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Vorstand: Prof. Dr. med. Detlef Schlöndorff

# Nitric oxide modulates the expression of matricellular genes involved in fibrosis in renal glomerular mesangial cells

Dissertation
zum Erwerb des Doktorgrades der Humanbiologie an der Medizinischen Fakultät der Ludwig-Maximilians-Universität zu München

Vorgelegt von
Javid Wani (M.Sc.)
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***FROM THE MEDICAL POLICLINIC LUDWIG MAXIMILIANS UNIVERSITY, MUNICH***

Director: Prof. Dr. med. Detlef Schlöndorff

# Nitric oxide modulates the expression of matricellular genes involved in fibrosis in renal glomerular mesangial cells

Dissertation

Submitted for the acquisition of the degree of Ph.D. Human Biology

Faculty of Medicine, Ludwig-Maximilians University, Munich

Presented by

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Kashmir, India

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*Dedicated to my parents,*
*To my wife, Sharifa,*
*And to my daughters,*
*Mutaharah and Jasmine*

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Praise be to Almighty Allah who is worth all the praises and from whom I seek help and guidance. Thanks to the All-Compassionate and the Most-Merciful who gave me the courage and the strength to complete my doctoral studies. Peace and blessings of Allah be upon Prophet Mohammad whose prophecies motivated me to choose biomedical research as my career. His following encounter with a person, 1500 years ago, was the source of my inspiration and made me to believe that sincere efforts of scientists to understand the basis of complex diseases will lead to better treatment strategies and will help alleviate the sufferings of mankind.

"A desert dweller once asked the Prophet Mohammad (peace be upon him):

What type of people are the best?

The Prophet (peace be upon him) replied: The best of them in moral character.

The man then asked: O' Messenger of Allah, should we seek medical treatment?

The Prophet (peace be upon him) replied: Seek medical treatment, for truly Allah does not send down a disease without sending down a cure for it. Those who have knowledge of the cure know it, and those who are ignorant of it do not *(reported in Musnad Ahmad)*".

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"Self-motivation makes a person a good candidate. You will never finish if you are not self motivated. Obstacles will likely be thrown in your path and you have to overcome them. Your

success depends on you. You need to be a hard worker. A Ph.D. is an endurance contest, many people do not finish....You can succeed in a masters program with intelligence alone, but a Ph.D. program requires intelligence, effort, and endurance".
--Dr. Bonnie Anderson, *Assistant Professor of Information Systems, Brigham Young University Provo, Utah, USA.*
Finally, I would like to thank the entire Clinical Biochemistry group for their roles in the making of this work.

## *Figures*

## *Abbreviations*

| | |
|---|---|
| BG | Biglycan |
| BH4 | Tetrahydro-L-biopterin (dihydrochloride); |
| BSA | Bovine serum albumin |
| COL1A1 | Procollagen type I alpha1 |
| COL1A2 | Procollagen type I alpha2 |
| COL4A2 | Collagen type IV alpha2 |
| COL5A1 | Collagen type V alpha1 |
| Cpm | Counts per minute |
| CTGF | Connective tissue growth factor |
| DETA NONOate | DiethylenetriaamineNONOate |
| DMEM | Dulbecco's modified Eagle's medium |
| DNA | Deoxyribose nucleic acid |
| Dpm | Disintergrations per minute |
| DTT | Dithiotheritol |
| ECM | Extracellular matrix |
| EDTA | Ethylendiaminotetraacetic acid |
| EGF | Epidermal growth factor |
| EGR-1 | Early growth response gene-1 |
| ELISA | Enzyme linked Immunosorbent Assay |
| eNOS | Endothelial nitric oxide synthase |
| FCS | Fetal calf serum |
| GAPDH | Glyceraldehyde-3-posphate-dehydrogenase |
| GSNO | S-Nitroso-L-glutathione |
| ICAM-1 | Intracellular adhesion molecule |
| IL-1β | Interleukin-1β |
| INF-γ | Interferon gamma |
| iNOS | Inducible nitric oxide Synthase |
| IRF | Interferon regulatory factor |
| Jak | Janus kinase |
| Kb | Kilobase |

| | |
|---|---|
| L-NIL | L-N6-(l-iminoethyl) L-lysinedihydrochloride |
| L-NMMA | N5-[imino(methylamino)methyl1]-L-Ornithine,citrate |
| LPS | Lipopolysaccharide |
| MC | Mesangial cells |
| NF-kB | Nuclear factor kB |
| nNOS | Neuronal nitric oxide synthase |
| NO | Nitric oxide |
| NOC-18 | 2, 2‘-(Hydroxynitrosohydrazino) bisethanamine |
| PBS | Phosphate buffered saline |
| RDA | Representational difference analysis |
| RNA | Ribose nucleic acid |
| Rnase | Ribonuclease |
| Rpm | Revolutions per minute |
| RT-PCR | Real time polymerase chain reaction |
| SEAP | Secreted alkaline phosphatase |
| SMAD | Mothers against DPP |
| SNAP | S-Nitroso-N-acetyl-dL-penicillamine |
| SOD | Superoxide dismutase |
| STAT | Signal transducers and activators of transcription |
| SV40 | Simian Virus 40 |
| TBE | Tris-Borate-EDTA |
| TF | Transcription factor |
| TGF-β | Transforming growth factor-β |
| TGF-βR | Transforming growth factor receptor |
| TIMP | Tissue inhibitor of metalloprotinases |
| TNFα | Tumor necrosis factor-α |
| TP53 | Tumor suppressor protein 53 |
| TSP-1 | Thrombospondin-1 |
| WT1 | Willm’s tumor |

# *Preface*

Nitric oxide (NO) is produced in response to stress and can lead to tissue injury because of its radical chemistry, or may be cytoprotective by destroying pathogenic microorganisms. NO causes the relaxation of vascular smooth muscles and has antiproliferative effects on mesangial cells (MC). Interactions of MC with components of the extracellular matrix (ECM) influence MC attachment, contraction, migration, survival and proliferation. ECM deposition leads to glomerular inflammation and fibrosis. Mesangial cells have proliferative and secretory potential which makes them important mediators of glomerular inflammation and fibrosis. In the presence of exogenous NO, adherent MC show detachment and exhibit disturbed organization of α-actin filaments and a reduction in the number of focal adhesions. NO has also been shown to inhibit the expression of intercellular adhesion molecule-1 (ICAM-1) in rat mesangial cells (Ikeda et al., 1996). Recent studies have established a direct role for NO in the regulation of gene expression in different cell types including MC. In MCs NO regulates the transcription of various genes such as MIP-2, MMP-9, SPARC, Biglycan, IAP, HO-1 and Cu/Zn- SOD. However, the extent and the level at which NO regulates the expression of ECM genes in MCs have not been systematically studied.

# *Introduction*

## Nitric Oxide

Nitric oxide (NO) is as a major signaling molecule in neurons and in the immune system, either acting within the cell in which it is produced or by penetrating cell membranes to affect adjacent cells. NO first captured the interest of biologists when this inorganic molecule was found to activate cytosolic guanylate cyclase and stimulate cyclic guanosine monophosphate (GMP) formation in mammalian cells. Further studies led to the finding that nitric oxide causes vascular smooth muscle relaxation and inhibition of platelet aggregation by mechanisms involving cyclic GMP and that several clinically used nitrovasodilators owe their biological actions to nitric oxide. NO is synthesized by vascular endothelium from the terminal guanido nitrogen atom(s) of the amino acid L-arginine (Moncada *et al.*, 1988; Torreilles and Guerin, 1995). Nitric oxide possesses physicochemical and pharmacological properties that make it an ideal candidate for a short-term regulator or modulator of vascular smooth muscle tone and platelet function. Nitric oxide is synthesized by various mammalian tissues including vascular endothelium, macrophages, neutrophils, hepatic Kupffer cells, adrenal tissue, cerebellum, mesangial cells and other tissues.

## 1.2. Synthesis of Nitric Oxide

Nitric oxide is synthesized from endogenous L-arginine by a nitric oxide synthase system that possesses different cofactor requirements in different cell types. Two primary steps have been identified. The first step, a two electron oxidation, is a hydroxylation of one of the guadino nitrogens of L-arginine requiring molecular oxygen and nicotinamide adenine diphosphate (NADPH) to form NG- hydroxy-L- arginine. The second step is a three electron oxidation, again requiring molecular oxygen and NADPH to perform an electron removal, oxygen insertion, and carbon-nitrogen bond cleavage to form L- citrulline and the free radical nitric oxide (Furchgott, 1993) (Fig.1).

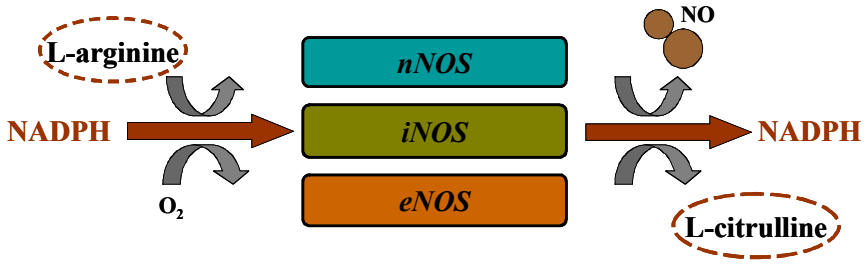



**Figure 1 Biosynthesis of NO catalyzed by NO synthase**

## 1.3. Nitric Oxide Signaling

Nitric oxide (NO) is a major signal transduction molecule in vertebrates. The NO formed diffuses out of its cells of origin and into nearby target cells, where it binds to the heme group of cytosolic guanylate cyclase and thereby causes enzyme activation (Ignarro, 1992). This interaction represents a widespread signal transduction mechanism that links extracellular stimuli to the biosynthesis of cyclic GMP in nearby target cells. The small molecular size and lipophilic nature of nitric oxide enable communication with nearby cells containing cytosolic guanylate cyclase. The extent of transcellular communication is limited by the short half-life of nitric oxide, thereby ensuring a localized response. Labile nitric oxide-generating molecules such as S-nitrosothiols may be involved as precursors or effectors (Ignarro, 1990; Ignarro, 1992). NO has effects on neuronal transmission as well as on synaptic plasticity in the central nervous system (Lipton, 1999). In the vasculature (Fig. 2), NO reacts with iron in the active site of the enzyme guanylyl cyclase (GC), stimulating it to produce the intracellular mediator cyclic GMP (cGMP), that in turn enhances the release of neurotransmitters resulting in smooth muscle relaxation and vasodilation (Murad, 1998).

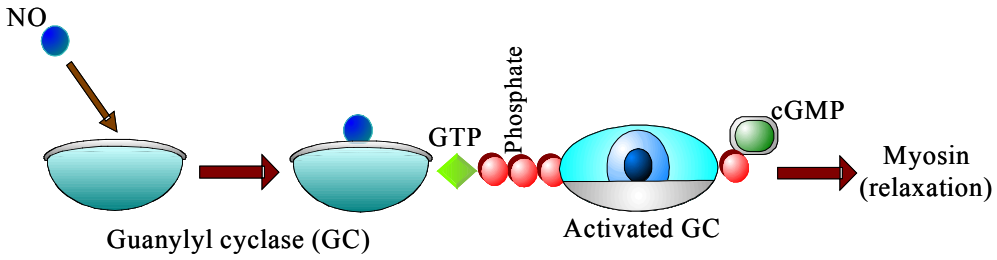



**Figure 2 Mechanism of NO signaling in the vasculature – a classical NO signaling mechanism**

NO has also been shown to activate diverse signaling pathways that affect gene expression in mammalian cells. A recent microarray study in NIH3T3 fibroblasts has shown that in addition to c-GMP NO employs different signaling pathways to activate gene expression (Hemish et al., 2003). These signaling pathways have been identified to be NF-kB-, PI3K-, PKC- and p53-dependent and the gene families targeted by NO were involved in the regulation of transcription, cell cycle, apoptosis, metabolism, oxidative stress, membrane transport, extracellular matrix and adhesion.

In addition to these pathways, different studies have suggested that all the major MAPK cascades including ERK, SAPK/JNK and p38 kinase cascades as well as JAK/STAT pathways were involved in the signaling by NO (reviewed in Beck et al., 1999). These cascade events then trigger the phosphorylation of key nuclear proteins, including transcription factors such as Egr-1 (Rupprecht et al., 2000; Cibelli et al., 2002), c-jun (Kim et al., 1997), ternary complex factors or STATs (Pfeilschifter et al., 2001 “b”) and, finally, lead to alterations in gene expression.

## 1.4. Nitric Oxide Metabolism

The discovery of the arginine- oxygenase pathway for nitric oxide (NO) biosynthesis was one of the greatest and the most surprising discoveries in modern biochemistry and won the Nobel Prize in 1998. The history of its discovery as a mediator is fascinating, and its role in mammalian biology and medicine is proving to be of fundamental importance. NO may also be involved in the regulation of protein activity through S-nitrosylation. In the extracellular milieu NO reacts with oxygen and water to form nitrates and nitrites. NO toxicity is linked to its ability to combine with superoxide anions (O2–) to form peroxynitrite (ONOO–), an oxidizing free radical that can

cause DNA fragmentation and lipid oxidation. In the mitochondria, ONOO– acts on the respiratory chain (I-IV) complex and manganese superoxide dismutase (MnSOD), to generate superoxide anions and hydrogen peroxide (H2O2), respectively (Brown, 1999) (Fig. 3).

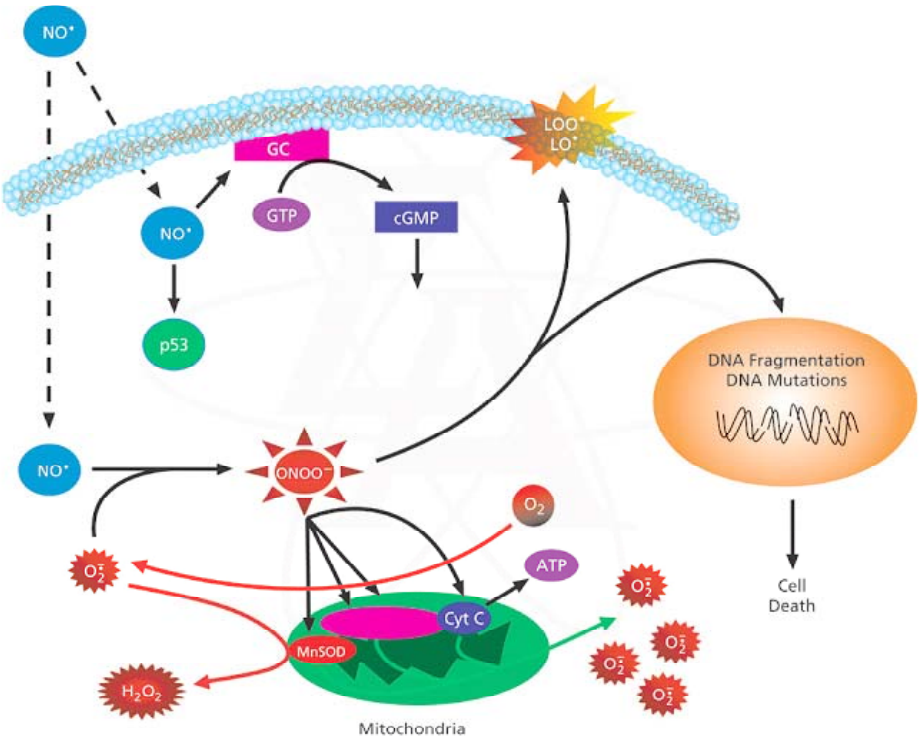



**Figure 3 Nitric Oxide metabolism – a schematic representation**

(Source : www.sigma-Aldrich.com)

## 1.5. Reactive nitrogen species (RNS) and NO

Under physiological conditions, NO reacts with molecular oxygen and reactive oxygen species (ROS) to produce intermediates known as reactive nitrogen species (RNS). The production of NO and RNS in the cell is controlled by hormones, neurotransmitters, cytokines, and growth factors. Hence NO and its derivatives act as secondary paracrine factors and transmit the signal from NO-producing to neighboring cells. Intracellular reception of NO and RNS is due to Src-related tyrosine protein kinases, G-protein Ras, cytochrome oxidase, and guanylate cyclase. Receptor proteins mostly contain heme, active thiol, or iron-sulfur groups, and are both on the plasma membrane and in internal cell compartments. Many of the NO receptors are key

components of cell regulatory systems controlling the transcription factors AP-1, HIF-1, NF-kappa B, and p53 and the expression of their target genes. Depending on the ROS level, NO activates different signal transduction pathways to induce (or suppress) different gene sets (Turpaev and Litvinov, 2004).

NO has received special attention ever since: besides its potent vasodilatory and vasoprotective effects, NO was identified as a key player in innate immunity and was found to act as an unconventional type of neurotransmitter.

## 1.6. NO and oxidative stress

Uncontrolled nitric oxide generation leads to oxidative stress by producing superoxide leading to the production of oxidants such as peroxinitrite, nitrogen dioxide and hydroxyl radicals. Overproduction of NO in response to bacterial endotoxins and cytokines has been shown to promote undesired increases in vasodilatation, which may account for hypotension in septic shock and during cytokine therapy. Excessive NO production can strongly inhibit S-nitrosylation of glyceraldehyde-3-phosphate dehydrogenase in a cGMP-independent mechanism resulting in reduced cellular energy production (Molina y Vedia *et al*., 1992).

On the other hand, high blood glucose levels, altered insulin signaling, reactive oxygen species (ROS), inflammation, and protein kinase C activation may lead to a decrease in NO bioavailability. Oxidative stress and decreased NO bioavailability can lead to vascular damage, such as endothelial dysfunction, vascular inflammation, atherosclerotic plaque formation and promotion of a prothrombotic state (Olson *et al.*, 1995;Lehr *et al.*, 2000; Suematsu *et al.*, 2002; Li and Shah, 2004). Possible sources of oxidative stress are reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, uncoupled NO synthase, and the mitochondria (Endemann and Schiffrin, 2004). LPS-induced overproduction of NO has been shown to inhibit cytochrome P450-dependent metabolism and to mediate the suppression of hepatic metabolism (Duval *et al.*, 1996; Kawada *et al.*, 1998; Heller *et al.*, 2000; Ding *et al.*, 2003). Moreover, NO synthetized in the peripheral nervous system is known to mediate nonadrenergic noncholinergic (NANC) neurotransmission (Boeckxstaens and Pelckmans, 1997). Overstimulation of NO synthases might therefore contribute to pathological states such as: reflux oesophagitis (Holzer, 1995; Giacoia, 1995; Martin *et al.*, 1997; Xiong *et al.*, 1999), asthma, adult

respiratory distress syndrome (ARDS) and chronic pulmonary artery hypertension (Barton *et al.*, 1995; Giacoia, 1995; Martin *et al.*, 1997; Camsooksai, 1997;Gitto *et al.*, 2001). NO-mediated biological functions also include the biological effects of NO-derivatives such as N-nitrosocompounds, which act as carcinogenic agents, or C-nitrosocompound which were recently used as "zinc-ejecting" agents to inhibit HIV-1 infectivity of human T-lymphocytes.

## 1.7. Nitric oxide in immune regulation

Nitric oxide (NO) and reactive oxygen species exert multiple modulating effects on inflammation and play a key role in the regulation of immune responses (Shah and Billiar, 1998). They affect virtually every step of the development of inflammation. Low concentrations of nitric oxide produced by constitutive and neuronal nitric oxide synthases inhibit adhesion molecule expression (Ruetten *et al.*, 1999; Kim *et al.*, 2001), cytokine and chemokine synthesis (Biswas *et al.*, 2001) and leukocyte adhesion and transmigration (Suematsu *et al.*, 2002). Large amounts of NO, generated primarily by iNOS can be toxic and pro-inflammatory (Hansen, Jr. *et al.*, 1998). Actions of nitric oxide are however not dependent primarily on the enzymatic source, but rather on the cellular context, NO concentration (dependent on the distance from NO source) and initial priming of immune cells (Wang *et al.*, 2003). These observations may explain difficulties in determining the exact role of NO in Th1 and Th2 lymphocyte balance in normal immune responses and in allergic disease. Similarly superoxide anion produced by NADPH oxidases present in all cell types participating in inflammation (leukocytes, endothelial and other vascular cells etc) may lead to toxic effects, when produced at high levels during oxidative burst, but may also modulate inflammation in a far more discrete way (Diefenbach *et al.*, 1998), when continuously produced at low levels by NOXs (non-phagocytic oxidases) (Catz and Sterin-Speziale, 1996). The effects of both nitric oxide and superoxide in immune regulation are exerted through multiple mechanisms, which include interaction with cell signaling systems like cGMP, cAMP, G-protein, JAK/STAT or MAPK dependent signal transduction pathways. They may also lead to modification of transcription factors activity and in this way modulate the expression of multiple other mediators of inflammation (Guzik *et al.*, 2003).

## 1.8. Nitric Oxide Synthases (NOS)

Three isozymes of nitric oxide synthase (NOS) have been identified. Their cDNA- and protein structures as well as their genomic DNA structures have been described. ncNOS (NOS I, originally discovered in neurons) and ecNOS (NOS III, originally discovered in endothelial cells) are low output, $Ca^{2+}$-activated enzymes whose physiological function is signal transduction. iNOS (NOS II, originally discovered in cytokine-induced macrophages) is a high output enzyme which produces toxic amounts of NO that represents an important component of the antimicrobial (Hibbs, Jr., 2002), antiparasitic (Fang, 2004) and antineoplastic (Drosten and Putzer, 2003) activity of these cells. Depending on the species, iNOS activity is largely (human) or completely (mouse and rat) Ca (2+)-independent. In the human species, the NOS isoforms I, II and III are encoded by three different genes located on chromosomes 12, 17 and 7, respectively. The amino acid sequences of the three human isozymes (deduced from the cloned cDNAs) show less than 59% identity. Across species, amino acid sequences are more than 90% conserved for NOS I and III, and greater than 80% identical for NOS II. All NOS produce NO by oxidizing guadino nitrogen of L-arginine utilizing molecular oxygen and NADPH as co-substrates. All isoforms contain FAD, FMN and heme iron as prosthetic groups and require the cofactor BH4. NOS I and III are constitutively expressed in various cells. Nevertheless, expression of these isoforms is subject to regulation. Expression is enhanced by e.g. estrogens (for NOS I and III), shear stress, TGF-beta 1, and (in certain endothelial cells) high glucose (for NOS III). TNF-alpha reduces the expression of NOS III by a post-transcriptional mechanism destabilizing the mRNA. The regulation of the NOS I expression seems to be very complex as reflected by at least 8 different promoters transcribing 8 different exon 1 sequences which are expressed differently in different cell types. Expression of iNOS is mainly regulated at the transcriptional level and can be induced in many cell types with suitable agents such as LPS, cytokines, and other compounds. Whether some cells can express iNOS constitutively is still under debate. Pathways resulting in the induction of the iNOS promoter may vary in different cells. Activation of transcription factor NF-kappa B seems to be an essential step for iNOS induction in most cells. The induction of iNOS can be inhibited by a wide variety of immunomodulatory compounds acting at the transcriptional levels and/or post-transcriptionally (Forstermann and Kleinert, 1995).

The functional NOS protein is formed of two identical sub-units. There are three distinct domains in each NOS sub-unit: a reductase domain, a calmodulin-binding domain and an oxygenase domain (Fig. 4)

- The reductase domain: This domain contains the FAD and FMN moieties and it acts to transfer electrons from NADPH to the oxygenase domain. It should be noted that the reductase domain transfers electrons to the oxygenase domain of the *opposite* sub-unit of the dimer, and not to the domain on the same sub-unit.
- Calmodulin binding: The binding of calmodulin is required for the activity of all the NOS isoforms. It detects changes in intracellular calcium levels, although its precise function is slightly different in each of the three isoforms.
- The oxygenase domain: This domain contains the binding sites for tetrahydrobiopterin, haem (heme) and arginine. The oxygenase domain catalyses the conversion of arginine into citrulline and NO.

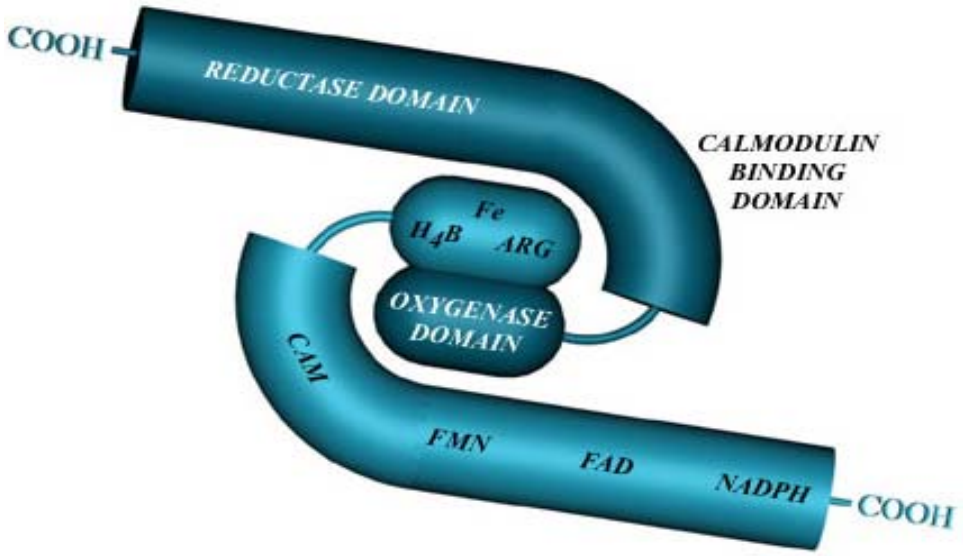



**Figure 4 General structure of the NOS enzymes**

## 1.9. Characteristics of Nitric Oxide Synthases

- Isoform : ncNOS (125-135 K Da monomer)
  Alternative names : Neuronal NOS/NOS typeI/NOS1
  Human cellular sources : Neurons, adrenal medulla, renal macula densa, glia and astrocytes
  Location: Membrane associated
  Presence: Constitutive
  Activation: Calcium increase leading to calmodulin binding
- Isoform : iNOS (155 K Da monomer)
  Alternative names : Inducible NOS/NOS type II/NOS2
  Human cellular sources : Macrophages, monocytes, leukocytes, endothelium, smooth muscle, neutrophils, retinal pigmented epithelium, astrocytes, microglial, hepatocytes, Küpfer cells, fibroblasts, mesangium
  Location: Cytosolic
  Presence: Inducible
  Activation: Transcriptional induction, Calcium independent, calmodulin always bound
- Isoform : ecNOS(135 K Da monomer)
  Alternative names : Endothelial NOS/NOS typeIII/NOS3
  Human cellular sources: Endothelium, platelets, smooth muscle
  Location: Membrane associated (inactive); cytosolic (active)
  Presence: Constitutive
  Activation: Calcium increase leading to calmodulin binding

*All enzymes active as dimmers.

