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Regulation of inducible nitric oxide synthase expression in rat mesangial cells and isolated glomeruli

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Regulation of inducible nitric oxide synthase expression in rat mesangial cells and isolated glomeruli. The presence of the inducible isoform of nitric oxide synthase (iNOS) in glomerular mesangial cells facilitates the synthesis of nitric oxide (NO) after stimulation with cytokines or lipopolysaccharide (LPS). As the role of NO within the glomerulus may be important in conditions such as glomerulonephritis, we have studied the effect of dexamethasone (DX) and pirrolidine dithiocarbamate (PDTC), an inhibitor of the nuclear transcription factor, NF-κB activation on the induced synthesis of NO in rat mesangial cells (RMC). LPS, tumor necrosis factor-alpha (TNF-α) and the combination of both were able to induce NO synthesis in a dose-dependent manner as measured with the determination of NO_2^- levels. Treatment with LPS (10 μg/ml) + TNF-α (100 ng/ml) for eight hours was the most potent stimulus for iNOS activity. DX (1 μM) had an inhibitory effect on LPS-, TNF-α- and LPS + TNF-α-induced NO synthesis (51.2, 42.5 and 68% of inhibition, respectively). The inhibitory effect of DX was confirmed using a reporter cell bioassay, whereas cGMP was measured as a reflection of bioactive NO. DX inhibited induced NO synthesis when RMC were exposed to this agent before (16 hr of pretreatment, 75.7% inhibition) or at the same time (8 hr of cotreatment, 61.2% inhibition) as TNF-α + LPS but not four hours after the stimuli. Northern blot analysis showed marked blunting of mRNA expression in RMC treated with DX, in concordance with functional studies. Both actinomycin D and cycloheximide significantly inhibited NO synthesis and iNOS mRNA expression. PDTC (100 μM) was able to inhibit the iNOS activity induced by LPS and TNF-α independently (56.8 and 49.9% inhibition, respectively), and in combination (79.1% inhibition). PDTC (1 to 100 μM) inhibited LPS + TNF-α-induced NO synthesis and iNOS mRNA expression in a concentration-dependent fashion (69 to 86% inhibition of NO synthesis and 50 to 100% inhibition of mRNA expression). Addition of PDTC four hours after exposure to TNF-α + LPS was still able to markedly inhibit NO synthesis. The effects of DX and PDTC were also demonstrated in isolated glomeruli, where two different combinations of inductive stimuli for NO synthesis were employed. Our results establish DX and PDTC as useful tools to study the regulation of NO synthesis in the mesangial cell and glomerulus, and suggest that NF-κB is involved in the transcriptional regulation of iNOS in RMC.

Mesangial cells are modified vascular smooth muscle cells in the renal glomerulus which participate in the regulation of glomerular filtration rate and macromolecular trafficking [1]. They are also crucial targets in several renal pathophysiological conditions [2] and are known to possess an inducible isoform of nitric oxide synthase. This enzyme catalyzes the conversion of L-arginine into L-citrulline and nitric oxide after the stimulation with cytokines or microbial wall products [3–5]. There are experimental data showing that glomeruli synthesize NO in diverse models of experimental glomerulonephritides [6–8], although its pathophysiological role and source are still under discussion. Recently, Shultz and Raij suggested that endogenous production of NO could play a role in the prevention of endotoxin-induced glomerular thrombosis [9, 10]. Thus, the regulation of glomerular iNOS becomes an important issue with potential implications in clinical situations such as septic shock [10].

The role of the ubiquitous messenger NO [11, 12] in the many tissues where it has been found is varied and dependent on the generator and effector cell types. The availability of cDNAs encoding for the different isoforms [13–15] made possible the correlation of their presence with functional studies, and hence almost every cell type or tissue synthesizing NO is associated with the expression of either a constitutive or an inducible isoform of nitric oxide synthase (cNOS or iNOS), with the probable exception of endothelial cells which may express both isoforms [16–18]. Initial work in rat mesangial cells showed their ability to synthesize NO under the influence of lipopolysaccharide and/or cytokines [3–5] and characterized the regulation exerted on this phenomenon by anti-inflammatory steroids and transforming growth factor-β [19, 20]. However, few studies have pursued the potential mechanisms involved in this regulation or correlated functional activity of iNOS with mRNA expression. In this work we have analyzed the effect of dexamethasone (DX), a well-known glucocorticoid anti-inflammatory agent, on the inhibition of iNOS in stimulated RMC, and studied its correlation with iNOS mRNA expression. Our interest in understanding regulatory pathways of NO synthesis in RMC led us to hypothesize that NF-κB, a nuclear transcription factor involved in the regulation of the expression of a plethora of genes related to inflammatory responses [21], could represent a potential transcriptional modulator of mesangial iNOS. In this study we have used an inhibitor of NF-κB activation with antioxidant properties, pirrolidine dithiocarbamate (PDTC) [22], as a tool to explore this hypothesis, and we provide data suggesting that NF-κB is involved in the transcriptional regulation of iNOS. Furthermore, we have extended these studies to isolated glomeruli and demonstrated an

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inhibitory effect of DX and PDTC on NO synthesis and iNOS mRNA expression.

Methods

Materials

Recombinant human tumor necrosis factor-alpha (rHuTNF-α, 9.8×10^6 U/mg) was a gift of Knoll Pharmaceuticals (Whippany, NJ, USA); recombinant murine interferon gamma (rMuIFN-γ, 10^7 U/mg) was from Genzyme (Cambridge, MA, USA); cell culture media were from Bio-Whittaker (Walkersville, MD, USA), fetal calf serum and media supplements were from ICN-Flow (High Wycombe, UK); cell culture plates were from Costar (Cambridge, MA, USA); Taq polymerase was from Promega (Southampton, UK) and dNTPs from Gibco, BRL (Middlesex, UK); deoxycytidine 5′-triphosphate [α-^{32}P] (3000 Ci/mmol) was from Amersham (Aylesbury, UK); guanosine 3′5′-cyclic monophosphate radioimmunoanalysis kit was from Biomedical Technologies Inc. (Stoughton, MA, USA). All other reagents including lipopolysaccharide *E. coli* strain 055:B5, dexamethasone and pirrolidine dithiocarbamate (PDTC) were from Sigma Chemical (St. Louis, MO, USA).

Glomerular isolation and cell culture

Rat mesangial cells and renal glomeruli were obtained and characterized as described [23, 24]. Cells were used in primary culture between 14 and 21 days after seeding or after one passage. They were cultured in 100 mm plates or 50 ml flasks with 5% CO_2 and fed every 48 hours with RPMI 1640 supplemented with 15% fetal calf serum, L-glutamine (1 mM), penicillin (50 U/ml), and streptomycin (50 μg/ml). RFL-6 cells, an established rat fetal lung fibroblast line, were obtained from American Type Culture Collection (ATCC CCL 192) and cultured as described [17]. Potential toxicity of reagents on RMC was ruled out by trypan blue exclusion (> 90% of cells were viable) and measurements of LDH release to the cell culture media (data not shown).

