Stimulation of vascular protein synthesis by activation of oestrogen receptor β

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Abstract

The objective of this study was to investigate the effects of oestrogen receptor (ER) β activation on vascular protein synthesis and protein expression. Nuclear immunoreactivity towards ER β was observed abundantly in vascular smooth muscle and endothelial cells of mouse aorta. No ER α -positive cell nuclei were observed. In aorta from ovariectomized mice, treatment with the selective ER β agonist genistein (100 nM) for 24 h increased [³H]leucine incorporation by about 30%. This effect was prevented by the ER blocker ICI 182780 (10 μ M). Although genistein treatment stimulated protein synthesis, it caused no change in total protein determined either by the Lowry method on tissue homogenate or by densitometric scanning of

protein bands (10–220 kDa) separated by SDS-PAGE. Separation of [³⁵S]methionine-labelled proteins by SDS-PAGE did not reveal the protein(s) stimulated by genistein. DNA synthesis was not affected by 100 nM genistein, suggesting that genistein-induced stimulation of protein synthesis is not part of a growth response. Protein expression, determined by SDS-PAGE, was similar in aorta from ER β -knockout and wild-type mice, suggesting that expression of vascular proteins does not depend solely on a functional ER β gene. We suggest that activation of vascular ER β stimulates synthesis of proteins and that this response is not associated with vascular growth.

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Introduction

Two oestrogen receptor (ER) subtypes (α and β) have been isolated and cloned (Green *et al.* 1986, Greene *et al.* 1986, Kuiper *et al.* 1996). Some domains of ER β cDNA are highly homologous to those of ER α cDNA and the overall homology between ERs β and α is about 50%. Upon ligand binding and activation, the receptor–ligand complex interacts with specific oestrogen response elements located in the promoter region of oestrogen-responsive genes, regulating their transcriptional activity (for reviews see Grandien *et al.* 1997, Muramatsu & Inoue 2000). Target genes for activated ER β and patterns of protein expression regulated by this receptor are important issues and are in the focus of current research.

Oestrogen increases uterine blood flow during pregnancy and during the oestrus cycle, but the mechanism(s) responsible for this effect remains unclear (Mendelsohn & Karas 1999). Vascular expression of mRNA for ER β has been demonstrated in rat aorta and carotid artery and in mouse aorta (Iafrazi *et al.* 1997, Lindner *et al.* 1998, Mäkelä *et al.* 1999). Recently, we have detected nuclear

expression of ER β protein in different vascular segments of female rats (Andersson *et al.* 2001). Abundant expression of ER β was detected in medial smooth muscle and endothelial cells. In contrast, ER α was observed only in occasional uterine vessel smooth muscle and endothelial cells. We have previously shown that both the maximal noradrenaline-evoked effect and the sensitivity to noradrenaline are similar in aorta from ER β -knockout and wild-type mice (Nilsson *et al.* 2000). These data thus suggest similar expression of vascular proteins in ER β -knockout and wild-type mice.

The aim of the present study was to investigate the importance of vascular ER β in the regulation of DNA and protein synthesis. Protein and DNA synthesis in aorta from ovariectomized mice was determined by incorporation of [³H]leucine, [³⁵S]methionine and [³H]thymidine after stimulation with the selective ER β agonist, genistein. Proteins were separated by SDS-PAGE and protein expression in vascular tissue from ER β -knockout female mice was compared with that in wild-type mice using SDS-PAGE. The results suggest that ER β activates vascular protein synthesis and that this is not part of a growth response.

Figure 1 Immunostainings with ER β antibody (dilution 1:1000). Nuclear expression of ER β was observed in medial smooth muscle and in endothelial cells of aorta from unoperated (A) and ovx (C) mice. Arrows show positively stained endothelial cells of the tunica intima (i); arrowheads show positively stained smooth muscle cells of the tunica media (m). No immunoreactivity was observed if the primary antibody was omitted (B and D). Bar represents 50 μ m (for A–D).

