

Different induction mechanisms of mRNA for inducible nitric oxide synthase in rat smooth muscle cells in culture and in aortic strips

Allan Sirsjö^{a,*}, Peter Söderkvist^b, Tommy Sundqvist^c, Maria Carlsson^a, Martin Öst^a, Anders Gidlöf^a

^aClinical Research Center, ^bDepartment of Occupational Medicine, ^cDepartment of Medical Microbiology, Faculty of Health Sciences, University of Linköping, S-581 85 Linköping, Sweden

Received 3 December 1993; revised version received 17 December 1993

Abstract

The expression of mRNA for the inducible form of nitric oxide synthase, (iNOS), was studied in rat aortic smooth muscle cells, (SMCs) in cell culture and in strips of rat aorta by reverse transcriptase coupled to the polymerase chain reaction. iNOS mRNA expression was weak in cultured SMCs when exposed to either interferon- γ (IFN γ) or lipopolysaccharide (LPS), but the combination LPS + IFN γ enhanced the expression. In aortic strips LPS alone induced a pronounced expression, with no further increase by IFN γ . Cycloheximide potentiated the expression of iNOS mRNA in SMCs in culture stimulated with LPS + IFN γ , but attenuated the response in aortic strips. The results indicate different cellular signaling pathways for the induction of iNOS mRNA by LPS and/or IFN γ , in cultured SMCs and in rat aortic strips.

Key words: Nitric oxide; Lipopolysaccharide; Interferon- γ ; Cycloheximide; Smooth muscle cells

1. Introduction

During severe experimental and clinical septicemia the resistance vessels of the vascular bed paradoxically relax, showing a reduced sensitivity to vasoconstrictors, causing progressive hypoperfusion and irreversible shock. This occurs in about 35% of all septicemia patients in emergency care. Its cause has been suggested to be due to a sustained production of nitric oxide in the vascular resistance vessels [1–3]. Administration of L-arginine analogs that block nitric oxide synthesis, both experimentally, in dogs after treatment with tumor necrosis factor (TNF α) [4], in rats after *E. coli* lipopolysaccharide (LPS) [5], and clinically in man [3,6], block this vasodilatation and restore the vascular smooth muscle contractile response to vasopressors [7,8]. Thus, the inducible form of nitric oxide synthase, iNOS, is expressed in the cells of many organs and in the vascular wall after exposure to LPS or to various cytokines, as demonstrated in vitro in endothelial cells [9,10], macrophages [11,12], rat renal tubular cells [13] and human mesangial cells [14]. It has also been shown in vitro that vascular

smooth muscle cells (SMCs) from rat aorta [15] and rat pulmonary artery [16] produce NO after exposure to cytokines and it was thus inferred that cytokines induce iNOS gene expression in vascular smooth muscle cells generally in resistance vessels [8].

The inducible form of NOS has recently been cloned from murine macrophages [17–19], rat vascular SMCs [20] and human hepatocytes [21] and has been identified as an approximately 4.4-kilobase mRNA on Northern blot analysis.

However, mature SMCs in the aortic wall and proliferating SMCs in culture express different phenotypes, contractile vs. synthetic [22]. The latter phenotype expresses growth factors that could possibly modulate the iNOS induction by LPS and cytokines [23–26]. Thus, results from studies of iNOS gene expression by cytokines in the synthetic phenotype may not be identical to the iNOS expression by cytokines in mature, non-cycling SMCs in the vasculature during clinical septicemia.

To investigate this we have studied iNOS mRNA expression in rat aortic SMCs in cell culture and in strips of rat aorta, in vitro, after exposure to either LPS or interferon- γ (IFN γ) or to both.

Our results disclose a difference in iNOS induction in synthetic, proliferating rat SMCs in culture and in mature smooth muscle cells in the rat aortic wall after exposure to cytokines and endotoxin in vitro. Thus, results from investigations of cultured SMCs in vitro may not simply be extrapolated to the clinical situation.

* Corresponding author. Fax: (46) (13) 12 74 65.

Abbreviations: iNOS, inducible nitric oxide synthase; SMC, smooth muscle cell; RT, reversed transcription; PCR, polymerase chain reaction; TNF, tumor necrosis factor; LPS, lipopolysaccharide.