Incubation procedure

On the day of the experiment, cells were washed twice with RPMI devoid of phenol red, as this compound may interfere with NO_2^- spectrophotometric measurement, and left in their usual medium except for the absence of serum and phenol red. Confluent plates of RMC were incubated with the different reagents as described for each experiment. At the end of the experiment supernatants were collected for NO_2^- measurements and cells from these same plates were used for RNA isolation as described below. Isolated glomeruli (aliquots of ≈1 to 2×10^4 glomeruli/plate) were placed in RPMI 1640 devoid of phenol red supplemented with HEPES 15 mM, sodium pyruvate (110 mg/liter), glucose (3.5 g/liter), penicillin (50 U/ml) and streptomycin (50 μg/ml) at 37°C during the experimental protocol. At the end of each experiment supernatants were collected for NO_2^- measurements and RNA was isolated from whole glomeruli.

NO_2^- determination

This procedure was based on the Griess reaction [25]. After completing the experimental protocol, 200 μl of culture supernatant were mixed with an equal volume of Griess reagent (0.1% N-(1-naphthyl)ethylenediamine in water; 1% sulfanilamide in 5% phosphoric acid, 1:1) and the absorbance was read at 540 nm. NO_2^- concentration was determined using sodium nitrite as a standard. Samples of RPMI 1640 that had not been exposed to mesangial cells were assayed for background levels of NO_2^- and these values were substracted from the values measured in the cell culture media.

RNA isolation and Northern blot analysis

Total cellular RNA was isolated from RMC with the guanidinium thiocyanate-phenol-chloroform method [26], size-fractionated by electrophoresis (10 μg/lane) through denaturing 1% agarose-0.66 M formaldehyde gels, transferred to Hybond hybridization transfer membranes (Amersham, UK), and UV cross-linked before hybridization as previously described [18]. As initial hybridizations using the full length cDNA from murine macrophage iNOS cDNA (MAC-NOS, gift of Drs. Lowenstein and Snyder) [27] resulted in a high background, a 700 bp fragment was obtained using the polymerase chain reaction (PCR) and the following primers: sense: 5′ GAG AGA TCC GAT TTA GAG TCT 3′; antisense 5′ GCA GAT TCT GCT GGG ATT TCA 3′. PCR was performed for 35 cycles, each consisting of one minute at 94°C, one minute at 55°C and one minute at 72°C. After validation with Southern blot analysis using MAC-NOS as a probe it was labeled with [α-^{32}P] dCTP using a commercial kit for random hexamer labeling (Boehringer Mannheim, Germany) and used as a probe for Northern analysis. Hybridization was performed at 42°C for 12 to 16 hours, membranes were then washed at final stringency conditions of 55°C, 0.2 × SSC, 0.1% SDS and exposed on XAR Kodak film, using an intensifying screen. In order to correct for uneven loading and to make sure differences in mRNA expression were specific for iNOS, every blot was stripped and rehybridized with rat β-actin [28]. However, because TNF-α has been shown to destabilize β-actin mRNA in some systems [29], we performed Northern blot analysis of β-actin mRNA expression in RMC treated with TNF-α at 0, 4, 8 and 24 hours. In these experimental conditions, we were unable to find significant differences in the expression of this gene in RMC (N = 2, data not shown). Densitometric analysis was performed on an Apple scanner using Image 1.3.1 (NIH, Bethesda, MD, USA). Results are expressed in arbitrary units as the ratio of iNOS/β-actin expression.

RFL-6 assay and cGMP quantitation

This procedure was based on the protocol described by Ishii et al [30] with slight modifications [31]. In this assay, the NO synthesized by a donor monolayer, RMC in this case, is sensed by a reporter cell monolayer, RFL-6 cells. NO is able to activate its natural ligand, soluble guanylate cyclase, and therefore changes in intracellular cGMP levels accurately reflect the amounts of NO generated by the donor cells. For this purpose RMC were cultured in flasks and RFL-6 cells in six-well dishes. At the end of the incubation period cells were placed on a 37°C water bath so that only the monolayers were in contact with the water. After a 10-minute equilibration period RMC were washed twice and left with 2 ml Locke's buffer (in mM:154 NaCl, 5.6 KCl, 10 Hepes, 2 $CaCl_2$, 1 $MgCl_2 \cdot 6H_2O$, 3.6 $NaHCO_3$ and 5.6 glucose) with 1 mM L-arginine. Superoxide dismutase (SOD) was added at a final concentration of 100 U/ml. RFL-6 cells were washed twice and left with 0.8 ml Locke's buffer with 3-isobuthyl-1-methylxanthine (IBMX) (0.3 mM final). After another 10 minutes, 0.8 ml conditioned medium from each flask of RMC were transferred to the

Table 1. LPS and TNF-α-induced NO synthesis in rat mesangial cells (RMC)

Treatment	Concentration	NO_2^- *nmol/10⁶ cells*
Control	—	4.09 ± 0.4
LPS *μg/ml*	0.1	4.38 ± 0.34
LPS *μg/ml*	1	8.76 ± 0.25[a]
LPS *μg/ml*	10	8.86 ± 0.4[a]
TNF-α *ng/ml*	0.1	4.22 ± 0.1
TNF-α *ng/ml*	1	9.42 ± 1.8[a]
TNF-α *ng/ml*	10	10.74 ± 0.8[a]
TNF-α *ng/ml*	100	13.13 ± 0.1[a]

RMC were incubated with vehicle (Control), LPS and rHuTNF-α for 8 hours at the concentrations shown above. NO_2^- levels were determined as described. Data represent the mean ± SE of 4 independent experiments with each condition duplicated per experiment.

[a] $P < 0.05$ vs vehicle

Table 2. Dexamethasone (DX) inhibits the LPS, TNF-α and LPS/TNF-induced NO synthesis in rat mesangial cells (RMC)

Treatment	NO_2^- *nmol/10⁶ cells*	
	NA	DX 8 hr
CT	3.47 ± 0.77	ND
LPS	10.88 ± 0.06[a]	6.86 ± 0.74[b]
TNF-α	11.22 ± 1.92[a]	7.92 ± 0.03[c]
LPS + TNF-α	17.29 ± 0.29[a]	9.16 ± 0.47[d]

RMC were incubated with vehicle (CT, 8 hr), LPS (10 μg/ml, 8 hr), rHuTNF-α (100 ng/ml, 8 hr) and LPS + TNF-α (8 hr) in the absence (NA) or presence of DX (10^{-6} M, 8 hr). NO_2^- levels were determined as described. Data represent the mean ± SE of 3 independent experiments with each experimental condition duplicated per experiment. ND is not done.

[a] $P < 0.05$ vs. vehicle
[b] $P < 0.05$ vs. LPS
[c] $P < 0.05$ vs. TNF-α
[d] $P < 0.05$ vs. LPS + TNF-α

RFL-6 cells and left for three minutes. At the end of this period, the medium was aspirated, 1 ml of 0.1 N HCl was added and cells were placed on ice for 20 minutes. Samples were then recovered and neutralized with 20 μl of 5 M NaOH and 20 μl of 5 M sodium acetate and kept at −20°C. Quantitation of RFL-6 cGMP content was performed with a commercial radioimmunoassay.

