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Oestrogenic Compounds Modulate Cytokine-induced Nitric Oxide Production in Mouse Osteoblast-like Cells

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Abstract

Nitric oxide (NO) is a mediator of bone metabolism with effects on both bone resorption and formation. Its production by both the constitutive and inducible isoforms of nitric oxide synthase (NOS) is affected by oestrogen in several types of cell and in tissues other than bone cells. Recently, oestrogens were found to increase basal NO production by osteoblasts via enhanced activity or expression, or both, of NOS-3. Inflammatory cytokines, however, increase NO by increasing the expression of NOS-2.

In this study we have examined whether cytokine-induced NO production by osteoblastic cells was affected by oestrogenic compounds by studying the effect of 17β-oestradiol and the anti-oestrogens ICI164,384 and 4-hydroxytamoxifen on cytokine-induced NO production in oestrogen receptor positive MC3T3-E1 osteoblast-like cells. Combinations of the inflammatory cytokines interleukin-1β, tumour necrosis factor-α, and interferon-γ with lipopolysaccharide stimulated NO production up to 11-fold. This cytokine-induced NO production was further increased dose-dependently by the anti-oestrogens ICI164,384 and 4-hydroxytamoxifen (133·3 $\pm$ 3·2% and 146·0 $\pm$ 13·2%, respectively). 17β-Oestradiol either had no effect on or slightly inhibited cytokine-induced NO production. It did, however, dose-dependently counteract the stimulatory effect of the anti-oestrogens. Concentrations of 17β-oestradiol needed to prevent the stimulatory effect of 4-hydroxytamoxifen were ca tenfold that of ICI164,384.

These findings show that, in addition to the stimulatory effect of oestrogen on basal NO production by NOS-3, cytokine-induced NO production is also affected by oestrogenic compounds in osteoblasts.

Nitric oxide (NO) is an ubiquitous molecule involved in the regulation of several biological processes. In bone, NO has been found to affect both osteoclastic bone resorption (MacIntyre et al 1991; Stern & Diamond 1992; Löwik et al 1994; Ralston et al 1995; Ralston & Grabowski 1996; Chae et al 1997) and osteoblast proliferation and activity in-vitro (Damoulis & Hauschka 1994; Ralston et al 1994; Hukkanen et al 1995; Riancho et al 1995a; Chae et al 1997). Osteoblasts synthesize small amounts of NO, but produce large amounts of NO when stimulated by cytokines (Damoulis & Hauschka 1994; Löwik et al 1994; Ralston et al 1994; Hukkanen et al 1995; Riancho et al 1995a, b; Chae et al 1997). NO is synthesized from L-arginine by three different isoforms of NO synthase (NOS)—the calcium-dependent neuronal and endothelial NOS (NOS-3 and NOS-1, respectively) and the inducible calcium-independent NOS (NOS-2). In osteoblasts, cytokine-stimulated NO production is caused by the induction of NOS-2 (Ralston et al 1994; Hukkanen et al 1995; Riancho et al 1995a, b; Ralston 1997).

Cytokines are important in the modulation of bone metabolism. It has been suggested that oestrogen, an important regulator of bone metabolism, alters the production of cytokines or changes the responsiveness of cells to these compounds, or both. NO as a mediator of bone loss induced by oestrogen deficiency was suggested by Wimalawansa et al (1996), who found that administration of the NO donor nitroglycerine prevented ovariectomy-induced bone loss, and that administration of the NOS inhibitor N^G-nitro-L-arginine

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Methyl Ester (L-NAME) abolished the preventive effect of 17β-oestradiol in rats.

The first observation of oestrogenic modulation of NO synthesis was reported by Hayashi et al (1992), who found basal NO release from aortic rings of female rabbits to be greater than that from those of male or ovariectomized female rabbits. In later studies they reported that oestrogen enhanced the activity of NOS-3 and, thereby, increased NO production by endothelial cells (Hayashi et al 1995, 1997). In-vivo, plasma levels of nitrate + nitrite, which reflect endogenous NO production, have been found to be positively affected by oestrogen both in man (Rosselli et al 1994, 1995; Ramsay et al 1995; Cicinelli et al 1996; Kawano et al 1996; Imthurn et al 1997) and in rats (van Bezooijen et al 1998). Stimulation of NO production by oestrogen has also been found in heart and skeletal muscle, and in the cerebellum (Weiner et al 1994; Hayashi et al 1995, 1997). In contrast, oestrogen has been found to inhibit NO production in hepatocytes (Pittner & Spitzer 1993), macrophages (Chao et al 1994; Hayashi et al 1997, 1998), uterine tissue (Yallampalli et al 1994) and vascular smooth muscle cells (Zancan et al 1999).

