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Nitric oxide production by human proximal tubular cells: A novel immunomodulatory mechanism?

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Nitric oxide production by human proximal tubular cells: A novel immunomodulatory mechanism? It is believed that human proximal tubular cells may possess immunological function and play an important role in a variety of renal disease states such as interstitial nephritis, allograft rejection and drug induced nephrotoxicity. The role of cytokines and nitric oxide in the human forms of these disease states is not clear. In this study we examined the effect of stimulation with the cytokines IL-1β, TNF-α and IFN-γ, individually and in combination, upon primary cultures of human proximal tubular cells. Nitric oxide production increased significantly within 24 hours following cytokine stimulation. This response was inhibited, in a dose dependent manner, by L-NMMA. PCR amplification of mRNA extracted from control and cytokine stimulated human proximal tubular cells revealed a NOS product with a >97% homology with human hepatocyte inducible nitric oxide synthase. The results of this study clearly show that human proximal tubular cells, in primary culture, are capable of producing nitric oxide in response to an immune challenge secondary to the induction of nitric oxide synthase.

The primary functions of proximal tubular epithelial cells (PTC) are the transport of electrolytes and water, together with the recovery of carbohydrate and proteins from the glomerular filtrate [1]. However, human proximal tubular (HPT) cells may exhibit immunological functions including processing and presentation of foreign antigen and production of cytokines [2]. The importance of these immunological actions of HPT cells are uncertain but are likely to be involved in diseases such as interstitial nephritis, allograft rejection, pyelonephritis and drug induced nephrotoxicity [3] where the HPT cell is a target for cytotoxic injury. Furthermore, HPT cells may play an active role in inflammatory diseases (as do mesangial cells [4]) and may possess other immunomodulatory functions. The recent discovery that nitric oxide (NO) has immunomodulatory and cytotoxic actions prompted us to examine the possible role of NO in the HPT cell.

NO was initially identified as a vasodilator (endothelium-derived relaxing factor, EDRF) [5] but has since been shown to act as a neurotransmitter, to possess immunomodulatory and cytotoxic effects [6–8] and to mediate tissue damage [8]. These cytotoxic actions of NO are mediated by high local concentrations of NO, produced by induction of a cytokine dependent, calcium independent enzyme-inducible NO synthase (iNOS) [9]. This contrasts with the vascular actions of NO which are mediated by low concentrations of NO produced by a calcium-dependent, constitutively expressed form of the enzyme [6, 9]. In macrophages induction of NO production has diverse actions, including killing of parasites and tumor cells [8, 10, 11], destruction of pancreatic islets in experimental streptozotcin-induced diabetes [8, 12, 13], and tissue injury in immune complex-mediated vasculitis [14]. Furthermore, in the experimental model of diabetes, inhibition of NO production attenuates the progression of disease [8, 12, 13]. Recent evidence suggests that NO is involved in the pathogenesis of glomerulonephritis (GN) [15–17]. Urinary nitrite excretion (a marker of NO production) is increased [14] in animal models of GN, glomeruli isolated from animals with experimental nephritis produce more nitrite [16], and isolated glomerular mesangial cells and infiltrating macrophages can produce NO in response to inflammatory cytokines [10, 17–20]. Thus there is substantial evidence that NO production is increased in GN, although the contribution to tissue damage remains to be established [17].

Since HPT cells are a target for immune mediated disease and possess immunomodulatory functions [3, 4], we investigated the ability of HPT cells in culture to produce NO. The aims of this study were: (i) to determine whether HPT cells can produce NO in response to cytokine stimulation, (ii) to identify the regulatory influences of cytokines implicated in inflammatory disease upon NO production, and (iii) to determine whether the inducible NO synthase gene is expressed by these cells when stimulated by cytokines.

Methods

Human proximal tubular cell preparation

Human proximal tubular cells (HPT) were prepared from the normal pole of human tumor nephrectomy specimens as previously described [21]. Briefly, 10 g of cortex was removed and minced in Hanks 1 buffer to remove remaining blood. Cortical fragments were then digested at 37°C for 60 minutes in Hanks II buffer containing 0.2% collagenase A (Sigma, UK). Following digestion the cell suspension was filtered through a 75 μm filter and the suspension pelleted and washed in Hanks III buffer before resuspension in Percoll buffer solution. This suspension was then centrifuged at 4°C for 30 minutes at 13,000 r.p.m. (20,000 g).

