Increased magnitude of relaxation to oestrogen in aorta from oestrogen receptor β knock-out mice

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Abstract

Micromolar concentrations of the biologically active oestrogen 17 β -oestradiol reduce agonist-induced force in vascular preparations through an unidentified mechanism. The aim of the present study was to investigate the importance of oestrogen receptor β (ER β) for oestrogen-induced vascular relaxation. 17 β -oestradiol was added to aortic rings from ER β knock-out (-/-) and wild-type (+/+) mice precontracted with noradrenaline. 17 β -oestradiol caused a concentration-dependent (1–100 μ M) relaxation of aortic rings from both -/- and +/+ animals of both sexes. Rings from male and female -/- mice were more sensitive to 17 β -oestradiol than those from

+/+ mice. Medial thickness, determined by computerized image analysis, was similar in rings from -/- and +/+ animals. Endothelium, as determined by immuno-cytochemistry, was present in -/- and +/+ aorta. Maximal noradrenaline evoked force and sensitivity to noradrenaline were similar in both groups. In summary ER β modulates vascular relaxation to μ M concentrations of oestrogen; lack of ER β renders the vascular wall supersensitive to 17 β -oestradiol. Lack of ER β caused no change in vascular wall morphology suggesting that this ER subtype is not involved in vascular structure development.

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Introduction

17 β -oestradiol causes, in μ M concentrations, reversible relaxation of precontracted vascular preparations (Jiang *et al.* 1992, Kitazawa *et al.* 1997). Relaxation is mainly evoked by 17 β -oestradiol while other oestrogens such as 17 α -oestradiol, oestriol or oestrone have a much weaker effect in equimolar concentrations (Kitazawa *et al.* 1997). Previous studies have reported that vascular relaxation to oestrogen is observed in endothelium-denuded preparations (Freay *et al.* 1997, Jiang *et al.* 1992, Kitazawa *et al.* 1997) showing that neither endothelial nitric oxide nor any other factor produced by the endothelium mediates relaxation. In addition, relaxation to oestrogen has been observed in the presence of the nitric oxide synthase inhibitors *N*^G-nitro-L-arginine and *N*^ω-nitro-L-arginine (Freay *et al.* 1997, Lydrup & Nilsson 1996, Ma *et al.* 1997). Thus, relaxation to μ M concentrations of oestrogen is evoked via a direct effect on vascular smooth muscle cell properties.

In vascular preparations precontracted with KCl or noradrenaline acute relaxation following exposure to 17 β -oestradiol is observed (Kitazawa *et al.* 1997). The effect of 17 β -oestradiol is potentiated with time of exposure (Freay *et al.* 1997, Lydrup & Nilsson 1996). Pretreatment with the protein synthesis inhibitor cycloheximide or the RNA synthesis inhibitor actinomycin D has no effect on 17 β -oestradiol-induced vascular relaxation (Freay *et al.* 1997, Kitazawa *et al.* 1997). The rapid onset of relaxation and the

lack of effect of protein and RNA synthesis inhibition suggest that vascular relaxation is a non-genomic effect of 17 β -oestradiol.

Two oestrogen receptor (ER) subtypes (α and β) have been identified. ER α expression, detected by RT-PCR has been found in rat uterus, testis, pituitary, ovary, kidney and adrenals (Kuiper *et al.* 1997). Kuiper and co-workers (1996) cloned a novel ER subtype (β) from rat prostate using Northern blot and RT-PCR. ER β expression was found in a number of organs from the rat such as prostate, ovary, testis, bladder, lung, uterus, small intestine and brain (Kuiper *et al.* 1996, 1997, Saunders *et al.* 1997). Recently, Saji *et al.* (2000) have shown high expression of ER β in rat mammary gland. Specific and high affinity binding sites for radiolabelled 17 β -oestradiol have been found in mouse and rat aorta (Freay *et al.* 1997, Lin & Shain 1985). In rat aortic smooth muscle cells ER α expression has been visualized using immuno-cytochemistry (Orimo *et al.* 1993). In both mouse and rat aorta expression of mRNA for ER β has been observed (Iafrafi *et al.* 1997, Lindner *et al.* 1998).

