of light is emitted that can be detected by a luminometrical detection. For analysis of gene expression, the MC (transiently transfected with a foreign promoter sequence) were washed with PBS before being harvested with 100 µl of cold lysis buffer and incubated on ice for 30 minutes. 50 µl of cell lysates were transferred into 5 ml Sarstedt tubes and subjected to luciferase measurement in a Luminometer (Berthold, Bad Wildbad, Germany). Following this step, 100 µl of stop & glo reagent that contains an inhibitor of firefly luciferase and the substrate for renilla luciferase, were injected and the light emission was measured again, representing the renilla luciferase activity.

16. Site-directed mutagenesis

In order to create a point mutation in the MMP-9 promoter region, site-directed mutagenesis technique was used. By this technique, two mutagenic primers with a length of 25-45 bases both containing the desired mutation and annealing to the same sequence on opposite strands of the plasmid are used for a PCR reaction using a highly efficient Turbo Pfu polymerase. For the PCR reaction, 50 ng dsDNA template, 5 µl 10 x PCR reaction buffer, 125 ng primer 1, 125 ng primer 2, 1 µl dNTP mix (10 mM each nucleotide), 1 µl Pfu Turbo DNA polymerase (2.5 U/µl) and H_2O (was added to 50 µl final volume) were mixed. The reactions were cycled under the following conditions : 30 seconds at 95°C before 18 cycles (denaturation: 95°C, 30 seconds; annealing: 55 °C, 30 seconds; elongation: 68°C, 10 minutes). Following temperature cycling, 1 µl of the Dpn-I restriction enzyme (10 U/µl) was added directly to the reaction to digest the methylated parental (non mutated) DNA template. The reaction was incubated at 37 °C for 1 hour. 1 µl of this reaction was transferred to an aliquot of competent cells for transformation as previously described on page 20.

17. Transcripition assay

Transcripitional assay is currently the most sensitive procedure for measuring specific gene transcripition as a function of cell status. In this assay, we combined the classical run-on protocol with that of the more sensitive RNase protection assay and proceded as follows. Nuclei were first isolated from cultured cells and frozen in liquid nitrogen. Nuclei were thawed and incubated with ^{32}P-labeled UTP and unlabeled NTPs to label nascent RNA transcripts. ^{32}P-labeled RNA was purified and used to detect specific RNA transcripts by hybridization to cDNA immobilized on nitrocellulose membranes.

Isolation of Nuclei : Cells were rinsed twice with 5 ml ice-cold PBS and scraped with a rubber policeman. Subsequently, cells were collected in a 15-ml centrifuge tube and centrifuged at 1500 rpm for 5 minutes at 4°C. The supernatants were removed and the cell pellets were resuspended by gently vortexing 5 seconds and 4 ml of NP-40 lysis buffer A were added during the vortexing. After lysis buffer was completely added, the cells were again vortexed gently for 10 seconds. The lysed cells were incubated on ice and a few microliters of cell lysate were examined microscopically to ensure that cells were uniformly lysed and nuclei appeared free of any cytoplasmic contaminants. The lysed cells were centrifugated 5 minutes at 1500 rpm at 4°C. The nuclear pellets were resuspended in 4 ml of NP-40 lysis buffer by gentle vortexing and centrifuged 5 minutes at 1500 rpm at 4°C. The nuclei were resuspended in 100-200 µl of glycerol storage buffer by gently vortexing and finally frozen in liquid nitrogen.

Nuclear runoff transcripition : 200 µl of frozen nuclei were thawed at room temperature and transferred to a 15-ml conical polypropylene centrifuge tube. 200 µl of 2x reaction buffer with nucleotides plus 10 µl of 10 mCi/ml [α-^{32}P] UTP were added and the tubes were incubated 30 minutes at 30°C with shaking. 40 µl of 1mg/ml RNase-free DNase I were mixed with 1 ml of HSB buffer. 0.6 ml of this solution was added to labeled nuclei, mixed by

pipetting up/down 10-15 times with a Pasteur pipet and incubated 5 minutes at 30°C. 200 µl of SDS/Tris buffer were mixed with 10 µl of 20 mg/ml proteinase K. 0.1 ml was added and incubated for 30 minutes at 42°C.

RNA isolation : The nuclear RNA was isolated by using the Tri-reagent and subsequent chloroform extraction followed by ethanol precipitation.

Hybridization of cDNA with radioactive RNA : A cDNA clone for rat MMP-9 (pKs-MMP-9 rat) was linearized with Pvu II and used as a template for MMP-9 antisense RNA. The MMP-9 antisense transcript was synthesized in vitro using T7 RNA polymerase and α-^{32}P UTP (3000 Ci/mmol). Nuclear RNA and 100,000 cpm of the labeled T7-derived MMP-9 antisense transcripit were co-precipitated by ethanol precipitation and hybridized at 42°C overnight in 30 µl of FAB hybridization buffer. Following hybridization, samples were digested with RNases A and T1 for 1 hour at 30°C. RNA samples were again extracted with phenol/chloroform and protected double-stranded RNA hybrids were ethanol precipitated. The protected fragments were separated on a 5% acrylamide/8M urea gel and analyzed using a phosphoimager.

18. In vitro degradation assay

Degradation assays were performed by following a protocol from Levy et al. (1996) with the following modifications. In stead of using *in vitro* transcribed radioactively labeled MMP-9 mRNA we used cytokine-induced total RNAs containing a high levels of MMP-9 transcripts. 20 µg of total RNA from one pool were aliquoted and subsequently incubated at room temperature with 130 µg of cytoplasmic extract derived from vehicle or from MC treated for different time points with different agents. At each time point the RNA from these incubations was isolated by standard procedures as described on page 25 and assessed by Northern blot analysis using a ^{32}P-labeled cDNA insert specific for rat MMP-9. The

remaining (undegraded) MMP-9 signals were visualized and quantified using an automated detector system BAS 1500 from Fujifilm (Raytest, Straubenhardt, Germany).

19. HuR-neutralization experiments

The impact of mRNA stabilizing protein (HuR) on the degradation of MMP-9 was tested by the addition of a neutralizing monoclonal anti-HuR antibody. 0.4 µg of the monoclonal antibody was added to the cytoplasmic extracts and preincubated at room temperature for 1 hour before addition of the RNA samples. To exclude any unspecific inhibitory effects achieved by the mouse serum we tested in parallel normal mouse serum. Subsequently the RNA was isolated and degradation monitored by in-vitro degradation assay.

20. Immunocytochemistry of HuR

At approximately 60-80% confluency, mesangial cells were rendered serum-free for 24 hours and thereafter stimulated with nucleotides for the indicated time periods. Subsequently, cells were washed with ice-cold phosphate-buffered saline (PBS) and incubated for 30min at -20°C with methanol containing 0.02% (w/v) EDTA. Cells were then washed twice with PBS, blocked for 1h in PBS containing 3% (w/v) BSA and incubated for 2 hours with monoclonal anti-HuR antibody. Cells were then washed several times with PBS and incubated for 1 h with an anti-mouse-Alexa 488-coupled secondary antibody and thereafter washed again with PBS. Cellular fluorescence was monitored using confocal microscopy (MicroRadiation, BIORAD, Hertfordshire, U.K.).

21. Statistical analysis

Results are expressed as means $\pm$ S.D. The data are presented as x-fold induction compared to control conditions (∗) or compared to IL-1ß stimulated values (#). Statistical analysis was

performed using studient's t test and ANOVA test for significance. P values < 0.05 (∗), (#) and < 0.01 (**), (##) were considered significant.

3. Results

1. Inhibition of cytokine-induced MMP-9 expression by PPARα agonists is indirect and is due to a NO-mediated reduction of mRNA stability

Introduction

Dysregulation of extracellular matrix (ECM) turnover is an important feature of glomerular inflammatory processes and may result in the loss of the mechanical and functional integrity of the glomerulus [Davis et al.,1992; Johnson et al.,1994; Fogo, 2001]. Physiologically, the balance between synthesis and degradation of matrix proteins is guaranteed by the action of a family of zinc-dependent, neutral proteinases, designated matrix metalloproteases (MMPs). A tight regulation of these proteases is accomplished by different mechanisms, including the regulation of gene expression, the processing of the inactive zymogenes by other proteases and, finally, the inhibition of the active enzymes by the action of endogenous inhibitors of MMPs, the TIMPs [for review see Nagase et al.,1999]. Cultured mesangial cells (MC) respond to proinflammatory cytokines such as tumor necrosis factor (TNFα) or interleukin-1ß (IL-1ß) with the production of several MMPs, including MMP-9 (gelatinase B) mainly due to an increase in gene transcription [Yokoo et al.,1996; Eberhardt et al., 2000]. The transcriptional regulation of the rat MMP-9 gene by proinflammatory cytokines is localized to a 0.7 kb region upstream from the transcriptional start site and critically depends on the binding sites for AP-1 and NF-κB transcription factors, respectively [Yokoo and Kitamura 1996; Eberhardt et al.,2000a; 2000b]. Besides MMP-9, MC under inflammatory conditions can express a variety of genes coding for potent inflammatory mediators, such as inducible nitric oxide synthase (iNOS), the cytosolic and secreted types of phospholipases A_2 (PLA_2), and cyclooxygenase 2, respectively [Pfeilschifter et al.,1991; 1993; Martin et al., 1994; Huwiler et al.,1997]. Metabolites of these enzymes themselves can have modulatory effects on MMP-9 expression as we have previously reported a NO-mediated suppression of

cytokine-induced MMP-9 expression [Eberhardt et al., 2000a]. Pharmacologically, the transcriptional activation of MMP-9 is modulated by agonists of the glucocorticoid receptor mainly through interference with NF-κB and members of the Ets/PEA transcription factor family (Eberhardt et al., 2002a). We now searched for a possible modulation of MMP-9 expression by agonists of an other member of the nuclear receptor superfamily, the peroxisome proliferator-activated receptors (PPARs) [for review see Corton et al., 2000]. Three distinct PPAR subtypes have been identified: PPARα, ß/δ, and γ, with each subtype showing a specific distribution, physiologic function and a specific set of endogenous as well as synthetic ligands. Physiologically, PPARs are key players in lipid and glucose metabolism and, therefore, disturbances of PPAR function have been implicated in a variety of metabolic disorders such as dyslipidemia and diabetes [Schoonjans et al., 1996]. In addition, PPARα, similar to PPARγ, has been reported to exert potent anti-inflammatory activities in various cell types by inhibiting the expression of inflammatory genes such as COX-2 [Staels et al., 1998], IL-6 [Delerive et al., 1999], endothelin-1 [Delerive et al., 1999] and various acute-phase proteins [Delerive et al., 2001] just to name a few of them. In accordance with these *in vitro* data, PPARα-deficient mice display an exacerbated response to inflammatory stimuli [Staels et al., 1998; Delerive et al., 1999]. Moreover, PPARα null mice exhibit a higher degree of kidney damage in response to ischemia/reperfusion injury when compared to wild type controls thus indicating that PPARα, in addition to its antiinflammatory actions, exhibits also cytoprotective properties in the kidney [Devchand et al., 1996; Portilla et al., 2000]. Interestingly, recent observations have demonstrated a role of glucocorticoids in PPARα gene expression in the development of rat kidney suggesting a cross-talk between both nuclear hormone receptor pathways [Djouadi and Bastin, 2001]. Although many studies have addressed the question how PPARs directly activate gene expression via PPREs, less information exists about negative mechanisms which mostly involve interference with

transcriptional activators. In this study we provide an additional mechanism by which PPARα agonists, independent of direct transcriptional effects, indirectly, via amplification of nitric oxide production can efficiently regulate the level of cytokine-induced MMP-9 expression.

Results :

Effect of the PPARα activator WY 14,643 on cytokine-induced MMP-9 activity and mRNA steady-state levels of MMP-9, TIMP-1 and TIMP-2

The family of gelatinases includes the two genetically distinct 72 and 92 kD type IV collagenases MMP-2 and MMP-9, respectively, which both are expressed in MC. Whereas MMP-2 shows a constitutive expression, MMP-9 expression is highly induced by proinflammatory cytokines such as IL-1ß [Eberhardt et al., 2000a; 2000b]. Since the expression of MMP-9 in MC after treatment with IL-1ß reaches a maximal level after 24h we chose incubation times of 24h. To evaluate possible effects of PPARα activators on the proteolytic activity secreted into the conditioned media from cytokine-treated cells, MC were treated with IL-1ß (2 nM) in the presence or absence of different concentrations of WY-14,643, a potent activator of PPARα [Corton et al., 2000]. The gelatinolytic content of conditioned medium of MC withdrawn after 24h of stimulation was tested by zymography using gelatine as a substrate. WY-14,643 reduced in a dose-dependent manner the IL-1ß-stimulated MMP-9 activity with a maximal inhibition seen at 100 µM. WY-14,643 alone had no effects on enzyme secretion (Fig. 3A). Higher concentrations used in 24h incubations caused cytotoxic effects as monitored by the MTT test (data not shown). The levels of the lytic activity of latent and active MMP-2, represented by the two lytic bands at 68-and 72 kD remained unchanged under all experimental conditions consitent with the constitutive expression of MMP-2 in rat MC [Eberhardt et al., 2000b]. To evaluate whether the reduction of the lytic content of MMP-9 is due to a decrease in the amount of the corresponding MMP-9 mRNA, we performed Northern blot analysis using a cDNA probe from the rat MMP-9 gene

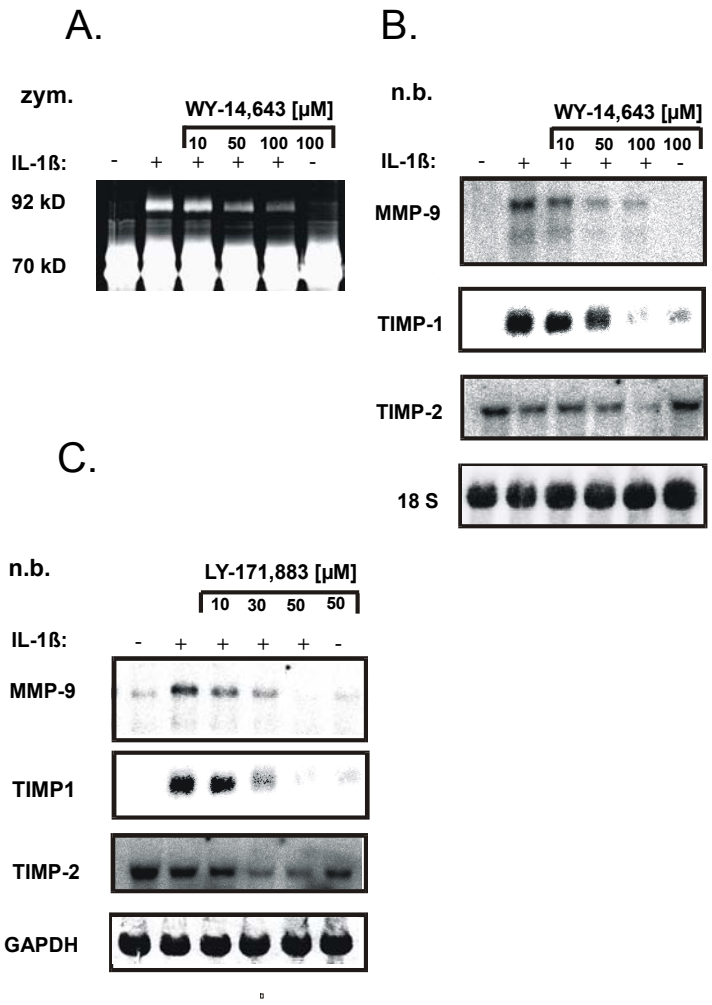

Figure 3. Inhibition of cytokine-induced MMP-9 and TIMP levels in MC by PPARα agonists. **(A)** Dose-dependent inhibition of the IL-1ß-induced gelatinase activity by WY-14,643. MC were simultaneously treated with vehicle (control) (-) or with IL-1ß (2 nM) (+) in the presence of the indicated concentrations of WY-14,643. 24 hours after stimulation 10 µl of cell supernatant was subjected to SDS-PAGE zymography(zym.) using gelatine as a substrate. The migrating properties of lytic bands corresponding to inactive pro-MMP-9 (92 kd) and the inactive and active froms of MMP-2 (72 and 68 kDa) are indicated by migration properties determined with molecular weight markers. The data are representative for three independent experiments giving similar results. (**B)** Northern-blot analysis (n.b.) demonstrating a dose-dependent modulation of IL-1ß-induced MMP-9, TIMP-1 and TIMP-2 mRNA steady-state levels by WY-14,643. MC were treated for 24 hours as indicated and subsequently extracted for total cellular RNA. 20 µg of total RNA were hybridized to ^{32}P-labeled cDNA inserts from rat MMP-9, TIMP-1 and TIMP-2 genes. Equivalent loading of RNA was ascertained by rehybridization to an 18S ribosomal probe. The data are representative for three independent experiments giving similar results. (**C)** Dose-dependent inhibition of the IL-1ß-induced MMP-9 mRNA steady-state level by LY-171,883. Quiescent MCs were coincubated for 24 hours with IL-1ß (2 nM) and the indicated concentrations of the PPARα activator LY-171,883. Total RNA (20 µg) were hybridized to ^{32}P-labeled cDNA inserts from rat MMP-9 and TIMP-1 and TIMP-2 cDNAs and equivalence of loading was ascertained by rehybridization to a GAPDH probe. The data are representative for three independent experiments giving similar results.

[Eberhardt et al., 2000a]. As shown in Fig. 3B WY-14, 643 dose-dependently attenuated the cytokine- induced MMP-9 mRNA level with a maximal inhibition seen at 100 µM WY. WY-14,643 given alone had no effects on the basal MMP-9 mRNA level. These data indicate that

the alterations of cytokine-induced zymogen activity by WY-14,643 predominatly result from changes in the MMP-9 expression levels. We next checked mRNA levels of TIMP-1 and TIMP-2, the endogenous inhibitors of MMP-9. Most interestingly, TIMP-1 followed a similar expression pattern as MMP-9 showing a strong increase of basal mRNA levels by IL-1ß and a dose-dependent attenuation of IL-1ß-induced steady-state mRNA level by WY-14, 643 (Fig. 3B). Similarily to MMP-9, basal TIMP-1 mRNA levels were not affected by WY-14, 643. In contrast, TIMP-2 displayed high basal steady-state mRNA levels which were attenuated by IL-1ß and furthermore attenuated by the highest concentration (100 μM) of WY-14, 643 (Fig. 3B). Furthermore, we tested other PPARα activators on MMP-9, TIMP-1 and TIMP-2 steady-state mRNA leves. The concentrations used were in a range known to maximally stimulate PPARα activation in cell culture. As shown in Fig. 3C, the prototypic PPAR ligand LY-171883, in a way similar to WY-14,643, dose-dependently inhibited the amounts of IL-1ß-induced MMP-9 and TIMP-1 steady-state mRNAs, respectively. The reduction of MMP-9 and TIMP-1 mRNA levels again was paralleled by a decrease of MMP-9 activity in the conditioned media (data not shown). In contrast, the high basal level of TIMP-2 mRNA, were reduced by IL-1ß and further attenuated by increasing concentrations of LY-171, 883 (Fig. 3C). These data demonstrate that the inhibition of MMP-9 levels is not paralleled by an increased expression of TIMPs.

WY-14,643 does not alter gelatinolytic activity of pro-MMP-9

To evaluate whether PPARα agonists are able to alter the activity of secreted MMP-9 we performed in-vitro zymography. Conditioned media from cytokine-treated MC were incubated for 16 hours with or without WY-14,643 (100 μM). The lytical band at 92 kD which is not detectable under control conditions but inducible by IL-1ß corresponds to the inactive proform of MMP-9 as is demonstrated by cleavage to the active 86-kDa form by treatment with different concentrations of p-amino phenylmercuric acetate (APMA) (Fig. 4B).

As shown in Fig. 4A, WY-14,643 does not alter the gelatinolytic activity of pro-MMP-9, thus indicating that the observed WY-mediated reduction of zymogen activity is due to a reduction of IL-1ß-mediated expression and secretion of pro-MMP-9.

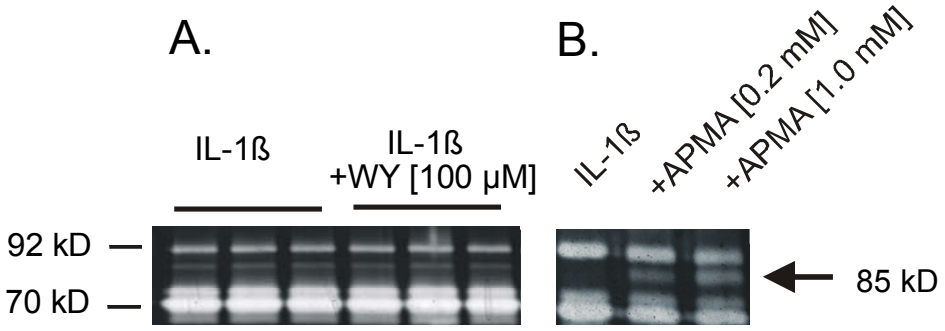

Figure 4. (A) WY-14, 643 does not affect extracellular IL-1ß-induced gelatinolytic activity of latent MMP-9. The conditioned media from MC stimulated for 24 h with IL-1ß were incubated for 16 h with WY-14,643 (100µM) at room temperature and subsequently assayed by SDS-PAGE zymography. **(B)** In vitro activation of latent MMP-9 by *p*-amino phenylmercuric acetate (APMA). The IL-1ß-inducible band corresponds to the inactive proform of MMP-9, which is cleaved to the active 86 kDa from by treatment with APMA. Supernatants form MC treated for 24 h with IL-1ß (2 nM) were incubated for an additional 3h with the indicated concentrations of APMA before being subjected to SDS-PAGE zymography. The results shown are representative for three independent experiments giving similar results.

Modulation of cytokine-induced MMP-9 promoter activity by PPARα agonists

To evaluate whether the PPARα-mediated inhibition of IL-1ß-induced MMP-9 steady-state mRNA level resulted from an inhibition of MMP-9 gene transcription, we cloned a 1.8 kb promoter fragment of the rat MMP-9 gene by genome walking using MMP-9 gene specific antisense primers as described in Materials and Methods. In addition to a multitude of putative elements involved in the cytokine-mediated regulation of MMP-9 expression we found by computational analysis two regions which displayed a high homology to PPRE-like motifs and which were denoted as "PPRE-1" and "PPRE-2", respectively (Fig. 5A). Transient transfection of MC with pGL-MMP-9 (1.8) comprising the 1.8 kb promoter fragment fused to a luciferase reporter gene was followed by a 24 h treatment with either vehicle, IL-1ß (2nM), WY-14,643 (100µm), or both compounds in combination and assayed for luciferase activity. IL-1ß significantly stimulated luciferase activity (2.67-fold, $p< 0.01$) and, surprisingly, this

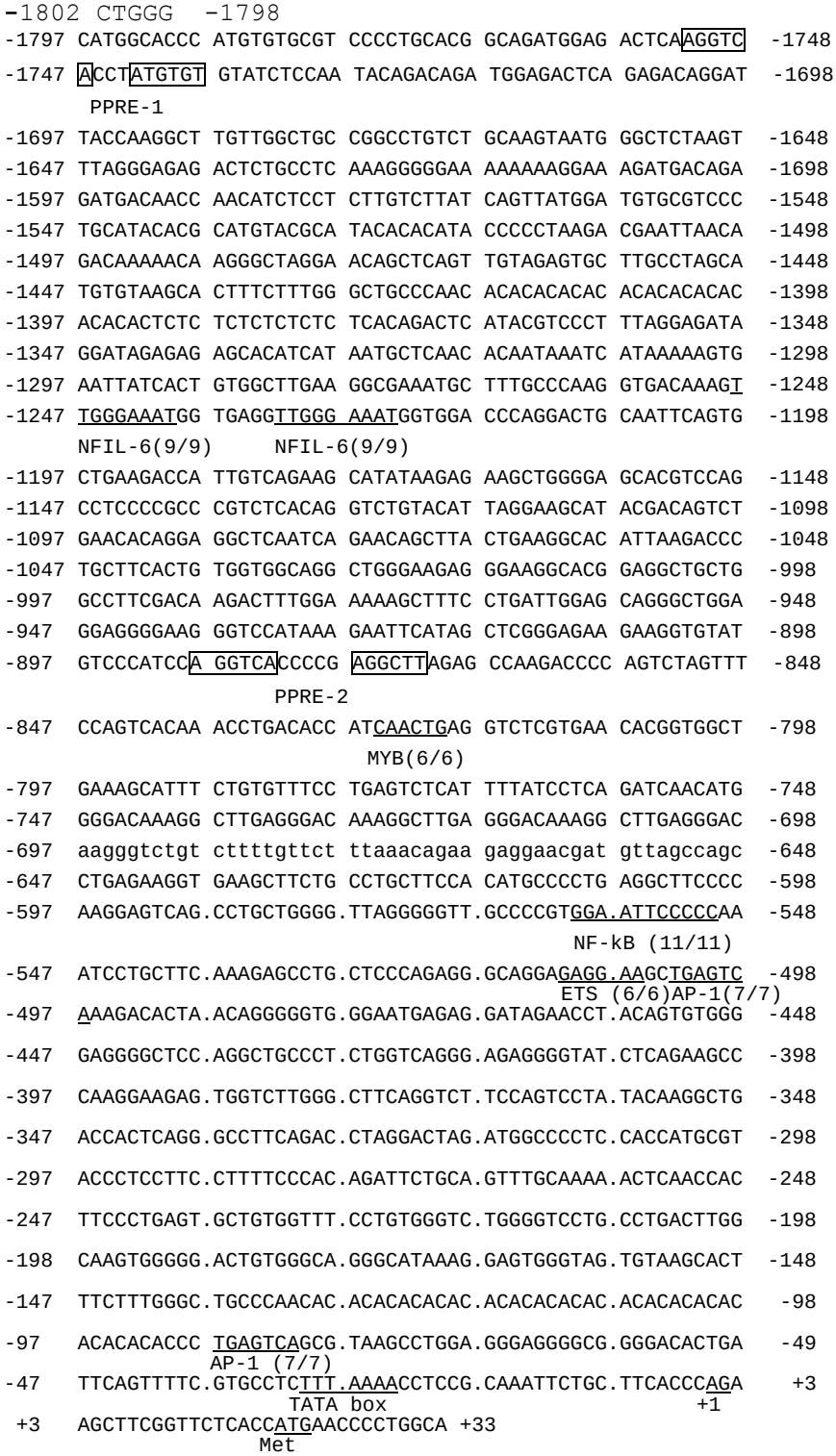

Figure 5. (A) Sequence of the upstream 1.8 kb of the rat MMP-9 gene promoter**.** Potential binding sites involved in the signaling of IL-1ß are underlined.

was further amplified by addition of WY-14,643 (4.1-fold, $p< 0.01$) which on its own had a moderate stimulatory effect on MMP-9 promoter activity (1.8-fold, $p< 0.01$, Fig. 5B). We recently have idenitified AP-1 and NF-κB-response elements, being crucially involved in the IL-1ß-mediated transcriptional activation of MMP-9 [Eberhardt et al., 2000b]. To test for a functional role of the two putative PPREs in the amplification of cytokine-induced MMP-9 promoter activity by PPARα ligands, each PPRE was point mutated by an exchange of 2 base pairs and tested for remaining promoter inducibility (Table 2). Mutation of the proximal PPRE at –888/ –872 ("PPRE-2") had no effect on promoter inducibility independent of which stimulus was used (Fig. 5C). In contrast, mutation of a distal laying PPRE at –1752/–1738 ("PPRE-1") prevented the potentiation of cytokine-induced MMP-9 promoter activity by WY-14,643 without affecting promoter activation by IL-1ß (Fig. 5D). These data suggest, that PPRE-1 in contrast to PPRE-2, is functionally involved in the amplification of cytokine-induced MMP-9 promoter activity by PPARα without affecting cytokine-induced transcription of MMP-9. Furthermore, these data demonstrate that the modulation of cytokine-induced mRNA steady-state level by PPARα agonists cannot be explained by regulatory events occuring in the upstream 1.8 kb MMP-9 promoter context.

PPARa activators enhance the cytokine-induced DNA-binding to PPRE-1

To further confirm the functionality of a cognate PPRE promoter binding site in the transcriptional amplification of MMP-9 we performed EMSA using a ^{32}P-labeled oligonucleotide comprising the critical PPRE-1 binding-site. Treatment of cells with IL-1ß (2 nM) or WY-14,643 (50, 100 μM) caused binding of a single slow migrating complex (Fig. 6A, left panel). Importantly, the intensity of DNA-binding was strongly increased when both reagents were given in combination (Fig. 6A, left panel). Similar results were obtained with a PPRE consensus oligonucleotide (data not shown). The identity of the DNA-bound complex

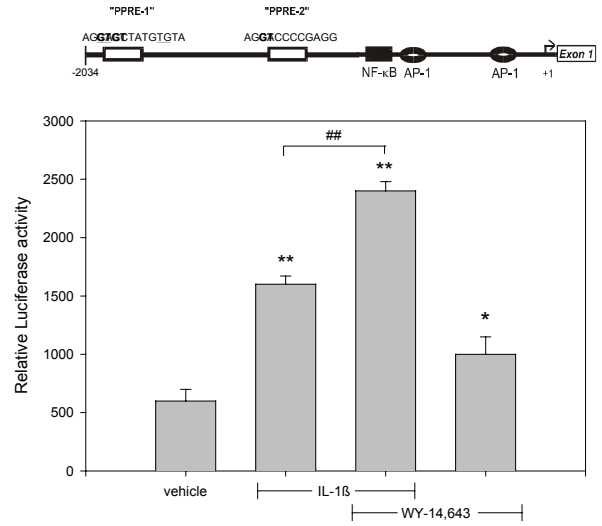

Figure 5 (B) A 1.8 kb wild type pGL-MMP-9 promoter construct and its corresponding promoter activation by IL-1ß and WY-14-643. Subconfluent MC were transiently cotransfected with 0.4 µg of pGL-MMP-9 (1.8) and with 0.1 µg of pRL-CMV coding for *Renilla* luciferase. After an overnight transfection MC were treated for 24 hours with vehicle (control), IL-1ß (2 nM), WY-14,643 (100 µM) or a combination of IL-1ß and WY-14,643 as indicated. The values for beetle luciferase were related to values for Renilla luciferase and are depicted as relative luciferase activities. Data are means ± S.D. (*n=6*). **P≤0.05; **P≤0.01* compared with vehicle, or IL-1ß-stimulated conditions (## P≤0.01).