2. Materials and methods

E. coli Lipopolysaccharide endotoxin, actinomycin D, cycloheximide, collagenase and ascorbic acid were all purchased from Sigma Chemicals (St Louis, MO, USA). Recombinant murine interferon- γ came from PharMingen (San Diego, CA, USA). Dexamethasone came from Calbiochem Corp (La Jolla, CA, USA), gentamycin sulphate, fungizone, fetal calf serum and L-glutamine were all purchased from Gibco Ltd (Renfrewshire, Scotland). Trypsin was purchased from Difco Lab (Detroit, MI, USA). All plastic labware was purchased from Costar Europe (Badhoevedorp, The Netherlands). Taq DNA polymerase came from Promega (Madison, WI, USA) and M-MLV reverse transcriptase from Gibco BRL (USA).

2.1. Preparation and culture of vascular smooth muscle cells and strips of aorta

The SMCs were prepared according to Thyberg et al. [27]. Aortas of adult Sprague–Dawley rats were stripped of adventitia, the endothelial cell layer of the intima was scraped off and the aorta was cut into small pieces and digested with 0.1% collagenase for one hour. The collagenase solution was exchanged for fresh collagenase and the aortic pieces were further digested for 16–20 h at 37°C. The digest was passed through a nylon filter. The cells were centrifuged and washed three times in complete medium and seeded out in 75 cm² flasks. The SMCs were grown in Ham's F-12 medium, supplemented with ascorbic acid (50 $\mu\text{g} \cdot \text{ml}^{-1}$), gentamycin sulphate (50 $\mu\text{g} \cdot \text{ml}^{-1}$), fungizone (2.5 $\mu\text{g} \cdot \text{ml}^{-1}$), L-glutamine (100 $\mu\text{g} \cdot \text{ml}^{-1}$) and 10% fetal calf serum. The flasks were kept at 37°C in an atmosphere of 5% CO₂ in humidified air (Forma Scientific Incubator).

Confluent cell cultures were treated with 0.25% trypsin in Ca²⁺- and Mg²⁺-free phosphate-buffered saline solutions containing 0.02% EDTA. The cells were washed three times with medium and seeded out in 100 mm² plates, in which they were cultured to confluence in Ham's F12 medium containing 10% calf serum in an atmosphere of 5% CO₂ in humidified air at 37°C. Cultures at passage 4 to 10 were used for the experiments.

Confluent cultures of SMCs were incubated for 12 h with medium containing 5% fetal calf serum at 37°C, before and during the treatment with the different agents, as given in the legends to figures.

Strips of aorta were prepared from excised aortic sections of male Sprague–Dawley rats, where the intima and the adventitia were stripped off by dissection. After excision, the aortic strips were incubated and treated the same as the cultured SMCs as described above.

Figs. 1–6 shows representative results from 3–5 independent experiments.

2.2. Preparation and isolation of RNA

Total RNA was prepared from SMCs and aortic strips using the guanidinium/phenol/chloroform method [28]. The RNA concentration and purity were estimated from the optical density at 260 nm and 280 nm (Perkin-Elmer, lambda 2 UV/Vis spectrophotometer).

2.3. Reversed transcription-polymerase chain reaction

Two μg of total RNA from each sample was reversely transcribed (RT) into cDNA by incubating with 1 μM oligo (dT)₁₈ primer, 3 mM MgCl₂, 400 U murine Moloney leukemia virus reverse transcriptase, 500 μM dNTP, 0.01 mM dithiothreitol, 75 mM KCl and 50 mM Tris-HCl pH 8.3 in a final volume of 20 μl at 37°C for 1 h.

The cDNA was amplified by the polymerase chain reaction (PCR) in a 40 μl reaction containing 100 ng of transcribed total RNA, 1.25 U of Taq DNA polymerase (Promega, Madison, WI, USA), 0.5 μM of primers and 1.5 mM MgCl₂ in 1 \times reaction buffer provided by the supplier (Promega). Amplification was performed in a Perkin-Elmer Thermal Cycler; with initial heating for 1 min at 94°C, followed by 25 cycles of 1 min at 94°C, 1 min at 60°C, 2 min at 72°C and a final extension for 7 min at 72°C.

Ten μl of the reaction mixture was mixed with loading buffer and separated by electrophoresis on 1.2% agarose gels containing ethidium bromide (0.5 $\mu\text{g}/\text{ml}$) and visualized by UV transillumination.