Oestrogenic modulation of NO production by osteoblasts has been reported only for osteosarcoma cells in man (Armour & Ralston 1998). In these cells, oestrogen enhanced the activity of a calcium-dependent isoform of NOS. In this work we have studied whether cytokine-stimulated NO production by osteoblasts is affected by oestrogenic compounds.

Materials and Methods

Chemicals

Alpha minimal essential medium (αMEM) was purchased from Gibco (Breda, The Netherlands), phenol red-free MEM from Sigma (Zwijndrecht, The Netherlands), penicillin and streptomycin from Flow Laboratories (Amstelstad, Zwanenburg, The Netherlands), and foetal calf serum from Integro (Zaandam, The Netherlands). Recombinant tumour necrosis factor-α (TNF-α) from man was purchased from Boehringer (Ingelheim, Germany) and lipopolysaccharide from *Escherichia coli* from Difco Laboratories (Detroit, MI). Recombinant rat interferon-γ and recombinant interleukin-1β from man were gifts from Dr P. H. van der Meide (Institute of Applied Radiobiology and Immunology, Rijswijk, The Netherlands) and from Dr M. Ponec (Department of Dermatology, Leiden University Hospital, The Netherlands), respectively. 17β-Oestradiol was provided by Organon (Oss, The Netherlands) and ICI164,384 (7α,17β-*N*-butyl-3,17-dihydroxy-*N*-methylestra-1,3,5(10)-triene-7-undecanamide) (Wakeling & Bowler 1987) was a generous gift from Dr A. E. Wakeling (Zeneca Pharmaceuticals, Macclesfield, UK). 4-Hydroxytamoxifen was purchased from Research Biochemicals International (Natick, MA).

Cell cultures

Mouse osteoblastic MC3T3-E1 cells were seeded at a density of 50 000 cells/1·8 cm^2 and cultured until confluence (4 days) in αMEM (500 μL) supplemented with 10% heat-inactivated foetal calf serum. At confluence, cells were pre-incubated for 24 h without or with 17β-oestradiol or anti-oestrogens, or both, in phenol red-free MEM (500 μL) supplemented with 10% heat-inactivated foetal calf serum. Identical results were obtained when cell cultures were pre-incubated for 24 or 72 h with 17β-oestradiol or anti-oestrogens, or both. After pre-incubation, cell cultures were treated by simul-taneous addition of 17β-oestradiol or anti-oestrogens, or both, and combinations of the cytokines interleukin-1β, TNF-α, and interferon-γ, and lipopoly saccharide. Charcoal-treated heat-inactivated foetal calf serum was not used because it reduced cytokine-induced NO production by 63·7% compared with cultures supplemented with heat-inactivated foetal calf serum (94·5 compared with 260 nmol (mg cellular protein)$^{-1}$, respectively). Whenever no 17β-oestradiol or no anti-oestrogens were added to the cell cultures, ethanol was added to furnish equal concentrations of ethanol (0·2%) in all experimental groups.

Nitrite production

NO was measured as nitrite production in culture media from confluent cultures. Although both nitrite and nitrate are stable end-products of NO, nitrite measurement has been shown to be a good reflection of in-vitro NO production and a strong relationship ($r = 0{\cdot}99$) between nitrite and nitrate production by osteoblast-like cells has previously been reported (Ralston et al 1995). Changes in NO production will, therefore, result in similar changes in nitrite and nitrate concentrations. The amount of nitrite released by cells was determined by use of Griess reagent comprising sulphanilamide (0·5%), naphthylethylenediamine dihydrochloride (0·05%), and H_3PO_4 (2·5%) (Green et al 1982). Briefly, culture supernatant (70 μL) was mixed with Griess reagent (70 μL) and incubated in a 96-well plate for 10 min at room temperature with continuous shaking (Ding et al 1988; Migliorini et al 1991). Nitrite concentration, proportional to OD_{550}

(optical density at 550 nm) was determined by use of a microtitre plate reader (Thermomax; Molecular Devices, Menlo Park, CA) with a 650-nm reference filter and with reference to a standard curve constructed after measurement of serial dilutions of sodium nitrite. Total cellular protein content was measured by means of the BCA protein assay (Pierce, Rockford, IL).