## 1.10. Mechanism of INF-γ-mediated inducible nitric oxide synthase (iNOS) activation

Interferons (IFNs) encode a large family of multifunctional secreted proteins that are involved in antiviral defense, the regulation of cell growth and modulation of the immune response. They are subdivided into two types that activate transduction pathways via different cell surface receptors. Binding of both IFN type I and II results in the differential activation of JAK (Janus kinases) that phosphorylate latent cytoplasmic transcription factors termed STATs (signal transducer and activator of transcription). Phosphorylated STATs translocate to the nucleus, bind specific DNA elements and direct transcription (Fig. 5). Type I IFN induces the phosphorylation of STAT1 and STAT2 proteins by tyrosine phosphorylation involving the type I IFN receptor-associated tyrosine kinases TYK2 and JAK1. Following phosphorylation, STAT1 and STAT2 form the transcriptionally active IFN-stimulated gene factor 3 (ISGF3) by association with a protein of the IFN regulatory factor (IRF) family, p48 (Darnell *et al.*, 1994).

IFN-γ, a cytokine that is secreted from activated T cells and macrophages, has been shown to enhance NO production (Lorsbach *et al.*, 1993). T helper cell (Th1) cytokines have been reported to play a pathogenic role in some types of experimental glomerulonephritis, such as crescentic glomerulonephritis and Heymann nephritis (Kitching *et al.,* 1997, 1998; Chadban *et al.,* 1997; Farrar and Schreiber 1993). Interferon-γ (IFN-γ), which is representative of the Th1 cytokines, elicits cellular immune responses activating cytotoxic T lymphocytes, natural killer (NK) cells, and macrophages (Martin *et al.,* 1989). Simultaneously, it also changes the phenotype of a variety of resident cells in renal tissue, inducing and/or enhancing the expression of multiple histocompatibility complex (MHC) class I and II, ICAM-1 (Baudeau *et al.,* 1994; Coers et al., 1994), inducible nitric oxide synthase (iNOS) (Mohaupt *et al.,* 1998), chemokines (Grandaliano *et al.,* 1994), or Fc receptors (Santiago *et al.,* 1991). These effects result in the initiation and promotion of inflammatory processes in glomeruli (Sakatsume *et al.*, 2000). In chronic glomerulonephritis, mesangial cells produce relatively large amounts of NO in response to the activation of iNOS by synergistic action of several proinflammatory cytokines, which may destroy renal tissue, worsen proteinuria, and produce deterioration of renal function. Although overproduction of NO is harmful to the body, complete inhibition of NO production is not beneficial because of the contribution of NO to homeostasis (Kone and Baylis, 1997).

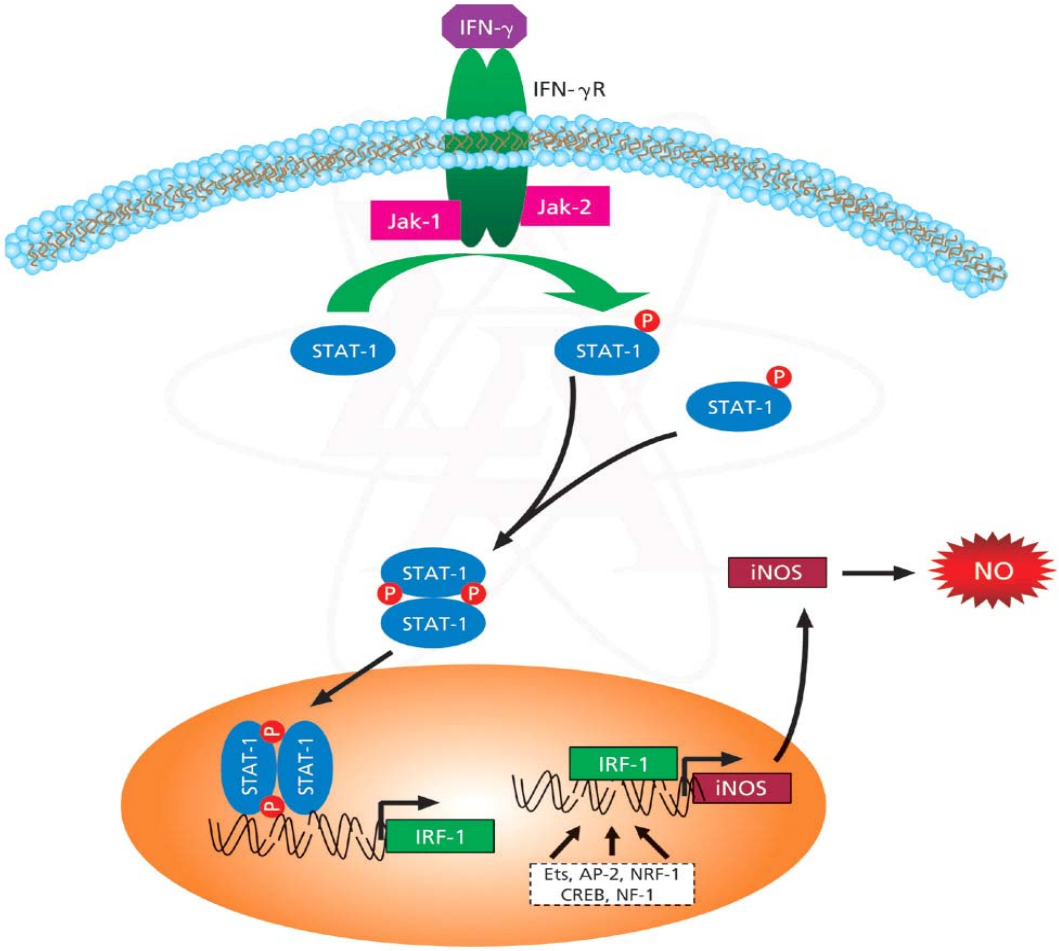



**Figure 5 Inducible nitric oxide synthase- signaling mediated by INF-γ**

(Source : www.sigma-Aldrich.com)

## 1.11. Nitric oxide and mesangial cells

NO causes the relaxation of vascular smooth muscles (Blatter and Wier, 1994) and has antiproliferative effects on mesangial cells (Rupprecht et al., 2000). Interactions of mesangial cells (MCs) with components of the extracellular matrix (ECM) influence MC attachment, contraction, migration, survival and proliferation. MCs by means of their proliferative and secretory potential are thought to be important mediators of glomerular inflammation and fibrosis. In the presence of exogenous NO, adherent MCs show detachment and exhibit disturbed organization of α-actin filaments and a reduction in the number of focal adhesions (Yao et al.,

1998). NO has also been shown to inhibit the expression of intercellular adhesion molecule-1 (ICAM-1) in rat mesangial cells (Ikeda et al., 1996). NO elicits changes in gene expression in rat MCs and regulates the transcription of genes such as MIP-2, MMP-9, SPARC, Biglycan, IAP, HO-1 and Cu/Zn- SOD (Bogdan, 2001; Pfeilschifter, 2002).

## 1.12. Nitric oxide and animal models of glomerular disease

In the healthy kidney, NO controls intrarenal hemodynamics, tubuloglomerular feedback response, pressure natriuresis, release of sympathetic neurotransmitters and renin, and tubular solute and water transport (Kone, 1997). In several animal models of chronic renal disease and glomerular inflammation, the administration of L-arginine, by increasing NO synthesis, has been shown to decrease the degree of glomerulosclerosis, reduce matrix score and TGF-β overexpression, ameliorate fibrotic changes in the tubulointerstitial compartment of the kidney, attenuate focal glomerulosclerosis and proteinuria and also to decrease the infiltration of the kidney by invading macrophages (reviewed by Groves and Wang, 2000).

Endogenously synthesized nitric oxide prevents endotoxin-induced glomerular thrombosis (Shultz and Raij, 1992). NO generated by the activation of iNOS has also been shown to have a protective role against tubulointerstitial injury and cytokine production in adriamycin nephropathy (Rangan et al., 2001). Inhibition of iNOS in a rat model of autoimmune interstitial nephritis shows host-protective effects of endogenously generated NO in an organ-specific manner (Gabbai et al., 1997). These results suggest that treatment modalities that increase nitric oxide formation might have a beneficial effect on the progression of cellular and molecular parameters of tubulointerstitial fibrosis and glomerular injury. However, chronic L-arginine supplementation, leading to a permanent increase in NO synthesis has been shown to be deleterious in renal ischemia, suggesting that excessive exposure to NO can be harmful (Peters et al., 1999).

## 1.13. Glomerular mesangial cells and glomerulonephritis

The glomerulus is a complex structure consisting of four cell types, namely, visceral epithelial, parietal epithelial, endothelial and mesangial cells. The glomerular mesangium consists of mesangial cells and extracellular matrix and plays a crucial role in maintaining structure and

function of the glomerular capillary tuft (Ardaillkjou, 1996). The mesangium is in direct contact with the fenestrated endothelium. Representing approximately one-third of the total number of glomerular cells, the turnover rate of MCs in the normal adult kidney is very low with a renewal rate of less than 1% (Pabst and Sterzel 1983). Hence, under normal conditions, quiescent MCs either face few mitogens or are unable to respond to mitogenic factors by downregulation of respective receptors or are protected by the presence of growth-inhibitory factors.

Glomerulonephritis is a common clinical condition that is caused by immune-mediated injury to the kidney and is characterized by dysfunction of the glomerular capillary filtration barrier. Inflammatory glomerular lesions are induced by circulating inflammatory cells or proliferating resident glomerular cells. In experimental models of glomerulopathy, increased MC proliferation often precedes the development of glomerulosclerosis with increased ECM deposition in the mesangium (Floege et al., 1992; Pesce et al., 1991). In experimental and human glomerular inflammatory diseases, two prominent histological features are (a) mesangial hyperplasia due to elevated proliferation rate or reduced cell loss of MCs and (b) altered and increased deposition of mesangial ECM. Mesangial reconstitution is required as part of the repair process during a destructive glomerular injury e.g., acute mesangiolysis or chronic diabetic glomerulosclerosis which is followed by the loss of MCs. Limitations in the renewal rate of MCs is compensated by the increase in extracellular matrix leading to sclerosis. On the other hand, growth of MCs may be inadequately controlled and results in chronic mesangioproliferative glomerulonephritis. In the second kind of lesion, MC proliferation is induced without obvious cell loss either due to mitogenic stimuli or by products of activated glomerular cells or recruited inflammatory cells. Regardless of the underlying mechanism, altered control of MC proliferation appears to play an important role in the pathogenesis of progressive glomerular abnormalities leading to glomerulosclerosis.

## 1.14. Mechanism of tissue response to injury

Scarring of soft internal organs such as liver, kidney and lung leads to loss of function and in certain circumstances death. Upon tissue injury inflammation occurs with platelets, fibroblasts, myofibroblasts, and eosinophils releasing transforming growth factor-beta (TGF-β) which stimulates fibroblasts and other reparative cells to proliferate and synthesize extracellular matrix components (Fig. 6). This leads to provisional repair, which under normal conditions results in involution, maturation, remodeling, reorganization, and regeneration. A continuation of provisional repair results in fibrosis and ultimately scarring.

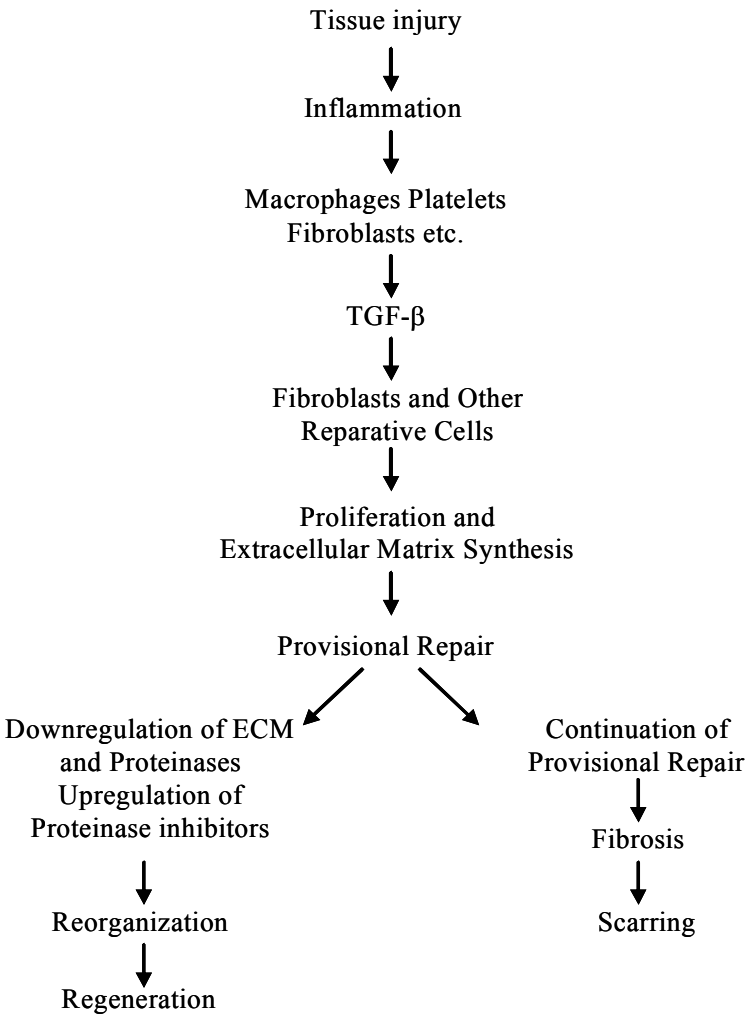



**Figure 6 Tissue response to injury**

## 1.15. Connective Tissue Growth Factor

CTGF is involved in extracellular matrix remodelling during development and in pathological conditions, and has increasingly been recognized as a pro-fibrotic factor e.g., in diabetic renal changes. It is a 36–38 kDa, cysteine-rich, secreted protein belonging to the CCN family of matricellular proteins. CTGF contains four modules: module 1 is an IGF-binding protein (IGFBP) domain, module 2 is a cysteine-rich von Willebrand type c (VWC) domain, module 3 is homologous to thrombospondin type 1 (TSP-1) and module 4 is a cysteine-rich C-terminal (CT) domain, found in several growth factors, including TGF-ß (Brigstock, 2003). CTGF's modular structure explains its multiple interactions with the cell surface, extracellular matrix and other growth factors. CTGF binds to integrins and heparan sulfate proteoglycans via module 4 (Gao and Brigstock, 2004) and low-density lipoprotein receptor-related protein (LRP) via module 3 (van Nieuwenhoven *et al.*, 2005). In addition, CTGF can interact with, and influence the signaling of, IGF-I (Lam *et al.*, 2003), VEGF (Hashimoto *et al.*, 2002) and TGF-ß and BMPs (Abreu *et al.*, 2002). Moreover, CTGF can be cleaved by metalloproteases (MMPs) and other proteases, and the resulting fragments have distinct biological activities (Brigstock, 2003). These properties and the fact that no signal transduction as a direct result of CTGF–receptor binding has been described make it likely that CTGF largely functions as a matricellular protein, modulating and integrating the role of other growth factors in extracellular matrix homeostasis.

## 1.16. Role of CTGF in kidney disease

Due to concerns regarding possible risks of long-term TGF-ß inhibition as a therapeutic approach to treat fibrotic diseases, CTGF was already recognized as a potential alternative target in 1997 (Franklin, 1997). In addition, targeting of CTGF as a possible therapy specifically for diabetic nephropathy has been proposed by several investigators (Goldschmeding *et al.*, 2000; Caramori *et al.*, 2000; Mason and Wahab, 2003). Some studies have been published in which CTGF inhibition was applied in an effort to attenuate renal fibrotic processes. *In vitro*, an antibody against CTGF partly inhibited the glucose-induced collagen production in human renal fibroblasts (Lam *et al.*, 2003), Glucose-induced elevated synthesis of fibronectin and plasminogen activator inhibitor-1 in human mesangial cell cultures was inhibited by CTGF antisense oligodeoxynucleotide (ODN) treatment (Wahab *et al.*, 2001). Transfection of CTGF

antisense ODN in cultured renal fibroblasts significantly attenuated TGF-ß-stimulated upregulation of fibronectin (Yokoi *et al.*, 2002). The same investigators showed that CTGF antisense ODN treatment *in vivo* attenuated renal fibrosis in rats after unilateral ureteral obstruction (Yokoi *et al.*, 2004 "b"). Moreover, in a very recent study, administration of a neutralizing CTGF antibody to db/db mice for 2 months showed beneficial effects in terms of reduced renal hypertrophy, UAE and hyperfiltration, while glomerular hypertrophy was unchanged (Flyvbjerg *et al.*, 2004) . Further, the diabetes-induced GBM thickening was significantly attenuated in CTGF-antibody treated mice (cf. attenuated GBM thickening in CTGF +/– STZ mice (Roestenberg *et al*., 2004 "b"), while the diabetes-associated increase in total mesangial volume was unaffected by the treatment (Flyvbjerg *et al.*, 2004). The safety and tolerability of the same CTGF antibody are currently being tested in a phase 1 clinical trial in patients with idiopathic pulmonary fibrosis. In addition to neutralizing antibodies and ODN, specific low molecular size inhibitors of CTGF are being developed and will be used to study the suitability of CTGF as a target for therapeutic intervention in diabetic nephropathy.

CTGF might modulate the signaling balance of key growth factors. CTGF contains four modules that associate with different growth factors, extracellular matrix proteins and cell surface proteins. The signaling activities of the different growth factors are influenced by binding to CTGF: IGF-I and TGF-ß1 signaling activity is enhanced, while BMP-4 and VEGF signaling activity is reduced by CTGF binding. The enhanced IGF-I and TGF-ß1 signaling is pro-fibrotic, while BMP signaling has been shown to reverse fibrosis (note that this has so far been shown for BMP-7 only, and binding of BMP-7 to CTGF has not been reported yet). VEGF signaling and angiogenic activity is inhibited by binding to CTGF, but is reactivated after cleavage of CTGF by MMPs. It thus appears that CTGF plays a key role in modulating the activity of several growth factors important in the development of diabetic kidney disease. In addition, CTGF might mediate cross-talk between signaling pathways by physical approximation of signaling receptors.

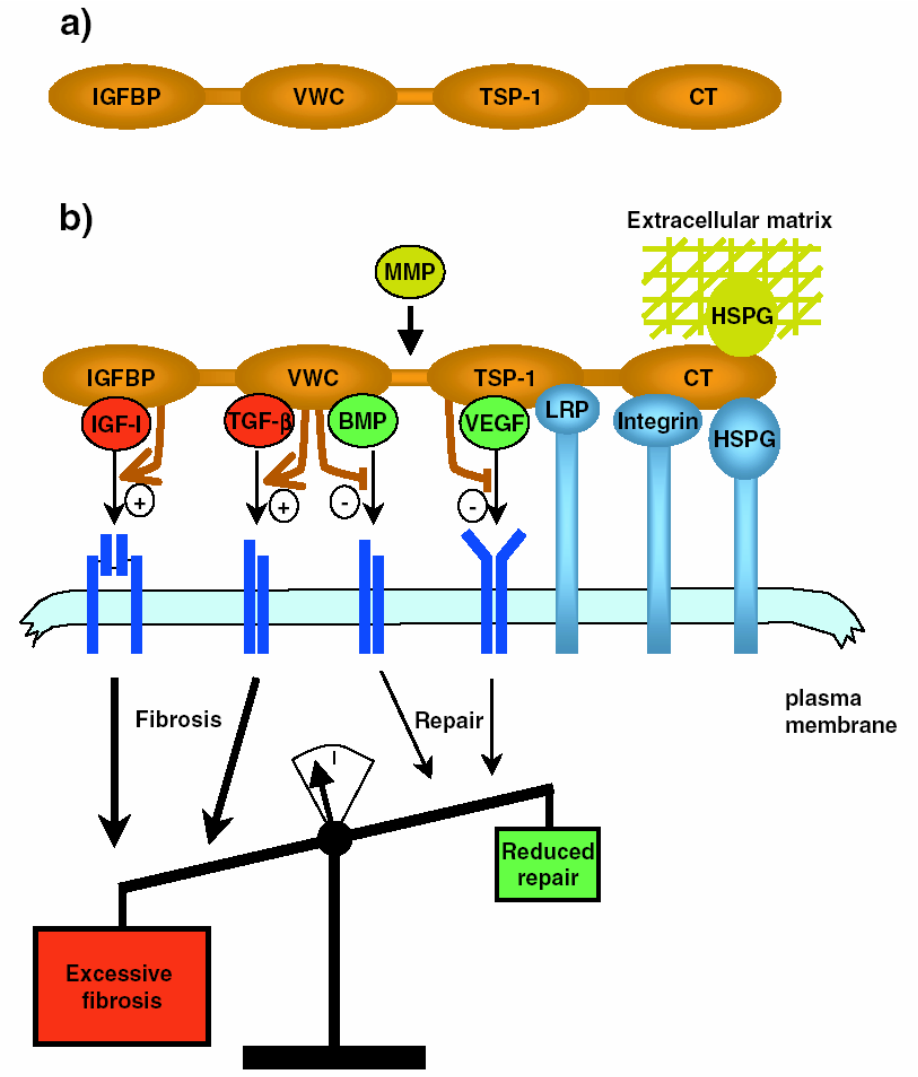



**Figure 7 Modular structure of CTGF (Nieuwenhoven *et al.*, 2005)**

Modular structure (Fig. 7) as proposed by recent studies (Nieuwenhoven *et al.*, 2005) is represented in the following figure IGFBP = insulin-like growth factor-binding protein domain; VWC = von Willebrand type c domain; TSP-1 = thrombospondin 1 domain; CT = C-terminal domain; MMP = matrix metalloprotease; IGF-I = insulin-like growth factor I; TGF-ß = transforming growth factor-ß; BMP = bone morphogenetic protein; VEGF = vascular endothelial growth factor; LRP = low-density lipoprotein receptor-related protein; HSPG = Heparan sulfate proteoglycan.

# *Aims and Objectives*

## 2.1. Hypothesis

The existing data on the beneficial role of NO in glomerular diseases together with previous studies from our laboratory that NO strongly inhibits MC proliferation by inhibiting serum-induced early growth response gene-1 (Rupprecht et al., 2000) and that MC play an important role in ECM deposition lead us to hypothesize that the potential antifibrotic effects of NO in renal glomerular inflammation and fibrosis could be mediated by its direct influence on matricellular gene regulation.

With this background in view, the purpose of this doctoral thesis was to study the role of NO in modulating the expression of genes in renal glomerular mesangial cells which can play a protective role during the course of fibrotic and inflammatory disorders in human kidney and to elucidate the fundamental mechanisms involved in the regulation of gene expression elicited by NO.

## 2.2. Objectives

### 2.2.1. To investigate the global effect of NO on gene expression in glomerular mesangial cells

- To determine the extent at which NO affects gene expression in MCs by using a polymerase chain reaction (PCR) – based representational differential analysis (RDA) (Hubank and Schatz, 1994; Pastorian et al., 2000) technique in S-Nitroso-L-glutathione (GSNO) – treated rat MCs.

### 2.2.2. To investigate and verify the effect of NO on extracellular matrix associated genes and proteins based on RDA results

a. To verify the results of RDA by dot blot analysis.
b. To underscore the significance of a set of downregulated genes involved in matrix accumulation and fibrosis.
c. To determine the chemical activity of various NO donors used in an expanded study.
d. To verify the downregulatory affects of NO on mRNA and protein levels of the genes involved in matrix accumulation and fibrosis in an expanded study entailing the use of exogenous NO donors with diverse potency.
e. To demonstrate that similar changes are induced by endogenously generated NO in response to the activation of iNOS by INF-γ in mesangial cells.
f. To determine whether some additional extracellular matrix associated genes linked to matrix accumulation are affected in a similar way by exogenous as well as endogenous NO stimulation in human mesangial cells.

### 2.2.3. To investigate the mechanism of NO-mediated gene regulation

g. To investigate the influence of NO- mediated downregulation at posttranscriptional level by carrying out mRNA stability measurements.
h. To determine whether one of the candidate firbrotic marker genes, connective tissue growth factor (CTGF), is transcriptionally regulated at the promoter level by NO.

# *Materials and Methods*

## 3.1. Mesangial cell (MC) culture

### 3.1.1. Primary rat mesangial cells

Rat MC were cultured as described earlier (Rupprecht et al., 1992), in Dulbecco's modified Eagle's medium (DMEM) containing 5.5mM glucose and supplemented with 10% heat inactivated (56°C, 30 min) fetal calf serum (FCS), 50 U/ml penicillin, 50 µg/ml streptomycin, 2 mM glutamin and 5 µg/ml insulin in a 95% air-5% $CO_2$ humidified atmosphere at 37°C. For serum starvation, rat MC were cultured in presence of 0.1% FCS. Except for Representational difference analysis (RDA), rat MC were further treated with 24.5nM of D-glucose and 5ng/ml TGF-β1 for additional 24 hours in starvation medium for all nitric oxide stimulations only. For the iNOS activation experiments, rat MC were serum starved for 24 hours and then treated with cytokine cocktail for different time points. Rat MC were used for experiments between passages 8 and 20.

### 3.1.2. Human mesangial cells

Human MC from a stable cell line source (Banas et al., 1999) were cultured in DMEM containing 5.5mM glucose and supplemented with 10% heat inactivated (56°C, 30 min) FCS, 50 U/ml penicillin, 50 µg/ml streptomycin in a 95% air-5% $CO_2$ humidified atmosphere at 37°C. For serum starvation, human MC were cultured in presence of 0.1% FCS for 24 hours. Cells were further treated with 24.5nM of D-glucose and 5ng/ml TGF-β1 for additional 24 hours in starvation medium for all nitric oxide stimulations only. For the iNOS activation experiments, human MC were serum starved for 24 hours and then treated with interferon gamma (INF-γ) and tetrahydrobiopterin (BH4) for 20 hours. Human MC were used for experiments between 30 and 50 passages.

## 3.2. NO donors, cytokines, growth factors and special reagents

S-nitroso-L-glutathione (GSNO), Spermine NONOate, DETA NONOate, 1400W (dihydrochloride), L-NMMA (citrate), L-NIL and tetrahydro-L-biopterin (dihydrochloride) ($BH4^{\cdot}$ 2HCL) was purchased from Cayman Chemicals (MI, USA). SNAP, NOC- 18, and Glutathione (GSH) was from Calbiochem (Germany). Human and rat recombinant interferon gamma (INF-γ), rat recombinant interleukin-1beta (IL-1β), rat recombinant tumor necrosis factor alpha (TNF-α) and human transforming growth factor-1beta (TGF- β1) was from Peprotech Inc.

