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# Effects of high glucose on cytokine-induced nerve growth factor (NGF) expression in rat renal mesangial cells

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**Abstract**

Nerve growth factor (NGF) accumulates at sites of inflammation and modulates local immune reactions. To characterize the mechanisms of cytokine-induced NGF expression under physiogical and pathophysiological conditions, we have used cultured glomerular mesangial cells, which play a key role in glomerular inflammatory diseases such as diabetic nephropathy. To study the effects of high glucose on cytokine-induced NGF expression, rat mesangial cells were treated with the cytokines interleukin-1β and tumor necrosis factor α under normal (1.0 g/L) and high (4.5 g/L) glucose concentrations. In the presence of high glucose concentrations, the cytokines drastically potentiated NGF protein but not mRNA expression when compared to physiological glucose levels. The specific protein kinase C inhibitors Ro31-8220 and CGP41251 suppressed cytokine-induced NGF expression. Moreover, blocking the oxidative activation of the protein kinase C pathway by *N*-acetylcysteine inhibited glucose effects on NGF synthesis. Neutralizing antibodies against transforming growth factor-β inhibited cytokine-induced NGF expression under normal glucose concentrations but not under high glucose conditions. Enhanced expression of NGF under high glucose conditions may contribute to kidney diseases such as diabetic nephropathy.


*Keywords:* Diabetic nephropathy; Hyperglycemia; IL-1β; Neurotrophins; TNF-α; Mesangial cells

## 1. Introduction

Increasing evidence implicates the neurotrophins including NGF in inflammatory processes [1]. Inflammatory mediators found at sites of injury such as IL-1, IL-6, TNF-α and TGF-β have been implicated in inducing NGF synthesis in peripheral organs including mesangial cells [2–5]. A cytokine–neurotrophin cascade could therefore be involved in inflammatory processes. The NGF system has been reported to critically participate in kidney development [6]. The low affinity NGF receptor (p75) is expressed during later stages of glomerulogenesis where it is limited to the mesangium. Its relevance for kidney development is documented by the use of antisense oligonucleotides which lead to abnormal kidney morphogenesis. The p75 neurotrophin receptor persists at a lower level in adults in glomeruli and a subpopulation of renal interstitial cells. The fact, that these receptors are upregulated during inflammatory kidney disease and diabetic nephropathy [7,8], suggests a pathophysiological role for neurotrophins.

At present, there are no data concerning a possible role of neurotrophins and their receptors in diabetic nephropathy. During kidney inflammation glomeruli have been

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*Abbreviations:* ANOVA, analysis of variance; CHM, cycloheximide; NGF, nerve growth factor; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; TGF-β, transforming growth factor-β; PDGF, platelet-derived growth factor; PDTC, pyrrolidine dithiocarbamate; bFGF, basic fibroblast growth factor; RT–PCR, reverse transcription–polymerase chain reaction; TPA, phorbol-12-myristate-13-acetate.

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demonstrated to synthesize and release growth factors like PDGF-β, TGF-β or bFGF, inflammatory cytokines like IL-1β, TNF-α, chemotactic factors, eicosanoids, nitric oxide and oxygen radicals [9–14]. These mediators released by invading immune cells and activated glomerular mesangial cells may further amplify local inflammatory cell function. Mesangial cells, which have been shown to play a key regulatory role in glomerular inflammatory diseases are responsive to and synthesize various growth hormones and cytokines including NGF [11]. Thus, inflammatory cytokines can regulate mesangial cell functions in an autocrine and paracrine manner, thereby enhancing the progress of glomerular injury.

To date, the activation of several isoforms of protein kinase C (PKC) is accepted as the main mediator of high glucose effects. In glomerular mesangial cells, several groups observed the translocation/activation of PKC-α, -δ, -ε and -ζ ([15–19], reviewed in [20]) under high glucose conditions. Several possibilities linking the high glucose effects to the PKC signaling pathway have been presented. Glucose, as a substrate for the polyol pathway increases the NAD/NADH ratio thereby generating diacylglycerol and phosphatitic acid that constitute typical activators of different PKC isoforms [21]. The conversion of glucose to fructose also leads to glutathione depletion via an increased NADP/NADPH ratio and subsequently to an increased production of reactive oxygen species (ROS) In turn, ROS are able to trigger PKC activity in mesangial cells [22].

Our finding that IL-1β and TNF-α elicited a marked increase in NGF mRNA and protein expression by rat mesangial cells implicate this neurotrophin in the pathophysiology of glomerular diseases [4,5,11]. We have now examined the role of glucose levels on mesangial cell NGF expression. The fact that glucose upregulates the NGF system in mesangial cells points to an involvement of a cytokine–NGF cascade in diabetic nephropathy.

