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NITRIC OXIDE (NO) MODULATES PLATELET-DERIVED GROWTH FACTOR RECEPTOR-α (PDGFR-α) EXPRESSION IN RAT RENAL MESANGIAL CELLS

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C O N T E N T S

I - I N T R O D U C T I O N

1.1 Glomerular Mesangial Cells – physiological functions, pathological implications

The nephron is the smallest functional unit of the kidney. It consists of the glomerulus, the proximal tubule, the loop of Henle, the distal tubule and the collecting duct. The glomerulus includes the blood vessel bundle between the afferent and efferent arteriole. But it also integrates the capsule of Bowman with all its interior and is used equivalently to the malphighian corpuscle. The mesangium is the intercapillary space within the glomerulus. Under physiological conditions only fluids or molecules smaller than 5-10 kDa enter the Bowman space freely. Larger components of the blood are effectively restrained by the glomerular barrier, which is composed of three sheets: the fenestrated endothelium of the arterioles, the glomerular basement membrane (GBM) and slit pores between foot structures of podocytes. But not the entire capillary surface is covered by the GBM, a certain part is enclosed by mesangial cells (MC) or MC-derived extracellular matrix (ECM). MC are smooth muscle-like pericytes, which modify the capillary surface area and thus contribute to the regulation of the glomerular filtration rate (GFR) (Stockand and Sansom, 1998).

The function of MC is not only restricted to mechanical or statical work, they participate also in immunological affairs and even have phagocytic function (Schlondorff, 1987).

MC built up one-third of the total number of glomerular cells in a normal adult kidney but the number of MC may alter under pathological conditions (Pabst and Sterzel, 1983). MC are able to respond to and to produce themselves mediators that induce MC proliferation and deposition of ECM (Pabst and Sterzel, 1983) – two characteristics of many inflammatory kidney diseases.

MC hyperplasia can be found in such diverse diseases like IgA nephropathy, lupus nephritis, membranoproliferative glomerulonephritis and diabetic nephropathy (Bohle et al., 1976) – each of them with a high risk to progress to glomerulosclerosis – the irreversible end stage of inflammatory kidney diseases with glomerular scarring and subsequent loss of function (Pesce et al., 1991; Floege et al, 1992). MC proliferation inhibitors like heparin, neutralizing antibodies to PDGF, angiotensin converting enzyme (ACE) inhibitors and others could reduce ECM accumulation and sclerotic changes in animal models (Kurogi, 2003), which emphasizes the critical role of MC proliferation in glomerulopathy.

On the other hand in some glomerular diseases like human poststreptococcal glomerulonephritis or certain types of lupus nephritis, MC proliferation is part of a controlled repair process, in which the transient increase of MC is followed by MC loss and

reconstitution of the glomerulus (Haas et al., 1999). A well characterized experimental model of a mesangioproliferative disease is the anti-Thy 1.1 GN in rats. The Thy-1.1 antigen is located on the cell surface of MC (Paul et al, 1984). Injection of complement activating antibodies against the Thy-1.1 antigen leads to an initial injury with up to 90 % loss of MC, which is followed by a mesangioproliferative phase with ECM accumulation and finally resolution with return to almost normal histology after several weeks (Bagchus et al., 1986; Ishizaki et al., 1986).

One way to attain the loss of MC is apoptosis. Apoptosis or the programmed cell death is regarded as the major mechanism to counteract mesangial hyperplasia (Baker et al., 1994; Shimizu et al., 1995). And it is indeed an effective weapon to oppose hypercellularity, but it is also responsible for incomplete cell repopulation and bares the risk of insufficient repair. Apoptotic cells are found in late stages of glomerulosclerosis - associated with glomerular cell deletion and abnormal ECM accumulation (Sugiyama et al., 1996).

Reactive oxygen species (ROS) and nitric oxide (NO) – both produced in the course of GN - are known mediators of programmed cell death. (Brüne et al., 1998). NO, a diffusible gas with various physiological and pathological properties, is synthesized by three forms of nitric oxide synthases (NOS) by the oxidation of L-arginine to citrulline and NO. Besides its apoptotic and cytotoxic features, it exerts many additional effects including stimulation of proliferation, cell-protection and regulation of gene-expression just to name some of them (Beck et al., 1999). In anti-Thy-1.1 GN there is a massive infiltration of neutrophils and macrophages into the glomerulus. Macrophages represent the majority of iNOS positive cells within the first 24 h after anti-Thy.1.1-injection (Yamamoto and Wilson, 1987). Inhibition of NO production by the arginine analogue L-NMMA results in almost complete abrogation of mesangiolysis (up to 90%), whereas addition of L-arginine – the substrate for NO generation - aggravates mesangiolysis (Narita et al., 1995). This accentuates the predominant role of NO as a central trigger to MC death. In the last decades a series of mediators of MC proliferation have been characterized. This includes autacoids, growth factors, hormones and cytokines that have been examined by *in vivo* and *in vitro* studies. In anti-Thy-1.1 GN MC proliferation is mediated primarily by platelets (Johnson et al., 1991) and the release of preformed bFGF from injured MC. Platelets are a rich source of promitogenic factors including platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), insulin-like growth factor I (IGF-1), extracellular nucleotides and vascular epithelial growth factor (VEGF), which are released upon activation. (Jefferson and Johnson, 1999).

Among these factors the most prominent and potent mitogen for MC proliferation is platelet derived growth factor (Abboud 1995; Heldin and Westermark 1999,). This soluble mediator exists in five different isoforms and is able to increase the thymidine incorporation rate of MC up to 15-fold depending on the isoform supplied. PDGF, which is also synthesized by MC induces its own mRNA expression, thus providing the basis for an autocrine growth mechanism. Under physiological circumstances glomerular PDGF synthesis and particularly PDGF receptor expression is low (Pfeilschifter and Hosang, 1991) but both parameters increase in experimental and human mesangioproliferative diseases (Abboud, 1993; Johnson et al., 1993).

It is worth mentioning that during glomerular injury counteracting mediators tackle glomerular cells: on the one hand NO, a potent inducer of MC death and on the other hand PDGF, the most potent effector of MC proliferation. Both, NO and PDGF are highly produced in inflammatory kidney diseases and exert partially cooperative and partially antagonising effects. A closure look to the regulatory interactions between NO and PDGF may help to understand the contradictory reports on NO under inflammatory conditions. In 1991, Pfeilschifter first reported that PDGF inhibits dose-dependently cytokine-induced NO-synthase activity in MC. My thesis particularly addresses the reciprocal control mechanisms of NO on the PDGF signalling system in glomerular MC.

1.2 Platelet-derived growth factor

Growth factors are polypeptides that serve as soluble intercellular signalling molecules. The ligation of the membrane-bound growth factor receptors activates intracellular signalling cascades resulting in proliferation, migration or differentiation of the target cells.

Platelet-derived growth factor (PDGF) is one of the most prominent representatives of the growth factor family with a tremendous mitogenic activity on mesenchymal cells including MC, fibroblasts, smooth muscle cells and other (Heldin et al., 1996, Raines et al., 1990).

1.2.1 Structure of PDGF

PDGF was originally isolated from the α-granules of human platelets (Antoniades et al.,1979; Heldin et al., 1979; Raines and Ross 1982) but have since been found in many normal as well as transformed cell types (Raines et al., 1990). It is an approximately 30 kDa cationic homo- or heterodimer, which consist of two disulfide-bonded polypeptides, termed A- (17 kDa) and B-chain (14 kDa). The combination of these two chains leads to the three well known isoforms PDGF-AA, PDGF-BB and PDGF-AB. The mature parts of PDGF-A- and B-chains

are approximately 100 amino acid residues long and show 60% amino acid sequence identity. Both chains are synthesized as precursor molecules and reveal the twofold molecular weight of the mature protein chain (Östman et al., 1991).

70% of the PDGF source in human thrombocytes is composed of PDGF-AB, 5-25 % of PDGF-BB and the rest of PDGF-AA (Hammacher et al., 1988; Raines et al., 1990).

In recent years two new members of PDGF-family were identified PDGF-CC (Li et al., 2000) and PDGF-DD (Bergsten et al., 2001; LaRochelle et al., 2001). The C- and D-chain do not combine for heterodimers.

1.2.2 Expression and regulation of PDGF

Positive regulators of PDGF production in MC are growth factors (PDGF, EGF, FGF, TGF-β, TGF-β, TNF), vasoactive compounds (angiotensin II, endothelin, thrombin), phospholipids, (phosphatidic acid, phosphatidylserine), lipoproteins (LDL, VLDL) and PKC agonists (Betsholtz, 1993; Abboud, 1995).

MC and other cells grown in culture secrete mainly the PDGF-AA and PDGF-AB isoforms and hardly PDGF-BB. One reason for the low amount of PDGF-BB-secretion is that MC predominantly express PDGF-A-chain mRNA and to a much lesser extent PDGF-B-chain-mRNA. (Betsholtz et al. 1986, Abboud et al., 1987, Pfeilschifter and Hosang, 1991). Furthermore, the amount of active PDGF A-chain and especially B-chain can be reduced by interaction with extracellular matrix molecules like different types of collagens (Somasundaram et al., 1996), thrombospondin (Hogg et al., 1997), SPARC (secreted protein acidic and rich in cysteine, also known as BM-40 or osteonectin) (Raines et al., 1992) and soluble proteins like α_2-macroglobulin (Lamarre et al., 1991).

1.2.3 Platelet-derived growth factor receptors

PDGF receptors (PDGFR) are tyrosine kinase receptors (RTK) that need to dimerize for activation (Heldin et al., 1989). Each subunit of the PDGF molecule binds to one receptor monomer. Therefore, two receptor monomers are needed to be activated by one PDGF isoform. Dimerization narrows the distance between the intracellular parts of the receptor monomers and allows for transphosphorylation of tyrosine residues through basal kinase activity between the two receptor subunits in the complex. There are two receptor subunits denoted as α-and the β-receptor with a molecular size of approx. 170 and 180 kDa, respectively (Heldin and Westermark, 1999).

Membrane-bound RTK´s are composed of an extracellular, a transmembrane and an intracellular domain. The extracellular part of PDGF receptors consists of five immunoglobulin-like domains, whereby the outer three domains are responsible for ligand binding. The transmembrane domain is followed by a 49 amino acids long juxtamembrane domain which is important for binding of e.g. src kinases or adapter proteins. The adjacent tyrosine kinase domain is interrupted through a characteristic 100 amino acids long sequence without catalytic activity. Also this domain has tyrosine residues that are autophosphorylated during activation (Claesson-Welsh et al, 1989; Matsui et al., 1989).

Both receptors show different binding abilities. Whereas the α-receptor only binds the B-chain, the α-receptor is able to bind both chains. Therefore binding of PDGF-AA results in dimerization of αα-receptor homodimers, PDGF-AB induces αα-receptor homodimers plus αβ-receptor heterodimers and PDGF-BB enables the formation of all three dimeric receptor combinations (αα-, αβ- and ββ-receptor dimers) (Seifert et al., 1989).

PDGF-DD is only known to bind to and to form PDGF receptor ββ complexes (Bergsten et al., 2001; LaRochelle et al., 2001), whereas PDGF-CC – similar to PDGF-AB - is able to induce dimerization of PDGFR-αα and PDGFR-αβ-complexes (Gilbertson et al., 2001).

Dimerization of different receptor combinations exert similar but not identical cell responses. These cell responses depend on the cell type and the amount of receptor present (Heldin and Westermark, 1999).

1.2.4 Regulation of PDGF receptor expression

The amount of receptor is not constant and can be increased during inflammation) and after hormone or cytokine treatment (Rubin et al., 1988. The regulation of the two subunits is mediated differentially and regulatory elements evoke diverse response patterns in distinct cell types and species. (Fukuoka et al., 1998; Xie et al., 1994).

The promoter of the α-receptor in human, rat or other species has been isolated (Wang and Stilles, 1994) and the molecular mechanisms of PDGFR-α gene transcription have been extensively studied. Kitami et al. (1995) found out that the promoter region between nucleotides –246 and –139 is responsible for the upregulation of PDGFR-α in hypertrophic vascular smooth muscle cells of genetically hypertensive rats. This domain includes an enhancer core sequence, which specifically interacts with CCAAT/enhancer binding protein (C/EBP) δ (Kitami et al., 1995; 1999; Fukuoko, 1999). C/EBP-δ is a member of the basic leucine zipper transcription factor family and is highly upregulated in inflammatory conditions and specifically in response to IL-1β and cAMP stimulation (Eberhardt et al.,

2000). However, soluble factors are not the only regulatory elements for PDGFR expression. Barrett et al. (1996) showed that cell context is even more effective to enhance PDGFR mRNA and protein-levels than cytokine treatment. By varying cell density, cell attachment and other culture conditions in *in vitro* studies of human fibroblasts, they observed an increase of both receptor subtypes up to 50-fold. Also cell culture in three-dimensional collagen gels upregulated the PDGFR expression. But the results are cell-specific. Rat MC even diminish their PDGFR-β expression, when cultured in three-dimensional collagen gels in comparison to ordinary monolayer cultures on plastic (Marx et al., 1993, 1994).
Nevertheless in cell culture conditions the expression of both receptor subunits is elevated when compared to *in vivo* models (Terracio et al., 1988), indicating that the PDGFRs are subjected to down-regulation by not yet characterized circumstances under physiological conditions in vivo.

1.2.5 Dimerization and autophosphorylation of the PDGF-Receptor

Binding of the ligand results in non-covalent dimerization of two receptor monomers, which leads to cross-phosphorylation of conserved tyrosine residues inside and outside the kinase domain. Phosphorylation of the latter sites enables downstream signal transduction molecules like STATs (signal transducers and activator of transcription), adaptor proteins and enzymes that contain SH2 domains to interact with PDGFRs (Heldin et al., 1998; Heldin and Westermark 1999).

1.2.5.1 STAT molecules that bind to PDGFR

STATs are cytosolic transcription factors that are important downstream effector molecules of interferon and other cytokine receptors. After interferon or cytokine treatment members of the Janus kinase family (Jaks) of cytoplasmic tyrosine kinases (Tyks) are activated and subsequently phosphorylate STAT proteins. The STATs can then dimerize with one another on their SH2 domain and the new formed homo- or heterodimers translocate to the nucleus and initiate transcription of target genes (Darnell, 1997). PDGFR can activate STATs indirectly through activation of Jaks (Vignais et al., 1996) or directly via binding of STATs to the receptor (Ghosh -Choudhury et al., 1998).

1.2.5.2 Enzymes and adapter proteins that bind to the PDGF receptors

Many enzymes associate with PDGFRs either directly or via SH-2 domains-containing adapter proteins like Shc, Nck, Grb7, Grb10, Grb14 or Crk, which are devoid of catalytic activity (Heldin and Westermark, 1999).

The most important effector molecule of PDGF-induced signal transduction in glomerular MC is probably phosphatidylinositol-3-kinase (PI3-K), since inhibition of PI3-K in human MC dose-dependently reduced PDGF-mediated DNA synthesis and migration to basal level (Ghosh-Choudhury, 1997).

PI3-K belongs to the family of enzymes that phosphorylate the inositol ring of phosphoinositides generating phosphatidylinositol phosphates. It consists of a p85 regulatory subunit and a p110 catalytic subunit. Binding of the regulatory subunit p85 with one of its SH2 domains to PDGFR-α or -β leads to a conformational change with subsequent activation of the enzyme (Panayotou et al., 1992). Activated PI3-K or its downstream effector substrates phosphorylate several other signal transducers that initiate diverse biological responses like proliferation (coactivation of PLCγ), inhibition of apoptosis (via activation of Akt/PKB) and cytoskeletal reorganisation with cellular migration (Kauffmann-Zeh et al., 1997; Vanhaesebroeck et al., 1997).

Once bond, the appropriate PDGF dimer leads to internalization of the ligand-receptor complex. The complex then dissociates and the receptor is either degraded or - to a much lesser extent -returns back to the cell membrane (Heldin and Westermark 1999).

1.2.6 Mesangial cell responses to the different PDGF isoforms

As indicated, a number of signal transduction pathways are activated through PDGFR dimerization. Some of these cascades overlap or differ from cell type to cell type, which complicates our understanding of the functions of PDGFR activation (Heldin and Westermark 1999).

In MC PDGF is able to stimulate proliferation, chemotaxis, contraction and its own expressional induction in an isoform-specific manner. The particular response may depend on the amount of receptors present on the cell surface.

Cultured human MC have about ten times more binding sites for PDGF-BB than for PDGF-AA. Abboud et al., (1994) counted 90000 binding sites for PDGF-BB, 30000 for PDGF-AB and 10000 for PDGF-AA.

The discrepancy between the amount of binding sites for PDGF-AA and PDGF-AB is not fully clarified, whereas some authors claim (low-affinity) formation of ßß- homodimers in

response to PDGF-AB treatment (Seifert et al., 1993) others deny the ability of PDGF-AB to dimerize ββ-receptors (Hammacher et al., 1989). However, about 100-fold larger concentrations of PDGF-AB are needed to evoke a slight mitogenic response in cells from patch-mice which have the α-receptor gene deleted (Seifert et al., 1993), so that the formation of PDGFR-ββ in response to PDGF-AB in vivo is neglectable.

Abboud et al. (1994) further demonstrated that PDGF-AA, which is a potent mitogen in Swiss 3T3 cells (a cell-type that expresses both receptors in similar amounts) failed to induce DNA synthesis and migration in cultured human MC, whereas PDGF-BB dose-dependently increased both parameters. One reason for the lack of mitogenic capacity of PDGF-AA, is the firm downregulation of the α-receptor due to constitutive secretion of PDGF-AA, and represents a mechanism to prevent autocrine growth. Inhibition of this endogenous PDGF secretion leads to a modest but significant increase of DNA-synthesis in response to exogenously applied PDGF-AA, giving rise to the theory that the amount of receptors dictates the particular effect (Abboud et al., 1994).

PDGF-AB, which has only one third of the binding sites of PDGF-BB exerts 75% of PDGF-BB induced mitogenic and chemotactic activity in human MC (Abboud et al., 1994; Ghosh-Choudhurry, et al. 2000). This has lead to the assumption that the formation of αβ-heterodimers might be even more potent than the ββ-homodimers. Cells which express equal numbers of α- and β-receptors give stronger responses to PDGF-AB treatment than to the homodimeric isoforms (Rupp et al., 1994; Heidaran et al., 1991). One reason for this observation is that autophosphorylation takes place at different sites in the hetero- as compared to the homodimer. Tyr-771 for example, a tyrosine residue that is able to bind and activate the ras inhibitor GAP, was found to be phosphorylated to a much greater extent in the homodimeric β-complex when compared to the heterodimeric complex. Therefore, the heterodimeric receptor promotes less negative regulatory mechanisms than the homodimeric receptor complex (Heldin and Westermark, 1999, Ekman et al., 1999).

1.2.7 Physiological and pathophysiological roles of PDGF

The role of PDGF begins with embryonic development. Knock-out studies in mice revealed the significance for both chains (A and B-chain) and both receptors, since deletion of each of these components was lethal. (Betsholtz et al., 2001).

Mice died during embryogenesis or perinatally. The phenotypes of PDGF-B chain or PDGFR-β knockout mice are very similar and start about embryonic day 16 (Levéen et al., 1994; Soriano, 1994). In both cases defective formation of the vascular system is

predominant. The failure of newly formed blood vessels to recruit vascular smooth muscle cells (VSMC) and pericytes leads to capillary dilatation with microaneurysms, placenta defects, general oedema, haemorrhage and abnormal renal glomeruli, with a total lack of MC. PDGF-B-chain or β-R deficient mice die just before birth due to these cardiovascular complications (Betsholtz et al., 2001).

The consequence of PDGF-A chain or PDGFR-α gene deletion is associated with different phenotypes (Boström et al., 1996; Soriano, 1997). Whereas the α-R knockouts die between embryonic day 8 to 10, a certain part of PDGF-A chain-deleted mice may survive up to six weeks after birth. However the majority dies either in embryonic stages or directly after birth due to respiratory insufficiency and a lack of alveolar myofibroblast. The malformations associated with the α-R knockouts are nevertheless more severe and include defects that are not seen in the PDGF-$A^{-/-}$ mice, like for example spina bifida, cleft face and skeletal abnormalities. Even PDGF-A and PDGF-B double knockout-mice are not able to imitate the consequences of PDGFR-α gene disruption. This emphasizes the role of other ligands activate the α-R. PDGF-C is such a supposed candidate, but future experiments have to verify this suggestion (Betsholtz et al., 2001).

Postnatally PDGF plays a major role in wound healing. The proliferative effects of PDGF and promising effects seen in PDGF-treated animal wound healing models led to the development of the drug Becaplermin, which is a recombinant PDGF-BB gel (Steed et al., 1995). Becaplermin enhances wound healing of human diabetic neuropathic ulcers slightly but significantly. However, despite its positive modulatory contribution to the wound-healing process, it did not fulfil the great expectations laid on its clinical efficacy.

1.2.8 Role of PDGF in glomerulonephritis

PDGF has the potency to mediate many symptoms that are associated with renal inflammation. It is an effective vasoconstrictor due to its mobilization of intracellular Ca^{2+} and contributes to the diminished GFR in renal diseases (Abboud et al., 1993).

Furthermore, it leads to hypercellularity of MC and subsequently to an increased deposition of extracellular matrix. Thereby, PDGF acts either directly or via the induction of other growth factors and cytokines like TGF-β (Abboud et al., 1993). These intraglomerular alterations may cause chronification of the disease process and finally result in glomerulosclerosis.