Statistics

Values are expressed as nmol NO (mg cellular protein)$^{-1}$ or as percentages of cytokine-induced NO production (means ± s.e.m.). Statistical differences between values were examined by one-way analysis of variance for multiple comparisons then by Fischer's protected least significant difference test.

Results

Confluent (4 days) MC3T3-E1 cells produced low levels of NO (17·5 nmol (mg cellular protein)$^{-1}$) which were not affected by treatment for 24 h with either 17β-oestradiol or the anti-oestrogens ICI164,384 and 4-hydroxytamoxifen at concentrations up to 10^{-6} M (data not shown). Stimulation for 24 h with a combination of the cytokines TNF-α (1 ng mL^{-1}), interferon-γ (10 units mL^{-1}), and interleukin-1β (1 ng mL^{-1}) with lipopolysaccharide (100 ng mL^{-1}) increased NO production by MC3T3-E1 cells 2·4-(TNF-α + interferon-γ) to 10·9-fold (TNF-α + interferon-γ + interleukin-1β), depending on the cytokine combinations used (Table 1). Simultaneous addition of 17β-oestradiol (up to 10^{-6} M) to the cytokine combinations had no effect on the production of NO (data not shown). In contrast, simultaneous addition of the anti-oestrogen ICI164,384 (10^{-7} M) increased NO production significantly (Table 1). This increase was prevented by addition of 17β-oestradiol (10^{-7} M).

ICI164,384 and 4-hydroxytamoxifen increased cytokine-induced NO production dose-dependently (Figure 1). 4-Hydroxytamoxifen was more potent—it increased NO production significantly even at a concentration of 10^{-11} M; with ICI164,384 this occurred at a concentration of 10^{-7} M. 17β-Oestradiol alone had no effect on cytokine-induced NO production, although a concentration of 10^{-6} M caused slight inhibition.

Further investigations were performed to determine whether the increase in cytokine-induced NO production by the anti-oestrogens ICI164,384 and 4-hydroxytamoxifen could be counteracted by 17β-oestradiol. The increase in cytokine-induced NO

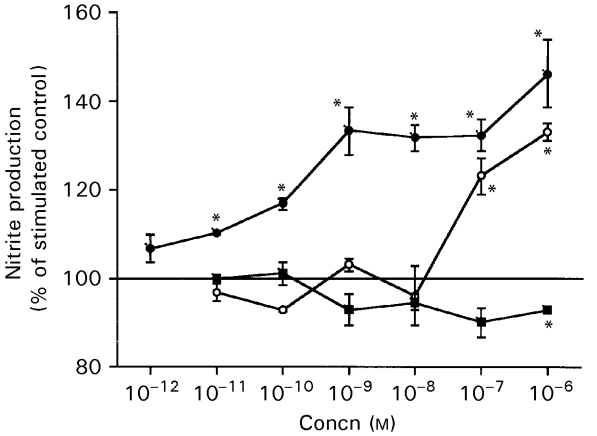

Figure 1. Dose-dependency of the effects of 17β-oestradiol (■) and the anti-oestrogens ICI164,384 (○) and 4-hydroxytamoxifen (●) on cytokine-induced nitric oxide production. After 4 days culture confluent MC3T3-E1 cells were pre-incubated for 24 h with increasing concentrations of 17β-oestradiol, ICI164,384 or 4-hydroxytamoxifen and subsequently stimulated for 24 h with the cytokine combination interleukin-1β, TNF-α and interferon-γ in the presence or absence of 17β-oestradiol, ICI164,384, or 4-hydroxytamoxifen. *$P < 0.05$ compared with stimulated cultures.

Table 1. Effects of the anti-oestrogen ICI164,384 and of 17β-oestradiol on cytokine-induced nitric oxide production.

	Concn nitrite (nmol (mg cellular protein)$^{-1}$)		
	Control	ICI164,384	ICI164,384 + 17β-oestradiol
Control	23 ± 1	n.d.	n.d.
Tumour necrosis factor-α + interferon-γ	57 ± 6	92 ± 12	56 ± 8
Tumour necrosis factor-α + interferon-γ + lipopolysaccharide	122 ± 2*	151 ± 7	129 ± 13
Tumour necrosis factor-α + interferon-γ + interleukin-1β	250 ± 34*	371 ± 31##	243 ± 18†
Tumour necrosis factor-α + interferon-γ + lipopolysaccharide + interleukin-1β	241 ± 14*	309 ± 4#	191 ± 3†