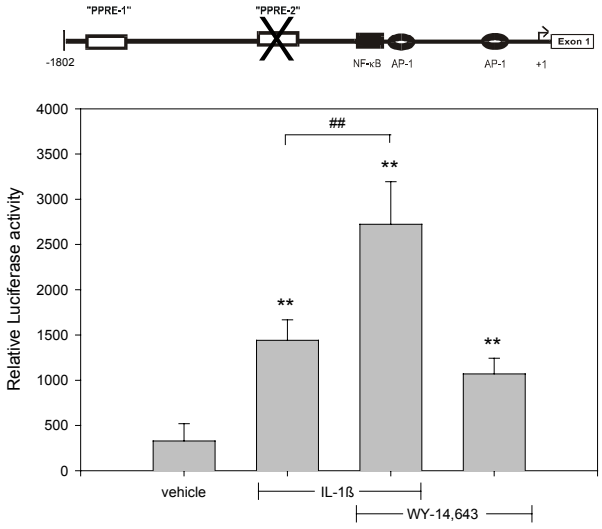

Figure 5 (C) pGL-MMP-9ΔPPRE-2 and its corresponding promoter activation by IL-1ß and WY-14-643. Subconfluent MC were transiently cotransfected with 0.4 µg of pGL-MMP-9 ΔPPRE-2 and with 0.1 µg of pRL-CMV coding for *Renilla* luciferase. After an overnight transfection MC were treated for 24 hours with vehicle (control), IL-1ß (2 nM), WY-14,643 (100 µM) or a combination of IL-1ß and WY-14,643 as indicated. The values for beetle luciferase were related to values for Renilla luciferase and are depicted as relative luciferase activities. Data are means ± S.D. (*n=6*). **P≤0.05; **P≤0.01* compared with vehicle, or IL-1ß-stimulated conditions (## P≤0.01)

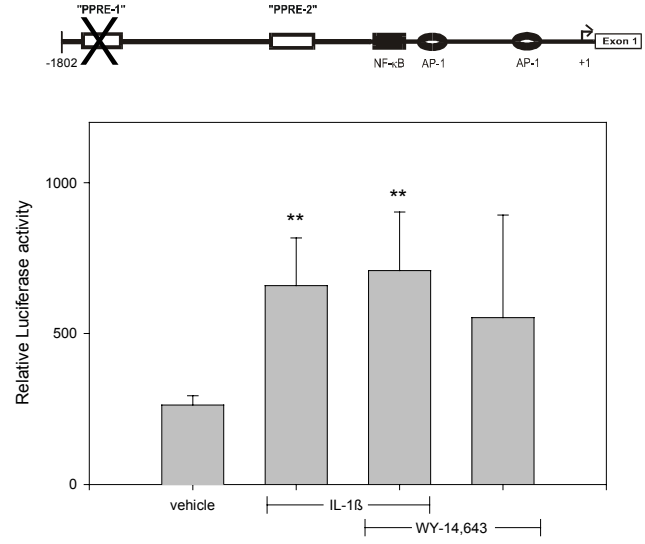

Figure 5 (D) pGL-MMP-9ΔPPRE-1 and its corresponding promoter activation by IL-1ß and WY-14-643. Subconfluent MC were transiently cotransfected with 0.4 µg of pGL-MMP-9 ΔPPRE-1 and with 0.1 µg of pRL-CMV coding for *Renilla* luciferase. After an overnight transfection MC were treated for 24 hours with vehicle (control), IL-1ß (2 nM), WY-14,643 (100 µM) or a combination of IL-1ß and WY-14,643 as indicated. The values for beetle luciferase were related to values for Renilla luciferase and are depicted as relative luciferase activities. Data are means ± S.D. (*n=6*). **P≤0.05; **P≤0.01* compared with vehicle, or IL-1ß-stimulated conditions (## P≤0.01).

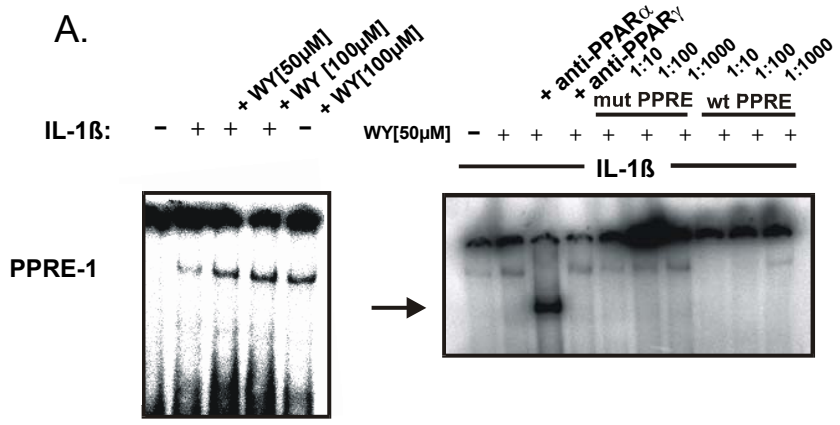

Figure 6.(A) Cytokine-induced PPAR binding is enhanced by WY-14,643. For EMSA serum-starved MC were stimulated with either vehicle (-), IL-1ß (+) (2 nM), WY-14,643 (50 and 100 µM) or IL-1ß plus WY (100 µM) as indicated for 5h before being harvested for nuclear extract preparations. The experiments were performed three times and representative experiments are shown. Supershift analysis identifying PPARα but not PPARγ as IL-1ß-/WY-inducible complex in MC (right panel). For supershift analysis the antibodies were preincubated overnight at 4°C before addition of the labeled oligonuclotide. The altered migration property of the supershifted complex is indicated by an arrow. The same gel shows a representative competition study by using different molar excess (depicted as different dilutions of an oligonucleotide stock solution) of unlabeled wild-type (wt PPRE) or mutated PPRE (mut PPRE) consensus oligonucleotides (sequences are shown in Table 2).

was confirmed by shifting the EMSA band through the addition of a PPARα-specific antibody (Fig. 6A) whereas the addition of a PPARγ-specific antibody had no effects on DNA binding (Fig. 6A, right panel). Interestingly, the disappearance of the PPRE-1 positive band after addition of the PPARα antibody was paralleled with the appearance of a strong band with faster migration properties (arrow in Fig. 6A). Probably, the binding of the antibody triggers a conformational change that allows for the binding of a further transcription factor to this promoter binding site which is normally competed by PPARα. Furthermore, the specificity of the DNA-bound complex was underlined by competition assays. Addition of different concentrations of unlabeled wildtype consensus PPRE oligonucleotide (wt PPRE) dose-dependently impaired DNA binding whereas the addition of cold mutant PPRE oligonucleotide only in a very high concentration (1:10) competed with the labeled PPRE-1 probe which is most probably due to an unspecific competition by the oligonucleotide (Fig. 6A, right panel).

WY-14,643 inhibits the IL-1- induced DNA-binding of NF-κB

PPARα agonists can inhibit the transcription of proinflammatory genes indirectly by interfering with the activation of NF-κB as it has been shown for the human IL-6 promoter [Delerive et al.,1999]. Previously we have demonstrated that NF-κB is critically involved in the activation of a 0.6 kb promoter portion of MMP-9 by IL-1ß [Eberhardt et al., 2000b]. To test whether activators of PPARα could modulate the activation of NF-κB binding in MC, we performed EMSA using a MMP-9 gene-specific oligonucleotide described in Table 2. Treatment of MC with IL-1ß (2 nM) induced DNA-binding of a single complex, and a weaker binding by WY-14,643 (Fig. 6B, left panel). Supershift analysis revelaed that the cytokine-induced complex containes both p50 and p65 subunits and the p65 antibody totally inhibited DNA binding. This indicates activation of a p50-p65 heterodimeric complex which mediates the potent transactivating activity of NF-κB (Fig. 6B, right panel). The formation of this IL-1ß-induced complex was markedly attenuated in cells which were simultaneously treated with WY-14,643 indicating that PPARα–dependent signaling cascades interfere with the DNA-binding of NF-κB (Fig. 6B, left panel). It should be noted that WY-14,643 alone induced a pronounced increase in NF-κB binding which, however, was significantly less than the effects observed with IL-1ß.

Table 2. Oligonucleotides used in EMSA and for point mutations generation. Consensus binding sites are underlined, and mutations are bold and italicized.

Oligonucleotides	Primer sequence (sense)
PPRE-1	5´-ATG GAG ACT CAA GGT CAC CTA TGT GT-3´
Δ-PPRE-1	5´-ATG GAG ACT CAA G***CA*** CAC CTA TGT GT-3´
PPRE-2	5´-TCC CAT CCA GGT CAC CCC GAG GCT TA-3´
Δ-PPRE-2	5´-TCC CAT CCA G***CA*** CAC CCC GAG GCT TA-3´
wt PPRE	5´-CAA AAC TAG GTC AAA GGT CA-3´
mut PPRE	5´-CAA AAC TAG ***CA***C AAA G***CA*** CA-3´
NF-κB (MMP-9)	5´-TTG CCC CGT GGA ATT CCC CCA AAT-3´

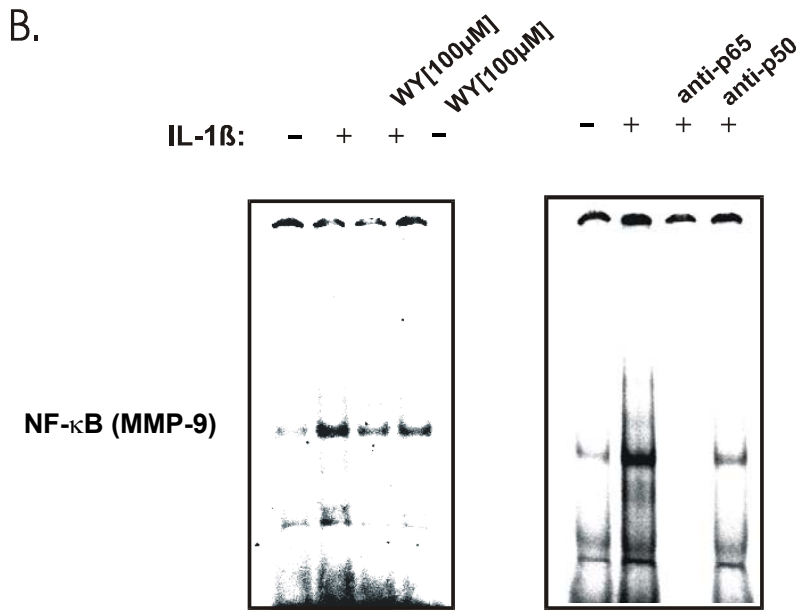

Figure 6. (B) Cytokine-induced NF-κB binding is inhibited by WY-14,643. Supershift analysis identifying an IL-1ß-inducible p50 and p65-containing complex (right panel).

Taken together these results demonstrate that activators of PPARα show mixed effects on NF-κB activity depending on the presence or absence of a simultaneous cytokine stimulus. However, their positive effects through PPRE dominate and result in the potentiation of MMP-9 gene expression.

Inhibition of cytokine-induced MMP-9 expression by PPARα agonists is switched to a potentiation in the presence of the NOS inhibitor L-NMMA

Since the data of the promoter studies do not reflect the reduction of the cytokine-induced MMP-9 mRNA steady-state level by PPARα agonists we hypothesized that PPARα activators may alter posttranscriptional events regulating MMP-9 mRNA stability. Previously, we have described the inhibitory effects of NO on the IL-1ß-induced MMP-9 steady-state mRNA level in rat MC. Correspondingly, we found that inhibition of iNOS leads to a marked potentiation of cytokine-induced MMP-9 mRNA levels thus proving a potent inhibitory action of endogenously produced NO [Eberhardt et al., 2000a]. To test whether the inhibitory effects of the PPARα activators on the IL-1ß-induced MMP-9 mRNA level depend

on NO production, MC were treated with IL-1ß and different PPARα agonists in the presence or absence of the NOS inhibitor L-NMMA. We first measured by zymography the gelatinolytic content of conditioned medium of MC withdrawn 24h after stimulation. Strikingly, the reduction of cytokine-induced MMP-9 content, most prominently seen with the highest concentration of the PPARα agonists WY-14, 643 (100 μM) or bezafibrate (500 μM), was not only reversed but even potentiated in the presence of L-NMMA (Figure 7A). The amplification of cytokine-mediated zymogen contents by L-NMMA was approximately 3-fold, demonstrating that PPARα activators in the absence of cytokine-triggered NO generation potently augment the cytokine-induced level of MMP-9 (Fig. 7A). Similar to the amplification of gelatinolytic contents, addition of PPARα agonists plus L-NMMA results in a strong amplification of the IL-1ß-mediated MMP-9 steady-state mRNA level (Fig. 7B) whereas L-NMMA by its own had no effects on the basal MMP-9 mRNA level (data not shown). Similarly to MMP-9, cytokine-induced TIMP-1 mRNA levels were inhibited by both PPARα agonists but strongly amplified in the presence of L-NMMA (Fig. 7B). In contrast, the high level of basal TIMP-2 mRNA was weakly reduced by IL-1ß and the reduction by IL-1ß was weakly enhanced by addition of WY-14,643 whereas bezafibrate had no further modulatory effect on this attenuation of TIMP-2 steady-state mRNA level. Similar to the amplification of gelatinolytic contents, addition of PPARα agonists plus L-NMMA results in a strong amplification of the IL-1ß-mediated MMP-9 steady-state mRNA level (Fig. 7B) Whereas L-NMMA by its own had no effects. Furthermore, the presence of L-NMMA had no effects on the TIMP-2 mRNA level, thus demonstrating that TIMP-1, but not TIMP-2 expression similarly to MMP-9 is negatively affected by PPARα agonists in a NO-dependent manner. To further confirm that the modulation of cytokine-induced MMP-9 mRNA level by PPARα activators negatively correlates with endogenous NO production we stimulated MCs with increasing concentrations of IL-1ß to gradually increase iNOS expression [Mühl et at .,

1993; Kunz et al., 1994] in the presence of a fixed concentration of WY-14,643 (100 µM) (Fig. 8). Whereas the low dose of IL-1ß (0.1 nM) was not able to induce detectable levels of MMP-9 mRNA, concentrations between 0.5 nM and 2 nM IL-1ß caused a strong induction of MMP-9 mRNA which was paralleled by a dose-dependent increase of nitrite accumulation in the cell culture supernatants (Fig. 8). Interestingly, although the addition of WY-14,643, to all IL-1ß concentrations caused a further increase in nitrite levels, WY differentially affected the

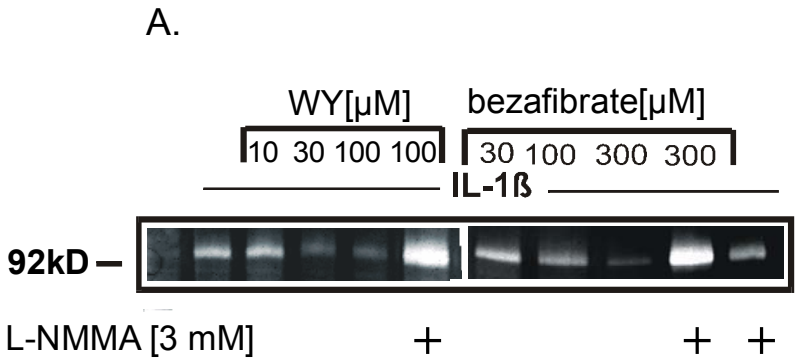

Figure 7. (A) MC were incubated for 24 hours with vehicle (control), IL-1ß (2 nM), IL-1ß plus the indicated concentrations of WY-14,643 or bezafibrate. In the presence of IL-1ß and the highest concentration of PPARα activator cells were additionally treated with (+) the NOS inhibitor L-NMMA (3 mM). 10 µl of aliquots from cell supernatants were assessed by SDS-PAGE zymography and migration property of the lytical band determined using standard m.w. markers.

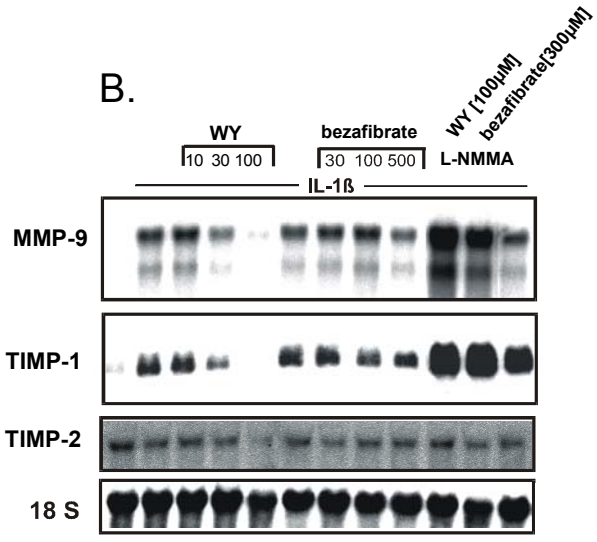

Figure 7 (B) MC were incubated for 24 hours with vehicle (control), IL-1ß (2 nM), IL-1ß plus the indicated concentrations of WY-14,643 or bezafibrate. In the presence of IL-1ß and the highest concentration of PPARα activator cells were additionally treated with the NOS inhibitor L-NMMA(3 mM) as indicated. Northern blot analysis was performed using 20 µg of total RNA from the very same cells assayed for lytical activity which was successively hybridized with specificDNA probes for rat MMP-9, TIMP-1 and TIMP-2, respectively. Equivalent loading of RNA was ascertained by a final rehybridization to an 18S ribosomal probe.

IL-1ß-induced MMP-9 mRNA level, depending on the concentration of IL-1ß applied (Fig. 8). Whereas at low concentrations of IL-1ß (0.1 and 0.5 nM) WY-14,643 clearly enhanced the cytokine-induced MMP-9 mRNA level in the presence of a higher concentration of IL-1ß (2 nM) WY-14,643 caused a significant reduction of cytokine-induced MMP-9 mRNA level.

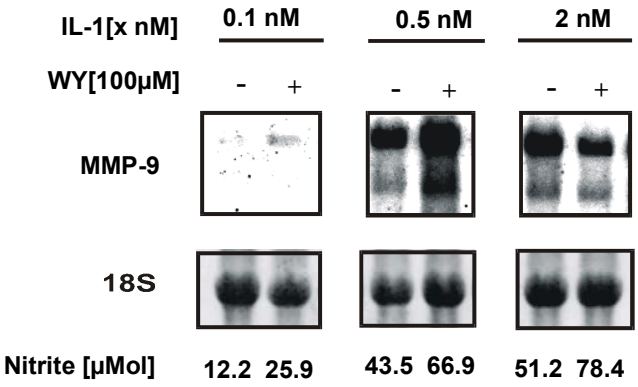

Figure 8. IL-1ß-induced MMP-9 steady-state mRNA levels are differentially modulated by WY-14,643, depending on the concentration of cytokine used for stimulation. Quiescent MC were coincubated for 24 hours with the indicated concentrations of IL-1ß in the presence (+) or absence (-) of a constant concentration of WY-14,643 (100 µM). Northern blot analysis demonstrates a dose-dependent modulation of cytokine-induced MMP-9 mRNA steady-state level. 20 µg of total RNA were hybridized to a ^{32}P-labeled cDNA insert from KS-MMP-9. Equivalent loading of RNA was ascertained by rehybridization to an 18S ribosomal probe. The stable endproduct of NO was measured by the Griess assay and the amount of total nitrite release by MC is given at the bottom of the Figure. The experiment was performed three times and one representative experiment is shown.

In summary, these data indicate, that WY-14,643 principally potentiates the level of cytokine-induced MMP-9 mRNA but this effect is antagonized by the negative effect of NO produced endogenously in large amounts upon stimulation with high concentrations of IL-1ß.

The cytokine-induced increase in nitrite production is amplified by WY-14,643 and is due to enhanced iNOS expression

The experiments with L-NMMA clearly demonstrate a causal role of NO in the PPARα-dependent repression of MMP-9 mRNA steady-state level. In order to elucidate whether activators of PPARα similarily to MMP-9, also influence iNOS expression we performed Western blot analysis using a N-terminal iNOS-specific antibody [Kunz et al., 1994].

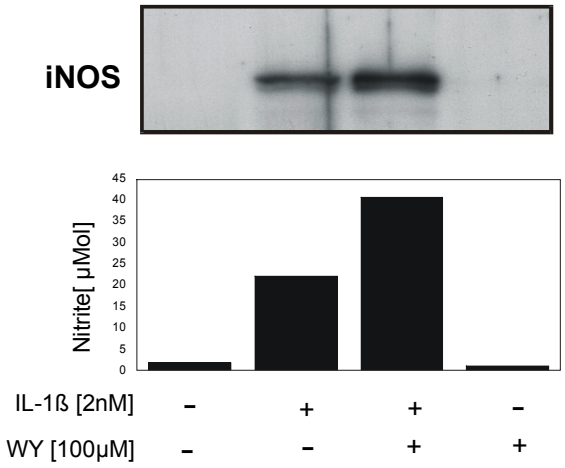

Figure 9. Cytokine-induced expression of inducible NO synthase is potentiated by WY-14,643 and results in an amplification of nitrite release. Quiescent MC were treated for 24 hours with vehicle (-), IL-1ß (2 nM), WY-14,643 (100 µM) or IL-1ß plus WY-14,643 as indicated. Total cellular protein lysates (50 µg) were subjected to SDS-PAGE and immunoblotted using an anti-iNOS N-terminal antibody [Kunz et al., 1994] (upper panel). The lower panel shows the nitrite levels in the corresponding cell supernatants as measured by the Griess assay. Equal loading of protein was ascertained by Ponceau S staining. Similar results were obtained in two independent experiments.

Treatment with IL-1ß for 24h results in the appearance of an iNOS band at 130 kD, which was not detectable in the extracts from untreated or WY-14,643-treated MC, respectively (Fig. 9, upper panel). When cells were simultaneously treated with IL-1ß plus WY-14,643 cytokine-triggered iNOS expression was strongly enhanced and paralleled by an amplification of nitrite production (Fig. 9, lower panel). These results indicate that PPARα activators potentiate cytokine-mediated iNOS expression and subsequent nitrite production.

The amplification of cytokine-induced MMP-9 mRNA level by WY-14,643 in the presence of L-NMMA is independent of the 1.8 kb-MMP-9 promoter context

We tested whether the modulation of MMP-9 promoter activity by WY-14,643 was affected by NO. To this end MC were transiently transfected with pGL-MMP-9 (1.8) and stimulated with IL-1ß to trigger endogenous NO formation or with DETA NONOate as an exogenous source of NO. As shown before, treatment of MC with IL-1ß in the presence of WY-14,643 caused a potentiation of cytokine-dependent pGL-MMP-9 luciferase activatity (Fig. 10A). Moreover,

the addition of either L-NMMA or DETA-NONOate did not affect cytokine or cytokine plus PPARα-induced MMP-9 promoter activity (Fig. 10A) and also had no effects on the basal promoter activity of pGL-MMP-9 (1.8) (data not shown). These data suggest that not a transcriptional but a posttranscriptional mechanism mediates NO suppression of cytokine-stimulated MMP-9 mRNA steady-state levels.

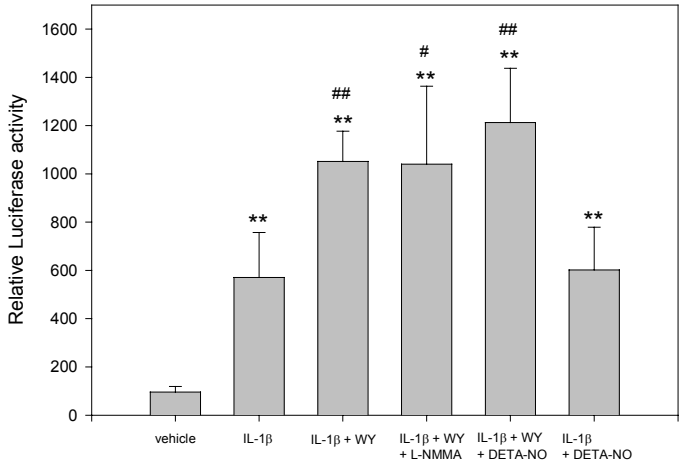

Figure 10. (A) Exogenous and endogenous NO does not affect the potentiation of cytokine- induced MMP-9 promoter activity by WY-14,643. Subconfluent MC were transiently cotransfected with 0.4 µg of pGL-MMP-9 (1.8) and with 0.1 µg of pRL-CMV coding for *Renilla* luciferase. After an overnight transfection MC were treated for 24 hours with vehicle or with IL-1ß (2 nM) in presence or absence of WY-14,643 (100 µM); L-NMMA (3 mM); DETA-NONOate (500 µM) as indicated. The values for beetle luciferase were related to values for Renilla luciferase and are depicted as relative luciferase activities. Data represent means ± S.D. (*n=6*). **P≤0.05; **P≤0.01* compared with vehicle, or IL-1ß-stimulated conditions (# P≤*0.05*; ## *P≤0.01*).

Nuclear MMP-9 mRNA level are increased by the PPARα agonsit WY-14,.643

The promoter studies indicate that PPARα agonists have a positive effect on the transcriptional activity of IL-1ß-triggered MMP-9. However, the use of partial promoter fragments in reporter gene assays does not necessarily reflect the real transcriptional regulation. Therefore, in addition to promoter studies we used a modified transcription assay with intact nuclei. MC were treated for 4 hours with either vehicle (control), or IL-1ß (2 nM), or WY-14,643 (100 µM) or both in combination before cells were lysed for nucelar fractionation. The quality of nuclei was tested by microscopy and only fractions containing pure and intact nuclei were used for the assay. We used a combination of classical nuclear run

on with that of RNase protection assay since the sensitivity of RNase protection assay is severalfold higher than that of conventional protocols involving hybridization of labeled RNA to blots containing immobilized cDNA probes. As shown in Fig. 10B, MMP-9 shows a relatively high basal expression which corresponds well with the high basal promoter activities of the MMP-9 promoter constructs and which suggests a strong posttranscriptional regulation that might explain the observed low basal MMP-9 steady-state mRNA level. Concomitantly, IL-1ß treatment caused a moderate increase of MMP-9 gene expression which was further amplified by addition of WY-14,643. In the protection assay we observed a second band with faster migrating properties (Fig. 10B, asterix) most probably reflecting a splicing variant of nascent MMP-9 mRNA also observed in the Northern blots (Fig. 8). In agreement with the data from reporter gene assays, WY-14,643 by its own caused an increase in nucelar MMP-9 mRNA level.

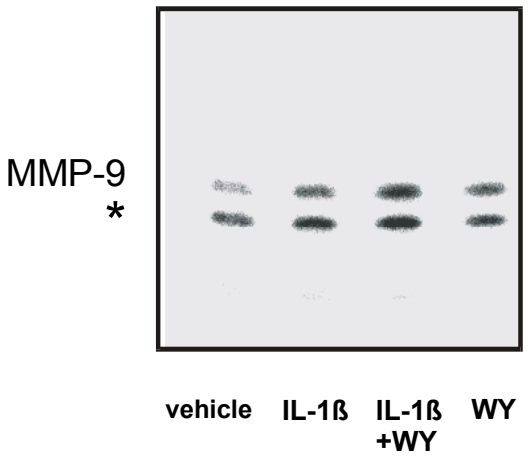

Figure 10 (B) WY-14,643 increases the content of nuclear MMP-9 mRNA. RNase protection assay demonstrating that the cytokine-induced content of nuclear MMP-9 mRNA is further amplified by WY-14,643. MC were either treated with vehicle (control) or stimulated with IL-1ß (2 nM), WY-14,643 (100 µM) or with both agents in combination for 4 h. The amount of nuclear MMP-9 transcripts in isolated nuclei was determined by hybridizing the RNA transcripts with a radiolabeled antisense RNA from rat MMP-9. Equal counts of radiolabeled samples were loaded on acrylamide/urea gels and analyzed by Phosphorimager analysis. A band which possibly corresponds to hybridization of a smaller MMP-9 splicing variant is indicated by an asterix. Similar results were obtained in two independent experiments.

Since posttranscriptional events regulating mRNA stability mainly occur in the cytoplasm these results clearly demonstrate that the increase in nuclear MMP-9 mRNA level results from an increased transcription rate. In summary, these data clearly demonstrate that the inhibitory effects by PPARα agonists on the cytokine-induced steady-state MMP-9 mRNA level result from indirect, posttranscriptional events.

Effect of NO on MMP-9 mRNA-stability

To test whether NO could affect the stability of cytokine-induced MMP-9 mRNA we used the transcription inhibitor actinomycin D. MC were stimulated for 20 hours with IL-1ß (2 nM) and subsequently cells were treated with vehicle or different NO donors. The reduction in MMP-9 mRNA observed under cytokine-stimulated conditions occurred with a half-life of approximately 8 h (Fig. 11A, upper panel). Interestingly, addition of NO by either DETA NONOate (Fig. 11A) or SNAP (Fig. 11B) markedly decreased MMP-9 mRNA stability with a half-life of approximately 4 h. This is most impressively documented by the complete loss of the MMP-9 transcripts 8h after addition of the NO donors. Importantly, WY-14,643 in a similar experimental setting did not affect the mRNA half-life of MMP-9 (data not shown). Furthermore, we addressed the question whether endogenously produced NO was, similar to exogenously added NO donors, able to reduce the stability of MMP-9 mRNA. To this end MC were treated with IL-1ß (IL-1ß) for 20 h either in the presence or absence of the NOS inhibitor L-NMMA (3 mM) before *de novo* transcription was blocked by actinomycin D (Fig.12A). As expected from the effects observed for exogenous NO, inhibition of endogenous NO production by L-NMMA caused a significant retardation of MMP-9 mRNA degradation (Fig. 12A). These data clearly demonstrate that endogenous NO levels similar to exogenous applied NO are sufficient to alter MMP-9 mRNA degradation. In a further approach we tested whether iNOS inhibition affects MMP-9 protein stability and /or activity. MC were treated for 20 h with IL-1ß (2 nM) in the presence or absence of L-NMMA (3 mM) before protein

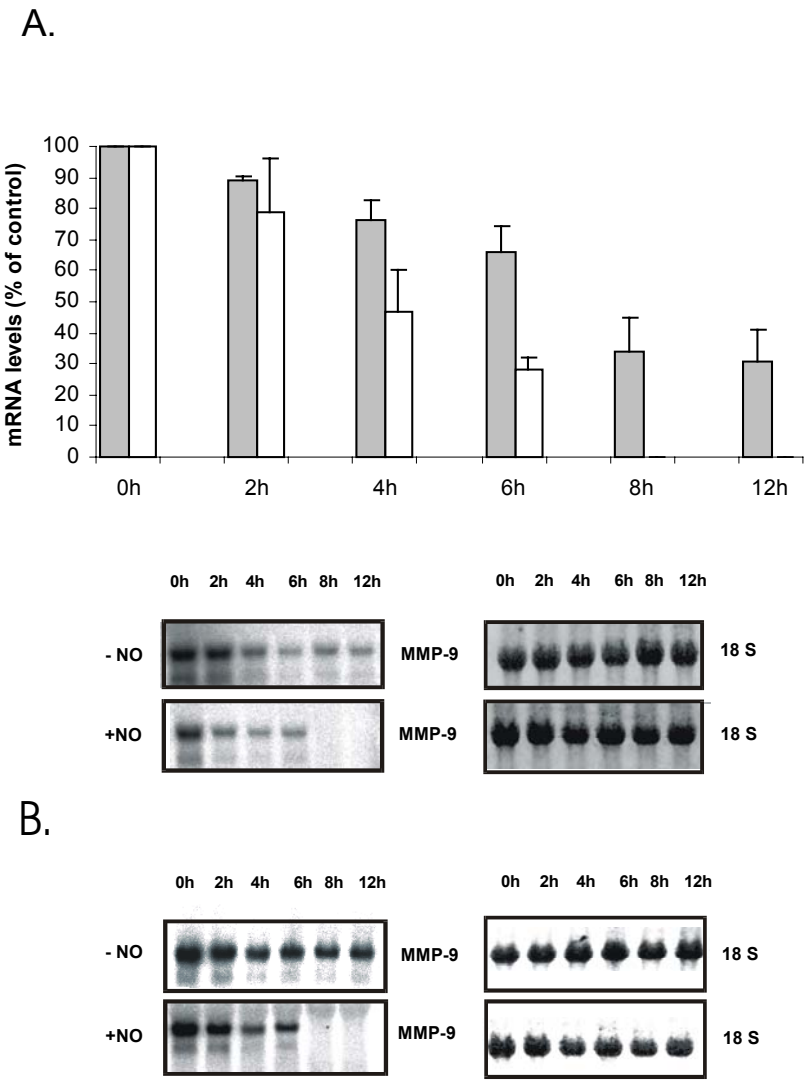

Figure 11. The stability of cytokine-induced MMP-9 mRNA is strongly diminished by the NO donors DETA-NONOate (A) and SNAP (B). Quiescent MC were treated for 20 hours with IL-1ß (2 nM). After incubation cells were washed twice and incubated with actinomycin D (5µg/ml). After a short preincubation of 30 min, cells were additionally treated for the indicated time points without (-NO) or with (+NO) the different NO donors (500 µM). At the time points indicated after start of actinomycin D treatment cells were harvested and extracted for total cellular RNA. 20 µg of total cellular RNA were hybridized to a ^{32}P-labeled MMP-9 probe. The equivalent loading of RNA was ascertained by subsequent hybridization with an 18 S rRNA probe. The densitometrical analysis of three independent experiments with DETA-NONOate is shown in the upper panel of Fig. 8A. The MMP-9 mRNA levels from MC treated with IL-1ß in the absence (filled bars) or presence (open bars) of NO donor after 30 min of actinomycin-D (0 hours) were set as 100%. Data in B. are representative for two experiments giving similar results.

synthesis was blocked by addition of cycloheximide (10 µg/ml). After the indicated time points conditioned media were collected for measurement of gelatinase activity and protein levels were assayed by Westernblot analysis using a specific MMP-9 antibody (Fig. 12 B).