2.4. PCR-primers

cDNA from each sample was amplified with specific primers for iNOS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in

separate tubes. The upstream and downstream primers for iNOS were: 5'-CCCTTCCGAAGTTTCTGGCAGCAG-3' and 5'-GGGCTCC-TCCAAGGTGTTGCCC-3', respectively, and gave a single band corresponding to a 473 base pair fragment from position 2973 to 3446 in rat iNOS cDNA, [20].

The upstream and downstream primers for GAPDH were: 5'-AC-CACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGC-TGTA-3', respectively. These yielded a single PCR-product corresponding to a 452 base pair fragment from position 586–1068 in the rat GAPDH cDNA and the primers were purchased from Clontech Laboratories, Inc. (Palo Alto, CA, USA).

2.5. Determination of nitrite

Nitric oxide production was assessed measuring its stable end product, nitrite, in the culture medium using a modification of Bell's method [29]. This method utilizes the diazotization of sulfanilic acid by nitrite at an acidic pH and its coupling to *N*-(1-naftyl)-ethylendiamine. Media from SMC cultures exposed to the different treatments were incubated with 1.0 mM sulfanilic acid, 0.4 M HCl and 0.5 mM *N*-(1-naftyl)-ethylendiamine at room temperature for 30 min and the coloured reaction product was quantitated at 548 nm (Beckman DU 68, spectrophotometer).

3. Results

Amplification of cDNA from cultured SMCs with specific iNOS primers generated a faint PCR-product of the predicted size, 473 bp, after exposure for 8 h to IFN γ or LPS as illustrated in Fig. 1. However, exposure of SMCs for 8 h to a combination of IFN γ + LPS revealed a pronounced increase in iNOS mRNA (Fig. 1).

Amplification of the same reversely transcribed RNA with primers specific for GAPDH was used as a control for equal RT-PCR processing and loading of samples (Figs. 1 and 3–6). RNA samples that were not processed with reverse transcriptase gave no detectable PCR product (results not shown).

In order to verify iNOS induction, we measured its activity by determination of nitrite, NO₂⁻, the end metabolite of nitric oxides, in the culture media. As shown in Fig. 2, neither single exposure to IFN γ nor LPS alone

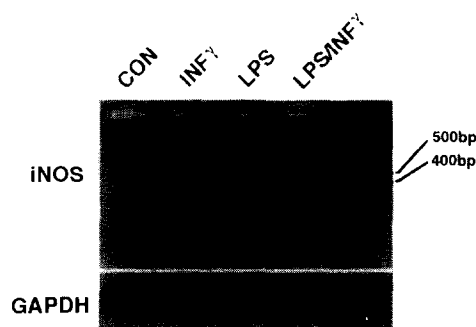


Fig. 1. Expression of iNOS mRNA in SMCs. Total RNA was isolated from SMCs and analyzed by the RT-PCR to measure the iNOS (upper panel) and GAPDH (lower panel) mRNA levels as described in Materials and Methods. An aliquot of the RT-PCR-amplified RNA from untreated cells (CON) or cells exposed for 8 h to 5 $\mu\text{g} \cdot \text{ml}^{-1}$ LPS (LPS), 300 U IFN $\gamma \cdot \text{ml}^{-1}$ medium (IFN γ) or 5 μg LPS + 300 U IFN $\gamma \cdot \text{ml}^{-1}$ medium (LPS/IFN γ) was separated on a 1.2% agarose gel and stained with ethidium bromide for comparison.

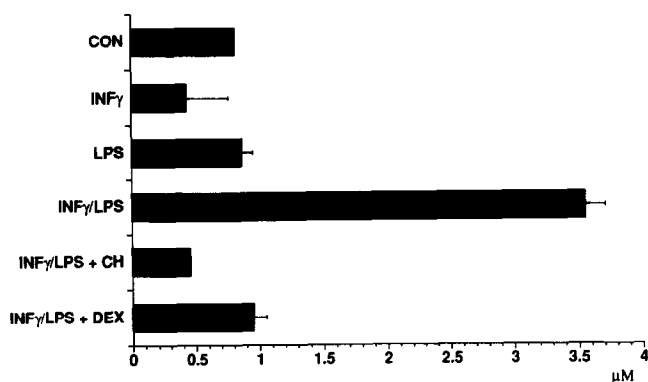


Fig. 2. Nitrite production by SMCs. Nitrite was measured as described in section 2 in medium from control cells (CON) and in medium where cells were exposed for 8 h to $5 \mu\text{g LPS} \cdot \text{ml}^{-1}$ medium (LPS), $300 \text{ U INF}_\gamma \cdot \text{ml}^{-1}$ medium (INF γ), $5 \mu\text{g LPS} + 300 \text{ U INF}_\gamma \cdot \text{ml}^{-1}$ medium (LPS/INF γ), $5 \mu\text{g LPS} + 300 \text{ U INF}_\gamma \cdot \text{ml}^{-1}$ medium together with $50 \mu\text{M}$ cycloheximide (LPS/INF γ + CH) or $0.5 \mu\text{M}$ dexamethasone (LPS/INF γ + DEX). Mean \pm S.D., $n = 4$.