Statistical analysis

Every experimental condition was duplicated within each experiment and each experiment was performed, at least three times. Comparisons were made with analysis of variance followed by Dunnett's modification of the *t*-test whenever comparisons were made with a common control and the unpaired two-tailed Student's *t*-test for other comparisons. This test was used whenever the number of total observations allowed to and after performing a Kolmogorov-Smirnov test to confirm the normal distribution of the data. When the number of observations was insufficient to perform adequate parametric tests, non-parametric tests were employed (Kruskal-Wallis). The level of statistically significant difference was defined as $P < 0.05$.

Results

Dexamethasone inhibits NO production in rat mesangial cells stimulated with LPS, TNF-α, and the combination of both

As both LPS and TNF-α had been shown to induce NO synthesis in mesangial cells [3, 5, 32] we explored the isolated and combined effect of these agents in RMC. Table 1 shows the effects of LPS and TNF-α on NO synthesis, measured as the NO_2^- level in RMC. Both compounds were able to significantly stimulate the synthesis of NO. In several experiments ($N = 3$ to 7) we were able to confirm that LPS induced NO synthesis approximately two- to fourfold, TNF-α two- to threefold and the combination of both at the highest concentrations shown for each of one, three- to sixfold (data not shown). Hence, the concentrations of 10 μg/ml for LPS and of 100 ng/ml for TNF-α were selected for the combined use of these agents in the rest of the experiments. Time course experiments using TNF-α and LPS simultaneously (L/T) were then performed. Although after four hours of treatment with L/T a significantly higher amount of NO_2^- was observed compared to control (0 hr, 5.28 ± 1.3; 4 hr, 16.91 ± 0.4 nmol/10^6 cells), a maximum was reached after eight hours of treatment, with no further significant increment in the amount of NO_2^- levels determined after 24 hours (8 hr, 27.6 ± 0.6; 24 hr, 30.7 ± 1.2 nmol/10^6 cells; $N = 4$). Thus, eight hours was selected as the period of stimulation with L/T for experiments concerning regulation of NO synthesis and iNOS expression.

We then explored the potential effect of DX on NO synthesis in RMC. Table 2 shows the effect of DX on NO_2^- levels in RMC treated with TNF-α, LPS and TNF-α + LPS. DX was able to inhibit the effect induced by TNF-α, LPS and L/T on NO synthesis. In several experiments described later, DX was unable to modify basal NO synthesis as studied with determination of NO_2^- or cGMP levels in a reporter cell assay.

NO_2^- and nitrate (NO_3^-) represent higher redox states of NO and their accumulation is widely accepted as a reflection of NO synthesis [33]. However, whether or not this NO is biologically active has not been established. Therefore, in order to confirm the effect of DX on NO synthesis in RMC, a coincubation bioassay with RFL-6 cells was used as described in *Methods*. Figure 1 depicts the effect of DX on LPS + TNF-α (L/T)-induced NO synthesis in RMC. DX clearly abrogated (>90%) the expected L/T-induction (4-fold) of NO synthesis without modifying basal values ($N = 2$). These results closely paralleled the observations made with the determination of NO_2^- levels. For this reason we have used this latter method in order to evaluate the activity of iNOS in RMC.

Incubation of stimulated RMC (LPS 10 μg/ml, 8 hr + rHuTNF-α 100 ng/ml, 8 hr) with two different NOS antagonists, L-NMA (N^G-methyl-L-arginine, 500 μM, 8 hr) and L-NNA (Nω-nitro-L-arginine, 500 μM, 8 hr), in separate experiments, completely abolished LPS + TNF-α-induced NO production (data not shown; $N = 2$).

Figure 2 shows the concentration and time dependence of the DX inhibitory effect. Pretreatment with DX significantly inhibited NO synthesis at two of the three concentrations tested (10 nM, 100 nM and 1 μM, 25.4 ± 5.9, 58.4 ± 8.4 and 74.2 ± 3.6% of inhibition, respectively, compared to induction by LPS + TNF-α after subtracting control values, $N = 6$). Only 16 hours of pretreatment or eight hours of cotreatment of RMC with DX had a significant inhibitory effect. As shown in Figure 2B, when DX was added four hours after LPS + TNF-α, NO_2^- levels were lower than stimulated ones, but did not reach statistical significance.

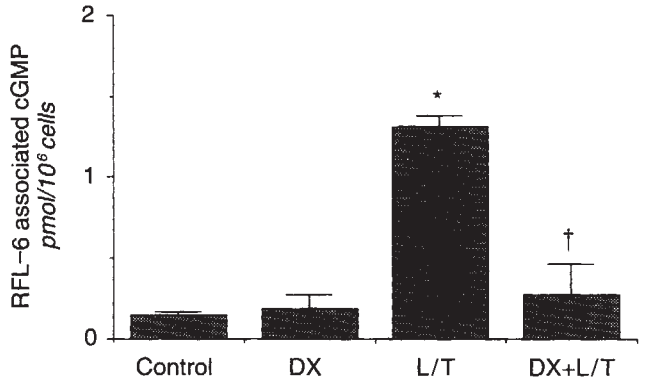

Fig. 1. *Effect of dexamethasone (DX) on iNOS activity in rat mesangial cells (RMC).* Cells were incubated with vehicle (CT, 24 hr), LPS (10 μg/ml, 8 hr) + rHuTNF-α (100 ng/ml, 8 hr) (L/T), DX (10^{-6} M, 24 hr) and DX (10^{-6} M, 24 hr) + L/T (8 hr). In this experimental procedure cGMP levels are determined using RFL-6 as reporter cells. Medium from RMC was transferred to RFL-6 cells in the presence of superoxide dismutase (SOD) (20 min, 100 U/ml). RFL-6 cells were incubated for 3 min with conditioned medium from RMC exposed to the different treatments in the presence of 0.3 mM IBMX. Columns represent the mean ± SE of 3 independent experiments with each experimental condition duplicated per experiment, * $P < 0.05$ vs. vehicle, † $P < 0.05$ vs. L/T.