Materials and Methods

Animals and tissue

Adult (3-month-old) NMRI female mice (24–30 g) were anaesthetized with pentobarbital sodium (75 mg/kg i.p.) and the ovaries removed bilaterally. After a 7-day period of recovery with food and water available *ad libitum*, the ovariectomized (ovx) mice were killed by cervical dislocation. Mice were fed a standard rodent diet (R36, Lactamin, Stockholm, Sweden). This diet contains about 5% soy bean. Although soy bean is rich in phytoestrogens such as genistein, this was unlikely to exert any influence, as the experiments were performed *in vitro*. The experiments were approved by the Ethics Committee at Lund University. The thoracic aorta from the diaphragm to the aortic arch was removed under sterile conditions and dissected free from fat and connective tissue in cold

Ca²⁺-free Krebs-buffered solution (for composition, see below). The aorta was cut open through the lumen and divided into three pieces of tissue. The tissues were incubated with or without genistein (10–10 000 nM) in Petri dishes with Ca²⁺-containing (2.5 mM) Krebs buffer or Dulbecco's Modified Eagle's Medium (Sigma Chemicals, St Louis, MO, USA) without phenol red at 37 °C in a water-jacketed tissue/cell incubator for 24 h. The pure ER blocker ICI 182780 was introduced 1 h before genistein and was then present throughout the incubation.

Adult (3-month-old) female mice lacking functional ER β (–/–) were generated by targeted disruption of exon three of the *ER β* gene as described previously (Krege *et al.* 1998). Their wild-type (+/+) litter mates served as controls. Lack of ER β in –/– mice was confirmed by RT-PCR for ER β mRNA and by Western blot showing

Figure 2 Immunostainings with ER α antibody (dilution 1:100). No expression of ER α was observed in aorta from unoperated (A) and ovx (B) mice. (C) Section of uterus with positively stained glandular epithelial and endometrial stroma cells. i, tunica intima; m, tunica media. Bar represents 50 μ m (for A–C).

the absence of ER β mRNA and protein in $-/-$ but not in $+/+$ mouse tissues.

Immunocytochemistry

Tissue specimens were fixed in 4% buffered formalin solution, dehydrated and then embedded in paraffin. The preparations were cut in 4- μ m transverse sections, deparaffinized and treated for 15 min with citrate buffer (pH 6.0) in a microwave oven before immunostaining (Shi *et al.* 1991). The ER β antibody (code no ER β 503) was raised in chicken and has been characterized previously (Saji *et al.* 2000, Andersson *et al.* 2001). The ER α antibody was a monoclonal mouse antibody (ER1D5; Dako A/S, Glostrup, Denmark). In preliminary experiments, the dilution of ER β and ER α antibodies was titrated to achieve optimal nuclear staining. The slides were stained in an automatic immunostainer TechMate 500 (Ventana Biotech, Tuscon, AZ, USA) with DAKO ChemMate Detection Kit peroxidase/3–3' diaminobenzidine. For ER β , the secondary antibody was a biotinylated rabbit anti-chicken IgY (1:5000, Jackson Immuno Research

Laboratories, West Grove, PA, USA). At each staining, a positive control (uterus) was run in parallel; for negative controls, the primary antibodies were omitted. No immunoreactivity was observed after omission of the ER β or the ER α antibody. Tissues for immunocytochemistry were obtained from five mice and at least two stained slides were analysed for each individual tissue.