induced any detectable increase of nitrite in the culture media following 8 h of exposure. However, when the SMCs were incubated with a combination of LPS + INF γ , a significant increase of nitrite was found in the media (Fig. 2).

iNOS mRNA induction was seen as early as after 4 h exposure to LPS + INF γ , and the induction was further enhanced at 8, 16, 24 h and still remained high at 36 h (Fig. 3).

To assess the role of on-going protein synthesis for the induction of iNOS mRNA in cultured SMCs after exposure to LPS + INF γ , the cells were exposed to the protein synthesis inhibitor cycloheximide. The expression of mRNA for iNOS increased after addition of cycloheximide together with LPS + INF γ , compared to the combination LPS + INF γ alone (Fig. 4). Furthermore, cycloheximide completely inhibited the LPS + INF γ induced nitrite accumulation in the media (Fig. 2), con-

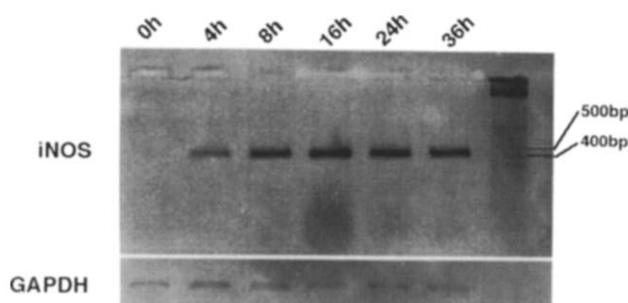


Fig. 3. Time course of induction of iNOS mRNA in SMCs. Total RNA was isolated from SMCs and analyzed by the RT-PCR to measure the iNOS (upper panel) and GAPDH (lower panel) mRNA levels as described in section 2. An aliquot of the RT-PCR-amplified RNA from cells treated with $5 \mu\text{g LPS} + 300 \text{ U INF}_\gamma \cdot \text{ml}^{-1}$ medium for 0, 4, 8, 16, 24, or 36 h was separated on a 1.2% agarose gel and stained with ethidium bromide for comparison.

firming the effectiveness of cycloheximide in blocking protein synthesis.

Addition of actinomycin D, an RNA synthesis inhibitor, to the combined inducer LPS + INF γ extinguished the induction entirely (Fig. 4). These findings suggest that the expression of iNOS in SMCs by LPS + INF γ is transcriptionally regulated.

Several reports in the literature have demonstrated the inhibition of NO synthesis by glucocorticoids [10,30]. The results shown in Fig. 4 demonstrate that dexamethasone decreased the LPS + INF γ induction of mRNA for iNOS and almost completely inhibited the accumulation of nitrite in the media (Fig. 2).

To mimic an in vivo situation, we exposed excised tissue strips of rat aorta in organ culture to INF γ or LPS alone or in combination. INF γ alone gave no induction of iNOS mRNA, but the combined exposure to LPS + INF γ induced a significant expression of iNOS mRNA by SMCs (Fig. 5). In contrast to cultured SMCs, LPS alone induced iNOS mRNA to approximately the same extent as the combined treatment with LPS + INF γ (Fig. 5).

The effects of cycloheximide on the induction of mRNA for iNOS by LPS and LPS + INF γ in the aortic tissue strips is shown in Fig. 6. Cycloheximide attenuated the induction both by LPS alone and by the combination of LPS + INF γ . This indicates that the induction of mRNA for iNOS in aortic tissue strips by LPS and by LPS + INF γ requires a certain de novo protein synthesis, not found necessary for the induction in cultured SMCs. In agreement with the results obtained with cultured SMCs, dexamethasone also decreased the induction of iNOS mRNA by LPS and LPS + INF γ in the aortic