## 2. Materials and methods

### 2.1. Cell cultures

Rat glomerular mesangial cells from male Sprague–Dawley rats (70–100 g body weight) were isolated and cultivated as described previously [23]. Single cells were cloned by limiting dilution and were grown in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum as described [24]. Confluent mesangial cells (about 106 cells/plate) were washed twice with PBS and stimulated with 4 mL medium (serum-free Dulbecco's modified Eagle's medium without glucose and phenol red (Sigma Chemical Co.) containing 1% antibiotic solution, supplemented with 0.1 mg/mL of fatty acid-free bovine serum albumin (Sigma)), supplemented with the different concentrations of D(+)-glucose (Sigma), with or without the cytokines and compounds as indicated. Recombinant human IL-1β was generously supplied by Dr. Christiane Rordorf (Novartis). TNF-α was a generous gift of Knoll AG, Ludwigshafen. The supernatant was removed and immediately assayed for NGF and TGF-β protein by a sensitive enzyme-linked immunoassay (ELISA) [25].

### 2.2. RNA preparation

Total cellular RNA was isolated from rat glomerular mesangial cells by of acid guanidinium thiocyanate/phenol/chloroform extraction and quantified spectrophotometrically by absorbance at 260 nm. The quality of RNA was checked by formaldehyde agarose gel electrophoresis.

### 2.3. RT–PCR

Total RNA of each sample was first reverse-transcribed into cDNA (Superscript™ II, Gibco-BRL) according to the manufacturer's protocol, which in turn was subjected to PCR amplification using primers specific for NGF, trkA, p75 and S12. The sequence of the NGF-specific PCR primers were: sense, 5′-CCAAGGACGCAGCTTTC-TAT-3′; antisense, 5′-CTCCGGTGAGTCCTGTTGAA-3′; of the S12-specific primers: sense, 5′-GGAAGGCATTGCT-GCTGG-3′; antisense, 5′-CTTCAATGACATCCTTGG-3′.

The numbers of cycles used to amplify each cDNA were chosen to allow the PCR to proceed in a linear range according to the Elongase™ enzyme mix-protocol (Gibco-BRL). PCR amplification of the constitutively expressed ribosomal protein S12 cDNA was used as a measure of input RNA. Controls using RNA samples without reverse transcription or controls without RNA were used to demonstrate absence of contaminating DNA. The amplification steps involved denaturation at 94° for 1 min, annealing for 50 s at 55° with specific primers and extension for 1 min at 68°. The PCR reactions (5 mL) were analyzed by electrophoresis in 1.5% agarose gels followed by alkaline blotting of the fragments onto nylon membranes and subsequent hybridization with specific digoxigenin-labeled DNA probes [26]. Detection was with CDP-Star™ (Tropix) as chemoluminescence substrate for alkaline phosphatase conjugated to anti-digoxigenin-antibodies (Roche Diagnostics) as indicated by the manufacturers. Appropriate exposures of Kodak X-Omat films were quantified using a video densitometer (Model 620, Bio-Rad). The identity of the PCR products was routinely confirmed by digestion with appropriate restriction enzymes and hybridization with specific probes [26,27]. Statistical evaluation of results was performed by ANOVA and the statistical error was indicated as the SEM (standard error of the mean).

### 2.4. Real-time RT–PCR

mRNA steady state levels of NGF were analyzed with real-time, quantitative RT–PCR (Applied Biosystems) according to the manufacturers protocol. Equal amounts

of total RNA were reverse transcribed with the reverse transcriptase (Invitrogen) using random hexamers. TaqMan assay oligonucleotide primers and probes for the quantification of rat NGF were designed using the Primer Express software (Applied Biosystems) with the sequence of the rat β-NGF gene (GenBank Accession No. M36589). The following oligonucleotides were used: forward primer, 5′-CAGAGGGAGACTCTGTCCCTGA-3′ (369–390); reverse primer, 5′-AGTGATGTTGCGGGTCTGC-3′ (502–484); TaqMan probe, 5′-ACTTCAGCATTCCCTTGACACAGCCC-3′ (406–431).

Each 50 ng of the reverse-transcribed cDNA were subjected to polymerase reaction using the GeneAmp 7700 sequence detection system (Applied Biosystems) and primers as well as probes for NGF and 18 s RNA (Applied Biosystems). The reactions were performed with 40 cycles (15 s at 95°; 1 min at 62°). Each sample was measured in triplicate. Relative quantification was performed according to the manufacturers suggestion.

### 2.5. Enzyme immunoassay

To measure immunoreactive NGF released into the culture medium, a two-site ELISA for the determination of NGF as described by the manufacturer was used (Roche Diagnostics). Briefly, polystyrene 96-well microtiter immunoplates (Nunc) were coated with either the monoclonal antibody against the β-subunit of NGF from mouse-hybrid cells (Roche Diagnostics) or a goat preimmune serum to determine nonspecific binding (Zymed). Each plate included a complete standard curve ranging from 0 pg/0.1 mL to 100 pg/0.1 mL. Fifty microliters of the cell culture medium were added to each well overnight at 4°. The plate was incubated with the β-galactosidase-coupled coating antibody. Specific signals were quantified using a chlorophenolred-β-D-galactopyranoside substrate solution (Roche Diagnostics). After a 3-hr incubation step at 37° the optical density was measured at 595 nm using an ELISA reader (Dynatech MR 700). For the analysis of TGF-$\beta_1$ protein the Quantikine ELISA Kit (R&D systems) was used.

