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Inhibition by tetranactin of interleukin 1β- and cyclic AMP-induced nitric oxide synthase expression in rat renal mesangial cells

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1 We have investigated whether tetranactin, a cyclic antibiotic produced by *Streptomyces aureus* with a molecular structure related to cyclosporin A, influences inducible nitric oxide synthase (iNOS; EC 1.14.13.39) induction in rat glomerular mesangial cells.

2 Previously we have shown that iNOS is expressed in renal mesangial cells in response to two principal classes of activating signals comprising inflammatory cytokines such as interleukin 1 (IL-1) or tumour necrosis factor α and agents that elevate cellular levels of cyclic AMP. Treatment of mesangial cells with IL-1β or the membrane-permeable cyclic AMP analogue, N^6, 0-2'-dibutyryladenosine 3',5'-phosphate (Bt_2 cyclic AMP) for 24 h induces iNOS activity measured as nitrite levels in cell culture supernatants by 44 fold or 33 fold, respectively. Incubation of mesangial cells with tetranactin inhibits IL-1β- and cyclic AMP-dependent production of nitrite in a dose-dependent fashion with IC_{50} values of 50 nM and 10 nM, respectively.

3 Western-blot analyses of mesangial cell extracts reveal that the inhibition of nitrite synthesis by tetranactin is due to a suppression of iNOS protein levels. This effect is preceded by a reduction of iNOS mRNA steady state levels as demonstrated by Northern blot analyses of total cellular RNA isolated from stimulated mesangial cells.

4 Thus, tetranactin is a potent inhibitor of iNOS expression in cytokine- and cyclic AMP-stimulated mesangial cells and represents a new class of iNOS inhibitors with IC_{50}s in the low nanomolar range. This compound may be useful in the therapy of diseases associated with pathological NO overproduction due to iNOS expression.

Keywords: Nitric oxide; nitric oxide synthase; tetranactin, interleukin 1; cyclic AMP

Introduction

In recent years nitric oxide (NO), generated from L-arginine by NO synthase (NOS) has become established as a diffusible universal messenger mediating cell-cell communication throughout the body (Moncada *et al.*, 1991; Nathan, 1992). Three main types of NOS isoforms have been characterized by molecular cloning and sequencing analyses (Knowles & Moncada, 1994; Nathan & Xie, 1994). The brain (type I) and endothelial (type III) enzymes are constitutively expressed and regulated by short-term elevations of intracellular free Ca^{2+}-concentration in response to hormone or neurotransmitter stimulation, thus triggering the production of minute amounts of NO for physiological regulatory processes. A third type of NOS (type II) has been cloned from murine macrophages. This enzyme is not constitutively expressed, but is induced by lipopolysaccharides and γ-interferon and is Ca^{2+}-independent. The macrophage inducible NOS (iNOS) is regulated at the transcriptional level and once induced, is active for hours and days and produces a multiple of NO synthesized by constitutively expressed NOS. The excessive production of NO not only mediates the bactericidal and tumouricidal actions of macrophages, but may also contribute to cell and tissue damage in immunological diseases (Anggard, 1994; Cattell & Cook, 1995).

Glomerular mesangial cells are a specialized type of vascular smooth muscle cell and participate in the regulation of the glomerular filtration rate (Pfeilschifter, 1989). The cells respond to endothelial cell-derived NO with cyclic GMP formation and subsequent relaxation. However, mesangial cells not only act as a target cell for NO, but also have the capacity themselves to synthesize this important signalling molecule. Recently, we and others have shown that inflammatory cytokines such as interleukin 1β (IL-1β) or tumour necrosis factor α induce a macrophage-type of NOS in mesangial cells with subsequent increase in cellular cyclic GMP concentration (Pfeilschifter & Schwarzenbach, 1990; Marsden & Ballermann, 1990; Pfeilschifter *et al.*, 1992). The excessive formation of NO and cyclic GMP in mesangial cells not only blocks contractile responsiveness of the cells (Pfeilschifter *et al.*, 1992), but may also contribute to tissue injury observed in the pathogenesis of certain forms of glomerulonephritis (Pfeilschifter, 1994; 1995; Cattell & Cook, 1995). Moreover, we have demonstrated that not only inflammatory cytokines but also cyclic AMP up-regulates iNOS expression in rat mesangial cells. The signal transduction pathways triggered by cytokines and cyclic AMP may act alone or interact in a synergistic fashion on the transcription of the iNOS gene (Kunz *et al.*, 1994a; Mühl *et al.*, 1994).