Determination of DNA and protein synthesis

After the preincubation with genistein, either radio-labelled thymidine or radiolabelled leucine was added. Ten μ Ci [methyl- 3 H]thymidine or L-[4,5- 3 H]leucine was present for the last 1 h (3 H]thymidine) or the last 18 h (3 H]leucine) of the 24 h incubation with genistein. The incubation was stopped by placing the Petri dishes on ice. The tissue was washed, weighed and homogenized by sonication for 4×10 s in 5 mM NaOH. Aliquots of the homogenate were precipitated with 5% trichloroacetic acid (TCA) on ice and centrifuged at 10 000 r.p.m. for 2 min at 4 °C. For every determination, double samples were analysed. The pellet was washed twice with 5%

Figure 3 Effects of genistein on protein synthesis (A) and on total protein content (B) in aorta from ovx mice. Protein synthesis was determined by [³H]leucine incorporation. The pure ER blocker ICI 182780 (10 μ M) was introduced 1 h before genistein (100 nM) and was present throughout the incubation. Incorporation of [³H]leucine was determined as d.p.m./mg tissue and data are presented as normalized values. Incorporation of radiolabelled leucine in controls, not receiving genistein, was set to 100%. Total protein data are presented as mg protein/mg tissue wet weight. Values are means $\pm$ S.E.M.; $n=3-6$.

TCA and centrifuged as above and then dissolved in Soluene 350 at room temperature for 2 h. A liquid scintillation cocktail was added and the radioactivity measured in a liquid scintillation counter (Beckman LS6500, Beckman Instruments Inc., CA, USA). Mean radioactivity (d.p.m.) in double samples was calculated. The amount of incorporated [³H]thymidine and [³H]leucine was computed as d.p.m./mg tissue.

Determination of total protein and protein separation

The tissue was weighed and pulverized in liquid N₂ and transferred to SDS sample buffer (for composition, see below). The sample buffer volume was 100 μ l/mg tissue.

Figure 4 Separation of vascular proteins by SDS-PAGE after stimulation of aorta from ovx mice with 100 nM genistein. (A) Silver-stained gel (one of three independent experiments). (B) Autoradiograph of [³⁵S]methionine-labelled proteins (one of six independent experiments). In this experiment, radiolabelled methionine was present for 18 h. Separation of proteins were performed on 7.5–15% gels to obtain good separation in the range 10–200 kDa. The optical density (OD) of the bands (OD/mm²) was determined using a GS-710 Imaging Densitometer (BioRad) and Quantity One quantitation software (BioRad).

Figure 5 Effects of genistein on DNA synthesis. DNA synthesis was determined by [³H]thymidine incorporation. Aorta from ovx mice was incubated with or without 100 nM genistein and [³H]thymidine was present for the last 1 h of incubation. Genistein had no effect on DNA synthesis. The well-known growth factor, fetal calf serum (FCS), was used as positive control. Values are means $\pm$ S.E.M.; $n=3-10$.

After incubation for 30 min on ice and sonication (10 s), the suspension was boiled for 3 min and centrifuged at 14 000 r.p.m. for 10 min at 4 °C. Supernatant was collected and total protein was determined by using a BioRad protein assay based on the Lowry method (Lowry *et al.* 1951). Proteins were separated using SDS-PAGE on a BioRad minigel system using 7.5% and 15% gels. Protein

Figure 6 Separation of proteins in aorta from ER β -knockout ($-/-$) and wild-type ($+/+$) mice using SDS-PAGE. Equal amounts of protein were added to each lane. Good separation of proteins between 6 and 200 kDa was obtained by using 7.5% and 15% gels. Homogenates from 5 $-/-$ and 5 $+/+$ mice were used and $-/-$ and $+/+$ samples were loaded on each gel in duplicate. In all, four 15% and three 7.5% gels were run. The optical density (OD) of the bands (OD/mm²) was determined using a GS-710 Imaging Densitometer (BioRad) and Quantity One quantitation software (BioRad).

concentration of the homogenate was determined as described above. Equal amounts of protein were always loaded on each lane. After electrophoresis, the gels were silver stained using a silver staining kit from Amersham Pharmacia Biotech AB, Uppsala, Sweden.

