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# Effect of 17β-oestradiol on cytokine-induced nitric oxide production in rat isolated aorta

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**1** Studies were performed on isolated aortic rings without endothelium to investigate the effect of 17β-oestradiol on cytokine-induced nitric oxide production by the inducible nitric oxide synthase (iNOS).

**2** Treatment of the isolated aortic rings with interleukin-1β (IL-1β, 20 μ ml$^{-1}$) led to the expression of iNOS mRNA and protein, as well as significant nitrite accumulation in the incubation media and suppression of phenylephrine (1 nM – 10 μM)-evoked contraction.

**3** Cycloheximide (1 μM), a protein synthesis inhibitor, prevented iNOS protein expression, nitrite accumulation and the suppression of contractility by IL-1β on the isolated aortic rings. 17β-oestradiol (1 nM – 10 μM) and the partial oestrogen receptor agonist 4-OH-tamoxifen (1 nM – 10 μM) produced concentration-dependent inhibition of IL-1β-induced nitrite accumulation and restored vasoconstrictor responsiveness to phenylephrine, similar to the iNOS inhibitor aminoguanidine (100 μM).

**4** Semiquantitative PCR demonstrated decreased iNOS mRNA in the IL-1β-induced and 17β-oestradiol-treated rings. Western blot analysis of rat aorta homogenates revealed that 17β-oestradiol treatment resulted in a reduction in IL-1ß-induced iNOS protein level.

**5** Incubation with tumour necrosis factor α (TNFα, 1 ng ml$^{-1}$) resulted in significant nitrite accumulation in the incubation media and suppression of the smooth muscle contractile response to phenylephrine, similar to IL-1β. The effects of TNFα were also inhibited by co-incubation of the rings with 17β-oestradiol and 4-OH-tamoxifen (1 μM).

**6** The anti-transforming growth factor-β1 (TGF-β1) antibody, which inhibited TGF-β1-induced suppression of nitrite production from IL-1β-treated vascular rings, did not affect the inhibitory action of 17β-oestradiol, suggesting that the effect of oestrogen on iNOS inhibition was not mediated by TGF-β1.

**7** These results show that the ovarian sex steroid, 17β-oestradiol is a modulator of cytokine-induced iNOS activity in rat vascular smooth muscle and its mechanism of action involves decrease of iNOS mRNA and protein.

**Keywords:** Inducible nitric oxide synthase; oestrogen; tamoxifen; vascular smooth muscle; IL-1β; TGF-β

## Introduction

Nitric oxide (NO) is a potent vasodilator (Furchgott & Zawadzki, 1980; Palmer *et al*., 1987) and besides the cardiovascular system, it is also an important physiological and/or pathophysiological mediator in the immune (Stuehr & Marletta, 1985; Xie *et al*., 1992) and nervous system (Lowenstein *et al*., 1992). NO is synthesized by the oxidation of the terminal guanidino-nitrogen atom of L-arginine, which is catalyzed by three different isoforms of nitric oxide synthase (NOS) (Förstermann *et al*., 1995). The constitutive isoforms of NOS are found predominantly in endothelial cells (ecNOS or NOS-III) (Schmidt & Murad, 1991) and in the brain (bcNOS or NOS-I) (Lowenstein *et al*., 1992). The inducible isoform, iNOS (or NOS-II), is expressed in several cell types, such as macrophages (Xie *et al*., 1992), hepatocytes (Billiar *et al*., 1990; Geller *et al*., 1993), chondrocytes (Palmer *et al*., 1992), as well as in vascular smooth muscle cells (Busse & Mülsch, 1990; Kanno *et al*., 1993; Koide *et al*., 1994) upon induction by lipopolysaccharide (LPS) or cytokines.

The ovarian sex steroid hormone, 17β-oestradiol is one of the few physiological regulators of ecNOS (Kauser & Rubanyi, 1994a; Harrison *et al*., 1995). It significantly enhances endothelial NO production in animals and man, an effect which contributes to its cardiovascular protective action (Kauser & Rubanyi, 1994a, 1997). However, little is known about the effect of oestrogens on excessive NO production by iNOS.

Expression of iNOS and excessive production of nitrite by vascular smooth muscle cells mediates endothelium-independent suppression of blood vessel contractility during septic shock contributing to the severe hypotension (Thiemermann & Vane, 1990). The simultaneous generation of large amounts of superoxide anion (Ohara *et al*., 1995) could lead to peroxynitrite formation (Hogg *et al*., 1993), which contributes to the cytotoxicity of excessive nitrite production (Beckmann *et al*., 1994). In a recent study we found that physiological substitution doses of 17β-oestradiol reduced nitrite production in LPS-treated ovariectomized rats (Kauser *et al*., 1997).

The present study was designed to study the effect of the ovarian sex steroid hormone, 17β-oestradiol, and the partial oestrogen receptor agonist 4-OH-tamoxifen, on interleukin-1β (IL-1β)-induced nitrite production and suppressed contractile reactivity in rat isolated aortic rings. The results showed, that in contrast to augmentation of endothelial NOS expression/activity, 17β-oestradiol decreased interleukin-1β (IL-1β)-induced iNOS mRNA and protein level, resulting in a significant suppression of excessive nitrite generation and restoration of contractile responsiveness in rat isolated aortic rings.