Vascular relaxation in response to μ M concentrations of oestrogen is not different in wild-type mice compared with mice lacking a functional ER α indicating that ER β might be responsible (Freay *et al.* 1997). The aim of the present study was to investigate the importance of ER β for relaxation caused by pharmacological concentrations of oestrogen by using arterial preparations from mice lacking functional ER β .

Materials and Methods

Adult female and male mice (6–7 months old) lacking functional ER β (-/-) and their wild-type litter mates (+/+) were used. The -/- mice were generated by targeted disruption of exon three of the ER β gene as described previously (Krege *et al.* 1998). Lack of ER β in -/- mice was confirmed by RT-PCR for ER β mRNA and by Western blot of nuclear extracts from lung and brain showing absence of ER β mRNA and protein in -/- but not in +/+ mice. In aorta of wild-type mice ER β mRNA is expressed, while it is not detectable in aorta of ER β knock-out mice (Karas *et al.* 1999). Mice were weighed and then sacrificed by cervical dislocation. The experiments were approved by the Animal Ethics Committee at Lund University. The abdominal aorta from the iliac bifurcation to the diaphragm was dissected free from fat and connective tissue and placed in cold Ca²⁺-free HEPES-buffered solution (for composition see below). The aorta was cut into about 20 uniform rings (0.5–0.8 mm in width) using a dissecting microscope. Rings were chosen randomly for either force measurements or prepared for morphology.

Force measurements

Rings were mounted on two stainless steel wires, one of which was connected to a force transducer (AE 801, SensoNor A/S, Horten, Norway) and instantly immersed in exchangeable Plexiglass cups filled with 400 μ l HEPES-buffered solution. The preparations were allowed to equilibrate at a passive tension of about 0.5 mN, corresponding to optimal length for active force, at 37°C for 60 min before experiments were started.

Experimental protocol

In one set of experiments sensitivity to 17 β -oestradiol was investigated by adding the steroid (1–100 μ M) cumulatively to a preparation precontracted with noradrenaline (10 μ M). Each concentration of 17 β -oestradiol was present for 15 min before a higher concentration was added. Force responses at 5, 10 and 15 min were normalized to those of control rings that received vehicle (ethanol) and were run in parallel. No run-down of force was observed in control rings. In another set of experiments concentration-response relationships for noradrenaline (1 nM–50 μ M) were determined. At the end of experiments the width of the rings were measured using a microscope equipped with an ocular scale. Force was expressed as relative and absolute (mN/mm) force.

Solutions

Dissection was performed in Ca²⁺-free and force measurements in Ca²⁺-containing HEPES buffered solution of the following composition (in mM): NaCl 135.5, KCl 5.9, CaCl₂ 2.5, MgCl₂ 1.2, N-2-hydroxyethylpiperazine-N'-2-ethane-sulphonic acid (HEPES) 11.6 and glucose 11.5.

Drugs

Noradrenaline was purchased from Apoteksbolaget, Sweden. All other chemicals were from Sigma Chemical (St Louis, MO, USA). 17 β -oestradiol was dissolved in ethanol.

Morphology

Rings were fixed overnight in a mixture of 2% formaldehyde and 0.2% picric acid in phosphate buffer (pH 7.2) followed by thorough rinsing in Tyrode's solution containing 10% sucrose. Specimens were then frozen on dry ice and cross sections were cut in a cryostat to a thickness of 10 μ m. Sections were processed for morphometry or immunocytochemistry.

Morphometric analysis

Automated measurements of the thickness of the media of aorta, were performed using a computerized image analyzing system (Leica, Q500MC). Measurements were performed on hematoxylin-eosin stained cross sections, at 3–4 different depths, from the aorta of -/- and +/+ mice ($n=3$ in each group). The width of the media was defined using a binary cursor. Data was presented as average of 30 measurements.