(USA). Aqueous solutions from the above reagents were prepared instantly as and when needed. D-(+)-Glucose solution was from Sigma-Aldrich (USA). Polyclonal anti-rabbit iNOS antibody was from Cayman Chemicals (USA). Human monoclonal anti-CTGF antibody (FG3019) was from Fibrogen Inc. CA, USA. Bovine serum albumin (BSA) fraction V was from Roche, Mannheim, Germany. Tri reagent® and FORMAzol were from Molecular Research Center Inc. (USA); Greiss Reagent System from Promega (Madison, WI, USA); NuPAGE 4-12% Bis-Tris gels from Invitrogen Life Technologies (CA, USA); Polyvinylidine difluoride (PVDF) membranes from BioRad (München, Germany); Bright star positively charged nylon membranes from Ambion; Great EscAPe fluorescent SEAP Detection kit from BD Biosciences (CA, USA); SuperFect Transfection Reagent, EndoFree Plasmid Max kit, Rneasy mini kit and Rnase-Free Dnase from Qiagen and Human TSP-1 EIA Kit from PromoKine, PromoCell GmbH, Heidelberg, Germany. Restriction enzymes were from Roche Diagnostics, Penzberg, Germany.

## 3.3. Buffers and solutions for bacterial growth and DNA

| | |
|---|---|
| Luria-Broth medium liquid | 10 g/l Tryptone; 5 g/l Yeast extract; 10 g/l Sodium chloride |
| solid | LB medium + 1.5% Agar |
| selection | 100 µg/ml Ampicillin; 40 µg/ml X-Gal; 0.2 mM/ml IPTG |
| for bacterial storage | 50% LB medium + 50% Glycerin |
| TE | 10 mM Tris-Cl ; 1 mM EDTA ; pH 8.0 |
| DNA-loading dye 6x | 0.25% Bromphenol blue; 0.25% Xylene cyanole FF; 30% Glycerol |
| TBE 10x | 1 M Tris; 0.5 M Boric acid; 20 mM EDTA; pH 8.3 |
| PAA: BAA 40% (19:1) | 380 g/l Acrylamide; 20 g/l N,N'- Methylenebisacrylamide |
| Sequencing-loading dye | 98% Formamide (deionized); 10 mM EDTA, pH 8.0; 0.025% Xylene cyanol FF; 0.025% Bromphenol blue |

## 3.4. Representational difference analysis

The representational difference analysis (RDA) of cDNA (Fig.8) as described by Hubank and Schatz (Hubank and Schatz, 1994) was performed with the Clontech PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, USA) according to the manufacturer's protocol. For this purpose, poly-$A^+$ RNA was extracted from GSNO-treated and controls MC and reverse transcribed into cDNA. The cDNA containing the specific (differentially expressed) transcripts was termed "tester" and the reference cDNA "driver". The tester and driver cDNAs were digested by Rsa I, a four-base-cutting restriction enzyme that yields blunt ends. The tester cDNA was then subdivided into two portions, and each was ligated with a different cDNA adaptor. The two adaptors had stretches of identical sequence to allow annealing of PCR primers once the recessed ends had been filled in. Two hybridizations were then performed. In the first, an excess of driver was added to each tester sample. The samples were then heat denatured and allowed to anneal. The concentration of high- and low-abundance sequences was equalized among the single strand molecules ligated to an adaptor because reannealing is faster for the more abundant molecules due to the second-order kinetics of hybridization. At the same time, these molecules were significantly enriched for differentially expressed sequences. During the second hybridization, the two primary hybridization samples and again an excess of driver were mixed together without denaturing. Now, only the remaining equalized and subtracted single-strand tester cDNAs could re-associate and form hybrid double-strand tester molecules with different ends, which correspond to the sequences of the different adaptors. The entire population of molecules was then subjected to PCR to amplify the desired differentially expressed sequences. Only the molecules, which had two different adaptors, could be amplified exponentially. These were the equalized, differentially expressed sequences. A secondary PCR amplification was performed using nested primers to further reduce any background PCR products and enrich for differentially expressed sequences.

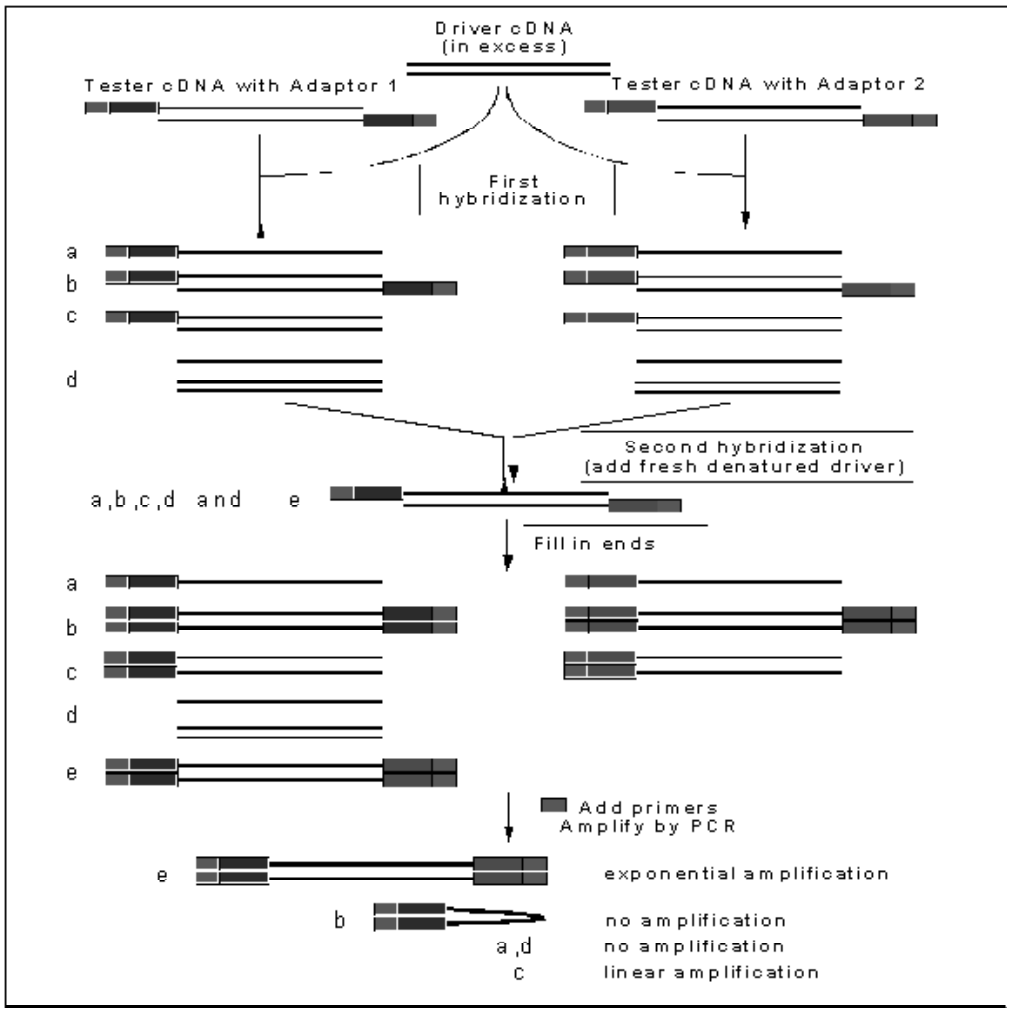



**Figure 8 Schematic representation of RDA analysis**

## 3.5. Reverse transcription

Reverse transcription was performed for 60 minutes at 42°C in a volume of 20 µl using a modified Moloney-murine leukemia virus (MMLV) reverse transcriptase (Superscript and respective buffer; Life Technologies, Karlsruhe, Germany). This was performed in the presence of 1 mmol/L dNTPs (Amersham Pharmacia, Freiburg, Germany), 40 U Rnase inhibitor (Rnasin; Promega, Mannheim, Germany), 2 µl dithiothreitol (DTT; Life Technologies), 2 µl random hexamers (Roche, Mannheim, Germany), and 7 µl of the above RNA solution.

## 3.6. PCR for the subtraction efficiency testing by GAPDH abundance

Subtracted and unsubtracted secondary PCR products were diluted 10-fold in $H_2O$. The following reagents were combined in a PCR reaction tube:

| | |
|---|---|
| cDNA (subtracted or unsubtracted) | 1.0 µl |
| 10 µM G3PDH 5' Primer (5'-ACCACAGTCCATGCCATCAC-3') | 1.2 µl |
| 10 µM G3PDH 3' Primer (5'-TCCACCACCCTGTTGCTGTA-3') | 1.2 µl |
| 10 x PCR reaction buffer | 3.0 µl |
| 10 mM dNTP mix | 0.6 µl |
| Sterile $H_2O$ | 22.4 µl |
| 50 x Advantage cDNA Polymerase Mix (Clontech, Palo Alto, USA) 0.6 µl | |
| Total volume | 30.0 µl |

The reagents were mixed by vortexing and briefly centrifuged. After denaturing the DNA for 5 min at 94 °C the following thermal cycling program was used for 18, 23, 28 and 33 cycles: 94 °C 30 s, 60 °C 30 s, 68 °C 2 min. At the end of the cycles the PCR-reactions were held at 68 °C for 10 min, cooled and stored at 4 °C. For analysis 5 µl of the PCR-reactions were size fractionated by electrophoresis in a 1.5% TBE-agarose gel containing ethidiumbromide. The stained DNA was visualized by UV-light.

## 3.7. Cloning of PCR-fragments

The PCR-products resulting after the subtraction procedure were blunt-end cloned into pCR-Script SK(+) vector (Stratagene, La Jolla, USA) and transfected into E.coli XL1-Blue MRF' supercompetent bacteria (Stratagene, La Jolla, USA). Positive clones were identified by a blue-white selection on LB/amp/IPTG/X-Gal plates. For plasmid preparation bacteria were grown up in 50 ml LB-medium containing 100 µg/ml Ampicillin. The plasmid DNA was isolated with plasmid-preparation kits from BioRad (München, Germany), Roche (Mannheim, Germany) or Qiagen (Hilden, Germany). For radioactive labeling the cDNAs were cut out of the vector with the restriction enzymes EcoRI and SacI.

## 3.8. Dot blot analysis

To identify differentially expressed RNAs, equal amounts of cloned cDNAs were dotted twice onto nylon membranes (Amersham LifeScience, Little Chalfont, UK) as recommended by the company (Clontech, Palo Alto, USA). The membranes were air dried and hybridized with $\alpha[^{32}P]$dCTP labeled forward- and reverse-subtracted cDNA probes of the GSNO-treated and untreated mRNA populations. Blots were exposed to Kodak Biomax MS or MR films (Eastman Kodak Company, Rochester, USA) at -80 °C. cDNAs indicating differentially expressed RNAs were used for further tests in Northern blot.

## 3.9. Sequencing of the cloned cDNAs

The cDNAs cloned into the pCR-Script vector were sequenced by the method of Sanger et al. (1977) with a T7-sequencing kit (Amersham Pharmacia Biotech, Little Chalfont, UK) and $\alpha[^{35}S]$-dATP. The used sequencing primers were T3 3' Primer: 5'-AATTAACCCTCACTAAAGGG-3' and T7 5' Primer (Universal-Primer): 5'-GTAATACGACTCACTATAGGGC-3'. The sequence-gels were vacuum dried on 3MM-paper and exposed to Kodak Biomax MS films (Eastman Kodak Company, Rochester, USA) at room temperature. The sequences were further verified by automated DNA sequencing done by Medigenomix, Martinsried, Munich, Germany.

## 3.10. Exogenous NO donors

There are a number of commonly used exogenous NO donors which differ from one another in their chemical structure, stability and mode of action, which dictates the rate of NO release from these compounds. NO donors like *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP), Hydroxy-nitrosohydrazino-bisethanamine (NOC's) and Spermine NONOates are essentially stable NO-amine complexes that release NO, without cofactors, under physiological conditions. The mechanism of NO release from donors like NOC's is very simple compared to other classical NO donors, such as nitroglycerin and nitroprusside, and the by-products do not interfere with cell activities. *S*-Nitrosothiols like s-nitroso-glutathione (GSNO) and s-Nitrosocysteine (CySNO) diffuse as such to the site of action. These are also stable compounds at 37 °C and pH 7.4 in the presence of transition metal ion chelators. The presence of trace transition metal ions (present in all buffers) stimulates the catalytic breakdown of *S*-nitrosothiols to NO and disulfide. Thiyl radicals are not formed as intermediates in this process.

## 3.11. Description of NO donors used in the expanded study

### 3.11.1. S-Nitroso-L-Glutathione

| | |
|---|---|
| Formal Name: | Glycine, N-(N-L-γ-glutamyl-S-nitroso-L-cysteinyl)- |
| Synonyms: | GSNO |
| MF: | $C_{10}H_{16}N_4O_7S$ |
| Solubility | PBS |
| Half-life | 10 hours at 37°C at pH 7.2 |

### 3.11.2. SNAP

| | |
|---|---|
| Formal Name: | S-Nitroso-N-acetyl-dL-penicillamine |
| Synonyms: | Diethylenetriaamine NONOate |
| MF: | $C_4H_{13}N_5O_2$ |
| Solubility | water |
| Half-life | 4.6 hours at 37°C at pH 7.4 |

### 3.11.3. Spermine NONOate

| | |
|---|---|
| Formula name: | 1, 3-Propanediamine, N-[4-[1-(aminopropyl)-2-hydroxy-2-nitrosohydrazino] butyl] |
| Molecular formula: | $C_{10}H_{26}N_6O_2$ |
| Solubility | water |
| Half-life | 39 minutes at 37°C at pH 7.4 |

### 3.11.4. DETA NONOate or NOC-18

| | |
|---|---|
| Formal Name: | (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl) amino] diazen-1-ium-1, 2-diolate |
| Synonyms: | Diethylenetriaamine NONOate |
| MF: | $C_4H_{13}N_5O_2$ |
| Solubility | water |
| Half-life | 20 hours at 37°C at pH 7.4 |

## 3.12. Nitrite Assay

Nitric oxide is metabolized into its stable and nonvolatile products nitrite and nitrate. One means to investigate nitric oxide formation is to measure nitrite (NO2–). This assay relies on a diazotization reaction that was originally described by Griess in 1879. The Griess Reagent System is based on a chemical reaction which uses sulfanilamide and N-1-napthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions. This system detects $NO2^-$ in a variety of biological and experimental liquid matrices such as plasma, serum, urine and tissue culture medium. The nitrite sensitivity is dependent on the matrix.

Conditioned media after stimulation with different NO donors or cytokines were collected over 24h and nitrite concentrations were measured using a colorimetric assay with the Griess reagent (Promega) using the protocol mentioned below. Absorbance at 550 nm was measured in GENios Plus Microtiter plate reader (TECAN GmbH, Austria) employing Xfluor4 software.

The following detailed protocol was used to perform nitrite assay using Griess reagent from Promega.

### 3.12.1. Preparation of a Nitrite Standard Reference Curve

- Prepare 1ml of a 500µM nitrite solution by diluting the provided 0.1M Nitrite Standard 5:1,000 in DMEM used for the experimental samples.
- Designate 3 rows (30 wells) in a flat bottom transparent 96-well microtiter plate for the Nitrite Standard reference curve. Dispense 50µl of DMEM into the wells in rows A2–C10.
- Add 100µl of the 500µM nitrite solution to the remaining 3 wells in rows A1-C1.
- Immediately perform 6 serial 2-fold dilutions (50µl/well) in triplicate down the plate to generate the Nitrite Standard reference curve (500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.9, 1.95, µM), discarding 50µl from the 1.56µM set of wells. Do not add any nitrite solution to the last set of wells (0µM). The final volume in each well is 50µl, and the nitrite concentration range is 0–100µM.

### 3.12.2. Nitrite Measurement

- Allow the Sulfanilamide Solution and NED Solution to equilibrate to room temperature (15–30 minutes).
- Add 50µl of each experimental sample to wells in duplicate or triplicate.
- Using a multichannel pipettor, dispense 50µl of the Sulfanilamide Solution to all experimental samples and wells containing the dilution series for the Nitrite Standard reference curve.
- Incubate 5–10 minutes at room temperature, protected from light.
- Using a multi-channel pipettor, dispense 50µl of the NED Solution to all wells.
- Incubate 5–10 minutes at room temperature, protected from light. A purple/magenta color will begin to form immediately.
- Measure absorbance within 30 minutes in a plate reader with a filter between 520–550nm.

## 3.13. Northern blotting

Total RNA was isolated by Tri Reagent or Rneasy mini kit according to the manufacturer's instructions. Total RNA (15µg) was subjected to electrophoresis on 1% formaldehyde agarose gels and transferred to positively charged Bright Star nylon membranes by downward alkaline transfer. Blots were hybridized in ExpressHyb (Clontech) solution with 2 x $10^6$ cpm/ml –labeled cDNA probes prepared by Prime-It® RmT Random Primer Labeling Kit, Single-Use $\alpha^{32}$P-dCTP-Labeling Reactions (Stratagene). Probes for 18S rRNA, CTGF and COL1A2 were obtained from the restriction digestion of RDA clones. Probe for TSP-1 (pTS-33) was purchased from ATCC (VA, USA) which yields a TSP-1 full length cloned cDNA fragment of 1.29 kb after restriction digestion with EcoRI. Membranes were either exposed to a Kodak Bio Max x-ray film or visualized after exposure to Phosphoimager screen and quantified by Image quantitation software (IQ Mac v 1.2) provided with the Phosphoimager Strom 840 (Molecular Dynamics). X-ray film blots were analyzed after scanning, by ImageJ version 1.32j image quantitation software (NIH, USA).

### 3.13.1. RNA isolation from mesangial cells

- Caution must be taken while handling RNA as it is prone to degradation by Rnases.
- After proper stimulation of mesangial cells cultured in 10 cm tissue culture dishes, below mentioned protocol was followed for RNA isolation.
- All steps except centrifugation were performed at room temperature in a fume hood.
- Remove media completely from the dish.
- Spread 1ml of the Tri Reagent on the monolayer of the cells.
- Leave unattended for 5 minutes.
- Spin the dish manually on a smooth surface to dislodge any attached cells for 2-3 minutes.
- Leave the dish in a tilted position for 5 minutes about till the cells in the reagent get collected at the bottom of the dish.
- Pipett the solution up and down forcefully through a 1ml blue tip 20 times to disrupt any intact cell membranes.
- Transfer the homogenate into a 1.5ml eppendorf.

- Incubate for 5 minutes.
- Add 100µl of bromochloropropane and subject to vigorous manual shaking and mixing.
- Incubate for 5 minutes.
- Centrifuge -15,000 rpm for 25 minutes at 4°C.
- Transfer aqueous phase (not more than 400µl to avoid any protein contamination) into a fresh tube.
- Add 0.5ml of room temperature isopropanol and mix well.
- Incubate for 10 minutes.
- Centrifuge -15,000 rpm for 25 minutes at 4°C.
- Mix RNA pellet with 1ml 75% ethanol by vortexing at least for two minutes.
- Centrifuge -10,000 rpm for 10 minutes at 4°C.
- Carefully drain the ethanol and wipe the insides of the tube with a clean paper towel without touching the pellet.
- Air dry the pellet for 5 minutes.
- Dissolve by pipetting in 75µl of FORMAzol (MRC, Inc. USA).
- Incubate at 60°C for 10 minutes.
- RNA in FORMAzol can be stored at -20°C until electrophoresis.

### 3.13.2. Buffers and solutions for RNA-Gel Electrophoresis

- DEPC Water

  0.001% (Diethyl Pyrocarbonate) DEPC in $ddH_2O$ or 1ml DEPC in 1L $ddH_2O$
- RNA- Gel Running Buffer (750 ml)

| | |
|---|---|
| 10X E Buffer | 75 ml |
| Formaldehyde | 60.75 ml |
| Water | 614.25 ml |

- 20X E Buffer (500 ml)

| | |
|---|---|
| 0.9 M Na2HPO4 | 25.56 g |
| 0.1 M NaH2PO4 | 2.76 g |

- 1.2 % RNA Denaturing Agarose gel (75ml)

| | |
|---|---|
| Agarose | 0.9 g |

| | |
|---|---|
| 10 X E Buffer | 7.5 ml |
| Water | 55.35 ml |

Melt agarose and let cool to 60°C

| | |
|---|---|
| Formaldehyde | 12.15 ml |

Add, mix and pour immediately

- 10X RNA Dye

| | |
|---|---|
| Bromophenol blue | 0.025% |
| Xylene cyanol FF | 0.025% |
| EDTA | 0.5mM |

- RNA- Loading buffer

| | |
|---|---|
| Formaldehyde | 81µl |
| 20 X E Buffer | 24µl |
| 10X RNA Dye | 48µl |
| EtBr (200ng/µl) | 48µl |
| Water | 39µl |
| Total volume | 240µl |

To each **10µl** RNA sample in FORMAzol, add **10µl** from the RNA- Loading buffer and mix by pipetting.

Denature RNA at 65°C for 15 minutes, cool and electrophorese at 7V/cm.

### 3.13.3. RNA transfer buffers

- High Salt Alkaline Transfer Buffer for RNA

| | |
|---|---|
| NaOH | 0.01N |
| SSC | 5X |

- 20X SSC Buffer (1L pH 7.0)

| | |
|---|---|
| NaCl | 175.3 g |
| Na Citrate | 88.2 g |

### 3.13.4. Downward transfer of RNA through a nylon membrane

Nucleic acid transfer methods include dot blot, slot blot, vacuum blot, colony or plaque lift, electrotransfer and capillary blotting. Traditionally, blotting is completed by upward transfer methodology. More recently, downward transfer methods were developed (Fig.9). The downward method makes use of gravity to speed nucleic acid transfer and requires less weight on the blotting stack, thus preventing damage to the gel. Downward transfer times of 15-20 minutes per mm gel thickness are recommended. Warm transfer solutions (35-45°C) soften the agarose gels allowing the nucleic acids to transfer faster and more efficiently for SSC or viscous transfer buffers. Alkaline transfers proceed very rapidly at room temperature, thus warming buffers is not recommended especially for RNA where fragmentation could occur. The membrane used for nucleic acid immobilization should be pre-wet (~5-15 minutes) in transfer buffer prior to transfer to equilibrate the membrane and remove any unattached surface particulate to which the nucleic acid could bind. Blotting paper porosity can affect wicking and transfer rates of nucleic acids. Quality grade blotting paper, ~4 sheets of Whatman 3MM, should be used for the wick and surround the gel and membrane. The quality blotting paper minimizes background contributions from lower grade papers. The blot stack must be carefully assembled to avoid air pockets that could disrupt the flow of transfer buffer, thus preventing nucleic acid capture. The wick should be thick enough to provide a continuous flow of liquid to the blot stack. The wick must also be carefully placed so that the wick only makes contact at the beginning sheet directing the flow through the gel to the membrane. The buffer system used here (0.01 N NaOH, 5X SSC) needs not more than 2 hours for a single RNA-Formaldehyde agarose gel of 15×10 cm dimension.

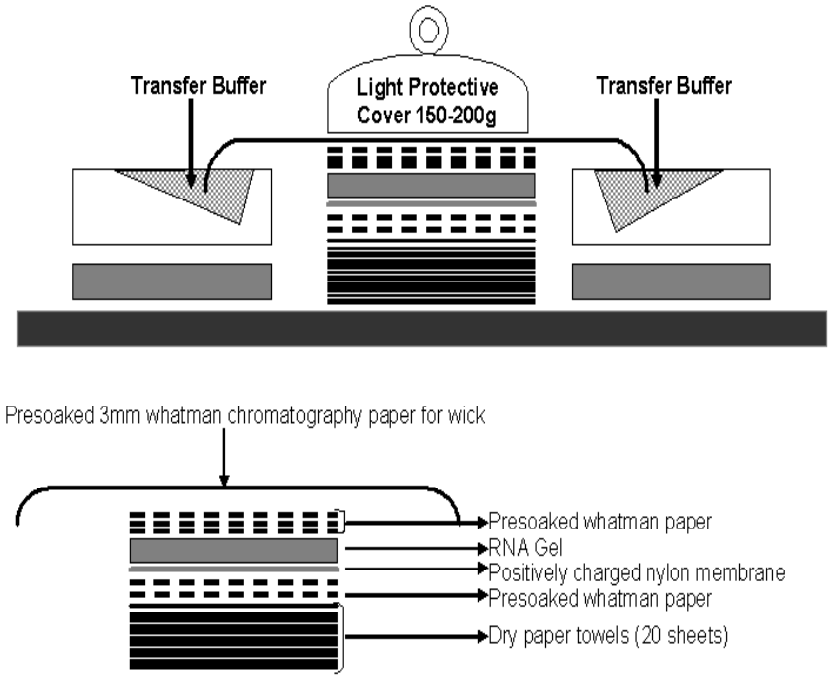



**Figure 9 Downward RNA transfer system – a schematic representation**

## 3.14. Radiolabeling of cDNA probes for northern blotting

### 3.14.1. Random Primer Labeling Protocol

The prime it® RmT random primer labeling kit uses random oligonucleotides as primers for labeling DNA to produce high-specific activity probes. The procedure relies on the ability of random 9-mers to anneal to multiple sites along the length of a DNA template. The primer–template complexes formed represent a substrate for the magenta DNA polymerase, a thermostable polymerase. The enzyme synthesizes new DNA by incorporating nucleotide monophosphates at the free 3´-OH group provided by the primer. The newly synthesized DNA is made radioactive by substituting radiolabeled [α-32p] dCTP for unlabeled dCTP in the reaction mixture. The resulting labeled DNA serves as a sensitive hybridization probe for northern blots. DNA labeling reaction components (random primers, dNTPs, buffers and cofactors) are supplied as a dehydrated reaction mixture, pre-aliquoted in 24 single-use reaction tubes.

### 3.14.2. Protocol for labeling cDNA probes

- Remove single-use reaction tubes from the reaction tube strips
- Add the following to each single-use reaction tube:

  $dH_2O$ to a final volume of 42 µl

  25–50 ng of DNA
- Boil the reaction for 5 minutes
- Centrifuge briefly to collect the condensate
- Add the following:
- 5 µl of labeled nucleotide
- 3 µl of magenta DNA polymerase (4 U/µl)
- Mix well and incubate at 37°C for 5–10 minutes
- Add 2 µl of stop mix

### 3.14.3. Measurement of Probe Specific Activity

To determine the specific activity of the radioactively labeled probes, the following steps were performed:

The final reaction volume was 52µl

- 1µl aliquot was removed and applied on a chromatography paper DE81 2.3cm diameter circle (Whatman, England)
- Remaining 51µl was applied to a desalting resin (Sephadex G-50) spin column (Roche) to remove any free nucleotides
- Centrifugation was performed at 2500g for 4 minutes
- 1µl from the resultant volume was again applied on another DE81 circle
- The DE81 circles were air dried for 15 minutes
- Radioactive incorporation as counts per minute (cpm) was measured in Liquid Scintillation Analyzer (Canberra Packard, Germany) by putting the circles containing radioactively labeled probes into small plastic scintillation vials (dry) and the counting was done by using Protocol 10 of the Scintillation Analyzer.
- Specific activity of the probe was determined according to the following formula:

$S_A = (\mu Ci)(2.2\times10^9)(P)/\ M_i\ [(1.3\times10^3)(P)(\mu Ci/\ S_a)]$

$S_A$ = the specific activity in disintegrations per minute per microgram (dpm/µg)

μCi = the amount of radiolabeled nucleotide in microcuries in the reaction mixture

*P* = the proportion of radiolabeled nucleotide incorporated into the probe DNA, calculated by dividing the average counts per minute counted on the washed Whatman DE81 filter paper disks divided by the average counts per minute counted on the unwashed Whatman DE81 filter paper disks

$M_i$ = the mass of input of the DNA template in nanograms (ng)

$S_a$ = the specific activity of radiolabeled nucleotide in curies per millimole (Ci/mmol) [curies per millimole (Ci/mmol) equals microcuries per nanomole (μCi/nmol)]

Multiply the microcuries (μCi) by 2.2 × 109 to calculate the total number of disintegrations per minute (dpm) in the reaction.