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## Methods

### *Interleukin-1β-induction of iNOS in rat aortic rings*

Thoracic aortae of adult, male Wistar rats (250–300 g) were dissected and cleaned of adherent connective tissue under sterile conditions. Aortae were cut into ~5 mm long rings and the endothelium was removed by gentle rubbing of the intimal surface by forceps. The endothelium-denuded segments were incubated in 150 $\mu$l final volume phenol red free, minimal essential medium (NPR-MEM) in the absence or presence of IL-1$\beta$ (20 $\mu$ ml$^{-1}$) on 96 well plates. Test substances such as aminoguanidine, cycloheximide, 17$\beta$-oestradiol, 4-OH-tamoxifen, transforming growth factor-$\beta$1 (TGF-$\beta$1) and anti-TGF-$\beta$1 antibody were added to the incubation media 1 h before to the cytokine treatment. The aortic rings were incubated for 12 h in a cell culture incubator in humidified air mixed with 5% $CO_2$ at 37°C. The rings were than suspended in Schuler organ chambers (Hugo Sachs, Germany) for isometric tension recording, according to protocol described in more detail earlier (Kauser & Rubanyi, 1994b). Contractility of the segments was assessed by isometric tension (expressed in g) generated by cumulative concentrations of phenylephrine (PE, 1 nM–10 $\mu$M). Effect of the NOS inhibitor, aminoguanidine, was studied in the organ chamber and it was added after the aortic rings were contracted by PE. The experiments in the organ chamber were performed in the presence of 10 $\mu$M indomethacin to block the production of vasoactive prostanoids (Rubanyi *et al*., 1986; Boulanger & Vanhoutte, 1993). At the end of the experiments the aortic rings were removed from the organ bath, blotted on filter paper and their wet weight was measured.

### *Measurement of nitrite production*

Nitric oxide synthase activity was assessed by measurement of nitrite accumulation in the organ culture media (collected at the end of the 12 h incubation period) with the Griess reagent (1% sulphanilamide in 5% $H_3PO_4$ and 0.1% naphthyl-ethylenediamine dihydrochloride, in a ratio of 1 : 1). One hundred microlitres of the incubation media were mixed with an equal volume of Griess reagent at room temperature for 30 min. The resulting coloured product was quantified spectrophotometrically at 540 nm. Nitrite accumulation per well was normalized to the total volume of the incubation media and to the wet weight of the aortic rings.

### *Semiquantitative PCR of iNOS mRNA*

Aortic rings from rats ($n=6$) were collected into liquid nitrogen after 12 h incubation in the absence or presence of IL-1$\beta$ (20 $\mu$ ml$^{-1}$) with or without 17$\beta$-oestradiol (1 $\mu$M). Total RNA was isolated from the pooled aortic rings according to the method of Chomczynski and Saachi (1987). Single-stranded cDNA was prepared by reverse transcription with the SuperScript preamplification System (Life Technologies, Gaithersburg, MD). Semiquantitative PCR was performed according to the method described by Murphy *et al*., (1990). To normalize signals from different RNA samples, cytochrome c oxidase subunit I (1A) was coamplified as an internal standard. cDNA, equivalent to 200 ng total RNA, was combined with 0.5 $\mu$ thermus flavus polymerase (Biozym, Hess. Oldendorf, Germany), 0.5 $\mu$Ci [$^{32}$P]-deoxy(d)-CTP (3000 $\mu$Ci mmol$^{-1}$), 200 $\mu$mol l$^{-1}$ dNTPs (ATP,GTP,CTP and TTP) and 500 ng of the respective oligonucleotide primers. The reaction volume was adjusted to 50 $\mu$l with 1 × PCR buffer (50 mM Tris-HCl(pH 9.0), 20 mM $(NH_4)_2SO_4$ and 2.5 mM $MgCl_2$). The reaction mixture was subjected to amplification cycles comprised of a 1-min step at 94°C for denaturation, a 1-min step at 58°C for annealing and a 1-min step at 94°C for denaturation. Amplification reactions were stopped in the exponential phase, i.e. iNOS 28 cycles and 1A 18 cycles. Amplification was performed with a Perkin-Elmer Cetus 9600 Thermal Cycler (Norwalk, CT). The primers were constructed based on the cDNA sequences available in the EMBL databank. The iNOS primers were, sense: 5′-CGTCTGCAGCACTTGGATCAA-3′; and antisense: 5′-TTCTGCAGGATGTCTTGAACG-3′. The 1A primers were, sense: 5′-CGTCACAGCCCATGCATTCG-3′; and antisense: 5′-CTGTTCATCCTGTTCCAGCTC-3′. The PCR-products were sequenced to verify their identity to corresponding cDNA sequences in the EMBL databank. PCR reaction products were separated on 6% polyacrylamide gels and detected by autoradiography. Autoradiograms were analysed by use of a Molecular Dynamics Densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

### *Western blot analysis of iNOS*

Aortic rings from rats ($n=6$) were collected into liquid nitrogen either immediately after isolation from the animals (not incubated) or after 12 h incubation in the absence or presence of IL-1$\beta$ (20 $\mu$ ml$^{-1}$) with or without cycloheximide (1 $\mu$M) or 17$\beta$-oestradiol (1 $\mu$M). The frozen tissues were powdered and homogenized in a buffer (62.5 mM Tris, pH 6.8, 10% glycerol, 1% SDS) containing enzyme inhibitors (5 mg ml$^{-1}$ aprotonin, 5 mg ml$^{-1}$ leupeptin, 1 mg ml$^{-1}$ pepstatin-A, 5 mg ml$^{-1}$ antipain, 100 mM pefabloc, 0.5 M, pH 8 EDTA). Protein contents of the samples were determined by the method of Bradford (1976). Equal amounts of samples (50 $\mu$g protein) were loaded on a 4–12% gradient Tris-Gly 1.5 mm gel (Novex, San Diego, CA) in the presence of 2-mercaptoethanol. After electrophoretic separation, samples were transferred onto nitrocellulose membranes (Novex, San Diego, CA) and blocked with 3% bovine serum albumin (BSA) in Tris (15 mM)-buffered saline solution (pH 7.5) at 4°C overnight. The immobilized iNOS protein was visualized by incubation of the membranes with 1 : 200 dilution of the polyclonal anti-rat iNOS antibody (kindly provided by Dr. H. Eshumi, National Cancer Center Research Institute, Tokyo, Japan) and a 1 : 1000 dilution of a secondary peroxidase conjugated goat anti-rabbit antibody (Amersham, Cleveland, OH). Signals were detected by the ECL detection system (Amersham, Cleveland, OH) and autoradiography films (Hyperfilm ECL, Amersham, Cleveland, OH). The films were than scanned with a densitometer (Computing Densitometer, Molecular Dynamics, Sunnyvale, CA) and a graph of the peak area was plotted against protein concentration.