In experimental and human glomerulonephritis a remarkable upregulation of PDGF and its receptors has been observed. Matsuda et al. (1995) studied the expression of PDGF-B and PDGFR-β in various forms of GN and noticed that the PDGF-B chain or the PDGFR-β were

hardly detected in normal glomeruli or minimal change nephropathy but were highly up regulated in mesangioproliferative forms of GN as is in Ig-A nephropathy, Henoch-Schönlein purpura nephritis and lupus nephritis. The amount of PDGF or its receptors correlated with the severity of the disease (Matsuda et al., 1995). Gesualdo et al. (1991, 1994) who made similar observations could furthermore, detect a slight upregulation of the PDGFR-α, indicating that both PDGFRs play a critical role in proliferative glomerulonephritis.
The expression pattern of PDGFR in these studies correlated somehow with that of iNOS in the studies of Furusu et al. (1998). Both proteins are highly upregulated in human mesangioproliferative forms of GN like IgA nephropathy and lupus nephritis and are more or less absent in minimal change nephropathy. In both studies there was an association between the severity of the disease and the amount of iNOS or PDGFR expression, respectively, which encouraged us to analyse the interaction between NO and PDGF/R expression and function .

1.3 Nitric Oxide - the discovery of an universal intercellular messenger

A beneficial effect of diluted nitro-glycerine on angina pectoris was already claimed by William Murrell from London's Westminster Hospital in 1879. But it took another 100 years until NO liberated by nitro-glycerine was identified as the mediator of smooth muscle relaxation (Palmer et al., 1987; Ignarro et al, 1987).
In 1977, Murad could demonstrate that exogenous applied NO relaxes smooth muscle cells via activation of guanylate cyclase (Arnold et al., 1977). Ignarro confirmed the NO effect two years later and furthermore showed that NO mediates a relaxation of isolated blood vessels (Gruetter et al., 1979).
In 1980, Furchgott, who was actually investigating the intracellular pathway of acetylcholine signalling, which led to dilatation of blood vessels in animal models, found out that acetylcholine does not work directly on vascular smooth muscle cells for relaxation, but via a substance produced by endothelial cells in response to acetylcholine stimulation (Furchgott and Zawadzki, 1980). He called this relaxing substance, which he was not yet able to identify, endothelial-derived relaxing factor (EDRF) and was now engaged to decode this unknown and very unstable molecule. It took several years until Salvador Moncada and Louis Ignarro sovereignly could verify the suspicion that EDRF and NO are one and the same molecule (Ignarro et al. 1987, Palmer et al, 1987).
It was rather surprising that NO, a well known atmospheric air pollutant (Schwartz et al., 1983) with its various toxic metabolites, would play a key role in the regulation of vascular tone. NO with its various biological activities was chosen as the "Molecule of the year" by the

magazine *Science* in 1992. And in 1998 three pioneers of NO research Furchgott, Ignarro and Murad were honoured with the Nobel Prize of Physiology and Medicine for their discovery of NO.

1.3.1 Physiological role of Nitric Oxide

NOS exists in at least three distinct isoforms: Two of them namely the endothelial and the neuronal forms (eNOS, nNOS, respectively) are constitutively expressed and the third form represents a cytokine-and endotoxin-inducible variant (iNOS). Endothelial NOS-derived NO plays an important role in the cardiovascular system (Marsh and Marsh, 2000). NO leads to relaxation of vascular smooth muscle cells via activation of the soluble guanylyl cyclase. Pathological conditions like arteriosclerosis disturb this highly balanced system. A subsequent reduced NO release is accompanied by enhanced vasoconstriction and may lead to cardiovascular diseases such as hypertension or angina pectoris (Shimokawa, 1999). Several NO releasing compounds have been developed to mimic the vasorelaxing effect of NO and to substitute for the lack of sufficient endogenous NO formation (Napoli and Ignarro, 2003).

NO is also involved in many other physiological procedures. Neuronal NOS-derived NO functions as a neurotransmitter in the central and peripheral nervous system (Crossin, 1991), whereas inducible NOS-derived NO participates in host defence (Nathan et al, 1991). However, in particular the latter type of NOS has been associated with diverse inflammatory diseases such as rheumatoid arthritis (Stichtenoth and Fröhlich, 1998), multiple sclerosis (Parkinson et al., 1997) and glomerulonephritis (Beck et al., 1999). Therefore NO research still is in motion and the list of its features grows continuously.

1.3.2 Biochemistry of Nitric Oxide

NO is a very unstable gas. The substrate for NO-generation is the amino acid L-arginine. Nitric oxide synthase (NOS) is the enzyme which catalyses the oxygen- and NADPH-dependent oxidation of a guanidino nitrogen on L-arginine to citrulline and NO.

Cofactors for the reaction are FMN, FAD, tetrahydrobiopterin, heme, Ca^{2+}/calmodulin and possibly Zn^{2+}-ions (Moncada et al. 1991; Stuehr, 1999).

NO reacts in biological systems with molecular oxygen (O_2) or other nitrogen species, superoxide (O_2^-), transition metals (M) and thiol group-containing proteins - forming products such as higher nitrogen oxides (NO_x; N_2O_x), peroxynitrite ($OONO^-$), metal nitrosyl adducts l(M-NO) and S-nitroso compounds. The metabolites of NO themselves undergo diverse

chemical reactions which may contribute to normal biological activity but also to cytotoxity (Stamler et al., 1992,1994).

One reason for the versatility of NO is the great capacity of nitrogen to form compounds in all oxidation states ranging from –3 to +5. The NO molecule contains nitrogen in the +2 oxidation state and consequently an unpaired electron in its valence shell. This unpaired electron facilitates both - reduction (forming NO^-, nitroxyl anion) and oxidation (forming NO^+, nitrosonium ion) of NO. NO^- and NO^+ have their own chemical properties like higher affinity to thiol groups which again enhances the multifarious reaction of NO (Wink et al., 1994).

1.3.3 Regulation of Nitric Oxide Synthases

The main difference between the constitutive isoforms and iNOS concerns the regulation of activity: Both constitutively expressed isoforms need Ca^{2+} calmodulin for activation. Their activation state depends on changes of intracellular free Ca^{2+} concentration. By contrast the inducible NOS already contains Ca^{2+} calmodulin bound to the enzyme and does not require an elevation of intracellular Ca^{2+}-level for activation (Cho et al., 1992).In general, the activity of iNOS depends on the amount of newly synthesized protein. The expression is induced by different inflammatory stimuli such as TNF-α, LPS, IFN-γ, IL-1βcAMP on the transcriptional level, but the response is highly cell- and species-specific (Beck et al., 1999).

Expression of the protein is increased after interaction of certain transcription factors with the iNOS promoter. In rat MC two very potent inductors for iNOS expression are cAMP and IL-1β (Kunz et al., 1994, Mühl et al., 1994).

Cyclic AMP induces the transcription factor CAAT/enhancer-binding protein (C/EBP), which binds and activates the iNOS promoter (Eberhardt et al., 1998). IL-1β acts via induction of nuclear factor κB (NF-κB) that activates the iNOS promoter as well (Eberhardt et al., 1994, 1998). Nevertheless, the vasopressor endothelin-1 is able to inhibit cytokine-induced iNOS expression without influencing NF-κB (Beck and Sterzel, 1996) indicating that further mechanisms contribute to cytokine-induced iNOS expression. Once expressed iNOS is active for hours to days and produces NO in much higher quantities than the constitutive isoforms eNOS and nNOS.

The effects of NO are diverse and depend on the amount of NO formed as well as the susceptibility of the target cell to NO. At low concentrations NO stimulates guanylyl cyclase activity and leads to the formation of cyclic guanine monophosphate (cGMP), an important

messenger molecule which regulates for example vascular homeostasis (Arnold et al., 1977; Katsuki et al., 1977).

Higher concentrations of NO produced by iNOS interact with thiol groups and transition metal-containing proteins and can alter protein function or gene expression. At high concentrations NO leads to cell damage, apoptosis and even necrosis – giving macrophages an effective weapon against infected cells and harmful micro-organisms. But also healthy cells can be destroyed. In particular, during inflammatory processes, that are often associated with pathologically high NO concentrations, the NO effect is mainly deleterious. The inhibition of iNOS in appropriate inflammatory animal models has been shown to exert beneficial effects. Therefore, it is of great interest to identify substances that inhibit cytokine-induced iNOS expression at the transcriptional level without affecting the constitutive isoforms eNOS or nNOS (Pfeilschifter et al., 1996, Beck et al., 1999) to avoid an imbalance of the physiological and beneficial effects of NO.

1.3.4 Signalling pathways and effects of Nitric Oxide

NO mediates its action by diverse mechanisms. The main signalling pathways leading to cell responses are initiated directly by interactions with certain enzymes, transcription factors and DNA or indirectly via activation of guanylyl cyclase and formation of cGMP (Beck et al., 1999).

1.3.4.1 Nitric Oxide and soluble guanylyl cyclase (sGC)

Exogenously applied or endogenously produced NO interacts with the heme moiety of soluble guanylyl cyclase (Ignarro, 1990) to form a ferrous-nitrosyl-heme-complex. This complex leads to a 200-fold induction of the enzyme activity, which catalyses the biosynthesis of cyclic 3´,5´-guanosine monophosphate (cGMP) from guanosine triphosphate.

The increase of the cGMP level modifies the activity of three main class of target proteins (Schmidt et al., 1993): cGMP-dependent protein kinases (PKG, responsible for the dilatative effect of NO in VSMC), cGMP-regulated phosphodiesterases (PDE, enzymes that modulate intracellular cyclic nucleotide concentrations) and cGMP-regulated ion channels, which play a major role in the sensory system (Dhallan et al., 1992; Breer and Shephard, 1993).

1.3.4.2 Nitric Oxide and gene expression

NO alters the expression of diverse genes by modulation of mainly redox-sensitive transcription factors like NF-κB or activator protein-1 (AP-1) (Sen and Packer, 1996;

Pfeilschifter et al., 2001). Furthermore, it influences mRNA stability (Beck et al. 1999, Akool et al., 2003) and evokes posttranslational modifications. Vodovotz et al., 1999; Bogdan, 2001; Franzen et al., 2002).

NF-κB is a homo- or heterodimeric transcription factor (consisting of p50/p50; p65/p65; p50/p65-dimers). In its inactive status it is bound to members of the IκB family of inhibitory proteins in the cytosol. It participates in immunological events and therefore is predominantly present in macrophages and lymphocytes. Activation of these cells by cytokines or oxygen radicals such as O_2^- or H_2O_2 results in activation of an IκB-kinase (IKK) which leads to phosphorylation, ubiquitination and degradation of IκB. Subsequently NF-κB is liberated, and then can enter the nucleus to initiate transcription of numerous target genes (Stancovski and Baltimore, 1997*)* like iNOS (Eberhardt et al., 1994; 1998).

NO, with both anti-oxidant and pro-oxidant properties can either stimulate (Lander et al., 1993; von Knethen et al., 1999) or inhibit (Peng et al., 1995) NF-κB activity depending on the cell-type and concentration of potential reaction partners such as superoxide and hydrogen peroxide or other reactive oxygen species (Beck et al., 1999). NO-mediated induction of NF-κB is comparable to the mechanisms of ROS: IKK is activated either directly or via the Ras/Raf cascade, resulting in depletion of NF-κB-inhibitor IκB (Lander et al., 1995;1996).

Whereas inhibition of NF-κB activity is attained via scavenging of O_2^- and thus reduction of H_2O_2 formation with the consequence of reduced IKK activation and reduced NF-κB liberation as shown for macrophage colony stimulating factor (Peng et al., 1995) and for monocyte chemoattractant protein 1 (Beck et al., 1999).

Furthermore, in vitro studies revealed that NO is able to inhibit NF-κB activity through direct S-nitrosation of a crucial cysteine residue on its DNA-binding domain, thus hindering NF-κB to contact its cognate DNA-binding site (Matthews et al., 1996).

A direct inhibitory effect by nitrosation of critical cysteines in the DNA-binding domain has also been shown in vitro for the transcription factor AP-1 (Nikitovic et al., 1998). AP-1 is a heterodimer that belongs to the basic domain leucine zipper family consisting of the two proto-oncogene products c-Fos and c-Jun. c-Fos and Jun B gene expression can be induced by NO through upstream regulatory events like activation of cGMP-dependent protein kinases (Haby et al., 1994; Pilz et al., 1995). AP-1 binds to phorbol ester responsive elements (TRE), which can be found in the promoter regions of many genes (Beck et al., 1999)

NO furthermore inhibits gene transcription via activation of a DNA methyltransferase (through S-nitrosation of critical cysteine residues), which leads to cytosine 5'methylation of

CpG islands and impedes further binding of transcription factors on the gene promoter (Hmadacha, et al., 1999).

NO can also exert posttranscriptional effects on gene expression. Recently, Eberhardt et al. (2002) observed a NO-dependent downregulation of the matrix metalloproteinase-9 gene. Analysis of mRNA stability revealed an effect of NO on the MMP-9 mRNA turnover in MC. Moreover this decrease in mRNA stability is due to an attenuated expression of the ELAV protein HuR (Akool et al., 2003).

NO exerts to posttranslational modifications of proteins like transforming growth factor-α. NO attenuates the expression of TGF-β. mRNA (Craven et al., 1997) but initiates positive posttranslational alterations of the molecule (Bogdan, 2001; Vodovotz et al., 1999). The amount of active TGF-β is therefore enhanced and leads for example to inhibition of iNOS expression. Furthermore**,** Franzen et al. (2002) reported an enhanced degradation of the neutral ceramidase by NO.

The fact that NO affects expression of protective and deleterious gene products complicates the pharmacological establishment of NOS inhibitors in the treatment of inflammatory diseases. For example, NO has beneficial properties as it inhibits extracellular matrix proteins like collagen I (Chatziantoniou et al., 1998) and mitogenic growth factors like vascular endothelial growth factor (VEGF) in VSMC (Tsurumi et al., 1997; Liu et al., 1998). But it also contributes to aggravation of inflammatory diseases since NO induces several potent proinflammatory cytokines like TNF-α (Zhang et al., 2000) and IL-8 (Villarette and Remick, 1995). Possibly the most challenging feature in NO-mediated gene regulation is the induction of its own biosynthesis. The subsequent excessive high NO formation may trigger apoptotic or even necrotic cell death with partially irreversible damage of the involved tissue (Mühl and Pfeilschifter, 1995).

1.3.5 Nitric oxide and apoptosis

Apoptosis – the programmed cell death - follows an universal pattern characterized by chromatin condensation, DNA cleavage, cell shrinkage, membrane blebbing and redistribution of phosphatidylserine from the inner to the outer leaflet of the cell membrane (Kerr et al., 1972). The major part of these morphological changes is caused by proteases of the caspase family (Alnemri et al., 1996).

These cysteine proteases stay at critical decision points of the apoptotic pathway and cleave distinct cellular proteins such as the poly(ADP-ribose)polymerase (PARP), nuclear lamin, endocnucleases, PKC-δ, MAPK kinase, MEK kinase 1 (MEKK1) and others at aspartic acid

residues, which can result either in the loss of function or in an activation of the target protein. There are two main pathways for apoptosis, namely the extrinsic and the intrinsic pathway. Both pathways direct to induction of the downstream effector caspase, caspase-3 (Hengartner, 2000; Reed, 2000).

In the extrinsic pathway activation of caspase-8 results after ligation of the appropriate ligand on the extracellular domain of the TNFR or FasR (Salvesen and Dixit, 1997). In the intrinsic or mitochondrial pathway, proapoptotic substances that are stored between the outer and the inner mitochondrial membrane like cytochrome c (activator of caspase-9) are released after changes in the homeostasis of Bcl-2 family members (Reed, 1997_a).

Bcl-2 family proteins could principally divided into two groups: the anti-apoptotic members (e.g. Bcl-2, Bcl-XL, Mcl-1) that stabilize the mitochondrial membrane potential and the pro-apoptotic members (e.g. Bax, Bad, Bim), that facilitate the discharge of the pro-apoptotic substances stored in the mitochondria (Reed, 1997_b, 1998) .

NO shifts the balance between the two groups through induction of the tumour suppressor p53 towards apoptosis. Normally the amount of p53 is low, but hypoxia or high amounts of NO lead to stabilization of the protein and therefore initiation of apoptosis (Meßmer et al., 1994; Meßmer and Brüne, 1996).The activity of Bcl-2, which is a potent antiapoptotic protein (Meßmer et al., $1996_{a,b)}$ can subsequently be inhibited by the nuclear phosphoprotein p53 by upregulation of its inhibitor Bax, (Bcl-2 associated protein x) which binds to and antagonizes Bcl-2. High amounts of NO or hypoxia further lead to nitrosative or oxidative DNA damage – another mechanism for p53 accumulation.

There are also p53 independent mechanisms described that lead to NO-induced apoptosis. NO affects the mitochondrial membrane directly - leading to cytochrome c release and inhibition of mitochondrial respiration (Meßmer and Brüne, 1996; Kim et al., 2001).

Furthermore, NO activates cJun N-terminal kinases1and 2 (JNK1/2). JNK- or stress activated protein kinases are known inducers of apoptosis(Xia et al., 1995; Pfeilschifter and Huwiler, 1996), which evoke accumulation of caspase 3.

During or prior the initiation of apoptosis also antiapoptotic proteins can be activated. Induction of antiapoptotic proteins explains at least partly the contradictory observations in NO-mediated cell death:

MC and RAW 264.7 macrophages respond to different NO donors time- and concentration-dependently with apoptosis (Brüne et al., 1998, Beck et al, 1999). While macrophages also die in response to endogenous NO production, MC stay resistant to cytokine mediated NO generation. (Duffield, 2000). This behaviour correlates with the fact, that the inhibitor of

apoptosis protein family (IAP) are downregulated in macrophages, while constant IAP protein levels can be measured in MC after cytokine treatment (Manderscheid et al., 2001). Another reason for this cell-type specific behaviour is the simultaneous generation of O_2^-. Cytokine treatment stimulates not only iNOS, but also NAD(P)H oxidases – the main producers of ROS in MC. O_2^-, which is on its own a potent proapoptotic substance in MC, reacts with NO and detoxifies it by forming peroxynitrite ($ONOO^-$) (Sandau et al., 1997). In most cell-lines the potent oxidant peroxynitrite mediates highly cytotoxic effects. Its protective role against NO plus ROS induced apoptosis in MC is due to the presence of reduced glutathione (GSH), an antioxidant that reacts rather with peroxynitrite and allows MC to cope with their toxic compound. Depletion of reduced GSH in MC induces apoptosis in the presence of both metabolites (NO and O^{2-}) (Sandau et al., 1999).

Also shifting the balance between NO and superoxides towards NO by additional supplementation of NO initiates MC death (Sandau et al., 1997).Another important method how cells protect themselves against NO mediated apoptosis is the generation of cytokines or growth factors (Mooney et al., 1997). Incubation with 10 % FCS was shown to protect MC completely against NO-mediated DNA fragmentation through phosphorylation of p42/44 MAPK (Sandau et al., 1999). Therefore the cellular response to even high amounts of NO does not necessarily lead to apoptosis. The balance between pro- and antiapoptotic stimuli determines at last whether cells can protect themselves against ROS, NO or hypoxia mediated apoptosis or not (Pfeilschifter et al., 2001).

1.3.6 Nitric Oxide and glomerulonephritis

GN can be classified by morphological, clinical and pathogenetic aspects. Disease induction is mediated e.g. by antibodies or T-cells, that affect directly (autoimmune diseases) or indirectly (immuncomplex nephritis) glomerular structures and initiate an inflammatory response with complement activation and/or infiltration of immune cells like macrophages and neutrophils. Macrophages play a critical role in human and experimental glomerulonephritis. The amount of invading macrophages in human lupus nephritis correlates with glomerular dysfunction (Alexopoulous, 1990) and makes the progression of e.g. human IgA nephropathy predictable (Ootaka et al., 1995). However an universal mechanism how macrophages contribute to renal injury still has to be elucidated. In 1988, Hibbs et al. declared NO as an important effector molecule of activated macrophages, which produce iNOS derived NO in high quantities. The gas NO and activated macrophages were able to impair L10 hepatoma cells in an indistinguishable manner. The presence of high amounts of nitrite in

experimental GN (Cattell et al., 1990), made NO, the precursor molecule of nitrite a potential candidate for macrophage-mediated cytotoxicity also in glomerulonephritis.

But the role of NO in GN still is controversially discussed. There are studies that claim a beneficial effect of NO. Ferrario et al. (1994) inhibited NOS activity with the unspecific NOS inhibitor L-NMMA in acute nephrotoxic serum nephritis. These authors reported that inhibition of NOS aggravates the course of the disease. One year later, in 1995, Narita et al. reduced the L-NMMA-concentration and chose the anti-Thy1 nephritis as an experimental model. They used lower concentrations of L-NMMA to avoid severe inhibition of the protective eNOS in renal vessels. They observed that inhibition of iNOS reduced mesangiolysis up to 90 per cent – which underlines the harmful, destructive role of iNOS-derived NO in GN. eNOS-derived NO on the other hand exert beneficial effects as it decreases blood pressure, and inhibits platelet adhesion and aggregation (Radomski et al., 1993). The lack of eNOS in accelerated anti-glomerular basement membrane nephritis worsens the course of the disease with increased mortality and morbidity of eNOS knock-out mice (Heeringa et al., 2000). In several types of experimental and human glomerulonephritis (e.g. IgA nephropathy, lupus nephritis) there was a significant downregulation of eNOS expression. The extent of eNOS expression inversely correlated with the degree of glomerular injury and with iNOS expression (Furusu et al., 1998).