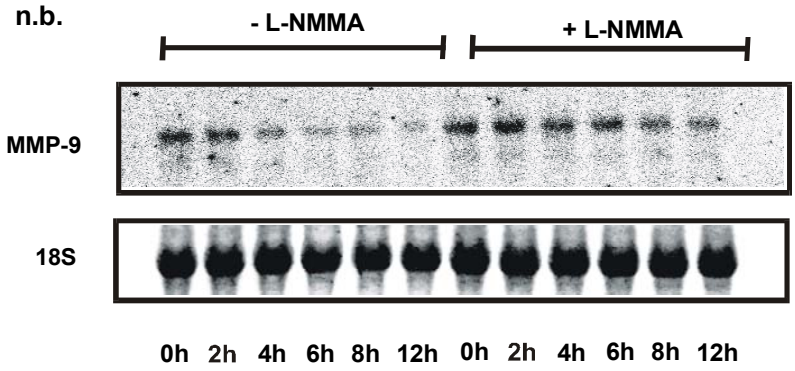

Figure 12. (A) The stability of cytokine-induced MMP-9 mRNA is inhibited by endogenously produced NO. Quiescent MC were treated for 20 hours with IL-1ß (2 nM) in the presence or absence of the NOS inhibitor L-NMMA (3 mM) as indicated. After incubation cells were washed twice and incubated with actinomycin D (5µg/ml) and harvested for RNA extraction after the indicated time points. 20 µg of total cellular RNA were hybridized to a ^{32}P-labeled MMP-9 probe. The equivalent loading of RNA was ascertained by subsequent hybridization with an 18 S rRNA probe. Similar results were obtained in two independent experiments.

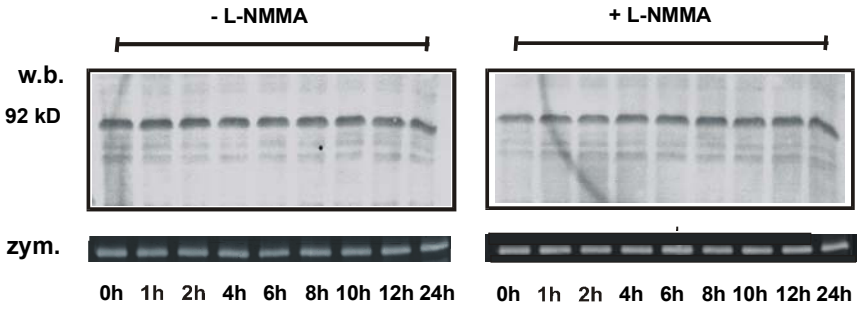

Figure 12.(B) The stability of MMP-9 protein and zymogen activity of secreted MMP-9 is not affected by endogenously produced NO. Quiescent MC were treated for 20 hours with IL-1ß (2 nM) in the presence or absence of the NOS inhibitor L-NMMA (3 mM) as indicated. After incubation cells were washed twice and incubated with cycloheximide (10µg/ml) and harvested for isolation of total protein lysates after the indicated time points. Protein lysates (100 µg) were subjected to SDS-PAGE and immunoblotted using a MMP-9 specific antibody (upper panel). Equal protein loading was ascertained by Ponceau S staining. The blot is representative of two independent experiments giving similar results. In parallel 10 µl of cell culture supernatants were collected after the indicated time-points and subjected to SDS-PAGE zymography (lower panel). The indicated band migrated at 92 kDa and corresponds to the latent form of MMP-9.

We observed that the stability of MMP-9 protein is quite high when compared to its corresponding mRNA since even a blockade of protein synthesis for 24 h did not change the total content of MMP-9 protein. Similarly to the cellular content of MMP-9, cycloheximide had no effects on the lytical content in the cell supernatants (Fig. 12B, lower panels). Most interestingly, there was no change in the MMP-9 protein contents when cells were grown in

the presence of the NOS inhibitor L-NMMA thus demonstrating that NO has no effects on the protein stability and gelatinolytic content of MMP-9.

Discussion

Increased expression of matrix metabolizing enzymes is a hallmark of many inflammatory processes and may lead to irreversible alteration of tissue architecture. Particularly MMP-9 has been shown to be centrally involved in the dysregulation of ECM turnover associated with severe pathologic conditions such as rheumatoid arthritis [Cawston, 1998] or fibrosis of lung, skin and kidney [Lemjabbar et al., 1999; Madlene et al., 1998; Davies et al., 1992]. The inflammatory cytokines IL-1ß and TNFα are among the most potent inducers of MMP-9 gene expression, mainly through the involvement of MAP kinase pathways leading to increased activity of NF-κB and AP-1 transcription factors [Yokoo et al., 1996; Eberhardt et al., 2000b]. Interference with these signaling cascades therefore has been suggested as a promising strategy to prevent aberrant matrix turnover. Here we have focused on possible modulatory effects of PPARα agonists on cytokine-induced MMP-9 expression since the PPARα signaling pathway exerts critical control functions in acute inflammation as shown by the exacerbated inflammatory response in PPARα null mice [Devchand et al., 1996]. We demonstrate that various structurally different PPARα agonists such as WY-14,643, LY-171883 and fibrates potently suppress cytokine-induced MMP-9 expression in renal MC. A downregulation of LPS-induced MMP-9 secretion by the PPARα agonist fenofibrate was recently documented in the human monocytic THP-1 cell line without addressing the underlying mechanisms [Shu et al., 2000]. The anti-inflammatory action by PPAR transcription factors is well established and in many cases occurs by antagonizing the pro-inflammatory action of nuclear factor κB (NF-κB) [Staels et al., 1998; Delerive et al., 1999; Poynter and Daynes 1998] as well as activator protein-1 (AP-1) [Delerive et al., 1999]. In line with these observations, we demonstrate that in MC the cytokine-induced binding of a

p50/p65 containing complex to a MMP-9-specific NF-κB binding site at –560/-550 is substantially reduced by PPARα agonists. However, the reduction of cytokine-induced NF-κB binding was not complete and somehow contrasts with the stimulatory effects on NF-κB binding by the PPARα agonist alone (Fig. 3B). Obviously, this reduced level of NF-κB activity is still sufficient to allow for a potentiation of the cytokine-triggered MMP-9 expression. Activation of NF-κB is crucially involved in IL-1ß-mediated MMP-9 promoter activation [Eberhardt et al., 2000b]. Whether the inhibition in DNA binding is caused by a physical interaction of PPARα with Rel-A transcription factors or indirectly, by the increased expression of the inhibitor of NF-κB (IκB) by PPAR-α activators remains to be evaluated. Recently, Delerive and coworkers have shown that both mechanisms of NF-κB activation are targeted by PPARα [Delerive et al., 1999]. In line with these considerations the inhibition of NF-κB DNA binding by PPARα agonists in the context of a 1.8 kb portion of rat MMP-9 promoter did not influence the PPARα-dependent amplification of IL-1ß-triggered MMP-9 promoter activity by the PPRE like binding-site at -1752/-1738. Interestingly, the PPRE sequence motif which is defined as two direct AGG(T/A)CA repeats and separated by one to five nucleotides is shared by other nuclear receptors, such as the thyroid hormone and the vitamin D receptors [Corton et al., 2000]. Like PPARs these receptors bind to direct repeats in a complex with the retinoic acid receptor (RXR). It is worth mentioning that although the PPRE like motif within the MMP-9 promoter contains only one half-site which completely matches a PPRE sequence motif, EMSA and supershift analysis indicate PPAR binding comparable to that observed with a complete consensus motif. The observation that PPARα agonists by their own increased the nuclear content of MMP-9 transcripts and have a weak stimulatory effect on MMP-9 promoter activity paralleled by increased DNA binding to PPRE and NF-κB sites somewhat contrasts with the finding that PPARα activators do not affect the basal MMP-9 mRNA steady-state and zymogen levels. Obviously, the PPARα-triggered

signals do not completely cover the cytokine-induced signaling pathways in rat MC. Moreover, in the complete promoter context additional regulatory events affecting transcriptional activators or repressors may explain the lack of MMP-9 mRNA increase in the absence of cytokine-induced signals. In line with these observations the potentiating effects of PPARα agonists on cytokine-induced sPLA2 promoter activity in rat MC which, similarily to their effects on MMP-9 expression, cause activation of the sPLA2 promoter without having significant effects on the basal sPLA2 mRNA steady-state level [Scholz-Pedretti et al., 2002]. In summary, these experiments clearly demonstrate that PPARα agonists synergistically with IL-1ß amplify MMP-9 promoter activity through a PPRE-dependent DNA binding despite a partially reduced NF-κB signaling. As found by a modified nuclear run-on assay, the PPARα-mediated decrease in MMP-9 mRNA steady-state and zymogen levels is not attributable to an inhibition of MMP-9 gene expression but points to possible posttranscriptional regulatory events. In addressing this hypothesis we most interestingly found that the reduction in cytokine-induced MMP-9 mRNA and zymogen levels by PPARα agonists is switched to strong potentiation in the presence of an inhibitor of NO synthesis. Furthermore, we demonstrate that treatment with PPARα agonists causes a strong increase of cytokine-induced iNOS expression and subsequent NO formation. These data suggest that PPARα-dependent effects on MMP-9 expression level primarily result from alterations in NO production. This is in a line with our previous finding that NO, either given exogenously or endogenously by stimulation of iNOS expression, potently inhibits the mRNA steady-state level of cytokine-induced MMP-9 in MC [Eberhardt et al., 2000a]. The negative modulation of MMP-9 expression has been confirmed in other cell types and suggests a general mechanism of NO-triggered tissue remodeling [Upchurch et al., 2001; Gurjar et al., 2001; Pfeilschifter et al., 2001]. When testing a 1.8 kb of the promoter region of the rat MMP-9 gene by reporter gene assay we found that NO had no direct effects on cytokine-induced MMP-9 promoter activity although the expression of many genes has been shown to be

transcriptionally modulated by NO in rat MC [Walpen et al., 2001; Pfeilschifter et al., 2001]. However, a transcriptional control of MMP-9 gene expression seems unlikely since in our hands none of the NO-sensitive candidate transcription factors, including AP-1 and NF-κB [Lander et al., 1993; Peng et al., 1995; von Knethen et al., 1999] were significantly affected by NO (data not shown). Whether regulatory regions upstream from -1.8 kb of the MMP-9 gene may be negatively influenced by NO is the subject of ongoing investigations. The 3´untranslated region (3´-UTR) of the rat MMP-9 gene bears several "AUUUA" motifs allowing for a posttranscriptional regulation of MMP-9 on the level of mRNA stability. In many genes AU-rich elements are specifically targeted by proteins of the ELAV-like protein family which has been implicated in the regulation of mRNA stability [Zhang et al., 1993; Ma et al., 1996; Rodriguez-Pascual et al., 2000]. Using actinomycin D, an inhibitor of eukaryotic gene transcription, we found that exogenous NO significantly reduced the half-life of MMP-9 mRNA (Figs. 9 and 10) whereas PPARα agonist had no influence on MMP-9 mRNA stability. Similar to MMP-9, the expression of transforming growth factor ß$_3$ (TGFß$_3$) is reduced by NO via destabilization of its mRNA [Abdelaziz et al., 2001]. Additional experimental work is required to more precisely evaluate the molecular mechanism of posttranscriptional regulation of MMP-9 expression by NO. In summary our study provides convincing evidence that PPARα agonists exert dominant negative effects on the steady-state level of MMP-9 mRNA and enzyme levels in addition to their positive transcriptional effects. The action of PPARα agonists on MMP-9 mRNA is indirect and due to an NO-triggered reduction of MMP-9 mRNA half-life. The overall effects of exogenous or endogenous PPARα activators on secretion of MMP-9 will critically depend on the simultaneous production of NO by cells exposed to an inflammatory environment. High-output levels of NO may account in part for the protective roles of PPARα agonists on the altered remodeling of ECM observed in many pathologies.

2. Nitric oxide increases the decay of MMP-9 mRNA by inhibiting the expression of mRNA stabilizing factor HuR

Introduction

Remodeling of extracellular matrix (ECM) is an important feature of normal growth and developmental processes. Consequently, an imbalance of ECM synthesis and degradation is associated with many diseases. Although changes in the synthesis of ECM may play a certain role in dysregulation of matrix turnover, recent studies have underlined the paramount role of ECM degradative systems. The main proteases regulating physiological degradation of ECM are the matrix metalloproteinases (MMPs), a family of zinc-dependent enzymes, which include the interstitial collagenases, stromelysins, elastases, membrane-type MMPs and gelatinases [Brinckerhoff and Matrisian. 2002; Nagase and Woessner 1999; Woessner,1998; Woessner, 1991]. A tight regulation of most of these proteases is accomplished by different mechanisms, including the regulation of MMP gene expression, the processing and conversion of the inactive zymogens by other proteases and, finally, the inhibition of active MMPs by endogenous inhibitors of MMPs, the tissue inhibitors of metalloproteinases (TIMPs) [for review see Nagase and Woessner, 1999]. Cultured mesangial cells (MC) respond to proinflammatory cytokines such as tumor necrosis factor α (TNFα) or interleukin-1ß (IL-1ß) with the production of several MMPs, including MMP-9 (gelatinase-B) mainly by an increase of gene expression [Yokoo and Kitamura**,** 1996 Eberhardt et al., 2000b]. In addition to MMPs, MC exposed to cytokines produce high levels of NO through the expression of the inducible NO synthase (iNOS) gene [Kunz et al., 1994; Pfeilschifter and Schwarzenbach, 1990]. Whereas the early and rapid actions of NO signaling affect post-translational modifications of cellular proteins, long-term regulation is executed primarily on the level of gene transcription [Pfeilschifter et al., 2002]. Interestingly, in MC NO can regulate the expression of a variety of ECM-related genes including secreted protein acidic

and rich in cysteine (SPARC) [Walpen et al., 2000], tissue inhibitor of metalloproteinase 1 (TIMP-1) [Eberhardt et al., 2000a], tissue plasminogen activator (tPA) [Eberhardt et al., 2002c], MMP-13 [Zaragoza et al., 2002] and MMP-9 [Eberhardt et al., 2000a]. However, the detailed mechanisms of these events are still unknown. By examining a 1.8 kb region of the rat MMP-9 promoter by reporter gene assays we could demonstrate that NO, either given by exogenous NO donors or endogenously produced after induction of the iNOS, has low effects on cytokine-induced MMP-9 promoter activity [Eberhardt et al., 2002b]. Moreover, in rat MC none of the candidate NO-sensitive transcription factors, including nuclear factor κB (NF-κB) or activated protein-1 (AP-1) [Lander et al., 1993; Pineda-Molina and Lamas, 2001; von Knethen and Brüne, 2000] are altered by NO, thus demonstrating that the NO-mediated effects on MMP-9 expression are mainly due to post-transcriptional effects. Post-transcriptional regulation of mRNA in the cytoplasm is recognized as an important control point in mRNA turnover and includes the localization and stability of mRNA but also translation of mRNA [Dreyfuss et al., 1996; Peng et al., 1998; Wang et al., 2002]. The control of selective mRNA degradation of many inducible genes including those for cytokines, nuclear transcription factors and proto-oncogenes seems to be localized within the 3´-untranslated regions (UTRs) of genes by sequence motifs which contain AU-rich sequences also denoted as AU-rich elements (AREs) [Chen and Shyu, 1995; Peng et al., 1998; Ross, 1995]. The 3´-untranslated region of the rat MMP-9 gene bears several copies of AUUUA motifs thus indicating that MMP-9, similarly to a variety of other genes expressed during inflammation, may also be regulated on the level of mRNA stability. By use of RNA-binding assays, several studies have identified proteins specifically binding to AREs, most interestingly proteins which play a role in stabilizing mRNAs. Most prominently are members of the embryonic lethal abnormal vision (ELAV) protein family [for review see Antic et al., 1999; Brennan and Steitz, 2001.] but also factors which can destabilize mRNA such as members of the AUF1 or hn RNPD families [Ehrenman et al., 1994; Wilson et al., 1999].

Members of both families have therefore been implicated in the regulation of mRNA turnover [Henics et al., 1994; Zhang et al., 1993]. We recently have reported that activators of the peroxisome proliferator-activated receptor α (PPARα) indirectly via increased induction of NO synthesis significantly reduce the stability of MMP-9 mRNA [Eberhardt et al., 2002b]. With regard to the presence of several AREs in the 3´-UTR of MMP-9 we now elucidated the involvement of the RNA stabilizing factor HuR (HuA), a member of the ELAV family in the modulation of MMP-9 mRNA stability by NO.

Results :

Nitric oxide reduces the mRNA stability of MMP-9

MMP-9 is synthesized in response to diverse stimuli including cytokines, growth factors, and hormones. Most importantly, the induced expression of MMP-9 can be modulated by oxidative and nitrosative stress conditions [Eberhardt et al., 2000a; 2000b]. We now demonstrate that the inhibitory effects of NO on the cytokine-induced levels of MMP-9 mRNA and gelatinolytic content of rat glomerular mesangial cells are mainly due to a reduction of the stability of MMP-9 mRNA since actinomycin D experiments indicated a potent acceleration in the MMP-9 mRNA degradation as demonstrated by Northern blot analysis (Fig. 13). MC were stimulated for 20 h with IL-1ß (2 nM) before being treated with the NO donor DETA NONOate (500 μM). The reduction of MMP-9 mRNA in the absence of NO occured with a half-life of ~ 7 hours. Interestingly, the addition of DETA NONOate markedly decreased MMP-9 mRNA stability, with a half-life of ~ 4 hours (Fig. 13, lower panel). The decrease in mRNA stability is most impressively documented by the complete loss of MMP-9 transcripts 8 hours after the addition of the NO donor (Fig. 13). Similarly, we found that the endogenous NO level produced by the induction of iNOS is sufficient to stimulate MMP-9 mRNA degradation [Eberhardt et al., 2002b]. Concomitantly, the presence

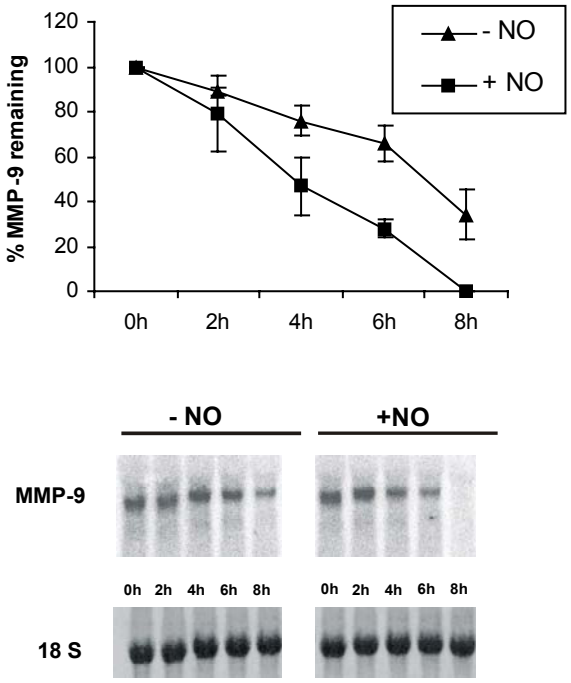

Figure 13. Accelerated degradation of cytokine-induced MMP-9 mRNA by DETA-NONOate. Quiescent MC were treated for 20 hours with IL-1ß (2 nM) and then washed twice before the addition of actinomycin D (5µg/ml). After a short preincubation of 30 min, cells were additionally treated for the indicated time points without (-NO) or with (+NO) DETA NONOate (500 µM) before being harvested and extracted for total cellular RNA. For Northern blot analysis 20 µg of total cellular RNA were hybridized to a ^{32}P-labeled MMP-9 probe. The equivalent loading of RNA was ascertained by subsequent hybridization with an 18 S rRNA probe. A representative experiment is shown in the lower part of the figure. The upper panel shows means ± S.D. (n=3) of three independent experiments and depicts the % of remaining MMP-9 transcripts after addition of DETA NONOate (NO).

of DETA-NONOate does not affect either cytokine-induced or basal promoter activity of a luciferase reporter gene encompassing 1.8 kb of the 5´-flanking region of the rat MMP-9 gene [Eberhardt et al., 2002b]. These data strongly suggest the involvement of posttranscriptional mechanisms in the regulation of MMP-9 expression by NO.

The 3´UTR of MMP-9 mRNA confers NO responsiveness to the stability of different mRNAs

An important paradigm for posttranscriptional regulation is the control of cytoplasmic mRNA stability which is mediated by the 3´-untranslated region (UTR) of mRNAs. Therefore, we focused on the 3´-UTR of the rat MMP-9 mRNA which contains four copies of AUUUA pentameric motifs potentially involved in the regulation of MMP-9 mRNA turnover (Fig. 14).

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                                2128 ACT AAGGCTCCTC TTTTGCTTCA GCGGTGCAGC
2161 GCAAGCCTCT AGAGACCACC CCTGAGGGGG AGGAGCTAGT TTGCCGGATA CAAACTGGTG
2221 ATCTCTTCTA GAGACTAGGA AGGAGTGGAG GCGGGCAGGG CCCTCTCTGC ACGCTGTCCT
2281 TTCTTGTTGG ACTGTTTCTA ATAAACACGG ATCCCCCAAC CTTTACCAGC TACTCGAACC
2341 AATCAGCTTG TCTGTAGTTG TATACACATC CAAGCCTGTG GTTGGTCAGA AGACAACTTT
2401 GTAGGGTCGG TTCTGACCTT TTGTTTTTAT GGGGCATCTG GGGATTGAAC TCAGCTGGCT
                                                        ARE-1
2461 TTTGTGACAA GTACTTCACC CGCTGCACCA CCTTACCGGC CCTTTTATTT ATTTATGTATG
                                ARE-2
2521 TGGTCATGTT CACACACATG TATTTAACCT ATAGAATGCT TACTGTGCGT CGGGCGCTGC
2581 TCCAACTGCT GTATAAATAT TAAGGTATTC AGTTACTCCT ACTGGAAGGT ATTATGTAAC
                                                     ARE-3
2641 CATTTCTCTC TTACATCGGA GGACACCACC GAGCTATCCA CTCATCAAAC ATTTATTGTG
                                                  ARE-4
2701 AGCATCCCTA GGGAGCCAGG CTCTCTACTG GGCATTAGGG ACAGAGGAAT TTATTGGATG
2761 TTGGTTCTTC CTTCAAGAAT CGCTCAGGGA TTCTCAGTGT CCTCAAAACA TGCTGAAACC
2821 GGACCCCAGA CTCCACTCTG GAGAATCCAA CCCACTCACA GGGGAAACAG CCCGTTTAAA
2881 GTGCATGTGT GCTAGAGAGG TAGGGTAGCC GAGATGATCC CTCAGTGGAA GGATAACCAA
2941 GTATCTTCCT CGGACACTCT TTTTTAAATA AATGAATAAA TGAAAA
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Figure 14. Sequence of the 3´-UTR of the rat MMP-9 gene (GenBank™ accession no U24441). Potential ARE binding sites involved in the posttranscriptional regulation of MMP-9 mRNA are depicted in boxes. The numbers of ARE were used to distinguish between the different ARE motifs within the MMP-9 UTR. The underlined sequence indicate the region encompassed by the RNA oligonucleotides used for EMSA.

The impact of this region on changes in mRNA stability by NO was investigated by transient transfection of a luciferase reporter gene driven by a 1.3 kb fragment of the rat MMP-9 promoter either containing the 3´-UTR of MMP-9 downstream of the luciferase reporter gene (3´-UTR-MMP-9pGL3MMP-9-1.3 kb) or no additional 3´-regulatory sequences (pGL3-MMP-9-1.3 kb). Therefore, the final luciferase activity measured in MC transiently transfected with luciferase constructs bearing the 3´-UTR of MMP-9 is not only the result of the activity of the passenger MMP-9 promoter but in addition will depend on the stability of luciferase mRNA regulated by the 3´-UTR of MMP-9. Transient transfection of MC with pGL3MMP-9-1.3 kb was followed by a 24-h treatment with either vehicle, IL-1ß (2 nM),

DETA NONOate (500 µM) or IL-1ß plus DETA-NONOate. Stimulation of MC with IL-1ß (2 nM) lead to a significant increase of MMP-9 promoter activity (2.4 –fold, $p \leq 0.05$) while NO had only weak modulatory effects on either cytokine-stimulated (3.1 –fold, $p \leq 0.01$) or basal (1.8 –fold, $p \leq 0.05$) MMP-9 promoter activities (Fig. 15A). Most interestingly, the same promoter construct under additional control of the 3´-UTR of MMP-9 displayed a strong reduction on MMP-9 promoter activity since the basal (6.2 –fold reduction, $p \leq 0.01$) as well as cytokine-induced MMP-9 promoter activity (2.1 –fold reduction, $p \leq 0.01$) was markedly reduced by addition of the NO donor (Fig. 15B). It is important to note that the basal promoter activity was higher in the 3´-UTR-MMP-9 pGL construct (2.42 RLU ± 0.54 compared to 0.32 RLU ± 0.02) which presumably results from the increase in mRNA stability confered by this regulatory region. The stronger inhibitory effects of NO on basal and IL-1ß-mediated luciferase activity suggests that NO can counteract the positive effects of IL-1ß on MMP-9 promoter activity only in the presence of the MMP-9-specific 3´-UTR. The negative posttranscriptional effects also exerted by endogenously produced NO observed upon IL-1ß stimulation may explain the weak induction of 3´-UTR-MMP-9-pGL3MMP-9-1.3 kb by IL-1ß in comparison to that observed with the pGL3MMP-9-1.3 kb construct (Fig. 15A and 15B). Furthermore, we tested whether the 3´-UTR of MMP-9 under control of a foreign promoter was still able to confer a NO-dependent negative regulatory function on luciferase gene expression. To this end, we chose the pGL3 control vector which contains the luciferase gene under the control of the constitutively active SV 40 promoter and enhancer sequences, confering a strong expression of luciferase when transfected into mammalian cells. A main advantage of this plasmid is that it displays a significant basal promoter activity without responding to cytokine stimuli. Therefore this reporter gene is most useful for monitoring the contribution of 3´-UTR regions on mRNA stability in the absence of any influence of an inducible enhancer region. Transient transfection of MC with 3´-UTR –MMP-9 pGL3-control comprising 600 bp of the MMP-9 3´-UTR downstream from the luciferase coding region

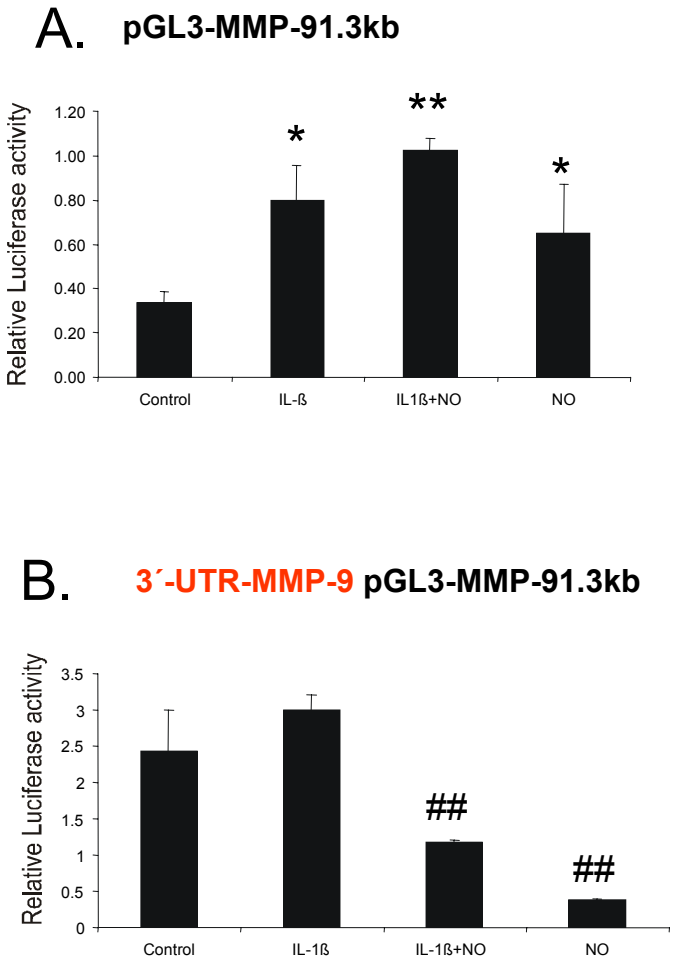

Figure 15. Influence of the 3´-UTR of MMP-9 on the MMP-9 promoter driven luciferase activities. Subconfluent MC were transiently cotransfected with 0.4 µg of pGL-MMP-9 (1.3) **(A)** or alternatively with 0.4 µg of 3´-UTR-MMP-9 pGL-MMP-9 (1.3) **(B)** containing additionally the 3´-UTR of MMP-9 downstream of the luciferase coding sequence. In each transfection 0.1 µg of pRL-CMV coding for *Renilla* luciferase was simultaneously added. After an overnight transfection MC were treated for 24 hours with vehicle (control) or with IL-1ß (2 nM) in presence or absence of DETA NONOate (500 µM) as indicated. The values for beetle luciferase were related to values for Renilla luciferase and are depicted as relative luciferase activities. Data (means ± SEM) represent the results of three different experiments. P=0.05 and P=0.01 compared with control conditions (*) and (**) or to IL-1ß-stimulated values (##). P=0.01

followed by a 24h treatment with DETA-NONOate (500 µM) resulted in a significant reduction of the basal promoter activity (53 % reduction, p≤0.05) of 3´-UTR-MMP-9 pGL3 control promoter (Fig. 16B) whereas the basal promoter activity of the same promoter (pGL3-control) but without the 3´-UTR of MMP-9 was not significantly affected by DETA NONOate (Fig. 16A). Taken Taken together these data indicate that the 3´-UTR of MMP-9 is a target of NO-mediated mRNA destabilization.

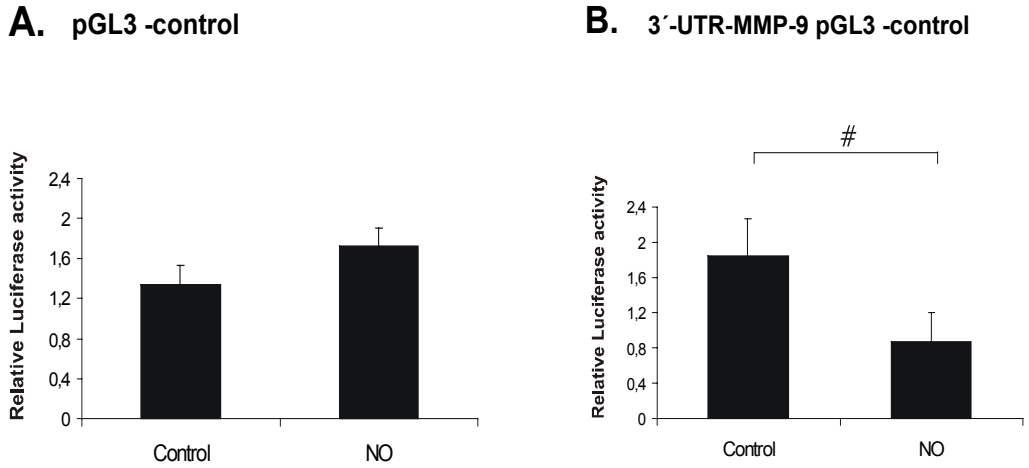

Figure 16. Influence of the 3´-UTR of MMP-9 on SV-40 driven Luciferase activities. Subconfluent MC were transiently cotransfected with 0.4 µg of empty pGL3-control vector **(A)** or with 0.4 µg of 3´-UTR-MMP-9 pGL3-control **(B)** containing additionally the 3´-UTR of MMP-9 downstream of the luciferase coding sequence. Each plasmid was cotransfected with 0.1 µg of pRL-CMV used to equilibrate differences in transfection efficiencies. After transfection MC were treated for 24 hours with vehicle or with DETA NONOate (500 µM) as indicated. Values for beetle luciferase were related to values for Renilla luciferase and are depicted as relative luciferase activities. Data (means ± SEM) represent the results of three different experiments. P=0.05 compared with control conditions (#).