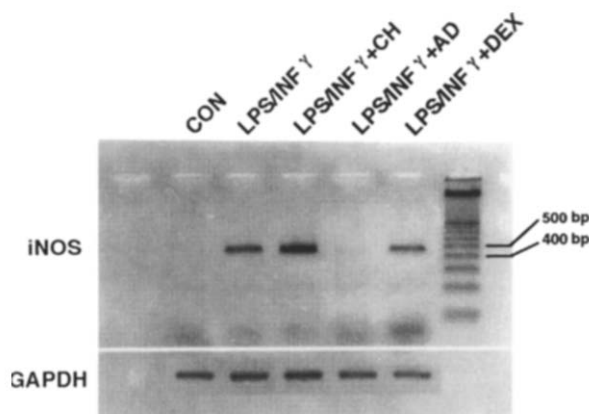


Fig. 4. Effects of cycloheximide, actinomycin D or dexamethasone on the expression of iNOS mRNA in SMCs. Total RNA was isolated from SMCs and analyzed by the RT-PCR to measure the iNOS (upper panel) and GAPDH (lower panel) mRNA levels as described in section 2. An aliquot of the RT-PCR-amplified RNA from untreated cells (CON) or cells exposed for 8 h to $5 \mu\text{g LPS} + 300 \text{ U INF}_\gamma \cdot \text{ml}^{-1}$ medium alone (LPS/INF γ) or together with $50 \mu\text{M}$ cycloheximide (LPS/INF γ + CH), $4 \mu\text{M}$ actinomycin D (LPS/INF γ + AD) or $0.5 \mu\text{M}$ dexamethasone (LPS/INF γ + DEX) was separated on a 1.2% agarose gel and stained with ethidium bromide for comparison.

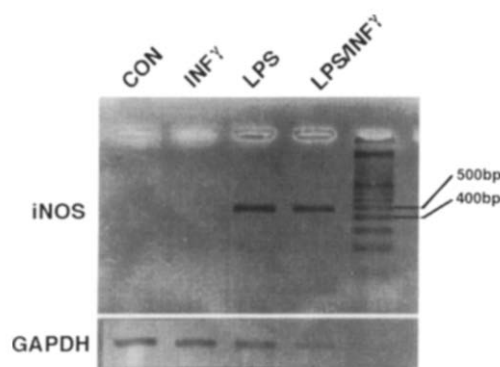


Fig. 5. Expression of iNOS mRNA in tissue strips of aorta. Total RNA was isolated from strips of aorta and analyzed by the RT-PCR to measure the iNOS (upper panel) and GAPDH (lower panel) mRNA levels as described in section 2. An aliquot of the RT-PCR-amplified RNA from untreated strips of aorta (CON) or after 6 h exposure to 5 $\mu\text{g LPS}\cdot\text{ml}^{-1}$ medium (LPS), 300 U $\text{INF}_\gamma\cdot\text{ml}^{-1}$ medium (INF_γ) or 5 $\mu\text{g LPS} + 300 \text{ U INF}_\gamma\cdot\text{ml}^{-1}$ medium (LPS/INF_γ) was separated on a 1.2% agarose gel and stained with ethidium bromide for comparison.

strips (Fig. 6). Under these experimental conditions we were unable to detect any nitrite accumulation in the culture media from aortic strips (results not shown).

4. Discussion

In this study we have demonstrated that vascular smooth muscle cells have the ability to express mRNA for the macrophage-type of inducible nitric oxide synthase, which has also been shown by others [20,31]. However, regulation of NO production may differ between different phenotypic forms of SMCs. Whereas SMCs in arterial wall media do express a contractile phenotype with an abundance of microfilaments, proliferating SMCs in vitro, originating and isolated from arteries, do express a so-called synthetic phenotype, with a higher volume fraction of cell organelles for de novo protein synthesis and supposedly thereby express growth and transcription factors [22].

The exact difference in sensitivity to inducing cytokines and possibly also in signal cross-talk between the signal transduction mechanisms in mature smooth muscle cells in tissue strips and in replicating SMCs in vitro still needs to be clarified.

However, our results suggest some caution when extrapolating in vitro results from cultures of SMCs to a clinical situation.

INF_γ alone did induce a weak expression of mRNA for iNOS but no NO synthesis in replicating SMCs in culture, the latter measured as nitrite in the medium. This is in conformity with recent findings [16, 32] in cultured SMCs.