The NGF or TGF-β content in the samples was determined by comparison with the standard curve. Statistical analysis was performed applying ANOVA and the statistical error was indicated as SEM. Previous studies [5] have shown that mesangial cell-derived immunoreactive NGF is also biologically active.

## 3. Results

Simultaneous addition of the human recombinant cytokines IL-1β and TNF-α (each 1 nM) to rat glomerular mesangial cells cultured at a physiological glucose levels of 1 g/L (5.5 mM) resulted in a time-dependent increase in NGF synthesis (Fig. 1). No differences in basal NGF synthesis under low or high glucose conditions were observed in mesangial cells treated without cytokines (data not shown). This effect was maximal after 12 hr (2.5-fold increase). With prolonged incubation there was a slow decrease over time and after 72 hr the amount of NGF protein reached control levels.

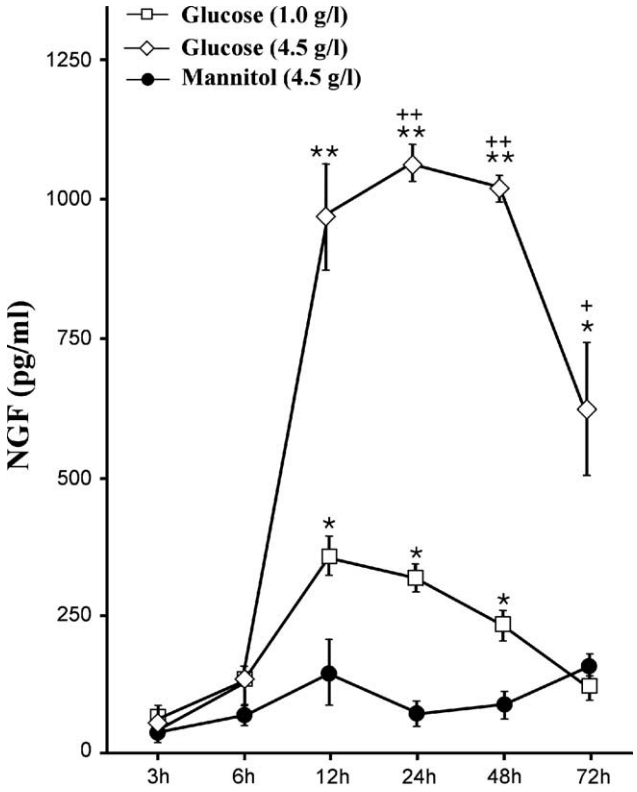


Fig. 1. Time-dependent stimulation of cytokine-induced NGF protein expression by glucose. Mesangial cells were incubated in the presence of the cytokines IL-1β/TNF-α (1 nM each) in a stimulation medium containing either mannitol (4.5 g/L) or different concentrations of glucose (1 or 4.5 g/L) as indicated. After the indicated incubation periods the medium was removed and NGF protein was measured as described in Section 2. Data are means ± SEM of three independent experiments. Statistical analysis was by ANOVA ($^{*}P < 0.05$, $^{**}P < 0.01$, compared with controls incubated in mannitol; $^{+}P < 0.05$, $^{++}P < 0.01$, compared with the cells incubated in low glucose (1 g/L) medium).

In high glucose medium (4.5 g/L; 25 mM) there was a markedly enhanced cytokine-induced increase in the amount of NGF protein secreted from rat glomerular mesangial cells (Fig. 1). Under these experimental conditions the cell number did not change significantly with respect to time, glucose concentration or cytokine stimulation. The NGF protein levels stimulated with IL-1β/TNF-α in high glucose medium began to increase after 6 hr, reaching a maximum after 24 hr (15.5-fold compared to controls) and remained elevated for 72 hr. In the high glucose medium the NGF protein levels secreted after 12–48 hr were significantly different ($P < 0.001$) from the two other treatment groups that were either stimulated in low glucose medium or in mannitol (4.5 g/L), which was used as an osmotic control to confirm that the effects observed were specific for glucose (Fig. 1). Elevation of

secreted NGF by cytokine-treated mesangial cells under high glucose conditions was also observed by immunoblotting (data not shown). The additional control experiments in the presence of 1 g/L glucose +3.5 g/L mannitol did not reveal significant differences to 1 g/L glucose alone, thus clearly excluding osmotic effects of high glucose (4.5 g/L) concentrations (data not shown).