Nitric oxide inhibits constitutive complex formation of AU-rich motifs (AREs) within the 3´-UTR of MMP-9

The stability of most short-lived mRNAs is modulated through the specific binding of proteins to the AU-rich motifs (AREs) present in the 3´-untranslated region (UTR) of these mRNAs [Chen and Shyu,1994; Ford et al., 1999; Ross, 1995.]. To elucidate whether i) the motifs within the 3´-UTR of MMP-9 display any RNA binding capacity and ii) RNA binding could be affected by treatment of MC with NO, we performed RNA gel shift assays using ^{32}P-labeled RNA oligonucleotides comprising the different AREs of MMP-9 denoted as ARE-1, ARE-2, ARE-3 and ARE-4 (Table 3). MC were either left untreated or treated for 4 h with different concentrations of DETA NONOate before being lysed for preparation of cytosolic fractions. We found that the cytosolic extracts of MC contained proteins which formed two major constitutive complexes of high electrophoretic mobility (Fig. 17, C1 and C2) with the ARE 1, ARE-2 and ARE 4 containing oligos but no complex binding to the oligonucleotide encompassing ARE-3 (data not shown). Although each of the four motifs consist of a single

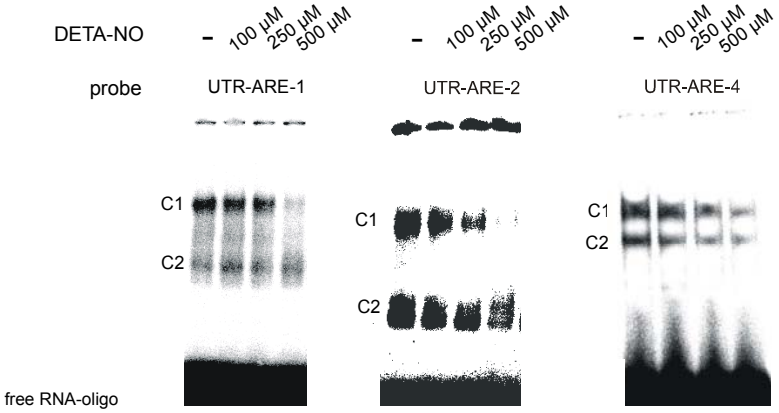

Figure 17 (A). DETA NONOate inhibits the constitutive RNA binding of cytoplasmic complexes to different ARE motifs from the 3´-UTR of MMP-9 .RNA binding was analyzed by EMSA using gene specific oligonucleotides (Table 3). MC were either left untreated (-) or stimulated for 4 hours with the indicated concentrations of DETA NONOate before being lysed for preparation of cytoplasmic extracts. Equal amounts of cytoplasmic extracts were incubated with a ^{32}P-labeled RNA probe derived from the corresponding AU-rich region of the 3´-UTR of rat MMP-9 (UTR-ARE-1,UTR-ARE-2 and UTR-ARE–4) and RNA binding assessed by using 6.0 % native PAGE gels. C1 and C2 indicate two main complexes constitutively binding to UTR-ARE-1, UTR-ARE-2 and UTR-ARE-4. The results shown in each panel are representative for three independent experiments giving similar results.

AUUUA pentanucleotide core sequence the composition of the 5´- flanking bases differs. Whereas ARE –1,-2 and ARE-4 are flanked by an upstream uracil or adenosine, ARE-3 at the 3´-position is flanked by the pyrimidine base cytosine (Table 3). A recent study elucidating the polymorphism of the 3´-UTR of TNFα mRNA has demonstrated the complexity of different HuR binding motifs [Di Marco et al., 2001]. Most interestingly, treatment of MC with DETA NONOate dose-dependently attenuated the constitutive RNA binding to all MMP-9-specific ARE motifs and a weak but significant reduction is seen already with a concentration of 100 µM DETA NONOate. Maximal inhibition was observed with a concentration of 500 µM DETA NONOate (Fig. 17A). Similar results were obtained with the NO-donor SNAP (data not shown). To further evaluate whether the observed inhibitory effects of NO on ARE binding are of physiological relevance, MC in addition to NO donating compounds, were alternatively treated with IL-1ß (2 nM), a potent activator of iNOS expression in rat MC [Pfeilschifter and Schwarzenbach, 1990]. Therefore MC were incubated in the presence or absence of the NOS inhibitor L- NMMA. MC were stimulated for 24 hours to ensure a high level of nitric oxide produced by the cells in response to IL-1ß [Kunz et al.,

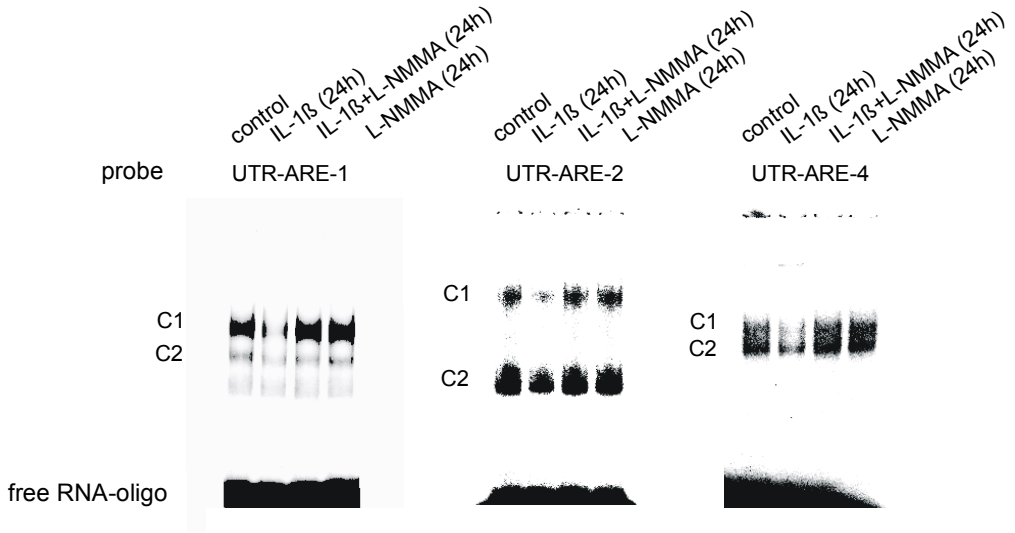

Figure 17 (B). Cytokine-mediated inhibition of constitutive RNA binding to different MMP-9-specific AREs is attenuated in the presence of L-NMMA. MC were either left untreated (control) or stimulated for 24 hours with IL-1ß (2 nM), L-NMMA (2 mM), or both in combination as indicated before being collected for extraction. Equal amounts of cytoplasmic extracts were incubated with the ^{32}P-labeled RNA probes specific for the 3´-UTR of rat MMP-9 depicted in Table 3. The conditions used for EMSA were described in A. The result shown is a representative experiment out of three giving similar results.

1994]. Treatment with IL-1ß (2 nM) resulted in a marked attenuation of constitutive RNA binding of the cytosolic fractions to the ARE-1, ARE-2 and ARE-4 motifs (Fig. 17B). In contrast, the two main complexes C1 and C2 from MC simultaneously treated with IL-1ß and L-NMMA retained a high constitutive RNA binding to ARE-1, ARE-2 and ARE-4 motifs whereas L-NMMA alone had no effects on the basal constitutive RNA binding capacities to any of the MMP-9-derived ARE probes (Fig 17B). These data indicate that NO, either given from an exogenous source or endogenously produced by the iNOS, potently inhibits the constitutive binding of complexes formed with AUUUA motifs from the 3´- UTR of the rat MMP-9 gene.

Complexes interacting with the ARE motifs of the 3´-UTR of MMP-9 contain the ELAV-like protein HuR (HuA) but no other members of the Hu protein family

In search for potential proteins possibly involved in the regulation of mRNA turnover we focused on the ELAV-like RNA binding protein HuR (HuA) which is able to specifically interact with AU- rich elements found in the 3´ UTR of many genes [Ma et al., 1996]. To test

Table 3. RNA oligonucleotides comprising the different AREs of MMP-9 denoted as ARE-1, ARE-2, ARE-3 and ARE-4.

UTR-ARE-1	2500	5′-CCCUUUU**AUUUA**UUAUGUAUG-3′ 2520
UTR-ARE-1mut	2500	5′-CCCUUUU**GGGCC**UGAUGUAUG-3′ 2520
UTR-ARE-2	2536	5′-ACAUGU**AUUUA**ACCUAUAGAA-3′ 2556
UTR-ARE-3	2683	5′-CAUCAAAC**AUUUA**UUGUGAGC-3′ 2704
UTR-ARE-4	2742	5′-CAGAGGAAUUUAUUGGAUGUU-3′ 2762

whether members of the Hu protein family were present in the complexes bound to the ARE motifs of the 3′-UTR of MMP-9, we performed supershift analysis using antibodies recognizing specific epitopes within the murine and rat Hu proteins, respectively. Furthermore, to exclude the possibility that the complex seen in the EMSA resulted from unspecific binding of any protein present in the extracts we additionally tested the effects of an antibody raised against the p65 subunit of the DNA binding transcription factor NF-κB Most interestingly, addition of the anti-HuR antibody resulted in a complete disappearance of the complexes C1 and C2 and the concomitant formation of two supershifted complexes (Fig. 18A, "UTR-ARE-1"). In contrast, addition of anti-HuB, anti-Hu-C, anti-Hu-D or anti-p65 (NF-κB) antibodies did not affect the constitutive RNA binding of C1 and C2 complexes (Fig. 18A). Supershift analysis with the UTR-ARE-2- and UTR-ARE-4-specific oligonucleotides similar to the supershifts obtained with UTR-ARE-1 caused a complete disappearance of both constitutive bound complexes upon addition of the anti-HuR antibody (Fig. 18A, "UTR-ARE-2", "UTR-ARE-4") although incubation with the UTR-ARE-2 containing oligonucleotide in some experiments caused no supershifted bands (Fig. 18A; "UTR-ARE-2"). Similar to the supershifts obtained with the UTR-ARE-1 oligonucleotide addition of anti-HuB, anti-Hu-C, anti-Hu-D or anti-p65 (NF-κB) did not affect the RNA-binding to UTR-ARE-2 or UTR-ARE-4-specific probes (Fig. 18A). These data indicate that in MC the ubiquitously expressed

ELAV-protein HuR, but not the Hu-family relatives Hu-B, Hu-C, and Hu-D, is the main constituent of complexes binding to AU-rich motifs of the 3´-UTR of MMP-9. Furthermore, the specificity of RNA-bound complexes present in the cytoplasmic extracts of MC was underlined by competition assays using wild-type or mutant UTR-ARE-1 oligonucleotides depicted in Table 3. For competition analysis different dilutions (1:10, 1:20, 1:100, 1:500) of either unlabeled wild-type or mutant UTR-ARE-1 oligonucleotide were added to the binding reaction 1h prior to the addition of the radiolabeled UTR-ARE-1 probe. As shown in Fig. 18B competition with the nonlabeled wild type oligo ("UTR-ARE-1wt") prevented binding of complex C1 when dilutions of 1:10, 1:20, and 1:100 of competitor wild-type oligonucleotide were added whereas binding of C2 was only weakly competed. In contrast, competitions with the unlabeled mutant oligonucleotide ("UTR-ARE-1 mut") in none of the dilutions tested were able to compete with the RNA binding to the radioactively labeled wild-type oligonucleotide. These data demonstrate that the AUUUA motif is necessary for retaining a full competition capacitiy of RNA binding. Similarly to UTR-ARE-1 RNA binding to UTR-ARE-2 and UTR-ARE-4 probes was competed by addition of cold wild-type ARE-1 competitor oligonucleotide suggesting a high redundancy between the different ARE motifs of 3´-UTR of MMP-9 (data not shown).

Recombinant HuR protein strongly binds to ARE-motifs of the 3´-UTR of MMP-9

In a next experiment we tested the RNA binding capacity of recombinant GST-coupled HuR protein to different ARE-motifs of the 3´-UTR of MMP-9. For EMSA 100 ng of GST-HuR was incubated with the radioactively labeled UTR-ARE-1, UTR-ARE-2 or UTR-ARE-4 oligonucleotides, respectively As shown in Fig. 18C GST-HuR formed one major complex with similar migration properties independent of which UTR-ARE oligo was used in the binding reaction. Similar to the findings obtained by EMSAs with cytoplasmic fractions,

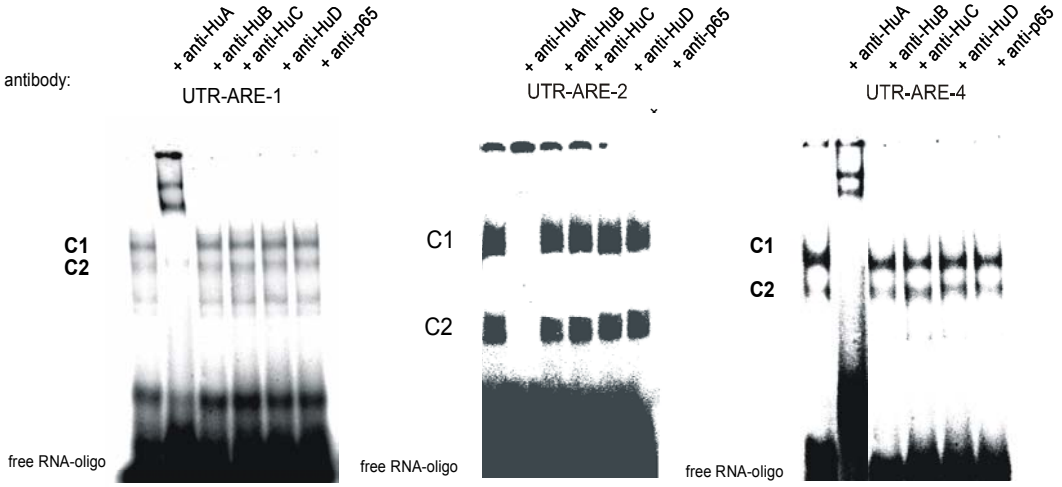

Figure 18.(A) HuR (HuA) is the main constituent of RNA-binding complexes within the cytoplasmic extracts of MC and binds with a high affinity to AU-rich elements of the 3´-UTR of MMP-9. For supershift analysis the ^{32}P radiolabeled UTR-ARE-1, UTR-ARE-2 and UTR-ARE-4 comprising oligonucleotides were incubated with 6 µg of cytoplasmic extract derived from resting MC in the presence or absence of different supershift antibodies indicated. The antibodies were added 15 min after addition of the radiolalabeled oligonucleotide and incubated for a further 15 min at room temperature.

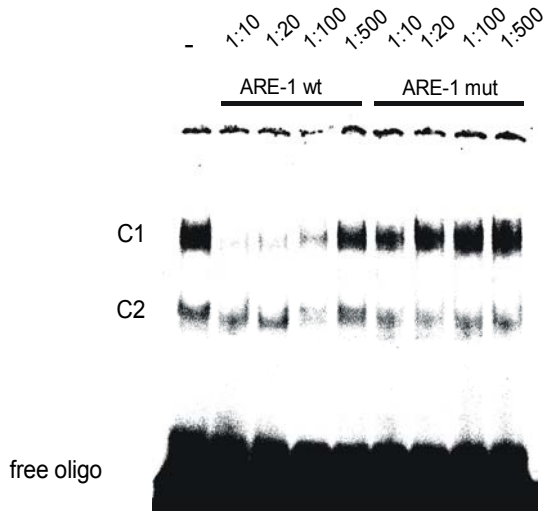

Figure 18 (B) Competition capacities of wild type and mutant UTR-ARE-1 oligonucleotides. The gel shows a representative competition study using different dilutions (as indicated) of either unlabeled wild type (UTR-ARE-1wt) or unlabeled mutated ARE-1 (UTR-ARE-1 mut) oligonucleotides (the sequences are shown in Table 3). The unlabeled competitor oligonucleotides were added 60 min before the addition of ^{32}P-labeled UTR-ARE-1 wt oligonucleotide.

addition of an HuR-specific antibody caused a complete disappearance of the RNA-bound complex and appearance of two supershifted complexes with reduced migration properties whereas antibodies specifically raised against HuB, HuC, HuD or p65 (NF-κB) did not affect the RNA binding of GST- HuR (Fig. 18C).

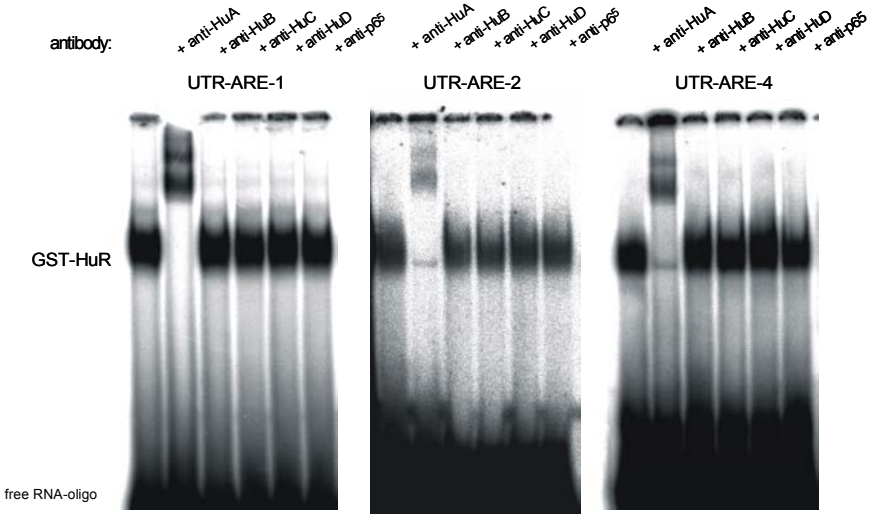

Figure 18 (C). GST-HuR binds with a high affinity to AU-rich elements of the 3´-UTR of MMP-9. EMSA analysis demonstrating that recombinant GST-HuR protein binds with a high affinity to ARE-elements of the 3´-UTR of MMP-9. For EMSA 200 ng of purified crude protein (GST-HuR) were incubated with the indicated oligonuclotides specific for different AREs of MMP-9 as described in table 3. For supershift analysis the ^{32}P radiolabeled UTR-ARE-1, UTR-ARE-2 and UTR-ARE-4 comprising oligonucleotides were incubated with the same amount of GST-HuR protein and 200 ng of supershift antibody as indicated. The antibodies were added 15 min after addition of the radiolabeled oligonucleotide and incubated for a further 15 min at room temperature. Similar results were obtained in three independent experiments.

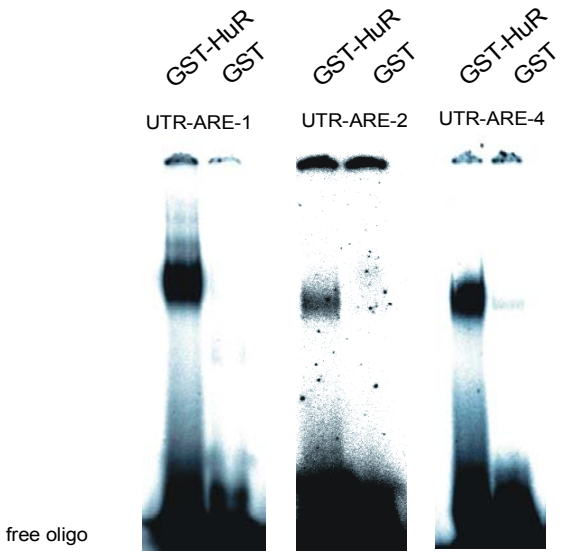

Figure 18 (D) EMSA analysis demonstrating that recombinant GST-HuR protein binds with a high affinity to ARE-elements of the 3´-UTR of MMP-9, whereas GST alone has only a weak unspecific RNA binding affinity.

These data document the high level of binding affinity of HuR to AREs of the 3´-UTR of MMP-9. In contrast to GST-HuR, the same amount of GST alone displayed only a very weak RNA binding independent of the ARE-motif used as probe (Fig. 18D). In summary, these data demonstrate that the ELAV-like protein HuR strongly can bind to AU-rich elements within the 3´-UTR of MMP-9.

The cytoplasmic fractions of NO-treated MC affect MMP-9 mRNA turnover

To further prove that the treatment of MC with exogenous NO correlates with the increased MMP-9 mRNA turnover we performed *in vitro* degradation assays. By this assay the time course of MMP-9 mRNA degradation exhibited by any trans-acting factor accumulated in the cytoplasmic extracts of MC treated for 4h with DETA NONOate (500 µM) was compared to the degradation profile derived from the incubation with cytoplasmic extracts from untreated MC. To this end, similar volumes of each extract (with a total protein content of 130 µg) were incubated with equal amounts of total RNA isolated from a common pool of cytokine-treated MC. Northern blot analysis performed after a second RNA isolation step revealed that primary undegraded MMP-9 transcripts exposed to the cytoplasmic fractions from NO-treated MC displayed an accelerated degradation when compared to the transcripts which had been exposed to cytoplasmic extracts from untreated cells (Fig. 19A). The differences in MMP-9 mRNA stability are clearly seen by the different time course of MMP-9 mRNA decay (Fig. 19A).

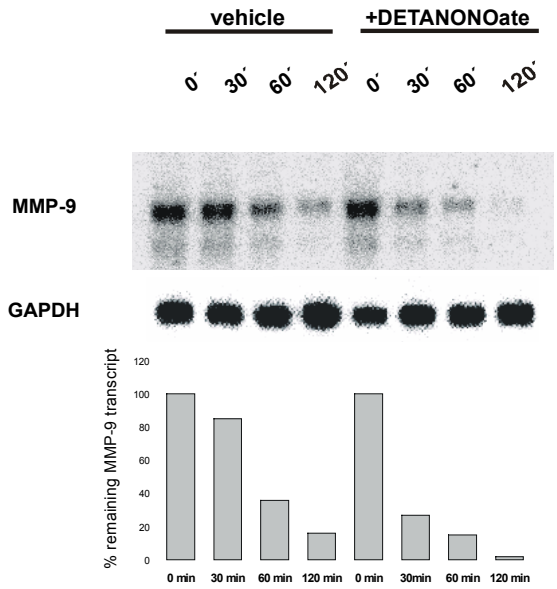

Figure 19 (A). HuR protects MMP-9 mRNA from a rapid decay within the cytoplasmic fractions of MC. For *in vitro* degradation assay portions of 20 µg of total RNA from a common pool of total RNA isolated from cytokine-stimulated MC were mixed with 130 µg of cytoplasmic extract derived from either untreated (vehicle) or alternatively, from MC treated for 4 hours with (+) DETA NONOate (500 µM) as indicated. After different time points (time in minutes) incubations were stopped by isolation of total RNA. RNA samples were collected and assessed for the amounts of remaining MMP-9 mRNA level by Northern blot analysis using a ^{32}P-labeled cDNA insert specific for rat MMP-9. Furthermore, the blots were rehybridized with a ^{32}P-labeled cDNA from GAPDH to prove equivalent mRNA amounts. The lower panel show a densitometric analysis of MMP-9-specific signals. Similar results were obtained in three independent experiments.

Additionally, the blot was rehybridized with a GAPDH-specific cDNA probe since the rat GAPDH mRNA contains no typical AU-rich elements within its 3´-UTR. In contrast to MMP-9, the level of GAPDH mRNA remained unchanged thus indicating that the changes in mRNA stability were specific for MMP-9 mRNA (Fig. 19A). Furthermore, these data suggest that endonucleolytic cleavage of MMP-9 transcripts is specifically attributable to factors predominantly present (or absent) in the cytoplasmic extracts of NO-treated MC.

Neutralization of HuR leads to an accelerated decay of MMP-9 mRNA in an in vitro degradation assay

As shown by RNA EMSAs the mRNA stabilizing protein HuR is a main constituent of the complexes constitutively bound to MMP-9-specific ARE motifs. To prove the functional relevance of HuR in MMP-9 mRNA stabilization we investigated by an *in vitro* degradation assay whether the half-life of MMP-9 mRNA is modulated by addition of a neutralizing anti-HuR antibody. To this end, cytoplasmic fractions from untreated MC were preincubated for 1h with a monoclonal anti-HuR antibody (a total amount of 400 ng antisera) and mixed with the total RNA from cytokine-treated MC for a further 60 min. As a negative control the same volume of mouse control serum was added to the cytoplasmic fractions instead of the antisera and also pretreated for 60 min. Additionally, to check for direct effects of the NO donors we tested whether cytoplasmic extracts derived from untreated MC but treated with NO after extraction procedures affect on the stability of MMP-9 mRNA. As demonstrated in Fig. 19B the degree of MMP-9 mRNA decay is increased when the cytoplasmic extracts had been pretreated with the neutralizing anti-HuR antibody (Fig. 19B, + anti-HuR (mAb)) whereas the mouse control serum on its own had no modulatory effects on the MMP-9 mRNA decay (Fig. 19B, + mouse serum). Similarly, extracts isolated from untreated cells which had been incubated with DETA NONOate after extraction had no modulatory effect on the MMP-9 mRNA amount (Fig. 19B, +DETA-NONOate). MMP-9 mRNA were exclusively confered by

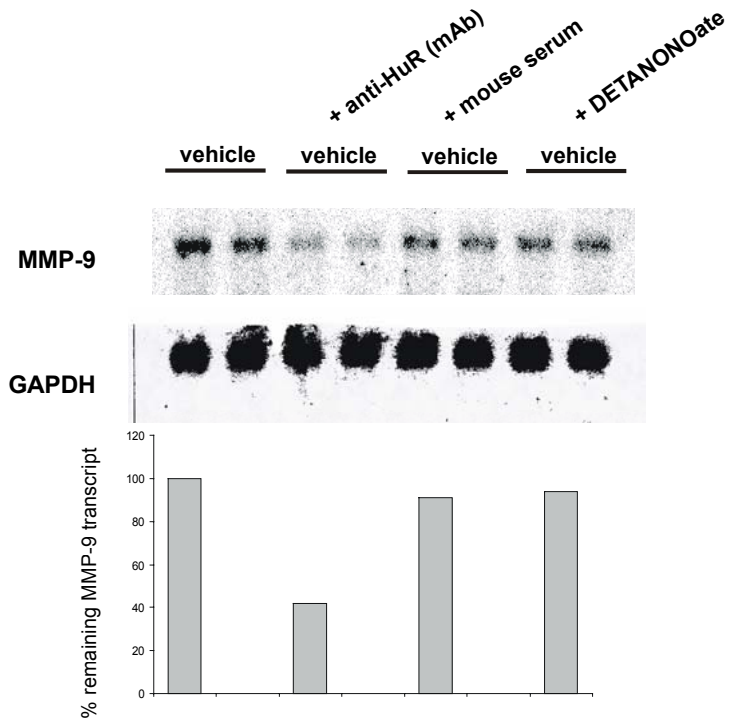

Figure 19 (B) The degradation of cytokine-induced MMP-9 transcripts is accelerated by a neutralizing anti-HuR antibody. Portions of 20 µg of total RNA from a common pool of total RNA isolated from cytokine-stimulated MC were mixed with 130 µg of cytoplasmic extract derived from untreated cells.The cytoplasmic extracts were kept either untreated (vehicle), or pretreated for 1 hour alternatively, with 0.4 µg of a monoclonal anti-HuR antibody (vehicle + anti-HuR) or with the same volume of vehicle (vehicle + mouse serum), or with DETA NONOate (500 µM) (vehicle + DETA NONOate) before the cytoplasmic extracts were incubated with the total RNA portions. Incubation with the RNA was stopped after 60 min before RNA was extracted for further Northern blot analysis. Samples derived from one cytoplasmic extract were subjected to RNA in duplicates. Furthermore, the blots were rehybridized with a ^{32}P-labeled cDNA from GAPDH to prove equivalent mRNA amounts. The lower panel shows a densitometric analysis of MMP-9-specific signals. The result shown is a representative experiment out of three giving similar results.

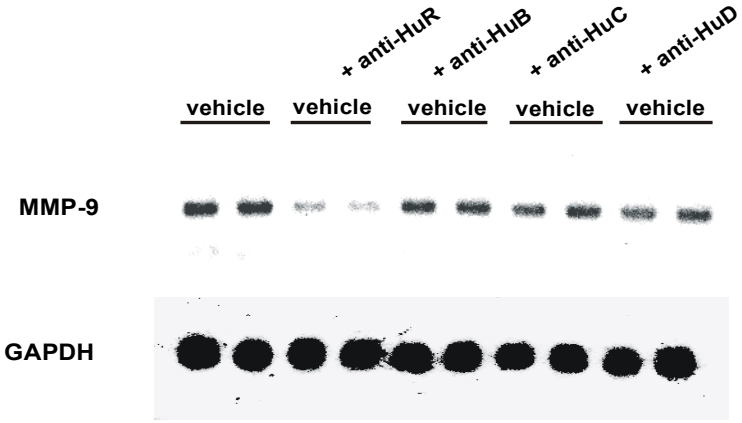

Figure 19 (C) Neutralization of HuR but not that of other Hu family members leads to a reduction in MMP-9 mRNA stability. Portions of 20 µg of total RNA isolated from cytokine-stimulated MC were mixed with 130 µg of cytoplasmic extract derived from untreated cells.The cytoplasmic extracts were kept either untreated (vehicle), or pretreated for 1 hour with 0.4 µg of the Hu-specific antibodies as indicated before the cytoplasmic extracts were incubated with the total RNA portions. Incubation with the RNA was stopped after 60 min before RNA was extracted for Northern blot analysis. Samples derived from one cytolasmic extract were subjected to RNA in duplicates. To prove specificity of the effects on MMP-9 mRNA blots were rehybridized with a ^{32}P-labeled cDNA from GAPDH. Similar results were obtained in two independent experiments.

HuR but not by other proteins of the ELAV- family (Fig, 19C). Finally, we tested whether the addition of recombinant HuR protein could modulate the stability of MMP-9 transcripts in the *in vitro* degradation assay As shown in Fig. 19D addition of GST-HuR protein (200ng) markedly inhibited the mRNA decay of MMP-9 (Fig. 19D; GST-HuR, 2h) whereas the addition of GST alone (200 ng) had no protective effects on the MMP-9 mRNA decay (GST, 2h).

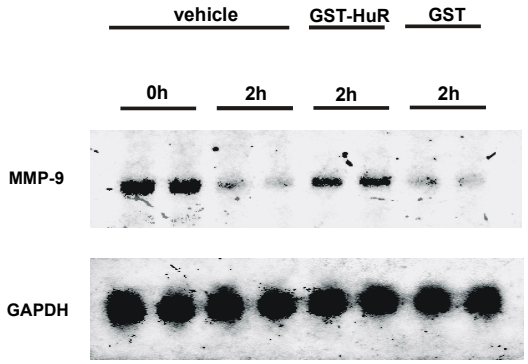

Figure 19 (D) GST-HuR protein protects MMP-9 mRNA from rapid decay. 20 µg of total RNA from cytokine-stimulated MC were mixed with 130 µg of cytoplasmic extract derived from untreated MC in the absence (vehicle) or presence of 200 ng of GST-HuR (GST-HuR) or GST (200 ng). After the indicated time points (0h and 2h) incubations were stopped by isolation of total RNA. Subsequently the RNA was assessed by Northern blot analysis using a MMP-9-specific probe. Furthermore, to prove specificity of the decay of MMP-9 mRNA blots were rehybridized with a ^{32}P-labeled cDNA from GAPDH .

Nitric oxide reduces HuR protein and mRNA levels

To further evaluate whether the reduction of HuR RNA binding caused by NO is due to a decrease in the amount of total HuR protein we performed Western blot analysis using the same monoclonal anti-HuR antibody used for supershift analysis. According to the time-course of NO-dependent MMP-9 mRNA decay (Fig. 13), MC were stimulated for 4 h with different concentrations of the NO donor DETA NONOate before cells were lysed for Western blot analysis. MC were found to express constitutively high levels of HuR protein. Interestingly, in addition to a major band migrating at 34 kDa the antibody detected a second minor band at 32 kDa which indicates a possible posttranslational modification of HuR.