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Following centrifugation the cell pellet was resuspended in ice-cold complete Dulbecco's modified Eagle's medium/Ham's F12 nutritional supplement (1:1, DMEM/HF12; Sigma UK). Cells were then plated onto 24 well plates at a density of 1×10^5 cells per well and incubated at 37°C (in 95% O_2, 5% CO_2) until confluence was achieved (approximately 7 days). Once confluent the cells were incubated for 24 hours [20] with the cytokines IL-1β (10 μ/ml), TNF-α (10 ng/ml), IFN-γ (100 μ/ml), PDGF (1, 10, 100 ng/ml), TGF-β (0.1, 1, 10 pg/ml) (all Boehringer, UK), IL-4 (0.1, 1, 10 IU/ml, Life Technologies, UK). Cytokines were used alone or in combination, and cytokines with potentially inhibitory effects (IL-4, TGF-β and PDGF) were studied in cells stimulated by a "cocktail" of IL-1β (10 μ/ml), TNF-α (10 ng/ml) and IFN-γ (100 μ/ml). Media was then removed for estimation of nitrite production. To demonstrate the presence of inducible nitric oxide synthetase cells were also incubated with the three cytokine "cocktail" in the presence of L-NMMA (Sigma; 1, 10, 100, 1000 μmol/liter) and nitrite production determined in the supernatant.

Nitric oxide production

NO production was determined by measurement of nitrite (as an end product of NO metabolism [5, 9]) in the culture supernatant using the Greiss reagent [5, 20]. Nitrite was determined by absorbance at 570 nm in a multichannel spectrophotometer (Titertek Multiscan MC, Titertek UK) and is expressed as nmoles per million cells per 24 hours (mean ± SD).

Molecular biology of inducible nitric oxide synthase

Human proximal tubular cells were seeded into two 75 cm^2 culture flasks (Costar, UK) at a density of 4×10^6 per flask and allowed to grow to confluence. Cells were then dissociated from the tissue culture flasks with 0.1% trypsin/5 nM EDTA, washed in cold phosphate buffered saline (PBS) and transferred to a microfuge tube. RNA was extracted by standard methods (MANIATIS). Cells were lysed in Nonidet P-40 (0.5% vol/vol) Tris-buffered saline at pH 8.6, with 20 mM vanadyl-ribonucleoside complexes (Sigma). Nuclei were removed by centrifugation (5 min at 150 g) and proteins digested with proteinase K (50 μg/ml, Boehringer Mannheim) in the presence of 2% SDS. Protein fragments were extracted with phenol:chloroform, and RNA collected by precipitation of the aqueous phase in ice-cold isopropanol. The pellet was washed three times in 70% ethanol, air dried and resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.6). Nucleic acid purity was measured by spectroscopy at 260 and 280 nM. RNA was then reverse transcribed by incubating the RNA template with four deoxynucleotides (Pharmacia; at concentrations of 500 μM), 500 ng/vol oligo dT, and 200 units of Superscript reverse transcriptase (Life Technologies, UK) in the supplied buffer (50 mM Tris-HCl, pH 8.3), 75 mM KCl and 3 mM $MgCl_2$ with 0.02 M dithiothreitol (Sigma). The reaction was incubated at 37°C for one hour. cDNA products were recovered by gel exclusion chromatography (Pharmacia nick columns; Pharmacia, UK) and phenol:chloroform extraction, followed by ethanol precipitation and resuspended in water.

The cDNA was amplified by the polymerase chain reaction (PCR) using degenerate primers for NOS and specific primers for β_2-microglobulin as a semi-quantitative control. For NOS, degenerate oligonucleotide primers were designed based on the known rat constitutive [22] and the mouse inducible [23] NOS cDNA sequences. The sequences derived were (degeneracies in brackets):

Upper: 5′ GG(CT) TGG TAC ATG (AG)GC AC(CT) GAG AT(CT) GG 3′

Lower: 5′ GTT (GC)A(AGC) CAT CTC CTG (AG)TG GAA (AGC) AC (AG)GG 3′

The position of the upper primer was from 1317-1342 and consisted of 26 base pairs, while the lower primer consisted of 27 base pairs at position 1652-1626. Using these primers the predicted size of the amplified cDNA product is 336 base pairs including primers or 283 bp excluding primers.