### *Materials*

Interleukin-1$\beta$ (IL-1$\beta$), transforming growth factor-$\beta$-1 (TGF-$\beta$1), anti-TGF-$\beta$1 antibody was obtained from Boehringer Mannheim Corp. (Indianapolis, IN). 17-$\beta$-oestradiol and 4-OH-tamoxifen were gifts from Schering AG. Chemicals and reagents for Western blot analysis and organ chamber studies were purchased from Sigma Chemical Co. (St. Louis, MO). Cell culture material was obtained from GIBCO Laboratories (Grand Island, NY).

*Data analysis*

Results are shown as means ± s.e.mean of 6–10 experiments performed on aortic rings isolated from different animals ($n$ represents the number of animals used). Statistical comparisons were performed by Student's $t$ test and analysis of variance (ANOVA) followed by Newmann test when more than two groups were compared. $P<0.05$ was considered to be statistically significant.

## Results

*Effect of IL-1β on iNOS mRNA and protein expression, nitrite production and contractile reactivity of rat isolated aortic rings*

Incubation of rat isolated aortic rings with 20 $\mu$ml$^{-1}$ IL-1$\beta$ for 12 h resulted in significant suppression of phenylephrine evoked contraction (Figure 1a) and accumulation of nitrite in the culture medium (Figure 1b). Semiquantitative PCR analysis of the total RNA, isolated from the IL-1$\beta$ incubated aortic rings, demonstrated the induction of iNOS mRNA in the cytokine-treated rings, compared to the expression in control rings incubated for the same period of time in control culture media (NPR-MEM) (Figure 2a). Western blot analysis of the incubated aortic tissue homogenates indicated the presence of iNOS protein (130 kD) in the IL-1$\beta$ treated vessels (Figure 2b). No iNOS protein was detected in the rat freshly isolated control aorta and in rings incubated in NPR-MEM without cytokine. Contractile reactivity to PE was restored by the iNOS inhibitor, aminoguanidine (100 $\mu$M), added to the

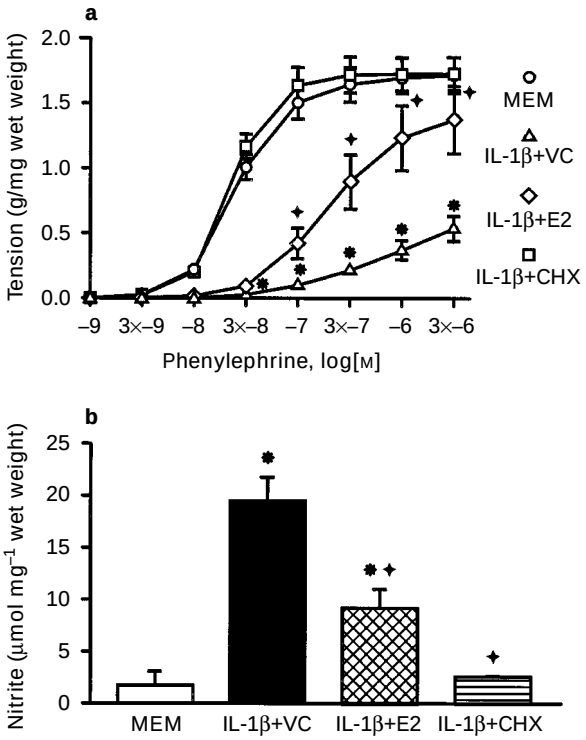


**Figure 1** Effect of 17$\beta$-oestradiol and cycloheximide on IL-1$\beta$-induced suppression of contractile reactivity to phenylephrine (**a**) and nitrite production (**b**) in isolated rat aortic rings. (**a**) Dose-response curves to phenylephrine (PE, 1 nM–10 $\mu$M) in control (MEM), IL-1$\beta$ (20 $\mu$ ml$^{-1}$) + vehicle (VC = ethanol), IL-1$\beta$ + cycloheximide (CHX, 1 $\mu$M) and IL-1$\beta$ + 17$\beta$-oestradiol (E2, 1 $\mu$M)-treated rings. IL-1$\beta$ significantly (*$P<0.05$) attenuated smooth muscle contraction of the aortic rings. 17$\beta$-Oestradiol treatment significantly (+$P<0.05$) reversed the effect of IL-1$\beta$. The protein synthesis inhibitor cycloheximide, (CHX) completely prevented the cytokine-evoked suppression of the PE response. The changes in nitrite accumulation in response to the different treatments. Nitrite levels in the media of the CHX-treated vessels was not different from control (MEM). IL-1$\beta$ induced significant (*$P<0.05$) nitrite production. 17$\beta$-Oestradiol (E2) significantly (+$P<0.05$) reduced nitrite accumulation in the supernatant of the cytokine treated vessels. Data represent means ± s.e.mean (vertical lines) of $n=10$ rings from different animals in each treatment groups.