Tetranactin is a hydrophobic and cyclic antibiotic produced by *Streptomyces aureus* with a molecular structure related to that of cyclosporin A, a compound that is used clinically to prevent graft rejection following organ transplantation. As for cyclosporin A, strong immunosuppressive activity has been reported for tetranactin (Tanouchi & Shichi, 1988; Teunissen *et al.*, 1992). Tetranactin has been shown to block the proliferation of T-lymphocytes as well as the generation of cytotoxic T-lymphocytes and activated killer cells (Callewaert *et al.*, 1988). In view of the structural similarity of cyclosporin A and tetranactin, it is likely that the pharmacological activities of these compounds may be mediated by similar mechanisms. Recently, we have shown that cyclosporin A and its derivatives cyclosporin G and cyclosporin H, but not FK 506, another potent immunosuppressive drug, inhibit IL-1β-induced iNOS

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expression in rat renal mesangial cells by down-regulation of steady state levels of iNOS mRNA and subsequent inhibition of nitrite formation (Mühl *et al.*, 1993; Kunz *et al.*, 1995).

The aim of the present study was to investigate a possible interference of tetranactin with the L-arginine-NO pathway in mesangial cells. We present data demonstrating that tetranactin is highly potent in inhibiting IL-1β- and cyclic AMP-dependent induction of NO synthase expression in renal mesangial cells.

Methods

Cell culture

Rat mesangial cells were cultured as described previously (Pfeilschifter *et al.*, 1992). In a second step, single cells were cloned by limited dilution using 96-microwell plates. Clones with apparent mesangial cell morphology were used for further processing. The cells exhibited the typical stellate morphology. Moreover, there was positive staining for the intermediate filaments, desmin and vimentin, which are considered to be specific for myogenic cells, positive staining for Thy 1.1 antigen, negative staining for the factor VIII-related antigen and cytokeratin, excluding endothelial and epithelial contamination, respectively. The generation of inositol trisphosphate upon activation of the angiotensin II AT_1 receptor was used as a functional criterion for characterizing the cloned cell line. The cells were grown in RPMI 1640 supplemented with 10% foetal calf serum, penicillin (100 units ml^{-1}), streptomycin (100 $\mu g\ ml^{-1}$) and bovine insulin at 0.66 units ml^{-1} (Sigma). For the experiments passages 6–16 of mesangial cells were used.

Nitrite analysis

Nitrite production by rat glomerular mesangial cells was measured as a read-out for NO synthase activity as described previously (Green *et al.*, 1982). Confluent mesangial cells in 24-well plates were washed twice with phosphate-buffered saline (PBS) and incubated in Dulbecco's modified Eagle's medium (DMEM) without phenol red and supplemented with 0.1 mg ml^{-1} of fatty-acid free bovine serum albumin, with or without agents for the indicated time periods. Thereafter, the medium was withdrawn and nitrite was measured by mixing 200 μl of the supernatant with 100 μl of Griess reagent. The absorbance at 550 nm was measured and the nitrite concentration was determined by use of a calibration curve with sodium nitrite standards. This assay does not include conversion of nitrate into nitrite, but we have previously shown that the amount of nitrate produced in IL-1β-stimulated cells is approximately 20–25% of that of nitrite (Mühl & Pfeilschifter, 1994).