After 4 days culture, confluent MC3T3-E1 cells were pre-incubated for 24 h with ICI164,384 (10^{-7} M) or with ICI164,384 (10^{-7} M) + 17β-oestradiol (10^{-7} M) and subsequently stimulated for 24 h with combinations of the cytokines interleukin-1β, tumour necrosis factor-α, interferon-γ and lipopolysaccharide in the presence of ICI164,384 or ICI164,384 + 17β-oestradiol. *$P < 0.0001$ compared with control. #$P < 0.005$, ##$P < 0.0001$ compared with non-treated cultures stimulated with the same cytokine combination. †$P < 0.0001$ compared with ICI164,384-treated cultures stimulated with the same cytokine combination. Results are means ± s.e.m. Similar results were obtained in four other experiments.

production elicited by ICI164,384 (10^{-7} M) was dose-dependently counteracted by 17β-oestradiol ($\geq 10^{-8}$ M) (Figure 2). To counteract the stimulatory effect of 4-hydroxytamoxifen (10^{-7} M), 10-fold higher concentrations of 17β-oestradiol were needed.

Discussion

These findings show that cytokine-induced NO production by mouse osteoblast-like MC3T3-E1 cells is modulated by anti-oestrogens and 17β-oestradiol. 17β-Oestradiol alone had no effect or slightly inhibited cytokine-induced NO production. This is in accordance with the previously reported lack of effect of 17β-oestradiol on basal and interleukin-1-induced NO production by MC3T3 cells and primary osteoblasts isolated from neonatal mouse calvariae (Riancho et al 1995b). In the current study, however, NO production induced by combinations of the cytokines TNF-α, interleukin-1β and interferon-γ with lipopolysaccharide was further, and dose-dependently, increased by the anti-oestrogens ICI164,384 and 4-hydroxytamoxifen. This stimulatory effect was counteracted by 17β-oestradiol, suggesting that it was mediated via the oestrogen receptor. Although 17β-oestradiol, 4-hydroxytamoxifen and ICI164,384 have comparable binding affinities for purified oestrogen receptors, concentrations of 17β-oestradiol needed to prevent the increase in NO production induced by 4-hydroxytamoxifen were ca 10-fold that needed to prevent the increase induced by ICI164,384. This might be because the relative binding affinity of ICI164,384 to cytosolic preparations of the oestrogen receptor protein was between one fifth and one tenth that of 4-hydroxytamoxifen and 17β-oestradiol (Pons et al 1984; Wilson et al 1990; Emmas et al 1992). The low relative binding affinity of ICI164,384 might be because of its extremely high non-specific binding in such preparations (Wilson et al 1990).

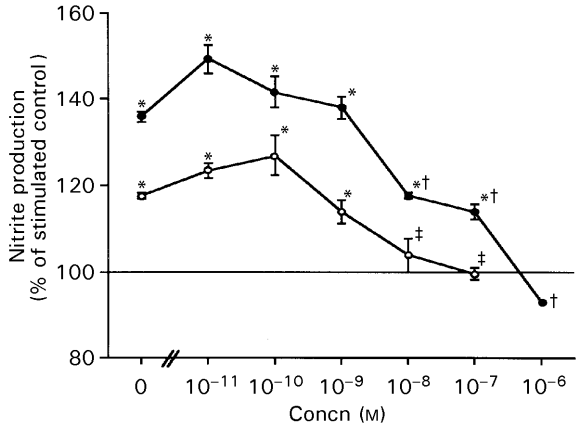

Figure 2. 17β-Oestradiol dose-dependently counteracted the effect of the anti-oestrogens ICI164,384 (○) and 4-hydroxytamoxifen (●) on cytokine-induced nitric oxide production. After 4 days culture confluent MC3T3-E1 cells were pre-incubated for 24 h with ICI164,384 (10^{-7} M) or 4-hydroxytamoxifen (10^{-7} M) in the presence or absence of increasing concentrations of 17β-oestradiol and subsequently stimulated for 24 h with the cytokine combination interleukin-1β, TNF-α and interferon-γ in the presence or absence of ICI164,384 or 4-hydroxytamoxifen and with or without increasing concentrations of 17β-oestradiol. *$P < 0.05$ compared with stimulated cultures. †$P < 0.05$ compared with stimulated cultures treated with 10^{-7} M 4-hydroxytamoxifen. ‡$P < 0.05$ compared with stimulated cultures treated with 10^{-7} M ICI164,384.