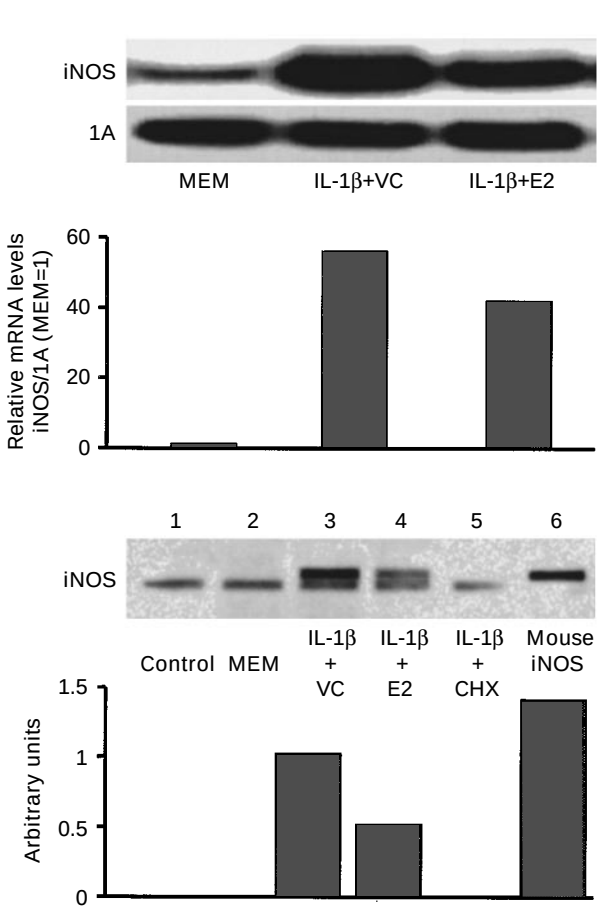


**Figure 2** iNOS mRNA and protein expression in rat isolated aortic rings. (**a**) The autoradiogram of the semiquantitative PCR analysis demonstrating mRNA expression of the inducible nitric oxide synthase (iNOS) and cytochrome c oxidase subunit I (1A) from total RNA of pooled thoracic aortic rings ($n=6$). Lanes from left to right: (1) 12 h incubated control (MEM), (2) IL-1$\beta$ (20 $\mu$ ml$^{-1}$) and vehicle-treatment (IL-1$\beta$ + VC) and (3) IL-1$\beta$ and 17$\beta$-oestradiol (1 $\mu$M) treatment (IL-1$\beta$ + E2). The signals were quantified by analysing the autoradiography with densitometer (Molecular Dynamics, Sunnyvale, CA). Histograms represent densitometric values of relative mRNA levels (iNOS/1A) from pooled RNA of rat thoracic aortic rings (6 aortae). 17$\beta$-Oestradiol treatment (lane 3) resulted in a decrease in mRNA level compared to IL-1$\beta$ treatment alone (lane 2). (**b**) Western blot and its quantitation by scanning densitometry. Lanes on the autoradiograph are from left to right: (1) control, freshly isolated rat aortic tissue homogenates, (2) 12 h incubated controls (MEM), (3) IL-1$\beta$ (20 $\mu$ ml$^{-1}$) and vehicle treated aortic tissues, (4) IL-1$\beta$ and 17$\beta$-oestradiol (1 $\mu$M)-treated aortic rings, (4) IL-1$\beta\rightarrow$ and cycloheximide (CHX, 1 $\mu$M) treated vessels and (6) mouse iNOS, as a positive control. Protein samples of lanes 1–5 were from pooled samples of 6 aortic rings isolated from different animals. Results were similar in three different experiments. In lanes 3 and 4 a 130 kD band appeared, similar to the iNOS control, indicating the expression of iNOS protein. The smaller band was not iNOS. It is a result of using a polyclonal antibody. It appeared in the first 5 lanes, but not in lane 6 which contained purified mouse iNOS protein. The level of iNOS protein was reduced by 33.9 ± 6.7% ($n=3$) in the oestrogen treated-rings.

organ bath (Figure 3a) suggesting that it is due to induction of iNOS and consequent excessive nitrite production in the vascular smooth muscle. Indeed, IL-1$\beta$-induced nitrite accumulation could also be attenuated by the addition of the NOS inhibitor into the incubation media (Figure 3b). Treatment of the aortic rings with the protein synthesis inhibitor cycloheximide (1 $\mu$M) prevented the IL-1$\beta$-induced suppression of the PE response (Figure 1a) and nitrite production by the rings (Figure 1b). Western blot analysis of cycloheximide treated rings confirmed the lack of induction of iNOS protein by IL-1$\beta$.

### *Effect of 17β-oestradiol on IL-1β-induced iNOS mRNA and protein expression*

Semiquantitative PCR analysis of the total RNA, isolated from pooled aortic rings incubated in the presence of IL-1$\beta$ (20 $\mu$ ml$^{-1}$) with 17$\beta$-oestradiol (1 $\mu$M), demonstrated down-regulation of iNOS mRNA in the 17$\beta$-oestradiol treated rings compared to mRNA levels detected in rings incubated with IL-1$\beta$ and vehicle alone (Figure 2a). The level of iNOS protein, isolated from pooled aortic rings, was also reduced by 33.9$\pm$6.7% ($n$=3) in the oestrogen treated rings (Figure 2b).