Northern blot analysis

Confluent mesangial cells were washed twice with PBS and incubated in DMEM, supplemented with 0.1 mg ml^{-1} of fatty acid-free bovine serum albumin, with or without agents for the indicated time periods. Cells were washed twice with PBS and harvested with a rubber policeman. Total cellular RNA was extracted from the cell pellets using the guanidinium thiocyanate/caesium chloride method (Sambrock *et al.*, 1989). Samples of 20 μg RNA were separated on 1% agarose gels containing 0.66 M formaldehyde prior to the transfer to gene screen membranes (New England Nuclear). After u.v.-cross-linking and prehybridization for 2 h, the filters were hybridized for 16–18 h to a ^{32}P-labelled Sma I cDNA insert from pMac-NOS. To correct for variation in RNA amount, the NOS probe was stripped with boiling 0.1 × SSC/1%SDS and the blots were rehybridized to the ^{32}P-labelled Bam H I/Sal I cDNA insert from clone pEX 6 coding for human β-actin. DNA-probes ($0.5-1 \times 10^6$ d.p.m. ml^{-1}) were radioactively labelled with [^{32}P]-dATP by random priming (Boehringer Mannheim). Hybridization reactions were performed in 50%(v/v) formamide, 5 × sodium chloride/sodium citrate buffer (SSC), 5 × Denhardt's solution, 1% (w/v) sodium dodecylsulphate (SDS), 10% (w/v) dextran sulphate and 100 $\mu g\ ml^{-1}$ salmon sperm DNA. Filters were washed 3 times in 2 × SSC/0.1% SDS at room temperature for 15 min, and then 2 times in 0.2 × SSC/1% SDS at 65°C for 30 min. Filters were exposed for 24–48 h to Kodak X-Omat XAR-film using intensifying screens (Kunz *et al.*, 1994a).

Western blot analysis

Confluent mesangial cells were stimulated with agents for 16 h and then washed with PBS and scraped into 1 ml of buffer A (50 mM Tris/HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 1 μM leupeptin, 0.1 mM phenylmethylsulphonyl fluoride, 1 mM dithiothreitol). Cells were homogenized in a Dounce homogenizer and centrifuged at 200'000 *g* for 30 min at 4°C. Protein concentration was determined by the method of Bradford (1976). The supernatants (3 mg of protein) were incubated for 30 min at 4°C with 400 μl of a 1:1 (v/v) slurry of 2',5'-ADP-agarose in buffer A with gentle agitation. After centrifugation for 10 min at 3'000 *g* the pellets were washed twice with buffer A, once with buffer A containing 0.5 M NaCl, and finally with buffer A only. The proteins were solubilized from the beads by heating for 5 min at 95°C in 0.1 ml of SDS-PAGE sample buffer (3.6% (w/v) SDS, 15% (w/v) glycerol, 120 mM Tris-HCl, pH 6.8, 0.125 M DTT) and subjected to SDS-PAGE (7.5% acrylamide gel). Immunoblotting was performed as described by Kunz *et al.*, (1994b) using a polyclonal anti-iNOS antibody at a dilution of 1:1000.

Chemicals

Recombinant human IL-1β was generously supplied by Dr C. Rordorf, Ciba-Geigy Ltd., Basel, Switzerland; tetranactin was kindly provided by Chugai Pharmaceutical Co., Ltd., Tokyo, Japan; Bt_2cyclicAMP was from Sigma, Buchs, Switzerland. The cDNA clone pMac-NOS, coding for the inducible macrophage NO synthase was kindly provided by Dr J. Cunningham, Boston, MA, U.S.A.; the cDNA clone pEX 6, coding for human β-actin, was a gift from Dr U. Aebi, Basel, Switzerland. Nylon membranes (Gene Screen) were purchased from DuPont de Nemours International, Regensdorf, Switzerland; [^{32}P]-dATP (specific activity 3000 Ci $mmol^{-1}$) was from Amersham, Dübendorf, Switzerland; cell culture media and nutrients were from Gibco BRL, Basel, Switzerland and all other chemicals were either from Merck, Darmstadt, Germany or Fluka, Buchs, Switzerland. Antibodies specific for iNOS were generated using the multiple antigen peptide (MAP) system. The peptide antigen based on a N-terminal sequence of mouse iNOS (NVPESLDKLHVT) is assembled stepwise on the MAP core resin. After complete peptide assembly, the MAP system is cleaved from the resin support and after dialysis used for immunization (Kunz *et al.*, 1994b).

Results

IL 1β and cyclic AMP stimulated iNOS activity

Mesangial cells were incubated for 24 h with IL-1β (2 nM) or the membrane-permeable cyclic AMP analogue, N^6, 0-2'-dibutyryladenosine 3',5'-phosphate (Bt_2 cyclic AMP, 10 mM). Cell-culture supernatants were assayed for nitrite production, one of the end-products of NO formation. Whereas unstimulated cells produced only low amounts of NO (14 ± 7 nmol nitrite mg^{-1} protein), treatment of the cells with IL-1β(2 nM) or Bt_2 cyclic AMP (10 mM) for 24 h dramatically increased the production of NO by 44 fold (617 ± 49 nmol nitrite mg^{-1} protein) or 33 fold (462 ± 28 nmol nitrite mg^{-1} protein) (means ± s.d., $n = 4$), respectively.