The most pronounced stimulatory effect of glucose on cytokine-induced NGF production was observed after 24 hr and was dose-dependent (Fig. 2). The basal cytokine-induced release of NGF protein observed in a medium without glucose or 4.5 g/L mannitol was already significantly ($P < 0.05$) upregulated in a low glucose medium containing 1 g/L glucose and maximally enhanced in a medium containing more than 3 g glucose/L ($P < 0.01$). The biological activity of secreted NGF protein was proven by a NGF bioassay using rat pheochromocytoma (PC12) cells as described [5]. PC12 cells are known to respond to NGF with neurite outgrowth. The specificity of biologically active mesangial cell-derived NGF was confirmed with specific blocking anti-NGF antibodies (data not shown).

To determine the mechanisms involved in the enhanced release of NGF protein upon cytokine-stimulation of mesangial cells cultured in high glucose we studied different antioxidants. Using ELISA and RT–PCR techniques, the effects of the antioxidant PDTC, known to prevent cellular reactive oxygen intermediate (ROI) production and cytokine-induced NF-κB activation in mesangial cells [27–29], and *N*-acetylcysteine (NAC) [30] were investigated.

Rat mesangial cells incubated with PDTC (30–100 μM) which alone does not influence basal NGF mRNA/protein expression levels [27] revealed the inhibitory effect of PDTC on cytokine-induced NGF protein release 24 hr after stimulation (Fig. 3). Moreover, the cytokine-induced NGF protein release was abolished by NAC (Fig. 3) suggesting that reactive radical intermediates are indeed involved in cytokine-induced mesangial cell NGF synthesis. Of special interest is that both inhibitors, PDTC and NAC, blocked the glucose-mediated enhancing effect on cytokine-induced NGF release (Fig. 3).

As shown by RT/PCR, the inflammatory cytokines TNF-α and IL-1β-stimulated mesangial NGF mRNA expression maximally 4 hr after stimulation (Fig. 4A). Interestingly, PDTC and NAC at maximal inhibitory concentrations [29,30] also reduced the cytokine-activated NGF mRNA expression. However, in contrast to NGF protein secretion, high glucose failed to enhance NGF mRNA expression. Additional experiments performed with the quantitative real-time RT–PCR method clearly exclude any effect of glucose on cytokine-induced NGF mRNA steady state levels (Fig. 4B). This indicates that a posttranscriptional

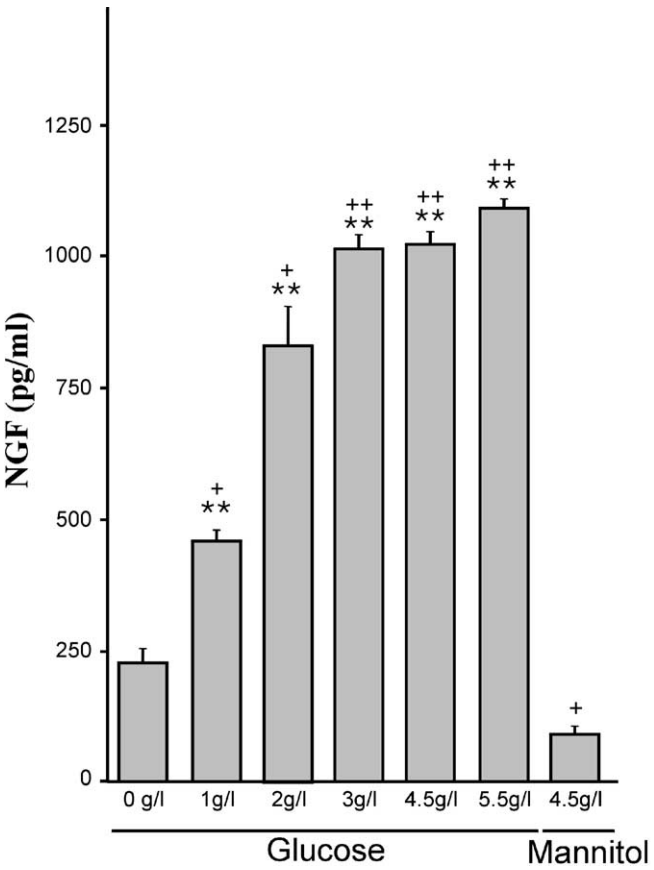


Fig. 2. Effect of 24 hr exposure to different glucose concentrations on NGF protein expression. Mesangial cells were incubated for 24 hr with the cytokines IL-1β/TNF-α (I + T, 1 nM each) in the presence of mannitol (4.5 g/L) or different concentrations of glucose as indicated. The medium was then removed and NGF protein was measured as described in Section 2. Data are means of three independent experiments. Statistical analysis was by ANOVA ($^{**}P < 0.01$, compared with controls incubated in mannitol; $^{+}P < 0.05$, $^{++}P < 0.01$, compared with the cells incubated in medium without glucose).