This calculation also converts the final value for *S*A from disintegrations per minute per nanogram (dpm/ng) to disintegrations per minute per microgram (dpm/μg).

Multiply the resulting value by *P* to calculate the proportion of disintegrations per minute (dpm) incorporated into the probe DNA.

To compute the *S*A, divide the value obtained above by the total amount of DNA present at the end of the reaction. The total amount of DNA present at the end of the reaction is the sum of the mass of input ($M_i$) DNA template plus the mass of the newly synthesized DNA. The latter value is obtained by multiplying the number of nanomoles of dCMP incorporated [(*P*)(μCi/ $S_a$)] by four times the average molecular weight of the four dNMPs [(4)(325) = $1.3 \times 10^3$].

## 3.15. Western blotting

Anti- iNOS rabbit polyclonal antibody from Cayman Chemicals was used in a dilution of 1:1,000. Anti-CTGF human monoclonal anti-CTGF antibody (isotype IgG- FG3019) was from Fibrogen Inc. CA, USA and used at a 1:250 dilution. Cell lysates (20 mg) were subjected to NuPAGE 4-12% Bis-Tris gel. After elctrophoresis, the gel was transferred to PVDF membranes. Membrane were blocked overnight in 2% BSA, incubated with anti-iNOS or anti-CTGF antibody for 1 hour and for 45 minutes in secondary horse radish peroxidase IgG antibody. Equal amounts of protein loading were confirmed by Coomassie brilliant blue staining before blotting. Proteins were finally visualized by the SuperSignal Chemiluminescent kit (Pierce, IL, USA).

## A. Preparation of cell lysates

- Collect confluent stimulated mesangial cells by trypsinization and spin.
- Lyse the pellet with 100 µl lysis buffer on ice for 10 min.
  (For 500,000 cells, lyse with 20 µl).
- Spin at 14,000 rpm in an Eppendorf microfuge for 10 min at 4°C.
- Transfer the supernatant to a new tube and discard the pellet.
- Determine the protein concentration (Bradford assay- Bio-Rad)
- Take 30µg protein and mix with an equal volume of 2x sample buffer.
- Boil for 5 min.
- Cool at RT for 5 min.
- Flash spin to bring down condensation prior to loading gel.

## B. Preparation of gel

- Assemble the glass plates and spacers (1.5 mm thick).
- Pour the running gel to about 1 cm below the wells of the comb (~20 ml).
- Seal with 1 ml water-saturated 1-butanol.
- When gel has set, pour off the butanol and rinse with deionized water.
- Pour the stacking gel (~5 ml) and insert the comb immediately.
- When the stacking gel has set, place in gel rig and immerse in buffer.
- Prior to running the gel, flush the wells out thoroughly with running buffer.

## C. Running the gel

- After flash spinning the samples, load into the wells.
- Use 15 µl Bio-Rad Kaleidoscope Prestained Standards directly.
- Run with constant current (35 – 37 mA with voltage set at > 300 V).
- Usual running time is about 2.5 hr.

## D. Using precast gels

- Assemble gel in gel rig (NuPAGE 4-12% Bis-Tris gels – Invitrogen Life Technologies).
- Prepare protein samples (10 µg will suffice).
- Use 5 µl of Kaleidoscope standard.
- Run at 200 V (constant voltage) for 30 min.

## E. Preparation of membrane

- Cut a piece of PVDF membrane (Pierce).
- Wet for about 30 min in methanol on a rocker at room temp.
- Remove methanol and add 1x Blotting buffer until ready to use.

## F. Membrane transfer

- Assemble "sandwich" for Bio-Rad's Transblot.
- Prewet the sponges, filter papers (slightly bigger than gel) in 1x Blotting buffer.
  Sponge – filter paper – gel – membrane – filter paper – sponge
- Transfer for 1 hr at 1 amp at 4°C on a stir plate.
- When finished, immerse membrane in Blocking buffer and block overnight.

## G. Antibodies and detection

- Incubate with primary antibody diluted in blocking buffer for 60 min at room temp.
- Wash 3 x 10 min with 0.05% Tween 20 in PBS.
- Incubate with secondary antibody diluted in Blocking buffer for 45 min at room temp.
- Wash 3 x 10 min with 0.05% Tween 20 in PBS.
- Detect with Amersham ECL kit (RPN 2106).

## H. Stripping blot

- Rinse blot off with 0.05% Tween 20 in PBS.
- Put blot into Kapak bag cut to slightly bigger size than blot.
- Add about 5 to 10 ml Stripping buffer.

- Remove as much air as possible and seal bag.
- Immerse into 80°C water bath and incubate for 20 min.
- Rinse blot off with 0.05% Tween 20 in PBS.
- Block for about 1 hr with 5% BSA/Tween 20, or overnight with 3% BSA/Tween 20.

### 3.15.1. Buffers and solutions for Western blotting

- Lysis buffer (10X)

  0.15 M NaCl

  5 mM EDTA, pH 8

  1% Triton X100

  10 mM Tris-Cl, pH 7.4

  Just before using add: 1:1000 5 M DTT

  1:1000, 100 mM PMSF in isopropanol

  1:1000, 5 M ε–aminocaproic acid

- 2x sample buffer

  130 mM Tris-Cl, pH8.0

  20% (v/v) Glycerol

  4.6% (w/v) SDS

  0.02% Bromophenol blue

  2% DTT

- 8x Resolving gel buffer (100 ml)

  0.8 g SDS (add last)

  36.3 g Trizma base (= 3 M)

  Adjust pH to 8.8 with concentrated HCl

- 4x Stacking gel buffer (100 ml)

  0.4 g SDS (add last)

  6.05 g Trizma base (= 0.5 M)

  Adjust pH to 6.8

- 10x Running buffer (1 L)

  30.3 g Trizma base (= 0.25 M)

  144 g Glycine (= 1.92 M)

  10 g SDS (= 1%)

  Do not adjust the pH!!

- 10x Blotting buffer (1 L)

  30.3 g Trizma base (= 0.25 M)

  144 g Glycine (= 1.92 M)

  pH should be 8.3; do not adjust

- 1x Blotting buffer (1L)

  200 ml Methanol

  100 ml 10x Blotting buffer

  700 ml water

- Blocking buffer (0.5 L)

  3% Bovine serum albumin (Fraction V)

  Make up in PBS and sterile filter.

  Then add 0.05% Tween 20.

- Stripping buffer (0.5 L)
  0.2 M Glycine, pH 2.5
  0.05% Tween 20
  Sterile filter solution and keep at 4°C

- Staining solution

| | |
|---|---|
| Methanol | 90 ml |
| $H_2O$ | 90 ml |
| acetic acid | 20 ml |
| Coomassie Brilliant Blue R250 | 0.25 g |

  Dissolve well and filtrate through a filter paper

- Destaining solution

| | |
|---|---|
| Isopropanol | 12.5% |
| glacial acetic acid | 10% |

  Addition of small pieces of used X-ray film with the gel will accelerate the destaining process.

## 3.16. Determination of secreted Thrombospondin-1 protein (TSP-1) by Enzyme-linked immunosorbent Assay (ELISA):

Quantitation of thrombospondin-1 protein (TSP-1) secreted into the culture medium by cells was performed using a competitive PromoKine Human TSP-1 enzyme immunoassay (EIA), which measures the natural and recombinant forms of TSP-1. With this assay system, goat anti-rabbit antibodies are used to capture a specific TSP-1 complex in each sample consisting of TSP-1 antibody, biotinylated TSP-1 conjugate, and sample/standard. Biotinylated TSP-1 conjugate (competitive ligand) and sample or standard form a competition reaction for TSP-1 specific antibody binding site. Therefore, as the concentration of TSP-1 in the sample increases, the amount of biotinylated TSP-1 captured by the antibody decreases. With the addition of streptavidin conjugated alkaline phosphatase (which binds only to the biotinylated TSP-1) followed by the addition of the colour reagent solution, the amount of biotinylated TSP-1 is detected. This results in an inverse relationship between optical density (OD) and concentration: the higher the OD the less TSP-1 in the sample. Human mesangial cells were plated in 24-well culture plates at 70,000 cells per well in growth medium and incubated for 1-3 days until subconfluent. Cells were then serum starved for 24 hours in Dulbecco's modified Eagles medium containing 30mM D-glucose and 5ng/ml TGF-β1. Thereafter, cells were treated with 50, 100 or 250μM of Spermine NONOate, DetaNONOate or NOC-18 for 12 and 24 hours in the same medium. The culture medium was then collected, clarified by centrifugation and used immediately for assay or stored at –70°C until assayed. TSP-1 protein was quantified and assayed from the conditioned media after a 1:1 dilution with starvation media in 96 well microtiter plates pre-coated with goat anti-rabbit antibody. 25μl of diluted rabbit anti-human TSP-1 polyclonal antibody was added to each 100μl of diluted sample for 3 hours at room temperature. Thereafter, 25μl of human TSP-1 conjugate was added to each well and incubated at room temperature for 30 minutes. Thereafter, 50μl of diluted streptavidin- alkaline phosphatase was dispensed into each well and incubated for 30 minutes at room temperature. After that, plates were washed several times and soaked for 10 minutes in wash buffer. After aspiring the wells, 200μl of the prepared color reagent solution was added to each well and incubated for 20 minutes. During these 20 minutes, the plate was frequently read at 490 nm with GENios Plus Microtiter plate reader (TECAN, Austria GmbH) employing Xfluor4 software,

until the OD of "growth medium only" reached to 1.6. At this time point, 20µl of the stop solution was added to each well and the plate was read again. A standard curve with recombinant TSP-1 protein standard was run at each setting. Starvation medium was used at a dilutant and as negative control. Data acquired was analyzed by making use of excel spreadsheet using Slope and Y-Intercept (LINEST) function.

## 3.17. Real-time quantitative RT-PCR

Real-time RT-PCR was performed on a TaqMan ABI 7700 Sequence Detection System (Applied Biosystems, Weiterstadt, Germany) using heat-activated TaqDNA polymerase (Amplitaq Gold, Applied Biosystems). After an initial hold of two minutes at 50°C and 10 minutes at 95°C the samples were cycled 40 times at 95°C for 15 seconds and 60°C for 60 seconds. The cDNA content of each sample was compared with another sample following the ΔCt technique. This procedure uses the formula $A_0/B_0 = (1+E)^{(Ct,B-Ct,A)}$, where $A_0$ is the initial copy number of sample A; $B_0$, initial copy number of sample B; E, efficiency of amplification; Ct,A, threshold cycle of sample A; and Ct,B, threshold cycle of sample B. The amplification efficiency was defined as 1 as all analyses were performed during the same runs including control dilution series. Similar amplification efficiencies for targets and housekeeping genes were demonstrated by analyzing serial cDNA dilutions showing an absolute value of the slope of log input cDNA amount versus ΔCT (Ct housekeeping gene –Ct target) of <0.1.

Commercially available human "TaqMan Gene Expression assays" with the following gene bank accession numbers and/or probe sequences were purchased from Applied Biosystems:

Collagen Type I alpha1 (COL1A1; NM_000088) ACGAAGACATCCCACCAATCACCT**;** Collagen Type IV alpha2 (COL4A2; NM_001846) GAGCGTCTTGGCGGGTGTGAAGAAG; Biglycan (BGN; NM_001711) CCCTCTCCAGGTCCATCCGCCAT; and GAPDH (M33197). Human procollagen type I alpha 2 (COL1A2) and human inducible nitric oxide synthase (INOS2) gene sequences and fluorescent probes were custom prepared by Applied Biosystems. They are: COL1A2- NM_053356.1, sense primer 5'-CACAGAAATAACACTGCAAAC-3', antisense primer 5'- CAGTGGTAGGTGATGTTCTG-3', fluorescence labeled probe 5'-GGCAGACCTGGCCCAATTGGCCCA-3'); INOS2- NM_000625 sense primer 5'-ACAACAGTAACCTACCAACTGACGG-3', antisense primer 5'- CCTCCCAATGCAGCGTG-3', fluorescence labeled probe 5'- TGAGCTCATCTTCGCCACCAAGCA-3'). All these assay

reagents do not amplify genomic DNA. This was verified using genomic human DNA samples. Controls consisting of $ddH_2O$ were negative in all runs.

## 3.18. Message stability assay

Quiescent human MCs grown in serum free medium containing 30mM glucose and 5ng/ml TGF-β1 for 24 hours were exposed to actinomycin D alone (10 μg/ml) or to actinomycin D (10μg/ml) and Spermine NO (500 μM). Total RNA from the stimulated cells (between 0 and 6 hours) was subjected to Northern blot hybridization using radiolabeled probes for CTGF and 18S rRNA to measure the rate of decay of CTGF mRNA. Measurement of the ratio of CTGF/18S rRNA at time = 0 (from actinomycin D treatment) in cells cultured in 500μM Spermine NONOate was assigned a relative value of 100%. Half-life was calculated according to the equation: $t ½ = \ln 2/ \lambda$ (where ln is natural logarithm and λ is the rate of decay of the message).

## 3.19. Transient Transfection and Reporter Assay

### 3.19.1. SEAP Reporter System

SEAP reporter system uses SEAP, a secreted form of human placental alkaline phosphatase (Berger et al., 1988) as a reporter molecule to monitor the activity of promoters and enhancers. We used here the BD Great EscAPe SEAP Reporter System 3 (BD Biosciences) for transfection studies in human and rat mesangial cells. A -805/+17bp sequence of CTGF promoter (kindly provided by Fibrogen Inc. CA, USA) was cloned into the pSEAP2-Basic Vector in front of the SEAP gene of the Reporter System 3. To analyze the promoter-reporter activities, we used the BD Great EscAPe™ SEAP Fluorescence Detection Kit. The fluorescent substrate, 4-methylumbelliferyl phosphate (MUP), enables to monitor expression of the SEAP reporter gene using simple, sensitive, nonradioactive assays of secreted phosphatase activity (Fig.10). The fluorescent assay is comparable to assays for firefly luciferase and is suitable for all systems. This assay is linear over a $10^4$ fold range of enzyme concentrations, which makes it particularly well suited for comparative analyses.

The SEAP reporter gene encodes a truncated form of the placental enzyme that lacks the membrane anchoring domain, thereby allowing the protein to be efficiently secreted from transfected cells. Levels of SEAP activity detected in the culture medium have been shown to be

directly proportional to changes in intracellular concentrations of SEAP mRNA and protein (Berger et al., 1988; Cullen & Malim, 1992). SEAP has the unusual properties of being extremely heat stable and resistant to the phosphatase inhibitor L-homoarginine (Cullen & Malim, 1992). Therefore, endogenous alkaline phosphatase activity can be eliminated by pretreatment of samples at 65°C and incubation with this inhibitor.

The secreted nature of SEAP provides several advantages for the use of this enzyme as a transcription reporter:

• Preparation of cell lysates is not required for analysis.

• The kinetics of gene expression can be studied simply by repeated collection of the culture medium from the same cultures.

• Transfected cells are not disturbed by measurement of SEAP activity in the medium, so a single set of cultures can be used both for the SEAP assay and for further investigations such as RNA and protein analysis.

• Background from endogenous alkaline phosphatase is almost absent in the culture medium following pretreatment.

• Sample collection of the culture medium can be automated by growing cultures and performing the assays in 96-well plates.

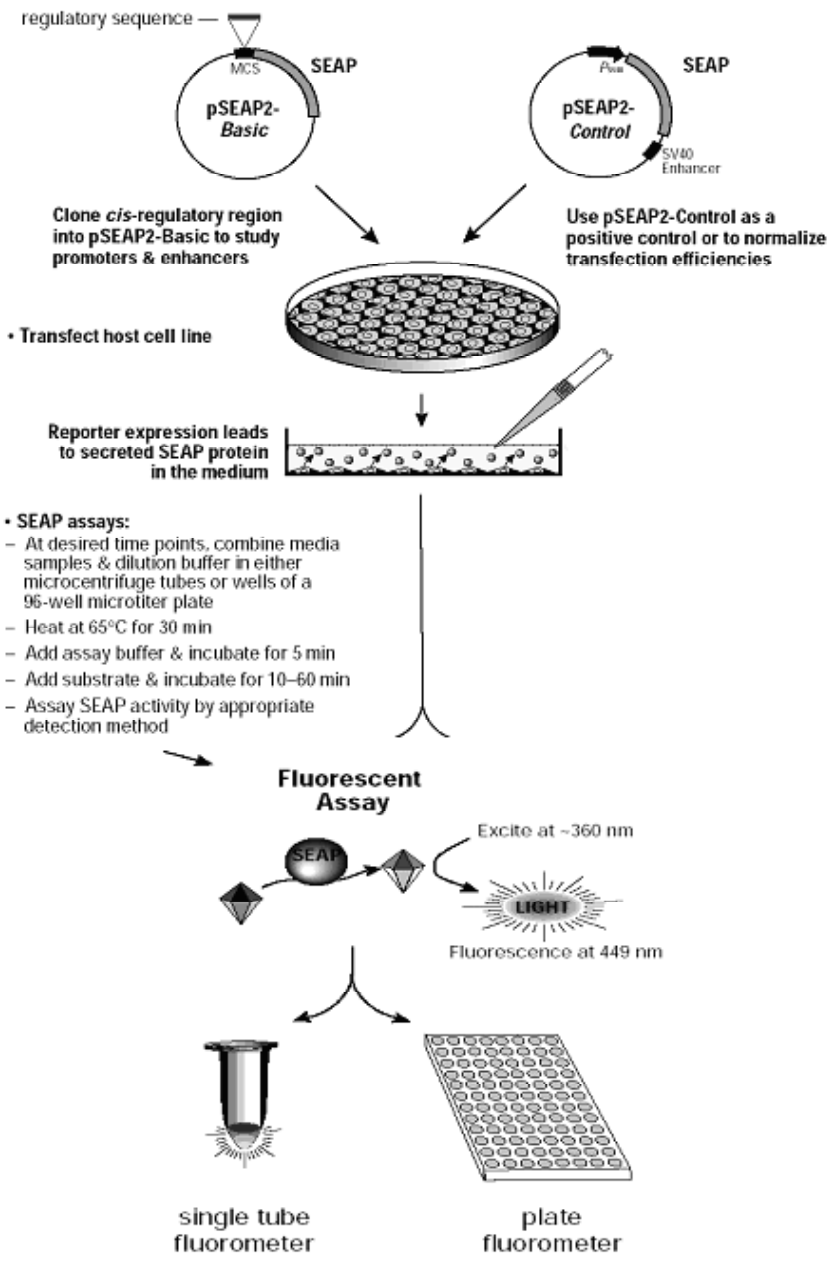



**Figure 10 Flowchart of the BD Great EscAPe™ SEAP Assay procedure**

### 3.19.2. Transient Transfection of Human Mesangial Cells

Human mesangial cells were transiently transfected using "Superfect Transfection Reagent" (Qiagen, Hilden, Germany) which is a specifically designed activated dendrimer. It possesses a defined spherical architecture with branches radiating from a central core and terminating at charged amino groups. The Superfect reagent assembles DNA into compact structures optimizing the entry of DNA into the cell. Superfect-DNA complexes possess a net positive charge which allows them to bind to negatively charged receptors on the surface of eukaryotic cells. Once inside, Superfect reagent buffers the lysosome after it has fused with endosome, leading to pH inhibition of lysosomal nucleases. This ensures stability of Superfect-DNA complexes and the transport of intact DNA to the nucleus.

Human mesangial cells were seeded at a density of 70- 80,000 cells per well of a 24 well plate. The cells were allowed to grow for 6 hours in Dulbecco's modified eagles medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (FCS) and 1% Pencillin/ Streptomycin (PS). After 6 hours, the medium was changed to 0.1% DMEM+ 1% PS for 12 hours. At this stage, the cell density had reached around 40%. After a brief wash with DMEM, the cells in each well were transfected with 1µg of pCTGF-SEAP, pSEAP-basic or promoter-less pSEAP circular vector DNA using 2µl of Superfect reagent for 2 hours in a final volume of 350µl per well. The DNA/Superfect complex formation was performed as described in the manufacturer's product manual. After transfection, cells were washed once with growth medium and incubated for next 6 hours in DMEM+ 10%FCS+ 1%PS+ 5ng/mlTGF-ß+ 25.5mM D-glucose. After 6 hours, the cells were stimulated in the presence or absence of increasing doses of different nitric oxide (NO) donors for 66 hours.

## 3.20. Statistical analysis

All results are expressed as means and SD unless otherwise stated. Student's unpaired *t*-test was used for statistical analyses. *P* values less than 0.05 were considered statistically significant.

# *Results*

## 4.1. Identification of genes differentially regulated by nitric oxide

To identify genes differentially expressed by NO, MCs were treated with 500 μM GSNO for 8 hours, a concentration which has been shown to inhibit MC proliferation, and at the same time is non-toxic and does not induce apoptosis of MC in the presence of serum (Rupprecht et al., 2000). GSNO is an external and potent NO-donor with a half-life of about 10 hours, which generates NO+ and NO radicals. It can be easily synthesized from glutathione and $NaNO_2$. MC were sub-confluent at the beginning of the experiment.

From control and GSNO-treated cells, poly $A^+$-RNA was isolated and reverse transcribed into cDNA. The cDNA was subtracted by RDA as described in materials and methods. To estimate the efficiency of the subtraction procedures the abundance of a known housekeeping gene, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, was compared by PCR before and after subtraction. PCRs of the subtracted and unsubtracted cDNAs with 18, 23, 28 and 33 PCR-cycles were performed. For the unsubtracted cDNA the GAPDH signal should be seen after 18 to 23 cycles. In the "Pool A" subtracted cDNA sample a GAPDH signal was seen after approximately 33 cycles corresponding to a roughly 1500-fold enrichment of potentially differentially expressed cDNAs (Fig. 11).

For the subtraction "Pool B" the difference was about 10 cycles, which corresponds to a roughly 400-fold enrichment (data not shown). Although some difference in subtraction efficiency was observed, both enrichments were suitable for the further steps of the procedure. Therefore, the amplified PCR-fragments from both subtractions pools were ligated into the pCR-Script SK (+) vector and transfected into E.coli XL1-Blue MRF' bacteria.

A total of 51 clones were isolated, 23 clones from the subtraction "Pool A" (identified as sequences representing upregulated genes) and 28 clones from the subtraction "Pool B" (identified as sequences representing downregulated genes). The cDNA inserts varied in size from 130 bp to up to 1.5 kb. To test for the possible differential expression of these cDNAs, two identical dot blots were prepared and hybridized with radiolabeld cDNA from both pools 'A and B' (summarized in table 1). A total of 45 clones showed regulation in the dot blots.

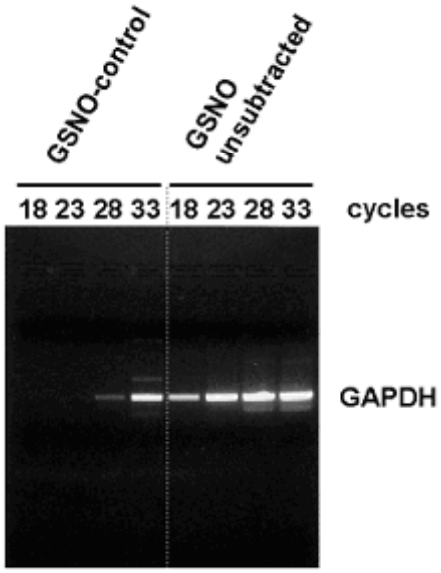



**Figure 11 Subtraction efficiency test of the RDA method**

Four PCRs of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA with 18, 23, 28 and 33 PCR-cycles comparing "GSNO minus control" subtracted (lanes 1 to 4) and unsubtracted (lanes 5 to 8) cDNA-samples were performed. Amplified cDNA was size fractionated on a 1.2% agarose gel and stained with ethidium bromide. The difference between the GAPDH-signals of subtracted and unsubtracted samples corresponds to a roughly 1500-fold enrichment of potentially GSNO-upregulated cDNAs.

### 4.1.1. Sequence comparison of the isolated clones

All 45 clones were sequenced and the acquired sequence data were aligned against the GenBank nucleotide (rat genome) database at the National Center for Biotechnology Information (National Institutes of Health, Bethesda, MD) using "Blastn" to search for sequence matches. 7 Gene sequences, identified from the pool "A" showed upregulation in dot blot and had 98-100% homologies to different genes of diverse physiological relevance (table 1) and 11 gene sequences from pool "B", showed downregulation in dot blot and had 98-100% homology to 4 well characterized matrix-associated genes and 7 genes with diverse biological significance (table 1). Several sequences from both pools were homologous to cloning vector and ribosomal RNA gene sequences (not listed).

### 4.1.2. List of differentially expressed genes of GSNO-treated rat mesangial cells

| **Pool "A": Upregulated genes** | **Genbank Accession #** |
|---|---|
| Pyruate kinase, muscle (Pkm2) | NM_053297 |
| Hemochromatosis (Hfe) | NM_053301 |
| ATPase synthase subunit 6 | AF504920 |
| Tyrosine 3- mono-oxygenase (Ywhab) | NM_019377 |
| Integrin beta 1 (Itgb1) | NM_017022 |
| Aldose reductase (Akr1b10) | NM_001013084 |
| Triosphosphate isomerase 1 (Tpi1) | NM_022922 |
| **Pool "B": Downregulated genes** | **Genbank Accession #** |
| Poly (A) binding protein 1(Pabpc1) | NM_134353 |
| ***Thrombospondin-1 (TSP-1)*** | AF309630 |
| ***Connective tissue growth factor (CTGF)*** | NM_022266 |
| ***Procollagen type I alpha1 (COL1A1)*** | Z78279 |
| ***Procollagen type I alpha2 (COL1A2)*** | NM_0253356 |
| Mitochondrial cytochrome B5 (Cyb5) | NM_022245 |
| G rich sequence factor-1(GRSF-1) | XM_223327 |
| Ornithine aminotransferase (Oat) | NM 22521 |
| Dimethylaminohydrolase 1(Ddah1) | NM_022297 |
| mRNA for astrocytic phosphoprotein (PEA-15) | AJ243949 |
| Translation initiation factor 5 (Eif5) | NM_020075 |

**Table.1 Differentially expressed genes identified by RDA.**

Rat MCs were incubated for 4 hours in the presence or absence of 500μM S-nitroso-glutathione. The subtraction library was generated using the cDNA representational difference analysis consisting of Pool "A" or upregulated and Pool "B" or downregulated genes. PCR products were cloned and sequenced. Sequence analysis was performed compared to public databases by using BLAST.