Tetranactin inhibition of IL-1β-and cyclic AMP-dependent nitrite formation in mesangial cells

Mesangial cells were simultaneously incubated with IL-1β or Bt_2 cyclic AMP together with increasing amounts of tetranactin for 24 h and cell-culture supernatants were assayed for nitrite production. As shown in Figure 1, tetranactin dose-dependently inhibited nitrite formation induced by IL-1β. Half-maximal inhibition was observed at 50 nM tetranactin. Similarly, as demonstrated in Figure 2, tetranactin suppressed Bt_2 cyclic AMP stimulated production of NO in a dose-dependent fashion with half-maximal inhibition at 10 nM tetranactin.

Inhibition of nitrite release due to decreased iNOS protein levels

Western blot analysis with an iNOS-specific polyclonal antibody demonstrated that treatment of mesangial cells with IL-1β(2 nM) or Bt_2 cyclic AMP(10 mM) for 24 h dramatically up-regulated the iNOS 130 kDa protein as shown in Figure 3. This band was not detected in unstimulated cells and also tetranactin alone had no effect on the iNOS protein expression (Figure 3). Co-incubation of IL-1β (2 nM) or Bt_2 cyclic AMP (10 mM) with tetranactin (100 nM) potently reduced iNOS protein expression (Figure 3).

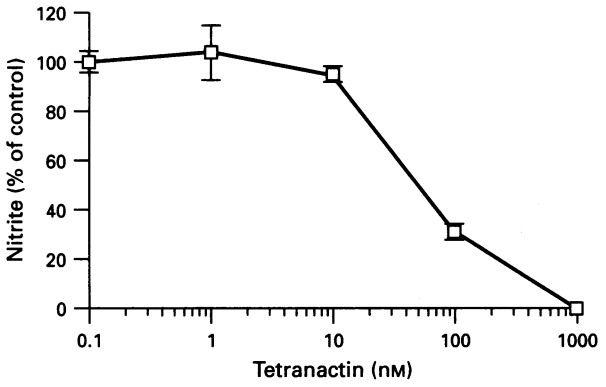

Figure 1 Inhibition of IL-1β-stimulated nitrite formation in mesangial cells by tetranactin. Mesangial cells were incubated for 24 h with IL-1β (2 nM) in the presence of the indicated concentrations of tetranactin. Thereafter, the medium was removed and used for nitrite determination. Data are means ± s.d. (n = 4).

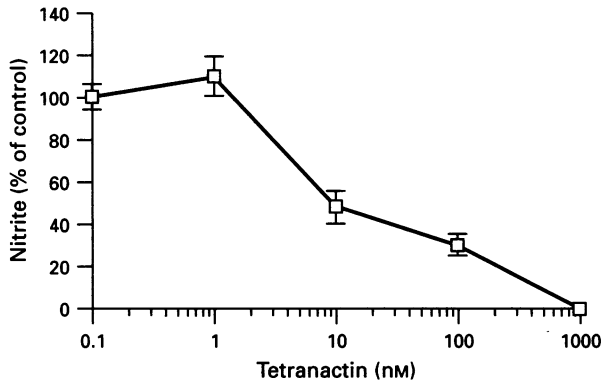

Figure 2 Inhibition of Bt_2 cyclic AMP-stimulated nitrite formation in mesangial cells by tetranactin. Mesangial cells were incubated for 24 h with Bt_2 cyclic AMP (10 mM) in the presence of the indicated concentrations of tetranactin. Thereafter, the medium was removed and used for nitrite determination. Data are means ± s.d. (n = 4).