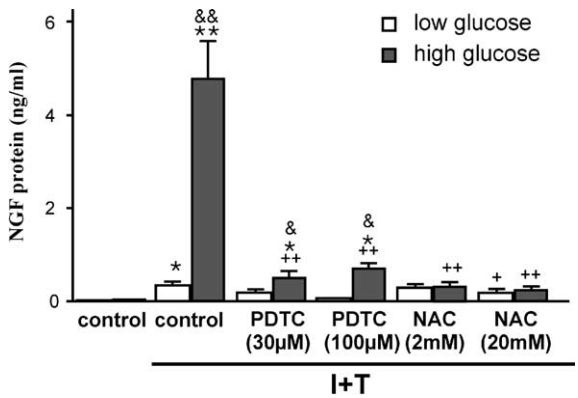


Fig. 3. Cytokine-induced NGF protein release is inhibited by various antioxidants—effect of glucose levels. Mesangial cells were incubated for 24 hr with the cytokines IL-1β and TNF-α (I + T, 1 nM each) and inhibitors in the presence or absence of high concentrations of glucose as indicated. The medium was then removed and NGF protein was measured as described in Section 2. Data are means of three independent experiments. Statistical analysis was by ANOVA ($^{*}P < 0.05$, $^{**}P < 0.01$, compared with unstimulated controls; $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$, compared with cytokine-activated cells; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$, compared with cells incubated in medium with low glucose concentrations).

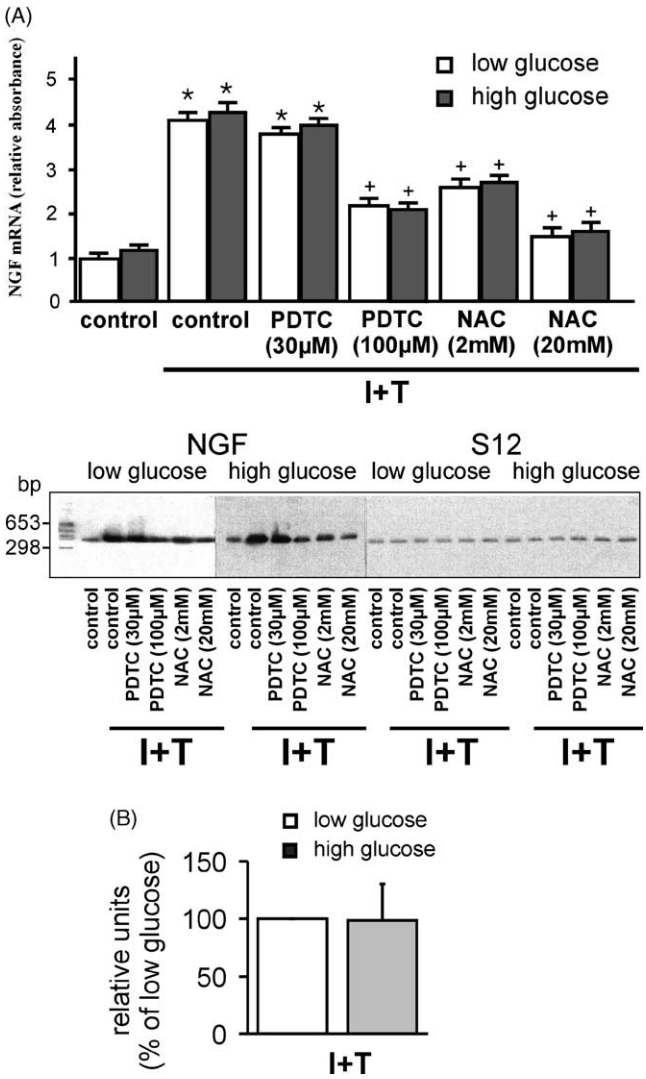


Fig. 4. Cytokine-induced NGF mRNA expression is inhibited by various antioxidants—effect of glucose levels. (A) *Top*: Quantitation of NGF transcripts. Values are the ratio of densitometric scores for NGF and S12 PCR-products from mesangial cells treated as indicated. Results are the mean of three independent experiments ± SEM. Statistical analysis was performed with ANOVA ($^{*}P < 0.05$, compared to unstimulated cells; $^{+}P < 0.05$, compared to cytokine-activated cells). *Bottom*: Representative Southern blot analysis of NGF and S12 PCR-products from mesangial cells incubated in the presence of the cytokines IL-1β/TNF-α (I + T, 1 nM each) in either high or low concentrations of glucose and various inhibitors as indicated. Total cellular RNA of three independent experiments was extracted after 4 hr and subjected to RT–PCR amplification as described in Section 2. (B) Comparison of cytokine-induced NGF mRNA-levels under low and high glucose conditions using the TaqMan quantitative RT–PCR method. Data are means ± SD from three independent experiments performed in triplicate. mRNA levels for low glucose were set to 100%.

mechanism is involved in amplification of cytokine-stimulated NGF release by glucose.