Autoradiography of [³⁵S]methionine incorporation

After preincubation with 100 nM genistein, 25 μ Ci radio-labelled ([³⁵S]) methionine was added. The isotope was present for the last 2, 6 or 18 h of the 24 h incubation with genistein. At the end of incubation, the reaction was stopped by placing the tissue on ice. After protein extraction and separation as described above the gels were dried in a Gel-drier and then exposed to X-ray film for 24 h.

Solutions

The Krebs-buffered solution had the following composition (in mM): KCl 4.7, NaHCO₃ 15.5, KH₂PO₄ 1.2, MgCl₂ 1.2, NaCl 122, glucose 11. The SDS sample buffer was of the following composition: Tris HCl 62.5 mM (pH6.8), 2% SDS, 10% glycerol, 1 mM phenylmethylsulphonylfluoride. For protein separation using SDS-

PAGE, 2-mercaptoethanol (final concentration 5%) and bromophenol blue (final concentration 0.001%) were added.

Chemicals

Genistein and 17 β -oestradiol were from Sigma Chemical Co. ICI 182780 was a kind gift from Zeneca Pharmaceuticals, Macclesfield, Cheshire, UK. L-[4,5-³H]Leucine, methyl-[³H]thymidine, and [³⁵S]methionine were from Amersham, Amersham, Bucks, UK. Genistein, 17 β -oestradiol and ICI 182780 were dissolved in dimethyl sulphoxide (DMSO). Controls received DMSO (<0.1%) as vehicle.

Statistics

Values are means $\pm$ S.E.M. Student's two-tailed *t*-test for unpaired comparisons was used to determine statistical significance. For multiple comparisons, the Bonferroni correction was used. *P* values less than 0.05 were considered to denote statistical significance.

Results

As seen in Fig. 1A, nuclear immunoreactivity towards ER β was observed in smooth muscle and in endothelial cells of mouse aorta. In ovx mice, vascular expression of ER β was similar to that of unoperated animals (Fig. 1C). No immunoreactivity was observed after omission of the ER β antibody (Fig. 1B, D). No immunoreactivity towards ER α was observed in either smooth muscle or endothelial cells of unoperated and ovx animals (Fig. 2A, B). In Fig. 2C, a positive control (uterus) for ER α is shown. Immunoreactivity towards ER α was observed in glandular epithelial cells and in stroma cells of the endometrium.

Activation of vascular ER β with genistein (100 nM) stimulated protein synthesis by 26% as measured by [³H]leucine incorporation (Fig. 3A). This effect was inhibited by the pure ER blocker ICI 182780. Total vascular protein concentration was not changed by genistein treatment (Fig. 3B). In addition, prolonged (48 h) treatment with genistein had no effect on total protein (0.054 ± 0.004 mg protein/mg tissue in genistein treated, compared with 0.046 ± 0.003 mg protein/mg tissue in controls; $n=6$). High concentrations (10 μ M) of genistein caused a decrease ($P<0.05$) in [³H]leucine incorporation of about 40% ($463\,000 \pm 64\,000$ d.p.m./mg tissue in treated mice and $768\,000 \pm 94\,000$ d.p.m./mg tissue in controls; $n=4$). Combination of 10 μ M genistein with 17 β -oestradiol (10 nM) had no effect ($717\,000 \pm 104\,000$ d.p.m./mg tissue in treated mice and $768\,000 \pm 94\,000$ d.p.m./mg tissue in controls; $n=4$).

The optical density of protein bands (10–200 kDa) separated by SDS-PAGE was not different in genistein-

treated and control tissues (Fig. 4A). In order to identify those protein bands in which new synthesis occurred after stimulation with genistein, separation of [35 S]methionine-labelled proteins (10–200 kDa) by SDS-PAGE was performed. The autoradiographs were analysed by determination of the optical density of the different protein bands. Radiolabelled methionine was present for 2, 6 and 18 h of the 24 h incubation time. At 2 and 6 h, weaker incorporation of radiolabelled methionine was observed compared with that at 18 h. No genistein-stimulated [35 S]methionine incorporation was detected at any time point (Fig. 4B).