IL-1β- and Bt_2 cyclic AMP-induced iNOS mRNA steady state levels suppressed by tetranactin

In order to assess whether the suppression of iNOS protein synthesis by tetranactin was associated with changes in the steady state mRNA levels coding for iNOS, Northern blot analyses were performed. Mesangial cells were stimulated with IL-1β or Bt_2 cyclic AMP in the presence of tetranactin for 24 h, thereafter total cellular RNA was isolated and subsequently used for Northern blot analysis. As shown in Figure 4, IL-1β induced iNOS mRNA steady state levels were suppressed by tetranactin in a dose-dependent manner and were completely abolished at a concentration of 300 nM tetranactin. Similar results were obtained for Bt_2 cyclic AMP-induced iNOS mRNA levels, as shown in Figure 5. At a concentration of 100 nM, tetranactin completely inhibited Bt_2 cyclic AMP-dependent formation of iNOS mRNA steady state levels.

Discussion

Cyclosporin A and FK 506 are potent immunosuppressive drugs used clinically for the prevention of graft rejection following organ transplantation. These drugs bind to intracellular receptor proteins, collectively termed immunophillins, that have peptidyl-prolyl *cis-trans* isomerase activity. The complex between drug and its cognate immunophilin inhibits the intracellular Ca^{2+}-dependent protein phosphatase, calcineurin. The activity of calcineurin is required for the activation and nuclear translocation of a subunit of nuclear factor of activated T-cells (NF-AT), known to be important in the transcriptional regulation of T-cell specific genes. Thereby, the drugs suppress Ca^{2+}-dependent T-cell responses, such as T-cell receptor mediated transcription of interleukin 2 (Schreiber & Crabtree, 1992). However, the therapeutic application of these drugs is limited by considerable side-effects, notably nephrotoxicity and neurotoxicity (Mason, 1989). Numerous reports on nephrotoxic activities of immunosuppressive drugs such as cyclosporin A suggest that these effects are due to its action on renal cells, thus interfering with important regulatory mechanisms in the kidney.

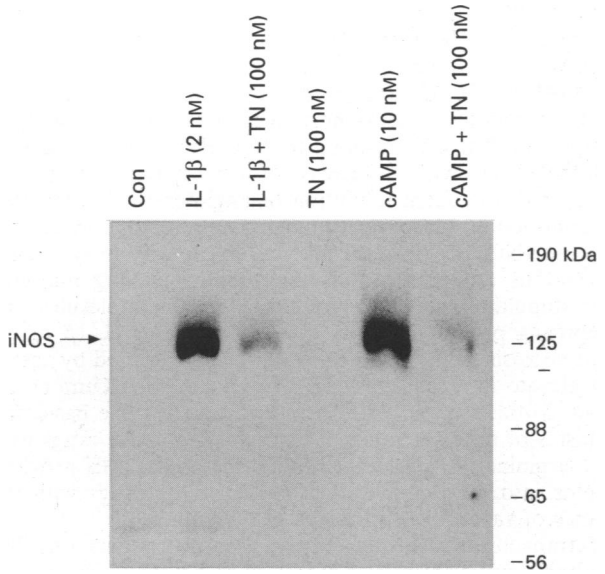

Figure 3 Inhibition of IL-1β- and Bt_2 cyclic AMP-stimulated iNOS protein expression in mesangial cells by tetranactin. Mesangial cells were incubated for 16 h with vehicle (control), IL-1β (2 nM), IL-1β plus tetranactin (100 nM), Bt_2 cyclic AMP (10 mM), Bt_2 cyclic AMP plus tetranactin (100 nM) and tetranactin (100 nM) alone as indicated. Samples were analyzed as described in the Methods section.