One reason for the downregulation of eNOS might be the inhibitory effect of large amounts of NO on eNOS (Rengasamy and Johns in 1993). Griscavage et al. (1995) showed that the constitutive expressed nNOS and eNOS isoforms were more susceptible towards NO than iNOS. From a certain concentration range NO even induces iNOS expression and therefore its own synthesis (Mühl and Pfeilschifter, 1995). However, higher concentrations of NO may also impair iNOS activity. This negative-feedback-mechanism is supposed to be regulated through NF-κB inactivation (Connelly et al., 2001) or by direct inhibition of iNOS enzyme activity (Mühl and Pfeilschifter 1995). But also other negative regulatory mechanisms in the course of diseases like the upregulation of PDGF might be responsible for the downregulation of iNOS expression.

Selective inhibition of iNOS by L-NIL (L-N_6-(l-iminoethyl)lysine) in a septic shock model leads to a stabilisation of the GFR. Whereas nonselective NOS inhibition attenuated GFR in LPS treated mice (Cheng et al., 2003). These data and eNOS-gene-knockout studies (Albrecht et al., 2003) underline the importance of eNOS-derived NO for glomerular function in physiological and pathophysiological circumstances.

The role of iNOS in inflammatory renal diseases is not that consistently characterized. Cattell et al. (1998) reported that iNOS knockout mice do not show differences in proteinuria or histology compared to wild type mice in accelerated nephrotoxic nephritis, indicating that iNOS-derived NO does not contribute to the course of disease. However, there are different types of glomerulonephritis. Furusu et al., (1998) investigated the expression and source of eNOS and iNOS in 4 distinctive types of human glomerulonephritis. The renal biopsies were taken from different patients with IgA nephropathy (IgAN), Lupus nephritis (LN), membranous nephropathy (MN) and minimal change nephritic syndrome (MCNS).

In MN, MCNS and control biopsies iNOS expression was hardly detected. Whereas significant changes of iNOS expression were perceived in IgAN and LN. In these proliferative glomerulonephritis types iNOS was highly upregulated and the extent of iNOS expression correlated positively with the severity of the disease. Furthermore, Furusu et al. (1998) reported that the major part of iNOS positive cells were glomerular resident cells and not macrophages.

Taken together, the role of NO in glomerulonephritis is not yet completely understood. NO with its diverse capacities may exert beneficial as well as harmful effects.

1.4 Aim of this study: Effects of Nitric Oxide on PDGF/PDGFR activity

Little is known about NO modulated effects on PDGF/PDGFR activity. Callsen et al. (1999) described that NO enhances PDGFR phosphorylation indirectly via inhibition of a tyrosine phosphatases. This NO modulated inhibition is due to interactions with cysteine residues in the active site motif of phosphatases and is also reproduced by application of superoxides.

Another indirect mechanism how NO affects PDGF activity is by a down-regulation of SPARC, which is a matrix constituent and potent scavenger of PDGF. Thus inhibition of SPARC expression leads to an enhanced availability of PDGF for its receptors and therefore increased activity (Walpen et al., 2000).

However, no direct NO-induced alterations in PDGFR mRNA or protein levels were demonstrated in either of these reports. Therefore, we analysed the effects of NO on the expression of the PDGF receptors subtypes. This study was performed to enhance our knowledge in the complex crosstalk of NO generating mechanisms and the PDGF signalling machinery in MC.

II - M A T E R I AL S & M E T H O D S

2.1 Materials

2.1.1 Apparatus

Agarose Gel Electrophoresis chamber	Sub Cell GT Agarose Gel Electrophoresis System, Bio Rad
Agarose Gel Dokumentation System	Gel Doc 1000, Amersham
Autoclave	Varioclave
Autoradiography Cassettes	Amersham Pharmacia Biotech
AutoLumat LB953	Berthold, Pforzheim
Blotting machine (for WB)	Trans-Blot° sDCell, No. 221BR
Centrifuge	Heraeus Megafuge 1.0, rotor 7570F Biofuge fresco, Heraeus instruments
Distillator	Milli-Q Plus PF UltraPure Water System (Millipore Waters,Bedford, USA)
Fuji film processor	Hyperprocessor, Amersham
Hybridisation-oven	Hereaus Instruments
Incubator	Heraeus BBD 6220 incubator
Microplate Reader Benchmark	BioRad
Microscope	Aciovert 25 Zeiss
PCR-cycler	Gene Amp PCR System 9700 PE Applied Biosystems
Photometer for DNA/RNA	Gene Quant II, Amersham Pharmacia Biotech
Phosphorimager	Fuji
Reverse Transcriptase Cycler	Reverse Transcriptase Mastercycler 5330
Semi-dry blotting apparatus	Bio-Rad, München, Germany
Scales	Sartorius research
Sequencer	ABI-PRISM™ Perkin Elmer 310 Genetic Analyzer
Sonifier	Sonifier „W-450“ (Branson)
Sterile bench	Heraeus, Kendro
Thermomixer	Eppendorf Thermomixer comfort Heidolph Duoax 1030
Vortexer	Vortex 2 genii scientific industries

2.1.2 Chemicals

Acetone	Merck, Darmstadt
Acrylamide / bisacrylamide-solutions	Roth, Karlsruhe
Agar	Gibco Life Technologies, Karlsruhe
Agarose	Biozym, Oldendorf
Ammoniumpersulfate	Sigma Aldrich Fine Chemicals, Deisenhofen

Ampicillin	Sigma Aldrich Fine Chemicals, Deisenhofen
Boric acid	Merck, Darmstadt
Bovine serum albumin	Sigma Aldrich Fine Chemicals, Deisenhofen
Bradford Reagent	BioRad
8-Br-cAMP	Sigma, Deisenhofen
Bromphenolblue	Serva
[α-^{32}P]CTP	Amersham Pharmacia, Freiburg
Chloroform	Merck
DETA NONOate (1-[2-(2-Aminoethyl)-N-(2-ammonioethyl)amino] diazen-1-ium-1,2-diolate)	Alexis, Läufelingen, Switzerland
Diethylpyrocarbonate (DEPC)	Sigma Aldrich Fine Chemicals, Deisenhofen
Diphenylamine	Sigma Aldrich Fine Chemicals, Deisenhofen
Dithiothreitol (DTT)	Sigma Aldrich Fine Chemicals, Deisenhofen
DMSO	Merck, Darmstadt
Dual-Luciferase® Reporter Assay System	Promega, Mannheim
ECL Detection Kit/Films	Amersham Pharmacia, Freiburg
EDTA	Sigma Aldrich Fine Chemicals, Deisenhofen
Effectene Transfection Reagent (4x 1ml)	Qiagen, Hilden
Ethanol	J.T.Baker
Ethidium bromide	Sigma Aldrich Fine Chemicals, Deisenhofen
EDTA	Sigma Aldrich Fine Chemicals, Deisenhofen
Fetal calf serum	Gibco Life Technologies, Karlsruhe
Formamid	J.T.Baker
Glycerol	Roth, Karlsruhe
Glycine	Merck, Darmstadt
HEPES	Roth, Karlsruhe
Immobilon™ –P Polyvinylidene Difluoride Transfer Membrane	Millipore, Eschborn
Insulin-Transferrin-Sodium Selenite Supplement	Roche Biochemicals, Mannheim
Interleukin-1β	Novartis Pharma, Basel
Isopropylalcohol	J.T.Baker
Isopropylthiogalactopyranoside (IPTG)	Roth, Karlsruhe
Loading Dye Solution 6x	MBI Fermentas
Methanol	J.T. Baker
β-Mercaptoethanol	Sigma Aldrich Fine Chemicals, Deisenhofen
Molecular weight markers (DNA)	MBI Fermentas, St. Leon-Rot
Molecular weight markers (protein)	Amersham Pharmacia, Braunschweig
N^G-monomethyl-L-arginine	Calbiochem, Schwalbach
Nucleotide triphosphates	PE Biosystems, Weiterstadt
NucleoSpin® Extract 2 in 1	Macherey & Nagel, Düren
Oligonucleotides	Roth, Karlsruhe

ODQ	Alexis, Grünberg
PDGF-BB	Hofmann La Roche, Basel
PDGF-AA	Upstate Biotechnologies (Lake Placid, USA)
Perchloric acid	Merck, Darmstadt
Ponceau S	Serva, Heidelberg
Potassium acetate	Merck, Darmstadt
Potassium chloride	Roth, Karlsruhe
Protein A-sepharose 4B CL	Amersham Pharmacia, Freiburg
Rediprime^{TM} Random Labelling System	Amersham Pharmacia, Freiburg
Skim milk (non fat)	Fluka, Deisenhofen
Salmon Spermine	Calbiochem, Schwalbach
SDS	Merck, Darmstadt
Sodium acetate	Merck, Darmstadt
Sodium chloride	Merck, Darmstadt
Sodium citrate	Merck, Darmstadt
Tetramethylethylendiamine (TEMED)	Sigma Aldrich Fine Chemicals, Deisenhofen
Triton-X-100	Sigma Aldrich Fine Chemicals, Deisenhofen
Tween 20	Sigma Aldrich Fine Chemicals, Deisenhofen
Xgal	Roth, Karlsruhe
Yeast extract	Gibco Life Technologies, Karlsruhe
YC-1	Alexis, Grünberg

All other solvents and salts were of the highest qualities available and supplied by Merck (Darmstadt), Roth (Karlsruhe) or Sigma Biochemicals (Deisenhofen).

2.1.3 Buffer-Solutions

2.1.3.1 Buffer for electrophoresis

TBE-buffer 10x	900 mM Tris borate, 20 mM EDTA
TAE-buffer 10x	400 mM Tris acetate, 10 mM EDTA

2.1.3.2 Buffer for cell culture

PBS-buffer 10x	1.5 M NaCl, 30 mM KCl, 15 mM KH_2PO_4, 60 mM Na_2HPO_4

2.1.3.3 Solutions for plasmid isolation from E.Coli-bacterials (Quiagen-method)

Resuspensionbuffer (B1, storage at 4 °C)	50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100µg/ml Rnase A
Lysisbuffer (B2)	200 mM NaOH, 200 mM SDS
Neutralisationsbuffer (B3):	3 M Potassiumacetat pH 5.5

2.1.3.4 Buffer for northern blot analysis

SSC-buffer 20x	3 M NaCl, 300 mM Na_2Citrat
10 x MOPS:	0.4 M MOPS, 0.1 M Na-Acetate, 10 mM EDTA
Denaturation-buffer:	66 µl 10 x MOPS, 316 µl Formamide, 117 µl Formaldehyde (37%)
20 x SSC	175.3 g NaCl, 88.2 g Na-citrate Dihydrate, adjust to pH 7.0 with HCL (10N) ad 1 l ddH_2O and autoclave

0.02% methylenbluebuffer in 0,5 M NaAcetatsolution	68.04 g Na-Acetate-3H_2O, solve in 500 ml ddH2O, adjust to pH 5.2 with CH_3COOH ad 1 l ddH_2O and autoclave, 200 mg methylenblue
Hybridisation-buffer	50 ml Formamid, 12.5 ml 20 x SSC, 5 ml 100 x Denhartz, 10 ml 10% SDS, 22.5 ml ddH2O, Herrings- sperm-DNA 100 µl to 10 ml solution, 10 g Dextransulfat
Washing-buffer I	2 x SSC, 0.1 % SDS
Washing-buffer II	0.2 x SSC, 1 % SDS

2.1.3.5 Buffer for western blot analysis

SDS-Electrophoresisbuffer 1x	25 mM Tris, 192 mM Glycine, 0.1 % SDS (pH 8.3)
Methanol-Transferbuffer 1x	25 mM Tris, 192 mM Glycine, 20 % Methanol (pH 8,3)
Upper-gel-buffer	125 mM Tris-HCl (pH 6.6), 0.1 % SDS
Lower-gel-buffer	375 mM Tris-HCl (pH 8.8), 0.1 % SDS

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

2.1.4 Primer

1. PDGFR-α	688 bp
PDGFR-α forward	ATG TTT CTA GAC TCG CAG CTC A
PDGFR-α reverse	ATA AAC AAA GGC AGT GAT ACA G
2. PDGFR-β	495 bp
PDGFR-β forward	TCC AGC TGT GCC TCA GGC TCT G
PDGFR-β reverse	GAC CAG TTC TAC AAT GCC ATC A
3. GAPDH	497 bp
GAPDH forward	CCT TCA TTG ACC TCA ACT AC
GAPDH reverse	GGA AGG CCA TGC CAG TGA GC
4. PDGFR-α-Promoter	1723 bp
PDGFR-α-Promoter forward	GGA GCT CTA AAT CTT GAC TTG CTT TTA ACA ACA GC
PDGFR-α-Promoter reverse	GGG ATC CTT GTT TCA CTC CCT CAA GCT CCA ACA GT

All primers have been purchased by Invitrogen life technologies

2.1.5 Antibodies

anti-PDGFR-α (951): sc-431, rabbit polyclonal	Santa Cruz Biotechnologies, Heidelberg
anti-PDGFR-β (958): sc-432, rabbit polyclonal	Santa Cruz Biotechnologies, Heidelberg
anti-pPDGFR-α (pTyr-720) sc-12911-R rabbit polyclonal)	Santa Cruz Biotechnologies, Heidelberg
anti-PKB-α (Akt-1), sheep polyclonal	Upstate Biotechnologies (Lake Placid, USA)
anti-pAkt (pSer-473): 4E2, rabbit Monoclonal	New England Biolabs, Frankfurt am Main

anti-rabbit IgG (horseradish peroxidase coupled)	Amersham Pharmacia, Freiburg

2.1.6 Enzymes

Pfu-DNA polymerase	Stratagene, Heidelberg
Restriction enzymes	MBI-Fermentas, St. Leon-Rot
Reverse transcriptase	MBI-Fermentas, St. Leon-Rot
Taq-DNA polymerase	MBI-Fermentas, St. Leon-Rot

2.1.7 Plasmids

pBluescript II KS (+)	Stratagene, Heidelberg
pGL3	Invitrogen, Heidelberg
pTOPO TA Cloning Kit	Invitrogen, Heidelberg

2.1.8 Eukaryotic cell lines

RMC, B1	Preparation of primary cells from rat kidney by J. Pfeilschifter, 1991 Frankfurt

2.1.9 Bacterial strains

E. coli TOP10F'	Invitrogene, Heidelberg

2.2 Methods

2.2.1 Cell-culture

2.2.1.1 Thawing of cells

Cloned mesangial cells (MC), originally isolated from rat kidneys (Pfeilschifter and Vosbeck, 1991), were used for all experiments. The cell stocks were stored in liquid nitrogen in FCS and 20% DMSO containing kryotubes (Nunc). Cells were thawed in a water-bath at 37°C and transferred to 175 cm^2 culture dishes (Greiner), containing 25 ml RPMI 1640 culture medium supplemented with 10 mM HEPES, 10 % fetal calf serum (FCS), 2 mM glutamine, 5 ng/ml insulin, 100 U/ml penicillin and 100 µg/ml streptomycin. The culture dishes were incubated at 37°C and 5 % CO_2. (Heraeus BBD 6220 incubator).The next day fresh medium was supplied and MC were incubated until confluency was achieved (3-4 days).

2.2.1.2 Cell passaging

For all experiments cells between passages eight and twenty were used. Before each passage, cells were rinsed with PBS (9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150 mM sodium chloride, pH 7.4) and then incubated with 4 ml trypsin-EDTA per 175 cm^2 culture-dish for 3 min at 37°C. Trypsinised MC were centrifuged for four minutes at 900 rpm (Heraeus Megafuge 1.0). Afterwards MC were re-suspended, counted using a Neubauer chamber and transferred in new culture dishes. For subcultivation 3.3 x 10^6 cells per 175 cm^2 culture dish were used.

2.2.1.3 Cell stimulation

The content of one confluent 175 cm^2 bottle was distributed on twenty 100 mm culture-plates. Cells were grown until confluence in 8 ml prepared RPMI 1640 culture medium.
One day before stimulation, cells were rinsed with PBS and incubated for 24 h with serum free Dulbecco's Minimal Essential Medium (DMEM) supplemented with 0.1 mg/ml of bovine serum albumin, 5 ng/ml insulin, 100 U/ml penicillin and 100 µg/ml streptomycin.
On the day of stimulation cells were rinsed with PBS and 5 ml prepared DMEM was added to each Petri dish. Afterwards, NO-donors or other reagents were supplied and cells were incubated for the indicated time-periods.

2.2.2 Isolation of total cellular RNA

Isolation of total cellular RNA was performed with Trizol Reagent™ (Sigma). MC cultured in 10-cm dishes were rinsed twice with PBS. After complete removal of residual PBS 1 ml Trizol (a mixture of guanidine thiocyanate and phenol) was added to each 10 cm-plate and cells were scraped with a rubber policeman. The lysates were transferred into new Eppendorf tubes. 200 µl chloroform were supplied and tubes were shaken for 15 sec and incubated for 5 min at room temperature. Then tubes were centrifuged for 15 min at 13000 rpm at 4 °C. The aqueous upper phase was carefully transferred into a new tube and an equal volume of isopropyl alcohol was added to precipitate RNA. The tubes were mixed thoroughly, incubated for 15 min at room temperature and centrifuged again (13000 rpm, 20 min., 4 °C). The supernatant was decanted, the RNA pellet was washed with 500 µl absolute ethanol and incubated at 4 °C for 10 min. Afterwards tubes were centrifuged again for 10 min (13000 rpm, 4 °C). Ethanol was decanted and the precipitated RNA pellet was air dried at 37 °C. The RNA was dissolved in 50 µl diethylpyrocarbonate treated water (DEPC-H_2O) and stored at -

80 °C. RNA concentration was quantified by photometry at a wavelength of 260 nm (GeneQuant, Pharmacia, Freiburg Germany).

2.2.3 Quantification of RNA/DNA

The absorption of RNA/DNA probes were determined with a photometer (Gene Quant II, Amersham Pharmacia) at a wavelength of 260 nm, corresponding to the maximal absorption rate of nucleic acids. There is a linear correlation between the absorption rate (optical density, OD) and RNA/DNA concentration. 50 µg/ml double-stranded DNA or 40 µg/ml single stranded DNA and RNA have an optical density (OD) of one (Sambrooke et al., 1989). The unknown concentrations of the probes could be calculated by measuring the respective OD rate. Proteins have their absorption maximum at wavelength of 280 nm. To control if there is any protein contamination in the RNA/DNA probe, the absorption rate at wavelength of 280 nm was measured as well and the ratio between OD_{260} and OD_{280} (OD_{260}/OD_{280}) was determined. A ratio between 1.8 and 2 stands for high purity of the RNA/DNA sample. Intactness of RNA and exactness of the measured concentration was confirmed on 1% agarose gel electrophoresis (3µg per probe).

2.2.4 Agarose gel electrophoresis

DNA or RNA at about neutral pH is negatively charged due to the phosphate groups of each nucleotide. There is a linear correlation between the amount of nucleotides and the ionisation state. This charge allows a size-dependent separation of DNA/RNA on agarose gel electrophoresis.

The gel concentration for all analyses was 1%. Agarose was dissolved in 1x TBE and ethidium bromide, an intercalating substance that binds to DNA/RNA, was added to a final concentration of 500 ng/µl. Ethidium bromide allows the visualisation of bound RNA/DNA since it fluoresces under ultraviolet light. The DNA/RNA probes were diluted with loading dye (6x loading buffer: 30 % glycerol v/v, 0.25 % bromophenolblue w/v, 0.25 % xylenecyanole w/v, 60 % 10x TAE buffer v/v) and loaded to the gel lanes. Electrophoresis was performed in 1x TBE buffer with a voltage of 5-10 V/cm gel. DNA fragment sizes were estimated using molecular weight markers (MBI Fermentas).

2.2.5 Reverse transcription of total cellular mRNA in cDNA

A DNA replica of mRNA is produced by the enzyme reverse transcriptase. Messenger RNAs have a long poly-adenosine tract at their 3'end. This position is used for a small oligonucleotide primer (oligo dT), consisting of thymidine nucleotides, to anneal and to give the enzyme a starting site to copy the mRNA template. The product is complementary DNA of total cellular mRNA.

5 µg RNA were mixed with the unspecific primer oligo dT (500 ng). Tubes were refilled to 12 µl with DEPC-H_2O centrifuged and incubated for 10 min at 70 °C in the thermocycler to enable the annealing of the oligonucleotide. Then probes were cooled on ice and in each tube a mixture of 1x First Strand Buffer, 0.1 M DTT, 2 mM dNTP-mix and 40 U Super Script Enzyme were added and tubes were incubated in the thermocycler for 60 minutes and 42 °C the optimal enzyme temperature. The cDNA probes were stored at –20 °C.

2.2.6 cDNA amplification of PDGFR -α, PDGFR -β and GAPDH

The polymerase chain reaction (PCR), established by Mullis and Faloona in 1987, allows the amplification of a known nucleotide sequence. For PCR two primers (oligonucleotides) are needed, in which one primer is complementary to each strand of the DNA double helix. The primers lie on opposite sides of the region to be amplified and determine the ends of the final DNA fragment.

For each probe 2 µl of the RT-product (3.4) was utilised. A mixture, which was composed of 39,5 µl PCR-H2O, 5µl PCR-Buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 50 mM KCl, pH 8.3) 1µl dNTP-mix (2 mM), 1µl forward primer (50 µM) , 1µl reverse primer (50 µM) and 0.5 µl Taq-polymerase (0.5 U) was added to each cDNA containing PCR-tube, to a final volume of 50µl. The tubes were mixed and centrifuged briefly before the thermocycler was started.