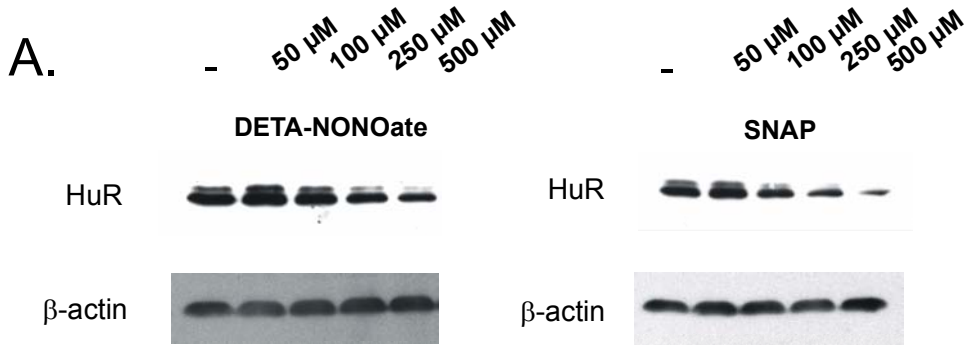

Figure 20. Inhibition of HuR expression in MC by different NO donors. MC were treated for 4 hours with different concentrations of either DETA NONOate (left panel) or SNAP (right panel) before cells were lysed for Western blot analysis 50 µg of total protein were subjected to Western blot analysis using a monoclonal antibody raised against full length HuR. Migration properties were determined using standard molecular weight markers. The Western blots shown in A were stripped and rehybridized to assess total ß-actin levels as a control for sample loading and protein transfer. Similar results were obtained in two independent experiments

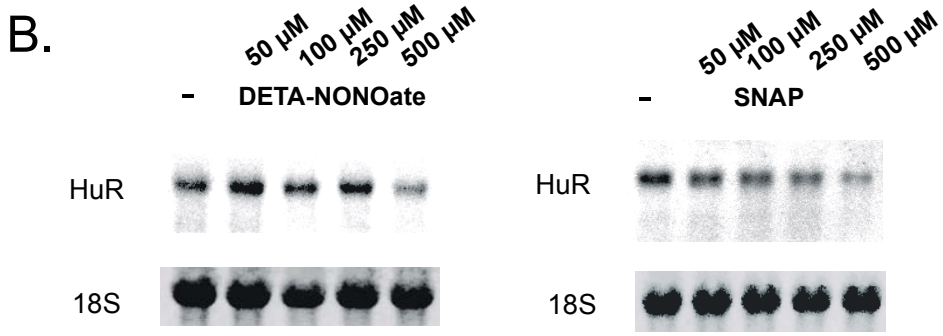

Figure 20 (B) Northern blot analysis demonstrating a dose-dependent modulation of HuR mRNA steady-state level by different NO donors. Total cellular RNA (20 µg) was hybridized to a ^{32}P-labeled cDNA insert from the plasmid "HuR9" and analyzed by Northern blot analysis. Equivalent loading of RNA was ascertained by rehybridization to a 18 S ribosomal probe. The blot shown is representative for two independent experiments giving similar results.

Expression of HuR was dose-dependently reduced by DETA NONOate with a maximal inhibition seen with 500 µM DETA NONOate (Fig. 20A, left panel). Similar to DETA NONOate, treatment of MC with SNAP, a chemically different NO donor, caused a dose-

dependent reduction of the HuR reactive bands (Fig. 20A, right panel). Furthermore, to determine whether the reduction of HuR protein was due to a decrease in the amount of the corresponding HuR mRNA, we performed Northern blot analysis using a probe from the human HuR cDNA [Ma et al., 1996]. As shown in Fig. 20B, both NO donors, DETA NONOate (left panel) and SNAP (right panel), dose-dependently attenuated the basal steady-state HuR mRNA level with a maximal inhibition achieved with 500 µM of each NO donor (Fig. 20B). These data demonstrate that the NO-mediated alterations in HuR RNA binding predominantly result from the changes in the HuR expression levels.

Endogenously produced nitric oxide inhibits the steady-state mRNA and protein levels of HuR

We recently have demonstrated that NO either given by exogenous sources or endogenously produced by the cytokine-evoked iNOS expression can reduce the cytokine-induced MMP-9 level [Eberhardt et al., 2000a]. To test whether endogenously produced NO also is able to inhibit the constitutive level of HuR, MC were treated for 24h with IL-1ß in the presence or absence of the NOS inhibitor L-NMMA. Measurement of nitrite, the stable endproduct of NO formation, in cell supernatants has revealed that the amounts of NO produced by the IL-1ß-stimulated MC are in the range released by the NO donors DETA NONOate and SNAP [Eberhardt et al., 2000a]. Stimulation of MC with IL-1ß caused a marked reduction of the basal HuR protein and mRNA levels as shown in Fig. 21A and Fig. 21B, respectively. The reduction of HuR mRNA level by IL-1ß was abrogated in the presence of the NOS inhibitor, which by its own had no effects on basal HuR levels (Fig. 21). These findings demonstrate that the cytokine-mediated attenuation of HuR expression depends on mesangial iNOS activity.

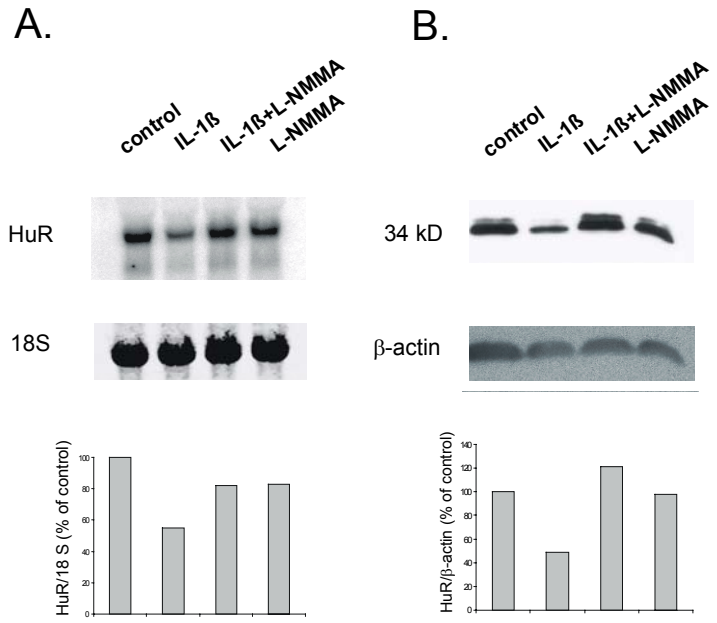

Figure 21. Cytokine-mediated inhibition of steady-state level of HuR mRNA **(A)** and protein **(B)** is blocked in the presence of the NOS inhibitor L-NMMA. Quiescent MC were treated for 24 hours with vehicle (control), IL-1ß (2 nM), L-NMMA (2 mM) or both in combination as indicated before being harvested for either total RNA isolation (A) or Western blot analysis (B) (A). 20 µg of total RNA was hybridized to a ^{32}P-labeled cDNA insert from the plasmid "HuR9" and analyzed by Northern blot analysis. Equivalent loading of RNA was ascertained by rehybridization to a 18 S ribosomal probe. The blot shown is representative for two independent experiments giving similar results. (B). 50 µg of total protein were subjected to Western blot analysis using a monoclonal HuR-specific antibody. The Western blot shown in B was stripped and rehybridized to assess the total ß-actin levels as a control for sample loading and protein transfer.

NO-mediated effects on HuR expression are mimicked by cGMP and depend on endogenous guanylyl cyclase activity

A broad spectrum of physiologic actions exerted by NO depends on the generation of cGMP and is mechanistically linked due to an activation of guanylyl cyclase through the direct binding of NO to the enzyme´s prosthetic heme group [for review see Schmidt and Walter, 1994]. To elucidate whether the inhibiton of HuR expression by NO is mediated by cGMP we tested for effects of the membrane-permeable analog 8-Bromo-cGMP. Similar to the NO donors, 8-Bromo cGMP in a dose-dependent manner reduced the total protein content of HuR with the maximal effect observed with 1 mM 8-Bromo-cGMP (Figure 22A). Concomitantly, 8-Bromo-cGMP dose-dependently reduced the steady-state level of HuR mRNA (data not shown). Finally, we elucidated whether the NO-mediated inhibitory effects on HuR expression depend on cGMP generation. Therefore, we tested whether NO could still reduce

HuR expression in the presence of LY-83583 (500 nM), a specific inhibitor of soluble guanylyl cyclase. As shwon in Fig. 22B, the reduction of HuR in DETA-NONOate-treated cells was strongly antagonized when cells were pretreated with LY-83583. LY-83583 had no effects on the basal HuR protein level (Fig. 22B). In summary, these data demonstrate that the reduction of HuR mRNA and protein steady-state level by NO primarily depend on the activity of guanylyl cyclase and the subsequent increase in cellular cGMP level.

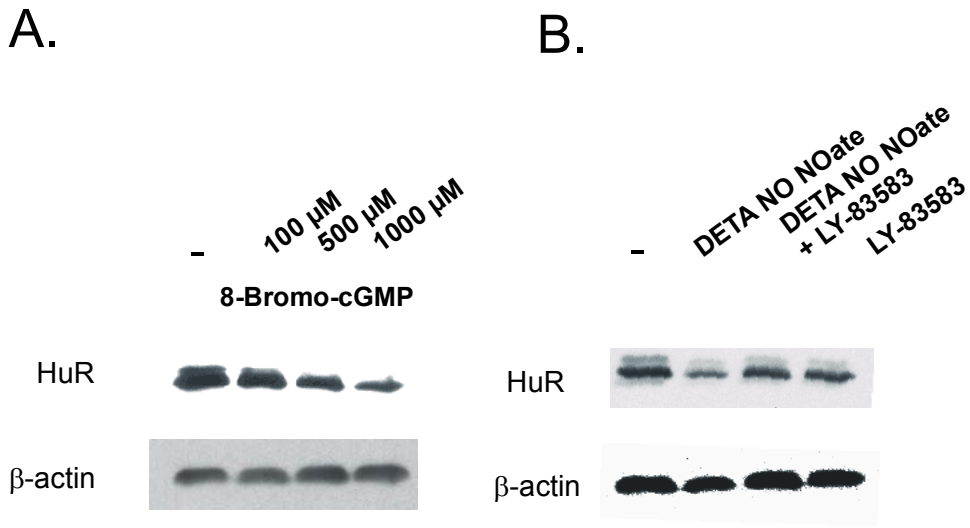

Figure 22: . **(A)**. Suppression of cellular HuR level by 8-Bromo-cGMP. MC were treated for 4 hours with vehicle (control, -) or with the different concentrations of the cGMP analog 8-Bromo-cGMP as indicated before cells were lysed. 50 µg of total protein were subjected to Western blot analysis using a monoclonal antibody raised against full length HuR. Migration properties were determined using standard molecular weight markers. **(B)**. The reduction of HuR protein level by DETA NONOate is antagonized in the presence of the guanylyl cyclase inhibitor LY-83583. Quiescent MC were treated for 4 hours with vehicle (control, -), DETA NONOate (500 µM), LY-83583 (500 nM) or both in combination as indicated before being harvested for preparation of total protein extracts. The level of total cellular HuR content was assessed with a monoclonal anti-HuR antibody. All Western blots were finally stripped and rehybridized to assess total ß-actin levels as a control for sample loading and protein transfer. Similar results were obtained in two independent experiments.

Discussion

MMP-9 is a protease which is critically involved in the regulation of ECM turnover and an imbalanced regulation is functionally correlated with numerous pathologic conditions associated with altered matrix turnover such as atherosclerosis, rheumatoid arthritis and fibrosis of lung, skin and kidney [for review see Brinckerhoff and Matrisian, 2002; Cawston,

1998; Davies et al.,1992]. Inflammatory cytokines such as IL-1ß and TNFα are among the most potent transcriptional inducers of MMP-9 gene expression, mainly through signaling via the MAP kinase pathway and triggering the activation of NF-κB and AP-1 transcription factors [Eberhardt et al., 2000b]. The free radical gas NO has been shown to modulate the expression and activity of different MMPs including MMP-9 [Eberhardt et al., 2000a; Murrell et al., 1995; Pfeilschifter et al., 2001; Trachtman et al., 1996; Zaragoza et al., 2002]. In this study we demonstrate that posttranscriptional mechanisms account for the NO-mediated inhibition of MMP-9 expression in rat MC. Using actinomycin D experiments we found that exogenously applied NO as well as endogenously produced NO significantly accelerates degradation of MMP-9 mRNA [Eberhardt et al., 2002b]. Furthermore, we demonstrate that cytoplasmic extracts derived from NO-treated MC displayed increased degradative properties upon MMP-9 mRNA transcripts when compared to the effects of cytosolic fractions from untreated cells. Similar to MMP-9, a recent study has demonstrated a NO-dependent attenuation of basal and angiotensin II-stimulated expression of transforming growth factor ß-3 by increased destabilization of the corresponding mRNA [Abdelaziz et al., 2001]. However, the underlying mechanisms of the NO-evoked mRNA decay had not been further characterized. Increasing evidence has accumulated demonstrating that selective mRNA turnover plays an important role in the tight control of eukaryotic gene expression [Brennan and Steitz, 2001; Chen and Shyu, 1995; Fan and Steitz, 1998; Grzybowska et al., 2001; Ma et al., 1996]. Regulation of mRNA stability has been demonstrated to contribute to the strong and rapid changes of RNA abundance observed mainly for genes of the inflammatory- and stress-induced response, among them mRNAs coding for TNFα [Biragyn and Nedospasov, 1995; Dean et al., 2001], IL-2 [Bohjanen et al., 1991; Henics et al., 1994], IL-3 [Nair et al., 1994], IL-8 [Stoeckle, 1991; Villarete and Remick, 1996], c-Fos [Chen et al., 1995; Perez-Sala et al., 2001], c-Myc [Dani et al., 1985, Rabbitts et al., 1985], cyclin A and B1 [Wang et al., 2000], granulocyte/macrophage colony stimulating factor (GM-CSF)

[Aharon and Schneider, 1993; Chen et al., 1995], vascular endothelial cell-derived growth factor (VEGF) [Levy et al., 1996], cyclooxygenase-2 (COX-2) [Dixon et al., 2001], and iNOS [Perez-Sala et al., 2001; Rodriguez-Pascual et al., 2000] just to name a few of them. The complex processes of transcript degradation in most cases includes a poly (A)-shortening endonucleolytic cleavage which is controlled by trans-acting factors binding to the cis-acting elements within the 3´-UTR of mRNAs [Chen and Shyu, 1994; Grzybowska et al., 2001; Ross, 1995; Xu et al., 1997]. Therefore, the half-lifes of most mRNAs are influenced by this region. We demonstrate that the 3´-UTR of MMP-9 confers a NO-dependent reduction of basal and cytokine-induced MMP-9-driven luciferase activity when fused downstream of the luciferase reporter gene. Similarly, the 3´-UTR of MMP-9 was able to convey a negative NO responsiveness to a constitutively active luciferase gene when fused downstream of the luciferase coding sequence. From these data it becomes evident that the reduction of luciferase activities induced by NO is conferred by the 3´-UTR of MMP-9. A common feature of most short-lived mRNAs is the presence of AU-rich elements (AREs) which are considered as cis-regulatory determinants of RNA turnover [Chen and Shyu, 1994; Malter, 1989; Xu et al., 1997]. Also the 3´-UTR of MMP-9 contains several copies of the pentameric AUUUA motif. This sequence motif was initially described as a destabilizing determinant in the 3´-UTR of GM-CSF [Aharon and Schneider, 1993; Chen et al., 1995] and the immediate early genes c-fos and c-myc [Chen et al., 1995; Rabbitts et al., 1985]. Meanwhile numerous ARE-binding proteins have been implicated in mRNA turnover, among them proteins which can destabilize mRNA such as AUF1 [Zhang et al., 1993] but interestingly also proteins which can reduce mRNA degradation, most prominently members of the embryonic lethal abnormal vision (ELAV) protein family [Levy et al., 1996; Ma et al., 1996; Peng et al., 1998]. By native RNA-EMSA we could identify HuR, an ubiquitously expressed member of the ELAV RNA-binding proteins, exhibiting a strong constitutive RNA binding to three different ARE motifs ("MMP-9-ARE-1, MMP-9-ARE-2, MMP-9-ARE-4") within the 3´-UTR of rat MMP-9.

Interestingly, a fourth AUUUA repeat ("MMP-9-ARE-3") did not display any binding affinity. Sequence comparison of the bases directly flanking the AUUUA pentamer indicates that the presence of a neighbouring uracil or adenosine base at the 3´-end of MMP-9-AREs seems critical for RNA binding. In line with these observations previous studies have suggested that AU-rich elements alone are necessary but not sufficient for mRNA destabilization and thereby underlined the importance of neighbouring sequences [Rodriguez-Pascual et al., 2000]. Similar to externally expressed HuR protein the crude cytoplasmic extracts of MC displayed a strong constitutive RNA binding to MMP-9-specific AREs. The identity of these complexes was confirmed by supershift analysis using antibodies raised against different members of the Hu protein family. Interestingly, only addition of anti-HuR resulted in the shifting of all complexes and appearance of two supershifted complexes whereas antibodies against HuB, HuC and HuD had no effects. We observed the appearance of two supershifted complexes independent of the source of protein (recombinant vs. MC-derived) used in the binding reaction. Obviously, HuR is the sole protein in both supershifted complexes and the different migration properties rather result from HuR oligomerization than from complexation with other ARE-binding proteins. Furthermore, we demonstrate that the constitutive RNA binding to MMP-9 ARE motifs by the HuR-containing complexes is drastically reduced in cells treated with different NO donors. Most importantly, these experiments displayed a dose-dependent reduction in RNA binding thus suggesting that RNA binding to the 3´-UTR of MMP-9 is susceptible even to low NO concentrations. Moreover, we prove the physiological relevance of these modulatory effects of NO on HuR binding as similar to exogenous NO, cytokine-treated MC exhibited a reduction in RNA binding dependent on endogenous NO formation. So far little is known about the mechanisms by which HuR can regulate mRNA stability. Unlike the other members of the ELAV family (HuB, HuC and HuD) which show a tissue-specific expression, HuR is ubiquitously expressed and shows a predominant nuclear distribution [Atasoy et al., 1998; . Dean et al.,

2001 ; Peng et al., 1998]. However, a change of distribution between the nuclear and cytoplasmic compartment is considered as the main mechanism by which HuR can modulate mRNA decay [Ford et al., 1999; Peng et al., 1998]. A previous study has demonstrated a downregulation of HuA in quiescent 3T3 fibroblasts but upregulated HuA levels after serum stimulation thus corroborating the important roles of ELAV proteins during cell growth and differentiation (Atasoy et al., 1998). Since we have chosen serum free preincubations for all experimental conditions it seems most unlikely that the rapid negative effects on HuR expression by NO or cGMP are indirectly due to negative growth effects. Recent studies have highlighted the role of the p38 MAP kinase pathway in the regulation of cytoplasmic HuR abundance [Winzen et al., 1999]. Using confocal fluorescence microscopy we found that HuR is most abundant in the nucleus and the nuclear distribution is not changed by treatment of MC with NO (W.Eberhardt and A.Huwiler, unpublished observations). In contrast, EMSA revealed that the RNA-binding properties exhibited by crude nuclear extracts were several times weaker than the RNA-binding exhibited by cytoplasmic fractions. These data suggest that although HuR is mainly localized in the nucleus, NO-mediated alterations in mRNA binding predominantly occur in the cytoplasmic compartment but are not attributable to a modulation of the nucleo-cytoplasmic shuttling properties of HuR. In this context, the recent finding that the AMP-activated kinase (AMPK) regulates the cytoplasmic HuR and consequently modulates the binding of HuR to its target mRNAs is of special interest [Wang et al., 2002]. Whether the activation of the AMPK or MAP kinase pathways is also involved in the regulation of MMP-9 mRNA turnover is currently investigated in our laboratory . Here we present an alternative mechanism by which a reduction of RNA binding is caused by the altered expression of HuR. We demonstrate that treatment of MC with different NO donors results in a significant reduction of the cellular HuR protein content. Furthermore, we found that the reduction in HuR protein is due to a reduction in the corresponding steady-state mRNA level. Similarly, the 24-hour exposure of cells to IL-1ß caused a significant reduction

of HuR on both, the protein and mRNA level, and this reduction could be blocked by the NOS inhibitor L-NMMA thus indicating that the cytokine-mediated inhibition of HuR expression is triggered by NO. These data demonstrate that the reduced RNA binding of HuR predominantly results from a decrease in the HuR expression levels. Consistent with our findings, alterations of HuR expression also determine the half-life of COX-2 mRNA and change COX-2 synthesis in colon cancer cells [Dixon et al., 2001]. Furthermore, an aberrant expression of HuR was demonstrated in human teratocarcinoma cells [Antic et al., 1999] and malignant brain tumors [Nabors et al., 2001]. The most common intracellular messenger system activated by NO is the soluble guanylyl cyclase/cGMP system which is activated by a direct binding of NO to the prosthetic heme group of the enzyme [Schmidt and Walter**,** 1994]. We demonstrate that the NO-mediated reduction of constitutive HuR expression level can be mimicked by the cGMP analog 8-Bromo cGMP. Furthermore, we found that the NO-dependent reduction of HuR is blocked in the presence of the specific guanylyl cyclase inhibitor LY-83583 thus demonstrating that the NO-mediated decrease in HuR expression depends on the activation of guanylyl cyclase and thus complements the mainly transcriptional regulation driven by NO in an cGMP-independent manner [Pfeilschifter et al., 2001; Schmidt and Walter, 1994]. In conclusion, the experiments presented here demonstrate for the first time that the expression of MMP-9, in addition to a transcriptional regulation, is controlled by post-transcriptional mechanisms including the modulation of mRNA stability by the mRNA stabilizing factor HuR. Moreover, NO reduces in a cGMP-dependent manner the expression of HuR. Whether the decrease of HuR mRNA in turn is due to transcriptional or posttranscriptional mechanisms remains to be elucidated. The excessive accumulation of extracellular matrix is an important feature in the progression of fibrotic pathologies and is most likely due to a reduced expression of matrix-metabolizing enzymes. In this context the increased production of NO often accompanying inflammatory processes may have a

causative role in the progression of fibrotic processes by altering matrix turnover via transcriptional as well as posttranscriptional mechanisms.

3. ATP potentiates IL-1ß-induced MMP-9 expression in mesangial cells via recruitment of the ELAV protein HuR

Introduction

The matrix metalloproteinases (MMPs) are members of a family of zinc-dependent endopeptidases which specifically degrade components of the extracellular matrix (ECM). Therefore, MMPs have mainly been implicated in various diseases accompanied with an altered turnover of ECM. Besides the altered synthesis of single ECM components the increased expression and/or activity of MMPs seems of paramount importance for pathological remodeling processes within the kidney such as acute proliferative glomerulonephritis [Edelstein et al., 1997; Lenz et al., 2000]. Mainly the altered expression of MMP-2 and MMP-9, which are also denoted as gelatinases, is crucially involved in the progression of glomerular inflammatory processes [Lenz et al., 2000; Davies et al., 1992]. In addition to various inflammatory cytokines, the expression of MMP-9 can be activated by many other stimuli such as mitogens, growth factors, and activators of the Ras oncogene [for review see: Woessner, 1991; Nagase and Woessner, 1999]. Although most of these stimuli can modulate gelatinolytic activity by influencing MMP-9 gene expression the regulation of MMP-9 activity is also achieved by the processing of the inactive proenzyme by the action of other proteases and by an inhibition of the active enzyme by its endogenous inhibitors, the tissue inhibitors of MMPs (TIMPs) [Woessner, 1991; Nagase and Woessner, 1999]. Previously, we have demonstrated an additional mode of posttranscriptional regulation of MMP-9 which involves the reduction of cytokine-induced MMP-9 via reduction of mRNA stability exerted by exogenous and endogenously produced NO [Eberhardt et al., 2002b]. A variety of inducible genes including proto- oncogenes, transcription factors, cell cycle regulating proteins and cytokines have been demonstrated to be regulated by a modulation of mRNA turnover. Recent evidence has revealed that AU-rich sequences also denoted as AU-

rich elements (AREs) located in the 3´-untranslated regions (UTRs) of these genes comprise specific cis-regulating elements which target mRNAs for rapid degradation [for review see: Chen and Shyu 1995; Ross, 1995; Fan and Steitz, 1998; Peng et al., 1998]. Several studies have identified proteins specifically binding to AREs, among them RNA stabilizing factors of the embryonic lethal abnormal vision (ELAV) protein family especially the ELAV-like protein HuR [Fan and Steitz, 1998; Peng et al., 1998; Brennan and Steitz, 2001]. Overexpression and subsequent binding of HuR was shown to result in an efficient stabilization of ARE-containing mRNAs *in vivo* [Fan and Steitz, 1998]. Besides the proinflammatory cytokines, MC are able to respond to a variety of other biological mediators including eicosanoids, growth factors, reactive oxygen species, NO and to extracellular nucleotides such as ATP and UTP [Pfeilschifter and Merriweather, 1993; Pfeilschifter et al., 2003]. Previously, we have reported that in renal mesangial cells, extracellular ATP via the $P2Y_2$ receptor can cause a mobilization of intracellular calcium and subsequent activation of protein kinases C [Pavenstädt et al., 1993; Pfeilschifter and Huwiler, 1996]. The cellular long-term responses towards ATP and UTP include a variety of pathophysiological key functions most importantly the inhibition of programmed cell death and an increase in cell proliferation [Huwiler et al., 2002; Schulze-Lohoff et al., 1998; Huwiler and Pfeilschifter, 1994]. Both processes, cell proliferation and the excessive ECM degradation are hallmarks of many chronic progressive glomerular diseases [Edelstein et al., 1997; Lenz et al., 2000; Davies et al., 1992; Fogo, 2001]. For this reason we tested whether extracellular nucleotides can influence the expression of MMP-9 in MC. Our data provide the first report that ATP and UTP can potentiate the cytokine-induced expression and activity of MMP-9. Furthermore, we implicate modulation of the nuclear-cytosolic shuttling of the RNA stabilizing factor HuR in the posttranscriptional regulation of MMP-9 by extracellular nucleotides.

Results :

Extracellular nucleotides potentiate IL-1ß-induced lytic activity and mRNA steady-state levels of MMP-9

To evaluate possible effects of extracellular nucleotides on the activity of MMP-9, MC were treated with IL-1ß for 24 h in the presence or absence of either ATP (30 µM) or UTP (30 µM). Furthermore, we tested for possible effects of adenosine, a degradation product of ATP, as well as γ-thio-ATP (γ -S-ATP), a stable ATP analog. The gelatinolytic contents in the conditioned medium of MC were tested by zymography using gelatin as a substrate [Eberhardt et al., 2000a]. We chose a 24 h treatment to allow accumulation of extracellular MMP-9 which is high enough to be detected by gelatine zymography [Eberhardt et al., 2000a]. The cytokine-mediated gelatinolytic content of latent MMP-9, which is represented by one lytic band at 92 kDa, is strongly increased when MC were cotreated with γ -S-ATP or UTP (Fig. 23A). No effect on the IL-1ß-caused lytic content is seen with ATP and its degradation product adenosine (Fig. 23A). We suggest that under cell culture conditions the rapid degradation by ectonucleotidases efficiently removes ATP and therefore prevents sustained signaling by ATP. We observed that the levels of lytic bands migrating at 68 and 72 kDa which corresponds to latent and active MMP-2 were not significantly changed which is consistent with the constitutive expression of MMP-2 in rat MC [Eberhardt et al., 2000a]. In the absence of IL-1ß, none of the tested nucleotides or nucleoside was able to induce MMP-9 activity (Fig. 23A). Furthermore, the stimulatory effect of γ-S-ATP occurred in a dose-dependent manner with a maximal effect seen with 30 µM of γ-S-ATP (Fig. 23B). In contrast, the higher concentrations did not further augment the cytokine-induced gelatinolytic content in the conditioned media (Fig. 23B, upper panel). To evaluate whether the increased level of gelatinolytic activity of MMP-9 is preceeded by an enhanced expression of MMP-9 mRNA, we performed Northern blot analyses using a cDNA from the rat MMP-9 gene. As shown in Fig. 23B (lower panel) similar to the changes in the gelatinolytic contents, γ-S-ATP dose-

dependently augmented the cytokine-induced MMP-9 mRNA level with a maximal effect seen at 30 µM γ-S-ATP whereas concentrations higher than 30 µM blunted the amplification of cytokine-induced MMP-9. No MMP-9 mRNA was detected in the absence of IL-1ß when stimulating with the nucleotide alone (Fig. 23B, lower panel). To test the time-dependency of cytokine-induced MMP-9 by γ-S-ATP we monitored the time-course of MMP-9 induction by Northern blot analysis.

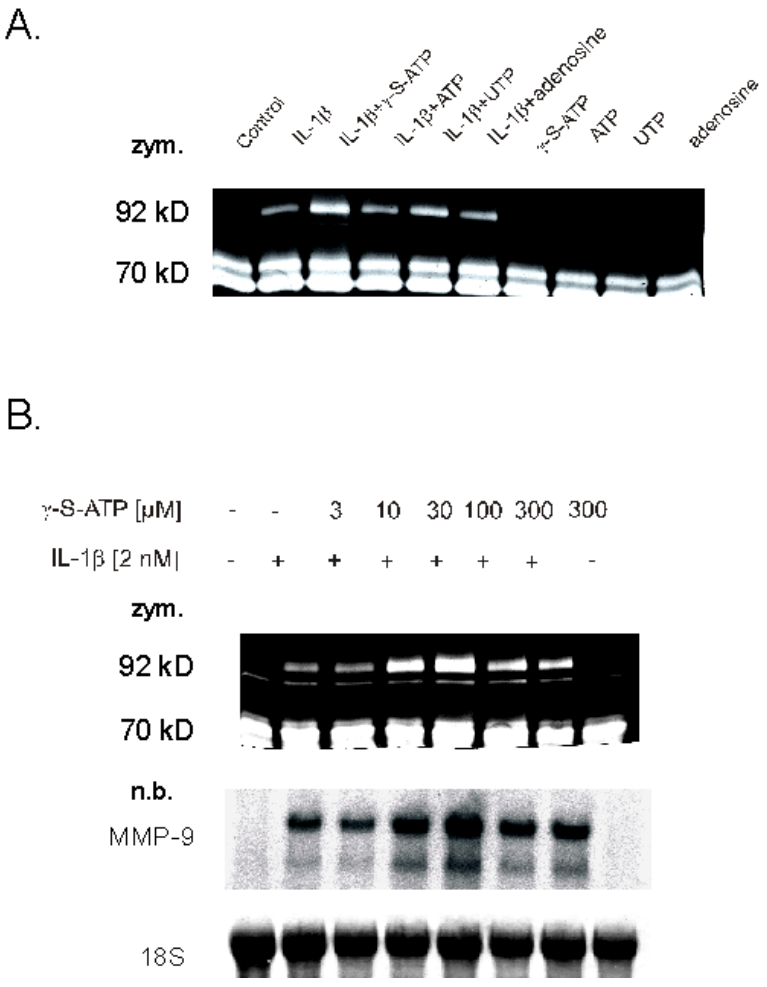

Figure 23 (A). Effects of extracellular nucleotides on IL-1ß-induced MMP-9 activity in mesangial cells. Quiescent mesangial cells were stimulated with either vehicle (Control) or IL-1ß (2nM) in the presence or absence of γ -S-ATP, ATP, UTP, and adenosine (each at 30µM). 24 hours after stimulation, 10 µl of supernatants were subjected to SDS-PAGE-zymography. The migration properties of lytic bands, corresponding to inactive pro MMP-9 (92 kD), and the inactive and active forms of MMP-2 (72 and 68 kDa) were determined using standard m.w. markers. The data shown are representative for three experiments giving similar results. **(B).** Dose-dependent amplification of IL-1ß-induced MMP-9 activity and MMP-9 mRNA level by γ-S-ATP. Quiescent mesangial cells were stimulated with either vehicle (-) or IL-1ß (2nM) (+) in the presence of the indicated concentrations (in µM) of γ-S-ATP. After 24 hours 10 µl of supernatants were collected for SDS-PAGE zymography (zym) and cell monolayers collected for mRNA isolation and Northern blot analysis (n.b.). Total cellular RNA (20 µg) was hybridized to a ^{32}P-labeled cDNA insert from KS-MMP-9. Equivalent loading of RNA was ascertained by rehybridization to a 18 S rRNA probe. Data are representative of 3 independent experiments giving similar results.