Several studies have reported that exposure of aortic

rings denuded of endothelial cells in vitro to LPS alone induces NO production [30], which is in agreement with our results at mRNA level. To our knowledge there are no studies addressing the ability of INF_γ to induce NO production and subsequent relaxation of aortic rings in vitro. Our results indicate however, that INF_γ is not required for the induction of iNOS mRNA in vascular smooth muscle cells in vivo, i.e. in situations such as septic shock.

The promotor region of the mouse gene encoding iNOS was recently cloned and shown to contain response elements and inducibility by INF_γ and LPS [33]. In view of these results it seems likely that the observed difference in response to INF_γ between cultured SMCs and aortic strips could be explained by differences in the number of INF_γ -receptors and/or differences in intracellular signaling between mature SMCs in the vessel wall and in cultures. In agreement with previous in vitro studies on endothelial cells [10], macrophages [34] and hepatocytes [35] we have demonstrated that dexamethasone attenuated the expression of iNOS mRNA and subsequent NO synthesis in cultured SMCs and in SMCs in aortic tissue strips in vitro after exposure to LPS alone or to LPS + INF_γ . As has been suggested by others [30,35], this seems to be the therapeutic mechanism by which glucocorticoids eliminate the hypotension and circulatory collapse during clinical septic shock. Our results demonstrated a major difference in the effects of cyclo-

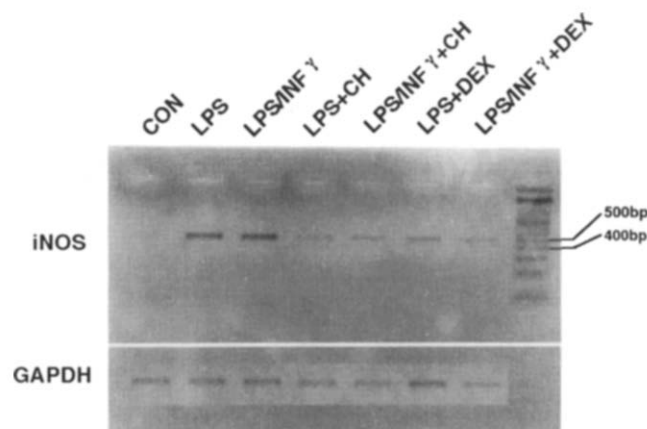


Fig. 6. Effects of cycloheximide or dexamethasone on expression of iNOS mRNA in tissue strips of aorta. Total RNA was isolated from strips of aorta and analyzed by the RT-PCR to measure the iNOS (upper panel) and GAPDH (lower panel) mRNA levels as described in section 2. An aliquot of the RT-PCR-amplified RNA from untreated strips of aorta (CON) or after 6 h exposure to 5 $\mu\text{g LPS}\cdot\text{ml}^{-1}$ medium (LPS), 5 $\mu\text{g LPS} + 300 \text{ U INF}_\gamma\cdot\text{ml}^{-1}$ medium (LPS/INF_γ), 5 $\mu\text{g LPS}\cdot\text{ml}^{-1}$ medium together with 50 μM cycloheximide ($\text{LPS} + \text{CH}$), 5 $\mu\text{g LPS} + 300 \text{ U INF}_\gamma\cdot\text{ml}^{-1}$ medium together with 50 μM cycloheximide ($\text{LPS/INF}_\gamma + \text{CH}$), 5 $\mu\text{g LPS}\cdot\text{ml}^{-1}$ medium together with 0.5 μM dexamethasone ($\text{LPS} + \text{DEX}$) and 5 $\mu\text{g LPS} + 300 \text{ U INF}_\gamma\cdot\text{ml}^{-1}$ medium together with 0.5 μM dexamethasone ($\text{LPS/INF}_\gamma + \text{DEX}$) was separated on a 1.2% agarose gel and stained with ethidium bromide for comparison.

heximide on iNOS mRNA induction between SMCs in culture and mature SMCs in aortic tissue strips.

In the former, cycloheximide together with LPS + IFN γ increased the induction levels of iNOS mRNA, in comparison to the induction with LPS + IFN γ . This implicates the involvement of either constitutively expressed labile repressor protein(s) and/or RNases.