## 4.2. NO donors are chemically active in mesangial cells

To investigate whether the NO donors used in an expanded study were chemically active, the stable and non-volatile metabolites of NO in the form of nitrites from the conditioned media were measured by Griess assay. Results from this study revealed that spermine NONOate, Deta NONOate, and NOC-18 under similar conditions produced nitrite as a stable metabolite of NO (Fig.12). Under our experimental settings, maximum nitrite production (280µM) was observed when human MCs were treated for 4 hours with 500µM of spermine NONOate which has a half-life of only 39 minutes. This level was reduced to 220µM after 24 hours. Nitrite accumulation with 500µM of Deta NONOate (half-life: 20 hours at 37°C) and NOC-18 (half-life: 56 hours at 37°C) was more pronounced at later time points with a maximum nitrite accumulation of 300µM with DetaNONOate and 290µM with NOC-18 at 24 hours. Under similar conditions unstimulated control human MCs showed a basal nitrite level of 2µM at 4, 8, 12 and 24 hours. These results indicate that all the NO donors tested were chemically active, although, the kinetics of NO release was different.

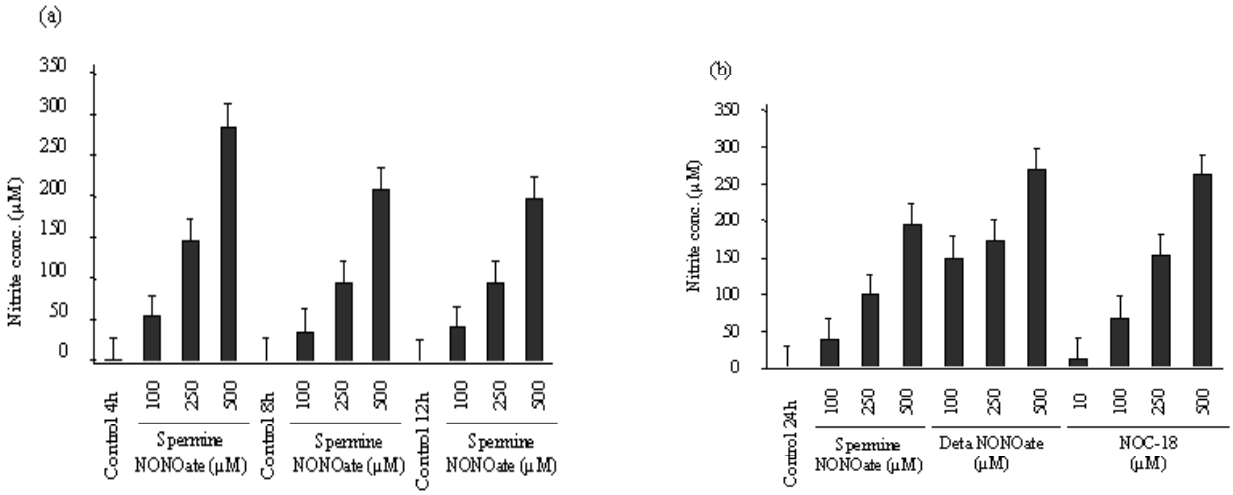



**Figure 12 Nitrite accumulations in human mesangial cells using different NO donors**

Quiescent human mesangial cells were incubated with medium containing 30mM glucose and 5ng/ml TGF-β1 for 24 hours before stimulation with or without spermine NONOate, DetaNONOate or NOC-18 at the indicated concentrations and time points. Supernatants were harvested and assayed for nitrite content by using the Griess assay (Promega). Data are the mean of three independent experiments.

## 4.3. Production of endogenous NO is stimulated by the addition of INFγ and BH4; Inhibitors of iNOS block the production of NO stimulated by INFγ and BH4

To investigate whether a combination of INFγ and BH4 (a NOS co-factor) can stimulate the release of NO in MCs, quiescent MCs were first treated for 24 hours with 30mM glucose and then stimulated with 200U/ml of INFγ alone or INFγ plus 10μM of BH4 for 0-24 hours in presence or absence of iNOS inhibitors- 1400W, L-NIL and L-NMMA. The media were harvested at different time points and assayed for the presence or absence of nitrite by Griess assay. Nitrite concentration in the medium of INFγ and BH4- stimulated cells was significantly higher (7μM ± SD; P< 0.001) than in medium from untreated cells in multiple experiments. There was a significant decrease (P< 0.001) in nitrite accumulation in the medium from cells treated with either L-NIL, 1400W or L-NMMA in presence of INFγ+BH4 (Fig.13). 1400W was chosen as the inhibitor of iNOS enzymatic activity for future experiments.

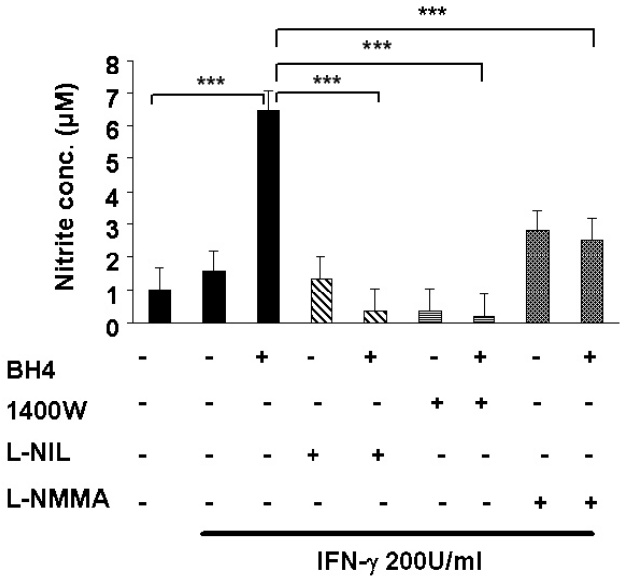



**Figure 13 Nitrite assay demonstrating the production of NO in response to INF-γ stimulation and inhibitory effects of iNOS inhibitors.**

Quiescent human mesangial cells were incubated with medium containing 30mM glucose for 24 hours. Cells were left untreated or treated simultaneously, in presence of 200U/ml INF-γ or 200U/ml INF-γ plus 10μM BH4, with 200μM 1400W, 100μM L-NIL, or 250μM L-NMMA. After 20 hours media were collected and assayed for nitrite content by using Griess assay. Data shown are mean of three independent experiments ± SD; ***P<0.001 compared to cells treated with 200U/ml INF-γ plus 10μM BH4.

## 4.4. INFγ and BH4 stimulation of human mesangial cells leads to the induction of iNOS mRNA and protein

Given that a combination of INFγ and BH4 are able to stimulate the production of NO (Fig 14a and b) in human mesangial cells and that a selective iNOS inhibitor, 1400W, is able to lower the NO production to a significant level, we next investigated whether INFγ and BH4 were also able to induce the expression of iNOS in MCs. For this purpose human MCs were treated with INFγ and BH4 and total RNA and protein was harvested for Northern and Western blot analysis respectively. Increased iNOS mRNA and protein expression was detected after 20 hours of stimulation with INFγ or INFγ plus BH4 in human (Fig. 14b) as well as rat MCs (data not shown). Controls did not show any expression at the mRNA or protein levels.

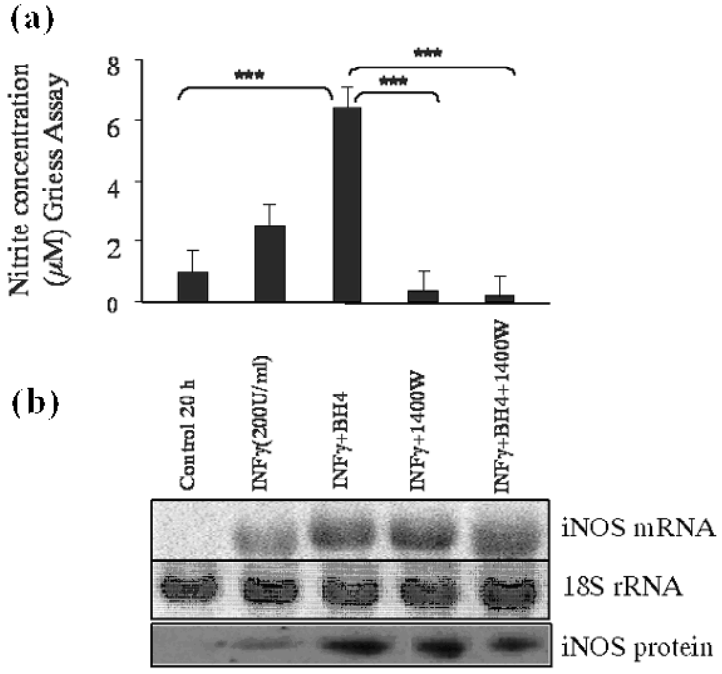



**Figure 14 INFγ and BH4 lead to the production of NO by inducing iNOS in human MCs**

(a) Quiescent human MCs grown in medium containing 30mM glucose for 24 hours were left untreated (lane 1) or treated with 200U/ml INF-γ (lane 2), 200U/ml INF-γ plus 10μM BH4 (lane 3), 200U/ml INF-γ plus 200μM 1400W (lane 4) or 200U/ml INF-γ plus 10μM BH4 plus 200μM 1400W (lane 5) for 20 hours and nitrite release was quantified by nitrite assay. (b) Northern blot analysis was performed with the RNA extracted from the stimulated cells and probed for iNOS or 18S rRNA. For the determination of iNOS protein, equal amounts of total protein was subjected to SDS-PAGE analysis and probed with an anti-rabbit iNOS polyclonal antibody as described in "materials and methods".

## 4.5. Induction of MC iNOS by pro-inflammatory cytokines

To demonstrate the induction of iNOS gene by a pro-inflammatory cytokine mixture, rat mesangial cells were treated with a mixture of cytokines (IL1-β 250U/ml; INF-γ 200U/ml; TNFα 500U/ml). Total RNA was harvested after 8, 12 and 24 hours and subjected to Northern blot hybridization with a radioactively labeled human iNOS cDNA probe. The cytokine cocktail led to a strong activation of iNOS mRNA at 12 and 24 hours. Treatment of the cells with 1400W resulted in a considerable inhibition of iNOS mRNA induction (Fig. 15).

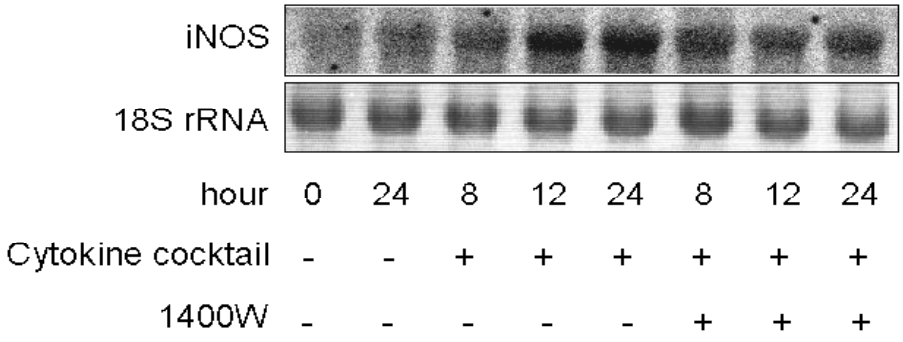



**Figure 15 A mixture of cytokines leads to the induction of iNOS in rat MCs**

Quiescent rat MCs were left untreated or treated with a cytokine cocktail (IL1-β 250U/ml; INF-γ 200U/ml; TNFα 500U/ml) with or without the iNOS inhibitor- 1400W for 8, 12 and 24 hours. 15µg of total RNA from the monolayer of stimulated cells was isolated at the indicated time points and subjected to Northern blot hybridization using probes for 18S rRNA and iNOS.

## 4.6. GSNO downregulates the expression of CTGF and TSP-1 in rat mesangial cells

To validate our RDA findings predicting the downregulation of CTGF and TSP-1, we analyzed total RNA extracted from GSNO stimulated rat MCs (treated with 5ng/ml TGF-β1 and 30mM glucose for 24 hours prior to NO stimulation) by Northern blot analysis using gene specific cDNA probes. Control cells were treated with equimolar amounts of GSH or decomposed GSNO (DGSNO) for 4 and 8 hours. A transient downregulation of CTGF and TSP-1 mRNA (Fig 16) was observed after 500μM of GSNO treatment. GSH and DGSNO alone had no effect on the downregulation of both the genes.

## 4.7. DETA NONOate downregulates the expression of COL1A1 and COL1A2 in rat mesangial cells

To validate our RDA findings predicting the downregulation of COL1A1 and COL1A2, total RNA extracted was analyzed from DETA NONOate stimulated rat MCs (treated with 5ng/ml TGF-β1 and 30mM glucose for 24 hours prior to NO stimulation) by Northern blot analysis using gene specific cDNA probes. Control cells were left untreated. A transient downregulation of COL1A1 and COL1A2 mRNA (Fig. 17) was observed after 500μM of DETA NONOate treatment for 8, 12 and 24 hours.

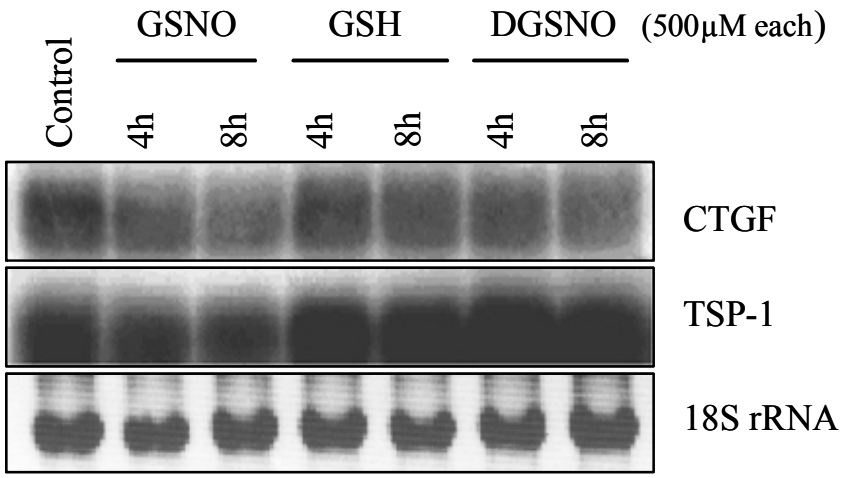



**Figure 16 CTGF ands TSP-1 are downregulated by GSNO in rat MCs**

Quiescent rat mesangial cells were incubated with medium containing 30mM glucose and 5ng/ml TGF-β1 for 24 hours before stimulation with or without 500μM each of GSNO, GSH or decomposed GSNO (DGSNO) at 4 and 8 hours. Total RNA (15μg) was isolated and the mRNA expression of CTGF and TSP-1 was analyzed by Northern blotting.

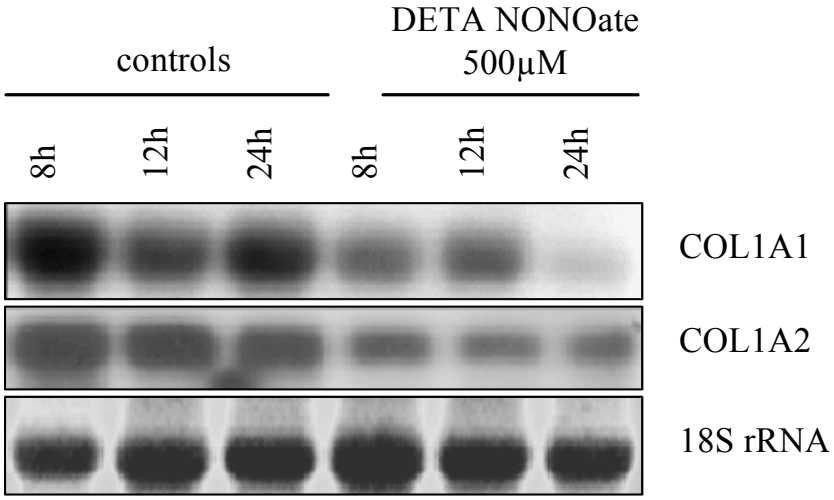



**Figure 17 COL1A1 ands COL1A2 are downregulated by DETA NONOate in rat MCs**

Quiescent rat mesangial cells were incubated with medium containing 30mM glucose and 5ng/ml TGF-β1 for 24 hours before stimulation with or without 500μM of DETA NONOate at the indicated time points. Total RNA (15μg) was isolated and the mRNA expression of COL1A1 and COL1A2 was analyzed by Northern blotting.

## 4.8. Spermine NONOate downregulates the expression of COL1A2 mRNA in rat mesangial cells

To investigate whether COL1A2 can also be downregulated by spermine NONOate, total RNA extracted was analyzed from spermine NONOate stimulated rat MCs (treated with 5ng/ml TGF-β1 and 30mM glucose for 24 hours prior to NO stimulation) by Northern blot analysis using gene specific cDNA probes. Control cells were left untreated. A prolonged downregulation of COL1A2 mRNA (Fig. 18) was observed after 500μM of spermine NONOate treatment for 4, 8, 12 and 24 hours.

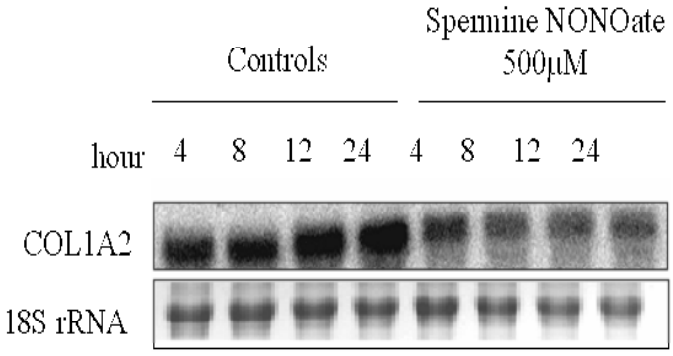



**Figure 18 Exogenous NO donor spermine NONOate downregulates mRNA expression of COL1A2 in rat mesangial cells**

Quiescent rat mesangial cells were incubated with medium containing 30mM glucose and 5ng/ml TGF-β1 for 24 hours before stimulation with or without spermine NONOate at the indicated concentrations and time points. Total RNA (15μg) was isolated and the mRNA expression of COL1A2 was analyzed by Northern blotting.

## 4.9. Spermine NONOate downregulates the expression of TSP-1 in human mesangial cells

To investigate the downregulation of TSP-1 in human mesangial cells, we analyzed total RNA extracted from spermine NONOate stimulated human MCs (treated with 5ng/ml TGF-β1 and 30mM glucose for 24 hours prior to NO stimulation) by Northern blot analysis using gene specific cDNA probes. A time and dose dependent downregulation of TSP-1 mRNA (Fig. 19) was observed after treatment with spermine NONOate.

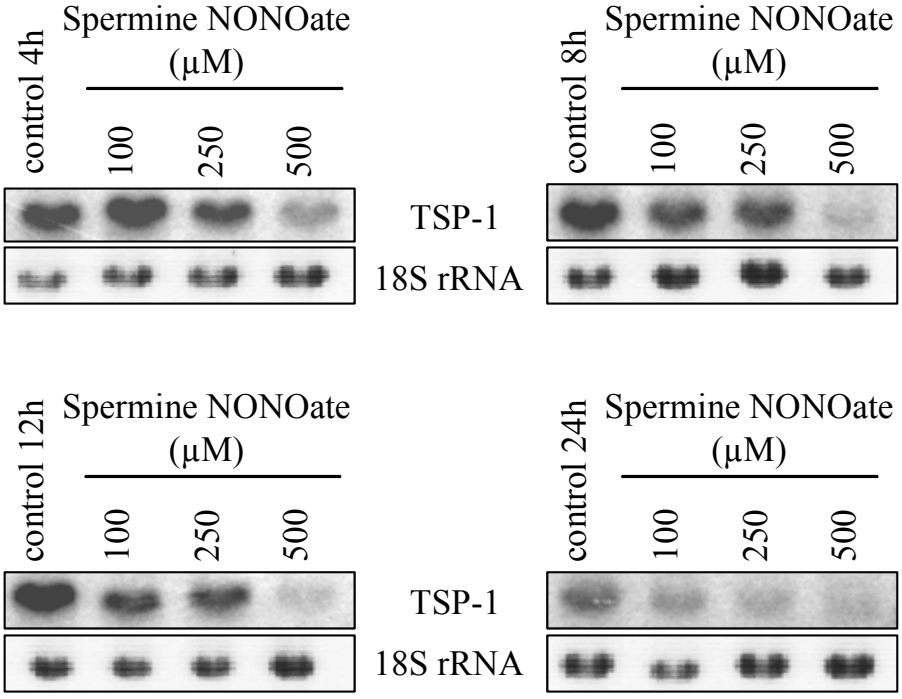



**Figure 19 Exogenous NO donor spermine NONOate downregulates mRNA expression of TSP-1 in human mesangial cells**

Quiescent human mesangial cells were incubated with medium containing 30mM glucose and 5ng/ml TGF-β1 for 24 hours before stimulation with or without spermine NONOate at the indicated concentrations and time points. Total RNA (15μg) was isolated and the mRNA expression of TSP-1 was analyzed by Northern blotting.

## 4.10. Spermine NONOate downregulates the expression of CTGF in human mesangial cells

To investigate the downregulation of CTGF in human mesangial cells, we analyzed total RNA extracted from spermine NONOate stimulated human MCs (treated with 5ng/ml TGF-β1 and 30mM glucose for 24 hours prior to NO stimulation) by Northern blot analysis using gene specific cDNA probes. Treatment with spermine NONOate resulted in a dose- and time-dependent downregulation of CTGF mRNA (Fig. 20).

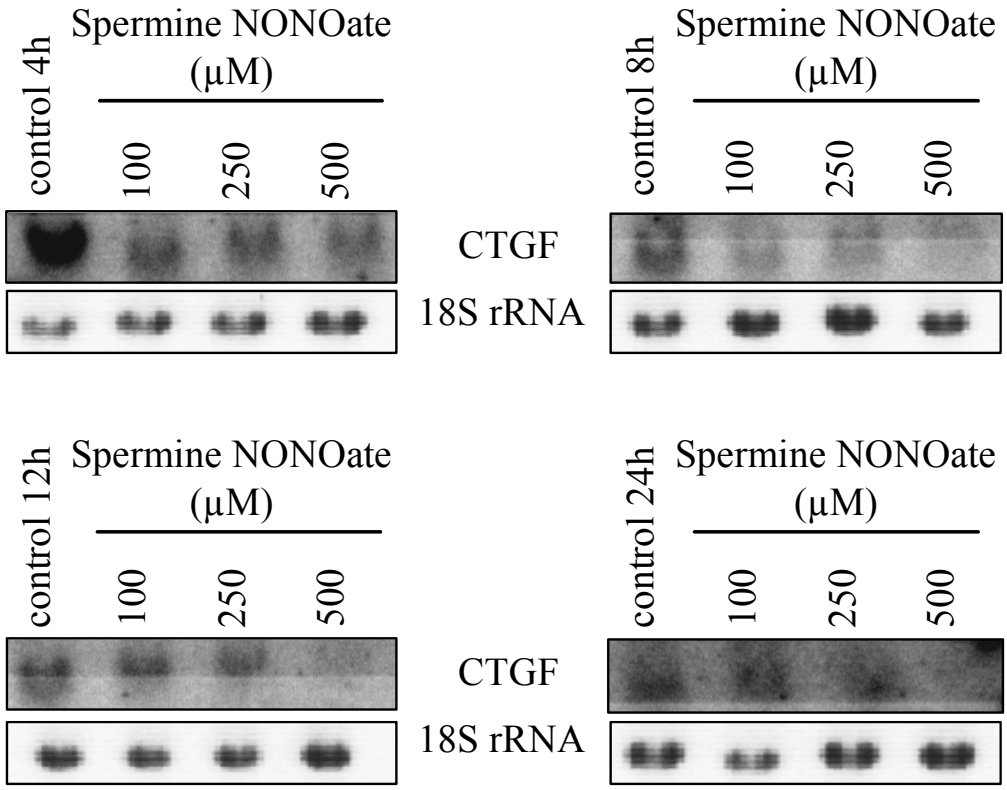



**Figure 20 Exogenous NO donor spermine NONOate downregulates mRNA expression of CTGF in human mesangial cells**

Quiescent human mesangial cells were incubated with medium containing 30mM glucose and 5ng/ml TGF-β1 for 24 hours before stimulation with or without spermine NONOate at the indicated concentrations and time points. Total RNA (15μg) was isolated and the mRNA expression of CTGF was analyzed by Northern blotting.

## 4.11. Endogenously generated NO downregulates CTGF and TSP-1 mRNA expression in human MC and COL1A2 mRNA in rat MC

To demonstrate that endogenously produced NO in response to iNOS activation by INFγ and BH4 in human MCs leads to the downregulation of CTGF and TSP-1, quiescent human MCs grown for 24 hours in medium containing 30mM glucose were treated with 200U/ml of INFγ and 200μM of BH4 or INFγ alone for 20 hours in the absence or presence of 1400 W. Control cells were left untreated. Total RNA was subjected to Northern blot hybridization with gene specific radioactively labeled probes. CTGF and TSP-1 mRNA were downregulated by INFγ alone or INFγ in combination with BH4 (Fig. 21a, lanes 2, 3). 1400W rescued the downregulatory effects, indicating that effects are NO specific.

To demonstrate that similar changes are induced in COL1A2 mRNA in response to NO produced enzymatically by NO synthase induction in rat mesangial cells, the expression of iNOS was stimulated by using a mixture of cytokines (IL1-β 250U/ml; INF-γ 200U/ml; TNFα 500U/ml). Total RNA was harvested after 8, 12 and 24 hours and subjected to Northern blot hybridization with a radioactively labeled iNOS cDNA probe. The cytokine cocktail led to a strong activation of iNOS mRNA at 12 and 24 hours (Fig. 15). Treatment of the cells with 1400W, a selective iNOS inhibitor, in presence of the cytokine cocktail reduced the iNOS mRNA levels by almost 50 percent (Fig. 15). Hybridization of the same membrane to COL1A2 radiolabelled probe showed that the COL1A2 mRNA was substantially downregulated at the 24 hour time point and 1400W had rescued the downregulatory affects of the endogenously produced NO (Fig. 21b).