For β_2-microglobulin the following primers were used:

Upper: 5′ CCA TGA TGC TGC TTA CAT GTC TC 3′

Lower: 5′ CCT TGA GGC TAT CCA GCG TAC TCC 3′

(All primers were synthesized by Oswell DNA services, Edinburgh, UK).

PCR amplification was performed using the Perkin Elmer Cetus Gene Amp kit and a Perkin Elmer Cetus DNA thermal cycler. Twenty-five microliter reactions containing reverse transcribed mRNA, nucleotides; 200 μM each dNTP, 1 unit of Taq DNA polymerase and primers (at 0.2 μM each primer) in amplification buffer were prepared in "Geneamp" reaction tubes. The samples were amplified through an amplification profile of 1 cycle at 94°C for two minutes, 55°C for one minute, and 72°C for 90 seconds. This was followed by 33 cycles of 94°C for 50 seconds, 55°C for 60 seconds, 72°C for 90 seconds. A final cycle consisted of 94°C for 50 seconds, 55°C for 60 seconds and 72°C for five minutes. The reaction products were separated by gel electrophoresis and stained in ethidium bromide. Using these degenerate primers a 336 bp product was amplified and sequenced directly using an automatic sequencer (Applied Biosystems); the product sequence was then compared with sequences for human inducible hepatocyte NOS, mouse inducible NOS and rat constitutive NOS held within the Genbank database using the GCG programs through the facility at the UK human genome mapping project.

Statistics

Results are expressed as mean ± SD. Comparisons are by two sample Student's t-test, with the Bonferroni correction for multiple comparisons where appropriate.

Results

Cell culture

Cultured HPT cells grew in monolayers to confluence over a period of approximately seven days and exhibited the typical morphology and dome formation characteristic of proximal tubular cells, except for a decrease in the density of brush border villi. We have previously demonstrated that such preparations of HPT cells maintain the sodium-dependent glucose, anion and cation transport mechanisms [21]. Human proximal tubular cells were also characterized according to their cAMP response to added PTH (10^{-6} M), vasopressin (10^{-6} M) and calcitonin (10^{-6} M). Only PTH stimulated the HPT cells to produce cAMP. Neither vasopressin nor calcitonin had any significant effect upon cAMP

Table 1. Enzyme and histochemical staining of confluent cultures of human proximal tubular cells and renal tissue sections

APAAP stain	Proximal tubular cells	Kidney sections
Acid phosphatase	Positive	Proximal tubule
Leucine amino peptidase	Positive	Proximal tubule
Glucose 3 phosphate dehydrogenase	Negative	Distal tubule
CALLA	Positive	Proximal tubule
vWF	Negative	Vascular endothelium
Thy 11	Negative	Mesangial cells, interstitial cells, fibroblasts
EMA	Negative	Glomeruli, Distal tubule

Abbreviations are: CALLA, common ALL antigen; vWF, von Willebrand's factor; Thy 11, cortical thymocyte; EMA, epithelial membrane antigen.

production when incubated with these HPT cells. Following incubation with PTH cAMP levels increased from a baseline value of 2.1 ± 1.3 to 8.3 ± 2.4 pmol/mg protein/hr ($P < 0.01$, $N = 6$) and the response was maintained for up to seven days [24]. Immunohistochemical and enzyme histochemical staining of the cells forming the confluent monolayers revealed a similar pattern, in close agreement, to the staining pattern found on fresh human kidney tissue sections prepared at the same time as the HPT cell isolation (Table 1).

Nitrite production

Incubation of human proximal tubular cells with individual cytokines IL-1β, TNF-α, or IFN-γ had no effect on nitrite production when compared to unstimulated cells (Fig. 1). Combinations IL-1β and TNF-α or IFN-γ and TNF-α were also ineffective in stimulating the production of NO. Prolongation of the stimulation for up to 48 hours also had no effect (data not shown). However, the combination of IL-1β and IFN-γ produced a significant rise in NO production from 3.7 ± 0.54 to 39.12 ± 9.7 nmol/million cells/24 hr ($P < 0.001$, $N = 6$). The combination of all three cytokines was more effective in stimulating NO production than this combination (49.04 ± 11.67 nmol/million cells/24 hr, $P < 0.001$).