### *Effect of 17β-oestradiol on IL-1β-induced suppression of contractility and nitrite production*

Co-incubation of the aortic rings with IL-1$\beta$ (20 $\mu$ ml$^{-1}$) and 17$\beta$-oestradiol (1 $\mu$M) resulted in a significant attenuation of the IL-1$\beta$-induced suppression of PE contraction (Figure 1a). 17$\beta$-oestradiol also significantly inhibited nitrite accumulation in the incubation media (Figure 1b). The inhibition of nitrite generation by 17$\beta$-oestradiol (9.1$\pm$3 $\mu$M) was less than the complete prevention by cycloheximide (Figure 1b), but it was equivalent to the inhibition observed after aminoguanidine treatment (9.9$\pm$2 $\mu$M) (Figure 3b). The effect of 17$\beta$-oestradiol on both the IL-1$\beta$-induced suppression of vascular smooth muscle contractile reactivity and nitrite accumulation was dose-dependent (1 nM – 10 $\mu$M) (Figure 4a).

### *Effect of 4-OH-tamoxifen on IL-1β-induced suppression of contractility and nitrite production*

Similar to 17$\beta$-oestradiol, the non-steroidal, partial oestrogen receptor agonist, 4-OH-tamoxifen, attenuated IL-1$\beta$-induced suppression of contractile reactivity (Figure 4a) and inhibited nitrite accumulation (Figure 4b) ($n$=8). Tamoxifen was somewhat less effective than 17$\beta$-oestradiol at restoring contractile reactivity, but they were equally effective in inhibiting nitrite production (Figure 4a and b).

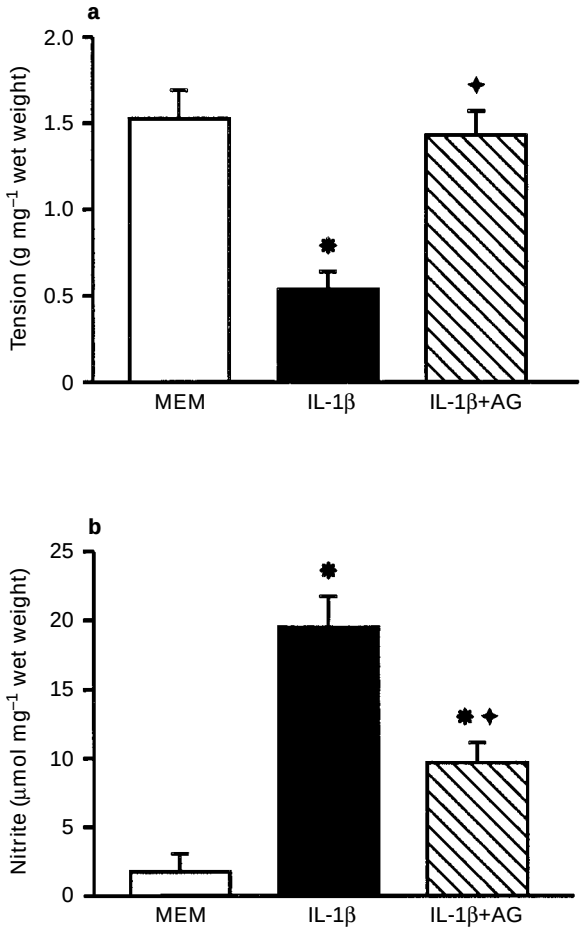


**Figure 3** Effect of aminoguanidine (AG) on IL-1$\beta$-induced suppressed contractility and nitrite production in isolated rat aortic rings. (**a**) Smooth muscle contractile responses to phenylephrine (PE) at 10 $\mu$M final concentration. IL-1$\beta$ (20 $\mu$ ml$^{-1}$) significantly (*$P<0.05$) attenuated PE contraction of the denuded rat aortic rings after 12 h incubation. The iNOS inhibitor AG (100 $\mu$M) reversed the IL-1$\beta$-induced suppression. (**b**) The accumulated nitrite levels in the incubation supernatant at the end of the 12 h incubation. Cytokine treatment induced significant (*$P<0.05$) nitrite production by the vascular rings. AG significantly (+$P<0.05$) attenuated the amount of nitrite accumulated in the media. Values represent means$\pm$ s.e.mean of $n$=10 rings from different animals in each treatment group.

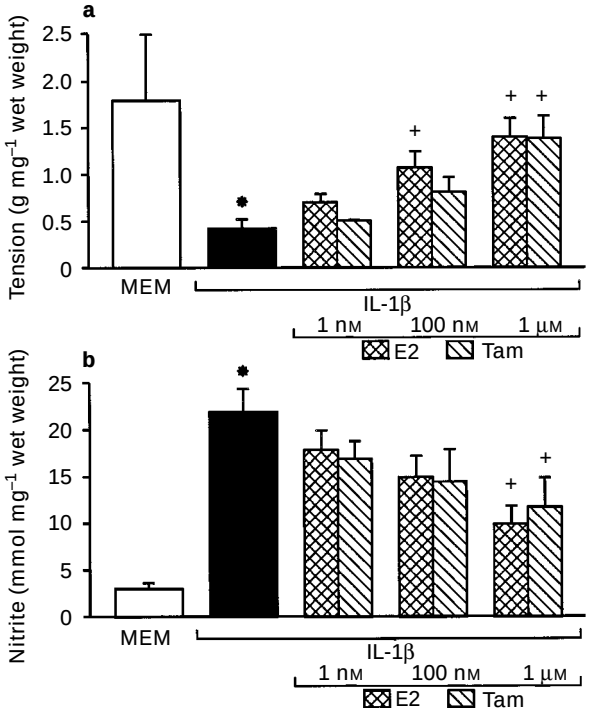


**Figure 4** Dose-response effects of 17$\beta$-oestradiol and 4-OH-tamoxifen on contractility and nitrite production in IL-1$\beta$-treated rat aortic rings. 17$\beta$-oestradiol (E2) and the partial oestrogen receptor analogue, 4-OH-tamoxifen (Tam), dose-dependently inhibited IL-1$\beta$(20 $\mu$ ml$^{-1}$-induced suppression of smooth muscle contractility (**a**) with a concomitant inhibition of nitrite production (**b**). Asterisks (*) and plus signs (+) indicate Significant ($P<0.05$) differences compared to control (MEM) or to responses of the IL-1$\beta$ treated segments, respectively. Values represent means$\pm$s.e.mean of $n$=8 rings from different animals in each treatment group.