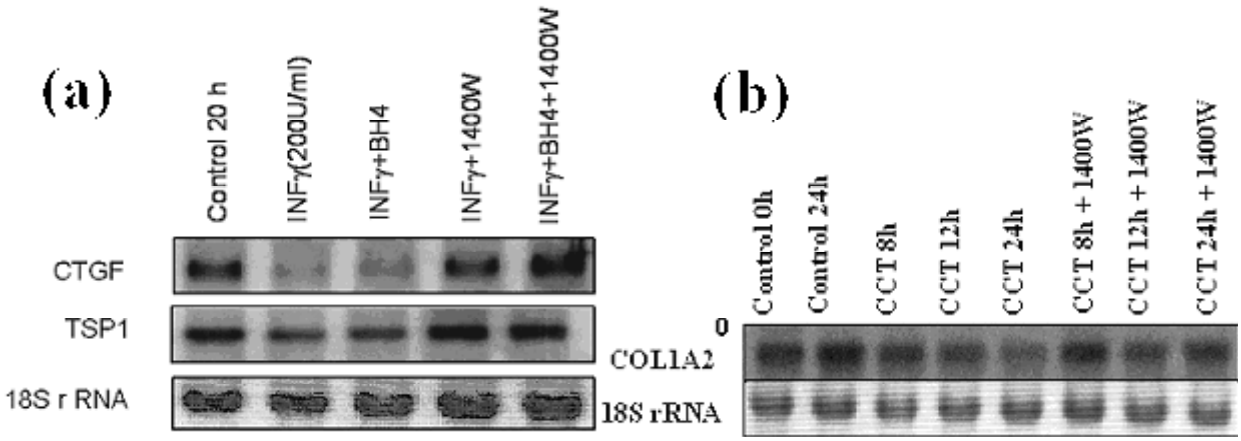



**Figure 21 Endogenously produced NO downregulates the expression of CTGF and TSP-1 in human MC and COL1A2 in rat mesangial cells**

(a) Quiescent human MCs grown in medium containing 30mM glucose for 24 hours were left untreated (lane 1) or treated with 200U/ml INF-γ (lane 2), 200U/ml INF-γ plus 10μM BH4 (lane 3), 200U/ml INF-γ plus 200μM 1400W (lane 4) or 200U/ml INF-γ plus 10μM BH4 plus 200μM 1400W (lane 5) for 20 hours. Northern blot analysis was performed with the RNA extracted from the stimulated cells and probed for CTGF, TSP-1 or 18S rRNA.

(b) Quiescent rat MCs were left untreated or treated with a cytokine cocktail (IL1-β 250U/ml; INF-γ 200U/ml; TNFα 500U/ml) with or without the iNOS inhibitor- 1400W for 8, 12 and 24 hours. 15μg of total RNA from the monolayer of stimulated cells was isolated at the indicated time points and subjected to Northern blot hybridization using probes for 18S rRNA and COL1A2.

## 4.12. Exogenous NO downregulates the expression of biglycan, COL4A2, COL1A1 and COL1A2 mRNA in human mesangial cells

To investigate whether Biglycan, COL4A2 (two additional ECM associated genes), COL1A1 and COL1A2 were also downregulated by spermine NONOate, total RNA extracted from Spermine NONOate stimulated human MCs (treated with 5ng/ml TGF-β1 and 30mM glucose for 24 hours prior to NO stimulation) was subjected to real time PCR (RT-PCR) analysis. Gene specific RT-PCR probes as described in materials and methods were used. Control cells were left untreated. A dose and time dependent downregulation of Biglycan, COL4A2, COL1A1 and COL1A2 gene expression (Fig. 22) was observed after 100, 250 and 500µM of spermine NONOate treatment for 4, 8, 12 and 24 hours.

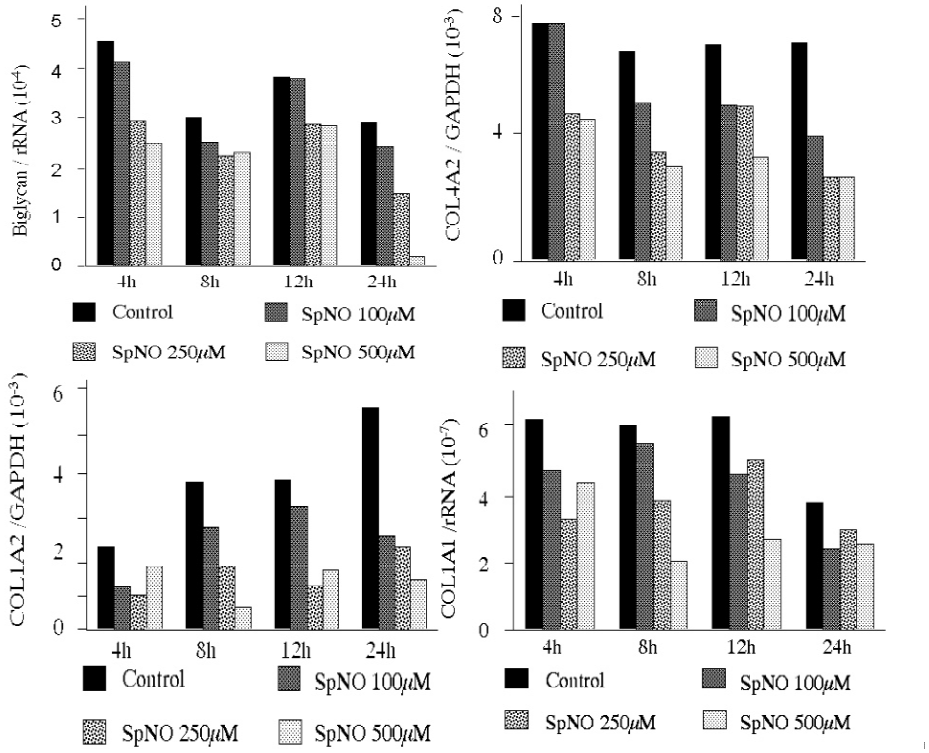



**Figure 22** **Exogenous NO donor Spermine NONOate downregulates mRNA expression of Biglycan, COL4A2, COL1A1 and COL1A2 in human mesangial cells**

Quiescent human mesangial cells were incubated with medium containing 30mM glucose and 5ng/ml TGF-β1 for 24 hours before stimulation with or without spermine NONOate at the indicated concentrations and time points. Total RNA was isolated and subjected to RT-PCR analysis by using gene specific RT-PCR probes as described in materials and methods.

## 4.13. Endogenously generated NO downregulates BGN, COL4A2, COL1A1 and COL1A2 gene expression in human mesangial cells

To demonstrate that endogenously produced NO in response to iNOS activation by INFγ and BH4 in human MCs leads to the downregulation of Biglycan, COL4A2 (two additional ECM associated genes), COL1A1 and COL1A2, quiescent human MCs grown for 24 hours in medium containing 30mM glucose were treated with 200U/ml of INFγ and 200µM of BH4 or INFγ alone for 20 hours in presence of 1400 W. Controls were untreated. Total RNA was subjected to RT-PCR analysis by using gene-specific RT-PCR probes. Results showed a significant downregulation of Biglycan, COL4A2 and COL1A1 mRNA in the samples treated with INFγ alone or INFγ in combination with BH4. The downregulation of COL1A2 was only significant in presence of $BH_4$ (Fig. 23, lanes 2, 3). 1400W, a selective iNOS inhibitor had rescued the downregulatory effects of the combination of INFγ plus BH4 indicating that effects are NO specific. Interestingly, the effects of INFγ alone were not rescued by 1400W.

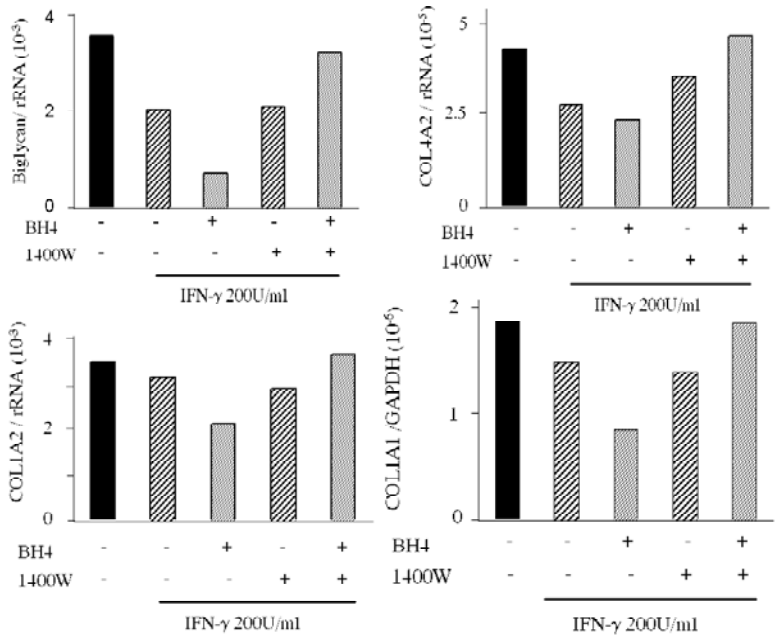



**Figure 23 Endogenously produced NO downregulates the expression of biglycan, COL4A2, COL1A1 and COL1A2 in human MC**

Quiescent human MCs grown in medium containing 30mM glucose for 24 hours were left untreated (lane 1) or treated with 200U/ml INF-γ (lane 2), 200U/ml INF-γ plus 10µM BH4 (lane 3), 200U/ml INF-γ plus 200µM 1400W (lane 4) or 200U/ml INF-γ plus 10µM BH4 plus 200µM 1400W (lane 5) for 20 hours. Total RNA was subjected to RT-PCR analysis by using gene-specific RT-PCR probes.

## 4.14. Exogenous NO donors downregulate CTGF protein expression

To investigate CTGF protein expression, human MCs were stimulated with 100 to 500μM of Spermine NO and NOC-18 for 24 hours and equal amounts of protein was subjected to SDS polyacralamide gel electrophoresis. CTGF protein was detected with a CTGF anti-human monoclonal antibody (FG-3019). Strong CTGF protein expression was detected in the control sample which was reduced in intensity in NO treated samples (Fig 24). There was a strong inhibition of CTGF protein expression in samples treated with 500 μM of both spermine NONOate and NOC-18.

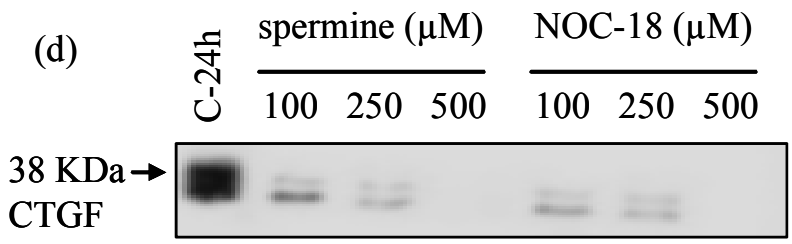



**Figure 24 CTGF protein expression is strongly downregulated by exogenous NO treatment.**

Quiescent human mesangial cells grown in medium containing 30mM glucose and 5ng/ml TGF-β1 for 24 hours were treated or not treated with indicated concentrations of spermine NONOate or NOC-18 for 24 hours. Total protein was harvested and subjected to Western blot analysis with human anti-CTGF antibody as described under "Experimental procedures".

## 4.15. Exogenous NO donors downregulate TSP-1 protein expression

To verify the effects of exogenous NO donors on TSP-1 and CTGF protein expression, quiescent human mesangial cells were first treated for 24 hours with 5ng/ml TGF-β1 and 30mM glucose. Three different NO donors, Spermine NONOate, SNAP and NOC-18 in increasing doses were used to stimulate cultured cells for an additional 12 or 24 hours. After 12 and 24 hours of incubation, conditioned media were harvested and TSP-1 protein concentrations were determined by ELISA. We observed a significant ($P< 0.001$) dose and time dependent decrease in the TSP-1 protein content of spermine NONOate, SNAP and NOC-18 treated cells at all time points and concentrations except for 100, 250 and 500μM of spermine NONOate at 12 hours (Fig. 25 – a, b, c).

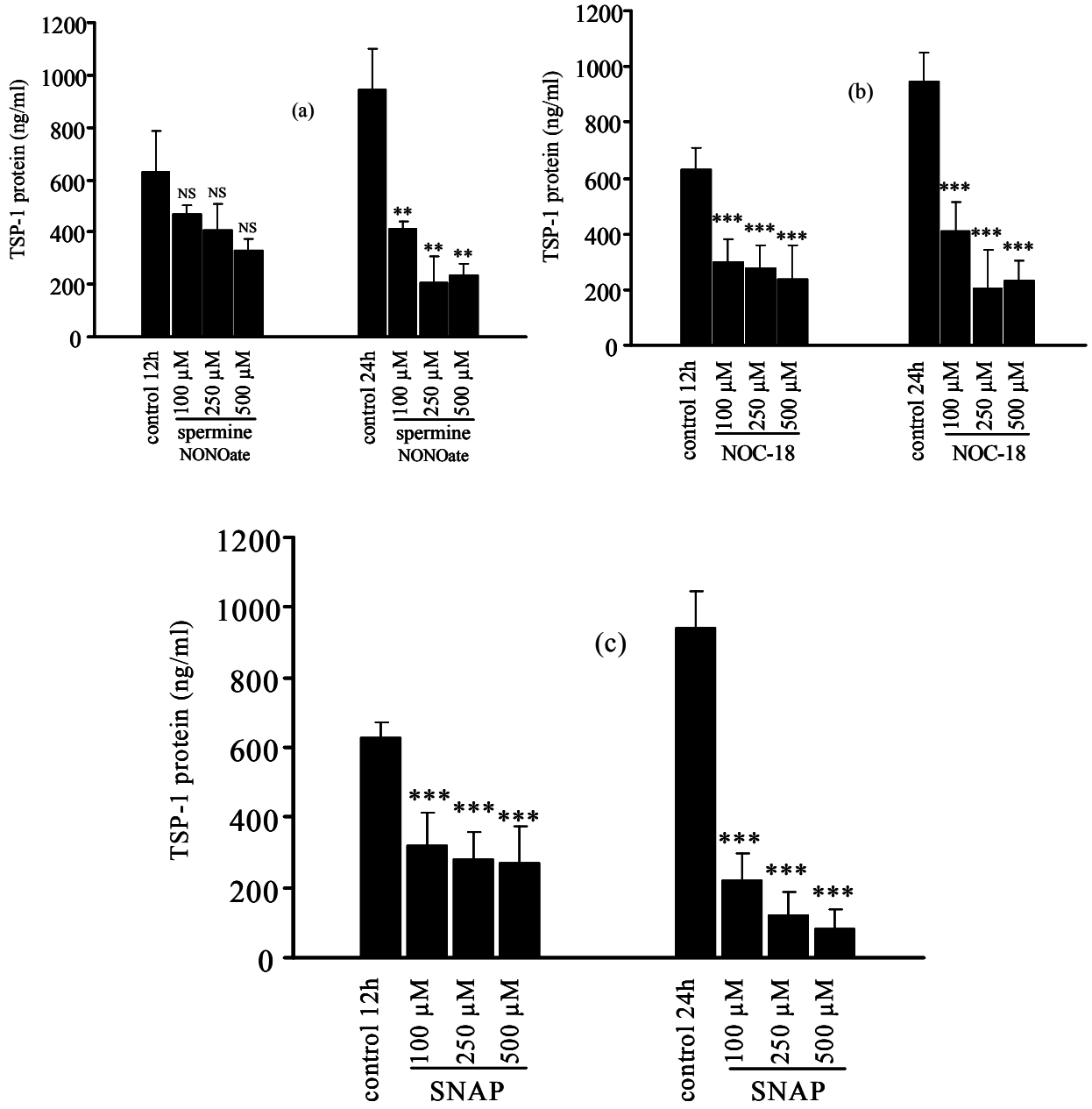



**Figure 25 TSP-1 protein expression is strongly downregulated by exogenous NO treatment.**

(a) Quiescent human mesangial cells were incubated with medium containing 30mM glucose and 5ng/ml TGF-β1 for 24 hours before stimulation with or without 100, 250 and 500µM of NOC-18, (b) Spermine NONOate and (c) SNAP for 12 and 24 hours. Conditioned media were collected after 12 and 24 hours and secreted level of TSP-1 were measured by enzyme-linked immunosorbent assay (ELISA) as described under "Experimental procedures". Data represent mean ± SD of 8 cultures for each condition. TSP-1 levels were decreased significantly (**P< 0.01 and (***P< 0.001) as compared to untreated controls with all indicated concentrations of spermine NONOate at 24 hours, NOC-18 at 12 and 24 hours and SNAP at 12 and 24 hours.

## 4.16. Downregulation of CTGF by NO is not mediated by changes in mRNA stability

To determine the effect of NO on CTGF mRNA stability; we performed Northern blot analysis of CTGF mRNA in human MCs treated with actinomycin D (10ng/ml) for up to 8 hours after incubation of MCs in the presence or absence of 500 μM of spermine NONOate. The rate of decay of the CTGF message was of equivalent magnitude in cells grown in spermine NO treated media (Fig. 26, a and b), and the average half-life of CTGF mRNA was calculated to be around 2 h in control and spermine NO-treated media (Fig.26c). We concluded that the decrease in the steady-state level of CTGF mRNA elicited by spermine NO treatment in MCs is unlikely to be due to decreased mRNA stability, although a small component of message stabilization cannot be excluded.

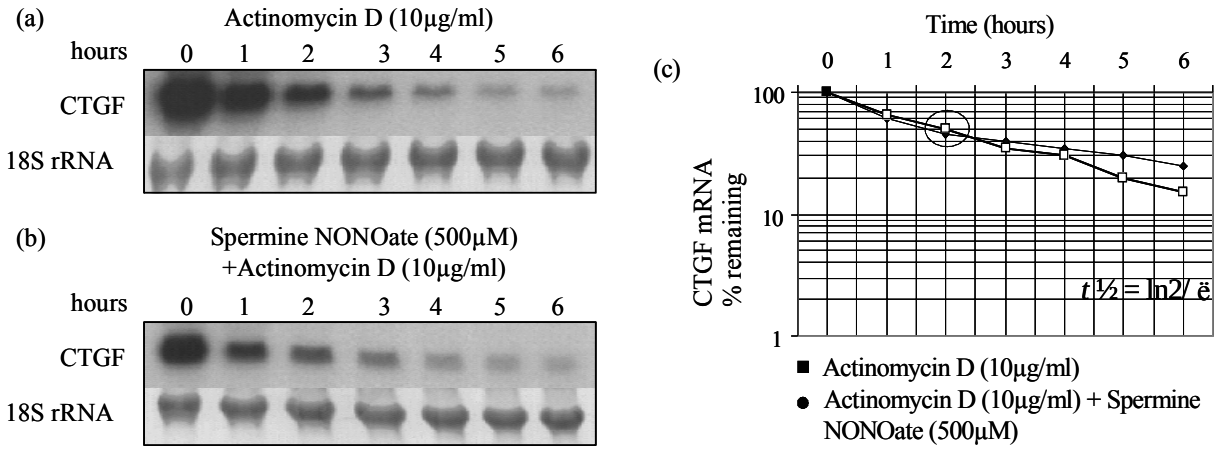



**Figure 26 CTGF mRNA stability is not affected by NO**

(a, b) Quiescent human MCs grown in serum free medium containing 30mM glucose and 5ng/ml TGF-β1 for 24 hours were exposed to actinomycin D alone (10 μg/ml) or to actinomycin D (10μg/ml) and Spermine NO (500 μM). Total RNA from the stimulated cells was subjected to Northern blot hybridization using radiolabelled CTGF probes. I Graphic profile of CTGF mRNA decay after treatment with actinomycin D for cells un- stimulated (□) or stimulated with 500 μM spermine NONOate (♦). Measurements at time 0 *(*from actinomycin D treatment) were assigned a relative value of 100%. The average half-life of the CTGF message was calculated to be around 2 hours in both treated and untreated samples.

## 4.17. Gene organization, cloning and transfection of CTGF promoter into human mesangial cells

CTGF protein contains four structural modules following the signal peptide (SP): insulin-like growth-factor binding (IGFB) domain, chordin-like cysteine rich (CR) domain, thrombospondin type 1 repeat (TSP-1), and a C-terminal cystineknot (CT).

The CTGF promoter (-805/17) was cloned into a pSEAP-basic vector and the promoter-reporter construct was transfected into human mesangial cells (Fig. 27).

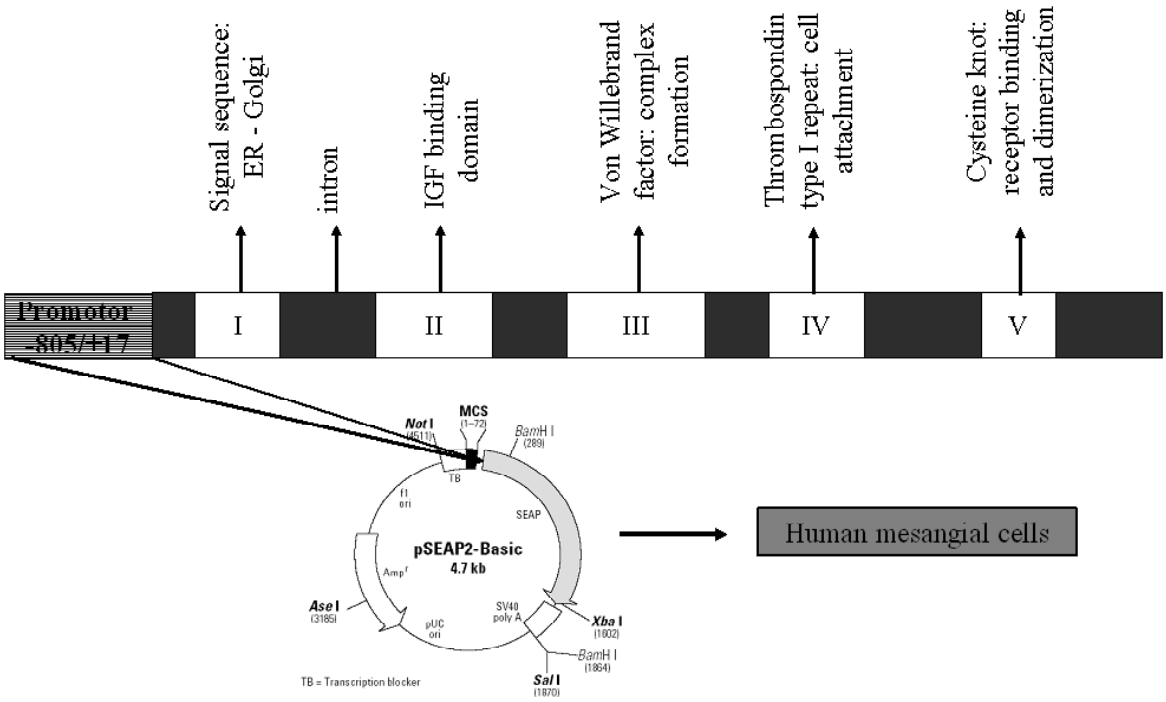
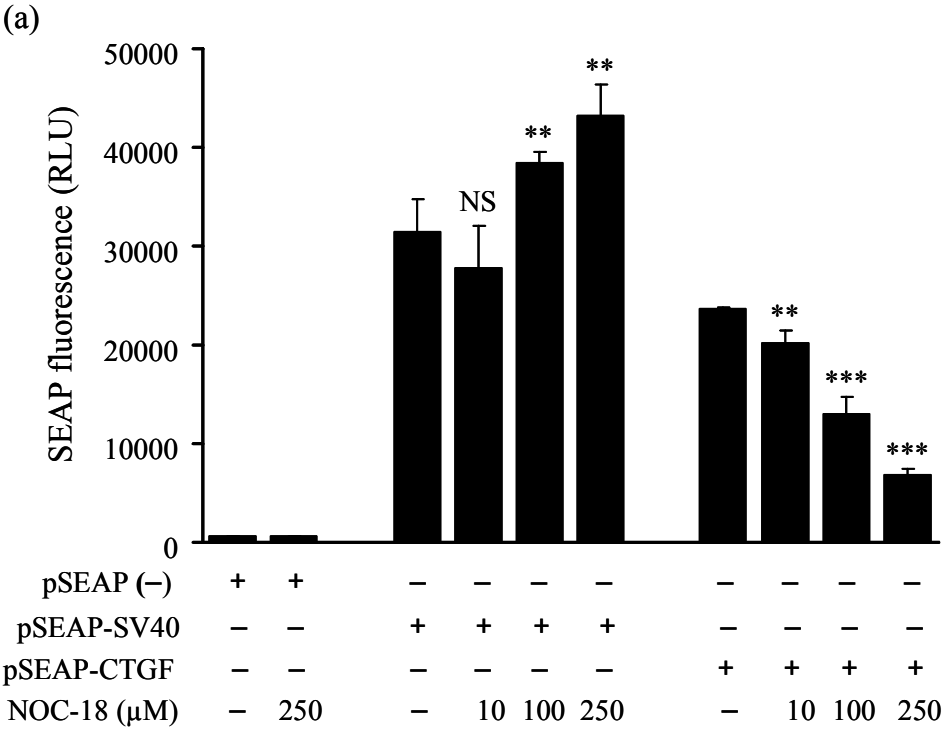



**Figure 27 CTGF gene organization and cloning strategy**

## 4.18. NO inhibits CTGF promoter activity in mesangial cell

To confirm that the decrease in the steady-state level of CTGF mRNA by exogenous NO is due to a decreased gene transcription rate, we performed reporter assays using a chimeric CTGF-promoter/SEAP- reporter construct. Human MCs were transiently transfected with a –805/+17 base pair long human CTGF promoter construct pCTGF-SEAP. Relative SEAP activity was measured 48 h after addition of 10, 100 or 250μM of NOC-18. Reporter analysis showed a significant decrease (Fig. 28a) in relative SEAP activity ($P< 0.001$) in NOC-18 treated cells as compared to untreated controls. Stimulation of pCTGF-SEAP- transfected cells with increasing doses of DetaNONOate and spermine NONOate (Fig. 28b) also showed significant decrease in the reporter protein (SEAP) expression ($P< 0.001$). pSEAP-SV40 transfected human MCs stimulated with different doses of NOC-18, and used as a positive control, showed surprisingly significant upregulation in the relative SEAP activity ($P< 0.01$) as compared to untreated control cells (Fig. 28a). These data suggest that NO acts at the promoter level to suppress TGF- β1 and glucose induced CTGF expression.