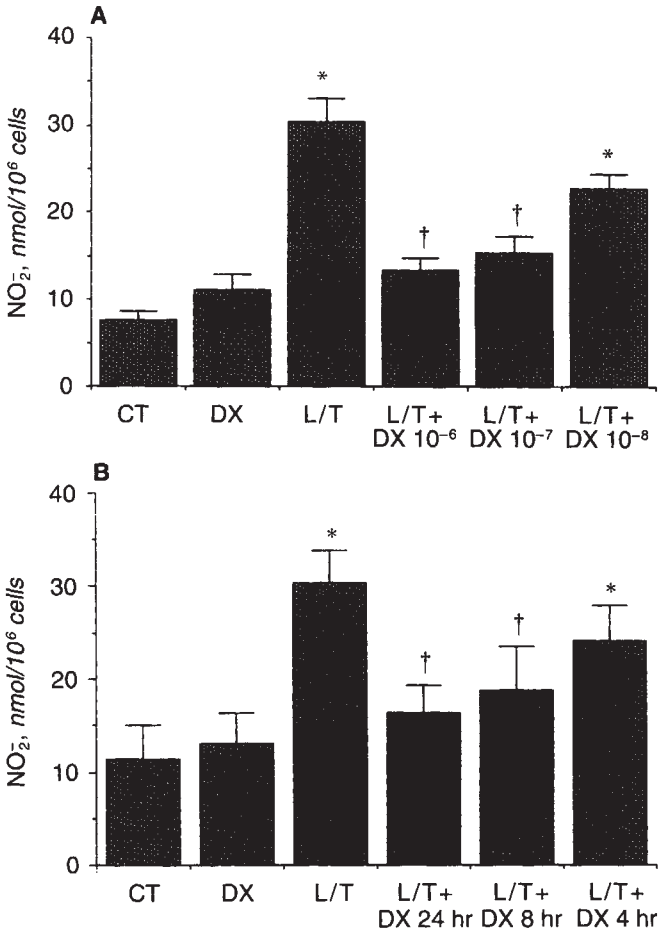

Fig. 2. *Concentration and time dependence of dexamethasone (DX) inhibition on LPS/TNF-α-induced NO synthesis in rat mesangial cells (RMC).* **A.** RMC were incubated with vehicle (CT, 24 hr), LPS (10 μg/ml, 8 hr) + rHuTNF-α (100 ng/ml, 8 hr) (L/T), DX (10^{-6} M, 24 hr) and DX (24 hr) at different concentrations (10^{-6}, 10^{-7} and 10^{-8} M) + L/T (8 hr). NO_2^- levels were determined as described. Columns represent the mean ± SE of 6 independent experiments with each experimental condition duplicated per experiment. * $P < 0.05$ vs. vehicle; † $P < 0.05$ vs. L/T. **B.** RMC were incubated with vehicle (CT, 24 hr), LPS (10 μg/ml, 8 hr) + rHuTNF-α (100 ng/ml, 8 hr) (L/T), DX (10^{-6} M, 24 hr) and DX (10^{-6} M) at different times (24, 8 and 4 hr) + L/T (8 hr). NO_2^-levels were determined as described. Columns represent the mean ± SE of 4 independent experiments with each experimental condition duplicated per experiment. * $P <$ 0.05 vs. vehicle; † $P < 0.05$ vs. L/T.

DX decreases iNOS mRNA expression in RMC stimulated with LPS + TNF-α

To explore the mechanism by which stimulation of NO_2^- levels takes place, Northern blot analysis of total RNA from RMC was performed. As shown in Figure 3A, LPS + TNF-α induced the expression of a 4.5 Kb transcript which corresponds to iNOS. No other bands were detected in these experiments, suggesting high specificity of the probe employed. This expression was blunted by DX (10^{-6} M) about 50%, an effect which was also concentration-dependent (control, 0.20 ± 0.09; L/T, 4.86 ± 0.64; DX 10^{-6} + L/T, 1.87 ± 0.09; DX 10^{-7} + L/T, 2.65 ± 0.21; DX 10^{-8} ± L/T, 2.53 ± 0.45 relative densitometric units; $N = 3$; Fig. 3A). Densitometric analysis of Northern blots showed that the DX inhibitory effect on iNOS expression was more powerful when it was added before (16 hr) than at the same time (8 hr) or after (4 hr) the addition of LPS/TNF-α (control, 0.30 ± 0.10; L/T, 5.1 ± 0.64; DX 24 hr + L/T, 0.94 ± 0.06; DX 8 hr + L/T, 2.61 ± 0.13; DX 4 hr ± L/T, 3.08 ± 0.45 relative densitometric units; $N = 3$; Fig. 3B), in concordance with the results obtained with NO_2^- measurements.

Nitric oxide synthesis induced by LPS + TNF-α in RMC is dependent on protein and mRNA synthesis

As we were interested in establishing potential regulatory mechanisms for iNOS expression in RMC, we investigated whether or not NO synthesis and iNOS expression were dependent on protein and mRNA synthesis. Cycloheximide (CX) (10 μg/ml, 8 hr), a well-known protein synthesis inhibitor, and actinomycin D (Act D) (10 μg/ml, 8 hr), an inhibitor of RNA synthesis, significantly blocked NO_2^- production in RMC stimulated by L/T (vehicle, 6.1 ± 0.6; L/T, 21.6 ± 0.4; CX + L/T, 9.2 ± 0.1; ActD + L/T, 12.4 ± 0.1 nmol/10^6 cells; $P < 0.05$ for vehicle vs. L/T, L/T vs. CX+L/T, and L/T vs. ActD + L/T; $N = 5$). CX and ActD did not significantly modify basal NO_2^- levels (data not shown). This effect was closely paralleled by iNOS expression in RMC (control, 0.32 ± 0.03; L/T, 5.01 ± 0.15; CX 10 + L/T, 0.86 ± 0.4; CX 1 + L/T, 3.28 ± 1.16; Act D + L/T, 0.20 ± 0.06 relative densitometric units; $N = 3$; Fig. 4).

Pyrrolidine dithiocarbamate (PDTC) inhibits stimulated nitric oxide synthesis in rat mesangial cells stimulated with LPS, TNF-α, and the combination of both

In an effort to identify transcriptional regulatory mechanisms implicated in the expression of RMC iNOS, we studied the role of NF-κB in the activation of iNOS transcription. As shown in Table