To further characterize the mechanism involved in the cytokine-induced changes of NGF protein, we have tested two potent PKC inhibitors, CGP41251 [31] and Ro31-8220 [32]. As demonstrated in Fig. 5 both PKC inhibitors blocked the cytokine-induced NGF protein release without

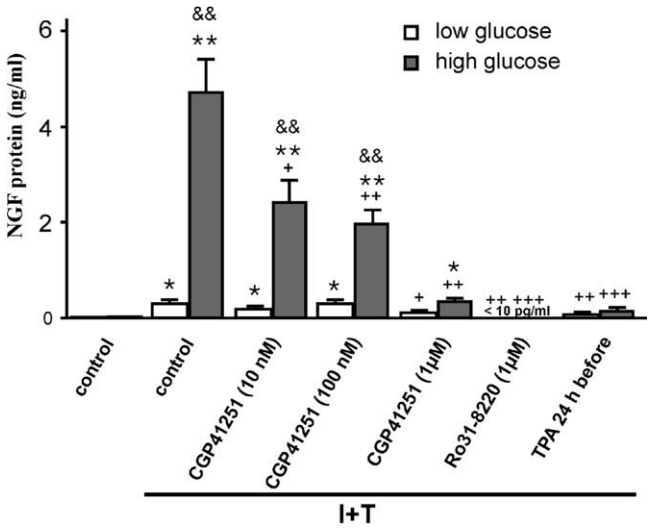


Fig. 5. Inhibition of cytokine-induced NGF release by various PKC inhibitors—effect of glucose levels. Mesangial cells were incubated for 24 hr with the cytokines IL-1β/TNF-α (I + T, 1 nM each) and inhibitors in low or high concentrations of glucose as indicated. The medium was then removed and NGF protein was measured as described in Section 2. Data are means of three independent experiments. Statistical analysis was by ANOVA ($^{*}P < 0.05$, $^{**}P < 0.01$, compared with unstimulated controls; $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$, compared with cytokine-activated cells; $^{\&\&}P < 0.01$, compared with cells incubated in medium with low glucose concentrations).

affecting mesangial cell phenotype or density (data not shown). Moreover, the dramatic (10–15-fold) enhancing effect of glucose on IL-1β/TNF-α-induced NGF release was abolished by these compounds in a strictly dose-dependent manner. Further evidence for a PKC-dependent mechanism contributing to glucose-amplification of cytokine-stimulated NGF protein release is provided by the observation that downregulation of PKC by a 24-hr pretreatment with TPA, a regimen that causes complete loss of PKC-α, -δ and -ε isoenzymes in mesangial cells [33] completely prevented cytokine-induced NGF secretion (Fig. 5), The fact that the different PKC inhibitors also reduced cytokine-induced NGF mRNA expression provides evidence for an additional transcriptionally controlled mechanism of cytokine-mediated NGF gene expression (Fig. 6). However, the failure of high glucose to further enhance IL-1β/TNF-α-activated NGF mRNA expression (Fig. 6) strongly suggests that a posttranscriptional mechanism is responsible for the high glucose-mediated NGF response.

To further evaluate the involvement of a posttranscriptional mechanism in glucose-amplified NGF expression we investigated the effect of the protein translation inhibitor CHM. As expected, CHM completely blocked IL-1β and TNF-α-induced mesangial cell NGF protein release both, under low and high glucose conditions. Moreover, NGF mRNA levels were markedly reduced in CHM-treated cells, thus excluding that NGF might function as a feedback regulator of NGF gene expression (data not shown).