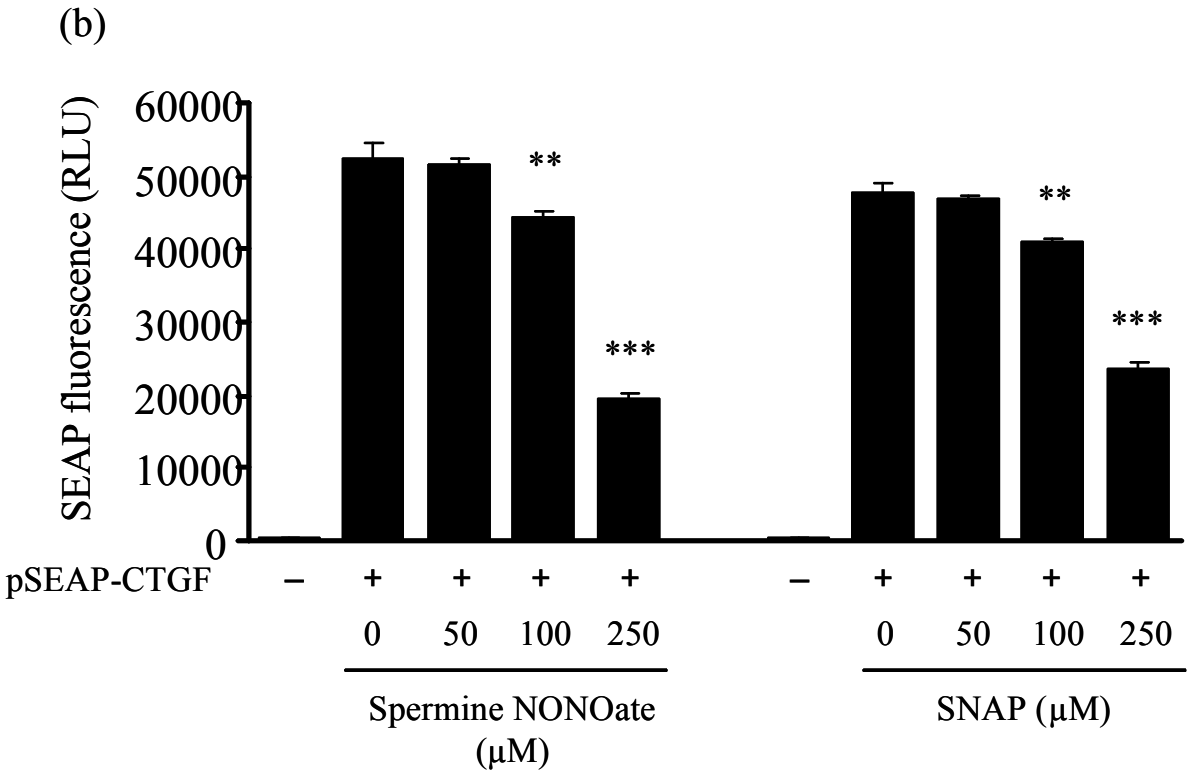



**Figure 28 NO donors downregulate the CTGF promoter-reporter activity in MC**

(a) Human MCs grown for 8 hours in DMEM containing 10% FCS were transfected with CTGF promoter (–805 to +17)/SEAP reporter plasmid (1.5 μg/well). After transfection, cells were incubated in serum free medium containing 30mM glucose and 5ng/ml TGF-β1 for 48 hours with or without 10, 100 and 250μM of NOC-18. Cells were co-transfected with SEAP-SV40 positive control plasmid (0.5μg/well) and treated with 250μM NOC-18 to show that NO does not exhibit any downregulatory effects on a different and unrelated promoter.

(b) Under similar conditions, human MCs were transfected with pCTGF-SEAP (1.5μg/well). Control cells were transfected with 1.5μg/well pSEAP (–) reporter plasmid having no promoter. Transfected cells were not treated or treated with 50, 100 or 250μM of spermine NONOate or SNAP for 48 hours as indicated. Data represent mean ± SD of three independent experiments with triplicate wells for each condition in each setting (Fig. 28 a, b). SEAP activity in relative light units was measured after 48 hours from the conditioned media by using SEAP fluorescent assay kit as described in "Experimental procedures". All experiments were performed in 12-well plates. Values show statistically significant differences in expression, **P<0.01 and *** $P$ <0.001 relative to cells transfected with pCTGF-SEAP but left untreated.

# 4.19. Protein interaction network and the regulatory pathways of CTGF, TSP-1, COL1A1, COL1A2, COL4A2, BGN and iNOS

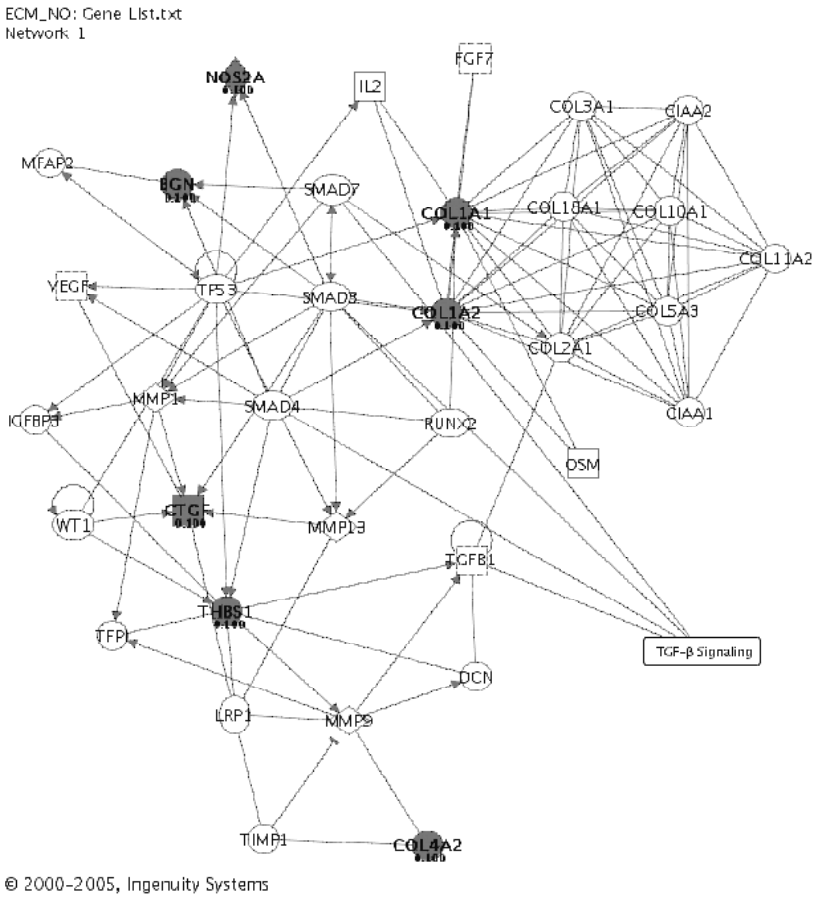



**Figure 29 Protein interactions and signaling pathways of ECM proteins**

This network diagram was generated by **Ingenuity Systems Software**, which is a software tool for biological pathways analysis, expansion and visualization of gene regulation networks and protein interaction maps based on data from PubMed and curated molecular interaction databases. Ingenuity identifies relationships among genes, small molecules and other objects, and draws pathway diagrams, linked to the original sources of information. The pathway generated shows a central role for TGF-β1 in regulating the expression of matricellular genes linked to fibrosis and links NO produced enzymatically by iNOS (as NOS2A in the diagram) to the signal transduction pathways mediated by SMADs.

# *Discussion*

## 5.1. RDA analysis successfully identified NO regulated genes

By using representational differential analysis, we have been able to add to our understanding of the complex role of NO in regulating gene expression in kidney mesangial cells. This systematic approach confirms the results of other investigators (Keil et al., 2002; Murphy et al., 1999) and provides novel observations regarding the biological role of NO in moderating gene expression in a fibrotic setting. Despite the limitations of the Respresentational Difference Analysis (RDA), we successfully identified several important matrix associated genes downregulated by treatment with S-Nitrosos-L-Glutathione (GSNO) in glomerular mesangial cells.

## 5.2. Mechanism of action and efficacy of exogenous NO donors

In this study we used GSNO, a nitrosothiol, as the initial donor for investigating the genes affected by exogenous NO. S-Nitrosothiols are compounds with the generic structure of RSNO. Under appropriate conditions these compounds decompose to liberate NO and the corresponding disulfide. It has been suggested that the formation and decay of low molecular weight S-nitrosothiols, such as S-nitrosoglutathione (GSNO) and S-nitrosocysteine (CySNO), may represent a mechanism for the storage or transport of NO (Myers *et al.,* 1990; Girard and Potier 1993). According to this proposal, *S*-nitrosothiols are synthesized chemically by reaction of NO with thiol. Subsequently, these compounds are diffused to the site of action. Decomposition of the *S*-nitrosothiol then leads to NO release and the corresponding biological effect. This hypothesis is mainly speculative and remains to be rigorously tested. Little is known about the reaction of NO with glutathione (GSH) *in vivo*; however, the direct reaction of GSH with NO does not generate GSNO but forms glutathione disulfide and nitroxyl anion (NO2) (Hogg *et al.,* 1996; DeMaster *et al.,* 1995). GSNO is formed only if NO is oxidized, by reaction with oxygen, to form NO2 and N2O3 (Wink et al., 1994). As intracellular oxygen concentrations at the tissue level are in the range of 10–20 μM (Smirnova *et al*., 1995) and as the rate of NO oxidation is proportional to the squared power of the NO concentration (Wink et al., 1993), it is likely that the oxidation of NO by oxygen *in vivo* is a slow and insignificant process. Evidence for the formation of *S*-nitrosothiols from endogenous NO remains scarce. Nevertheless, nitrosylation of protein thiols has been implicated in the NO-dependent regulation of many enzymes, including protein kinase C (Gopalakrishna *et al.,* 1993) and glyceral-3-phosphate dehydrogenase (Clancy

*et al.,* 1994). It has been reported that normal human serum contains *S*-nitroso-serum albumin (Stamler 1992 "a/b") which has been proposed to act as an endogenous regulator of vessel tone (Scharfstein 1994). Although the physiological relevance of *S*-nitrosothiols remains to be established, these compounds have been used as donors of NO (Ignarro *et al.,* 1981; Asahi et al., 1995; Radomski et al., 1992). The most commonly employed compounds are *S*-nitroso-*L*-glutathione (GSNO) and *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP). Such compounds have been shown to have diverse and remarkable biological effects. For example, SNAP is a potent vasodilator (Ignarro *et al.,* 1981) and low concentrations of GSNO have been shown to provide significant protection to the ischemic myocardium (Konorev *et al*., 1995). It is generally assumed that *S*-nitrosothiols decompose by cleavage of the *S*-N bond.

$$RSNO \rightarrow RS+NO$$

This process generates NO and a thiyl radical, RS (Josephy *et al.,* 1984). However, this assumption has not been effectively tested under physiologically relevant conditions. It has been established that *S*-nitrosothiols are sensitive to both photolytic (Singh *et al.,* 1995; Sexton *et al.,* 1994) and transition metal ion-dependent breakdown (McAninly et al., 1993) but are stable in the presence of transition metal ion chelators in the dark. The biological activity of *S*-nitrosothiols may not be exclusively dictated by the release of NO as the chemistry of these compounds is complex. *S*-Nitrosothiols have also been shown to form NO2, which under appropriate conditions can lead to the formation of either nitrous oxide (Hogg *et al.,* 1996; DeMaster *et al.,* 1995; Pryor *et al.,* 1982) or peroxynitrite (Hogg *et al.,* 1996; Bonner *et al.,* 1986). *S*-Nitrosothiols can also undergo nitrosonium ($NO^+$) transfer to other cellular thiols by a process referred to as transnitrosation (Hogg N, 2000).

In this study, we studied the effects of a novel class of NO donors known as NONOates on gene expression and regulation in mesangial cells. NONOates otherwise known as diazeniumdiolates, are a novel group of NO donors. They are complexes of NO with nucleophiles (*X*) and have the general formula, *X*N ($O^-$) N=O (Maragos *et al.,* 1991). These compounds are generally stable as solids, but decompose in solution to generate NO (Keefer *et al.,* 1996). Decomposition occurs at a predictable rate that depends on pH, temperature and the nature of the nucleophile (Maragos *et al.,* 1991). NONOates were described in the chemical literature over 30 years ago (Longhi *et al.,*

1962; Ragsdale *et al.,* 1965) but it is only recently that their potential as sources of NO, with biological properties, has been studied (Maragos *et al.,* 1991; Morley *et al.,* 1993). NONOates have been found to be effective vasodilators both in vitro (Maragos *et al.,* 1991; Morley *et al.,* 1993) and in vivo (Diodati *et al.,* 1993; Vanderford *et al.,* 1994). However, the in vitro effects of this novel group of NO donors have not previously been evaluated specifically on mesangial cells. As potential therapeutic agents, NONOates have an advantage over other NO donors in that the choice of nucleophile can determine the duration of biological action (Homer and Wanstall, 1998).

We used two different NONOates, Spermine NONOate and DETA-NONOate (NOC-18). The total amount of NO that a NONOate can generate varies for different compounds. The theoretical maximum value is 2 mol of NO per mol of parent compound (Maragos *et al.,* 1991) but at neutral pH it is usually less than this (Feelisch and Stamler, 1996). DETA-NONOate is a 1-substituted diazen-1-ium-1, 2-diolates, containing a $[N (O) NO]^-$ group, generally referred to as diazenium diolates (Keefer *et al.,* 1996). It has a half-life of 20 hours and releases NO without prior biotransformation (Hanson *et al.,* 1995). Spermine NONOate also belongs to the same class of chemical compounds but differs in the rate of release of NO. Spermine NONOate has a half-life of 39 minutes at 37°C and at pH 7.4. In our experiments, we measured nitrite as the stable metabolites of NONOates and found that nitrite concentrations in the medium were reduced by almost 50% as compared to the initial dose of parent compound with no time dependence (Fig.12). In experiments with mRNA or protein expression of different matricellular genes studied, we did not find any notable differences in the effects of either DETA-NONOate or Spermine NONOate. No remarkable differences were found either on CTGF promoter activity or the reporter gene expression during transfection analysis while working with DETA-NONOate or Spermine NONOate (Fig. 28b). However, Spermine NONOate at 12 hours did not show a significant change in the TSP-1 protein level (Fig. 25). Conclusively, the effects seen in each set of experiments in this study were dose- and time- dependent.

## 5.3. Genes upregulated by NO – general biological functions

The genes upregulated in the assay include **Pyruvate kinase (PK)**, a rate-controlling glycolytic enzyme, which catalyses the transfer of a phosphoryl group from phosphoenolpyruvate to ADP, yielding ATP and pyruvate. PK exists in the cytosol and is functional as a tetramer. Mammalian PK exists as four isoenzymes, which are referred to as the L-, R-, M1-, and M2-types, respectively (Imamura *et al.,* 1986). The L-, R-, and M1-PK isoenzymes are tissue-specific, while L-PK is predominantly expressed in the liver but is also present in the kidney, small intestine, and in pancreatic b-cells (Imamura *et al.,* 1986; Noguchi *et al.,* 1991). R-PK is only expressed in erythrocytes. M1-PK is expressed in skeletal muscle, heart, and brain. In contrast, M2-PK is a ubiquitous isoenzyme and is found in nearly all tissues or cells.

PK was expressed in MC with very low abundance and was approximately 6-fold induced by GSNO. This finding is, to our knowledge, the first description of a regulation of PK expression by nitric oxide. Interestingly, the transcription rate of PK has been described to be induced in muscle cells subjected to hypoxia, facilitating glycolysis under anaerobic or hypoxic conditions when oxidative metabolism is repressed (Kress et al., 1998).

**Aldose reductase (AR)** another upregulated gene belongs to aldo/keto reductase family. This gene family includes a number of K+ ion channel beta chain regulatory domains. These are reported to have oxido-reductase activity. AR catalyzes the NADPH-mediated reduction of aldo-sugars and aldehydes to their corresponding alcohols. AR-catalyzed reduction of glucose to sorbitol constitutes the first and rate-limiting step of the polyol pathway. Aldose reductase (AR) is a widely expressed aldehyde-metabolizing enzyme. The reduction of glucose by the AR catalyzed polyol pathway has been linked to the development of secondary diabetic complications. Although treatment with AR inhibitors has been shown to prevent tissue injury in animal models of diabetes, the clinical efficacy of these drugs remains to be established. Recent studies suggest that glucose may be an incidental substrate of AR, which appears to be more adept in catalyzing the reduction of a wide range of aldehydes generated from lipid peroxidation. Moreover, inhibition of the enzyme has been shown to increase inflammation-induced vascular oxidative stress and prevent myocardial protection associated with the late phase of ischemic preconditioning. On the basis of these studies several investigators have ascribed an important antioxidant role to the enzyme. Additional studies indicate that the antioxidant and signaling

roles of AR are interlinked and that AR regulates the PKC-NF-kappaB via redox sensitive mechanisms (Srivastava *et al.*, 2005). Our result confirms the investigation of Seo (Seo *et al.,* 2000) which provided the first evidence that AR gene expression is induced by NO or NO-derived chemical species in the vascular cells and a macrophage cell line. The major findings demonstrated in this study were that: 1) NO derived from NO donors as well as from endogenous iNOS up-regulated the expression of AR; 2) this effect of NO involved de novo mRNA synthesis, whereas neither guanylate cyclase nor tyrosine kinase activity mediated the signaling pathway; and 3) inhibition of AR activity under normal glucose conditions exacerbated the cytotoxic effect of NO. Methylglyoxal (MG), a reactive dicarbonyl produced during glucose metabolism, is known as a preferred substrate of AR that concomitantly catalyzes the reduction of glucose in the polyol pathway. MG modifies cellular proteins to form cross-links of amino groups, generating so-called advanced glycation end products. Increased rates of MG formation under hyperglycemic conditions and ensuing high serum levels of MG are reported in diabetic patients. A study published by Yabe-Nishimura (Yabe-Nishimura *et al.,* 2003)) suggests that exposure to high concentrations of MG may be among the causative factors that accelerate cellular oxidative stress under such pathological conditions as diabetes. Induction of AR expression by MG was significantly suppressed when they pre-incubated aortic smooth muscle cells (SMC) with NAC, a thiol antioxidant as well as a precursor of GSH. On the other hand, pretreatment of SMC with BSO, a reagent that depletes intracellular GSH, further augmented the MG-induced increase in AR mRNA level. As GSH also serves as a cofactor of the glyoxalase system that catalyzes the metabolic disposal of MG, interpretation of these observations is not straightforward. However, the determination of intracellular levels of ROS using a peroxide-sensitive fluorophore demonstrated that MG-induced a significant increase in intracellular ROS level prior to the elevation of AR mRNA. The molecular mechanism(s) underlying the MG-induced augmentation of intracellular oxidative stress remains unclear. ROS may be generated during glycation reactions between MG and amino acid residues in proteins. Depletion of GSH due to increased metabolic load of MG through the glyoxalase system may further decelerate elimination of ROS in the cell. This observation that intracellular ROS were elevated prior to the change in AR mRNA level suggested that the effect of MG on AR expression may be attributable to increased oxidative stress caused by MG treatment. Findings of these investigators together with our results indicate that induction of AR by NO is the consequence of an adaptive

response to protect cells from oxidative stress and cytotoxic effects of MG. Accordingly, the primary role of AR may be a detoxification enzyme that degrades reactive aldehydes for cell survival.

**Triosephosphatase isomerase (TPI)** gene, encoding another glycolytic enzyme, which catalyses the conversion of dihydroxyacetone phosphate into glyceraldehyde 3 Phosphate, an induction of transcription in response to ischemia has been reported (Liaud *et al.*, 2000). Astrocytes exposed to hypoxia showed a decrease of intracellular glucose levels in parallel to the upregulation of various glycolytic enzymes, including TPI, which seemed to prevent cell death (Niitsu *et al.*, 1999). In our assay TPI was upregulated by GSNO-treatment. Although hypoxia is not comparable with increased NO-levels, both environments represent an oxidative stress for the cells and various genes are similarly regulated under both conditions. Conclusively the upregulation of PK, AR and TPI by GSNO suggests an increased requirement for glycolytic enzymes of MC under conditions of high NO availability.

The **Hemochromatosis gene (Hfe)** is a membrane protein involved in iron metabolism. Defects in the human homolog lead to hereditary hemochromotosis, an iron storage disorder (Holmstrom *et al.*, 2003; Zhang *et al.*, 2004).

**ATPase synthase subunit 6** is a mitochondrial gene involved in electron transport and oxidative phosphorylation (Huang *et al.*, 2004). Nitric oxide specifically and reversibly inhibits cytochrome oxidase (complex IV), nitrosothiols inactivate complex I, while peroxynitrite ($ONOO^-$) has multiple effects on different respiratory complexes and can activate the permeability transition pore, which may trigger apoptosis (Brown 2001). In physiological concentrations, the effects on complex IV are probably most important and provide a mechanism by which nitric oxide may inhibit or regulate oxygen consumption. It is possible that the local generation of nitric oxide within or close to mitochondria may tonically inhibit oxygen consumption.

**Tyrosine 3- mono-oxygenase (Ywhab)** is a member of the 14-3-3 protein family. Its functions are not well known but are thought to mediate signal transduction by binding to phosphoserine-containing proteins and also play a role in cell cycle regulation (Cavet *et al.*, 2003).

**Integrin beta 1 (Itgb1)** belongs to beta 1-integrin family of cell-surface receptors. These receptors mediate cell-matrix interactions that play a critical role in tissue development and tissue remodeling after injury (Juliano *et al.,* 1994). The integrins are a major family of cell

surface receptors that mediate attachment to the ECM (Ruoslahti *et al.,* 1995; Clark *et al.,* 1995). They are heterodimeric transmembrane glycoproteins that consist of various combinations of noncovalently bound α- and β-chains. Sixteen α and 8 β-chains have been described to date. The α-subunit largely determines substrate specificity with the ECM proteins (Venstrom and Reichardt *et al.,* 1995), while the intracytoplasmic tail of the β-chain is mainly responsible for its interaction with the cell cytoskeleton (Yamada *et al.,* 1991). The $\beta_1$-subunit contains sites of tyrosine phosphorylation in the cytoplasmic domain, suggesting they have a potential role in signal transduction (Juliano *et al.,* 1994; Clark *et al.,* 1995). The normal mesangium possesses $\alpha_1$ $\beta_1$, $\alpha_2$ $\beta_1$, $\alpha_3$ $\beta_1$, $\alpha_5$ $\beta_1$, $\alpha_8$ $\beta_1$, and $\alpha_v$ $\beta_3$ integrins (Gauer *et al.,* 1997 ; Cosio *et al.,* 1990 ; Cosio 1992). Mesangial cell survival and death may be regulated by ECM via $\beta_1$-integrin molecules of cell surface receptors (Sugiyama *et al.,* 1998). At present, it remains unclear which of $\beta_1$-containing integrins promote mesangial cell survival. Distinct $\beta_1$-integrins can mediate cell survival in mammary epithelial cells and Chinese hamster ovary cells, whereas $\alpha_v$ $\beta_3$ integrin can mediate the survival of vascular endothelial cells and melanoma cell survival in three-dimensional collagen (Zhang *et al.,* 1995; Brooks *et al.,* 1994). The integrin required for cell survival and integrin-dependent survival signals may be cell type specific. Changes in integrin expression in glomerular disease have been reported (Kagami *et al.,* 1993). Several investigators have shown that the expression of $\beta_1$-integrin decreases within areas of glomerular scarring in biopsies of patients with IgA nephropathy (Hillis *et al.,* 1995) and rapidly progressive glomerulonephritis (Baraldi *et al.,* 1995). These studies conclude that there was no evidence of an altered repertoire or increased intensity of staining for integrin receptors on mesangial cells or on apparently preserved glomerular capillary tufts. In contrast, the expression of several β1 integrins was enhanced on damaged tubules, areas of tubulointerstitial scarring and sclerotic glomerular tufts. Our observation that Itgb1 is upregulated by NO supports the view that NO might prove to be a potential therapeutic molecule in treating glomerular fibrosis.

## 5.4. Genes downregulated by NO – a link to fibrosis

Among the identified downregulated targets of NO were both the structural genes of Type I collagen – COL1A1 and COL1A2, COL4A2, OAT, TSP-1, biglycan, and CTGF.

**Type I collagen** is a major structural component of the ECM, which is synthesized by fibroblasts and vascular smooth muscle cells and in the kidney by glomerular mesangial cells. Type I collagen is considered to be centrally involved in progressive glomerular ECM accumulation and is associated with chronic sclerotic processes (Ortega-Velazquez et al., 2004).

**Ornithine aminotransferase (OAT)** is an enzyme of the alternative metabolic pathway for L-arginine and generates proline (Burcham *et al.,* 1988). It has been demonstrated that L-arginine metabolism through the arginase pathway is also increased in experimental acute nephritis (Cook *et al.,* 1994; Jansen *et al.,* 1992) and may play a role in matrix accumulation (Jansen *et al.,* 1992). The metabolites of arginine produced through the activation of OAT are associated with cell proliferation and tissue repair (Smith and Phang 1978). In addition, Proline is a precursor of collagen, which is one of the major extracellular matrix proteins present in the mesangium of sclerotic glomeruli (Striker et al., 1984). A study published by Koga (Koga *et al.*, 2003) demonstrated that NO generated by the induction of iNOS did not change the mRNA levels of OAT substantially in retinal pigment epithelial cells in culture. Our finding that OAT is downregulated by GSNO in mesangial cells is a unique observation made so far. Inhibiting the transcription or the enzymatic activity of OAT by NO in the activated arginase metabolic pathway may have substantial therapeutic potential in reducing extracellular matrix accumulation during the course of glomerular inflammation.

**Thrombospondin-1 (TSP-1)** has been shown to be an important activator of TGF β in vivo (Crawford et al., 1998). TSP1 is involved in numerous biological functions, probably attributable to its multiple domains and cell-surface receptors as well as its ability to act as either a soluble or matrix-bound factor. In our studies, treatment of MCs with high glucose and TGF β resulted in an increased basal level of TSP-1 which was strongly downregulated by NO stimulation. This observation is consistent with the results from the study published by Wang (Wang et al., 2002) in rat MC. Our results show that different NO donors in a dose and concentration-dependent

manner prevent increases in steady state TSP-1 mRNA and result in a decrease in TSP-1 protein levels in human MCs, suggesting that NO signaling might affect high glucose and TGF-β – mediated TSP1 gene expression either by changing mRNA stability or regulating gene transcription.

The observation that **biglycan (BGN)** is also a target of exogenous and endogenous NO in human MCs is consistent with studies by Schaefer (Schaefer et al., 2003), wherein they report that BGN is a NO-regulated gene in rat MCs both *in vitro* and *in vivo* and that it is involved in the modulation of the extent of adhesion, proliferation and survival of MCs. Biglycan is a member of small, leucine-rich repeat glycoproteins/proteoglycans (SLRPs), which are primarily considered to play a role as organizers of extracellular matrices (Hocking et al., 1998). Overexpression of BGN is found in advanced stages of glomerulosclerosis (Davies, 1995). BGN is capable of inhibiting the proliferation of MCs induced by PDGF-B, a major mitogenic growth factor frequently involved in glomerular disease (Blom et al., 2001; Schaefer et al., 2003).