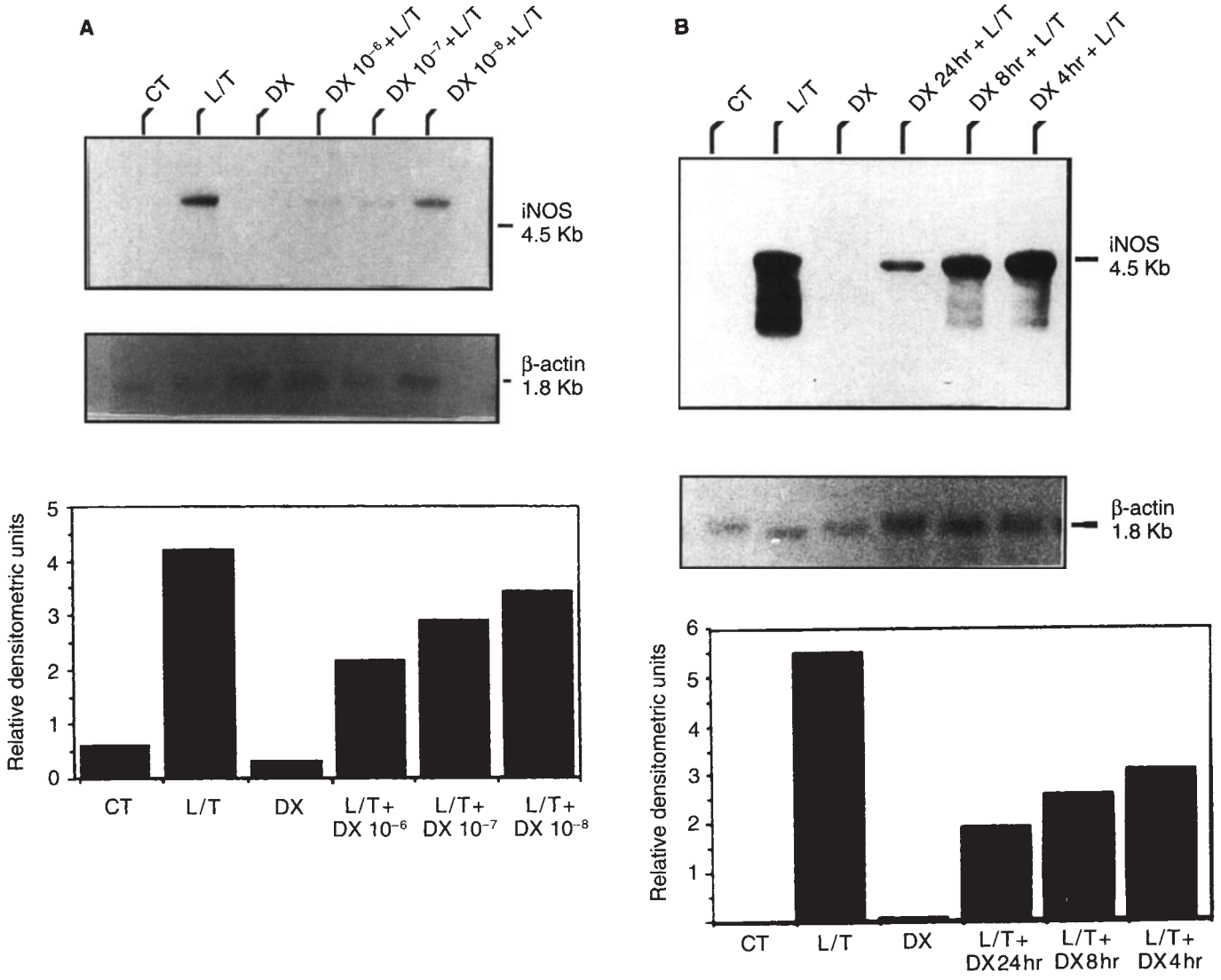

Fig. 3. *Effect of dexamethasone (DX) on iNOS mRNA expression in rat mesangial cells (RMC) stimulated with LPS + TNF-α.* Northern blot analysis of total RNA (10 μg/lane) from RMC probed with a 700 bp fragment of MAC-iNOS. An iNOS transcript (4.5 Kb) was clearly visible in RMC treated with L/T. Expression of rat β-actin in the same membrane is shown in the middle half of the panel and the densitometric analysis of the iNOS Northern blot corrected for β-actin expression is shown below. **A.** RMC were treated with vehicle (CT, 24 hr), LPS (10 μg/ml, 8 hr) + rHuTNF-α (100 ng/ml, 8 hr) (L/T), DX (10^{-6} M, 24 hr) and DX (10^{-6} to 10^{-8} M, 24 hr) + L/T (8 hr). **B.** RMC were incubated with vehicle (24 hr), LPS (10 μg/ml, 8 hr) + rHuTNF-α (100 ng/ml, 8 hr) (L/T), DX (10^{-6} M, 24 hr) and DX (10^{-6} M, 24, 8 and 4 hr) + L/T (8 hr). Shown are representative data of three independent experiments.

3, PDTC, a relatively specific inhibitor of NF-κB activation [22], significantly blunted NO_2^- production by RMC after treatment of these cells with LPS, TNF-α and the combination of both. This inhibition was dependent on the concentration but was clearly significant with the three concentrations tested (1, 10 and 100 μM, 69.4 ± 18.9, 79.8 ± 8.1 and 86.6 ± 8.1% inhibition, respectively, of LPS + TNF-α-induced NO synthesis after subtracting control values, $N = 4$; Fig. 5A). Both pretreatment for 16 hours and simultaneous cotreatment (8 hr) with PDTC markedly abrogated NO_2^- production (91.5 ± 9.5 and 82.5 ± 16.6% respectively, $N = 4$; Fig. 5B). However, in contrast with DX, PDTC was still able to significantly inhibit NO_2^- production when RMC were exposed to this agent four hours after LPS + TNF-α stimulation (61 ± 8.9% of inhibition, $N = 4$; Fig. 5B). PDTC alone did not significantly modify basal NO_2^- levels (Fig. 5).

Pyrrolidine dithiocarbamate (PDTC) inhibits iNOS mRNA expression in rat mesangial cells stimulated with LPS and TNF-α

Northern blot analysis also demonstrated that pretreatment with PDTC was able to completely blunt iNOS mRNA expression in stimulated RMC (Fig. 6), an effect which was also concentration-dependent (control, 0.39 ± 0.1; L/T, 8.02 ± 0.1; PDTC 100 + L/T, 0.4 ± 0.06; PDTC 1 + L/T, 1.79 ± 0.03 relative densitometric units; $N = 3$). PDTC alone did not modify basal NO_2^- levels or mRNA expression.

Whole isolated glomeruli treated with LPS + cytokines synthesize NO and express iNOS and this synthesis and expression is abrogated by DX and PDTC

As shown in Table 4 isolated glomeruli were able to synthesize NO after stimulation with LPS + TNF-α or LPS + IFN-γ. We

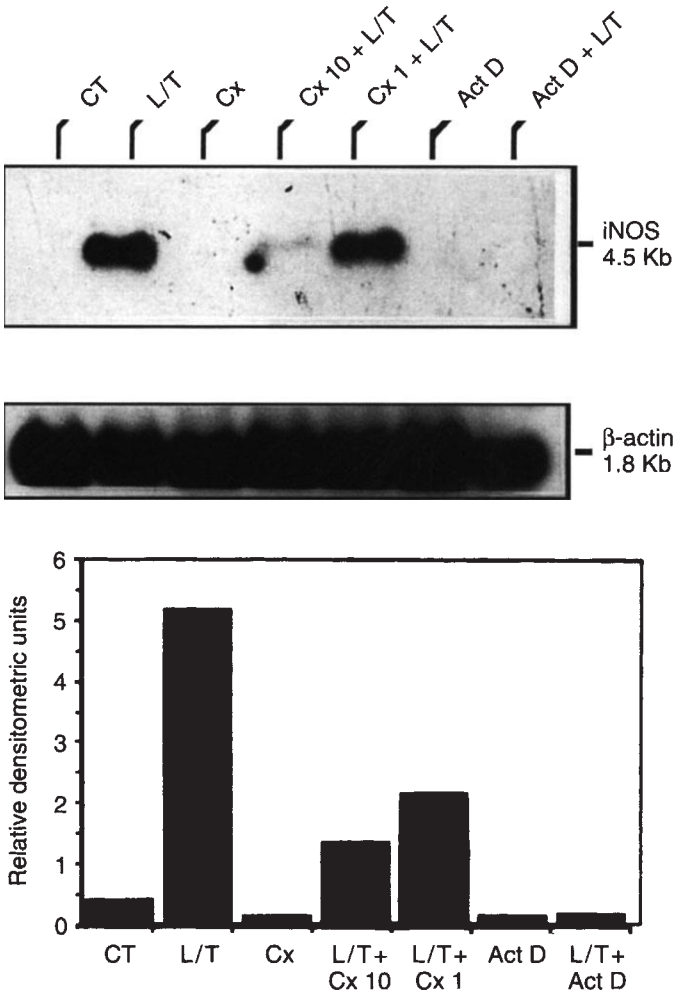

Fig. 4. *Effect of cycloheximide (CX) and actinomycin D (Act D) on iNOS mRNA expression in rat mesangial cells (RMC) stimulated with LPS + TNF-α.* Northern blot analysis of total RNA (10 μg/lane) from RMC probed with a 700 bp fragment of MAC-iNOS. Expression of rat β-actin in the same membrane is shown in the middle half of the panel and the densitometric analysis of the iNOS Northern blot corrected for β-actin expression is shown below. RMC were incubated with vehicle (CT, 24 hr), LPS (10 μg/ml, 8 hr) + rHuTNF-α (100 ng/ml, 8 hr) (L/T), CX (10 μg/ml, 8 hr), Act D (10 μg/ml, 8 hr), CX (1 and 10 μg/ml, 8 hr) + L/T and Act D (10 μg/ml, 8 hr) + L/T. Shown are representative data of three independent experiments.