Our reaction began with heating of the probes for 5 min at 94 °C (1 cycle) to denaturate the DNA completely. Then the actual 3-phased-PCR-cycle began with the denaturing phase in which probes are heated for 30 sec at 94 °C, followed by the annealing phase for 45 sec at 53 °C and in the last step of the cycle, temperature is elevated for 60 sec to 72 °C – the optimal working temperature of Taq polymerase and elongation was initiated. These 3 phases were repeated 23 times for the PDGFR-β primers and 25-times for PDGFR-α primers, PDGFR-α-promoter primers and for GAPDH primers. The sequence of these primers are listed in chapter 2.1.4. The reaction was completed by heating the probes for 7 min at 72 °C (1 cycle). The amplification products were stored at 4 °C, centrifuged and analysed by agarose gel electrophoresis.

2.2.7 DNA-cloning (by TOPO TA Cloning®)

In this experiment bacteria were transfected with a plasmid that includes the DNA insert, which has to be amplified. The product serves e.g. as a hybride for northern blot analysis. Plasmids are small circular DNA molecules that replicate independently of the genome. The plasmids used for DNA cloning models often encode for a resistance factor against antibiotics. E. coli cells that carry such a recombinant plasmid develop into a colony on an agar stained with antibiotics. By inoculating a single colony of interest into a liquid culture, a large number of identical plasmid DNA molecules - each containing the same DNA insert - could be gained.

2.2.7.1 Transfection of E. coli bacteria with PCR-product-containing vectors

For transfection TOPO TA Cloning Kit (Invitrogen) was utilised. 4 µl PCR-product were mixed with 1µl buffer solution and 1 µl TOPO vector. The mixture was incubated for 5 min at room-temperature. The PCR product was inserted into the plasmid (TOPO vector) by supplemented enzymes. Meanwhile E. coli bacteria (TOP10F'strain) were thawed on ice. 2 µl of the 6 µl plasmid mixture was supplemented to 1µl E. coli bacteria and mixed briefly. The cell plasmid solution was then incubated for 5 min on ice. Afterwards cells were heated for 30 sec at 42 °C to make the cell membrane permeable for the plasmids. Then 250 µl SOC medium (2 % Tryptone, 0.5 % Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was supplied and tubes were shaken for 1 h at 37 °C. 100 µl of this cell solution were transferred on agar-plates (agar 15 g/l) that were treated with ampicillin (50 µl/ml), 50 µl XGAL (1 mg) and 50 µl IPTG (1.25 mg). Plates were incubated over night at 37 °C. A resistance factor against ampicillin is inoculated in the plasmid. Therefore only plasmid-containing E. coli bacteria were able to grow on the agar plates. Furthermore the insertion of PCR-products can be confirmed by the colour of the E. coli colonies. Successful insertion interrupts a gene that encodes for an enzyme, which in its intact state metabolises the substance X-Gal, leading to a characteristic blue coloration of the colony. Infected E. coli colonies that contain a PCR product insert in their plasmids beware their natural white colour. For further procedures one white colony was picked up and transferred in a glass tube with 2 ml N3Y-Broth Medium and ampicillin (50µg/ml).The glass tubes were shaken over night at 37 °C.

2.2.7.2 Mini-preparation of plasmid DNA from E. Coli by Quiagen

This method serves the separation of amplificated pDNA of experiment 2.2.7.1

The content of the glass tube of method 2.2.7.1 was transferred into an Eppendorf tube and centrifuged for one minute. The liquid phase was discarded and the pellet was re-suspended in 100 µl resuspension buffer P1 (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 µg/ml RNase A). 200 µl lysis buffer (200 mM NaOH, 200 mM SDS) were added to the resolved E. Coli-cell-solution and the tube was mixed briefly. Then 150 µl neutralisation buffer P3 (3 M potassium acetate pH 5.5) was supplied, that lead to precipitation of the proteins. The solution was centrifuged for 15 min at 4 °C and the liquid phase was decanted into a new Eppendorf-tube. 800 µl propanol were added to the liquid phase and the tube was centrifuged again for 15 min at 4 °C. Propanol leads to precipitation of DNA. The liquid phase could be discarded. The pellet was again treated with alcohol - 100 µl 96 % ethanol to clean the DNA- and centrifuged for 10 min at 4 °C. The supernatant was discarded and the pellet dried at 37 °C. Thereafter the pellet was resolved in 50 µl ddH_2O and DNA concentration was measured as described.

2.2.7.3 Digestion of mini-Prep-DNA with EcoR I

This method tests, whether the DNA fragment inserted in the plasmid DNA molecule actually is the gene of interest. Restriction enzymes that cut DNA at certain nucleotide sequences were used to liberate the cloned insert from the whole plasmid-DNA prepared in experiment 2.2.7.2 After digestion probes were run on agarose gel electrophoresis together with a DNA molecular weight ladder and the size of the liberated DNA fragment was compared with the former inserted PCR product. 2µl pDNA, 0.5 µl ECORI, 2 µl Buffer and 15.5 µl ddH2O are mixed and the tube is incubated for one h at 37 °C. 4 µl 6x loading dye is applied to the 20 µl volume and the probe was run on 1 % agarose gel electrophoresis.

2.2.7.4 DNA extraction of agarose gel

In method 2.2.8.2 the DNA fragment inserted in the plasmid has been cut out by enzyme digestion and the DNA fractions were separated by size via agarose gel electrophoresis. Now the DNA fragment of interest was excised out of the gel with a scalpel. To separate pure DNA from gel remnants NucleoSpin Extract protocol (NucleoSpin Extract 2 in 1 by Macherey & Nagel), was used as described by the manufacturer. The DNA concentration was measured as described.

2.2.8 Promoter analysis

For promoter analysis a PCR product of about 1723 bp (forward primer: GGA GCT CTA AAT CTT GAC TTG CTT TTA ACA ACA GC; reverse primer: GGG ATC CTT GTT TCA CTC CCT CAA GCT CCA ACA GT) was used.

2.2.8.1 Cloning of PCR products in cloning vector (pTOPO) and luciferase vector (pGL3)

The RT-PCR products were cloned into the TOPO-Cloning Vectors (pCR® II-TOPO®, Invitrogen) as described. After preparation of plasmid DNA, PCR inserts were restricted by type II endonucleases and separated from these cloning vectors by gel electrophoresis and subsequent gel elution using a column gel extracting kit (NucleoSpin Extract 2 in 1 by Macherey & Nagel), followed by ligation into the prepared luciferase vector pGL3.These constructs were used to transfect MC.

2.2.8.2 Reporter gene assay

To study gene expression, the dual-luciferase reporter assay system from Promega was used. For this purpose DNA sequences carrying putative regulatory elements were cloned in front of a reporter gene, the luciferase enzyme. By transfection with Effectene transfection reagent, newly generated constructs were transferred into the eukaryotic target cells. After transfection and stimulation, cells were harvested to measure the amount of synthesized reporter gene products. To assess the influence of a certain sequence on PDGFRα-promoter gene regulation, reporter gene expression in stimulated and unstimulated cells were compared.

2.2.8.3 Transient and stable transfection of MC

Transfection of cells was performed using the Effectene transfection reagent (Qiagen, Hilden, Germany). Effectene represents a new class of lipid-based transfection reagent that spontaneously forms micelle structures. One day before transfection $2x10^5$ cells per well were seeded in 6-well plates. On the day of transfection MC were 50-80% confluent. The cells were washed with PBS before 1.6 ml of DMEM medium 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. For stable transfection the cells were transfected with the prepared pGL3 plasmids and mixed with the DNA condensation buffer (EC) and 3.2 µl of enhancer. The DNA mixture was vortexed for 1 second 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 and down. Finally, the transfection complex was added drop-wise onto the cells. The plates were swirled gently and incubated for 18 hours at 37°C and 5% CO_2. MC were washed with PBS and new medium with stimulating reagents was added for 24 hours and thereafter MC were harvested. For stable transfection, one day after transfection the medium containing the complexes was replaced with fresh complete medium. For this purpose, two days after transfection the appropriate antibiotic was added to select for expression of the transfected antibiotic-resistance gene.

2.2.8.4 Luciferase assay

The luciferase of the Firefly (*Photinus pyralis*) catalyses the oxidation of luciferin. During this reaction photons are released at a wavelength of 562 nm. By employing a luminometer (AutoLumat LB953, Berthold) a quantitative measurement of the emitted light was performed. Cells were transfected with a luciferase construct, stimulated and harvested in 1x Reporter-Lysisbuffer (Promega) as described by the manufacturer. The solutions needed for measurement were prewarmed at room temperature. 50 µl of cell extract were transferred into 5 ml Sarstedt tubes. Subsequently, the reaction was started in the Luminometer by injection of 100 µl Luciferase-Assay-Reagent (Promega) (20 mM Tricin, 1.07 mM $(MgCO_3)Mg(OH)_2$ x 5 H_20, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 µM Coenzym A, 470 µM Luciferin, 530 µM ATP pH 7.8). The emitted light was measured for 10 s with a Photomultiplier and expressed in Relative Light Units short (RLUs) by the luminometer, representing the firefly luciferase activity. 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, now representing the renilla luciferase activity.

2.2.9 Sequence analysis by ABI-PRISM™

DNA sequencing was performed by the four-colour ABI-Prism 310 Genetic Analyser (PE Biosystems) based on the dideoxynucleotide chain termination method (Sanger et al., 1977). In the termination labelling 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 colours corresponding to the four nucleotides can be detected and distinguished within a single run. Raw data were evaluated by the Abi Prism sequencing analysis software on a Power G3 Macintosh computer.

For each PCR-tube 250 ng plasmid DNA, 10 pmol primer and 2 µl BigDyeTerminator Premix were mixed with ddH2O to a final volume of 10 µl. The sequencing reaction, was performed in a thermocycler (GeneAmp 2400, PE Biosystems) with 25 cycles of the following temperature steps: denaturation: 96°C for 10 sec, annealing: 55°C for 10 sec, elongation: 60°C for 4 min. After termination of the PCR the tube was centrifuged for 15 minutes at maximum speed, washed with 250 µl 70% ethanol and air-dried at 37 °C. The pellet was resuspended in 25µl template suppression reagent TSR and heated to 95°C for 2 minutes. The probe was then transferred into a sequencing reaction tube and sequencing was started.

2.2.10 Northern blot analysis

Total cellular RNA was isolated as described in chapter 2.2.2. 20 µg RNA diluted in 15µl H_2O and equal volume denaturing solution (500 µl formamide, 162 µl 37% formaldehyde, 100 µl 10x MOPS [3-(N-morpholino)-propanesulfonic acid]), were incubated for 15 min at 60 °C and mixed with 6 µl loading dye. Total RNA was separated according to the size in 0.8 % agarose formaldehyde gel (200 ml gel consisting of 5 % formaldehyde and 95 % 1x MOPS) at 80 V for 4 hours in 1x MOPS buffer. Afterwards gel was washed two times with 2x SSC for 15 min and transferred to a nylon membrane by capillary drag over night at room temperature.

Next day RNA on the nylon membrane was immobilised with a UV transilluminator (254 nm, 150 mjoule). The membrane was stained with 0.02 % methylene-blue-solution to control the intactness of RNA. The nylon membrane was completely decoloured with washing buffer II, placed in a tube with 5 ml hybridisation solution (2.5 ml formamide, 0.5 ml 20x SSC, 0.5 ml 10 % sodium dodecyl sulfate, 1.5 ml H_2O) and incubated in a hybridisation oven with rotation for 3 hours at 42 °C. Blots were hybridised with a 688 bp RT-PCR product for PDGFR-α (forward primer: ATG TTT CTA GAC TCG CAG CTC A; reverse primer: ATA AAC AAA GGC AGT GAT ACA G) and a 497 bp RT-PCR product for GAPDH (forward primer: CCT TCA TTG ACC TCA ACT AC; reverse primer: GGA AGG CCA TGC CAG TGA GC). The cDNA was amplified by Midi Prep Culture. For radio-labelling the “ready prime” labelling system (Amersham Pharmacia Biotech) was used and RT-PCR products were labelled with

[α-^{32}P]-dCTP according to the manufacturers instructions. Hybridisation was performed over night (14 h) at 42 °C and continuous rotation. Next day the membrane was washed 2x with washing buffer I at 42 °C, 2x with washing buffer II at 65°C for 15 min and exposed on a Phosphorimager (Fuji).Membranes were re-hybridised with GAPDH.

2.2.11 Western blot analysis

The Western or immunoblot analysis is used to separate and detect certain proteins out of a complex mixture. In the first step the mixture of cellular proteins are separated by size in SDS-gel electrophoresis and transferred to a nitrocellulose membrane. Next the membrane is incubated with a specific antibody (AB1) that recognises the requested protein. Thereafter the membrane is washed several times to remove non-bound AB1 and a second antibody (AB2) that binds the bound-AB1 is supplied. AB 2 is labelled with an enzyme e.g. horseradish peroxidase that catalysis the supplemented substrate ECL. The chemiluminescent reaction blackens a Kodak film and makes the protein bands that are connected to the AB1-AB2-complex visible.

2.2.11.1 Isolation of proteins

After the indicated time periods stimulated cells (10 cm dishes) were cooled on ice to stop further reactions. Medium was decanted and cells were rinsed with ice-cold PBS.

600µl of a lysis-buffer-proteinase-inhibitor-mix (62.5 mM Tris pH 6,8, 2% SDS, 5% Glycerol, 1mM NaF, 1mM PMSF, 1mM Na_3VO_4 ,10µg/ml Leu, 10µg/ml Pep (Pepstatin), 10µg/ml Apro, 10µg/ml Tryp) was added to the cells and the plates were shaken (Heidolph duomax 5330) for 5min on ice to incubate the whole plate. The cell-lysates were then scraped with a rubber-policeman, transferred to heat-resistant eppendorf-tubes and boiled for 5 min. After denaturation tubes were cooled-down on ice. Each tube was sonicated (Sonifier „W-450“Branson) 3 times for 15 seconds to reduce the viscosity and centrifuged at 13000 rpm for five min. The pellet was discarded and supernatant transferred to a new tube. 20 µl was aliquoted for protein concentration measurement while the rest was mixed with β-mercaptoethanol (5 % of total protein-volume) a substance that breaks sulphur-linkages in the proteins. Then protein samples were aliquoted and stored at – 80 °C until use.

2.2.11.2 Quantification of protein

The protein concentration was determined by the Bradford protein assay on 96-well plates (Bio-Rad, München, Germany). A standard-curve with 5 different concentrations of BSA

reaching from 10 to 50 µg/ml was created. 1µl of every protein sample was diluted in 99 µl ddH_2O. For determination of the zero value we used the lysis-buffer proteinase-inhibitor mix. To each well 100 µl BCA-reagent was supplied, which dyes the solution dependent on the protein-concentration. The plate was incubated for 20 min at 56 °C. Thereafter absorption was measured by the microplate reader (Biorad) at a wavelength of 595 nm. The standard curve was constructed and the concentrations of the protein samples were extrapolated by the Microplate Manager 4.0 software (Bio Rad).

2.2.11.3 SDS Polyacrylamid Gel Electrophoreses

Proteins are charged negatively or positively according to their net amount of charged amino acids and the pH of the environment. To obtain an uniform charge protein samples were incubated with SDS. SDS is a negatively charged detergent that binds and unfolds protein molecules. The extended polypeptide chains uptake SDS according to their size. The inserted SDS highly overwhelms the proteins' intrinsic charge and makes them migrate toward the positive electrode in SDS polyacrylamide gel electrophoresis. In the meshes of polyacrylamid gel, proteins with high molecular weight run slower than the small ones. Therefore SDS polyacrylamide gel electrophoresis allows a size-dependent separation of different cellular proteins. Electrophoretic separation of proteins was carried out for SDS polyacrylamide gels as originally described by Laemmli (1970).

The indicated protein amounts (20-50µg) were diluted with equal volume of 2x Laemmli-buffer (1 ml glycerol, 3 ml 10% SDS, 1.25 M Tris-HCl, pH 6.7 and 1mg bromphenol blue), denaturated for 4 min at 95 °C and cooled on ice. Thereafter the samples were loaded to the gel lanes. The gel consists of two different concentrated sheets, the upper and the lower gel. The upper gel (125 mM Tris-HCl pH 6.8, 0,1% SDS, 0,65 ml polyacrylamide, 25µl 10% APS, 5 % Temed) is only half as dense as the lower gel. It enables also high molecular weight proteins to move and reach the lower gel almost as fast as smaller-sized polypeptides. The lower gel is the actual protein-separating matrix. For the PDGFR we used 8% polyacrylamide gels (2,5 ml lower-gel-buffer, 2,7 ml polyacrylamide (30 %), 4,8 ml ddH2O, 50 µl APS, 5 µl TEMED). Electrophoresis was performed for 2 ½ h at 25 mA in SDS electrophoresis buffer (25 mM Tris, 192 mM Glycine, 0,1 % SDS).

2.2.11.4 Protein transfer to nitrocellulose membrane

For protein transfer from gel to membrane a semi-dry electro-blotting apparatus was used (Trans-Blot SD, Bio Rad). After electrophoresis the upper-gel was removed and the lower gel

was rinsed in transfer buffer (20 % methanol, 25 mM Tris, 192 mM glycine; pH 8.3). 2x 6 Whatman paper and the polyvinylidene difluoride membrane were cut to the size of the lower gel and soaked one after another in transfer buffer. The first 6 whatman papers were placed on the anode side of the blotting apparatus, followed by the methanol activated membrane. Next the gel was positioned on the membrane and the last six whatman papers covered the gel. The sandwich was flattened with a rolling pin. The apparatus was closed with the cathode to the upper side and the negatively charged proteins migrated down towards the anode and were stopped on the nitrocellulose membrane. Blotting was performed at 23 V for 30 min. After blotting the success of transfer and was controlled by Ponceau S dye staining (0.2% Ponceau-S w/v, 3% TCA w/v). Afterwards the membrane was discoloured with ddH2O.

2.2.11.5 Blocking

After blotting the membrane was exposed to protein containing blocking buffer (10 mM Tris, 300 mM NaCl, 0,5 % Tween 20, 2 % BSA) to avoid non-specific binding of the antibodies. Blocking was performed by shaking the membrane for one hour at room temperature.

2.2.11.6 Immunodetection

The membrane was incubated with the primary antibody diluted in blocking buffer (rabbit anti-PDGFR-α (Santa Cruz, Heidelberg, Germany) in a dilution of 1:200; α-Actin 1:2000, β-Tubulin 1:2000) and shaken for 16 h at 4°C. Then the blot was washed 5 times for 10 min with TBS-T (10 mM Tris, 300 mM NaCl , 0.05% Tween 20) at room temperature and the secondary antibody diluted 1:10000 in blocking buffer was supplied. The membrane was shaken for one hour at room temperature. Again the blot was washed 5 times for 10 min with TBS-T at room temperature. Thereafter, the substrate (ECL) for the enzyme (horseradish peroxidase) linked to the secondary antibody was applied on the membrane and a Kodak film was positioned hereon. The enzyme metabolises the substrate leading to a chemiluminescent reaction that blackens the film. The film was developed (Hyperprocessor, Amersham Pharmacia) and scanned (GS 700 Imaging Densitometer, Bio Rad). Scanned film was analysed by the Molecular Analyst software from Bio Rad.

2.2.12 In vivo analysis

All in vivo analysis was performed by Dr. Liliana Schäfer from the University of Münster, Germany.

2.2.12.1 Experimental design of anti-Thy.1.1 Glomerulonephritis

The anti-Thy-1 glomerulonephritis (anti-Thy 1-GN) was induced in adult male Wistar rats weighing 180-200 g (Charles-River, Sulzfeld, Germany) by a single intravenous injection of mouse anti-rat Thy-1.1 IgG, clone OX-7 (BioTrend, Cologne, Germany) dissolved in 18 mM sodium phosphate, pH 7.4/0.15 M NaCl (PBS), at a dose of 1 mg/kg b.w. Control animals were receiving a single intravenous injection of PBS only. Kidneys were harvested at 2, 4, 8, 16 and 24 h after induction of Thy-1 nephritis.

L-N^6(1-iminoethyl)lysine(dihydrochloride), (L-NIL; Alexis Biochemicals, Gruenberg, Germany) a selective inhibitor of iNOS was administered intravenously at a dose of 5mg/kg b.w. to control and nephritic rats 45 min before anti-Thy 1-GN was induced. Kidneys were harvested (n = 3 animals per group) 16 h after injection of the anti-Thy 1.1 antibody or PBS. Systolic blood pressure was monitored by tail plethysmography (Byrom et al., 1938). Animals were anesthetized with hexobarbital (150 mg/kg). Kidneys were removed and glomeruli were isolated by differential sieving according to the method of Spiro (Spiro et al, 1984). Glomeruli from each kidney preparation were washed three times, examined by light microscopy (purity of isolates >95 %), counted thrice in a Fuchs-Rosenthal chamber and processed for Western blotting. Protein concentration was determined using the BCA Protein Assay (Pierce, Rockford, IL, USA).

2.2.12.2 Western blot analysis of isolated glomerular tissue

Glomerular homogenates from each individual rat kidney containing equal amounts of glomeruli were heated for 5 min at 95°C in Laemmli buffer, separated on SDS-PAGE (7.5% acrylamide gel) and transferred (BioRad, Hercules, CA, USA) to nitrocellulose membranes. The membranes were blocked with 3% casein, 1% goat serum and 0.002% Tween 20 in 10 mM Tris/HCl, pH 7.4/0.15 M NaCl (TBS). The membrane was incubated with the primary antibody rabbit anti-PDGFR-α (Santa Cruz, Heidelberg, Germany; in a dilution of 1:500 with TBS/1% BSA), for 16 h at 4°C. The secondary antibody, horse-radish peroxidase-coupled goat anti-rabbit IgG (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany, dilution 1:5000) was applied for 2 h at ambient temperature. The samples were visualised by using the ECL Western blotting reagent kit (Amersham Pharmacia Biotech Europe GmbH)

and analysis was performed with IQ Solutions Image Quant software (Molecular Dynamics, Uppsala, Sweden). Values are given as means ± SEM from three Western blots representing the content of PDGFR-α in 10^3 of glomeruli.