In our study with **Connective tissue growth factor** (**CTGF)**, we observed a dose and time dependent decrease in the mRNA and protein expression with different NO donors. Downregulation of CTGF mRNA was also evident when endogenous NO release was stimulated by the activation of iNOS with INFγ and BH4 in human MCs. These data are consistent with the observations made by Keil (Keil et al., 2002) in rat MC. CTGF, a secreted protein (Kireeva et al., 1997) is a member of the CCN (CTGF, Cyr61, and Nov) family of proteins that promote angiogenesis, cell migration, and cell adhesion (Fan et al., 2000; Ivkovic et al., 2003). CTGF upregulation is an important factor in the pathogenesis of mesangial matrix accumulation and progressive glomerulosclerosis, acting downstream of TGF-β (Riser et al., 2000). CTGF is induced by TGF-β in fibroblasts and acts synergistically with TGF-β to promote sustained fibrosis (Leask et al., 2002). The induction of fibrosis by high glucose is thought to be mediated at least in part by TGF- β. However, increased CTGF expression has also been observed in cultured mesangial cells under high glucose conditions (Wahab et al., 2001).

Our promoter studies using a CTGF-SEAP reporter construct demonstrate that NO acts at the transcriptional level to downregulate high glucose and TGF-β –mediated CTGF gene expression in both rat as well as human MCs. These results were also supported by mRNA half-life

experiments where NO had no effect on CTGF mRNA stability. CTGF expression has been shown to be differentially regulated depending on the cell type and condition.

## 5.5. Activation of iNOS and the effects of endogenously generated NO in mesangial cells

In nitrite assay experiments with human or rat MCs, we recorded a basal level of 2 ± 0.45 μM of nitrite content in the medium and an increase to 7 ± 0.8 μM of nitrite in INFγ + BH4 or 15 ± 1.45 μM in cytokine mix treated MCs. This observation is consistent with the observation made by Prabhakar, 2000 in murine MCs. The amount of endogenously generated NO by the activation of iNOS after cytokine stimulation was sufficient to downregulate mRNA expression of type I collagen – COL1A1 and COL1A2, COL4A2, Biglycan, CTGF and TSP-1. Northern blot analysis demonstrated that CTGF and TSP-1 were downregulated by endogenously generated NO in response to iNOS activation by INFγ alone or INFγ in combination with tetrahydrobiopterin (BH4) – a co-factor for iNOS in human MCs. Treatment with a selective iNOS inhibitor, 1400W, rescued the downregulatory effects of both INFγ and the combination of INFγ+BH4 indicating that the downregulatory effects on CTGF and TSP-1 mRNA expression are NO- specific (Fig. 21a – Northern blot). However, in a real time PCR assay, INFγ alone did not downregulate COL1A1, COL1A2, COL4A2, and Biglycan expression significantly. The activation of iNOS by INFγ alone or INFγ in combination with tetrahydrobiopterin resulted in the induction of iNOS at both mRNA and protein levels. Moreover, INFγ treatment alone resulted in less iNOS mRNA and protein activation for 20 hours than did the combination of INFγ+BH4. Co-stimulation with 1400W resulted in the inhibition of mRNA, protein and nitrite levels in INFγ+BH4-treated cells. The activation of iNOS in rat MCs was achieved with a combination of bacterial endotoxin (LPS), TNF-α and IL-1β. This treatment resulted in the downregulation of COL1A2 mRNA which was rescued by the treatment of 1400W. 1400W significantly reduced iNOS mRNA levels over an extended priod of time. INFγ alone was able to induce iNOS expression at both mRNA and protein levels but did not generate significant levels of nitrite above base levels. INF-γ is a known activator of iNOS and acts through well defined pathways to activate iNOS. INF-γ has the potential to direct inflammatory responses by inhibiting production of pro-inflammatory IL-1 and IL-8, by upregulating the production of

cytokine antagonists such as IL-1Ra and IL-18BP, by inducing expression of members of the SOCS family of regulatory proteins, and by induction of apoptosis in leukocytes and local resident cells. These anti-inflammatory properties of the principally pro-inflammatory cytokine INF-γ may be essential in order to fine-tune and control the extent of inflammatory conditions (Mühl and Pfeilschifter 2003). Our results indicate that the anti-inflammatory properties of INF-γ can also be attributed to its potential to activate the production of endogenous NO by stimulating the activation of iNOS. We used $BH_4$ to stimulate human MCs in combination with INFγ because $BH_4$ has been shown to contribute to cytokine induction of iNOS expression in human mesangial cells through the stabilization of iNOS mRNA (Saura *et al.*, 1996). In addition, $BH_4$ availability has been demonstrated to be a limiting factor for iNOS activity in many cell types (Werner-Felmayer *et al.,* 1990; Gross and Levi 1992; Schoedon *et al.,* 1993). The intracellular levels of $BH_4$ are determined by the activity of two different biosynthetic pathways: the *de novo* synthesis from GTP and the regeneration of $BH_4$ from dihydropterins through a pterin salvage pathway (Nichol *et al.,* 1985). The first enzyme in the *de novo* pathway is GTP cyclohydrolase I. Cytokines have been reported to stimulate potently the *de novo* synthesis of $BH_4$ in several cell types through the induction of GTP cyclohydrolase I (Werner-Felmayer et al., 1993; Hattori and Gross 1993; Nakayama *et al.,* 1994). In fact, expression of GTP cyclohydrolase I and iNOS appears to be regulated coordinately (Hattori and Gross 1993; Werner *et al.,* 1993). This phenomenon has been interpreted previously as a mechanism to ensure an adequate supply of $BH_4$ for the activity of cytokine-induced NOS (Werner-Felmayer *et al.,* 1990; Gross and Levi 1992; Schoedon *et al.,* 1993; Werner *et al.,* 1993). In addition to its catalytic role, $BH_4$ has been reported to protect NOS from feedback inhibition of NO *in vitro* (Mühl and Pfeilschifter 1994; Hyun *et al.,* 1995), and to stabilize the structure of both the macrophage and the neuronal NOS proteins (Giovanelli *et al.,* 1991; Baek *et al.,* 1993). In experiments, performed to determine the effects of various iNOS inhibitors on nitrite levels in human MCs treated with INFγ alone or INFγ+BH4, we observed a significant decrease in nitrite accumulation in the medium of cells simultaneously treated with 1400W and L-NIL (selective inhibitors of iNOS) and L-NMMA – a non selective inhibitor of iNOS. The nitrite levels after treatment with 1400W and L-NIL were decreased below the basal nitrite levels observed in unstimulated cells. However, L-NMMA treatment resulted in an increased nitrite production as compared to untreated control cells but was significantly decreased as compared to nitrite levels in cells treated with INFγ+BH4. This

observation that L-NMMA induces nitrite production in the cells is in agreement with the data published by (Hong *et al.*, 2005) in RAW 264.7 Cells and by (Nicolson *et al.,* 1993) in human mesangial cells. The investigators argue that the increase in nitrite above base levels may be due to the decomposition of the arginine analogue in the culture medium over time. We stimulated human MCs with 30mM glucose prior to cytokine stimulation to augment the effects of INFγ and $BH_4$ on iNOS activation. Exposure of murine mesangial cells and macrophages to high glucose in presence of INFγ and LPS has been shown to enhance stimulation of iNOS message and iNOS activity (Sharma *et al.,* 1995). High glucose has also been shown to enhance growth factor-stimulated NO production by cultured rat MCs (Trachtman *et al.,* 1998). The investigators have suggested that high glucose effect can be protein kinase C (PKC)-mediated.

## 5.6 Transcriptional Regulation of Gene Expression by NO

Regulation of signal transduction and gene expression is a multifaceted process involving ligands, receptors, and second messengers that trigger cascades of protein kinases and phosphatases and propagate the signal to the nucleus to alter gene expression. NO is one of the powerful intra- and extracellular messenger that mediates diverse signaling pathways in target cells and is known to play an important role in many physiological processes. A classical example for the signaling effects of NO is the relaxation of vascular smooth muscle cells when exposed to NO produced by adjacent endothelial cells. This effect, which was the first function of NO to be discovered (Hibbs and Bastian, 1999), is due to the activation of the soluble isoform of guanylyl cyclase (sGC), the formation of cGMP and the subsequent activation of cGMP-dependent ion channels and kinases (Zhao *et al.,* 1999; Bellamy *et al.,* 2000). The signaling cascades activated by NO operate in part through the redox-sensitive regulation of transcription factors (Bogdan C, 2001; Pfeilschifter *et al.,* 2001 "a"; Marshall *et al.,* 2002). Cross-communication with other pro-oxidant or antioxidant mediators will critically influence the fate of a cell under pathologic conditions when inducible NOS is expressed. Once primed and activated by inflammatory cytokines such as INF-γ, IL-1β and TNF-α, most cells, including renal mesangial cells, co-produce NO and $O_2^-$ also known as reactive oxygen species ( ROS). The interaction of NO and $O_2^-$ is thought to be highly relevant to the regulation of gene expression (Pfeilschifter *et al.,* 2001). A number of NO-regulated genes are also targeted by ROS (Pfeilschifter *et al.,* 2001). Whereas certain genes are regulated in a coordinated manner by NO

and $O_2^-$ (Mühl and Pfeilschifter, 1995; Beck *et al.,* 1998), others are affected in a contrasting manner (Eberhardt *et al.,* 2000 "b"). The simultaneous generation of NO by many cells exposed to an inflammatory environment and the opposite effects of both radicals on certain genes may provide a genetic switch-like mechanism, with a subtle change in the $O_2^-$/NO ratio resulting in dramatic changes in enzyme expression. A prominent group of target genes regulated in this way by NO and ROS are the extracellular matrix proteins and their metabolizing enzymes the matrix metalloproteinases (MMP) and plasminogen activators (PA), such as MMP-9 and t-PA (Eberhardt *et al.,* 2000 "b" and 2001), and their endogenous inhibitors like tissue inhibitors of matrix metalloproteinase-1 and plasminogen activator inhibitor-1, respectively (Eberhardt *et al.,* 2001., Bouchie *et al.,* 1998). In the kidney, accumulation of extracellular matrix is often a hallmark of chronic disease, eventually leading to the development of glomerulosclerosis. In this context, the coordinated expression of proteases and their inhibitors by inflammatory cytokines and NO will allow the fine-tuned regulation of tissue proteolysis and protect against overwhelming tissue destruction. NO also modulates the expression of major matrix components such as collagen, fibronectin, and laminin (Bouchie *et al.,* 1998; Craven et al., 1997), which may also be important for tissue remodeling in chronic inflammatory kidney diseases. Recently, NO was found to inhibit the expression of another matrix protein, secreted protein acidic and rich in cysteine (SPARC; also known as BM-40 or osteonectin) (Walpen et al., 2000). The highly glycosylated SPARC protein has a variety of biologic activities, and its action as a scavenger of PDGF may be relevant in the course of glomerulonephritis. By modulating SPARC expression, NO may subsequently affect mesangial cell proliferation in the course of glomerular inflammation. In addition, NO up- or downregulates a heterogeneous set of gene products including protective mediators, proinflammatory mediators, chemokines and cytokines, adhesion molecules, growth factors, hormones, receptors, and signaling devices (for a review, see (Pfeilschifter et al., 2003)). Many genes targeted by NO share roles in common physiologic and pathophysiologic processes. NO might affect gene transcription by interfering with transcription factor translocation and binding to their cognate sequences on the promoter. A characteristic feature of many transcription factors is their remarkable redox sensitivity. NO has been shown to inhibit the DNA-binding activity of NF-kB through S-nitrosylation of a crucial cysteine residue within the p50 subunit (Matthews *et al.,* 1996). Interestingly, in a recent study, NO was shown to inhibit MMP-2 expression in endothelial cells via the induction of transcription factor ATF3

(Chen and Wang 2004). Previous studies from our group have shown that NO inhibits DNA binding of transcription factor Egr-1 (Rupprecht *et al.,* 2000). A potential mechanism by which NO inhibits DNA binding of Egr-1 is by interfering with the zinc-finger domains that establish the DNA contacts. Our present data that NO regulates CTGF gene expression at transcriptional level reveals a direct role for NO in gene regulation. Data from different groups discussed above strengthens the existing view that NO employs diverse signaling pathways or may act directly to regulate gene transcription however, an NO-responsive DNA element in a prokaryotic or a eukaryotic promoter has yet to be identified.

## 5.7. CTGF as a candidate target gene for therapeutic intervention in diabetic nephropathy

In the diabetic environment, the balance between the signaling activities of different growth factors involved in renal matrix homeostasis is shifted towards a pro-fibrotic state. This leads to matrix accumulation and fibrosis, and eventually contributes to the development of diabetic nephropathy. The special role CTGF appears to play in this process most probably relates to its capacity to modulate the signaling activity of other growth factors critically involved in renal response to injury. More specifically, the CTGF-induced stimulation of IGF-I and TGF-ß signaling, together with a decrease in BMP and VEGF signaling, might contribute significantly to the diabetes-related response to injury and adverse remodeling of the diabetic kidney. Restoring the balance of these growth factor signaling disturbances in the development of diabetic nephropathy by targeting CTGF might be more attractive than addressing individual growth factor signaling pathways. The first studies that have been performed so far suggest beneficial effects of CTGF inhibition in processes leading to renal fibrosis and mesangial matrix accumulation (Wahab *et al.,* 2001); GBM thickening, glomerular hyperfiltration and renal hypertrophy (Roestenberg et 2004 “a”; Flyvbjerg *et al.,* 2004); albuminuria and declining renal function (Gilbert *et al.,* 2003; Roestenberg *et al.,* 2004 “a”; Andersen *et al.,* 2004; van Nieuwenhoven *et al.,* 2004); and Interstitial fibrosis (Lam *et al.,* 2003; Yokoi *et al.,* 2004 “a”). To assess the possible suitability of CTGF as a target for therapeutic intervention in diabetic nephropathy, we need more extended studies with NO, CTGF-neutralizing antibodies and antisense ODNs, and studies in genetic animal models in which CTGF expression can be

conditionally disrupted. Ultimately, such studies will reveal whether CTGF is indeed more than just another factor in glomerular kidney diseases, and qualifies as a suitable target for therapeutic intervention.

## 5.8. A novel role for NO in glomerular inflammation and fibrosis

In conclusion, we propose a model describing a novel role for NO in glomerular inflammation and fibrosis on the basis of our findings. High glucose and TGF-β upregulation results in the inhibition of intracellular NO necessary to counteract the accumulation of matricellular gene products leading to fibrosis. Application of NO donors or endogenous generation of NO as a result of iNOS activation by interferon gamma (INFγ) and tretrahydrobiopterin (BH4) may result in a partly direct interference with the accumulation of extracellular matrix components thus moderating the course of glomerular fibrosis (Fig. 30).

These findings provide evidence that NO is capable of down-regulating COL1A2, COL4A2, BGN, CTGF and TSP-1 gene expression particularly in human MCs. Using a series of NO-donors we could show that in the case of CTGF this regulation occurred at the transcriptional level in a time- and concentration-dependent manner. These data further demonstrate that the downregulation of gene expression by endogenously generated NO was not caused by a direct cytokine effect, since 1400W, a selective inhibitor of iNOS, abrogated the NO effect. Moreover, we demonstrated that this NO- mediated downregulation is transcriptional in nature, however, the exact mechanism and NO- responsive cis- regulatory elements in the CTGF promoter are yet to be identified. Previous work from our group has indicated that Egr-1 is regulated by NO at the transcriptional level (Rupprecht et al., 2000) and that Egr-1 might be important for mesangial cell proliferation and matrix accumulation. Theses results together with previous observations from our laboratory that NO has an antiproliferative effect on MCs, suggest an important role for NO in regulating the expression of profibrotic and promitotic genes. Moreover, treatment strategies employing optimal intervention with exogenous NO donors might prove beneficial in certain diseases conditions with glomerular fibrosis.

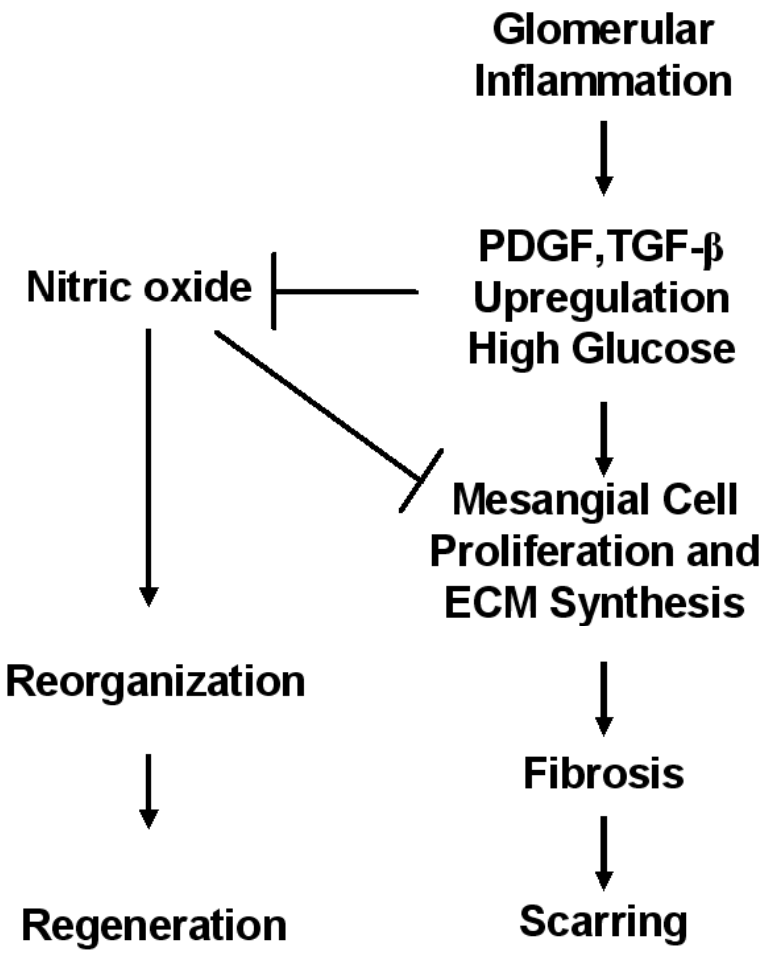



**Figure 30 Model for the direct role of NO- regulation of matricellular genes linked to glomerular fibrosis.**

# Summary

By means of their proliferative and secretory potential glomerular mesangial cells are thought to be important mediators of glomerular inflammation and fibrosis. Recent studies have established a direct role for NO in the regulation of gene expression in different cell types including mesangial cells. Representational difference analysis was used to investigate changes in gene expression elicited by the treatment of S-Nitroso-L-glutathione in rat mesangial cells. We identified 7 upregulated and 11 downregulated genes. Four out of 11 downregulated genes, connective tissue growth factor, thrombospondin-1, collagen type I alpha 1 and collagen type I alpha 2, are matricellular genes linked to inflammation and fibrosis of different organs including the kidney. Results were verified by using Northern blot analysis, quantitative real time PCR and protein analysis methods in human mesangial cells treated with a series of NO donors. We validated our findings by inducing endogenous NO production by cytokine stimulation. Real time PCR analysis showed that two additional matrix related genes, biglycan and collagen type IV alpha 2 are also downregulated by NO. Connective tissue growth factor promoter studies in mesangial cells demonstrated that NO acts at the transcriptional level to suppress gene expression. These results reveal a complex role of NO in regulating gene expression in mesangial cells and suggest an antifibrotic potential for NO.

# *Zusammenfassung*

Glomeruläre Mesangialzellen gelten wegen ihres proliferativen und sekretorischen Potentials als bedeutende Mediatoren glomerulärer Entzündungen und fibrotischer Erkrankungen. In neueren Studien wurde für Nitric oxide (NO) eine direkte Beteiligung an der Regulation der Genexpression in unterschiedlichen Zelltypen, darunter auch Mesangialzellen, nachgewiesen. Zur Untersuchung der von S-Nitroso-L-Glutathion induzierten Veränderungen der Genexpression in Ratten-Mesangialzellen wurde die Representational-Difference-Alalysis-Methode eingesetzt. Damit konnten 7 hochregulierte und 11 herabregulierte Gene identifiziert werden. 4 dieser 11 herabregulierten Gene sind Zellmatrix-Gene, die an Entzüdungsprozessen und fibrotischen Erkrankungen in unterschiedlichen Organen, einschließlich Niere, beteiligt sind. Die Ergebnisse wurden mit Northern-Blot-Analyse, Quantitativer Real-Time-PCR sowie Protein-Analyse-Methoden in humanen Mesangialzellen unter Anwendung einer Reihe von NO-Donoren erfolgreich überprüft. Diese Daten konnten ebenfalls bestätigt werden, wenn die endogene NO-Produktion durch Cytokin-Gabe induziert wurde. Eine Real-Time-PCR-Analyse ergab für 2 weitere Matrix-assoziierte Gene, Biglycan und Collagen-Type-IV-alpha-2, ebenfalls eine NO-induzierte Herabregulation. Wie in Connective-Tissue-Growth-Factor-Promoter-Reporter-Gen Studien gezeigt, führt NO auf Transkriptionsebene zur Suppression der Genexpression. Diese Ergebnisse weisen NO eine komplexe Rolle in der Regulation der Genexpression in Mesangialzellen zu und lassen ein anti-fibrotisches Potential für NO vermuten.

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# *Curriculum Vitae*

# *Javid A. Wani*

## Personal Details


- **Sex:** Male
- **Nationality:** Indian
- **Date of Birth:** 01.07.1966
- **Marital Status:** Married
- **E-mail:** *javid.wani@gmail.com*


## Career Profile

- **March 1993 – Mar. 1996** Master of Science (Zoology), University of Kashmir, India
- **July 1997 – Nov.1998** Training Assistant Fisheries, University of Agricultural Sciences and Technology, RARS, Leh, J&K
- **Dec.1998 – July 2000** Junior Research Fellow, Jawaharlal Nehru University, New Delhi, India
- **Aug. 2000 – May 2001** Project Associate, Center for DNA Fingerprinting and Diagnostics (CDFD), Hyderabad, India
- **July 2001 – Apr. 2002** Guest Scientist, Institute for Human Genetics, GSF National Research centre for Environment and Health, Neuherberg, Germany
- **May 2002 – Sep. 2005** Ph.D. Human Biology at the Medical Policlinic, Medical Faculty, Ludwig-Maximilians University, Munich, Germany (Thesis submitted for the award of the doctoral degree).

## Awards

1. National Talent Search Award for 1982
2. Department of Science and Technology (Govt. of J&K) fellowship for advanced course in biotechnology 1998
3. Department of Biotechnology, Govt. Of India Junior research fellowship for 1999-2000
4. Department of Biotechnology, Govt. Of India Project Associate ship for 2000-2001
5. DGF fellowship for 2001July –April 2002
6. German Research Foundation Funding from May 2002- June 2005.

## Conferences attended

- 10th International Immunology Congress in New Delhi, 1998
- 5th International Congress on the "Molecular epidemiology of evolutionary and infectious diseases (MEEGID-5)" Hyderabad, India April 2001 as one of the Organisers.
- Nephrology Congress in Düsseldorf Germany, July 2002
- Satellite symposium to the "World Congress of Nephrology" in Kloster Seon Germany, August 2003
- International Symposium of the British Society for Matrix Biology, University of Liverpool, UK, March 2005

## Poster presentations

1. "Nitric oxide-induced changes in gene expression in glomerular mesangial cells" presented at the German Nephrology Congress in Düsseldorf, Germany, July 2002.
2. "Nitric oxide metabolites moderate the expression of matricellular genes involved in fibrosis in glomerular mesangial cells" presented at the British Society for Matrix Biology meeting in Liverpool, UK, March 2005.

## Abstracts

1. "Nitric oxide metabolites moderate the expression of matricellular genes involved in fibrosis in glomerular mesangial cells". Wani J, Rupprecht HD (Accepted for the British Society for Matrix Biology held in March 2005 in Liverpool, Great Britain and for publication in the "*International Journal of Experimental Pathology*").
2. "Changes in gene expression mediated by Nitric oxide can play a protective role during the course of fibrotic and inflammatory disorders in human Kidney". Wani J, Rupprecht HD (Accepted for poster presentation at the FASEB meeting in April 2-6, 2005 at San Diego, USA – meeting not represented in person).

## Special Courses

- "International English Language Testing System (IELTS)" – 2004; Band Score 7.0
- "How to write a scientific research paper in English"- a 2 day course sponsored by *Roche Pharma,* Mannheim Germany- Jan. 2005

## Publications

Nitric oxide Modulates Expression of Matrix-associated Genes Linked to Fibrosis in Kidney Mesangial Cells.
Wani J, Nelson P, Carl M, Henger A, and Rupprecht HD
(Manuscript to be communicated)!

## Laboratory Experience

### *Cell Biology*

- Dissection of mammalian eye for the isolation of retinal neuronal cells
- Characterization and *in vitro* expansion of retinal cells
- Primary culture of photoreceptor and müller glia cells
- Primary culture of glomerular mesangial cells
- Cell culture of mesangial, fibroblast, endothelial and embryonic cell lines
- Transfection of primary and transformed cell lines
- Live and Dead assay of primary cultures and co-cultures
- Protein ELISA and Immuno-histochemistry (IHC) of cultured cells
- *In vitro* gene transfer techniques like electroporation, liposome and non-liposome-mediated transfection into mammalian cells using plasmid vectors
- Luciferase, GFP, secreted alkaline phosphatase (SEAP) and ß-gal assays
- Cell proliferation and apoptosis assay
- Fluorescent and light microscopy including digital Leica DM IRE2 inverted research microscope with imaging software

### *Molecular Biology*

- Molecular cloning, sub-cloning and expression in E.coli and mammalian cells
- PCR (Long range, Nested, Allele specific, Degenerate, Touchdown and QRT-PCR)
- Agarose and polyacrylamide gel electrophoresis
- Autoradiography and phosphor imaging
- Northern blotting, in-vitro transcription and Ribonuclease protection assay
- Western blotting and co-immunoprecipitation
- Single Stranded Conformational Polymorphism Analysis (SSCP) & HD analysis
- DNA isolation from whole tissues, blood, plasmids and bacteriophages
- Bacteriophage lambda cloning, propagation and screening

- Restriction analysis and mapping
- Promoter analysis
- Packaging of viruses
- Automated and classical Gene Sequencing
- Site-directed mutagenesis
- Cloning, characterization and expression of eukaryotic genes in yeast two hybrid and common cloning vectors
- Molecular cloning by using gateway cloning technology
- Adequate knowledge of computer applications (Windows and Mac OS, MS Office and different graphic software applications)
- Database mining and Software experience: All public and curated databases related to nucleotide, protein and vector sequences and some commercial like Genomatix for promoter analysis and Ingenuity for pathway analysis including primer, vector designing by using commercial and free softwares.
- Image analysis by using Image quant, ImageJ and phospohoimaging softwares