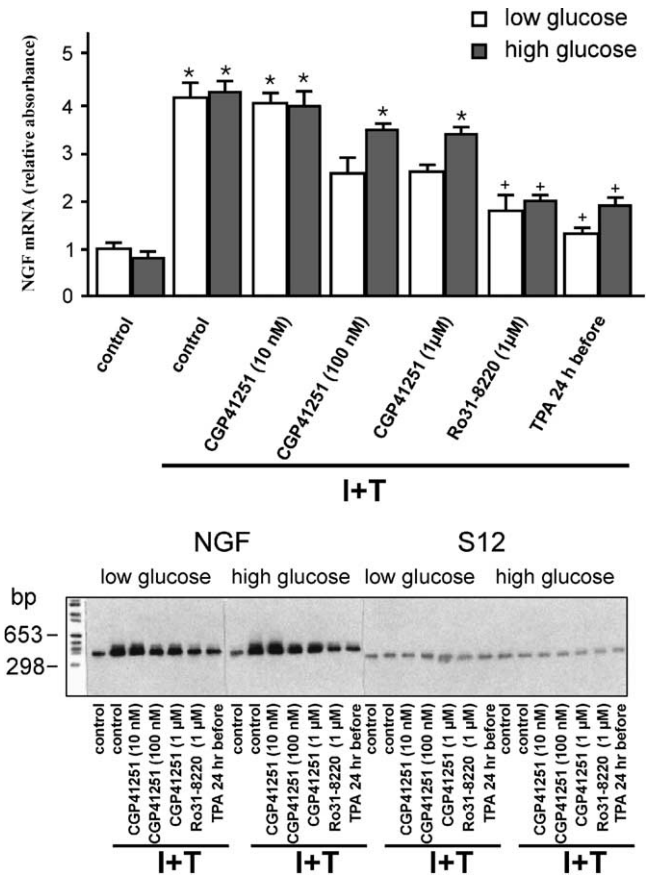


Fig. 6. Inhibition of cytokine-induced NGF mRNA expression by various PKC inhibitors—effect of glucose levels. *Top*: Quantitation of NGF transcripts. Values are the ratio of densitometric scores for NGF and S12 PCR-products from mesangial cells treated as indicated. Results are the mean of three independent determinations ± SEM. Statistical analysis was performed with ANOVA ($^{*}P < 0.05$, compared to unstimulated cells; $^{+}P < 0.05$, compared to cytokine-activated cells). *Bottom*: Representative Southern blot analysis of NGF and S12 PCR-products from mesangial cells incubated in the presence of the cytokines IL-1β/TNF-α (I + T, 1 nM each) in either high or low concentrations of glucose and various PKC inhibitors as indicated. Total cellular RNA of three independent experiments was extracted after 4 hr and subjected to RT–PCR amplification as described in Section 2.

Previous studies have shown that mesangial cells cultured under high glucose conditions display an increased expression of TGF-β [34,35]. Therefore, we tested whether amplification of NGF expression in mesangial cells is mediated by TGF-β. Experiments with neutralizing anti-TGF-β antibodies revealed that cytokine-induced NGF expression is mediated to a great extent by endogenously produced TGF-β (Fig. 7A). However, under high glucose conditions the involvement of TGF-β in the amplifying effect is less pronounced ($P > 0.05$). Obviously, cytokine-induced TGF-$\beta_1$ expression is elevated in high glucose compared to low glucose conditions (Fig. 7B). These increased amounts of TGF-$\beta_1$ may not sufficiently be neutralized by the amounts of anti-TGF-β antibodies used in this study. However, TGF-β-induced NGF protein expression was only slightly enhanced under high glucose conditions but this effect reached no significance in our experiments (Fig. 7C). It is worth noting that TGF-β alone increased NGF protein expression under high glucose conditions (Fig. 7C) but did not further potentiate IL-1β plus TNF-α-stimulated NGF expression neither under low glucose nor under high glucose conditions (Fig. 7C).

## 4. Discussion

Increasing evidence identifies NGF as a signaling molecule modulating inflammatory processes and associated with tissue repair and fibrosis [1,36,37]. The fact that inflammatory cytokines including IL-1β and TNF-α are potent stimulators of NGF synthesis in renal mesangial cells points to an active role of mesangial cell-derived NGF in glomerular inflammation [4,5].

Here we report that IL-1β/TNF-α-induced mesangial cell NGF production is modulated by glucose. High glucose levels (4.5 g/L), comparable to plasma glucose levels found in diabetic patients, drastically augmented cytokine-induced NGF protein release in rat mesangial cells. Surprisingly, the dramatic changes in glucose-modulated NGF protein release are not accompanied by a concomitant increase of NGF mRNA levels indicating that a posttranscriptional mechanism is responsible for this observation. Various posttranscriptional events, either alone or in combination, could contribute to this effect: (i) an increased rate of NGF translation; (ii) an increased stability of the NGF protein; (iii) an increased NGF release; (iv) a diminished NGF protein metabolism.

Our present results provide evidence for the involvement of oxidative stress and PKC activation in mediating glucose-induced NGF release. The finding that NAC and the PDTC blocked the potentiating effect of high glucose on cytokine-stimulated NGF secretion provides evidence that radicals are implicated in glucose-mediated actions. However, it cannot be ruled out that a NF-κB-dependent pathway contributes to the observed glucose actions, since it as been shown that PDTC also blocks NF-κB activation [27–29].

Elevated glucose levels have a well-documented impact on gene expression [38]. Moreover, for example, high glucose increases *de novo* synthesis of diacylglycerol in mesangial cells which subsequently can activate PKC, thereby initiating various protein phosphorylation reactions [39–41]. Kikkawa et al. [15] reported activation of PKC-α and -ζ isoenzymes in diabetic renal disease. Therefore, it was of interest to study whether the effects of glucose on NGF release are mediated by PKC. To this end we have used the potent PKC inhibitors Ro31-8220 [32] and CGP41251 [31] as well as a PKC depletion approach [33]. In this context it is important to note that CGP41251 displays a strong preference for the $Ca^{2+}$-dependent classical PKC isoenzymes such as PKC-α, -β and -γ [19,42–44]. Inhibition of PKC resulted in suppression of glucose action on NGF expression and release in a concentration-