As shown in Fig. 23C the indcution of MMP-9 mRNA as caused by the treatment of MC with either IL-1ß or with IL-1ß plus γ-S-ATP did not occur at the early time points tested (4h and 12h) thus indicating that γ-S-ATP can not induce MMP-9 mRNA expression by its own. Furthermore, these data indicate that the alterations of cytokine-induced gelatinolytic activity by γ-S-ATP predominantly result from changes in the MMP-9 expression levels.

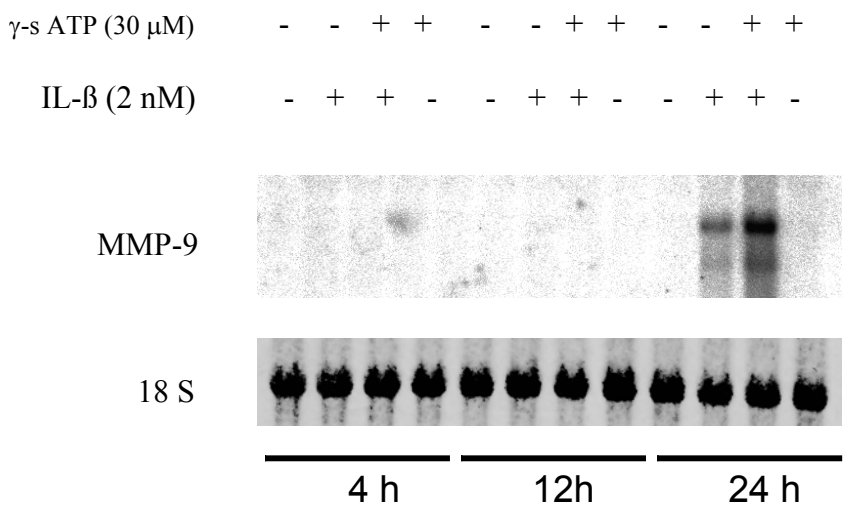

Fig.23 (C). Time-course of IL-1ß-induced MMP-9 steady-state mRNA level in the presence or absence of γ-S-ATP. Mesangial cells were stimulated with the IL-1ß (2nM) with or without γ-S-ATP (30 μM) for the indicated time periods before mRNA was isolated for Northern blot analysis. Total cellular RNA (20 μg) was hybridized to a ^{32}P-labeled cDNA insert from KS-MMP-9. Equivalent loading of RNA was ascertained by rehybridization to a 18 S rRNA probe. Data are representative of 2 independent experiments giving similar results.

Involvement of the P2Y$_2$ receptor in the amplification cascade of MMP-9 by ATP

Many of the physiologcial actions exerted by ATP involve the G-protein coupled P2Y$_2$ subtype of purinoceptors. We therefore tested by a pharmacological approach for the involvement of the P2Y$_2$ purinoreceptor in the amplification of cytokine-induced MMP-9 expression by use of suramin, a putative antagonist of P2Y$_2$ but not of P2Y$_4$ purinoceptors [Boarder and Hourani, 1998]. As shown in Fig. 24, suramin dose-dependently inhibited the γ-S-ATP-mediated potentiation of IL-1ß gelatinolytic activity of MMP-9 without affecting the

cytokine-caused gelatinolytic content of MMP-9. This suggests an involvement of the $P2Y_2$ subtype of purinoceptor in the ATP signaling of MMP-9 expression.

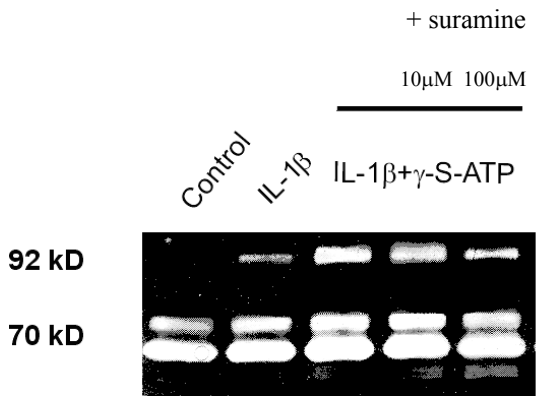

Fig. 24. The γ-S-ATP-triggered amplification of cytokine-induced gelatinolytic activity of MMP-9 is inhibited by suramin. Quiescent mesangial cells were simultaneously treated for 24 hours with either vehicle (control) or IL-1ß (2nM), IL-1ß (2nM) plus γ-S-ATP (30 μM) in the presence of the indicated concentrations of suramin before 10 μl of cell supernatants were subjected to SDS-PAGE zymography. Migration properties of lytic bands were determined with molecular weight markers. The data are representative for two independent experiments giving similar results.

Nucleotides have no effects on cytokine-induced MMP-9 promoter activity

To further evaluate whether the ATP-mediated amplification of IL-1ß-induced MMP-9 expression resulted from an increase in MMP-9 gene transcription we assessed promoter activites derived from a 1.3 kb fragment of the rat MMP-9 promoter region (pGL-MMP-9 1.3 kb) by luciferase reporter gene assays. This promoter region contains several functional elements necessary for cytokine-mediated regulation of MMP-9 expression most important one NF-κB binding site and one AP-1 response element which is close to a functional Ets-1 binding site [Eberhardt et al., 2002a]. Transient transfection of MC with pGL-MMP-9 1.3 kb comprising the 1.3 kb promoter fragment fused to the luciferase reporter gene, was followed by a 24 h treatment with either vehicle, IL-1ß (2 nM), with or without either γ-S-ATP (30 μM), or UTP (30 μM) or with each nucleotide alone and subsequently assayed for luciferase activity (Fig. 25). IL-1ß significantly stimulated luciferase activity (1.9-fold, $p< 0.01$) but the promoter activity was only weakly enhanced by either nucleotide. These data demonstrate that

the amplification of cytokine-induced mRNA steady-state levels by γ-S-ATP can not be located to the upstream 1.3 kb MMP-9 promoter context.

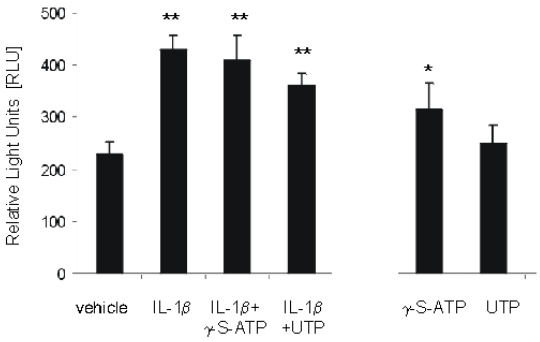

Figure 25. The extracellular nucleotides γ-S-ATP and UTP do not affect cytokine-mediated promoter activity of a 1.3 kb portion of rat MMP-9 promoter. MC were transiently cotransfected with 0.4 µg of pGL-MMP-9 1.3 kb and with 0.1 µg of pRL-CMV coding for *Renilla* luciferase. After overnight transfection, MC were treated for 24hours with vehicle (control), IL-1ß (2 nM), γ-S-ATP (30 µM), UTP (30 µM) or a combination of IL-1ß and the nucleotides as indicated before being harvested for measurement of dual luciferase activities, as described in *Material and Methods*. Values for beetle luciferase were related to values for *Renilla* luciferase and are depicted as relative light units (RLU). Data are the means ± S.D. (n=6) **, $p \leq 0.01$, compared to control.

γ-S-ATP inhibits the decay of cytokine-induced MMP-9 mRNA

To test whether the ATP effects relay to some posttranscriptional events we performed actinomycin D experiments. MC were stimulated for 20 h with IL-1ß (2 nM) before transcription was blocked by actinomycin D (5 µg/ml). Subsequently, cells were either directly homogenized (vehicle , 0h) or left untreated (vehicle, 12 h) or alternatively treated with the stable ATP analog γ-S-ATP (30 µM). After 12 h cells were homogenized for the isolation of total RNA. The MMP-9 mRNA from untreated MC displayed a strong reduction by almost 80 % ($p< 0.005$) in the mRNA steady-state level (Fig. 26A). Most interestingly, the decay of MMP-9 mRNA was completely blocked in the presence of γ-S-ATP, thus indicating that γ-S-ATP can stabilize the IL-1ß-induced MMP-9 mRNA (Fig. 26A). Furthermore, time-course experiments revealed that the mRNA stabilizing effects on MMP-9 transcripts by γ-S-ATP already occurred at 4 h and were maximal 12 h after the blockade of transcription by actinomycin D (Fig. 26B).

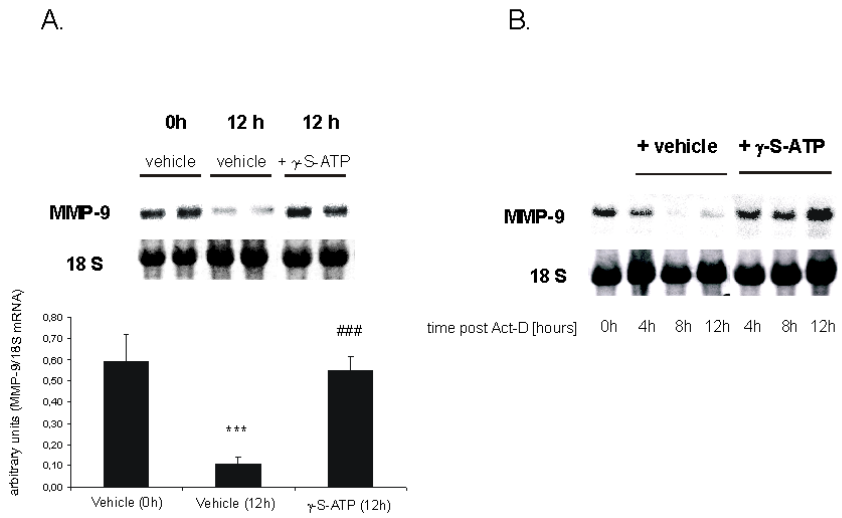

Figure 26 : (A). γ-S-ATP prevents the decay of cytokine-induced MMP-9 mRNA. Quiescent MC were treated for 20 hours with IL-1ß (2 nM) to reach maximal level of MMP-9 transcripts. Thereafter cells were washed twice and incubated with actinomycin D (5 µg/ml) for 30 min. This time point was set as 0 hours and cells were additionally treated for either 12 hours or left untreated (vehicle 12h). **(B)** Quiescent MC were treated for 20 hours with IL-1ß (2 nM) to reach maximal level of MMP-9 transcripts. Thereafter cells were washed twice and incubated with actinomycin D (5 µg/ml) for 30 min. This time point was set as 0 hours and cells were additionally treated for the indicated time points without (vehicle) or with 30 µM γ -S-ATP before being harvested and extracted for total cellular RNA. 20 µg of total cellular RNA were hybridized to ^{32}P-labeled MMP-9 or 18S cDNA probes, respectively. The lower panel of Fig. 26A shows a densitometric analysis of three independent experiments.

Recently it has been reported that transcription blockage by actinomycin D can induce a redistribution of the mRNA stabilization factor HuR from the nucleus to the cytoplasm and therefore may cause an increase in the mRNA half-life [Peng et al., 1998]. However, in our experiments all cells were equally treated with actinomycin D and, therefore, the net effect on MMP-9 decay exclusively depends on the presence or absence of extracellular γ-S-ATP. In contrast, IL-1ß by itself had no effect on the stability of cytokine-induced MMP-9 mRNA since the addition of IL-1ß(2nM) after transcriptional blockade did not change the mRNA decay of MMP-9 (Fig. 26C). In summary, these data indicate that γ-S-ATP can augment the cytokine-induced MMP-9 expression by an increase of MMP-9 mRNA stability.

The cytoplasmic fractions of ATP-treated MC stabilize MMP-9 mRNA in vitro

The involvement of cytoplasmic factors exerting MMP-9 mRNA stabilizing properties was furthermore tested by an *in vitro* degradation assay. By this assay the time-course of degradation of purified MMP-9 mRNA exhibited by any trans-acting factor accumulated in

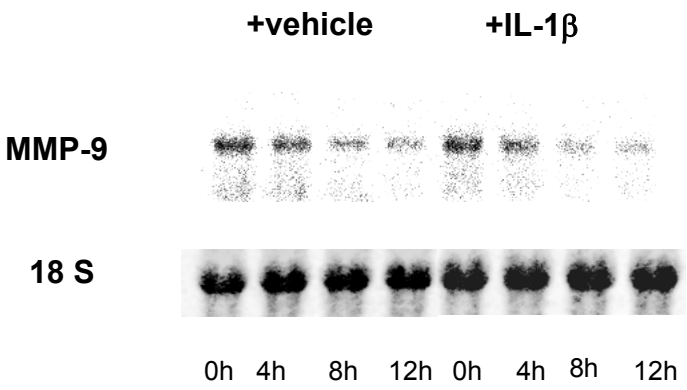

Figure 26 (C) Quiescent MC were treated for 20 hours with IL-1ß (2 nM) before cells were washed twice and incubated with actinomycin D (5 µg/ml) for 30 min. This time point was set as 0 hours and cells were additionally treated for the indicated time points without (+vehicle) or with 2 nM IL-1ß (+IL-1ß) before being harvested and extracted for total cellular RNA. 20 µg of total cellular RNA were hybridized successively to ^{32}P-labeled MMP-9 or 18S cDNA probes, respectively. The blot is representative for two independent experiments.

the cytoplasmic extracts from γ-S-ATP-treated MC was compared to the degradation profile derived from untreated MC. Similar volumes of each cytoplasmic extract (with a total protein content of 130 µg) were incubated with 20 µg of total RNA isolated from a common pool of cytokine-treated MC containing a high level of MMP-9 mRNA. Subsequently, we isolated total RNA after the indicated time points and performed Northern blot analysis using a probe from the rat MMP-9 cDNA (Fig. 27). As shown before γ-S-ATP alone is not able to induce endogenous MMP-9 mRNA levels (Fig. 23). Therefore, most of the MMP-9 mRNA detected by degradation assay is derived from the exogenous pool of MMP-9 mRNA but not from endogenous MMP-9 transcripts within the cytoplasmic extracts. We observed that the primary undegraded MMP-9 transcripts which had been exposed to the cytoplasmic fractions from ATP-treated MC displayed a delayed degradation when compared to MMP-9 transcripts exposed to cytoplasmic extracts from untreated cells which is indicated by the different time-courses of decay of MMP-9 mRNA (Fig. 27). In contrast, the amount of the stable GAPDH mRNA was not affected by none of the extracts tested, thus suggesting that the effects on mRNA decay are specific for MMP-9 mRNA. These data indicate that the cytoplasmic

extracts from γ-S-ATP-treated MC contain RNA protective factors responsible for the delayed decay of MMP-9 mRNA.

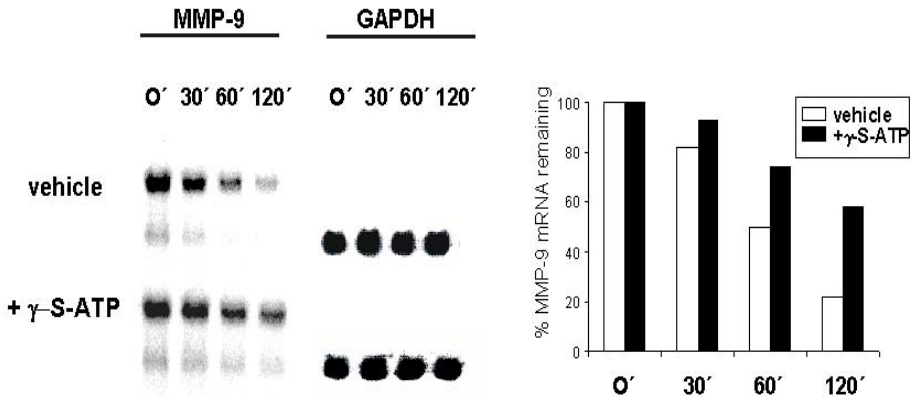

Figure 27. Cytosolic lysates of ATP-treated MC exert anti-degradative properties on MMP-9 mRNA in an *in vitro* RNA degradation assay. Portions of 20 µg of total RNA from a common pool of total RNA isolated from cytokine-stimulated MC were mixed with 130 µg of cytoplasmic extract derived from either untreated (vehicle) or γ-S-ATP-treated MC (+γ-S-ATP). After the indicated time periods incubations were stopped by isolation of total RNA. RNA samples were collected and assessed for the amounts of remaining MMP-9 mRNA level by Northern blot analysis using a ^{32}P-labeled cDNA insert specific for rat MMP-9. In order to prove specificity of the effects we furthermore hybridized the blots with a ^{32}P-labeled cDNA from GAPDH. Similar results were obtained in three independent experiments.

The 3´-UTR of the MMP-9 gene specifically confers an ATP-dependent increase in promoter activity

Regulation of mRNA stability is in many cases determined by AU-rich sequences (AREs) within the 3´-UTR of genes [Chen and Shyu, 1995; Ross, 1995; Brennan and Steitz, 2001]. Since MMP-9 contains four copies of ARE motifs within its 3´-UTR (ARE1, ARE2, ARE3, ARE4 in fig. 14) we tested whether the increase in MMP-9 mRNA stabilitization by γ-S-ATP depends on the presence of these ARE motifs downstream of the coding region of the gene (CDS). To this end the promoter activitiy of a luciferase reporter gene, which was driven by the pGL-MMP-9 1.3 kb upstream promoter region (Fig. 28A), was compared to that derived from transient transfection of a similar MMP-9 promoter construct containing an additional 662 bp from the 3´-UTR of the MMP-9 gene cloned downstream of the Luciferase CDS (3´-UTR- pGL MMP-9-1.3kb ín Fig. 28B). Transient transfection of MC with both MMP-9 reporter genes was followed by a 20h treatment with either vehicle (control), IL-1ß (2 nM), γ-

S-ATP (30 µM) or both in combination. Stimulation of MC with IL-1ß leads to a significant increase of MMP-9 promoter activity (3.4-fold, p< 0.005) (Fig. 28A). Addition of γ-S-ATP did not further increase the IL-1ß-induced pGL-3-MMP-9 luciferase activity (3.5-fold, p< 0.005) but caused a significant enhancement of cytokine-triggered promoter activity when the luciferase coding region was under the additional control of the 3´-UTR of MMP-9 (Fig. 28B). Similarly, stimulatory effects on basal luciferase activity by γ-S-ATP alone were significantly increased when the luciferase gene was under the additional control of the 3´-UTR of MMP-9 (from 1.6 ± 0.02-fold, p<0.05; to 2.3 ± 0.015-fold induction; p<0.005; mean ± SD, n=3). Most probably this is due to the stabilization of the constitutively expressed luciferase mRNA by γ-S-ATP via the inserted 3´-UTR of MMP-9. Accordingly, the high level of basal promoter activities measured with all pGL-MMP-9 reporter constructs to some extent may cover the strong stimulatory effects by IL-1ß (in the presence or absence of γ-S-ATP) observed for the endogenous MMP-9 expression.

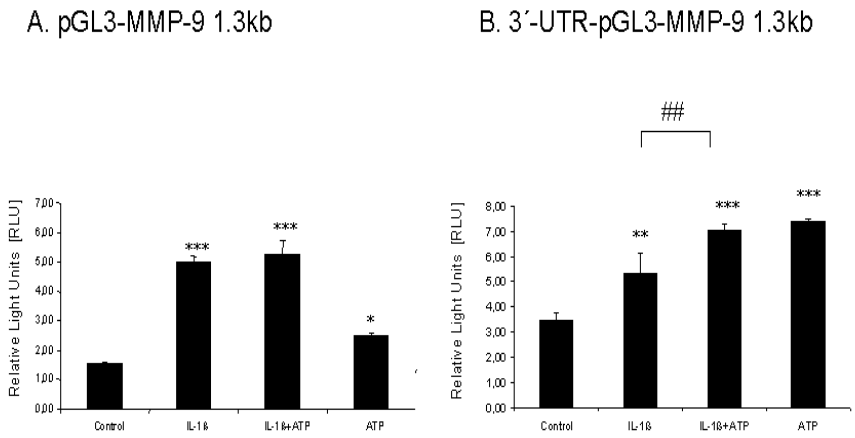

Figure 28. Influence of the MMP-9 3´-UTR on MMP-9 promoter-driven Luciferase activity. **(A).** Subconfluent MC were transiently cotransfected with 0.4 µg of pGL-MMP-9 -1.3kb and 0.1 µg of pRL-CMV coding for *Renilla* luciferase. After an overnight transfection MC were treated for 20 hours with vehicle (control) or with IL-1ß (2 nM) or γ-S-ATP (30 µM) or both in combination as indicated. **(B).** To assess the influence of the 3´UTR of the MMP-9 mRNA MC were alternatively transfected with 0.4 µg of 3´-UTR-MMP-9 pGL-MMP-9 -1.3kb containing additionally the 3´-UTR of MMP-9 downstream of the luciferase coding sequence before being treated with vehicle, IL-1ß (2 nM), or ATP (30 µM) or both in combination. The values for beetle luciferase were related to values for Renilla luciferase and are depicted as relative light units (RLU). Data (means ± SEM) are the results of triplicates and are representative for three independent experiments. (*) *P≤ 0.05,* (**) *P≤0.01,* (***) *P≤0.005* compared with unstimulated control values and or to IL-1ß-stimulated values (##) *P≤0.01*

In contrast, the low basal expression of endogenous MMP-9 may explain the lack of ATP effects on basal MMP-9 levels demonstrated by Northern blot and zymography, respectively (Fig. 23).

Mutagenesis studies with MMP-9 reporter genes bearing mutated AREs

To investigate the impact of each single ARE-motif on the ATP-dependent mRNA stabilization we generated different point mutations by replacing the pentameric "AUUUA" motif of ARE-1, ARE-2 and ARE-4 to an inactive "ACCCA" sequence (table 4) which impairs its RNA-binding affinity. As shown in Fig. 29, MC transiently transfected with 3´-UTR-MMP-9 promoter constructs bearing one mutated ARE motif displayed similar induction profiles by ATP and IL-1ß as those derived from the wild-type 3´-UTR pGL MMP-9 promoter although the absolute rates of luciferase activities differed between single constructs (compare RLU in Fig. 29 with RLU in Fig. 28B). In contrast, mutation of all three putative ARE-motifs (3´-UTR-ΔARE-1-2-4 pGL3-MMP-9) resulted in a complete loss of ATP-dependent effects on reporter gene activities (Fig. 29) similar to those observed with the wild-type MMP-9 promoter construct lacking the 3´-UTR (Fig. 28A). These data indicate that none of the ARE motifs within the 3´-UTR of MMP-9 is indispensable for the ATP-dependent MMP-9 mRNA stabilization. However, mutation of all three ARE motifs results in a complete loss of the ATP-dependent increase in luciferase activities without affecting the stimulatory effects of IL-1ß. This indicates that the ATP-dependent effects via 3´-UTR are mainly attributable to AREs but not to the presence of 3´-enhancer elements.

ATP augments binding of HuR protein to AU-rich elements (AREs) within the 3´-UTR of MMP-9

The stability of many short-lived mRNAs is modulated through specific binding of proteins to the AREs present in their 3´-UTR. We therefore used electrophoretic gel mobility shift assay

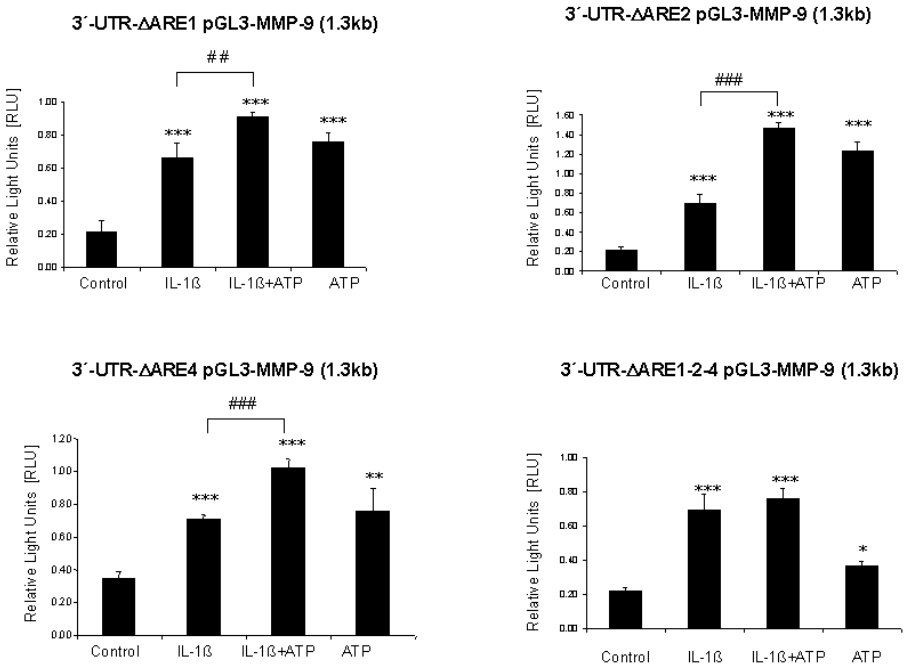

Figure 29 Relative Luciferase activities of mutated 3´-UTR –MMP-9 promoter constructs. Subconfluent MC were transiently cotransfected with 0.4 µg of the indicated point-mutated 3`-UTR-MMP-9 promoter constructs and 0.1 µg of pRL-CMV coding for *Renilla* luciferase. After an overnight transfection MC were treated for 20 hours with vehicle (control) or with IL-1ß (2 nM) or γ-S-ATP (30 µM) or both in combination as indicated.Values for beetle luciferase are related to values for Renilla luciferase and are depicted as relative light units (RLU). Data are (means ± SEM) for three independent experiments. (*) *P≤ 0.05,* (**) *P≤0.01,* (***) *P≤0.005* compared with unstimulated control values and or to IL-1ß-stimulated values (##) *P≤0.01,* (###) *P≤0.005.*

Table 4:

UTR-ARE-1	2500	5´-CCCUUUU**AUUUA**UUAUGUAUG-3´	2520
UTR-ΔARE-1		5´-CCCUUUU**ACCCA**UUAUGUAUG-3´	
UTR-ARE-2	2536	5´-ACAUGU**AUUUA**ACCUAUAGAA-3´	2556
UTR-ΔARE-2		5´-ACAUGU**ACCCA**ACCUAUAGAA-3´	
UTR-ARE-4	2742	5´-CAGAGGA**AUUUA**UUGGAUGUU-3´	2762
UTR-ΔARE-4		5´-CAGAGGA**ACCCA**UUGGAUGUU-3´	

(EMSA) to determine if treatment of cells with γ-S-ATP would cause an increase in the constitutive binding of HuR-containing complexes bound to different ARE motifs within the 3´-UTR of MMP-9 [Akool et al., 2003]. The protein binding to MMP-9-specific AREs was monitored by using ^{32}P-labeled RNA oligonucleotides comprising the different wild-type or mutant AREs of MMP-9 (denoted as UTR- ARE or UTR-ΔARE as depicted inTable 4). MC were either left untreated or treated for 4 h with different concentrations of γ-S-ATP and subsequently lysed for cytosolic fractions. Using an RNA oligonucleotide containing the wild-type ARE 1 motif from the 3´-UTR of MMP-9 we observed the constitutive binding of three major complexes of high electrophoretic mobility and, interestingly, the binding of all complexes was dose-dependently increased when cells had been treated with γ-S-ATP (Fig. 30A). Similar to ARE-1, a constitutive binding of two main complexes to ARE-2 and ARE-4 encompassing oligos was dose-dependently increased by γ-S-ATP and a maximal binding capacity was observed with 30 μM γ-S-ATP (Fig. 30B and C). As we have described previously, an ARE-3 containing RNA oligo displayed no *in vitro* binding capacity most probably due to the different bases flanking this ARE-motif [Akool et al., 2003]. Additionally, the binding affinity of RNA oligos bearing a mutated ARE motif (ACCCA in stead of AUUUA, Tab. 4) was strongly reduced when compared to oligos bearing the wild-type ARE (Fig. 30A-C) demonstrating the functionality of each ARE motif. In contrast to the effects observed with γ-S-ATP, the short-term treatment of MC with IL-1ß (4 hours) did not modulate the constitutive binding to any of the ARE-motifs tested (Fig. 30D).

ATP-inducible complexes binding to MMP-9-specific AREs contain the mRNA stabilizing factor HuR

We have described previously that the ELAV-like RNA binding protein HuR is critically involved in the regulation of MMP-9 mRNA stability [Akool et al., 2003]. To test whether the γ-S-ATP-inducible complexes interacting with the ARE motifs of MMP-9 contain any HuR-

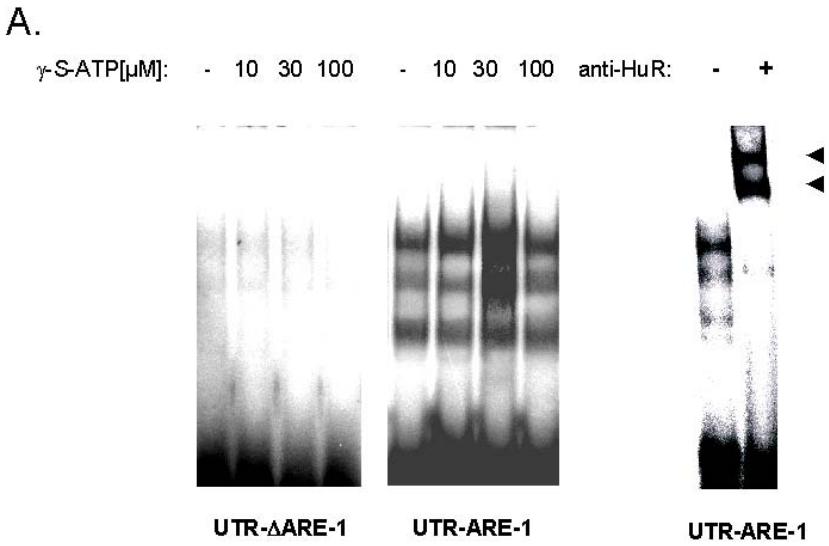

Figure 30 (A). γ-S-ATP causes an increase in the constitutive RNA binding of HuR to AU-rich motifs within the 3´-UTR of MMP-9. RNA binding was analyzed by EMSA using gene specific wild-type oligonucleotides (UTR-ARE) or oligos bearing mutations of three bases within the AU-rich elements (UTR-ΔARE) as depicted in Table 4. MC were either left untreated (-) or stimulated for 4 h with the indicated concentrations of γ-S-ATP before cells were lysed for preparation of cytoplasmic extracts. 4 μg of cytoplasmic extracts were incubated with a ^{32}P-labeled RNA probe derived from the corresponding AU-rich region of the 3´-UTR of rat MMP-9. UTR-ARE-1 and RNA binding was assessed in 6.0 % native PAGE gels. The results shown in each panel are representative for three independent experiments giving similar results. Supershift analysis was done by incubating the ^{32}P radiolabeled oligonucleotides with 6 μg of cytoplasmic extract derived from γ-S-ATP-treated MC (30 μM) in the presence of 1 μl (200 ng) of a monoclonal anti-HuR antibody (anti-HuR). The antibody was added 15 min after addition of the radiolabeled oligonucleotide and incubated for a further 15 min at room temperature. Arrowheads indicate the supershifted complexes. Similar results were obtained in three independent experiments.