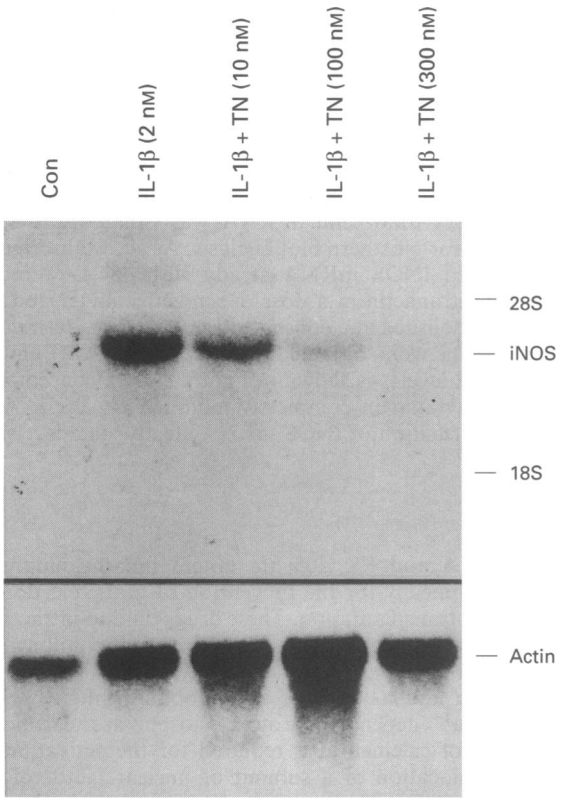

Figure 4 Inhibition of IL-1β-stimulated induction of iNOS mRNA in mesangial cells by tetranactin. Mesangial cells were incubated for 24 h with vehicle (control), IL-1β (2 nM), or IL-1β plus the indicated concentrations of tetranactin. Total RNA (20 μg) was successively hybridized to ^{32}P-labelled iNOS and β-actin cDNA probes as described in the Methods section.

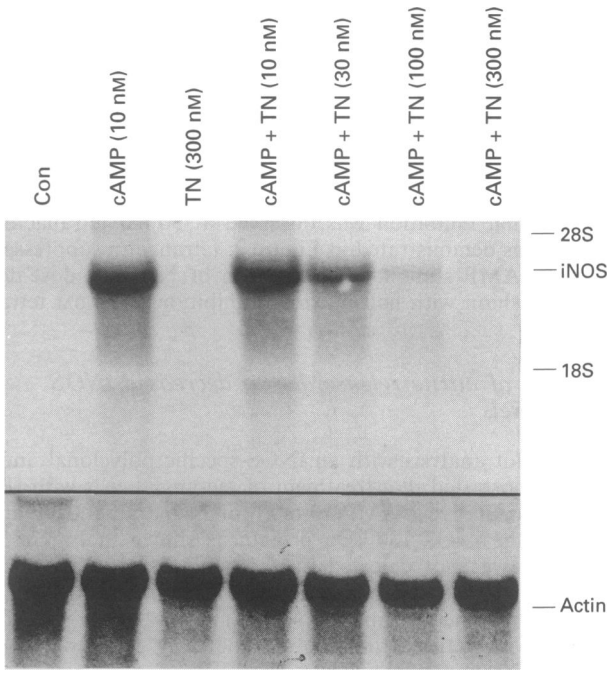

Figure 5 Inhibition of Bt_2 cyclic AMP-stimulated induction of iNOS mRNA in mesangial cells by tetranactin. Mesangial cells were incubated for 24 h with vehicle (control), Bt_2 cyclic AMP (10 mM), tetranactin (300 nM) or Bt_2 cyclic AMP plus the indicated concentrations of tetranactin. Total RNA (20 μg) was successively hybridized to ^{32}P-labelled iNOS and β-actin cDNA probes as described in the Methods section.

Among the cell types present in the glomerulus, mesangial cells are being increasingly recognized as a major determinant in the regulation of glomerular filtration rate and blood flow. Furthermore, recent reports have made it obvious that the L-arginine-NO pathway plays an important role in the cross-communication between glomerular cells and thus helps to preserve structure and function of the glomerulus (Pfeilschifter, 1994; 1995). Mesangial cells are able to contract upon stimulation with vasoactive hormones such as angiotensin II or vasopressin. The cells respond to NO released by glomerular endothelial cells with an increase of intracellular cyclic GMP-levels and subsequent inhibition of angiotensin II-stimulated cell-contraction. Moreover, mesangial cells do not only act as a target for NO, but have themselves the capacity to synthesize tremendous amounts of this important signalling molecule upon stimulation with two groups of signalling molecules, one comprising proinflammatory cytokines such as IL-1β or tumour necrosis factor α and the other being triggered by agents that elevate intracellular levels of cyclic AMP (Kunz *et al.*, 1994a; Mühl *et al.*, 1994). Therefore, to study the basic mechanisms of the interaction of immunosuppressive drugs with the L-arginine-NO pathway in renal mesangial cells provides insights into the possible interference of the drugs with the balance of vasoactive substances in the kidney.