observed that the combination of LPS + IFN-γ was more potent than LPS + TNF-α (4.5-fold vs. 2-fold), in contrast with results obtained in RMC where the two combinations were equally potent (data not shown). Also in this case, DX and PDTC clearly inhibited NO_2^- production (Table 4), an effect again paralleled by iNOS expression (control, 1 ± 0.01; L/T, 2.78 ± 0.46; L/IF, 5.04 ± 0.11; DX + L/T, 0.68 ± 0.05, DX + L/IF, 1.55 ± 0.01; PDTC + L/T, 0.23 ± 0.08; PDTC + L/IF, 0.18 ± 0.06 relative densitometric units; $N = 4$; Fig. 7).

Discussion

Our study addresses potential pathways of regulation of iNOS expression in RMC. The induced synthesis of NO in mesangial cells is a well established fact at least in three different species

Table 3. Pirrolidine dithiocarbamate (PDTC) inhibits LPS, TNF-α and LPS + TNF-α-induced NO synthesis in rat mesangial cells (RMC)

Treatment	NO_2^- *nmol/10⁶ cells*	
	NA	PDTC *8 hr*
CT	3.47 ± 0.77	ND
LPS	10.88 ± 0.06[a]	6.67 ± 0.56[b]
TNF-α	11.22 ± 1.92[a]	7.35 ± 0.01[c]
LPS + TNF-α	17.29 ± 0.29[a]	6.77 ± 0.47[d]

RMC were incubated with vehicle (CT, 8 hr), LPS (10 μg/ml, 8 hr), rHuTNF-α (100 ng/ml, 8 hr) and LPS + TNF-α (8 hr) in the absence (NA) or presence of PDTC (100 μM, 8 hr). NO_2^- levels were determined as described. Data represent the mean ± SE of 3 independent experiments with each experimental condition duplicated per experiment. ND is not done.

[a] $P < 0.05$ vs. vehicle
[b] $P < 0.05$ vs. LPS
[c] $P < 0.05$ vs. TNF-α
[d] $P < 0.05$ vs. LPS + TNF-α

[3–5, 34]. We have demonstrated that DX and PDTC directly inhibit iNOS activity and expression in RMC treated with TNF-α, LPS and a combination of both. Furthermore, we believe this is one of the first reports which suggests the participation of a transcription factor, NF-κB, in the activation of iNOS in RMC. Finally we have extended our studies to isolated glomeruli in an effort to correlate our findings with histologically more complex structures but probably more suited for pathophysiological interpretations.

The fact that CX and Act D inhibited iNOS activity and mRNA expression in RMC is consistent with current concepts of iNOS regulation in other tissues, including rat vascular smooth muscle cells [35], which, given their common mesenchymal origin, one would predict to behave in a similar fashion as RMC. Two different groups have recently described that cycloheximide is able to induce the expression of iNOS in vascular smooth muscle cells and macrophages [36, 37]. Cycloheximide may increase the levels of inducible mRNAs in several ways [38], including the inhibition of the synthesis of an mRNA destabilizing factor, the stabilization of polysome-associated mRNAs, the inhibition of the synthesis of a transcriptional inhibitor, and the influence on the activity of nuclear protein kinases that may activate transcription factors. Whether some or all of these effects take place according to cell or species differences remains to be established. Our results are in agreement with reports from other groups who studied regulation of iNOS by cycloheximide in different cell types [35, 39, 40]. Although the concentrations employed in our study may be regarded as high, significant toxicity was excluded by cell viability and enzyme release studies.

The time course exhibited by the DX-induced inhibition of iNOS suggests that this agent needs to be present for a long period of time in order to inhibit iNOS expression. In this sense, DX was unable to abrogate NO production when cells had already been exposed to LPS + rHuTNF-α for four hours. This implies that it may act on an event which is proximal to the transcriptional activation of iNOS and possibly other genes related to inflammatory responses. This may help to understand why, in clinical trials of DX aimed to evaluate its therapeutic role in situations such as septic shock, it might not represent a useful agent, as its administration should precede the clinical expression of sepsis [41].

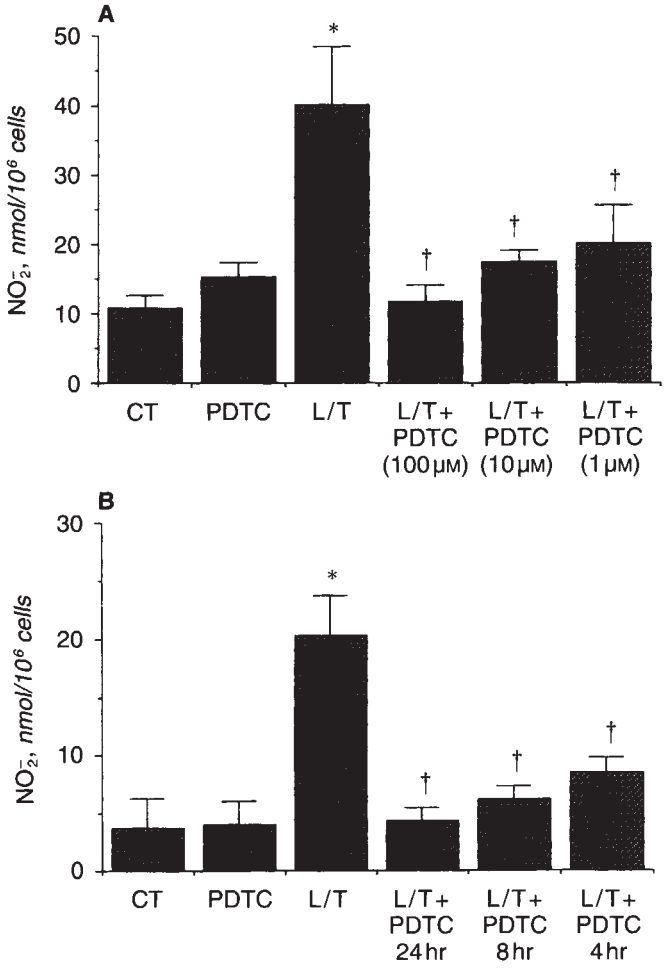

Fig. 5. *Concentration and time dependence of pirrolidine dithiocarbamate (PDTC), inhibition on LPS/TNF-α-induced NO synthesis in rat mesangial cells (RMC).* **A.** RMC were incubated with vehicle (CT, 24 hr), LPS (10 μg/ml, 8 hr) + rHuTNF-α (100 ng/ml, 8 hr) (L/T), PDTC (100 μM, 24 hr) and PDTC (1 to 100 μM, 24 hr) + L/T (8 hr). NO_2^- levels were determined as described. Columns represent the mean ± SE of 4 independent experiments, with each experimental condition duplicated per experiment. *$P < 0.05$ vs. vehicle; †$P < 0.05$ vs L/T. **B.** RMC were incubated with vehicle (24 hr), LPS (10 μg/ml, 8 hr) + rHuTNF-α (100 ng/ml, 8 hr) (L/T), PDTC (100 μM, 24 hr) and PDTC (100 μM) at 24, 8 and 4 hr + L/T (8 hr). NO_2^- levels were determined as described. Columns represent the mean ± SE of 4 independent experiments, with each experimental condition duplicated per experiment. *$P < 0.05$ vs. vehicle; †$P < 0.05$ vs. L/T.