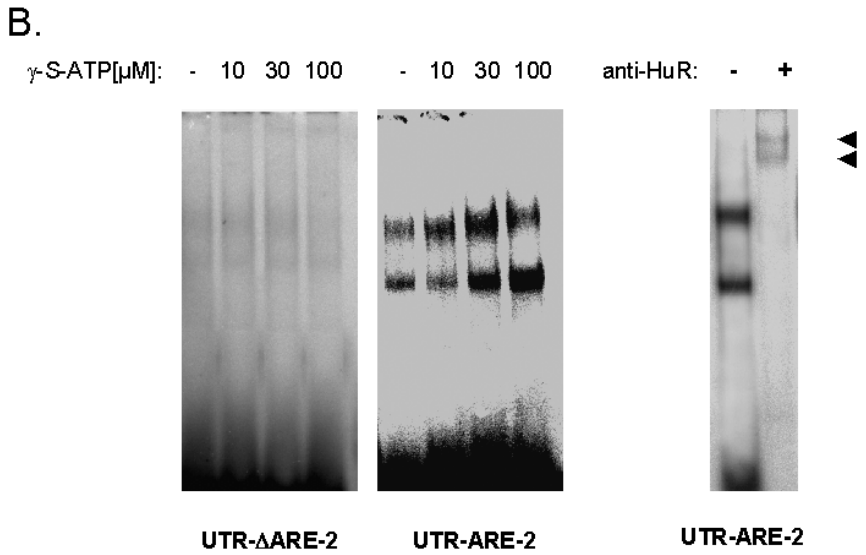

Figure 30 (B). γ-S-ATP causes an increase in the constitutive RNA binding of HuR to AU-rich motifs within the 3´-UTR of MMP-9. RNA binding was analyzed by EMSA using gene specific wild-type oligonucleotides (UTR-ARE) or oligos bearing mutations of three bases within the AU-rich elements (UTR-ΔARE) as depicted in Table 4. MC were either left untreated (-) or stimulated for 4 h with the indicated concentrations of γ-S-ATP before cells were lysed for preparation of cytoplasmic extracts. 4 μg of cytoplasmic extracts were incubated with a ^{32}P-labeled RNA probe derived from the corresponding AU-rich region of the 3´-UTR of rat MMP-9. UTR-ARE-2 and RNA binding was assessed in 6.0 % native PAGE gels. The results shown in each panel are representative for three independent experiments giving similar results. Supershift analysis was done by incubating the ^{32}P radiolabeled oligonucleotides with 6 μg of cytoplasmic extract derived from γ-S-ATP-treated MC (30 μM) in the presence of 1 μl (200 ng) of a monoclonal anti-HuR antibody (anti-HuR). The antibody was added 15 min after addition of the radiolabeled oligonucleotide and incubated for a further 15 min at room temperature. Arrowheads indicate the supershifted complexes. Similar results were obtained in three independent experiments.

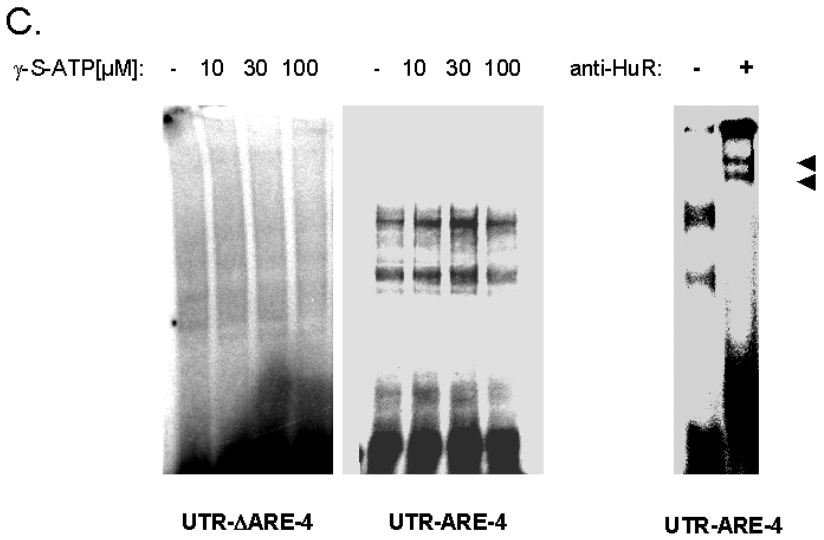

Figure 30 (c). γ-S-ATP causes an increase in the constitutive RNA binding of HuR to AU-rich motifs within the 3´-UTR of MMP-9. RNA binding was analyzed by EMSA using gene specific wild-type oligonucleotides (UTR-ARE) or oligos bearing mutations of three bases within the AU-rich elements (UTR-ΔARE) as depicted in Table 4. MC were either left untreated (-) or stimulated for 4 h with the indicated concentrations of γ-S-ATP before cells were lysed for preparation of cytoplasmic extracts. 4 μg of cytoplasmic extracts were incubated with a ^{32}P-labeled RNA probe derived from the corresponding AU-rich region of the 3´-UTR of rat MMP-9. UTR-ARE-4 and RNA binding was assessed in 6.0 % native PAGE gels. The results shown in each panel are representative for three independent experiments giving similar results. Supershift analysis was done by incubating the ^{32}P radiolabeled oligonucleotides with 6 μg of cytoplasmic extract derived from γ-S-ATP-treated MC (30 μM) in the presence of 1 μl (200 ng) of a monoclonal anti-HuR antibody (anti-HuR). The antibody was added 15 min after addition of the radiolabeled oligonucleotide and incubated for a further 15 min at room temperature. Arrowheads indicate the supershifted complexes. Similar results were obtained in three independent experiments.

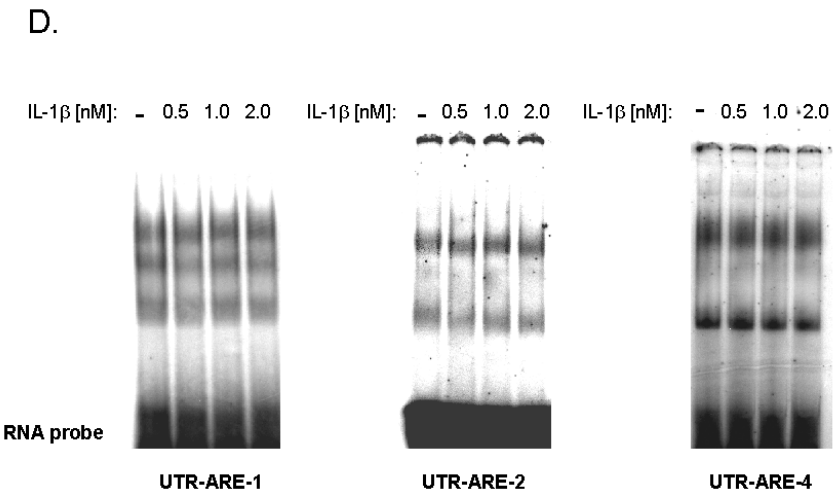

Figure 30 (D). Short-term stimulation with IL-1ß does not affect the constitutive binding to AREs within the 3´-UTR of MMP-9. MC were either left untreated (-) or stimulated for 4 h with the indicated concentrations of IL-1ß before cells were lysed for preparation of cytoplasmic extracts. The procedures for EMSA were similar to those described in (A). The different RNA probes (UTR-ARE-1, UTR-ARE-2 and UTR-ARE-4) were derived from the corresponding AU-rich region of the 3´-UTR of rat MMP-9. The EMSAs shown are representative for two independent experiments giving similar results.

like protein we performed supershift analysis. We focused on HuR (HuA) which participates in the regulation of ARE-mediated RNA turnover [Peng et al., 1998] and which has been identified as a target of NO-dependent MMP-9 mRNA decay in rat MC [Akool et al., 2003]. As shown in Fig. 30 A-C (right panels), the addition of anti-HuR antibody caused the appearance of two supershifted bands (arrowheads) independent of which ARE-oligonucleotide was used in the binding assay. Most interestingly, the addition of supershift-antibody results in an almost complete shift of all complexes indicating that HuR is a main constituent of the ATP-regulated complexes. Similar to the EMSAs with cytoplasmic fractions, recombinant HuR protein displays a strong *in vitro* binding affinity to all three wild-type ARE motifs from rat MMP-9 mRNA (UTR-ARE-1, UTR-ARE-2 and UTR-ARE-4) [Akool et al., 2003]. These data indicate that γ-S-ATP by increasing the constitutive HuR binding to AREs stabilizes the cytokine-induced MMP-9 mRNA.

The mRNA stabilizing factor HuR (HuA) is critically involved in nucleotide-induced MMP-9 mRNA stabilization

In a previous study we have demonstrated that neutralization of HuR by addition of anti-HuR antisera results in the accelerated decay of MMP-9 mRNA whereas addition of recombinant HuR has an opposite effect on the mRNA decay of MMP-9 [Akool et al., 2003]. To further test the functional role of HuR in the γ-S-ATP-mediated stabilization of MMP-9 mRNA we examined the effect of the neutralizing Anti-HuR antibody by *in vitro* degradation assays. To this end, the cytoplasmic fractions from γ-S-ATP-treated MC (which confer protective properties towards cytokine-induced MMP-9 mRNA) were preincubated for 1h with a monoclonal anti-HuR antibody (a total amount of 400 ng antiserum) before the decay of MMP-9 RNA was monitored by Northern blot analysis. Total cellular RNA was isolated after a further 2h coincubation with total cellular RNA, a time point where the stabilizing effects by γ- S-ATP are most obvious (Fig. 27). As a negative control the same volume of control

immunoglobulins (mouse IgG) was preincubated. As shown in Fig. 31 the level of MMP-9 mRNA was reduced when HuR was neutralized by the anti-HuR antibody. In contrast, mouse IgG on its own had no effects on the MMP-9 level (Fig. 31). Again, the steady-state mRNA level of GAPDH was not affected in the degradation assay thus indicating that the modulation of mRNA stability by HuR is not observed with a none-ARE-containing mRNA.

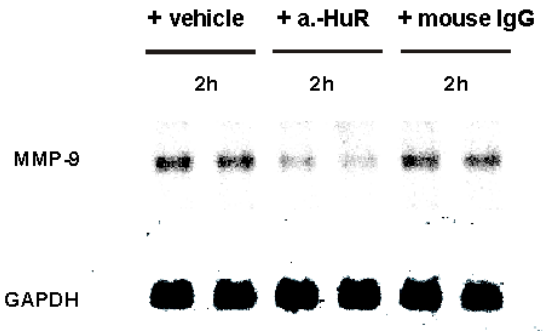

Figure 31. Neutralization of HuR reduces the stability of MMP-9 mRNA. Portions of 20 µg of total RNA from a common pool of total RNA isolated from cytokine-stimulated MC were mixed with 130 µg of cytoplasmic extract derived from MC treated for 4h with γ-S-ATP. The cytoplasmic extracts were kept either untreated (vehicle), or, alternatively pretreated for 1 h with 0.4 µg of a monoclonal anti-HuR antibody (+ a HuR) or with the same volume of mouse IgG (+ mouse IgG) before the cytoplasmic extracts were incubated with the total RNA portions. Incubation with the RNA was stopped after additional 120 min before RNA was extracted for Northern blot analysis. Equal transfer and loading of total RNA was ascertained by hybridization to a GAPDH probe. Samples derived from one cytoplasmic extract were subjected to RNA in duplicates. Similar results were obtained in two independent experiments.

The ATP-induced nucleo-cytoplasmic shuttling of HuR is inhibited by suramin

We next investigated the subcellular localization of HuR by confocal microscopy. Several reports have documented a predominant nuclear localization of HuR [Fan and Steitz, 1998; Peng et al., 1998; Keene, 1999] although fractions of HuR protein have also been found in the cytoplasm [Antic et al., 1999]. Under basal conditions (0 min, Fig. 32A) we observed a strong fluorescence in the cell nuclei but almost no staining within the cytoplasm thus indicating that HuR shows an almost exclusive nuclear distribution. Treatment of MC with 30 µM γ-S-ATP triggered a substantial increase in cytoplasmic HuR as is indicated by the appearance and increased fluorescence in punctated structures outside the nucleus (Fig. 32A, upper panel) A maximal density of immunopositive grains in the cytoplasm was observed after 2 hours of

treatment with γ-S-ATP. A granular distribution of HuR within the cytoplasm has also been shown for the ELAV homologous proteins Hel-N1 and Hel-N2 [Gao and Keene, 1996]. Incubations of longer than 2h reduced the degree of cytosolic staining (Fig. 32A). These results indicate that the rise in binding to UTR of MMP-9 is paralleled by an increased nuclear- cytoplasmic shuttling of the RNA stabilizing protein HuR. To investigate whether the $P2Y_2$ purinoreceptor subtype is involved in the γ-S-ATP-dependent increase in cytoplasmic staining of HuR we tested the effects of the putative $P2Y_2$ antagonist suramin [Boarder and Hourani, 1998]. Interestingly, the γ-S-ATP-mediated effects on HuR translocation were inhibited at all time points tested (Fig. 32A, lower panel) which again indicates the involvement of $P2Y_2$–dependent signaling in the regulation of HuR by extracellular ATP. The subcellular localization of endogenous HuR was also monitored by Western blot analysis. MC were treated for 4 h with different concentrations of γ-S-ATP (3, 10 and 30 μM) and subsequently fractionated into nuclear and cytoplasmic fractions. We observed that untreated MC contain low HuR protein levels in the cytoplasm (Fig. 32B) which is consistent with the weak fluorescence in the perinuclear region observed by confocal microscopy (Fig. 32A). Stimulation with γ-S-ATP caused a dose-dependent increase in the level of cytosolic HuR with a maximal effect seen at 30 μM γ-S-ATP (Fig. 32B, left panel). The addition of suramin to ATP (30 μM) resulted in a strong reduction of HuR accumulation again demonstrating a $P2Y_2$-dependent mechanism (Fig. 32B, left panel). Finally, we tested for possible γ-S-ATP-induced changes in the nuclear HuR content. We observed that the nuclear amount of HuR is dramatically higher than that of cytoplasmic HuR (Fig. 32C). Therefore, no significant change in the nuclear HuR level was detectable when cells were treated with γ-S-ATP (Fig. 32B, right panel) despite the marked changes in cytoplasmic HuR. In summary, our data indicate that the γ-S-ATP-dependent rise in the IL-1ß-induced MMP-9 expression in MC is functionally linked to mechanisms which involve increased RNA-binding of HuR.

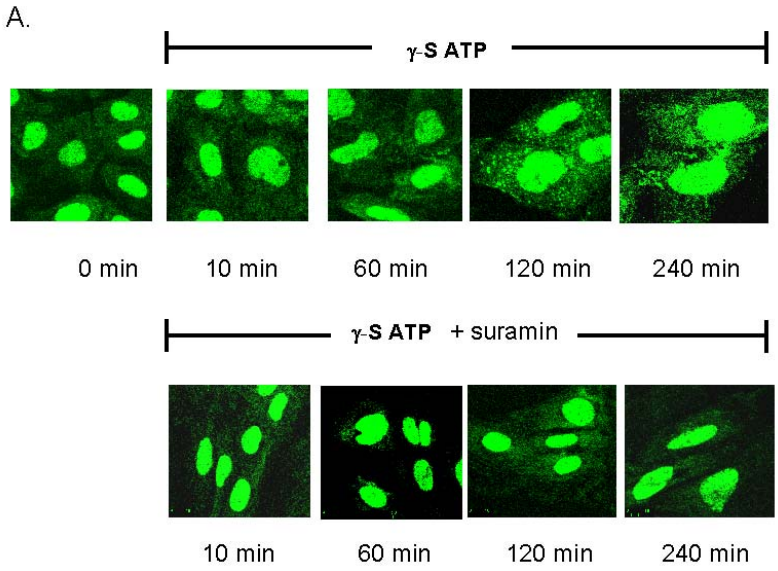

Figure 32 (A). Suramin inhibits the γ-S-ATP-triggered nucleo-cytoplasmic shuttling of the ELAV-protein HuR. Indirect immunofluorescence showing the changes in the localization of HuR during stimulation of cells with γ-S-ATP. Quiescent mesangial cells were stimulated for the indicated time periods with either vehicle (0 min) or γ-S-ATP (30 µM) in the absence (upper panel) or presence of 100 µM suramin (lower panel). Thereafter cells were fixed and stained with an anti-HuR antibody and anti-mouse-Alexa 488 as a secondary antibody.

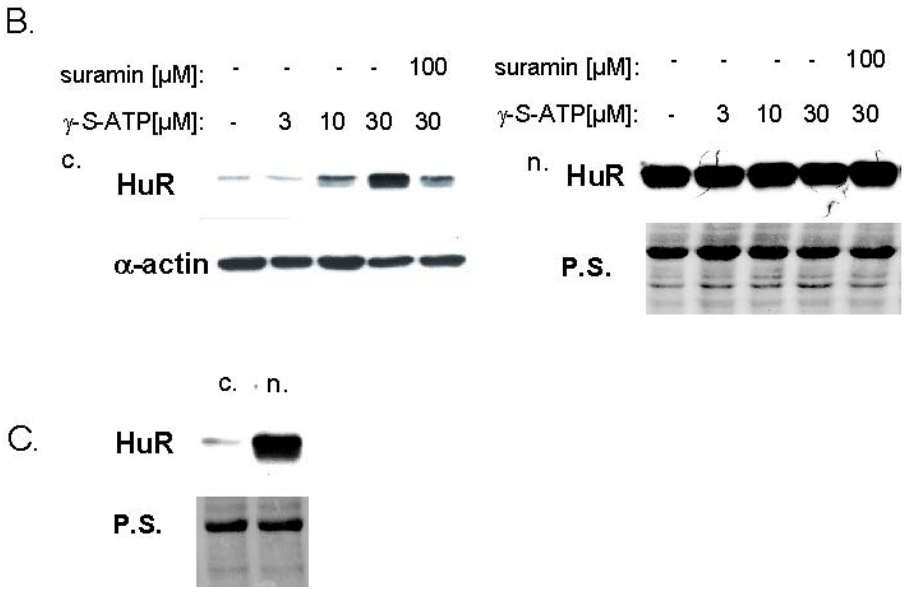

Figure 32 (B,C) : B. Western blot analysis showing ATP-induced redistribution of HuR from the nucleus to the cytoplasm. MC were stimulated for 4 h with different concentrations of γ-S-ATP as indicated and then lyzed for cellular fractionation. Protein lysates (100 µg) from cytoplasmic (left panel) or nuclear (right panel) fractions were subjected to SDS-PAGE and probed with a HuR-specific monclonal antibody. To correct for variations, the blot was either stripped and incubated with an α-actin antibody (left panel) or loading of equal amounts of total protein was proven by Ponceau-S staining (P.S., right panel). Data are representative of two independent experiments giving similar results. **C.** Relationship between the cytoplasmic and nuclear levels of HuR. Equal amount of total protein extracts (100 µg) from nuclear (n.) or cytoplasmic (c.) fractions from untreated MC were subjected on SDS-PAGE and probed with a HuR-specific antibody. Loading of equal amounts of total protein was proven by Ponceau-S staining (P.S.).

Additionally, the increase in RNA-binding by ATP is paralleled by an increase in the nucleo-cytoplasmic shuttling of HuR.

Discussion

MMP-9 is a protease which is strongly induced by inflammatory cytokines such as IL-1ß and tumor necrosis factor α in many cell types including glomerular MC. Here we have focused on possible modulatory effects on MMP-9 by extracellular ATP and UTP since both nucleotides can mediate a variety of cell responses in MC including cell growth and inhibition of apoptosis [Schulze-Lohoff et al., 1992; Huwiler and Pfeilschifter, 1994]. Moreover, extracellular nucleotides have also been reported to modulate experimental forms of glomerulonephritis *in vivo* [Poelstra et al., 1992]. We demonstrate that the stable ATP analog γ-S-ATP as well as UTP potently amplify the IL-1ß-mediated expression of MMP-9. The stimulatory effects of ATP on MMP-9 expression could be potently inhibited by the addition of suramin, a potent although none selective antagonist of $P2Y_2$ purinoceptors. The occupation of $P2Y_2$ receptors in rat MC can activate different isoforms of protein kinase C (PKC) [Pfeilschifter and Huwiler, 1996; Boarder and Hourani, 1998] but also different MAPK pathways including the extracellular signal-regulated kinases (ERKs) [Huwiler and Pfeilschifter, 1994] the stress-activated protein kinase (SAPK) and p38-MAPK cascade [Huwiler et al., 1997; 2000]. Interestingly, several studies have implicated MAPK pathways as well as PKC-dependent signaling cascades in the up-regulation of MMP-9 expression [Eberhardt et al., 2000b; 2002a; Simon et al., 1998; Esteve et al., 2002; Yokoo and Kitamura,1996] mainly via the activation of NF-kB and AP-1 transcription factors [Eberhardt et al., 2000b; 2002a; Simon et al., 1998; Esteve et al., 2002; Yokoo and Kitamura,1996]. In rat MC none of the nucleotides was able to induce MMP-9 expression on its own. Obviously, in MC none of the signaling pathways affected by extracellular nucleotides is sufficient to transcriptionally activate MMP-9 and an additional cytokine-triggered signal is essentially

required. The assumption that cytokines and extracellular nucleotides can activate two independent signaling pathways is further underlined by the observation that both ATP and UTP have only marginal effects on the cytokine-evoked activity of a 1.3 kb fragment of the rat MMP-9 promoter, although this promoter region is sufficient to mediate a strong activation by IL-1ß in rat MC [Eberhardt et al., 2002a]. In line with these suggestions we found a substantial contribution of posttranscriptional regulation to the ATP-mediated amplification of cytokine-induced MMP-9 expression. However, we cannot exclude the additional involvement of transcriptional regulation by γ-S-ATP attributable to some enhancer elements upstream from the proximal 1.3 kb of MMP-9. Therefore, a possible contribution of transcriptional regulation exerted by extracellular nucleotides has to be checked by additional reporter gene experiments using larger fragments of the rat MMP-9 promoter. By use of actinomycin D experiments and *in vitro* RNA degradation assay we further demonstrate that treatment of cells with γ-S-ATP significantly increased the stability of MMP-9 mRNA. The 3´-UTR of rat MMP-9 contains several copies of AU-rich elements which are considered as important determinants of RNA turnover [Chen and Shyu, 1995; Ross, 1995; Malter, 1989 ; Xu et al., 1997] Concomitantly, we demonstrate that the 3´-UTR of MMP-9 confers an ATP-dependent increase of basal and cytokine-induced MMP-9 driven luciferase activity when fused downstream of the luciferase reporter gene. Focusing on the responsible cis regulatory regions we found four copies of AUUUA pentameric motifs within the 3´-UTR of the rat MMP-9 mRNA potentially involved in the regulation mRNA decay of MMP-9. Interestingly, these motifs have been implicated in the rapid turnover of many mRNA species. Proteins functionally targeting these AU-rich elements (AREs) include destabilizing factors such as AUF 1 [Zhang et al., 1993] as well as mRNA stabilizing factors, most prominently members of the embryonic lethal abnormal vision (ELAV) protein family [Peng et al., 1998; Levy et al.,1996; Ma et al., 1996]. By gel shift analysis we demonstrate binding of complexes to three different ARE motifs of the 3´-UTR of MMP-9 (“MMP-9-ARE-1”, “MMP-9-ARE-2”,

"MMP-9-ARE-4") the binding of these complexes being substantially increased in cells treated with γ-S-ATP but not with IL-1ß. We focused on the ELAV–like RNA-binding protein HuR which is known to bind AREs with a high affinity thereby increasing the stability of respective mRNAs [Peng et al., 1998; Levy et al., 1996; Ma et al., 1996]. The binding affinity to the MMP-9 specific AREs was strongly impaired when the uracil base within the AUUUA pentameric motif was replaced by cytosine which confirms the functionality of the AU-rich elements within the 3´-UTR of MMP-9. Concomitantly, the presence of HuR in the cytoplasmic extracts from ATP-treated MC was confirmed by supershift analysis. In addition, we have previously demonstrated that the MMP-9-specific ARE displays a high binding affinity to recombinant HuR protein thus demonstrating a functional binding capacity of HuR to these ARE motifs within the 3´-UTR of MMP-9 [Akool et al., 2003]. The RNA binding of HuR has been shown to parallel the *in vivo* ability of ARE sequences to direct mRNA degradation [Myer et al., 1997]. Consistent with these findings we here demonstrate the functional importance of HuR by *in vitro* degradation assays. The addition of a neutralizing HuR antibody to the lysates of ATP-treated MC results in a significant acceleration of the decay of MMP-9 mRNA but not of GAPDH mRNA which lacks AU-rich elements within its 3´-UTR (Fig.31). Our findings suggest that ATP triggers an increase in HuR binding to the 3´-UTR of MMP-9 mRNA and thus conveys protection of MMP-9 transcripts from rapid degradation. It is interesting to note that in the in vitro degradation assays we did not observe degradation fragments of MMP-9 mRNA. The gradual shortening of the poly (A) tail of mRNA followed by a rapid exonuclease-dependent 3´- to –5´-digestion is thought as a major mechanism of mRNA decay within eukaryotic cells [Van Hoof and Parker, 2002]. Moreover, the rate of deadenylation seems an important control point for the rate of mRNA degradation. The disappearance of the MMP-9 positive band without detectable intermediates observed in our experiments indicates a rapid 3´-to-5´-degradation which is consistent to the findings of Chen et al. [Chen et al., 2001]. Since the cDNA insert

used for Northern blot analysis in this study covers a 3´-region of MMP-9 mRNA (1227-1939) this probe is not optimal for monitoring mRNA intermediates. So far little is known about the exact mechanisms by which HuR regulates mRNA stability. Unlike the other members of the ELAV family (HuB, HuC, or HuD) which all show a tight tissue-specific regulation, HuR is ubiquitously expressed and is predominantly located in the nucleus [Peng et al., 1998; Brennan and Steitz, 2001; Ma et al., 1996; Myer et al., 1997; Good, 1995; King et al ., 2000; Dean et al., 2001]. Mechanisms of HuR dependent mRNA decay can involve a nuclear-cytoplasmic movement [Pinol-Roma and Dreyfuss, 1992; Atasoy et al., 1998; Keene, 1999] but also changes in the HuR expression level [Akool et al., 2003; Klöss et al., 2003]. Using confocal fluorescence microscopy we demonstrate that HuR is predominantly nuclear but partially distributes into distinct punctuate structures in the cytoplasm following treatment with γ-S-ATP. This is in agreement with a recent study which has shown that ATP in higher concentrations (1 mM) inhibits the activity of AMP-activated kinase (AMPK) and thereby elevates the HuR level in the cytoplasm of colorectal carcinoma RKO cells [Wang et al., 2002]. AMPK is an enzyme which is considered to act as a cellular defense mechanism to protect cells from ATP depletion [Moore et al., 1991]. Most intriguingly, the expression of HuR was shown to be downregulated in quiescent cells but increased following serum stimulation. Therefore, ATP as a proliferative stimulus may affect a default pathway of mRNA decay thereby regulating the expression of a variety of growth regulatory proteins. Besides AMPK the activation of PKC plays a cardinal role in the enhanced stability of mRNAs coding for p21 and IL-1ß [Park et al., 2001; Gorospe et al., 1993]. Mapping of the protein sequence has revealed that HuR contains a multitude of putative PKC phosphorylation sites which makes it tempting to speculate that ATP might regulate HuR via PKC. Other studies have implicated the involvement of MAPK pathways in the regulation of the nucleo-cytoplasmic shuttling of HuR [Winzen et al., 1999; Ming et al., 2001]. Whether changes in HuR phosphorylation either by different PKCs or by the MAPK pathway account for the

ATP-induced HuR redistribution in MC is currently investigated in our laboratory. In this context it is noteworthy that ATP has been shown to activate different PKC isoenzymes [Pfeilschifter and Huwiler, 1996] as well as the three major MAPK cascades [Huwiler and Pfeilschifter, 1994; Huwiler et al., 1997; 2000]. The excessive degradation of extracellular matrix is an important feature in the progression of many acute inflammatory diseases accompanied by a cellular hyperproliferation. Our present findings present a molecular mechanism which may explain how extracellular nucleotides transiently potentiate the cytokine-mediated cellular capacities to degrade extracellular matrix without having any effects on MMP-9 expression in resting cells. To the best of our knowledge this is the first time that the stable ATP analog γ-S-ATP is shown to regulate the expression of MMPs by posttranscriptional mechanisms. Furthermore, our data emphasize the importance of modulation of mRNA stability in the tight regulation of MMP-9.

Summarizing discussion

MMPs play a pivotal role in the regulation of ECM turnover in the glomerulus. Various forms of glomerular diseases are characterized by a profound shift in the balance between matrix synthesis and degradation [Arthur, 1998]. While in the scarring process the balance is tilted toward increased synthesis, excess degradative activity promotes glomerular destruction in inflammatory diseases. Generally, glomerular diseases remain the most common causes of progressive renal failure and in late stages in many cases require dialysis or kidney transplantation [Pfeilschifter, 1994]. Most forms of progressive glomerular diseases are characterized by decreased turnover of extracellular matrix (ECM) and accumulation of interstitial collagens in the glomeruli. This increase in collagen mRNA may not merely mirror existing sclerosis, but may have a predictive value early in the disease. A lesser degree of structural damage in another model showed a correspondingly lesser elevation of type IV collagen [Peten et al., 1993]. In human renal tissue, correlation was also found between level of glomerulosclerosis and expression of collagen type IV mRNA [Peten et al., 1992]. Based on the knowledge that the activated MC itself can synthesize and release a myriad of inflammatory mediators, including growth factors, cytokines and high expression levels of gelatinases (MMP-2 and MMP-9), I focused on the question how and by which mechanisms these mediators may orchestrate the tissue remodeling in glomerular MC. The inflammatory cytokines IL-1ß and TNFα are among the most potent transcriptional inducers of MMP-9 gene expression [Yoko and Kitamura, 1996; Eberhardt et al., 2000a]. Searching for targets interfering with the cytokine-induced MMP-9 expression I investigated the effects of activators of the nuclear receptor superfamily the peroxisome proliferator-activated receptors (PPARs) [for review see Corton et al., 2000] . PPAR activators have been shown to exert anti-inflammatory activities in various cell types by inhibiting the expression of proinflammatory genes such as cytokines [Delerive et al., 1999] and acute phase proteins [Delerive et al., 2001]. Three subtypes of PPARs have been identified: PPARα, ß/δ, and γ each one showing a

specific distribution and physiologic function. I focused on the α-subtype of PPAR receptors since a variety of other inflammatory genes has been shown to be modulated by PPARα [for review see: Delerive et al., 2001; Daynes and Jones, 2002]. In the present work, I found that the structurally different PPARα agonists WY-14, 643, LY-171883 and bezafibrate potently inhibit the cytokine-induced MMP-9 on both, the mRNA and the protein level. Most interestingly, the negative effects by PPARα agonists on cytokine-induced MMP-9 mRNA steady-state and zymogen levels is not attributable to an inhibition of MMP-9 gene transcription but results from some indirect, posttranscriptional regulatory events by NO generated via activation of PPARα by different PPARα agonists. This increase in NO production results from potentiation of cytokine-induced stimulatin of iNOS expression by the PPARα agonists. Previously, it has been reported that NO, given endogenously by stimulation of iNOS expression, potently inhibits the mRNA steady-state levels of cytokine-induced MMP-9 in mesangial cells [Eberhardt et al., 2000a]. Meanwhile, the negative modulation of MMP-9 expression has been confirmed in several other cell types thus suggesting a general mechanisms of NO-triggered tissue remodeling [Pfeilschifter et al., 2001; Upchurch et al., 2001; Gurjar et al., 2001]. In contrast to the negative modulation of MMP-9 by NO, it has been reported recently that NO increases the expression of MMP-9 in vascular smooth muscle [Marcet-Palacios et al., 2003]. These conflicting results on amplification or inhibition of MMP-9 demonstrate the complexity of the interactions of NO with cell signaling cascades. Many factors may contribute to some of the differences observed such as cell type, stimulation time and stimulating agents. Searching for the underlying mechanisms, most interestingly, I found that NO significantly reduced the half-life of MMP-9 mRNA [Eberhardt et al., 2002b]. From this observation, we suggest that the tight cross-talk between NO and PPAR-triggered signaling pathways found in MC, may contribute to the anti-inflammatory effects of PPARα agonists observed in some pathologies. Our findings

furthermore imply that in the renal glomerulus the overall effect of PPARα activators on MMP-9 expression may critically depend on the simultaneous production of NO by cells exposed to an inflammatory environment. Future studies should investigate whether this NO-dependent effects mediated by PPARα activation also occurs in other cells or tissues or even *in vivo*.