### *Effect of 17β-oestradiol and 4-OH-tamoxifen on TNFα-induced suppression of contractility and nitrite production*

Incubation of the rat isolated aortic rings with 1 ng ml$^{-1}$ TNFα resulted in significant suppression of phenylephrine evoked contraction (Figure 5a) and accumulation of nitrite in the culture medium (Figure 5b), similar to that observed with IL-1$\beta$ induction of iNOS (Figure 1a and b). 17$\beta$-oestradiol (1 $\mu$M) and 4-OH-tamoxifen (1 $\mu$M) significantly inhibited both the suppression of contractility and the production of nitrite induced by TNFα (Figure 5a and b).

### *Effect of anti-TGF-β1 antibody on 17β-oestradiol evoked inhibition of IL-1β-induced nitrite production*

Co-incubation of aortic rings with TGF-$\beta$1 (10 ng ml$^{-1}$) and IL-1$\beta$ (20 $\mu$ ml$^{-1}$) significantly inhibited Il-1$\beta$-induced nitrite accumulation ($n=6$) (Figure 6a). Anti-TGF-$\beta$1 antibody (10 $\mu$g ml$^{-1}$) prevented the inhibitory effect of TGF-$\beta$1 on IL-1$\beta$-induced nitrite production (Figure 6a). However, anti-TGF-$\beta$1 antibody (10 $\mu$g ml$^{-1}$) had no effect on the inhibition by 17$\beta$-oestradiol (Figure 6b).

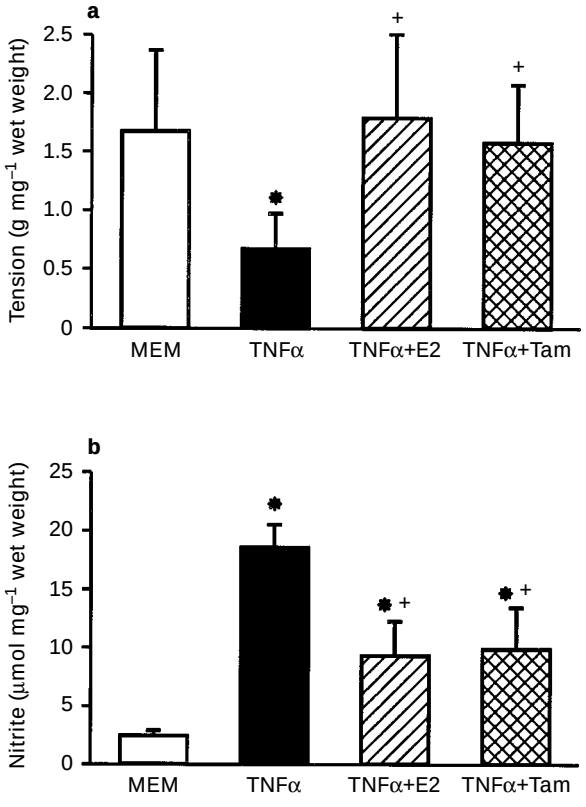


**Figure 5** Effect of 17$\beta$-oestradiol and 4-OH-tamoxifen on contractility and nitrite production in TNFα-treated rat aortic rings. (**a**) Contraction to phenylephrine (PE, 10 $\mu$M) in control (MEM), TNFα (1 ng ml$^{-1}$) + vehicle (VC = ethanol), TNFα + 17$\beta$-oestradiol (E2, 1 $\mu$M and TNFα + 4-OH-tamoxifen (4-OH-Tam, 1 $\mu$M. TNFα significantly (*$P<0.05$) attenuated smooth muscle contraction of the aortic rings. 17$\beta$-oestradiol and 4-OH-tamoxifen treatment significantly (+$P<0.05$) reversed the effect of TNFα. (**b**) Changes in nitrite accumulation in response to TNFα and the co-treatment with oestrogen and tamoxifen. Nitrite levels in the media of TNFα incubated rings were significantly (*$P<0.05$) increased. 17$\beta$-oestradiol and 4-OH-tamoxifen significantly (+$P<0.05$) reduced nitrite accumulation in the supernatant of the cytokine treated vessels. Data represent means ± s.e.mean of $n=6$ rings from different animals in each treatment group.