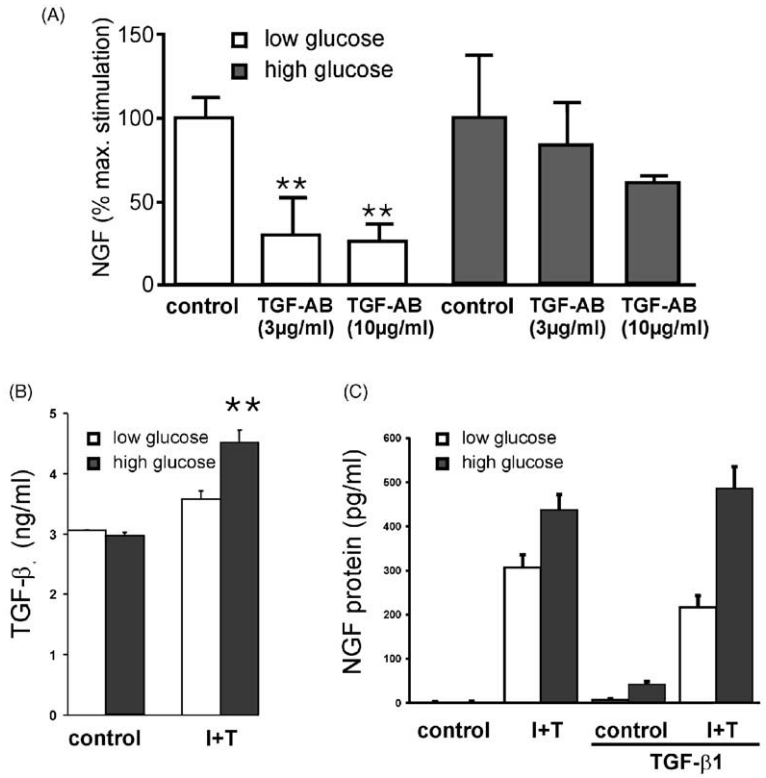


Fig. 7. Effect of TGF-β and anti-TGF-β-antibody on cytokine-induced NGF release—effect of high glucose on TGF-β expression. (A) Mesangial cells were incubated for 12 hr with the cytokines IL-1β/TNF-α (I + T, 1 nM each) alone or in presence of 3 or 10 μg/mL of anti-TGF-β antibody (TGF-β-AB) in low or high concentrations of glucose as indicated. Thereafter, medium was removed and NGF protein was determined as described in Section 2. Data are means ± SEM of three independent experiments. Results are expressed as percent maximal stimulation of NGF release. Cytokine-stimulated NGF protein concentrations amounted to 280 ± 31 and 479 ± 18 pg/mL in low or high glucose medium, respectively. Statistical analysis was by ANOVA ($^{**}P < 0.01$, compared with cytokine-activated cells in medium with normal (low) glucose medium). (B) Mesangial cells were stimulated for 24 hr with the cytokines IL-1β/TNF-α (I + T, 1 nM each) in low or high concentrations of glucose as indicated. TGF-β was activated in the conditioned media by acidification. Thereafter TGF-β protein content was analyzed by ELISA ($^{**}P < 0.01$ for I + T + high glucose vs. I + T + low glucose). (C) Mesangial cells were stimulated for 24 hr with the cytokines IL-1β/TNF-α (I + T, 1 nM each) and with or without TGF-β (10 ng/mL) in low or high concentrations of glucose as indicated. NGF content was analyzed from the conditioned media by ELISA.

dependent manner. In addition, we have found evidence that in the presence of normal glucose levels the PKC-α isoenzyme, the only known $Ca^{2+}$-dependent PKC isoform expressed in rat mesangial cells [33,44,45] controls the IL-1β/TNF-α-induced NGF expression. The key role of PKC-signaling in cytokine-stimulated NGF expression is further supported by the observation that phorbol ester-mediated downregulation of all sensitive PKC isoforms, i.e. PKC-α, -δ and -ε by 24 hr TPA-pretreatment [33,44,45] completely abolished IL-1β/TNF-α-triggered NGF expression in mesangial cells (Fig. 5).

Results with a neutralizing anti-TGF-β antibody revealed that endogenously produced TGF-β at least partially mediates cytokine-induced NGF expression (Fig. 7A). The much weaker effect of anti-TGF-β antibodies in downregulating NGF expression under high glucose conditions may be due to the higher amounts of TGF-β produced by cytokines under high glucose conditions. We conclude that TGF-β is one of several players responsible for the immense enhancing effect of high glucose on cytokine-stimulated NGF release in mesangial cells. Cytokine- and glucose-induced superoxide production followed by an oxidant-mediated PKC activation may be the main signaling pathway that leads to enhanced NGF formation by mesangial cells.

In conclusion, we have shown that cytokine-mediated NGF release by mesangial cells is dramatically enhanced by high glucose levels. Free radicals and PKC-α-dependent signaling pathways control the glucose-modulated NGF response by proinflammatory cytokines. With respect to the role of endogenous NGF in human diabetic complications such as diabetic nephropathy [8] or neuropathy [46–49], it remains to be elucidated whether mesangial cell-derived NGF also acts in the kidney as a proinflammatory cytokine or is part of a protective defense system. Thus, further analysis of the cytokine–NGF cascade and its role in diabetic nephropathy is necessary before new therapeutic approaches interfering with the NGF/NGF receptor system can be initiated.

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