The stimulatory effect of the anti-oestrogens on cytokine-induced NO production suggests a basic activity of the oestrogen receptor of the MC3T3-E1 cells, possibly because of the oestrogenic activity of foetal calf serum (Bondy & Zacharewski 1993) or because of non-oestrogenic agents that activate the oestrogen receptor in a ligand-independent way (Power et al 1991; Aronica & Katzenellenbogen 1993), or both. In the current study foetal calf serum was not charcoal-treated because this reduced, by 63·7%, the capacity of cytokines to induce NO production. This decrease was not because of loss of oestrogens, because addition of exogenous 17β-oestradiol did not restore the capacity of cytokines to induce NO production (data not shown). Charcoal treatment probably removes from the foetal calf serum other co-factors that are needed for maximum production of NO in response to cytokine stimulation.

Previously reported effects of oestrogen on NO production include stimulation of NO production by constitutive isoforms of NOS in heart, kidney, skeletal muscle, cerebellum (Weiner et al 1994) and endothelial cells (Hayashi et al 1995) and inhibition of NO production in hepatocytes (Pittner & Spitzer 1993), macrophages (Chao et al 1994; Hayashi et al 1997, 1998), uterine tissue (Yallampalli et al 1994), and vascular smooth muscle cells (Zancan et al 1999). In hepatocytes, macrophages and vascular smooth muscle cells, NO production was catalysed by NOS-2, whereas the rapidity of induction of relaxation by L-arginine in uterine tissue was indicative of the involvement of a constitutive isoform of NOS. In summary, NO production by the constitutive isoforms of NOS has been reported to be both increased and inhibited by oestrogen, whereas NO production by NOS-2 has been found to be inhibited only.

In-vivo, positive correlation has been found between nitrate + nitrite plasma levels and serum oestrogen levels in man (Rosselli et al 1994, 1995; Ramsay et al 1995; Cicinelli et al 1996; Kawano et al 1996; Imthurn et al 1997). Similarly, we have recently reported that systemic endogenous NO production is reduced after ovariectomy and can be

increased by treatment of ovariectomized rats with oestrogen (van Bezooijen et al 1998). Together with the inhibitory effect of NO on osteoclastic resorption and its stimulatory effect on osteoblast proliferation and activity, these findings support the hypothesis that reduced NO production might be involved in the bone loss induced by oestrogen deficiency. If NO is involved in the protective effect of oestrogen on bone, it must be produced locally within the bone microenvironment, for example by osteoblasts, because systemic endogenous NO production does not correlate with trabecular bone mineral density (van Bezooijen et al 1998).

NO as a mediator of the protective effect of oestrogen on bone was suggested by Wimalawansa et al (1996). They reported that treatment with the NO-donor nitroglycerine prevented bone loss in ovariectomized rats. Furthermore, the protective effect of oestrogen against bone loss after ovariectomy was abolished by the NOS inhibitor L-NAME, although L-NAME did not further reduce bone mineral density in ovariectomized rats. Similarly, L-NAME did not induce bone loss when given to non-operated rats (Tsukahara et al 1996). In that study, however, L-NAME at a higher dose of 80 mg day^{-1} did slightly reduce bone mineral density. Administration of aminoguanidine, a relatively selective inhibitor of NOS-2 (Misko et al 1993; Joly et al 1994), caused bone loss in rats, irrespective of their oestrogen status (Kasten et al 1994). In elderly women Jamal et al (1998) reported a positive association between bone mineral density and nitrate use. Intermittent nitrate use was more associated with greater benefits than daily use. These findings suggest that NO production catalysed by calcium-dependent isoforms of NOS, rather than by NOS-2, is involved in bone loss induced by oestrogen deficiency. In accordance with this is the observation that 17β-oestradiol increased basal NO production by osteosarcoma TE85 cells in man (Armour & Ralston 1998). This increase was reversed by the calcium chelator EGTA, implying activation of a calcium-dependent isoform. Further mRNA analysis implicated the involvement of NOS-3.

In physiology and during hormone replacement therapy it is important to realize that other factors, for example progesterone, might also affect NO production. Progesterone has been reported to affect NO production both in-vitro (Pittner & Spitzer 1993; Chao et al 1994; Zancan et al 1999) and in-vivo (Rosselli et al 1994, 1995; Ramsay et al 1995; Cicinelli et al 1996; Kawano et al 1996; Imthurn et al 1997; van Bezooijen et al 1998).

In conclusion, cytokine-induced NO production by MC3T3-E1 osteoblast-like cells is affected by oestrogenic compounds. The anti-oestrogens ICI164,384 and 4-hydroxytamoxifen increased cytokine-induced NO production, and 17β-oestradiol dose-dependently prevented this increase.

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