2.2.12.3 Immunohistochemistry

Kidney tissue samples were fixed in 4% formaldehyde in PBS and embedded in paraffin. Sections (4µm) were stained with periodic acid-Schiff reaction and processed for immunohistochemical studies by immunoperoxidase techniques (Schaefer et al., 2002). Prior to incubation with the primary antibody, endogenous peroxidase was blocked with 0.2% hydrogen peroxidase in methanol and endogenous biotin was blocked using the Avidin/Biotin Blocking Kit (Vector Lab, Burlingame, CA, USA). Primary antibodies included affinity-purified rabbit anti-human PDGFR-α and anti-human PDGFR-α (Tyr720) phosphorylated at Tyr720, which both react with the respective forms of PDGFR-α of rat origin (both from Santa Cruz, dilution 1:200, applied for 1h at 37°C). Unspecific staining was blocked with PBS/1% BSA/20% goat serum followed by incubation for 1h at 37°C with an affinity purified biotinylated goat anti-rabbit IgG (Vector Lab, dilution 1:200). To complete the sandwich technique the conjugate of extravidin-peroxidase (Sigma, Munich, Germany, dilution 1:500) was applied for 1h at 37°C and sections were developed with the Diaminobenzidine Substrate Kit (Vector Lab). Counterstaining was with Mayer's hemalaun (Sigma). The specificity of immunostaining was tested by omitting the primary antibody and by using non-immune serum "unspecific" IgG.

2.2.13 Statistical analysis

Means and Standard Deviations were determined. Students' t-test or ANOVA analysis were used to test for statistically significant differences (P values < 0.05).

III - R E S U L T S

3.1 Effects of Nitric Oxide on PDGFR expression

The PDGFR is an important signal generator that triggers proliferation and migration of glomerular MC (Heldin and Westermark, 1999). Under inflammatory conditions particularly the β-receptor is highly upregulated and contributes to the sclerotic modification of the glomerulus (Matsuda et al., 1995). The α-receptor is barely detectable in healthy MC. There are strong down-regulatory mechanisms that inhibit the presence of this receptor on the cell-surface (Abboud et al., 1994). However, glomerular inflammation leads to an increase of PDGFR-α expression, indicating a particular role for the alpha receptor under pathological conditions (Gesualdo et al., 1991, 1994). Another important mediator participating in the course of inflammatory kidney diseases is NO. This molecule regulates many genes and proteins (Pfeilschifter et al., 2001; Huwiler and Pfeilschifter, 2003).

3.1.1 Nitric Oxide time-dependently induces PDGFR-α mRNA expression

To analyse whether the NO pathway also modulates PDGFR expression we stimulated MC for different time-periods with or without the NO donor DETA-NO. As shown in Figure 1 DETA-NO treated cells revealed a marked increase of PDGFR-α mRNA steady-state levels, whereas the β-receptor was not or only marginally affected by NO. Therefore, the β-receptor subunit was not further analysed in my thesis.

Fig. 1a

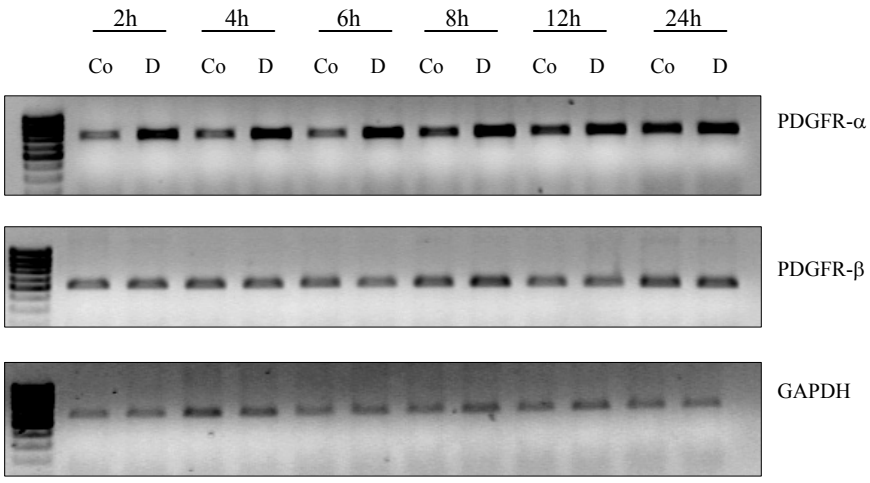
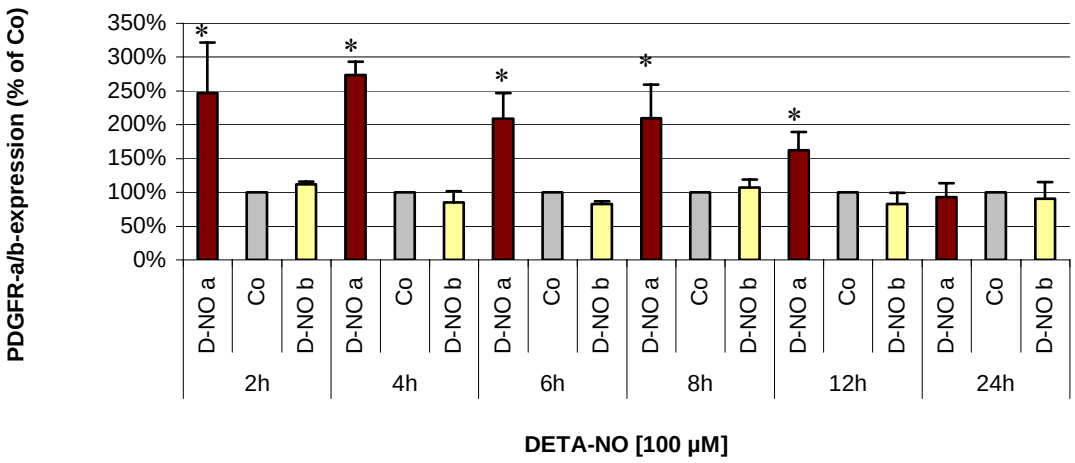


Fig. 1 : Time-dependency of NO-induced PDGFR-α expression

Quiescent rat mesangial cells were stimulated with the NO donor DETA-NO (D, 100μM) for the indicated time periods and compared to control cells (Co). Total cellular RNA was isolated and reverse transcriptase-PCR was performed for PDGFR-α (upper panel), PDGFR-β (middle panel) and GAPDH (lower panel) as described. Results are representative for at least three independent experiments giving similar results (Fig. 1a) and are depicted as means +/- SD. * $P < 0.05$ for D-NO versus Co (Fig. 1b).

Fig. 1b



3.1.2 Nitric Oxide concentration-dependently induces PDGFR-α-mRNA expression

We started our analysis with DETA-NO concentrations between 62,5 µM and 500 µM. As presented in Figure 2 and 3 the lowest concentration of 62,5 µM DETA-NO was sufficient to increase transcription PDGFR-α-mRNA in PCR and Northern blot analysis.

Fig. 2

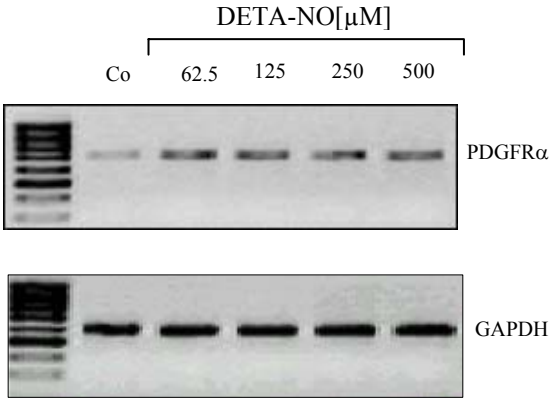
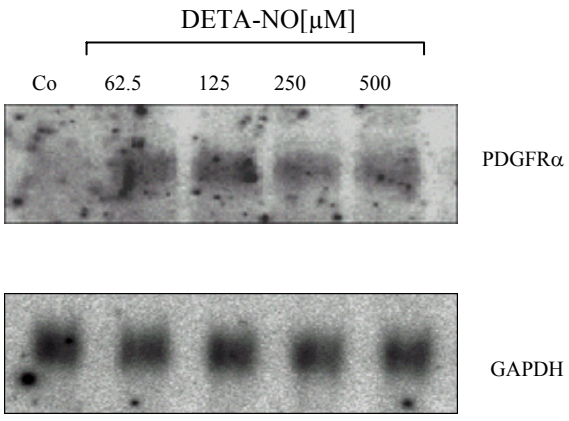


Fig. 2: Concentration-dependency of nitric oxide induced PDGFR-α mRNA steady-state level
Quiescent rat mesangial cells were stimulated with either vehicle (co) or with the indicated concentrations of DETA-NO for 6 hours. Total cellular RNA was isolated and reverse transcriptase-PCR was performed for PDGFR-α (upper panel), and GAPDH as described. Results are representatives of at least three experiments giving similar results.

Fig. 3: Concentration-dependency of nitric oxide induced PDGFR-α mRNA steady-state level
Quiescent rat mesangial cells were stimulated with either vehicle (co) or with the indicated concentrations of DETA-NO for 8 hours. Total cellular RNA was isolated and Northern blot analysis was performed for PDGFR-α (upper panel) as described. To correct for variations in RNA amount blots were rehybridized with GAPDH (lower panel) (n=1).



Since the Northern blot technique revealed only weak signals also after a long exposition period further experiments for the analysis of PDGFR-α mRNA expression were performed exclusively by RT-PCR.

To investigate the consequences of lower doses of DETA-NO we repeated the experiment with concentrations between 5 and 500 µM DETA-NO (14 h treatment). As depicted in Figure 4 even 5 µM DETA-NO were able to induce PDGFR-α-m-RNA significantly. Higher concentrations of DETA-NO reversed the effect or partially even inhibited PDGFR-α- mRNA level. This could be due to a potentially cytotoxic effect of high doses of DETA-NO.

Fig. 4a

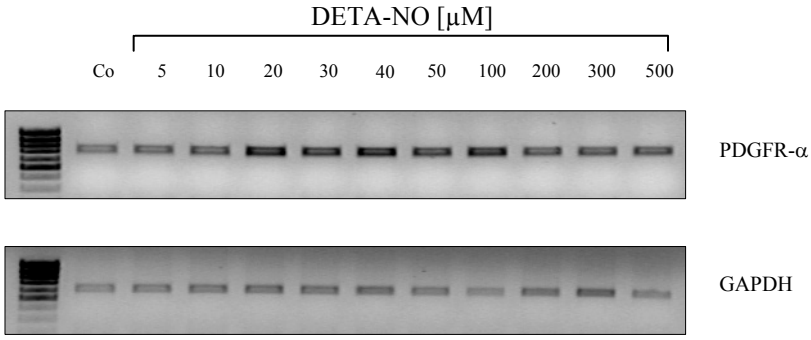


Fig. 4b

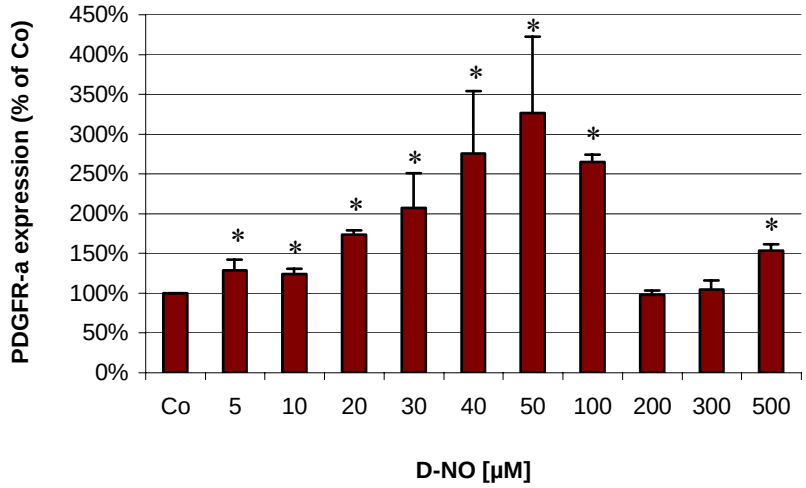


Fig. 4: Concentration-dependency of nitric oxide-induced PDGFR-α m RNA levels

Quiescent rat mesangial cells were stimulated with or without the NO donor DETA-NO (D) for 14 h with the indicated concentrations. Total cellular RNA was isolated and reverse transcriptase-PCR was performed for PDGFR-α (upper panel) and GAPDH (lower panel) as described. Signals were quantified densitormetrically. Results are representative for at least three independent experiments giving similar results (Fig. 4a.) Values were depicted as means +/- SD. * $P < 0.05$ for D-NO versus Co (Fig. 4b).

3.1.3 Nitric Oxide time-dependently increases PDGFR-α protein-level

The first part of this work demonstrated that DETA-NO elevates PDGFR-α mRNA steady-state levels. The next step was to analyse whether the surplus of mRNA is also translated into protein. Therefore we stimulated cells for different time-periods with 100 µM DETA-NO. As demonstrated in Figure 5 there is a significant increase of PDGFR-α protein already after 4 h, and peak values are obtained at 12 hours. Afterwards, the amount of PDGFR-α declines back to basal level (24h). However, MC do not stringently reply to NO stimulation in the same manner. The strength of response for the different time points varies leading to the immoderate standard deviations (e.g. the 6h time point). But nevertheless a constant increase of PDGFR protein expression in NO-exposed cells in comparison to control was always observed between 6h and 16h of treatment when compared to control cells.

Fig. 5a

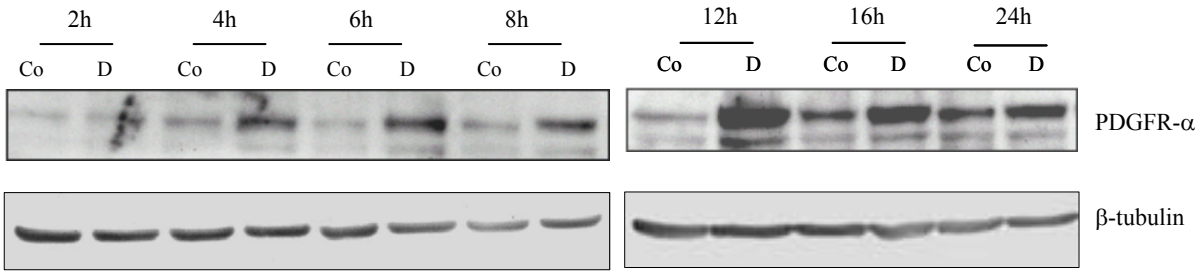


Fig. 5b

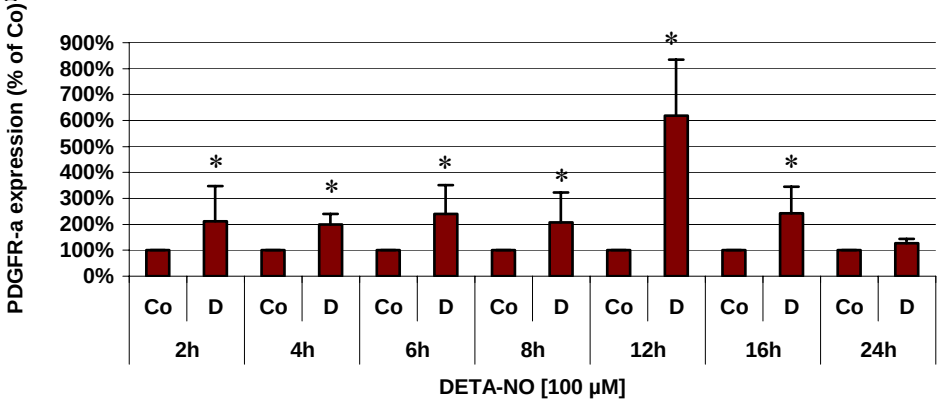


Fig. 5: Time-dependent induction of PDGFR-α protein expression by nitric oxide

Quiescent rat mesangial cells were stimulated for the indicated time periods either with vehicle (Co) or with 100 µM DETA-NO. Total cellular protein was isolated as described. The protein lysates were subjected to SDS-PAGE (8% acrylamid) and transferred onto a nitrocellulose membrane. Western blot analysis was performed using a specific PDGFR-α antibody and a β-Actin antibody at a dilution of 1:500 and 1:2000 respectively. Bands were detected by the ECL method according to the manufacturer's recommendation and relative units between PDGFR-α and β-tubulin were calculated. Values were depicted as percentage of control probes. Data are representatives of three independent experiments giving similar results (Fig. 5a) and are expressed as means +/- SD. * P<0,05 for D-NO versus Co (Fig 5b).

3.1.4 Nitric Oxide concentration-dependently increases PDGFR-α protein levels

MC were stimulated with different concentrations of DETA-NO for 14 h.

As depicted in Figure 6 DETA-NO enhances PDGFR-α protein level with a maximal effect between 50 and 100 µM.

Fig. 6a

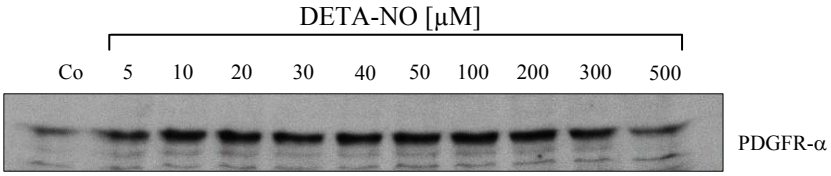
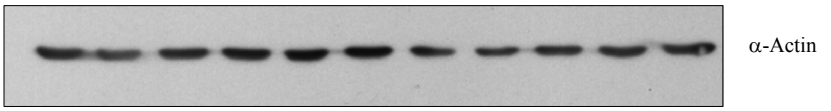


Fig. 6b



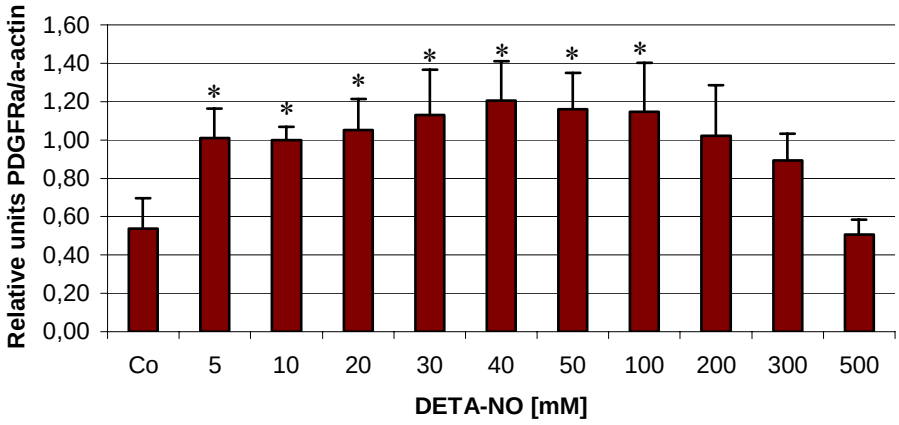


Fig. 6: Effects of DETA-NO on PDGFR-α protein expression

Quiescent rat mesangial cells were pretreated either with vehicle (Co) or with the indicated concentrations of DETA-NO for 14 h. Total cellular protein was isolated. Protein lysates were subjected to SDS-PAGE (8% acrylamide) and transferred to a nitrocellulose membrane. Western blot analysis was performed using a specific PDGFR-α antibody and an α-Actin antibody at a dilution of 1:500 and 1:2000 respectively. Bands were detected by the ECL method according to the manufacturer's recommendation and relative units between PDGFR-α and α-Actin were calculated. Data are representatives of three independent experiments giving similar results (Fig. 6a) and are expressed as means +/- SD. * $P<0.05$ for DETA-NO versus Control (Fig.6b).

3.2 IL-1ß induces PDGFR-α expression in part by the synthesis of Nitric Oxide

PDGFR-α is significantly upregulated by exogenously applied NO on the mRNA and protein level (Figures 1-6).

In vivo NO derives predominantly either from macrophages or is synthesized by the inducible NO synthase of MC, since MC do not express eNOS or nNOS (Pfeilschifter et al., 1992). MC are able to induce the iNOS after IL-1ß stimulation that leads to the synthesis of high amounts of NO. (Pfeilschifter and Schwarzenbach, 1990) To verify that the induction of PDGFR-α by DETA-NO is mimicked by endogenous NO production, cells were stimulated with IL-1β.

This pro-inflammatory cytokine is one of the major inducers of iNOS expression in rat MC. The most important feature for IL-1β mediated iNOS stimulation is the activation of the transcription factor NF-κB (Eberhardt et al., 1994; 1998). Also the activity of other transcription factors like C/EBPβ and C/EBPδ (CCAAT/enhancer binding protein) is triggered by IL-1ß and contribute to iNOS expression and subsequent NO formation (Eberhardt et al., 1998).

Not only iNOS but also PDGFR-α expression is under the transcriptional control of IL-1β. Kitami et al. (1995), reported that the IL-1β mediated induction of PDGFR-α in vascular smooth muscle cells (VSMC) is due to the activation of the transcription factors C/EBPδ and (to a much lesser extent) C/EBPβ (Fukuoka et al., 1999).

Consequently, there are two IL-1β-mediated molecular mechanism for PDGFR-α induction. One comprises the IL-1β-iNOS-NO pathway, the other induction of transcription factors which directly associate with the PDGFR-α promoter.