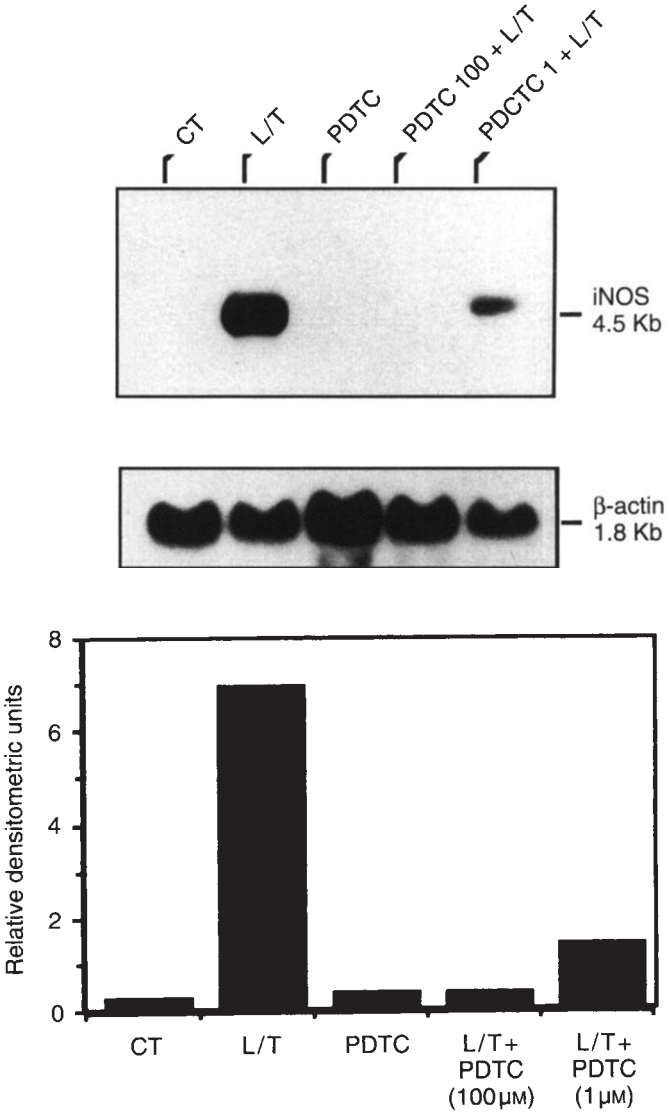

Fig. 6. *Effect of pirrolidine dithiocarbamate (PDTC) on iNOS mRNA expression in RMC stimulated with LPS + rHuTNF-α.* Northern blot analysis of total RNA (10 μg/lane) from RMC probed with a 700 bp fragment of MAC-iNOS. Expression of rat β-actin in the same membrane is shown in the middle half of the panel and the densitometric analysis of the iNOS Northern blot corrected for β-actin expression is shown below. RMC were incubated with vehicle (CT, 24 hr), LPS (10 μg/ml, 8 hr) + rHuTNF-α (100 ng/ml, 8 hr) (L/T), PDTC (100 μM, 24 hr) and PDTC (100 μM and 1 μM, 24 hr) + L/T (8 hr). Shown are representative data of three independent experiments.

Whether DX destabilizes iNOS message or inhibits newly transcribed mRNA remains to be elucidated. Available promoter analysis of iNOS in macrophages does not show an apparent 5′ glucocorticoid response element [42, 43]. This fact suggests that DX could have an alternative mechanism of inhibition, such as an intermediate transregulatory acting element or intracellular alteration of protein synthesis or signal transduction pathways, which remain to be explored. Interestingly, a similar phenomenon has been described in relation with the transcriptional activation of endothelial-leukocyte adhesion molecule (ELAM-1) [44]. Our results are in agreement with previous functional studies in macrophages [45] and RMC [19] and with reports showing the effect of DX on iNOS mRNA expression in rat hepatocytes [39].

Among the potential candidates for the regulation of iNOS transcriptional activation we considered NF-κB. This nuclear factor regulates the expression of multiple genes in inflammatory responses of eukaryotic organisms [21]. Carefully conducted studies have shown that dithiocarbamates are potent inhibitors of NF-κB activation in intact cells [22]. Their mechanism of action is probably related to both their dual capability as metal chelators and strong antioxidants, their molecular action being related with the inhibition of the release of Iκ-B from the rest of the

Table 4. Dexamethasone (DX) and pirrolidine dithiocarbamate (PDTC) inhibit LPS/TNF-α- and LPS/IFN-γ-induced NO synthesis in isolated glomeruli

	NO_2^- *nmol/10² glomeruli*		
Treatment	NA	DX	PDTC
Control	2.0 ± 0.1	2.0 ± 0.2	2.4 ± 0.3
L/T	4.2 ± 0.9[a]	2.8 ± 0.5[b]	2.7 ± 0.1[b]
L/IFN	9.5 ± 0.6[ab]	5.1 ± 1.4[c]	3.6 ± 1.3[c]

Aliquots of ≈1 to 2 × 10^4 glomeruli/plate were incubated with vehicle (control, 24 hr), LPS (10 μg/ml, 24 hr) + rHuTNF-α (100 ng/ml, 24 hr) (L/T), LPS (10 μg/ml, 24 hr) + rMuIFN-γ (100 U/ml, 24 hr) (L/IFN), DX + L/T, DX + L/IFN, PDTC + L/T and PDTC + L/IFN (same concentrations as above, 24 hr). NO_2^- levels were determined as described. Values represent the mean ± SE of 3 independent experiments, duplicate determinations.