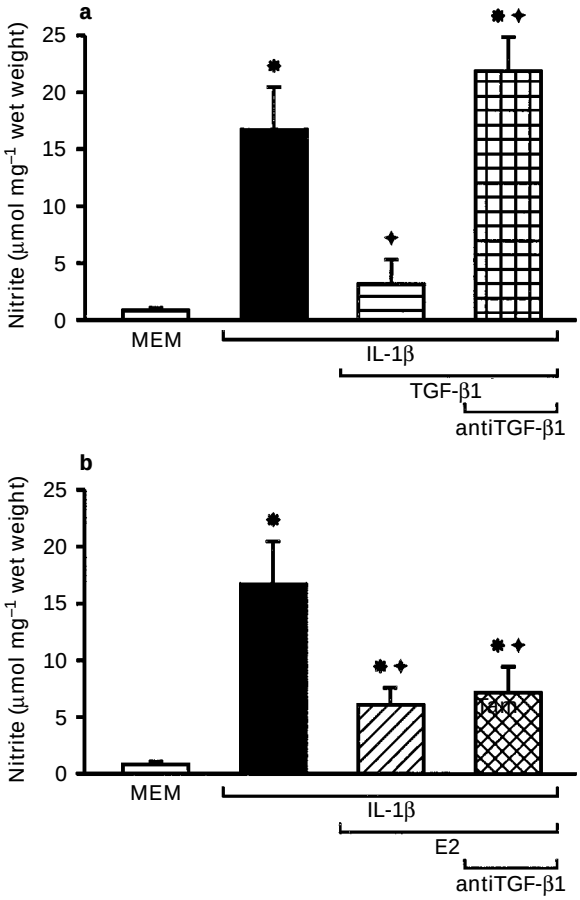


**Figure 6** Effect of anti-TGF-$\beta$1 antibody on 17$\beta$-oestradiol evoked inhibition of nitrite production in IL-1$\beta$-treated rat aortic rings. (**a**) Control experiment, in which 10 ng ml$^{-1}$ TGF-$\beta$1 inhibited IL-1$\beta$ (20 $\mu$ ml$^{-1}$) -induced nitrite production. This effect of TGF-$\beta$1 could be reversed by 10 $\mu$g ml$^{-1}$ anti-TGF-$\beta$1 antibody (**b**) The same concentration of anti-TGF-$\beta$1 antibody was not able to reverse the inhibitory effect of 17$\beta$-oestradiol (1 $\mu$M) on IL-1$\beta$-induced nitrite production. Asterisks (*) indicate significant ($P<0.05$) differences compared to control (MEM). Plus signs (+) show significant ($P<0.05$) differences compared to responses of the IL-1$\beta$ incubated aortic rings. Values represent means ± s.e.mean of $n=6$ rings from different animals in each treatment group.

## Discussion

The present study demonstrated for the first time that 17$\beta$-oestradiol decreases cytokine-induced nitrite accumulation and restores depressed contractile reactivity in rat isolated, endothelium-denuded aortic rings, by decreasing the IL-1$\beta$–induced, increased level of iNOS mRNA and protein in the vascular tissue. Tamoxifen, a partial oestrogen receptor agonist had similar effects. The effect of oestrogen was investigated earlier on the spontaneous nitric oxide production in cultured hepatocytes (Pittner & Spitzer, 1993) and rat peritoneal macrophages (Chao *et al*., 1994). However, its effect on cytokine-induced iNOS activity and expression has not yet been studied.

IL-1$\beta$ is an effective inducer of iNOS in vascular smooth muscle (Busse & Mülsch, 1990; Kanno *et al*., 1993; Koide *et al*., 1994). In the present study, 12 h incubation of the rat isolated aortic rings with IL-1$\beta$ and TNF resulted in significant accumulation of nitrite in the incubation media and suppression of phenylephrine-induced contraction. Induction of iNOS by IL-1$\beta$ was confirmed by the detection of the iNOS

mRNA and protein via semiquantitative PCR and Western blotting, respectively. The suppression of the contractile response was inhibited by the iNOS inhibitor, aminoguanidine, suggesting that it was mediated via nitric oxide production. Since the endothelium was removed from the vessels before cytokine treatment, endothelial production of nitric oxide via the endothelial constitutive nitric oxide synthase (ecNOS) could not account for the attenuation of contraction. Preincubation of the rings with the protein synthesis inhibitor cycloheximide prevented the accumulation of nitrite, indicating that its production is due to *de novo* synthesis of nitric oxide synthase. Indeed, Western blot analysis confirmed the absence of the iNOS-protein in the cycloheximide treated, IL-1$\beta$-incubated aortic rings.

Similar to the iNOS inhibitor, aminoguanidine, co-incubation of the aortic rings with the cytokines and 17$\beta$-oestradiol significantly reduced nitrite accumulation and increased contractile reactivity to phenylephrine of rat aortic rings. These observations on isolated vascular rings in organ culture agree well with our recent observations *in vivo*, where physiological doses of 17$\beta$-oestradiol inhibited excessive nitrite accumulation in the blood of LPS-treated ovariectomized rats (Kauser *et al*., 1997).

It is unlikely that 17$\beta$-oestradiol exerts a specific inhibitory effect on the IL-1$\beta$-stimulated pathway, since similar effects of TNF$\alpha$ on vascular smooth muscle contractility and nitrite accumulation were also inhibited by oestrogen. Furthermore, oestradiol had no effect on leukocyte adhesion to IL-1$\beta$ stimulated endothelial cells, although it enhanced adhesion to TNF$\alpha$ treated cells (Cid *et al*., 1994).

In the search for the potential mechanism of action of 17$\beta$-oestradiol-induced inhibition of excessive nitrite production by iNOS, several sites of regulation seem possible. These include (1) inhibition of iNOS gene transcription or protein expression, (2) modulation of iNOS enzyme activity and (3) interaction with NO itself.