Searching for the precise mechanisms of the NO-evoked mRNA decay, I found that the untranslated region (3´-UTR) of the MMP-9 gene is able to confer a NO-dependent reduction of cytokine-induced MMP-9 mRNA. The 3´-UTR of rat MMP-9 contains several copies of AU-rich elements which are considered as important determinants of RNA turnover [Chen and Shyu, 1995; Ross, 1995; Malter, 1989 ; Xu et al., 1997] Meanwhile numerous ARE-binding proteins have been implicated in the regulation of mRNA turnover, among them proteins which can destabilize mRNA such as AUF1 [Zhang et al., 1993] but interestingly also proteins which can reduce mRNA degradation, most prominently members of the embryonic lethal abnormal vision (ELAV) protein family [Levy et al., 1996; Ma et al., 1996; Peng et al., 1998]. Unlike the other members of the ELAV family (HuB, HuC and HuD) which show a tissue-specific expression, HuR is ubiquitously expressed and shows a predominant nuclear distribution [Atasoy et al., 1998; . Dean et al., 2001 ; Peng et al., 1998]. However, a change of distribution between the nuclear and cytoplasmic compartment is considered as the main mechanism by which HuR can modulate mRNA decay [Ford et al., 1999; Peng et al., 1998]. Here I present an alternative mechanism by which a reduction of RNA binding is caused by the altered expression of HuR. Furthermore, I found that the NO-dependent reduction of HuR is blocked in the presence of the specific guanylyl cyclase inhibitor LY-83583 thus demonstrating that the NO-mediated decrease in HuR expression depends on the activation of guanylyl cyclase and thus complements the mainly transcriptional regulation of other genes driven by NO in an cGMP-independent manner [Pfeilschifter et al., 2001; Schmidt and Walter, 1994]. Similar to MMP-9, the 3´-UTR of HuR

contains several copies of AU-rich elements (AREs) implying a possible posttranscriptional regulation of HuR itself. Future studies are needed to investigate whether the change in HuR expression is mediated via transcriptional or posttranscriptional regulation. From the previous experiments we can conclude that the expression of MMP-9, in addition to a transcriptional regulation, is controlled by post-transcriptional mechanisms including the modulation of mRNA stability by the mRNA stabilizing factor HuR. The increased production of NO which often does accompany the overall inflammatory responses may have a causative role in the progression of fibrotic processes by altering matrix turnover via transcriptional as well as posttranscriptional mechanisms. Future work using animal models of inflammatory as well as fibrotic diseases should provide further insight to the *in vivo* relevance of this mechanism.

Besides the proinflammatory cytokines, MC are able to respond to a variety of other biological mediators including eicosanoids, growth factors, reactive oxygen species, NO and to extracellular nucleotides such as ATP and UTP [Pfeilschifter, 1990; Pfeilschifter and Merriweather, 1993; Pfeilschifter et al., 2003]. In the last part of this thesis I focused on the modulatory effects on MMP-9 expression exhibited by extracellular ATP, since nucleotides can mediate a variety of cell responses in MC including cell growth and inhibition of apoptosis [Schulze-Lohoff et al., 1992; Huwiler and Pfeilschifter, 1994]. Moreover, extracellular nucleotides have also been reported to modulate experimental forms of glomerulonephritis *in vivo* [Poelstra et al., 1992]. I found that the stable ATP analog γ-S-ATP potently amplifies the IL-1ß-mediated expression of MMP-9. In contrast to the degradative effects on MMP-9 mRNA by NO, I found that γ-S-ATP significantly can increase the stability of MMP-9 mRNA. As mentioned before, the 3´-UTR of rat MMP-9 contains several copies of AU-rich elements which are considered as important determinants of RNA turnover [Chen and Shyu, 1995; Ross, 1995; Malter, 1989 ; Xu et al., 1997]. Again, I focused on the ELAV–like RNA-binding protein HuR which critically contributes to the posttranscriptional

regulation of MMP-9 in rat MC [Akool et al., 2003]. Using confocal fluorescence microscopy I demonstrate that HuR is predominantly nuclear but partially distributes in distinct punctuate structures in the cytoplasm following treatment with γ-S-ATP. This is in agreement with a recent study which has shown that ATP in higher concentrations (1 mM) inhibits the activity of AMP-activated kinase (AMPK) and thereby elevates the HuR level in the cytoplasm of colorectal carcinoma RKO cells [Wang et al., 2002]. Our findings suggest that ATP triggers an increase in HuR binding to the 3´-UTR of MMP-9 mRNA and thus conveys protection of MMP-9 transcripts from rapid degradation. This establishes an additional way of how signaling cascades affect the activity of HuR. However, the exact mechanisms by which HuR regulates mRNA stability is still poorly studied. HuR dependent changes in mRNA decay either can involve a nuclear-cytoplasmic movement (as shown by ATP) [Pinol-Roma and Dreyfuss, 1992; Atasoy et al., 1998; Keene, 1999] and/or changes in the HuR expression level as described for NO [Akool et al., 2003; Klöss et al., 2003]. The present findings present a molecular mechanism which may explain how extracellular nucleotides transiently potentiate the cytokine-mediated cellular capacities to degrade extracellular matrix without having any effects on MMP-9 expression in resting cells. Furthermore, our data emphasize the importance of modulation of mRNA stability in the tight regulation of MMP-9. Future *in vivo* studies investigating the modulatory effects exerted by different inflammatory mediators and nuclear hormon receptors such as PPARs may provide new targets to pharmacologically manipulate the abarrent expression of matrix degradative enzymes observed in various diseases.

With the knowledge that MMPs not only regulate many physiological processes such as cell growth, apoptosis, angiogenesis, and immune responses but are also involved in different diseases such as arthritis, renal fibrosis, cancer, atherosclerosis and heart failure, it was very interesting to learn more details about the regulation of MMP activity. Meanwhile, it is known that MMP activity is tightly regulated at several levels (transcription, processing of the

proenzyme, and inhibition by TIMPs) all of them providing new strategies to block or potentiate the effects mediated by these enzymes. Many clinical trials have been designed and currently are tested to target these key regulatory points. The expression of MMP genes can be inhibited by targeting extracellular factor, cell-surface receptor, signal transduction pathways and nuclear factors that activate the expression of many of the inducible MMP genes. For example, the blocking of signaling induced by cytokines or growth factors that upregulate MMPs could be selected for therapy. One prominent example is the clinical use of monoclonal antibodies against TNF-α or soluble TNF receptor which both are effectivly used in the therapy of rheumatoid arthritis. Also, selective inhibition of p38 MAPK activity can abolish the expression of MMP-1,9 and 13 in different cell types [Simon et al., 1998; Johansson et al., 2000]. A future focus to manipulate the expression of MMPs will be transcription factors such as AP-1 and NF-κB which control the expression of most of the inducible MMP genes. Recently, it has been reported that some natural flavonoids have the ability to inhibit AP-1 binding activity and thus were able to suppress the production of MMP-1 and MMP-9 by human fibrosarcoma cells resulting in decreased invasive properties of these cells. Also, many of the well known antiinflammatory drugs such as glucocorticoids can block the production of some MMPs by negative interference with the NF-κB pathway [Eberhardt et al., 2002a]. The activation of MMPs can be controled by targeting other proteases that cleave or activate MMPs as has been demonstrated by the successful inhibition of the proteolytic activity of some MMPs by use of neutralizing MT1-MMP antibodies [Galvez et al., 2001]. Another strategy to inhibit MMP activity is the use of TIMPs which were among the first approaches to block MMP activities. Those studies have reported that TIMPs can inhibit tumor growth in transgenic mouse models. However, the clinical application seems to be limited because of the general poor pharmacokinetics of TIMPs [Kruger et al., 1997; Martin et al., 1999]. Therefore, in the last few years many trials have tried to develop synthetic MMP-inhibitors (MMPIs) for the treatment of different diseases

most prominent rheumatoid arthritis and cancer. In this thesis, I present the posttranscriptional regulation of MMP-9 gene expression which possibly could provide a new target for modulating the expression of many genes involved in inflammation and tumor progression since mRNA stability plays an important role in the regulation of many other genes such as TNF α [Biragyn and Nedospasov, 1995; Dean et al., 2001], IL-2 [Bohjanen et al., 1991; Henics et al., 1994] and VEGF [Levy et al., 1996]. Meanwhile, different studies have shown that the regulation of factors which are critically involved in the stability of mRNA will affect not only the expression of MMP-9 but also of other genes that are similar to MMP-9, regulated at the level of mRNA stability. Most interestingly, recent studies could demonstrate that the expression of several genes regulated via the 3´-UTR are changed in the course of cardiovascular diseases [Misquitta et al., 2001]. Prominent example includes the $ß_1$ and $ß_2$ adrenergic receptors [Blaxall et al., 2000; Danner et al., 1998], eNOS which is mainly expressed in vascular endothelium and in the endocardial endothelium of the heart [Forstermann et al., 1998] as well as the inducible type of NOS (iNOS) which is mainly expressed in macrophages [Galea and Feinstein,1999]. A further study could document that the level of AUF-1 protein is higher in individuals suffering from myocardial failure [Pende et al., 1996]. In summary, these data indicate that posttranscriptional regulation may be a mechanism which is responsible for aberrant expression of some genes functionally involved in cardiovascular diseases. Similar to cardiovascular diseases, changes in the expression level of factors involved in mRNA stability are involved during tumorigenesis. E.g., cyclooxygenase-2 and VEGF mRNA which both contain AREs were reported to be abnormally stable in colon carcinoma cells indicating the role of the ARE-binding and RNA-stabilizing factor HuR in cancer progression [Dixon et al., 2001].

In conclusion, the posttranscriptinal regulation of gene expression may represent an attractive target to manipulate or modulate the expression of genes which in addition to transcriptional regulation display a modulation via mRNA stability.

Summary

Remodeling of extracellular matrix (ECM) is an important physiologic feature of normal growth and development. In addition to this critical function in physiology many diseases have been associated with an imbalance of ECM synthesis and degradation. In the kidney, dysregulation of ECM turnover can lead to interstitial fibrosis, and glomerulosclerosis. The major physiologic regulators of ECM degradation in the glomerulus are the large family of zinc-dependent proteases, collectively refered to matrix metalloproteinases (MMPs). The tight regulation of most of these proteases is accomplished by different mechanisms, including the regulation of MMP gene expression, the processing and conversion of the inactive zymogen by other proteases such as serine proteases and finally the inhibition of active MMPs by endogenous inhibitors of MMPs, denoted as tissue inhibitors of metalloproteinases (TIMPs). Namely, the MMP-9 has been shown to be critically involved in the dysregulation of ECM turnover associated with severe pathologic conditions such as rheumatoid arthritis or fibrosis of lung, skin and kidney. In the present work I searched for a possible modulation of MMP-9 expression and/or activity in glomerular mesangial cells which are thought as key players of many inflammatory and non-inflammatory glomerular diseases. I found that various structurally different PPARα agonists such as WY-14,643, LY-171883 and fibrates potently suppress the cytokine-induced MMP-9 expression in renal MC. Furthermore, I demonstrate that the inhibition of MMP-9 expression by PPARα agonists was paralleled by a strong increase of cytokine-induced iNOS expression and subsequent NO formation, suggesting that PPARα-dependent effects on MMP-9 expression level primarily result from alterations in NO production which in turn reduces the MMP-9 mRNA half-life. Searching for the detailed mechanism of NO-dependent effects on MMP-9 mRNA stability, I found that NO either given from exogenous sources or endogenously produced increases the MMP-9 mRNA degradation by decreasing the expression of the mRNA stabilizing factor HuR. Furthermore, I demonstrate a reduction in the RNA-binding capacity of HuR containing complexes to MMP-

9 ARE motifs in cells treated with NO. Since the reduction of HuR expression can be mimicked by the cGMP analog 8-Bromo-cGMP, I suggest that NO reduces in a cGMP-dependent manner the expression of HuR. Finally, I elucidated the modulatory effect of extracellular nucleotides, mainly ATP, on cytokine-triggered MMP-9 expression. Interestingly, I found that in contrast to NO, γ-S-ATP the stable analog of ATP potently amplifies the IL-ß mediated MMP-9 expression. The increase in mRNA stability was paralleled by an increase in the nuclear-cytosolic shuttling of the mRNA stabilizing factor HuR. Furthermore, I demonstrate an increase in the RNA-binding capacity of HuR containing complexes to the 3´-UTR of MMP-9 by ATP.

In summary, the data presented here may help to find new targets (posttranscriptional regulation) that could be used to manipulate or modulate the expression of not only MMP-9 but also other genes regulated on the level of mRNA stability.

6. References

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7. Abbreviations

AP-1	activator protein 1
APMA	p-aminophenylmercuric acetate
AREs	AU-rich elements
ATP	adenosine triphosphate
BCA	bicinchonic acid
BSA	bovine serum albumin
bFGF	basic fibroblast growth factor
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
COX-2	cyclooxygenase 2
CTP	cytidine triphosphate
DDT	dithiothreitol
DEPC	diethylpyrocarbonate
DETA-NO	diethylene triamine nitric oxide
DMEM	Dulbecco's modified Eagle's media
dNTPs	deoxynucleotides
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis (ß-aminoethyl ether)-N,N,N´,N´-tetraacetic acid
ELAV	embryonic lethal abnormal vision
EMSA	electromobility shift assay
e NOS	endothelial nitric oxide synthase
ERK	extracellular signal-regulated kinase
FCS	fetal calf serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GST	glutathione transferase
GTP	guanosine triphosphate
IFN-γ	interferon γ
IGF	insulin-like growth factor

IkB	inhibitor of kB
IL-1ß	interleukin 1ß
iNOS	inducible nitric oxide synthase
JNK	c-Jun-N-terminal kinase
LB	Luria Bertani
L-NMMA	N^G-monomethyl-L-arginine
LPS	lipopolysaccharides
MAPK	mitogen activated protein kinase
MC	mesangial cell
M-MLV	Moloney murine leukemia virus
MMPs	matrix metalloproteinases
NF-κB	nuclear factor κB
n NOS	neuronal nitric oxide synthase
NO	nitric oxide
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PKC	protein kinase C
PMSF	phenylmethylsulfonyl fluoride
PNK	polynucleotide kinase
PPAR-α	peroxisome proliferator activated receptor α
PPRE	peroxisome proliferator responsive element
PVDF	polyvinylidene difluoride
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
γ-S-ATP	γ-thio-ATP
SDS	sodium dodecyl sulfate
SNAP	S-nitroso-N-acetyl penicillamine
$sPLA_2$	secretory phospholipase A_2
SSC buffer	saline-sodium citrate buffer
STAT	signal transducer and activator of transcription
sv 40	simian virus 40
TGF-ß	transforming growth factor ß

TIMPs	tissue inhibitor of metalloproteinases
TNF-α	tumor necrosis factor-α
TSR	template suppression reagent
UTP	uridine triphosphate
UTR	untranslated region
VEGF	vascular endothelial growth factor

8. List of publications

A. Journal publications :

Akool, El-S.,* Eberhardt, W.,* Rebhan, J., Frank, S., Beck, K.F., Franzen, R., Hamada, F.M., and Pfeilschifter, J. Inhibition of cytokine-induced matrix metalloproteinase 9 expression by peroxisome proliferator-activated receptor α agonists is indirect and due to a NO-mediated reduction of mRNA stability.
J. Biol. Chem. (2002), 277: 33518-33528.

* Both authors contributed equally.

Akool, El-S., Kleinert, H., Hamada, F.M.A., Abdelwahab, M.H., Forstermann, U., Pfeilschifter, J., and Eberhardt, W. Nitric oxide increases the decay of MMP-9 mRNA by inhibiting the expression of mRNA stabilizing factor HuR.
Mol. Cell. Biol. (2003), 23: 4901-4916.

Akool, El-S.,* Huwiler, A.,* Aschrafi, A., Hamada, F.M.A., Pfeilschifter, J., and Eberhardt, W. ATP potentiates IL-1ß-induced MMP-9 expression in mesangial cells via recruitment of the ELAV protein HuR.
J. Biol. Chem. [Epub ahead of print, Oct 2003].

* Both authors contributed equally.

B. Poster presentation :

El-Sayed Akool, Jorg Rebhan, K.F.Beck, Farid M:A:Hamada, Josef Pfeilschifter and wolfgang Eberhardt
Inhibition of cytokine-induced MMP-9 expression by PPARα is indirect and is due to a NO-mediated reduction of mRNA stability.
43. Spring meeting of DGPT, Mainz, 2002, N-S Arch Pharmacol 365 :27 Suppl.

El-Sayed Akool, Hartmut Kleinert, Josef Pfeilschifter and Wolfgang Eberhardt
Differential regulation of MMP-9 expression under nitrosative and oxidative stress conditions in rat renal mesangial cells
Satellite Symposium to the ISN-ERA/EDTA-world congress of Nephrology 2003 on "glomerulonephritis and progression to renal insufficiency"
at Kloster Seeon, Bavaria, Germany, June 12-15, 2003.

9. Acknowledgements

I am grateful and very thankful to **Prof. Dr. Josef Pfeilschifter** for giving me the opportunity to work in his laboratory and his scientific support.

I especially want to thank **Dr. Eberhardt** as my direct supervisor for giving me the possibility to work on this topic and for his kind supervision, enthusiastic help and always having time for discussion.

I thank **Prof. Dr. Manfred Schubert-Zsilavecz** for his readiness to give his export opinion on my work and to be the official supervisor of this thesis.

Furthermore, I thank all the members of our group who have accompained and helped me through the years. I thank Rowitha Müller for helpful assistance and a pleasant working atmosphere.

I would like to thank Dr. Armaz Aschrafi for performing the confocal microscopy experiments.

Further thanks to all members of the Institute for fruitful discussions, many scientific help and a pleasant working atmosphere.

Deutsche Zusammenfassung

Umbauprozesse der Extrazellulären Matrix (ECM) spielen eine wichtige Rolle für normale Wachstums-und Entwicklungsprozesse. In der Niere kann ein nicht adäquater Umsatz von ECM beispielsweise zur interstitiellen Fibrose und Glomerulosklerose führen. Diese gelten als gemeinsame Endstrecke einer Vielzahl von entzündlichen Erkrankungen der Niere unterschiedlicher Genese. Progressive interstitielle Fibrosen sind u.a. durch eine Akkumulation von Leukozyten und Fibroblasten, durch eine Atrophie des Tubulus sowie durch eine massive Anhäufung von Extrazellulären Matrix (ECM) im Glomerulus charakterisiert. Zu den wichtigsten physiologischen Regulatoren des Abbaus von ECM im Glomerulus zählen Zink-abhängige Proteasen, die zur Familie der Matrixmetalloproteasen (MMPs) zusammengefasst werden. Hauptproduzenten der renalen MMPs sind glomeruläre Mesangiumzellen, die neben der MMP Synthese an zahlreichen anderern physiologischen und pathologischen Prozessen der Niere beteiligt sind. Die genau abgestimmte Regulation dieser Proteasen wird über verschiedene Mechanismen erreicht, u.a. über die Regulation von Genexpression, über die Prozessierung und Aktivierung des inaktiven Proenzyms durch andere Proteasen (z.B. Serinproteasen) und schliesslich über die Hemmung der aktiven MMPs durch die endogenen MMP Inhibitoren, die als „tissue inhibitors of metalloproteases“ (TIMPS) zusammengefasst werden. Zu den potentesten Aktivatoren der MMP-9 Expression zählen proinflammatorische Zytokine wie Interleukin 1β und tumor necrose factor α (TNFα) als auch Tumorpromotoren wie Phorbolester und das virale src Onkogen (v-src).

Das Hauptziel der vorliegenden Arbeit galt der Aufklärung der molekularen Mechanismen der MMP-9 Expression und/oder MMP-Aktivität in glomerulären Mesangiumzellen der Ratte. Mesangiumzellen gelten als Hauptakteure von glomerulären Erkrankungen mit entzündlicher- als auch nicht entzündlicher Genese. Im Verlauf der glomerulären Entzündung können im Mesangium durch den Einfluss von Zytokinen und Wachstumsfaktoren auch grössere

Mengen reaktiver Sauerstoffverbindungen (reactive oxygen species: "ROS") und Stickstoffmonoxid (NO) gebildet werden. ROS und NO können neben ihrer rein zelltoxischen Wirkung z.B. durch Schädigung von DNA oder Strukturproteinen über eine Aktivierung redox-sensitiver Signalkaskaden auch als Botenstoffe der Zelle wirken und damit spezifische Zellantworten, wie z.B die Gentranskription oder das Auslösen von Apoptose beeinflussen. Die Bedeutung dieser Zellantworten bei den oben aufgeführten pathologischen Prozessen ist bislang noch wenig bekannt. In Mesangiumzellen kann die über Zytokine induzierte Expression von MMP-9 durch exogenes und endogen gebildetes NO gehemmt werden, ohne dass die Aktivität von MMP-9 verändert wird. Mittlerweile ist eine Regulation der MMP-9 Expression durch NO nicht nur in Mesangiumzellen, sondern auch in einer Reihe anderer Zellsysteme wie z.B. glatten Muskelzellen oder in Gefässendothelzellen nachgewiesen worden.

Im ersten Teil meiner Arbeit untersuchte ich die molekularen Mechanismen der über eine Aktivierung der Peroxisomen Proliferation aktivierten Rezeptoren (PPAR)-vermittelten MMP-9 Expression in glomerulären Mesangiumzellen. Aus vorherigen Studien war bekannt, dass Liganden von nukleären Hormonrezeptoren wie z.B. Glukokortikoide, über eine Hemmung der Transkriptionsfaktoren „nuklear factor κB" (NF-κB) und „activated protein-1" (AP-1) die Zytokin-induzierte MMP-9 Expression stark herabsetzen können. Als weitere nukleären Hormonrezeptoren, die als Modulatoren der MMP-9 Expression wirken können, haben wir Liganden der PPARs identifizieren können. PPARs können durch die Bindung von sowohl physiologischen (z.B. Retinolsäure, langkettige Fettsäuren und Eikosanoide) als auch pharmakologischen Liganden (z.B. Fibrate und Glitazone) aktiviert werden. Von besonderem Interesse ist, dass Aktivatoren der PPARs neben ihren Funktionen beim Fettabbau, zusätzlich wichtige antiinflammatorische Wirkungen vermitteln können. Mechanistisch kann die Aktivierung der PPARs über eine direkte Bindung an spezifische DNA-Erkennungsmotive, sogenannte „PPAR-responsible elements" („PPREs"), oder indirekt, durch die

Wechselwirkungen mit anderen Transkriptionsfaktoren ohne einen direkten Kontakt zur DNA, zu einer veränderten Transkriptionsrate der über PPAR-regulierten Gene führen. Wie ich zeigen konnte sind unterschiedliche Aktivatoren des PPARα Rezeptortyps wie beispielsweise WY-14,643, LY-171883 oder Bezafibrat in der Lage, die Zytokin-induzierte MMP-9 Expression in Mesangiumzellen in potenter Weise zu hemmen. Im Gegensatz hierzu haben „Nuclear run-off" Experimente und Promotoranalysen gezeigt, dass die expressionshemmende Wirkung der PPARα Aktivatoren nicht auf eine Hemmung der Transkriptionsrate zurückgeführt werden können. Auf der Suche nach weiteren PPAR-modulierten Genen der Entzündungskaskade fand ich die induzierbare NO Synthase (iNOS), deren Expression durch PPARα Aktivatoren verstärkt wird was letzlich zu einer gesteigerten NO Freisetzung führt. In Mesangiumzellen kann die Expression der iNOS, ähnlich wie die Expression der MMP-9, durch Stimulaton mit proinflammatorischen Zytokinen um ein Vielfaches gesteigert werden. In der vorliegenden Arbeit konnte die Existenz eines funktionellen „Crosstalks" zwischen diesen beiden prominenten inflammatorischen Genprodukten (NO und MMP-9) nachgewiesen werden. Dieser Crosstalk äussert sich darin, dass die MMP-9 hemmenden Effekte der PPARα Aktivatoren in der Gegenwart eines iNOS Aktivitäthemmstoffes zu einer massiven Verstärkung der Zytokin-induzierten MMP-9 Expression konvertiert werden können. Die Umkehrung in Gegenwart des NO Synthesehemmers zeigt, dass die PPARα-vermittelten Effekte in erster Linie durch die Hemmung der NO Synthese hervorgerufen werden. Auf der Suche nach den mechanistischen Grundlagen dieser NO Effekte konnte ich mit Hilfe des Transkriptionshemmstoff Actinomycin D zeigen, dass sowohl exogen zugeführtes NO als auch durch eine Induktion der iNOS entstandenes NO in der Lage ist, den Abbau der Zytokin-induzierten MMP-9 mRNA zu beschleunigen. Weiterhin konnte der Nachweis erbracht werden, dass die Expression des mRNA stabilisierenden Faktors HuR durch NO sowohl auf Protein- als auch auf mRNA Ebene signifikant gehemmt wird. HuR ist ein Protein der ELAV Genfamilie (embryonic lethal

abnormal vision), deren Mitglieder als RNA-bindende Proteine bei der Regulation des zellulären mRNA Abbaus und damit bei der Regulation der posttranskriptionellen Genexpression massgeblich beteiligt sind. Die über HuR vermittelte Stabilisierung von mRNA erfolgt über eine hochaffine Bindung des HuR Proteins an AU-reiche Sequenzmotife innerhalb des untranslatierten 3´-Bereichs der mRNA (3´-UTR) .

Durch die Behandlung der Zellen mit einem membranpermeablen cGMP Analogon (z.B. 8-Bromo-cGMP) konnte ich nachweisen, dass die NO-abhängige Stabilisierung von MMP-9 mRNA über cGMP-aktivierte Signalkaskaden vermittelt wird. Entsprechend konnte durch die Zugabe eines spezifischen Hemmstoffes der löslichen Guanylatzyklase (z.B. LY-83,583) die über NO-vermittelte Hemmung der HuR Expression signifikant gehemmt werden.

Über die Klonierung des etwa 800 Basen langen 3´-UTR des MMP-9 Gens gelang es mir, die für die Stabilisierung der MMP-9 mRNA verantwortlichen „cis-regulatorischen“ DNA-Bereiche mit Hilfe der Punktmutagenese zu identifizieren. Die funktionelle Bedeutung konnte nachfolgend mit der Hilfe von transienten Transfektionen von Mesangiumzellen mit verschiedenen mutierten Reportergenkonstrukten demonstriert werden.

Die funktionelle Konsequenz einer über NO -vermittelten Stabilitätsminderung von MMP-9 mRNA konnte ausserdem mit der Hilfe von *in vitro* RNA-Degradationsexperimenten belegt werden. Diese Experimente haben gezeigt, dass die endogene MMP-9 mRNA nach der Inkubation mit zytoplasmatischen Zellextrakten von NO behandelten Zellen einem im Vergleich nach Inkubation mit Zellhomogenaten aus unbehandelten Mesangiumzellen, beschleunigten RNA Abbau unterliegt. Entsprechend konnte durch Neutralisierung des HuRs mit einem spezifischen Antikörper der über NO vermittelte Abbau der MMP-9 mRNA nahezu vollständig blockiert werden, während durch die Inkubation mit einem Kontrollserum keine Verzögerung der mRNA Degradation beobachtet werden konnte.

Auf RNA-Ebene konnte ich mit Hilfe von RNA-Gelschift und Superschiftanalysen eine starke, konstitutive Bindung von HuR-haltigen Proteinkomplexen an MMP-9-genspezifische

AU-reiche Sequenzmotive nachwiesen. Die hohe Bindungsaffinität des HuRs an diese AU-reichen Sequenzmotife zeigt sich auch anhand der hohen Bindungsaffinität des in *E.coli* exprimierten HuR Proteins. Durch Mutation der MMP-9-spezifischen AU-reichen Sequenzmotive, konnte die RNA-Bindugsaffinität des HuRs (unabhängig davon ob fremdexprimiert oder aus Mesangiumzellen isoliert) stark vermindert werden. Die hier zusammengefassten Untersuchungen zeigen nach unserem Wissen erstmalig die Möglichkeit einer durch NO-vermittelten Genexpressionsänderung von MMP-9 durch Modulation der mRNA-Stabilität.

In einem weiteren Projekt untersuchte ich, ob extrazellulären Nukleotide in der Lage sind, einen modulierenden Einfluss auf die Zytokin-induzierte MMP-9 Expression auszuüben. Extrazelluläre Nukleotide wie das Adenosintriphosphat (ATP) und Uraciltriphosphat (UTP) sind nicht nur für die Nukleinsäuresynthese und den Energiestoffwechsel der Zelle von zentraler Bedeutung, sondern sind über eine Bindung an zellmembranständige Rezeptoren (sogenannte Purin Rezeptoren) in der Lage, eine Reihe weiterer zellphysiologischer Prozesse wie z.B. Apoptose oder Zellproliferation zu steuern. Letztere ist zusammen mit dem extensiven Abbau der ECM ein Hauptcharakteristikum vieler chronisch entzündlichen Erkrankungen der Niere. In den hier vorgestellten Untersuchungen konnte ich zeigen, dass das chemisch stabile ATP-Analogon γ-S-ATP in der Lage ist, einen über IL-1β-vermittelten Anstieg der MMP-9 Expression noch weiter zu verstärken. Interessanterweise korreliert der Anstieg der MMP-9 mRNA Stabilität mit einer Zunahme des intrazellulären Transports von HuR aus dem Zellkern in das Zytoplasma. Der verstärkte Export von HuR aus dem Zellkern ist auch mit einer Zunahme der RNA Bindungsaffinität der HuR-haltigen Komplexe an die AU-reichen Sequenzenmotife innerhalb des 3´-untranslatierten Bereichs (3´-UTRs) des MMP-9 Gens verbunden.

Im Unterschied zu der im ersten Teil meiner Arbeit beschriebenen Veränderung der MMP-9 mRNA Stabilität durch eine über NO-hervorgerufene Expressionsänderung von HuR, beruht die durch ATP-induzierte Stabilitätszunahme von MMP-9 mRNA hauptsächlich auf einem verstärkten intrazellulären Transport des HuR Proteins, ohne dass dabei seine Expression verändert wird. Jeder dieser Mechanismen könnte für das Verständnis der molekularen Ursachen von Krankheiten, die u.a auf fehlerhafte Matrixumbauprozesse in der Niere oder anderen Organen wie Leber oder Lunge zurückzuführen sind , von grosser Bedeutung sein.

Die in der vorliegenden Arbeit zusammengefassten Erkenntnisse können ausserdem dazu beitragen, neue insbesondere posttranskriptionelle Ansätze der spezifischen Manipulation von MMP-9 und möglicherweise anderer auf Ebene der mRNA Stabilität regulierten Gene zu finden.

11. Curriculum vitae

Personal Data: El-Sayed Ibrahim M.S. Akool
Born on 22.09.1967 in El-sharkia, Egypt.

Marrital status: married, two children

Education

Transcript : secondary education at the secondary school in El Karin, Egypt.

University: 1985 Studies of Pharmacy at Al- Azhar University, Cairo, Egypt.
1990 Bachelor Science of Pharmacy (excellent) at Al-Azhar University, Cairo, Egypt.
1999 Master degree in "Pharmacology" at Al-Azhar University.
title of master thesis: "Modulatory effect of melatonin in cancer chemotherapy"

Since 2001: Ph.D student at "Pharmazentrum Frankfrurt" (Institut für Allgemeine Pharmakologie und Toxikologie, Klinikum der Johann Wolfgang Goethe-Universität Frankfurt/M., Germany (chair: Prof. Dr. J.Pfeilschifter)

Teaching: **1996-1999:** "Demonstrator" (teaching of advanced students of pharmacy in pharmacology & toxicology) at Al-Azhar University, Cairo, Egypt.
1999-2001: "Assistant lecturer" (teaching of advanced students of pharmacy in pharmacology &toxicology) at Al-Azhar University, Cairo, Egypt.