To discriminate between both signalling cascades, cells were costimulated with N^Gmonomethyl-L-arginine (L-NMMA), which is an L-arginine analogue, with a substituted guanidine nitrogen group. It inhibits all forms of NO synthases. L-NMMA binds to the same binding site as the natural substrate and hinders competitively arginine to associate with the enzyme, leading to a dose-dependent reduction of NO formation (Leiper and Vallance, 1998).

As shown in Figure 7, IL-1β upregulates PDGFR-α protein level. Blocking iNOS activity by L-NMMA diminishes the cytokine effect down to 60 % of total activity, but as expected also higher concentrations of L-NMMA do not completely reverse the observed induction.

These results clearly demonstrate that the IL-1β-mediated PDGFR-α induction is at least partly due to iNOS induction and subsequent NO formation.

Fig. 7a

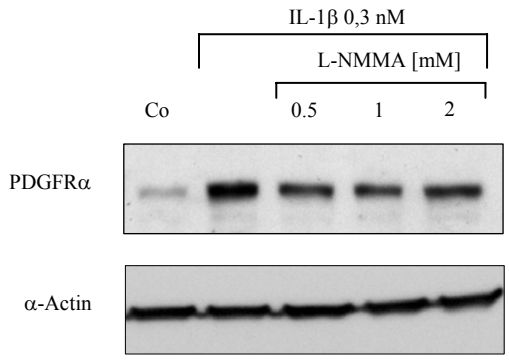


Fig. 7b

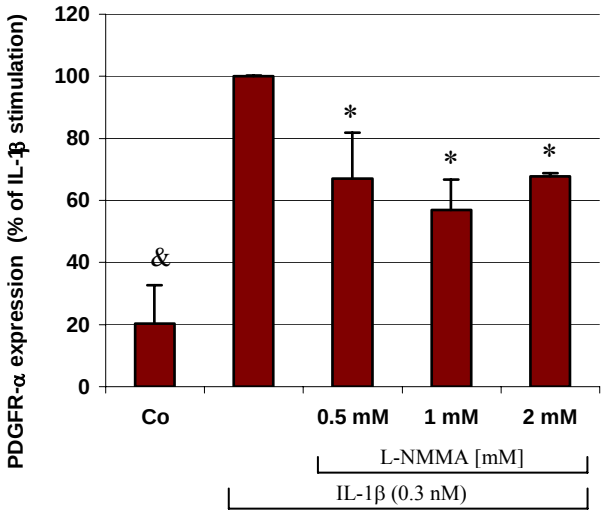


Fig. 7: Effects of IL-1β and L-NMMA stimulation on PDGFR-α protein expression

Quiescent rat mesangial cells were stimulated for 24 h with either vehicle (Co) or IL-1β (0.3 nM) in the absence or presence of the indicated concentrations of the NOS inhibitor L-NMMA. Total cellular protein was isolated as described. 60 µg of protein were subjected to SDS-PAGE (8% acrylamide) and transferred to a nitrocellulose membrane. Western blot analysis was performed using specific PDGFR-α and α-actin antibody at a dilution of 1 : 500 and 1 : 2000 respectively. Bands were detected by the ECL method according to the manufacturer's recommendation. Data are representative for three individual experiments (Fig. 7a). PDGFR-α and α-actin-bands were evaluated densitometrically and relative units were determined. Values were depicted as percentage of IL-1 β stimulation and are expressed as means +/- SD. * $P < 0.05$ for Il-1β + L-NMMA versus IL-1β, $^{\&}P < 0.05$ for control versus IL-1ß (Fig. 7b).

3.3 Molecular mechanisms of Nitric Oxide-triggered PDGFR-α expression.

NO exerts its actions by using different signalling pathways. It affects genes either directly or indirectly via induction or activation of certain mediators. At low concentrations a common mechanism is the activation of soluble guanylyl cyclase (sGC). NO binds to iron of a prosthetic heme group and induces a conformational change with a 200-fold stimulation of the enzyme activity (Stone et al., 1994). The subsequent formation of cGMP causes many of the well established effects of NO.

3.3.1 Nitric Oxide induces PDGFR-α expression via activation of the sGC

To examine whether the induction of PDGFR-α is mediated via the NO-sGC-cGMP axis, cells were stimulated with 3-5'Hydroxymethyl-2'-furyl-1-benzylindazole (YC-1). YC-1 was identified as a NO independent activator of sGC (Ko et al., 1994) and leads to a tenfold increase of enzyme activity. Furthermore, YC-1 sensitises NO-responsive sGC towards NO by retarding the deactivation of NO-stimulated sGC (Russwurm et al., 2002).

The quinoxalin derivative 1H[1,2,4]oxadiazolo[4,3-a]-quinoxalin-1-one (ODQ) is a well known inhibitor of NO-sensitive sGC (Garthwaite et al., 1995) and enables the differentiation between cGMP-dependent and cGMP-independent effects of NO. ODQ inhibits sGC irreversibly by oxidation of its heme iron (Schrammel et al., 1996).

If the NO mediated induction of PDGFR-α expression is triggered by cGMP formation, YC-1 will reproduce and ODQ application will reverse the NO effect on PDGFR-α. To address this issue MC were stimulated for 14 hours with DETA-NO which elevates PDGFR-α-protein expression (Figure 8). The DETA-NO effect was inhibited when MC were preincubated with the sGC inhibitor ODQ (200 μM) 15 min before DETA-NO application. YC-1 stimulation on the other hand enhanced PDGFR-α protein level, indicating that NO indeed uses the cGMP pathway for PDGFR-α-induction.

Interestingly, PDGFR-α protein translation was stronger in response to YC-1 than to DETA-NO, although NO is the more effective inducer of sGC activation. This result implies once again that there might be other, potentially simultaneously acting negative regulatory elements by which NO influences PDGFR-α-activity.

Fig. 8a

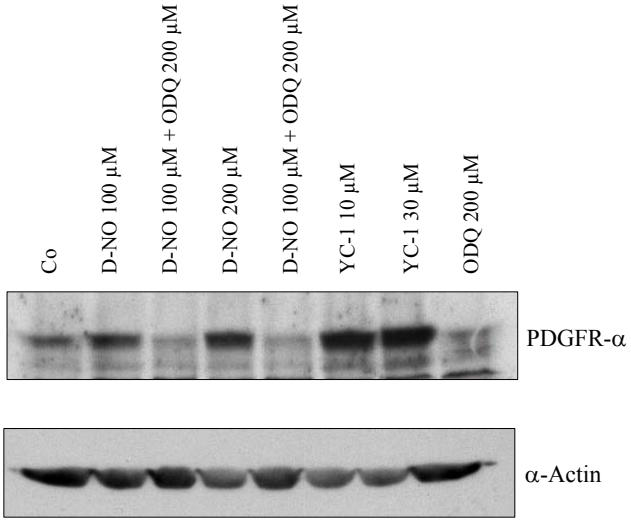


Fig. 8b

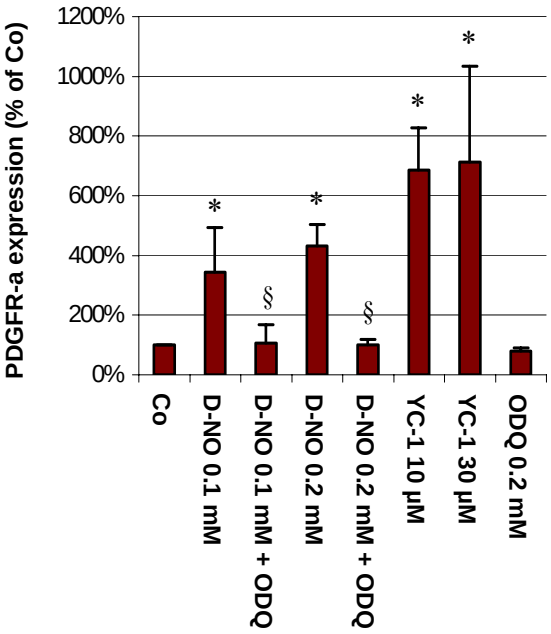


Fig. 8: Effects of the sGC inhibitor ODQ and the sGC activator YC-1 on PDGFR-α protein expression

Quiescent rat mesangial cells were stimulated for 14 h with either vehicle (Co), YC-1 (10 µM and 30 µM) or the indicated concentrations of DETA-NO with or without the sGC inhibitor ODQ (200 µM). Total cellular protein was isolated as described. Protein lysates were subjected to SDS-PAGE (8 % acrylamide) and transferred to nitrocellulose. Western blot analysis was performed using a specific PDGFR-α antibody at a dilution of 1 : 500. To correct for variations in protein amounts blots were stripped and furthermore treated with a α-actin antibody at a dilution of 1 : 2000. Bands were detected by the ECL method according to the manufacturer's recommendation. Data are representative for three individual experiments (Fig. 8a). PDGFR-α and α-actin-bands were evaluated densitometrically and their ratio was determined. Values were depicted as percentage of control cells and are expressed as means +/- SD. * $P < 0.05$ for D-NO, D-NO + ODQ, YC-1 versus Co, and § $P < 0.001$ for D-NO + ODQ versus D-NO (Fig. 8b).

3.3.2 Analysis of Nitric Oxide-mediated PDGFR-α transcription

NO affects many genes on the transcriptional level via activation of transcription factors (Beck et al., 1998; Pfeilschifter et al., 2001; Eberhardt et al., 2003). To examine whether the induction of PDGFR-α expression is mediated at the transcriptional level we cloned the PDGFR-α promoter and performed transfection analysis.

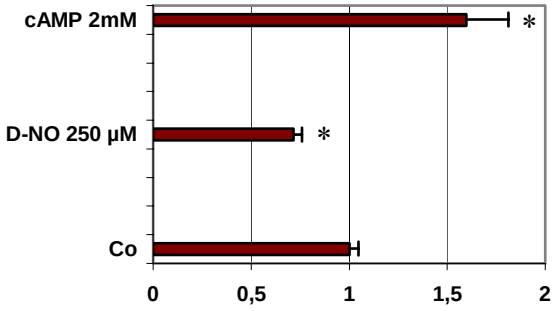
The PDGFR-α promoter sequence of rat has been deciphered by Kitami et al. (1995). The promoter is inducible by cyclic AMP and IL-1ß. The induction of promoter activity by cAMP is mediated via a C/EBP recognition site. We designed primers that amplify a fragment of about 1723 bp genomic DNA related to the 5`sequences of the PDGFR-α gene. The addition of recognition sequences for the restriction enzymes SacI and BamII facilitated direct cloning into the respective sites of the pGL3 vector that allows to bring the luciferase gene under the transcriptional control of the PDGFR-α promoter. The promoter/luciferase construct was further on referred to as promPDGFR-α/PGL3.

MC were transiently transfected with promPDGFR-α/PGL3. Transfected MC were treated either with vehicle or with DETA-NO (250 µM). As a positive control we stimulated cells with 2mM 8Br-cAMP**,** a substance that releases cAMP**,** which is a known activator of PDGFR-α-promoter (Fukuoka et al. 1999, Kitami et al., 1999).

As depicted in Figure 9, cAMP leads to slight but constant increase of PDGFR-α-promoter driven luciferase activity. This verifies that the cloned promoter fragment is functional. NO on the other hand does not induce the PDGFR-α promoter activity. By contrast, NO has a slight but significant negative regulatory effect on the PDGFR-α promoter activity. These results indicate that the NO-dependent induction of PDGFR-α is probably mediated posttranscriptionally. Alternatively the 5´flanking region of 1723 bp might be not sufficient to trigger NO-induced promoter activity.

Fig. 9: Effects of NO on PDGFR-α-promoter activity
Quiescent MC were transfected with the plasmid PDGFRα/pGL3 and with the renilla luciferase containing plasmid pRL-TK that includes the constitutive TK promoter. Thereafter, cells were treated with 8Br-cAMP (cAMP), DETA-NO (D-NO) or vehicle(Co) as indicated for 24 hours. MC were then harvested and beetle and renilla luciferase activities were assayed using the dual luciferase kit in a luminometer. Luciferase values were corrected for renilla activity. Values were depicted as percentage of control cells and are expressed as means +/- SD. * P < 0.05 for cAMP, DETA-NO versus Control.

Fig. 9



3.4. Functional relevance

NO regulates PDGFR-α expression on the mRNA and the protein level. The activity of the newly synthesized protein was examined by analysing the phosphorylation status of the receptor and of the downstream effector molecule PKB/Akt.

3.4.1 Induction of PDGFR-α protein expression permits its enhanced phosphorylation

Binding of the ligand leads to dimerization and autophosphorylation of PDGF receptors. In general, the number of PDGFR-α in rat MC is very low and therefore, the amount of the phosphorylated form (pPDGFR-α) was also expected to be hardly detectable, when exposed to PDGF. An upregulation of the protein might also increase the accessibility of the receptor for its ligand and cause an enhanced autophosphorylation and activation of downstream signalling molecules.

To confirm the functionality of the newly synthesized PDGFR-α cells were preincubated with the indicated concentrations of DETA-NO for 14 h. Thereafter PDGF-BB that binds and activates both receptor subunits was applied to all plates for the next 15 min. Western blot analysis was performed with an antibody specific for the phosphorylated tyrosine residue 720 of PDGFR-α. As depicted in Figure 10 DETA- NO preincubation leads to an increase of pPDGFR-α, demonstrating that higher expression of the PDGFR-α is followed also by a higher phosphorylation rate.

Fig. 10

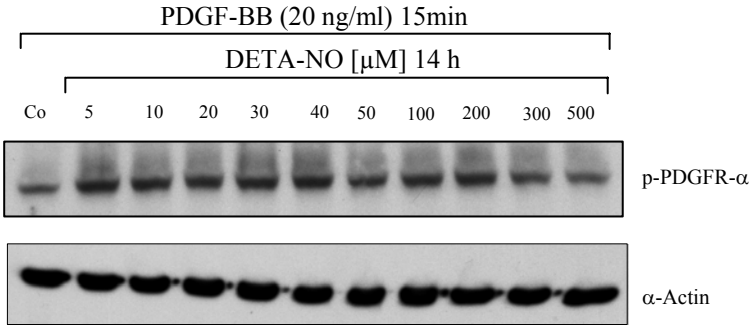
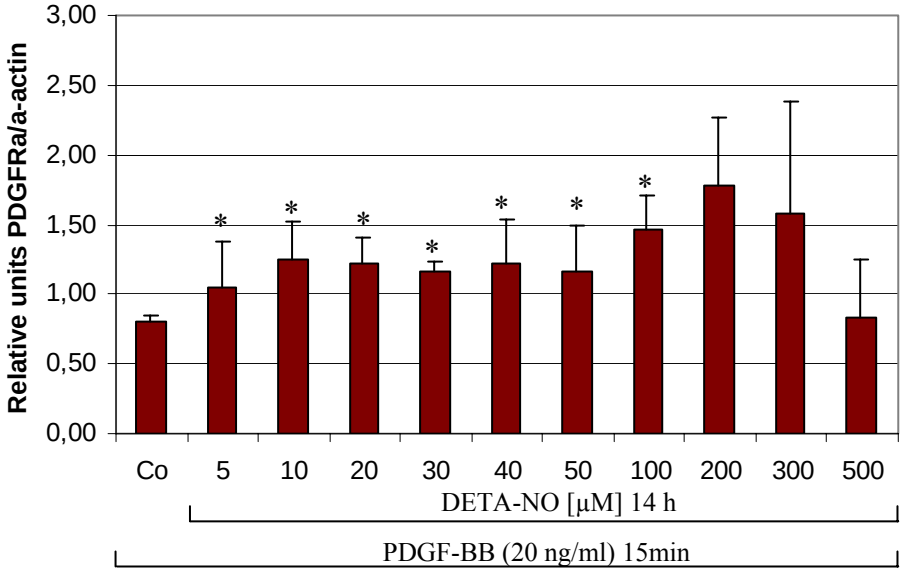


Fig. 10: Nitric oxide triggers PDGFR-α protein expression enables and its phosphorylation

Quiescent rat mesangial cells were pretreated either with vehicle (Co) or with the indicated concentrations of DETA-NO for 14 h. Thereafter cells were stimulated with PDGF-BB (20 ng/ml) for 15 min. Total cellular protein was isolated as described. The protein lysates were subjected to SDS-PAGE (8% acrylamide) and transferred to nitrocellulose. Western blot analysis was performed using a specific antiphospho-Tyr^{720} PDGFR-α antibody and a α-Actin antibody at a dilution of 1 : 500 and 1 : 2000, respectively. Bands were detected by the ECL method according to the manufacturer's recommendation and relative units between PDGFR-α and α-actin were calculated. Data are representatives of three independent experiments giving similar results (Fig. 10a) and are expressed as means +/- SD. * P< 0,05 for DETA-NO versus Control (Fig. 10b).

Fig. 10b



3.4.2 Nitric Oxide-triggered induction of PDGFR-α augments PKB phosphorylation

The NO induced increase of basal PDGFR-α protein level enhances the amount of phosphorylated receptor, when incubated with an appropriate ligand and consequently more docking sites for potential target molecules including PI3K become available.

PI3K is one of the most important mediators of PDGF-induced MC proliferation. Inhibition of PI3K dose-dependently diminished mitogenic and migrative effects of PDGF down to basal level (Ghosh-Choudhury et al., 1997). Activation of PI3K is essential for phosphorylation of protein kinase B (Franke et al., 1995). PKB (also referred to as Akt) is a serine/threonine protein kinase that exists as three distinct isoforms, PKB-α, PKB-β and PKB-γ Since the PKB-α isoform is the most common in MC, immunoblot analysis were performed with a PKB-α specific antibody.

Once phosphorylated PKB in turn catalyses the phosporylation and inactivation of the proapoptotic substrate Bad and caspase-9 and thus promotes survival signals (Coffer et al., 1998). To examine whether NO might alter the phosphorylation status of PKB by induction of PDGFR-α, cells were preincubated with the NO donor DETA-NO or vehicle for 14 h and then stimulated with PDGF-AA (30ng/ml) for 8 min. Since PDGF-AA is incapable to activate PDGFR-β, the obtained effect can only be caused by PDGFR-α.

Western blot analysis was performed with an antiphospho-antibody specific for the phosphorylated serine-473 of PKB and with an antibody against PKB-α, to correct for variations in protein loading. Figure 11 reveals that there are very low amounts of phosphorylated PKB in untreated MC. NO alone does not change pPKB level either.
PDGF-AA on the other hand is a potent initiator of PKB phosphorylation. It leads to a twofold increase of the pPKB level. Preincubation with NO in combination with PDGF-AA treatment amplifies the PDGF-AA effect threefold, indicating that the surplus of docking sites gained by transcriptional induction of PDGFR-α is actually utilized and functionally active.

Fig. 11a

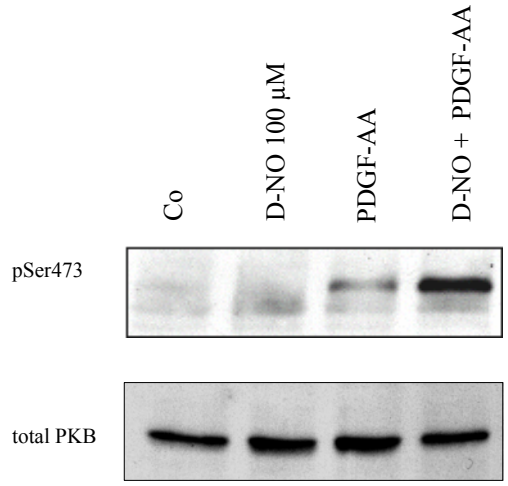


Fig. 11b

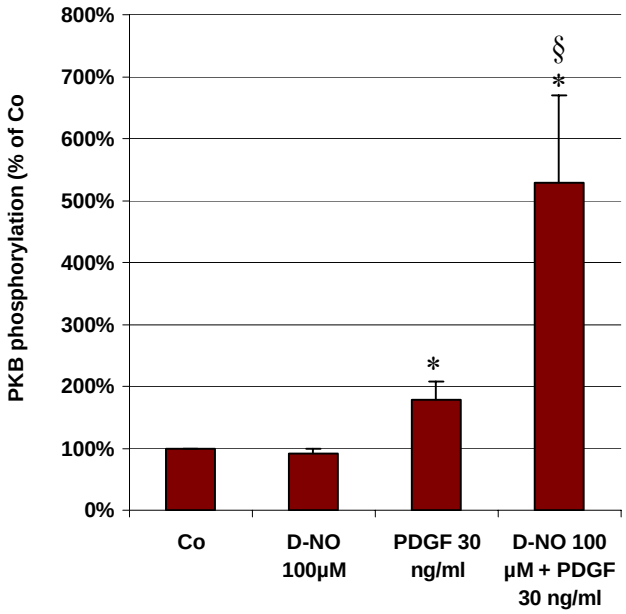


Fig. 11: Effects of NO prestimulation on PDGF-AA-induced PKB-α phosphorylation

Quiescent rat mesangial cells were pretreated with 100 µM DETA-NO or vehicle for 14 hours, prior to stimulation with either vehicle or PDGF AA (30 ng/ml) for the lasting 8 min. Total cellular protein was isolated as described. 60 µg of protein were subjected to SDS-PAGE (10 % acrylamide) and transferred to a nitrocellulose membrane. Western blot analysis was performed using specific antiphospho-Ser^{473} –PKB and total PKB-α antibodies at dilutions of 1 : 1000 and 1 : 2000, respectively. Bands were detected by the ECL method according to the manufacturer's recommendation. Relative units between pPKB and PKB were determined. Values are depicted as percentage of control probes. Data are representatives of three independent experiments giving similar results (Fig. 11a) and are expressed as means +/- SD. * $P < 0.05$ for NO, PDGF, NO + PDGF versus Control and § $P< 0.05$ for NO + PDGF versus PDGF (Fig. 11b).