Inhibition of nitrite production

Incubation of human proximal tubular cells with the three cytokine cocktail in the presence of incremental concentrations of a specific inhibitor of NO synthase-L-NMMA (1 to 1000 μmol/liter) produced a dose-dependent reduction in NO production. Nitrite production fell progressively from 44.8 ± 2.2 (cytokine cocktail) to 4.2 ± 2.1 (1000 μM L-NMMA, $P < 0.001$, $N = 6$) approaching control levels at this dose (Figs. 2 and 3).

Co-incubation of cytokine stimulated cells with increasing doses of IL-4, PDGF and TGF-β also inhibited nitrite production. IL-4 decreased nitrite production progressively from 49.0 ± 11.7 (cytokine cocktail) to 29.2 ± 11.2 (100 IU/ml, $P < 0.001$, $N = 6$).

Treatment with PDGF decreased nitrite production from 53.6 ± 8.1 to 22.6 ± 10.1 (100 ng/ml, $P < 0.001$, $N = 6$). TGF-β decreased nitrite to 30.3 ± 4.1 (10 pg/ml, $P < 0.001$, $N = 6$).

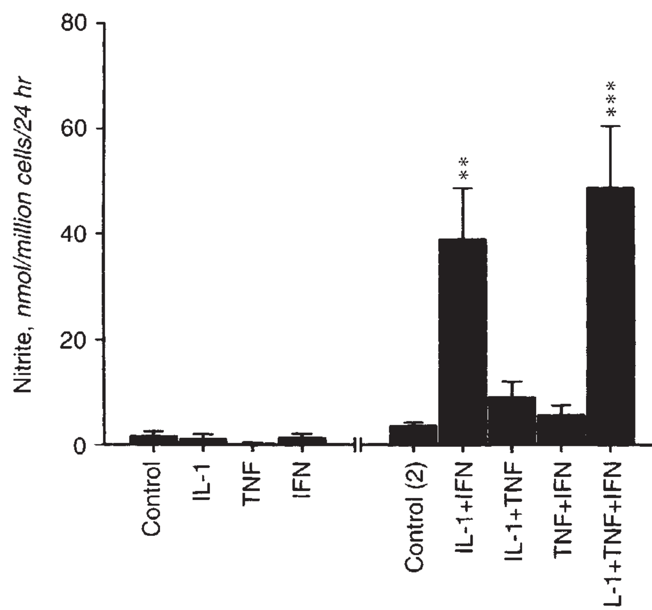

Fig. 1. *Stimulation of nitrite (nitric oxide) production by the individual cytokines IL-1β, TNF-α and IFN-γ and combinations of cytokines IL-1β and TNF-α, IL-1β and IFN-γ, IFN-γ and TNF-α; and a "cocktail" of IL-1β, TNF-α and IFN-γ.*

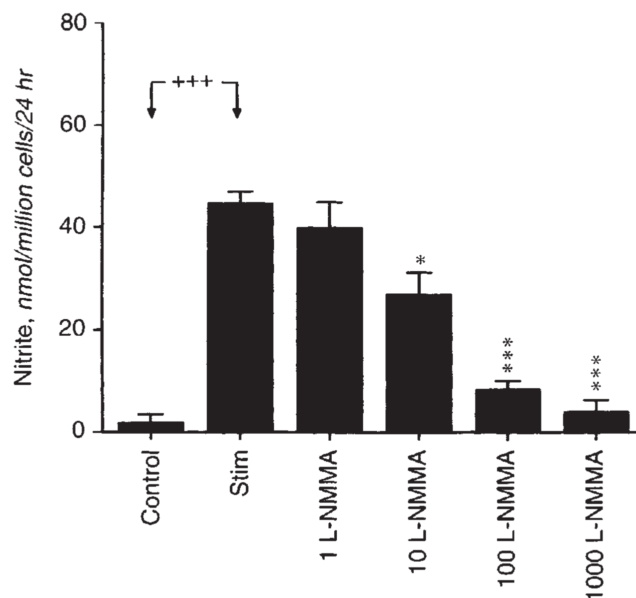

Fig. 2. *The inhibition of cytokine stimulated nitrite production by L-NMMA.*

PCR results

The results of amplification of mRNA extracted from control and cytokine stimulated PCT are shown in Figure 4. There was no change in the expression of the control (β_2-microglobulin) mRNA but a 336 bp product was detected only in stimulated cells using the degenerate primers based on NO synthase. Sequence analysis of the NOS product obtained (Fig. 5) revealed more than 97%