The cycloheximide-dependent induction has also been demonstrated in mouse peritoneal macrophages for the genes induced by interferon- γ , TNF α , interleukin-1 and urokinase [36]. LPS and cycloheximide induction have also been described for the melanoma growth stimulatory activity factor gene [37], the type 1 plasminogen activator inhibitor gene [38] and the manganese superoxide dismutase gene [39]. In aortic tissue strips cycloheximide attenuates the induction seen by LPS alone or by LPS + IFN γ . This indicates that the induction of iNOS mRNA in SMCs in aortic tissue strips requires de novo protein synthesis, protein(s) or transcription factors that seems to be constitutively expressed in SMCs in culture. ATTTA sequences are present in the iNOS cDNA of murine macrophages [17–19] and human hepatocytes [21], that have been implicated to confer instability to the transcriptional regulation of gene expression by many cytokines, protooncogenes and transcription factors [40,41]. However, ATTTA sequences have not been found in rat iNOS cDNA [20]. This might imply that the rat iNOS cDNA was incomplete, which could explain the effects of cycloheximide in this study. However, mRNA instability would also prevail if the synthetic SMC type implied higher levels of specific or non-specific RNases compared to the non-replicating SMCs in aortic strips. However, we cannot exclude the possibility that the differences found could also be dependent on contributions from contaminating non-smooth muscle cells within the aortic strips. Electron microscopic [42] and histologic [43] studies have demonstrated the presence of a substantial population of lymphocytes and monocytes within the aortic intima.

In conclusion, our results indicate for different requirements for iNOS mRNA induction in rat smooth muscle cells in culture and in mature smooth muscle cells in aortic strips in organ culture. These differences impose a certain restriction to the extrapolation of clinical conditions that may be drawn from in vitro experiments with proliferating smooth muscle cells in culture.

Acknowledgements: This work was supported by grants-in-aid from the Swedish Medical Research Council (B92–17X-02042–25A). We thank Camilla Lindgren for excellent technical assistance.

References

- [1] Kilbourne, R.G., Jubran, A., Gross, S.S., Griffith, O.W., Levi, R., Adams, J. and Lodato, R.F. (1990) *Biochem. Biophys. Res. Commun.* 172, 1132–1138.
- [2] Ochoa, J.B., Udekwu, A.O., Billiar, T.R., Curran, R.D., Cerra, F.B., Simmons, R.L. and Peitzman, A.B. (1991) *Ann. Surg.* 214, 621–626.
- [3] Petros, A., Bennet, D. and Vallance, P. (1991) *Lancet* 338, 1557–1558.
- [4] Kilbourne, R.G., Gross, S.S., Jubran, A., Adams, J., Griffith, O.W., Levi, R. and Lodato, R.F. (1990) *Proc. Natl. Acad. Sci. USA* 87, 3629–3632.
- [5] Thiernemann, C. and Vane, J.C. (1990) *Eur. J. Pharmacol.* 182, 591–595.
- [6] Nava, E., Palmer, R.M.J. and Moncada, S. (1991) *Lancet* 338, 1555–1557.
- [7] Moncada, S. and Higgs, E.A. (1990) *Eur. J. Clin. Invest.* 21, 361–374.
- [8] Moncada, S. (1992) *Acta Physiol. Scand.* 145, 201–227.
- [9] Kilbourne, R.G. and Belloni, P. (1990) *J. Natl. Cancer Inst.* 82, 772–776.
- [10] Radomski, M.W., Palmer, R.M.J. and Moncada, S. (1990) *Proc. Natl. Acad. Sci. USA* 87, 10043–10047.
- [11] Hibbs, J.B., Vavrin, Z. and Taintor, R.R. (1987) *J. Immunol.* 138, 550–565.
- [12] Stuehr, D.J. and Marletta, M.A. (1987) *Cancer Res.* 47, 5590–5594.
- [13] Markewitz, B.A., Michael, J.R. and Kohan, D.E. (1993) *J. Clin. Invest.* 91, 2138–2143.
- [14] Nicolson, A.G., Haites, N.E., McKay, N.G., Wilson, H.M., MacLeod, A.M. and Benjamin, N. (1993) *Biochem. Biophys. Res. Commun.* 193, 1269–1274.
- [15] Busse, R. and Mulsch, A. (1990) *FEBS Lett.* 275, 87–90.
- [16] Nakayama, D.K., Geller, D.A., Lowenstein, C.J., Davies, P., Pitt, B.R., Simmons, R.L. and Billiar, T.R. (1992) *Am. J. Respir. Cell. Biol.* 7, 471–476.
- [17] Xie, Q.-W., Cho, H.J., Calaycay, J., Mumford, R.A., Swiderek, K.M., Lee, T.D., Ding, A., Troso, T. and Nathan, C. (1992) *Science* 256, 225–228.
- [18] Lyons, R.C., Orloff, G.J. and Cunningham, M. (1992) *J. Biol. Chem.* 267, 6370–6374.
- [19] Lowenstein, C.J., Glatt, C.S., Bredt, D.S. and Snyder, S.H. (1992) *Proc. Natl. Acad. Sci. USA* 89, 6711–6715.
- [20] Nunokawa, Y., Ishida, N. and Tanaka, S. (1993) *Biochem. Biophys. Res. Commun.* 191, 89–94.
- [21] Geller, D.A., Lowenstein, C.J., Shapiro, R.A., Nussler, A.K., Di Silvio, M., Wang, S.C., Nakayama, D.K., Simmons, R.L., Snyder S.H. and Billiar, T.R. (1993) *Proc. Natl. Acad. Sci. USA* 90, 3491–3495.
- [22] Thyberg, J., Hedin, U., Sjölund, M., Palmberg, L. and Bottiger, B.A. (1990) *Arteriosclerosis* 10, 966–990.
- [23] Sjölund, M., Hedin, U., Sejersen, T., Heldin, C.-H. and Thyberg, J. (1988) *J. Cell. Biol.* 106, 403–413.
- [24] Scott-Burden, T., Schini, E., Elizondo, V.B., Junquero, D.C. and Vanhoutte, P.M. (1992) *Circ. Res.* 71, 1088–1100.
- [25] Schini, V.B., Durante, W., Elizondo, E., Scott-Burden, T., Junquero, D.C., Schafer, A.I. and Vanhoutte, P.M. (1992) *Eur. J. Pharmacol.* 216, 379–383.
- [26] Junquero, D.C., Scott-Burden, T., Schini, V.B. and Vanhoutte, P.M. (1992) *J. Physiol.* 454, 451–465.
- [27] Thyberg, J., Palmberg, L., Nilsson, J., Ksiazek, T. and Sjölund, M. (1983) *Differentiation* 25, 156–167.
- [28] Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.* 162, 156–159.
- [29] Ignaro, J.R., Bug, G.M., Wood, K.S. and Byrns, R.E. (1987) *Proc. Natl. Acad. Sci. USA* 84, 9265–9269.
- [30] Rees, D.D., Cellet, S., Palmer, R.M. J. and Moncada, S. (1990) *Biochem. Biophys. Res. Commun.* 173, 541–547.
- [31] Koide, M., Kawahara, Y., Tsuda, T. and Yokoyama, M. (1993) *FEBS Lett.* 318, 213–217.
- [32] Geng, Y.-J., Hansson, G.K. and Holme, E. (1992) *Circ. Res.* 71, 1268–1276.