3.5 In vivo examination of PDGFR-α-expression in an inflammatory model of anti-Thy1.1 nephritis

To investigate whether the *in vitro* observed data could also be confirmed *in vivo* a cooperation with Dr. L. Schäfer from the University Münster was started. Dr. Schäfer is familiar with the inflammatory model of anti-Thy.1.1 nephritis and performed all *in vivo* analyses.

The anti-Thy-1 glomerulonephritis (anti-Thy 1-GN) was induced in adult male Wistar rats by a single intravenous injection of mouse anti-rat Thy-1.1 IgG, whereas control animals were receiving PBS only. L-N^6-1-iminoethyl lysine dihydrochloride, (L-NIL), a selective inhibitor of iNOS, was either omitted or administered intravenously to control and nephritic rats 45 min before anti-Thy 1-GN was induced. Kidneys were harvested (n = 3 animals per group) 16h after injection of the anti-Thy 1.1 antibody or PBS and glomeruli were isolated and processed as described (Spiro, 1984).

3.5.1 L-NIL decreases PDGFR-α protein expression in anti-Thy.1.-GN

Western blot analysis was performed with a PDGFR-α-antibody. As depicted in Figure12 injection of anti-Thy.1.1 antigen induces PDGFR-α expression by approximately twofold. Coapplication of the selective iNOS inhibitor L-NIL diminishes PDGFR-α protein level (Figure 12), indicating that iNOS acts as the major inductor of PDGFR-α expression in this GN model.

Fig. 12

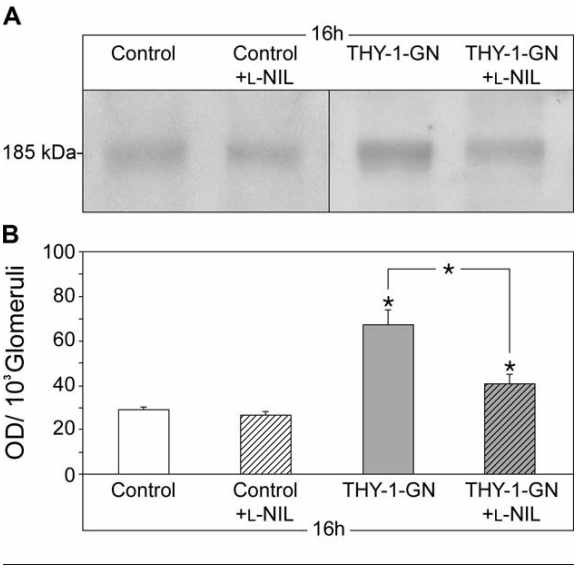


Fig. 12: Western blot for PDGFR-α in isolated glomeruli from control and THY 1-GN rats

Panel A shows a Western blot for PDGFR-α in equal amounts of isolated glomeruli from control and nephritic rat kidneys with and without administration of L-NIL as indicated. In panel B the quantification of Western blots for PDGFR-α (three samples in each group) is given as optical density (OD) from 10^3 glomeruli (means ± SEM). Significant differences between THY 1-nephritic and non-nephritic glomeruli are indicated by an asterisk positioned directly over the bar. The asterisk between grey and and striped grey bars indicates statistical differences between THY 1-nephritic glomeruli with or without L-NIL treatment, $p<0.05$.

3.5.2 L-NIL decreases the phosphorylation rate of PDGFR-α in anti-Thy1- GN

Immunohistochemistry was performed for phosphorylated and unphosphorylated PDGFR-α-protein. As shown in Figure 13 expression of PDGFR-α-and pPDGFR-α-protein is very low in glomeruli of control animals. Thy 1.1 glomerulonephritis causes a strong staining within the capillary tuft, which apparently represents GMC expressing PDGFR-α protein.

Again coapplication of the iNOS inhibitor L-Nil was able to reduce PDGFR-α and pPDGFR-α expression in rats treated with Anti-Thy.1.1-IgG.

In particular, the increase of PDGFR-α phosphorylation points to a critical role of PDGFR-α in GN. Figure 13 clearly shows that this upregulation is mainly due to enhanced iNOS expression, since inhibition of iNOS counteracts PDGFR-α protein elevation.

In summary, the work of Dr. Schäfer verifies our previously observed *in vitro* data and demonstrates that NO acts as potent mediator of PDGFR-α gene expression also *in vivo*.

Fig. 13

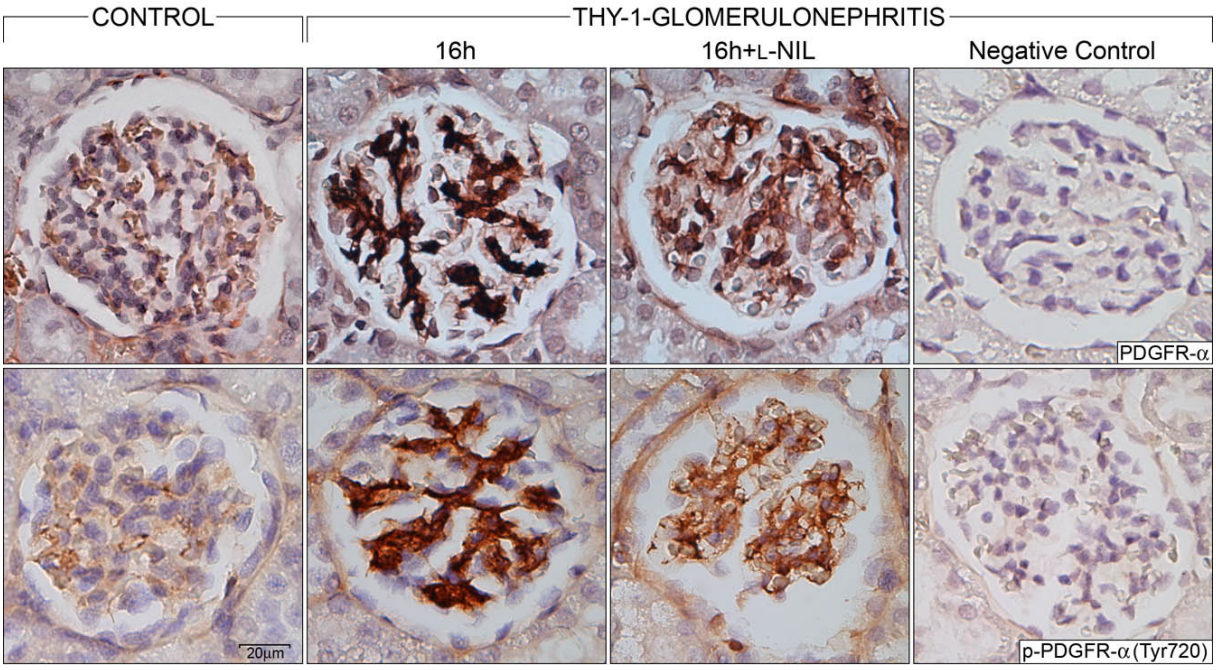


Fig. 13: Immunostaining for PDGFR-α (upper panel) and p-PDGFR-α (Tyr720) (lower pannel) in glomeruli from control and THY 1-GN kidneys 16h after the induction of nephritis.

In THY 1 nephritis there was increased mesangial staining for PDGFR-α (upper panel, brown color) and its phosphorylated form (lower panel, brown color) 16h after induction of disease. Pretreatment with L-NIL reduced the intensity of staining for the phosphorylated and unphosphorylated PDGFR-α. There was no difference in controls receiving L-NIL or vehicle. Negative controls, shown here in a tissue section from a THY 1 nephritic kidney as an example, were performed by omitting the primary antibodies. The bar indicates magnification**.**

IV – DISCUSSION

NO is one of the most versatile molecules involved in inflammatory processes. Due to its diverse reaction partners like other inorganic molecules, DNA structures, prosthetic groups of proteins such as enzymes and transcription factors, NO impacts on many signalling pathways and exerts a wide range of effects (Beck et al., 1999; Bogdan, 2001; Huwiler and Pfeilschifter, 2003). The role of NO in inflammatory processes is controversially discussed. Beneficial as well as detrimental consequences of NO-presence in inflammatory diseases have been reported (Beck et al., 1999). We believe that the variability of NO action empowers this molecule not only to interfere in the disease process but also to regulate and initiate crucial turning points in the course of glomerulonephritis (Pfeilschifter et al., 2002; Pfeilschifter, 2002).

The experimental model of anti-Thy.1.1 nephritis is a convenient model to analyse the mechanisms of inflammatory disease progression. In principal, it is composed of three phases: mesangiolysis (first day after disease induction), mesangioproliferation (day 4-10) and resolution of the disease (weeks 4-8) (Goto et al., 1995). The mesangiolytic phase is associated with high concentrations of NO (Goto et al, 1995). At this stage NO acts as a potent inducer of MC death (Narita et al., 1995) and is capable to reduce survival signalling e.g. by inhibition of PKB phosphorylation (Rölz et al., 2002). Nevertheless, NO may also simultaneously deliver proliferative signals, as it diminuates significantly the expression of the potent PDGF scavenger SPARC (Walpen et al., 2000), with a maximal protein reduction at a time when maximal NO concentrations can be measured (day 2 after disease induction) (Pichler et al., 1996; Walpen et al. 2000). Inhibition of SPARC leads to enhanced availability of the potent proliferative growth factor PDGF. Therefore, NO does not only trigger MC loss in the mesangiolytic phase but might also be able to prepare the next phase of anti-Thy.1 nephritis.

The mesangioproliferative phase begins with the immigration of extraglomerular MC of the juxtaglomerular apparatus into microaneurysmal areas in the glomerulus (Hugo et al, 1996). Subsequently mesangial hyperplasia can be observed with a maximal quantity of cells at day five after disease induction. The MC number at this stage of the disease exceeds the normal values two or three times (Jefferson and Johnson, 1999). A lot of mediators have been identified that induce MC proliferation, whereby PDGF is regarded as the most potent stimulant (Johnson et al., 1992; Jefferson and Johnson, 1999). Mechanisms that lead to the decrease of MC number and resolution of the disease are not so well investigated so far. One

way to restrict further MC proliferation comprises the inhibition of pro-inflammatory or the induction of anti-inflammatory mediators that neutralize or antagonize inflammatory signals. The increase of SPARC at day 5 after disease induction and the subsequent reduction of available PDGF is just one example for a self-defence process of the glomerulus (Pichler et al., 1996; Walpen et al., 2000). A vast number of other anti-inflammatory substances have been described that contribute to or maintain the onset of the glomerular self-defence system, whereby NO participates in regulating the gene-expression of numerous of these mediators (Kitamura and Fine, 1999). Additionally, a continuous elimination of MC by apoptosis can be observed. This latter mechanism is essential for the diminution of MC cell number back to normal values and enables the restructuring of the glomerulus (Tomooka et al., 1992; Baker et al., 1994; Jefferson and Johnson, 1999).

Elucidating the control mechanisms that lead to this orderly repair process and determining the relevance of NO action in this context is part of the research effort of the Institute of Pharmacology and Toxicology in Frankfurt.

Generally low amounts of NO are regarded as protective against inflammatory tissue damage (Pfeilschifter et al., 1995, 2001), whereas high doses of NO are known to induce apoptosis and necrosis in many cell types (Meßmer et al., 1994; Meßmer and Brüne, 1996; Mühl et al., 1996; Brüne et al., 1998). However, during inflammatory diseases the concentration range of NO is wide and resulting effects of NO do not simply depend on the measured absolute amount of the molecule, but also of its temporal and spatial distribution (Grisham et al., 1999; Pfeilschifter et al., 2001; 2002). Since NO is a gas it diffuses from its source easily and dilutes its concentration proportionally to the distance (Grisham et al., 1999). Therefore, also high concentrations of NO as produced by iNOS under inflammatory conditions are able to exert or initiate somehow protective effects by affecting targets outside of the high-dosed toxic area.

Another important aspect for the resulting net effect of NO concerns the availability of potential reaction partners such as reactive oxygen species (ROS). The reaction of NO with ROS like superoxide (O_2^-) or hydrogen peroxide (H_2O_2) shifts the NO action towards the so called indirect effects (nitrosation, nitration, oxidation) that are supposed to be responsible for the destructive proinflammatory features of NO (Grisham et al., 1999). Nevertheless the consequences of NO/ROS interaction depend mainly on the ratio between NO and ROS and does not have to be necessarily harmful. NO is even able to counteract the oxidative stress imposed by ROS to the cellular system (Sandau et al, 1997; Pfeilschifter et al, 2002).

In MC, ROS are mainly produced by enzymes that are constitutively expressed, e.g. xanthine oxidase, cyclooxygenases, nicotinamide adenine dinucleotide phosphate-oxidase (NADPH-oxidase) but also by iNOS (Pfeilschifter et al., 2001).

Importantly, the production of ROS and NO does not arise simultaneously. In anti-Thy.1.1 GN the generation of ROS starts briefly after disease induction, whereas synthesis of iNOS commences with a delay of a few hours (Goto et al., 1995). With the increase of NO-production, ROS synthesis declines and direct NO modulated effects dominate (Pfeilschifter et al., 2002). In this context the diminution of ROS-levels is directly mediated by NO altering the cellular redox-system: NO downregulates the main ROS producing enzymes NADPH-oxidase (Clancy et al., 1992), xanthine-oxidase (Lee et al., 2000) and induces protective antioxidant enzymes like copper/zinc superoxide dismutase (Cu/ZnSOD) (Frank et al., 1999) or manganese SOD (Keller et al., 2003). Another mechanism how NO supports the cellular protection system is by inhibition of the iNOS enzyme activity. Although NO is an effective inducer of iNOS expression, it inhibits particularly at high concentrations the NOS enzymatic activity and limits, therefore, its own synthesis (Mühl and Pfeilschifter, 1995).

An important mediator that contributes to the inhibition of iNOS expression is PDGF. PDGF, that is either released by platelets or secreted by mesangial and other glomerular cells, impedes iNOS activity (Pfeilschifter 1991) via activation of protein kinase C (Mühl and Pfeilschifter, 1994; Kunz et al., 1997).

My present study was done to elucidate the role of NO on the PDGF signalling system, the main mediator of inflammatory proliferation of rat MC (Johnson et al., 1993; Abboud 1995). To this end, the regulation of PDGF ligands and receptors by NO was examined. The only component of the PDGF system that was significantly modulated by NO in its expression was the PDGFR-α. This induction of PDGFR-α expression is mediated via activation of the NO-sensitive soluble guanylyl cyclase (sGC). The newly synthesized receptor is functionally active and responds in turn to its natural ligand with autophosphorylation and activation of downstream effector molecules like protein kinase B (PKB). PKB is a major initiator of survival and antiapoptotic signalling (Kaufmann-Zeh et al., 1997). The NO-modulated induction of PDGFR-α expression was confirmed in an *in vivo* model of anti-Thy.1.1 nephritis and clearly demonstrates that inhibition of iNOS by the iNOS-specific inhibitor L-NIL reduces PDGFR-α expression as analysed by immunohistochemistry and Western blot analysis.

Taken together, we showed that NO consistently induces the expression of PDGFR-α *in vitro* and *in vivo*. By contrast, the PDGFR-β is not or only marginally affected by NO. The

differential regulation of both receptors is of pathophysiological importance, since MC produce and secrete mainly PDGF-AA and PDGF-AB (Betsholtz et al. 1986, Abboud et al., 1987, Pfeilschifter and Hosang, 1990). Both ligands require the α-subunit for receptor dimerization (Heldin and Westermark, 1999). Under physiological conditions MC express very low amounts of the α-subunit (Abboud et al., 1994). The induction of this receptor in combination with the continuously expressed ligands may permit autocrine stimulation and consequently proliferation of MC. Interestingly, the NO-mediated up-regulation of PDGFR-α expression is attained at low doses of DETA-NO, which usually characterizes processes evoked by cGMP (Moncada et al., 1991).

Our in vitro and in vivo study revealed that MC respond to NO stimulation with significant upregulation of PDGFR-α mRNA and protein expression within 4 hours. The increase of the PDGFR-α protein enables PDGF-AA to dimerize and activate PDGFR-αα complexes.

PDGF-AA is regarded as an ineffective mediator of proliferation, migration and autoinduction of PDGF chains in human MC (Abboud et al., 1994, Ghosh-Choudhury et al, 2000). However, Abboud and colleagues investigated unstimulated MC, in which the α-receptor is chronically downregulated by a constant PDGF-AA secretion. The induction of the receptor, that occurs for example upon inflammation, may change the potency of PDGF-AA action in MC.

As shown here, induction of the α-receptor creates more binding sites and leads to enhanced delivery of down-stream signals e.g. activity of PI3K. The observation that PKB phosphorylation by PDGF-AA was more effective in NO stimulated cells than in the unstimulated control cells, reveals that the magnitude of the proliferative potency of PDGF-AA is limited somehow by the amount of receptors expressed on the cells.

Whether the NO-mediated elevation of PDGFR-α subunit is actually sufficient to initiate proliferation or migration in response to PDGF-AA stimulation has to be clarified in further experiments.

PDGF-AA is not the only isoform that necessitates PDGFR-α monomers to associate. PDGF-AB as well requires the α-receptor for high affinity binding. It dimerizes PDGFR-ββ complexes only at unphysiological high concentrations (Seifert et al., 1993). PDGF-AB is a potent mitogenic growth factor for MC. It delivers proliferative and migrative actions and induces PDGF-A and B-chain expression thereby setting the basis for an autoinductive stimulation circle in MC (Abboud et al., 1994).

Furthermore, PDGF-AB is the predominant isoform in human serum in contrast to other species, in which PDGF-BB is the main but not the only isoform present (Bowen-Pope et al.,

1989). Although, platelets and macrophages synthesize PDGF, the MC is regarded as the primary source of PDGF in GN (Jefferson and Johnson 1999).

Most PDGF-producing cell types secrete PDGF-AA or PDGF-AB and to a much lower extent, if at all, PDGF-BB. MC express constitutively PDGF-β receptors, but secrete inadequate amounts of its ligand PDGF-BB. In addition, they sufficiently secrete PDGF-AA or –AB but barely express the α-receptor. It seems as if there is a natural, reciprocal expression pattern between receptors and their corresponding ligands. The increase of PDGF-BB or PDGFR-α expression may easily disturb this balance and shift it towards proliferation and cell survival. Since PDGF-BB is only secreted inadequately at least at the beginning of the disease, induction of PDGFR-α and subsequent activation by PDGF-AB and PDGF-AA might be of pathophysiological significance. However, the PDGFR-β subunit is, once activated, definitely the stronger mitogenic signal device in MC and most other cell types (Abboud et al., 1994; Ghosh-Choudhurry et al., 2000).

The biological activity of the somewhat less potent α-receptor is also less well investigated so far. Nevertheless, a consistent upregulation of PDGFR-α under inflammatory diseases has been observed (Gesualdo et al., 1994). The pathophysiological relevance of the α-receptor in the course of mesangioproliferative glomerulonephritis is gaining recognition.

The recent discovery of the novel PDGF isoform PDGF-CC (Li et al., 2000) contributed to the growing interest. PDGF-CC binds and dimerizes PDGFR-αα and -αβ subunits, but it is unable to activate ββ complexes. The mitogenic capability of this isoform is comparable to that of PDGF-AB with the difference that higher concentrations are needed for receptor activation (Gilbertson et al., 2001).

The activation of PDGFRs by PDGF-CC might differ from the known other isoforms, since the phenotype of PDGFR-α gene deleted mice diverges widely from PDGFR-$\beta^{-/-}$ or PDGF-A chain and B chain double knockouts, indicating that eventually this new isoform evokes other effects (Betsholtz et al. 2001).

Eitner et al. (2002) investigated the gene expression of PDGF-C chain in normal and diseased adult rat kidney. They demonstrated that in the healthy kidney PDGF-C chain expression is limited to several cell types including vascular smooth muscle cells and tubulointerstitial cells of the collecting duct. By contrast, in the course of various inflammatory kidney diseases e.g. the anti-Thy-1.1 nephritis, PDGF-C chain expression is markedly up-regulated also in other cells. With the beginning of the mesangioproliferative phase MC are a major source for PDGF-C chain synthesis, indicating the participation of this novel PDGF isoform in the course of the disease. The functional relevance of PDGF-C chain still is under investigation.

Apart from its potential mitogenic and fibrogenic effects, PDGF-C is also known to stimulate effectively angiogenesis and may contribute to oncogenicity in several sarcoma cell lines (Zwerner et al., 2001).

All effects exerted by PDGF-CC are due to activation of at least one PDGFR-α subunit. The induction of the weakly expressed PDGFR-α by NO provides the basis for PDGF-CC promoted activation of PDGFRs. The functional relevance of PDGF-CC in the course of glomerulonephritis definitively needs to be clarified in future investigations.

In contrast to many human forms of mesangioproliferative glomerulonephritis the animal model of anti-Thy.1.1 nephritis completely resolves several weeks after injection of the antibody. The scheme composed of the three disease phases: mesangiolysis - mesangioproliferation – resolution, with complete restitution of the glomerulus occurs always in the same ordered manner. Understanding the control mechanisms that lead to the organized reconstitution of the glomerulus and their temporal and spatial appearance in the course of the disease may provide the basis for the establishment of effective pharmacological interventions in inflammatory kidney diseases.

One pharmacological approach to dampen inflammatory disease activity in mesangioproliferative glomerulonephritis was attempted via the inhibition of the PDGF system. The high mitogenic potency of PDGF initiated the development of multiple PDGF-antagonists that affect either the ligand, its receptor or downstream signalling molecules (Heldin et al., 1998). Among them the specific PDGF-B aptamer (Ostendorf et al., 2001), widely attained attention. Glomerulonephritis and chronic inflammatory renal diseases still are the most common causes for renal failure requiring dialysis or organtransplantation. Obviously inhibition of the most potent PDGF isoform is not sufficient to stop the progression of disease.