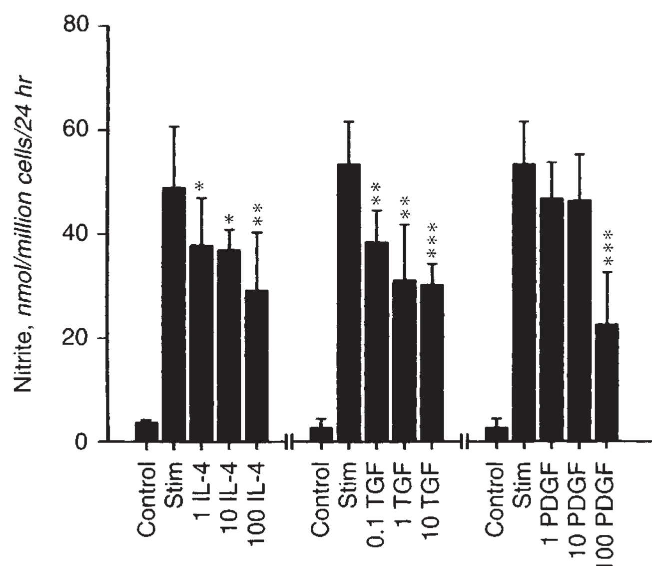

Fig. 3. *Inhibition of cytokine stimulated nitrite production by IL-4, PDGF and TGF-β.*

homology with the recently published sequence for human hepatocyte inducible NOS, corresponding to positions 1317-1652 of human hepatocyte iNOS cDNA [24]. There was less (78%) homology with the human brain constitutive NOS sequence [25], the mouse inducible NOS (88%) and rat constitutive (83%) NOS held within the Genebank database.

Discussion

This study demonstrates that human proximal tubular cells in culture can be reproducibly induced to secrete nitric oxide. Production of NO is regulated by cytokines involved in immune mediated disease and is inhibited by the NO synthase antagonist L-NMMA, the pattern of regulation being similar to that reported in other human cell lines. In addition, using RT-PCR, we have shown that cytokines stimulate nitrite production by induction of iNOS mRNA [26, 27].

Induction of NOS

Single cytokines had no effect on the production of nitrite; combinations of two of TNF-α, IL-1β or IFN-γ were also without effect with the exception of the combination of IL-1β and IFN-γ; the use of all three cytokines produced a consistent (Fig. 1 to 3), twentyfold increase in nitrite production. This pattern of stimulation is similar to that which we have previously found for human mesangial cells in culture [20], and human hepatocytes [25]. Non-human cell lines appear to be less stringent in their stimulatory requirements [10, 11, 18, 19]. For example, rodent macrophages require only IFN-γ to produce large amounts of NO [10, 11, 28]. Renal cell lines have also been studied; rat mesangial cells can be induced to produce NO in response to IFN-γ alone [18, 19] whereas tubular epithelial cells require a combination of high doses of TNF-α and IFN-γ (150 U/ml and 500 U/ml, respectively) for reproducible stimulation. The most striking differences appear to be between macrophages: those of rat origin are easily induced [10, 11, 28], whereas there are several reports of the inability of human macrophages to produce NO [29]. The reasons for these differences are not clear. It is possible that there are true differences in the regulation of inducible NOS between cell types within species and certainly between species. However, an alternative hypothesis is that several stimulatory influences may be required in all cells and that the differences may lie in the ability of some cells to produce additional cytokines which then act in an autocrine manner. For example, rodent macrophages may produce TNF-α and IL-1 in response to IFN-γ [30]. In the present study the relatively minor effect of addition of TNF-α to IL-1β and IFN-γ, may reflect the ability of HPT cells to produce TNF-α when stimulated by IL-1β [31]. More detailed studies on the inducible NOS gene expression will be required to address the regulation of iNOS. *In vivo*, and particularly in inflammatory conditions, it is unlikely that PTC would be exposed to single cytokines in isolation. All three of the cytokines which we have found necessary for NOS induction will be produced by infiltrating lymphocytes, polymorphs and macrophages at sites of inflammation [30, 32] and, despite the requirements for multiple cytokines, induction of NOS in HPT cells is likely to be of pathophysiological relevance.