In order to investigate the effect of genistein on vascular growth, we measured incorporation of [3 H]thymidine into DNA. As seen in Fig. 5, genistein (100 nM) had no effect on DNA synthesis, whereas the well-known growth factor, fetal calf serum, stimulated [3 H]thymidine incorporation by two to three times.

Vascular protein expression in ER β -knockout (–/–) and wild-type (+/+) mice was tested by SDS-PAGE. Expression of 10–200 kDa proteins was similar in aorta from –/– mice and in aorta from their wild-type litter mates (Fig. 6). Analysis of protein bands by determination of optical density did not reveal any differences in protein expression between –/– and +/+ animals.

Discussion

ER β seems to be the predominant ER isoform expressed in the rat and human vascular wall (Hodges *et al.* 2000, Andersson *et al.* 2001, Critchley *et al.* 2001). As shown in the present study, this seems also to be the case in mouse vascular tissue. We report here that activation of mouse aorta ER β with the selective ER β agonist, genistein, in nM concentration caused a 30% increase in protein synthesis as determined by [3 H]leucine incorporation, but no change in total protein determined either by a modified Lowry method on tissue homogenate or by densitometric scanning of protein bands separated by SDS-PAGE. The increase in protein synthesis was ER-dependent, as it could be blocked by the ER antagonist, ICI 182780. Protein synthesis determination using [35 S]methionine labelling and SDS-PAGE did not disclose any protein bands with increased incorporation. Thus it remains an open question which proteins in the vascular wall are regulated by ER β . Genistein had no effect on DNA synthesis, showing that activation of ER β causes no stimulation of vascular growth. This suggests that activation of ER β causes a specific stimulation of protein synthesis, which is not part of a growth response.

Genistein is an isoflavonoid phytoestrogen present in, for example, dietary soy. Genistein binds to ER β nearly as well as does 17 β -oestradiol, whereas its affinity for ER α is much lower than that of 17 β -oestradiol (Kuiper *et al.* 1998). Genistein has more than 20 times greater affinity

for ER β than for ER α , and studies based on competition between genistein and radiolabelled 17 β -oestradiol for ER α and β have shown that nM concentrations of genistein preferentially activate ER β (Kuiper *et al.* 1998). It may be argued that genistein not only activates ER β but also inhibits protein tyrosine kinases. In vascular smooth muscle cells, genistein blocks tyrosine kinase-dependent events such as proliferation and contraction (Epstein *et al.* 1997, Nelson *et al.* 1997, Schonherr *et al.* 1997). To obtain these effects, however, one needs much greater concentrations (>10 μ M) of genistein than those necessary for activation of ER β . Here, we have observed that genistein increased protein synthesis in nM concentrations, involving activation of ER β , but decreased protein synthesis in μ M concentrations, involving effects on both ER and tyrosine kinases. Interestingly, the effect of high concentrations of genistein was counteracted by 17 β -oestradiol. The stimulation of protein synthesis by nM concentrations of genistein, observed in this study, was prevented by the ER blocker ICI 182780, strongly suggesting that genistein exerted its effect through ER β and not through tyrosine kinase inhibition.

Protein separation by SDS-PAGE using gels that give good separation of proteins in the range of 10–200 kDa did not reveal any differences in protein expression, as determined by analysis of band optical density, between aorta from ER β -knockout and wild-type mice. One explanation could be that ERs β and α regulate expression of the same proteins, and that ER α takes over if ER β is absent. We have previously shown that aorta from ER β -knockout and wild-type mice, respectively, is equally sensitive to noradrenaline (Nilsson *et al.* 2000). ER β is thus not the sole crucial factor for the expression of proteins involved in the regulation of vascular contractility, indicating some degree of redundancy of ER α and ER β function in the vessels. Further studies using ER α -ER β double-knockout mice should help clarify this issue.

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