Tetranactin is a member of the group of polynactins, hydrophobic cyclic antibiotics produced by *Streptomyces aureus*. The chemical structure is similar to that of the cyclic polypeptide, cyclosporin A, suggesting that tetranactin may have comparable properties. Strong immunosuppressive activity was described for tetranactin (Callewaert *et al.*, 1988; Teunissen *et al.*, 1992). The authors demonstrated that tetranactin, similar to cyclosporin A and with a comparable potency, blocked the initiation of interleukin 2-dependent proliferation of human T-lymphocytes as well as the generation of cytotoxic T lymphocytes and activated killer cells in mixed lymphocyte cultures.

We show here that tetranactin inhibits the IL-1β- and cyclic AMP-dependent induction of iNOS expression in mesangial cells. Stimulation of the cells with IL-1β or Bt_2 cyclic AMP strongly induces iNOS activity and the production of NO, as determined by measuring the amount of nitrite, the stable end product of NO formation, in culture-supernatants. The IC_{50} values for inhibition are in the nanomolar range suggesting that tetranactin is highly potent in exerting its inhibitory effects in mesangial cells. The reduction of nitrite release is due to decreased levels of iNOS protein as shown by Western blot analysis. Northern blot analysis strongly suggests that the observed reduction of iNOS protein levels is the consequence of decreased steady state levels of iNOS mRNA. Whether this phenomenon is caused by a decreased transcriptional activity of the iNOS gene or a decreased stability of iNOS mRNA or a combination of both remains to be elucidated. The most widely studied compounds used for inhibition of iNOS activity are undoubtedly the guanidino mono- or di-substituted derivatives of L-arginine like N^G-monomethyl-L-arginine (L-NMMA) and N^G-nitro-L-arginine (L-NOARG) and its methyl ester (L-NAME). The analogues of L-arginine like L-NMMA are potent inhibitors of NO synthesis by all informs of NOS. Such a non-specific inhibitor may be used in treating patients with septic shock; however, for treating inflammatory diseases the use of L-NMMA and related compounds is limited by their actions on eNOS and the resulting hypertensive side effects. Corbett *et al.*, (1992) reported that aminoguanidine is equipotent to L-NMMA as an inhibitor of cytokine-stimulated NO production in an insulinoma cell line, but has only mild effects on mean arterial blood pressure. These data suggest that aminoguanidine is a selective inhibitor of iNOS that can be used in diseases characterized by pathological NO formation by iNOS (Misko *et al.*, 1993; Griffith *et al.*, 1993). Potent and selective inhibition of human iNOS with a 190 fold selectivity

versus eNOS has been reported for certain isothiourea derivatives (Garvey *et al.*, 1994). These compounds show beneficial effects and improved survival in rodent models of septic shock (Szabo *et al.*, 1994). An alternative approach for the treatment of NO overproduction is to interfere with the induction process of the high output iNOS. This will result in a very specific inhibition of NO from iNOS leaving NO production by the constitutive enzymes untouched. So far few substances have been identified that inhibit the expression of iNOS. Glibenclamide, an inhibitor of ATP-sensitive potassium channels, has been reported to block iNOS expression in cultured J774.2 macrophages and in the anaesthetized rat (Wu *et al.*, 1995). Similarly, N-acetyl-5-hydroxytryptamine was shown to inhibit iNOS expression in RAW 264.7 macrophages and LPS-treated rats *in vivo* (Klemm *et al.*, 1995) and tricyclodecan-9-yl-xanthogenate (D609), an inhibitor of phosphatidylcholine-specific phospholipase C suppressed LPS and interferon-γ-stimulated nitrite production in J774A.1 macrophages (Tschaikowsky *et al.*, 1994). However, all these compounds require micromolar concentrations to result in half-maximal inhibition of NO production. In contrast, tetranactin blocks IL-1β- and cyclic AMP-induced expression of iNOS with IC_{50}s in the low nanomolar range comparable to the potency of glucocorticoids like dexamethasone (Pfeilschifter, 1991; Kunz *et al.*, 1996). It will be a fascinating task to unravel the signalling cascades affected by tetranactin and to pinpoint in more detail the level of interaction with the induction of iNOS in mesangial cells. Moreover, it is of importance to test the effects of this substance under conditions of increased iNOS expression in *in vivo* studies.

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