Nitrite is a stable end-product of NO metabolism [5] and therefore provides a useful marker for NO production in the absence of a generally available direct assay. The ability of L-NMMA, a specific NO synthase antagonist, to inhibit nitrite generation indicates that nitrite is produced by NO synthase; this finding and the dose-response relationship are consistent with findings for inducible forms of NO synthase in other tissues [9].

Having established that NO synthesis can be induced in PTC we sought to identify potentially inhibitory influences. TGF-β, PDGF and IL-4 have established inhibitory actions on inducible NOS in other tissues [28, 33–36] and are involved in the pathogenesis of inflammatory diseases including glomerulonephritis [4, 37, 38] and allograft rejection [39]. PDGF inhibits NO synthesis by rat mesangial cells [33], TGF-β and IL-4 both have potent inhibitory actions on the production of NO by macrophages [28, 34, 35]. The ability of all three to inhibit nitrite generation in HPT cells suggests that inducible NOS is regulated in a similar manner to other cell types.

At the time when this study was conceived there were no published data on the sequence of inducible forms of human NOS. We therefore designed degenerate oligonucleotide primers based on knowledge of the conserved areas in mouse macrophage inducible [23] and rat brain constitutive forms of NO synthase [22], based on the assumption that human forms of the enzyme would be likely to share similarities. The sequence for hepatocyte inducible NOS has since been published [25], and our degenerate primers share homology with the equivalent areas of mRNA for this enzyme. Although the polymerase chain reaction may be criticized on the basis that it is difficult to quantify, it is clear from our data that only in cells stimulated with the three cytokine "cocktail" reveal a band (Fig. 4). Thus, it is apparent that the gene is "switched on" in response to cytokine stimulation.

The PCR product was sequenced using an automated analyzer and fluorescine end labeling [26]. The product obtained shows high homology with the inducible mouse enzyme and near identity with the human hepatocyte inducible NOS. Therefore, although the primers used may have detected either form of the enzyme, it is apparent that only the inducible form is amplified in stimulated cells.

NO produced by proximal tubular cells may have several

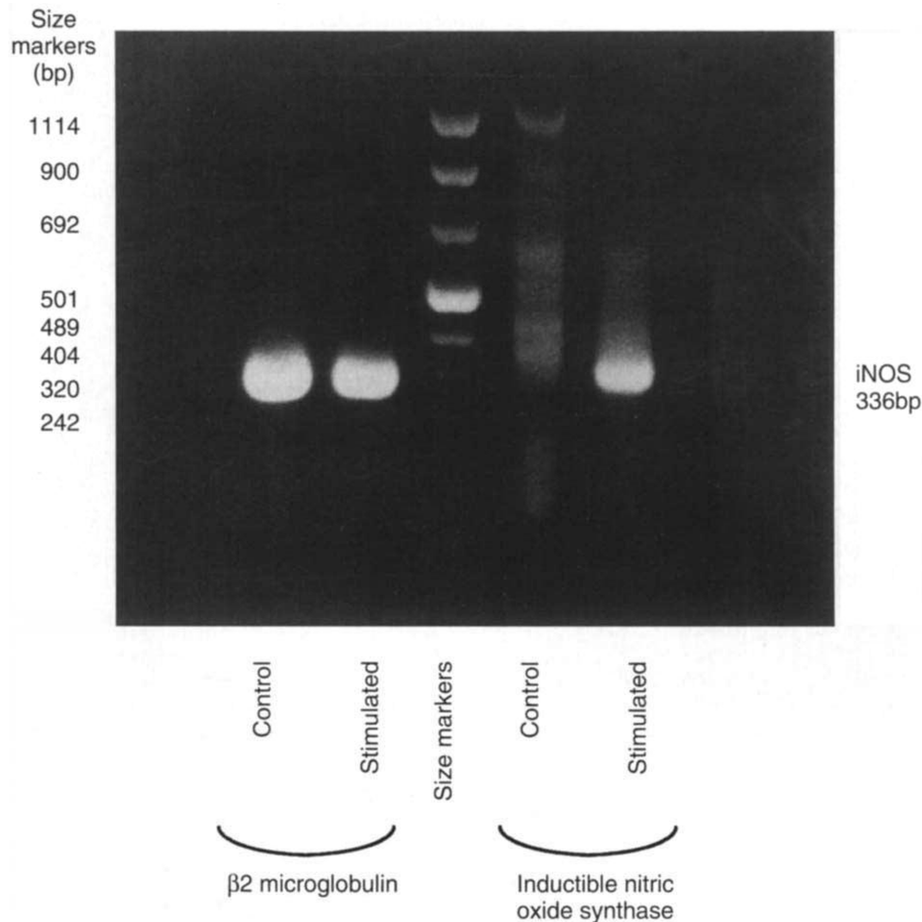

Fig. 4. *Ethidium bromide gel of the PCR product obtained in stimulated and control cells.*