[a] $P < 0.05$ vs. vehicle
[b] $P < 0.05$ vs. L/T
[c] $P < 0.05$ vs. L/IFN

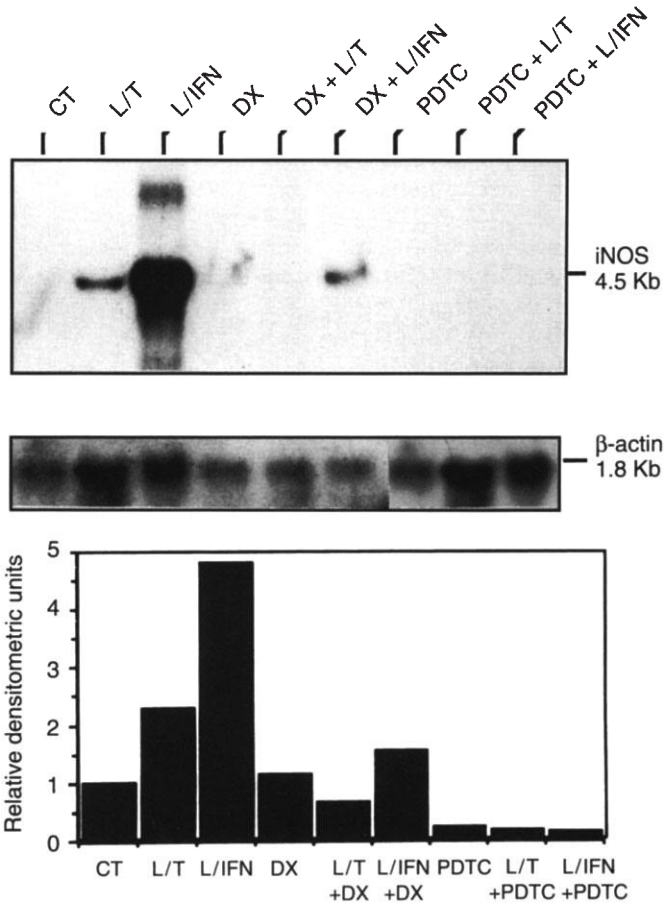

Fig. 7. *Effect of DX and PDTC on iNOS mRNA expression in isolated rat glomeruli stimulated with LPS + rHuTNF-α or LPS + recombinant murine interferon-γ (rMuIFN-γ).* Northern blot analysis of total RNA (10 μg/lane) from isolated rat glomeruli probed with a 700 bp fragment of MAC-iNOS. Expression of rat β-actin in the same membrane is shown in the middle half of the panel and the densitometric analysis of the iNOS Northern blot corrected for β-actin expression is shown below. Glomeruli were incubated with vehicle (CT, 24 hr), DX (10^{-6} M, 24 hr), PDTC (100 μM, 24 hr), LPS (10 μg/ml, 24 hr) + rHuTNF-α (100 ng/ml, 24 hr) (L/T), LPS (10 μg/ml, 24 hr) + rMuIFN-γ (100 U/ml, 24 hr) (L/IFN), Dx + L/T, DX + L/IFN, PDTC + L/T and PDTC + L/IFN (all at the above concentrations, 24 hr). Shown are representative data of three independent experiments.

cytoplasmic NF-κB complex, thus preventing its translocation to the nucleus and subsequent binding to DNA [22]. Our results in RMC evidence that NF-κB may be involved in iNOS gene activation as treatment with PDTC inhibited both iNOS expression and activity. This is in agreement with recent observations made in other cell types [46, 47]. Although two consensus sequences for NF-κB binding have been recently described in the promoter region of the iNOS gene in mouse macrophages [42, 43] and NF-κB interaction with the iNOS promoter has proven to be a potential mechanism for transcriptional activation [48], other mechanisms cannot be excluded. First of all, iNOS cDNA from RMC has not been cloned or characterized, nor has its promoter region. Although most probably it will be encoded by the same gene as the macrophage and vascular smooth muscle isoforms [27, 49–51] this remains to be stablished. Second, NF-κB seems to be involved in the activation of genes by reactive oxygen intermediates (ROI) [52], and PDTC is an agent with proven antioxidant activity. Participation of these mediators in the cascade of cytokine activation in RMC has been described [53] and therefore this potential pathway of PDTC intervention cannot be totally excluded. Of note, gene transcription and expression of vascular cell adhesion molecule-1 (VCAM-1) in human endothelial cells is regulated by an antioxidant-sensitive mechanism [54], thus suggesting that transcription of genes encoding for inflammation-related molecules could share common features. Although our data are consistent with the effect of PDTC on iNOS transcriptional activation, the time course exhibited by the PDTC inhibition was somewhat surprising. Treatment of RMC with PDTC after four hours of stimulation was still able to significantly abrogate LPS + TNF-α-induced NO synthesis, suggesting that PDTC is also able to act on a post-transcriptional level of iNOS activation. Studies directed towards promoter analysis of iNOS in RMC, including deletions of specific consensus sequences for the potential NF-κB *cis*-regulatory region, should be able to confirm and extend these observations.

Intracellular signaling pathways of LPS and TNF-α responsible for iNOS activation are not well defined. It is well accepted that activation of macrophages by LPS results in release of numerous potent immune mediators, including TNF-α and NO [55]. Protein tyrosine phosphorilation has been suggested as a mechanism for macrophage activation with LPS [56]. Other investigators have found that G-proteins could differentially regulate two distinct biochemical pathways in mouse macrophages, one facilitating the synthesis of TNF-α and the other one of NO [57]. TNF-α alone is able to trigger the expression of inflammatory molecules through protein kinase C-dependent and -independent pathways [56, 58]. In RMC both LPS- and TNF-α-induced NO synthesis was individually inhibited by DX and PDTC. Even when these two agents are known to act on different pathways [22, 59] recent observations suggest that there might be interaction between the glucocorticoid receptor and NF-κB [60, 61], which is also recognized as a common target for LPS and TNF-α [21]. Thus, and in agreement with our results, it seems reasonable to suggest, at the present level of complexity, that both LPS and TNF-α and DX and PDTC could use differential and common stimulatory and inhibitory pathways, respectively, responsible for the regulation of iNOS.

Mesangial cells are intermingled inside the glomerulus in an

strategic position to exert regulatory functions of macromolecular trafficking and ultrafiltration surface. However, it is the whole glomerulus which represents a structure-function unit and is the target of several forms of acute and chronic disease. For this reason, we were interested in demonstrating the synthesis of NO and expression of iNOS in isolated glomeruli, which are histologically complex structures, where the relevance of any finding more closely reflects the *in vivo* situation. The results obtained in whole isolated glomeruli are qualitatively similar and consistent with those obtained in RMC. In the former, however, a different combination of cytokines (IFN-γ + LPS) was more potent than the one shown in RMC. Although it is certain that RMC are responsible for part of the NO synthesized by isolated glomeruli, we cannot exclude the participation of glomerular endothelial cells or resident macrophages, which, as proposed by Cattell et al, would be the main source of NO production in inflammatory conditions such as glomerulonephritis [8]. Cytokine sensitivity of this alternative cellular source of NO synthesis is predictably different and has been demonstrated in a murine macrophage cell line [40], thus offering a reasonable explanation for our results. Hence, the regulatory effect of DX and PDTC on induced NO synthesis in isolated glomeruli further reinforces the observations obtained in RMC and suggests potential pathways of therapeutic intervention in the cascade of events present in the renal dysfunction of sepsis.

We believe our study contributes to clarify the mechanism of glucocorticoid inhibition of iNOS in RMC and incorporates new information regarding regulation of the iNOS gene by transcription factors, namely NF-κB.

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Note added in proof

While this work was under review, two different groups reported similar effects of dexamethasone (Shultz et al, *Kidney Int* 46:683–689, 1994) and pirrolidine dithiocarbamate (Eberhart et al, *Biochem Biophys Res Comm* 200:163–170, 1994) on the regulation of nitric oxide synthesis in rat mesangial cells.

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