- [33] Xie, Q.-W., Whisnant, R. and Nathan, C. (1993) *J. Exp. Med.* 177, 1779–1784.
- [34] Di Rosa, M., Radomski, M., Carnuccio, R. and Moncada, S. (1990) *Biochem. Biophys. Res. Commun.* 172, 1246–1252.
- [35] Geller, D.A., Nussler, A.K., Di Silvio, M., Lowenstein, C.J., Shapiro, R.A., Wang, S.C., Simmons, R.L. and Billiar, T.R. (1993) *Proc. Natl. Acad. Sci. USA* 90, 522–526.
- [36] Collart, M.A., Belin, D., Vassalli, J.D., De Kossodo, S. and Vassalli, P. (1986) *J. Exp. Med.* 164, 2113–2118.
- [37] Wen, D., Rowland, A. and Derynck, R. (1989) *EMBO J.* 8, 1761–1766.
- [38] Sawdey, M., Podor, T.J. and Loskuton, D.J. (1989) *J. Biol. Chem.* 264, 10396–10401.
- [39] Visner, G.A., Dougall, W.C., Wilson, J.M., Burr, I.A. and Nick, H.S. (1990) *J. Biol. Chem.* 265, 2856–2864.
- [40] Shaw, G. and Kamen, R. (1986) *Cell* 46, 659–667.
- [41] Caput, D., Beutler, B., Hartog, K., Brown-Scheimer, S. and Cerami, A. (1986) *Proc. Natl. Acad. Sci. USA* 83, 1670–1674.
- [42] Joris, I., Stetz, E. and Majno, G. (1979) *Atherosclerosis* 34, 221–231.
- [43] Freudenberg, N. and Riese, K.H. (1976) *Beitr. Path. Bd.* 159, 125–142.