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HEP iNOS 1301 AGGGTGCCCC TTCAATGGCT GGTACATGGG CACAGAGATC GGAGTCCGGG 1350
PT  iNOS                                                   AGTCCGGG

HEP iNOS 1351 ACTTCTGTGA CGTCCAGCGC TACAACATCC TGGAGGAAGT GGGCAGGAGA 1400
PT  iNOS      ACTTCTGTGA CGTCCAGCGC TACAACATCC TGGAGGAAGT GGGCAGGAGA

HEP iNOS 1401 ATGGGCCTGG AAACGCACAA GCTGGCCTCG CTCTGGAAAG ACCAGGCTGT 1450
PT  iNOS      ATGGGCCTGG AAACGCACAA GCTGNCCTCG CTCTGGAAAG ACCAGGCTGT

HEP iNOS 1451 CGTTGAGATC AACATTGCTG TGATCCATAG TTTTCAGAAG CAGAATGTGA 1500
PT  iNOS      CGTTGAGATC AACATTGCTG TGCTCCATAG TTTCCAGAAG CAGAATGTGA

HEP iNOS 1501 CCATCATGGA CCACCACTCG GCTGCAGAAT CCTTCATGAA GTACATGCAG 1550
PT  iNOS      CCATCATGGA CCACCACTCG GCTGCAGAAT CCTTCATGAA GTACATGCAG

HEP iNOS 1551 AATGAATACC GGTCCCGTGG GGGCTGCCCG GCAGACTGGA TTTGGCTGGT 1600
PT  iNOS      AATGAATACC GGTCCCGTGG GGNCTNCCCG GCAGACTGGA NTTGGCTGGT

HEP iNOS 1601 CCCTCCCATG TCTGGGAGCA TCACCCCCGT GTTTCACCAG GAGATGCTGA 1650
PT  iNOS      CCCTCCCATG TCTGGGAGCA TCACC

HEP iNOS 1651 ACTACGTCCT GTCCCCTTTC TACTACTATC AGGTAGAGGC CTGGAAAACC 1700
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Fig. 5. *Nucleotide sequence of human hepatocyte iNOS and human proximal tubule PCR iNOS product showing the region of homology.* Underlined bases indicate the primer sites. The human proximal tubule iNOS shows 98.5% homology with human hepatocyte iNOS.

potentially conflicting actions. It is likely to have vasodilator effects on adjacent vessels thus increasing blood flow and the delivery of infiltrating cells to the tubular interstitium. *In vitro* and in the rejecting allograft it has been suggested that NO may limit the proliferation of infiltrating T lymphocytes [39, 40]. NO and other nitrosovasodilators are known to limit proliferation of other cell types [41]. Consistent with the antiproliferative actions of NO is the observation that inhibition of NOS in experimental allograft

rejection increases the cellular infiltrate and the severity of the rejection process [7, 39, 40]. It is possible that NO produced by PTC, and other cells which are the target for immune-mediated injury, acts to limit the proliferation of infiltrating cells. The findings that PDGF, TGF-β and IL-4 reduce NO production is also consistent with this hypothesis. In inflammatory states TGF-β and PDGF are likely to be derived from infiltrating cells and platelets [37, 38]; IL-4 is produced by T lymphocytes of the Th-2 subtype [32]. In inflammatory conditions these cytokines might act to inhibit NO production by target cells and therefore permit increased proliferation of infiltrating T cells. The other potential action of NO is cytotoxic. There is abundant evidence that NO production by macrophages is involved in destruction of parasites and tissue [7, 8, 10–12] in immune-mediated disease. There is also evidence that NO may cause auto-destruction of cells [42], and therefore induction of NO synthesis in PTC may contribute to damage to these and other renal cell types [17]. Inhibition of NO synthesis may therefore have potentially deleterious or beneficial effects in inflammatory conditions involving the HPT cell; future studies will be required to determine the role of NO and of pharmacological NOS inhibition.

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