Semiquantitative PCR and Western blot analysis revealed the presence of iNOS mRNA and protein in IL-1$\beta$ treated aortic rings, confirming the origin of excessive NO production, which leads to significant suppression of contractility and accumulation of nitrite during the incubation. 17$\beta$-oestradiol treatment resulted in a decrease in the IL-1$\beta$-induced expression of iNOS mRNA and protein, which may contribute to the reduced nitrite accumulation and increased contractile reactivity observed after 17$\beta$-oestradiol treatment. Direct inhibition of iNOS enzyme is not probable, since 17$\beta$-oestradiol has no effect on the activity of recombinant iNOS enzyme (J. Parkinson, unpublished observations). Although alternative mechanisms cannot be ruled out, inhibition of substrate or cofactor availability is highly unlikely, as these would inhibit endothelial NO production as well, which was augmented, rather than suppressed in oestrogen-treated rat aorta (Goetz *et al*., 1994; Kauser & Rubanyi, 1995). Oestrogen can also modify superoxide anion production (Keaney *et al*., 1994). However, decreases in the amount of superoxide free radicals would result in an increase in bioavailable NO (Rubanyi & Vanhoutte, 1986). This would cause greater suppression of contractility, opposite to the finding in our study. Reduced iNOS mRNA and protein level can be the result of inhibition of iNOS gene transcription and/or increased mRNA or protein degradation by 17$\beta$-oestradiol. The present data do not allow us to distinguish between these possibilities.

Glucocorticoids have been shown to prevent cytokine-induced iNOS gene expression in vascular cells (Radomski *et al*., 1990; Di Rosa *et al*., 1990). Dexamethasone is a potent inhibitor of excessive nitrite production in LPS-treated rats, when it is given before iNOS induction (Knowles *et al*., 1990; Kauser *et al*., 1997). Synergism between oestrogen and glucocorticoids has been demonstrated (Ankenbauer *et al*., 1988), but it is unlikely that inhibition of iNOS expression by oestrogen was mediated via a mechanism which is shared by glucocorticoids, since 17$\beta$-oestradiol does not activate glucocorticoid receptors (Klock *et al*., 1987). However, the effect of 17$\beta$-oestradiol may be mediated by activation of the oestrogen receptor, since another ligand of the oestrogen receptor, the non-steroidal, partial oestrogen-receptor agonist, 4-OH-tamoxifen, also inhibited nitrite production and attenuated the suppression of phenylephrine contraction. Indeed, the presence of the oestrogen receptor has been confirmed in the rat aorta (Lin *et al*., 1986; Knauthe *et al*., 1996) and the role of oestrogen receptor activation has been demonstrated in 17$\beta$-oestradiol-induced regulation of rat aortic vascular smooth muscle growth (Orimo *et al*., 1995).

It is possible that the effect of oestrogen is mediated by the production of a substance which leads to the downregulation of iNOS protein. Such a factor could potentially be transforming growth factor-$\beta$1 (TGF-$\beta$1), which has been shown to mediate the effect of 17$\beta$-oestradiol in bone tissue by modulating osteoclast activity (Oursler *et al*., 1991). Oestrogen is a potent upregulator of TGF-$\beta$1-expression in the rat uterus, where tamoxifen has also been shown to have a similar effect (Sartor *et al*., 1995). Tamoxifen has been shown to upregulate TGF-$\beta$1 in the mouse aorta (Grainger *et al*., 1995). TGF-$\beta$1 is a likely candidate for the mediation of the effect of 17$\beta$-oestradiol, since it has been demonstrated to decrease the amount of iNOS mRNA and protein in several biological systems (Vodovotz *et al*., 1993; Perrella *et al*., 1994; Pinsky *et al*., 1995). To analyse whether the effect of oestrogen was mediated by subsequent increase in TGF-$\beta$1 production by the blood vessel in response to 17$\beta$-oestradiol treatment, cytokine-treated rings were co-incubated with anti-TGF-$\beta$1 antibody in the presence of oestrogen. The TGF-$\beta$1-antibody, which was able to reverse the effect of TGF-$\beta$1, did not affect the inhibitory effect of 17$\beta$-oestradiol on nitrite production, indicating that the effect of oestrogen on iNOS was not mediated by TGF-$\beta$1.

Induction of iNOS has been suggested to contribute to the pathomechanism of a variety of diseases, such as septic shock (Wizemann *et al*., 1994; Szabo *et al*., 1995), rheumatoid arthritis (Kaur & Halliwell, 1994), inflammatory bowel-disease (Rachmilewitz *et al*., 1993; Ma *et al*., 1995), multiple sclerosis (Okuda *et al*., 1995) and atherosclerosis (White *et al*., 1994). Inhibition of excessive nitric oxide production has been shown to provide some therapeutic benefits (Nava *et al*., 1992). However, non-specific inhibition of nitric oxide synthases in general can be detrimental (Nava *et al*., 1991; Harbrecht *et al*., 1992). Therefore, there is a need for isoenzyme-specific inhibition of iNOS. 17$\beta$-oestradiol has been shown to upregulate endothelial nitric oxide synthase in cultured endothelial cells (Hayashi *et al*., 1995; Hishikawa *et al*., 1995) as well as *in vivo* (Weiner *et al*., 1994). It has been postulated that the well documented vasculoprotective effect of 17$\beta$-oestradiol is mediated at least in part via increased NO production by endothelial nitric oxide synthase (Kauser & Rubanyi, 1994a; 1997).

In conclusion, the present data show that in contrast to upregulation of endothelial NO production in the same tissue (Goetz *et al*., 1994; Kauser & Rubanyi, 1995), 17$\beta$-oestradiol inhibits excessive NO production by iNOS in rat aorta by decreasing iNOS mRNA and protein expression. These

properties make this ovarian sex steroid hormone an ideal candidate for NOS isoenzyme regulation in pathological conditions associated with excessive NO production, where inhibition of iNOS expression/activity without reduction in endothelial NO production is a therapeutic goal.

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