It is worth noting, that PDGF also exerts beneficial effects, since it initiates tissue remodelling and repair (Abboud, 1995). The excessive upregulation of PDGF and its receptors by cytokines, growth factors and other inflammatory mediators in a pathological milieu leads to the well-known pathogenic effects of platelet-derived growth factor. Therefore, detection and neutralization of the regulatory elements responsible for overshooting PDGF activity may prevent the problem straight from the beginning.

Nevertheless a certain degree of MC proliferation is required to repair glomerular injury. As indicated, the physiological procedures that regulate the switch between MC loss and MC hyperplasia (or vice versa) are incompletely understood so far. NO is supposed to play a key-role in triggering this process (Pfeilschifter et al. 2002). At the early phases of the anti-Thy1.1

nephritis NO is produced in high concentrations. At this stage NO induces effectively cytotoxic and anti-proliferative signalling. One can imagine that NO counteracts its own effects by changing the expression of diverse proliferative and protective mediators that reconstitute the glomerulus and initiate the proliferative state of the disease. The observed induction of PDGFR-α by NO described in my thesis may be one mechanism underlying the controlled repair process responsible for the switch between mesangiolysis and mesangioproliferation in anti-Thy.1.1 nephritis.

Since NO is regarded as one of the major players in mesangioproliferative glomerulonephritis, inhibition of iNOS became a central mission in NO-research.

Numerous non-selective as well as several more or less selective iNOS inhibitors were identified (Pfeilschifter et al, 1996; Alderton et al., 2001).

One of the earliest iNOS inhibitors found were the glucocorticoids (Pfeilschifter & Schwarzenbach 1990). Glucocorticoids are potent immunosuppressive agents, that inhibit inflammatory mediators like IL-1 (one of the main inducers of iNOS expression) generally on their transcriptional level. Interestingly, the glucocorticoid mediated inhibition of iNOS is additionally attained by post-transcriptional and posttranslational modifications, including a decreased protein translation and an increased degradation of iNOS protein. The beneficial effects of glucocorticoids in several non-infectious inflammatory kidney diseases are well documented (Friedmann et al., 1982) and the observed glucocorticoid-mediated iNOS-inhibition may contribute to these positive effects in glomerulonephritis.

Cyclosporin which is a further immunosuppressive agent was also found to inhibit iNOS expression (Mühl et al., 1993; Kunz et al., 1995). Cyclosporin A, cyclosporin G and even cyclosporin H, a derivate that is devoid of immunosuppressive activity, are able to suppress IL-1β-induced elevation of iNOS mRNA level (Pfeilschifter et al., 1996). There are lots of other substances that diminish NO production (Pfeilschifter et al, 1996; Alderton, et al., 2001), manipulation of the L-arginine-NO pathway is the most common mechanism to attenuate NO synthesis in experimental models. L-arginine is the natural substrate of all NOS isoforms. Substrate depletion, attained via the administration of arginase, an enzyme that also metabolises L-arginine (Waddington et al., 1996) or competitive inhibition of NOS by non-active arginine analogues like N^G-monomethyl-L-arginine (L-NMMA) or L-arginine-methylester (L-NAME) that compete with the natural substrate for binding (Drapier et al., 1986; Palmer et al., 1987), are two effective methods to block NO synthesis. The unspecific NOS inhibition by both inhibitors, worsens the course of the disease (Waddington et al., 1996). The reason for the aggravation of glomerulonephritis was searched in the

simultaneously occurring inhibition of eNOS and the subsequent diminuation of GFR (Goligorsky, 1999).

The development of NOS inhibitors that bind iNOS with higher affinity than the constitutive NOS isoforms like aminoguanidine (Corbett et al., 1992; Misko et al., 1993) or L-N^6-(1-iminoethyl)lysine (L-NIL) (Moore et al., 1994) and their examination in *in vivo* models of glomerulonephritis strengthened the idea that the specific inhibition of iNOS ameliorated disease progression (Bremer et al, 1997). However, there is increasing evidence that the consequences of iNOS-downregulation do not always have to be positive. Gabbai et al. (1997) reported that the administration of aminoguanidine or L-NIL in an *in vivo* model of interstitial nephritis increased tubular injury, which could be confirmed also by others and in additional nephritis models (Gopala et al., 2001; Westenfeld et al., 2002). Moreover Gabbai et al., (2002) assumed a biphasic effect of iNOS derived NO in the course of disease, with an aggravation at the beginning and an amelioration at the later phases of glomerulonephritis. These findings emphasize the protective role of iNOS for the resolution of disease. Moreover, they are in line with reports from our Institute of Pharmacology and Toxicology in Frankfurt (Frank et al., 2000) and illustrate once again that uncoordinated inhibition of NOS without regarding the pathophysiological background will not provide an optimal strategy to treat glomerulonephritis.

V – S U M M A R Y

Nitric oxide (NO) modulates Platelet-derived growth factor receptor-α (PDGFR-α) expression in rat renal mesangial cells

NO, a diffusible gas with various physiological and pathological properties, is synthesized by three different forms of nitric oxide synthases (NOS). The effects of NO depend particularly on the actual NO concentration. At low concentrations, NO participates in many physiological processes, whereas high concentrations of NO, produced e.g. by the inducible NOS in the course of inflammatory diseases, initiate apoptosis and necrosis.

PDGF, a potent growth factor, is able to counteract NO-production by inhibition of iNOS expression and activity. This work analyses the reciprocal control mechanisms of NO on the PDGF signalling system in rat glomerular mesangial cells (MC).

In the first part of my work, the effect of NO on the expression of the PDGF ligands and their receptors was analysed. MC were stimulated with the NO delivering agent DETA-NO. PDGFR mRNA levels were analysed in polymerase chain reaction (PCR) for both receptor subunits. PDGFR-α mRNA expression was time- and dose-dependently induced by NO, whereas mRNA expression of the β-subunit was not significantly affected by DETA-NO. The time- and dose-dependent induction of PDGFR-α mRNA expression by NO could also be confirmed on the protein level by Western blotting.

In the second part, the influence of endogenously produced NO on PDGFR-α -protein expression was investigated. MC were stimulated with the cytokine IL-1β, which is a known inducer of iNOS expression in rat MC. Consequently, IL-1β induces PDGFR-α expression. To discriminate between the IL-1β-iNOS-NO pathway and direct IL-1β mediated promoter activation, cells were costimulated with the NOS inhibitor L-NMMA. L-NMMA was not able to completely reverse the IL-1β mediated induction of PDGFR-α protein expression, but it diminished the effect significantly down to 60 %. This result reveals, that IL-1β involves iNOS activation to a certain degree for PDGFR-α induction.

There are multiple mechanisms described for NO induced signalling. At subtoxic concentrations the most common pathway is mediated via activation of the soluble guanylyl cyclase (sGC). To examine whether NO uses the sGC pathway to induce PDGFR-α expression, DETA-NO stimulated MC were cotreated with the sGC inhibitor ODQ. ODQ reversed almost completely the NO-mediated effect. Additionally, MC were incubated with the sGC activator YC-1. YC-1 was able to mimic PDGFR-α protein induction concentration-

dependently. Taken together both experiments indicate that the NO induced stimulation of PDGFR-α expression is due to sGC activation.

Since NO affects many genes also on a transcriptional level, PDGFR-α promoter was cloned and promoter analysis was performed by the dual-luciferase reporter assay system. MC, transfected with a PDGFRα promoter/luciferase construct were stimulated with DETA-NO or with 8-Bromo-cAMP, a known inducer of PDGFR-α transcription. 8-Bromo-cAMP but not DETA-NO was able to induce PDGFR-α promoter transcription, indicating that the NO-dependent stimulation of PDGFR-α expression is mediated posttranscriptionally or, alternatively, that the putative NO responsive element in the PDGFR-α promoter is not included in the cloned genomic DNA fragment.

In the last part of my work, the functionality of NO on PDGFR-α expression was tested by analysis of the phosphorylation state of the α-receptor and of the downstream effector molecule protein kinase B (PKB). MC were prestimulated with DETA-NO and thereafter incubated for 15 min with PDGF-BB to activate the receptor. Western blot analysis, performed with anti-pTyr720-PDGFR-α-specific antibody, revealed that the NO-induced elevation of basal PDGFR-α protein level also increases PDGFR-α phosphorylation. Furthermore, an enhanced phosphorylation level of PKB was detected in DETA-NO pretreated cells, which were co-incubated with PDGF-AA, a PDGF isoform that selectively activates the PDGFR-α subunit.

In co-operation with Dr. L. Schäfer (University of Münster), the NO-modulated induction of PDGFR-α expression was confirmed in an *in vivo* model of anti-Thy.1.1 nephritis. Inhibition of iNOS by the iNOS-specific inhibitor L-N^6-(1-iminoethyl)lysine (L-NIL) reduced PDGFR-α expression as shown by immunohistochemistry and Western blot analysis.

In conclusion, I have demonstrated that exogenously applied or endogenously produced NO induces the expression of PDGFR-α protein and that this increase permits enhanced phosphorylation of downstream effector molecules, which indicates that the actual amount of the receptor dictates the strength of the mitogenic effects of PDGF. NO induces the expression of PDGFR-α *in vitro* and *in vivo*. The pathophysiological significance of the NO-mediated upregulation of PDGFR-α expression has yet to be clarified in further *in vivo* experiments.

VI – R E F E R E N C E S

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V I I – S U P P L E M E N T

7.1 Abbreviations

ACE	angiotensin converting enzyme
8-Br-cAMP	8-bromo-adenine monophosphate
BFGF	basic fibroblast growth factor
BSA	bovine serum albumin
CDNA	complementary DNA
CGMP	cyclic guanine monophosphate
Co	Control
Crk	chicken tumour virus no.10 regulator of kinases
DETA-NO	1-[2-(2-Aminoethyl)-N-(2-ammonioethyl) amino] diazen-1-ium-1,2-diolate
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
ECM	extracellular matrix
E. coli	Escherichia coli
ECL	enhanced chemiluminescence
EGF	epidermal growth factor
EDRF	endothelial derived relaxing factor
et al.	et alter
Fig	Figure
GAP	GTPase activating protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBM	glomerular basement membrane
sGC	soluble guanylyl cycylase
GF	growth factor
GMC	glomerular mesangial cell
GN	glomerulonephritis
GFR	glomerular filtration rate
H	hour
IκB	inhibitor of κB
IκB-K	IκB-kinase
IL-1β	Interleukin 1-β
IPTG	isopropylthioglactoside
KDa	kilo Dalton
L-NIL	L-N^6-(1-iminoethyl)lysine
L-NMMA	N^G-monomethyl-L-arginine
Min	minute
mRNA	messenger RNA
NF-κB	nuclear factor-κB
NO	nitric oxide
NOS, eNOS, iNOS, nNOS	nitric oxide synthase, endothelial NOS, inducible NOS, neuronal NOS
OD	optical density
ODQ	1H[1,2,4]oxadiazolo[4,3-a]-quinoxalin-1-one
PBS	phosphate-buffered saline
PCR	polymerase chain reaction

PDE	phosphodiesterase
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
pPDGFR	phosphorylated PDGF
PI3-K	Phosphatidylinositol 3'-kinase
PKB	protein kinase B
pPKB	phosphorylated PKB
PKG	protein kinase G
PLC-γ	phospholipase C-γ
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
ROS	reactive oxygen species
Rpm	Rounds per miniute
RT	reverse transcriptase
RTK	receptor tyrosine kinase
SDS	sodium dodecyl sulfate
Sec	seconds
SH-2	Src homology domain 2
SPARC	secreted protein acidic and rich in cysteine
STAT	signal transduction and activator of transcription
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF-β	transforming growth factor-β
VEGF	vascular endothelial growth factor
v/v	volume per volume
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
YC-1	3-5'Hydroxymethyl-2'-furyl-1benzylindazole

7.2 Zusammenfassung

Stickoxid (NO) moduliert die Regulation des Plättchenwachstumsfaktorrezeptors α (PDGFR-α) in renalen Mesangiumzellen (MZ) der Ratte

NO ist ein gasförmiges Molekül, das durch drei verschiedene NO-Synthasen hergestellt werden kann. Die Signalkaskaden von NO sind multipel und sehr stark von der jeweiligen Konzentration abhängig. In niedrigen Dosen ist NO an der Regulation physiologischer Prozesse beteiligt, wohingegen hohe NO-Spiegel, wie sie von der induzierbaren NO Synthase (iNOS) im Verlauf von entzündlichen Erkrankungen produziert werden, zytotoxische Effekte wie Apoptose und Nekrose bedingen können. Der Wachstumsfaktor PDGF kann durch Inhibition der iNOS Expression, dieser hohen NO Produktion entgegen wirken. Ob NO im Gegenzug auch eine Wirkung auf das PDGF-System aufweist, sollte mit dieser Arbeit geklärt werden. Da die Aktivität von PDGF letztendlich von der Rezeptormenge abhängt, wurde die expressionsmodulatorische Wirkung von NO auf der PDGF-Rezeptorebene untersucht.

Im ersten Teil der Arbeit wurden MZ mit dem NO-Donator DETA-NO stimuliert. Mittels PCR-Analyse konnte gezeigt werden, dass NO die PDGFR-α-mRNA Expression zeit- und dosisabhängig induziert. Die Expression von PDGFR-β wird hingegen nicht wesentlich beeinflusst. Western-Blot-Analysen (WB) bestätigten die Regulierbarkeit des PDGFR-α auch auf Translationsebene. Als nächstes sollte geprüft werden, ob die durch exogene Applikation eines NO-Donatoren hervorgerufene Induktion des PDGFR-α auch durch eine endogene NO-Produktion imitiert werden kann. Hierzu wurden MZ mit dem Zytokin IL-1β inkubiert. IL-1β steigert die iNOS und auch die PDGFR-α-Expression durch Induktion von Transkriptionsfaktoren. Die IL-1β bedingte PDGFR-α-Expression könnte dabei über zwei Mechanismen reguliert werden: Einerseits über die gesteigerte Synthese von NO durch iNOS und andererseits durch direkte Interaktionen der IL-1β-induzierten Transkriptionsfaktoren mit dem PDGFR-α-Promotor. Um die NO bzw. iNOS-vermittelte von der Promotor-vermittelten Wirkung zu unterscheiden, wurden MZ zusätzlich mit dem NOS-Inhibitor L-NMMA inkubiert. L-NMMA war im Stande die durch IL-1β hervorgerufene Erhöhung der PDGFR-α Proteinmenge signifikant auf 60% zu reduzieren, was die Beteiligung der iNOS bzw. von NO an der IL-1β vermittelten Regulation des PDGFR-α impliziert.

NO entfaltet seine Wirkung über verschiedene Signalkaskaden. Der klassische Weg verläuft über die Aktivierung der löslichen Guanylatzyklase (sGC). Um die Beteiligung der sGC an der NO-vermittelten Induktion des PDGFR-α zu untersuchen, wurden DETA-NO stimulierte MZ zeitgleich mit ODQ, einem Inhibitor der sGC inkubiert. Die durch NO verursachte

Erhöhung der PDGFR-α-Proteinmenge konnte durch die gleichzeitige Zugabe von ODQ komplett gehemmt werden. Die Behandlung von MC mit dem sGC-Aktivator YC-1 imitierte andererseits den NO-Effekt. Beide Versuche zusammengenommen beweisen die Notwendigkeit der sGC-Aktivierung zur NO-vermittelten Induktion des PDGFR-α.

Da viele Gene schon auf Transkriptionsebene durch NO beeinflusst werden, wurde der an einen Vektor gebundene PDGFR-α-Promotor vor das Luziferase-Gen kloniert und MZ mit diesem Konstrukt transfiziert. Die transfizierten MZ wurden mit DETA-NO oder 8-Bromo-cAMP stimuliert. cAMP erhöht die Aktivität des PDGFR-α-Promotors und diente somit als Positivkontrolle. Die Promotoraktivität wurde indirekt über das Luziferase-Renilla-System bestimmt. Da NO im Gegensatz zu 8-Bromo-cAMP die Promotoraktivität nicht erhöhte, ist davon auszugehen, dass die NO-abhängige Induktion des PDGFR-α posttranskriptionell erfolgt oder, dass das NO-responsive Element nicht in unserem Konstrukt enthalten war.

Im letzten Abschnitt meiner Arbeit wurde die Funktionsfähigkeit des neusynthetisierten PDGFR-α Proteins bestätigt. Hierzu wurde die Phosphorylisierung vom PDGFR-α und von einem weiteren in der PDGF-Signalkaskade angeordneten Enzym, der antiapoptotisch wirksamen Proteinkinase B (PKB) untersucht. Mit DETA-NO vorbehandelte MZ wurden mit PDGF-BB stimuliert und eine Nachweisanalyse mit einem Phospho-spezifischen Antikörper der gegen pTyr720-PDGFR-α (pPDGFR-α) gerichtet ist, durchgeführt. Der Vergleich mit nichtvorbehandelten MZ belegt eindeutig, dass die NO vermittelte Erhöhung der basalen PDGFR-α-Proteinmenge auch zu einer Zunahme an detektierbarem pPDGFR-α führt, die dann wiederum eine Verstärkung der Signaltransduktion zur Folge hat. So konnten in DETA-NO vorbehandelten MZ, die mit dem α-Rezeptor spezifischen Liganden PDGF-AA stimuliert wurden, eine vergleichsweise erhöhte PKB-Phosphorylierung festgestellt werden.

In einer Kooperation mit Dr. L. Schäfer (Universität Münster) konnte ferner gezeigt werden, dass die NO-Abhängigkeit der PDGFR-α Proteinexpression auch im Krankheitsverlauf eines experimentellen Glomerulonephritismodells, zu beobachten ist. Durch WB-Analysen und immunhistologischen Färbungen konnte dargelegt werden, dass die Vorinjektion des iNOS-spezifischen Inhibitors L-NIL die Produktion von phosphoryliertem und unphosphoryliertem PDGFR-α Protein in Anti-Thy.1.1-behandelten Ratten signifikant hemmt.

Zusammenfassend weisen die Ergebnisse darauf hin, dass NO über eine Aktivierung der sGC, die Produktion von funktionsfähigem PDGFR-α- Protein *in vitro* und *in vivo* steigert. Die pathophysiologische Bedeutung der NO-vermittelten Induktion des PDGFR-α im Krankheitsprozess der GN, wird gegenwärtig in weiteren in vivo Experimenten untersucht.

7.3 Publications

This work has been presented in the following journal and congress-meetings:

Publication:
Beck, K.-F., Güder, G., Schaefer, L., Pleskova, M., Mihalik, D., Apel, M., Schaefer, R. M. and Pfeilschifter, J. (2004) Nitric oxide upregulates induction of Platelet-derived growth factor receptor in rat renal mesangial cells and in anti-Thy 1 glomerulonephritis. *J. Am. Soc. Nephrol.* **(In Revision).**

Congress-meetings:
Güder, G., Beck, K.-F., Pleskova, M. and Pfeilschifter, J. (2002) Expression of the PDGF receptor α in rat mesangial cells is up-regulated by nitric oxide. *43. Frühjahrstagung der Deutschen Gesellschaft für experimentelle und klinische Pharmakologie und Toxikologie in Mainz, Germany, 12.-14. March.*

Güder, G., Beck, K.-F., Pleskova, M. and Pfeilschifter, J. (2002) Expression of the PDGF receptor α in rat mesangial cells is up-regulated by nitric oxide. *53. Mosbacher Kolloquium, in Mosbach, Germany, 5. - 7. April.*

Beck, K.-F., Güder, G., Pleskova, M., Apel, M. and Pfeilschifter, J. (2004) Nitric oxide causes induction of PDGF receptor α expression and downstream signalling events. *45. Frühjahrstagung der Deutschen Gesellschaft für experimentelle und klinische Pharmakologie und Toxikologie in Mainz, Germany, 9. - 11. March.*

Beck, K.-F., Güder, G., Schaefer, L., Pleskova, M., Mihalik, D., Apel, M., Schaefer, R. M. and Pfeilschifter, J. (2004) Stickoxid induziert die Expression des PDGF Rezeptors-alpha in Rattern Mesangiumzellen und im Verlauf der Anti-Thy.1 Glomerulonephritis. *35. Kongress der Gesellschaft für Nephrologie, Basel, Switzerland; 18. - 21. September.*

Beck, K.-F. Güder, G., Schaefer, L., Pleskova, M., Mihalik, D., Apel, M., Schaefer, R.M. and Pfeilschifter, J. (2004) Stickoxid steigert die Expression des PDGF Rezeptors α in vitro und in vivo. *NO-Forum deutschsprachiger Länder in Mainz, Germany, 5.- 6. Oktober.*

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S P R A C H E N

- Türkisch: Muttersprache
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- Französisch: Fließend

7.6 Ehrenwörtliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin zur Promotionsprüfung eingereichte Arbeit mit dem Titel

„Nitric Oxide (NO) Modulates Platelet-derived Growth Factor Receptor-α (PDGFR-α) Expression in Rat Renal Mesangial Cells"

am Institut der Allgemeinen Pharmakologie und Toxikologie unter der Leitung von Herrn Prof. Dr. Josef Pfeilschifter, mit Unterstützung durch Dr. Karl-Friedrich Beck,

ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe.

Ich habe bisher an keiner in- oder ausländischen Medizinischen Fakultät ein Gesuch um Zulassung zur Promotion eingereicht noch die vorliegende Arbeit als Dissertation vorgelegt.

Vorliegende Arbeit wurde in folgendem Publikationsorgan
"Journal of the American Society of Nephrology"
veröffentlicht.

Hanau, den 10.11.2004