Immunocytochemistry

Sections were incubated overnight with a polyclonal antiserum raised in rabbit against factor VIII (von Willebrand factor, dilution 1:640, code No A 082, Dakopatts, Copenhagen, Denmark) in order to determine the presence of endothelium in aorta of -/- and +/+ mice. The site of the antigen-antibody reaction was visualized by fluorescein isothiocyanate (FITC)-conjugated antibodies to rabbit Ig G raised in pig (Dakopatts, Copenhagen, Denmark).

Statistics

Values are presented as means $\pm$ S.E.M. Statistical significance was determined by Student's two-tailed *t*-test for unpaired comparisons. *P*-values less than 0.05 were regarded as statistically significant. For multiple comparisons, the Bonferroni method for determination of statistical significance was used.

Results

The weights of ER β knock-out (-/-) male and female mice were similar to corresponding wild-type (+/+) animals (Fig. 1, panel A). Females had a lower body weight compared with males (26.1 $\pm$ 0.8 vs 32.4 $\pm$ 2.0 g, $n=6$, $P<0.05$). Maximal noradrenaline-evoked force was similar in aortic rings from -/- and +/+ animals (Fig. 1, panel B). No difference in noradrenaline sensitivity was observed between aortic preparations from -/- and +/+ males or females. The EC₅₀ values for -/- and +/+ animals were 1.32 $\pm$ 0.40 μ M ($n=11$) and 1.94 $\pm$ 0.45 μ M ($n=8$), respectively.

In Fig. 2 (upper panel) cross sections of hematoxylin-eosin stained aortic preparations from -/- and +/+ female mice are

Figure 1 (A) Body weights of wild-type (+/+) and ER β knock-out (-/-) male and female mice ($n=3$ in each group) and (B) maximal noradrenaline-evoked force (mN/mm) in aortic rings from +/+ and -/- mice ($n=15-19$). Values are means $\pm$ S.E.M.

shown. No visible difference in vascular wall structure was observed between sections from -/- and +/+ irrespective of sex. The thickness of the media was determined using a computerized image analyzing system. The average width was $43.1 \pm 1.4 \mu\text{m}$ ($n=30$) in -/- animals compared with $41.7 \pm 1.2 \mu\text{m}$ ($n=30$) in +/+ animals. In both -/- and +/+ animals intact endothelium could be visualized using immunocytochemistry for von Willebrand factor (Fig. 2, lower panel). Morphological data thus show unaltered aortic wall structure in -/- animals.

Cumulative addition of 17 β -oestradiol (1-100 μM) to aortic rings of male and female mice precontracted with 10 μM noradrenaline caused a concentration-dependent relaxation. Force was evaluated at 5, 10 and 15 min of exposure to each concentration of 17 β -oestradiol and normalized to that of

Figure 2 Cross sections of aorta from wild-type (+/+) and ER β knock-out (-/-) female mice stained with hematoxylin-eosin (upper panel) or with an antiserum against von Willebrand factor (lower panel). Arrows indicate immunoreactive endothelium; elastic fibers in the media show autofluorescence. Bar 20 μm .

control rings. Relaxation in response to each concentration of the steroid was potentiated with time. In Fig. 3 summarized data on force-responses obtained at 5 min after addition of 1-100 μM 17 β -oestradiol to aortic rings from -/- or +/+ mice of both sexes are shown. Relaxation to 17 β -oestradiol was more pronounced in preparations from -/- than from +/+ mice. This effect was observed in mice of both sexes. Rings from both wild-type and knock-out female mice tended, however not statistically significant, to be more sensitive to 17 β -oestradiol than the corresponding ones from males. Vascular supersensitivity to 17 β -oestradiol in -/- mice was observed also after longer time periods (10 and 15 min) of exposure to each concentration of the steroid.

Discussion

In the present study we observed a more pronounced vascular relaxation by μM concentrations of 17 β -oestradiol in ER β deficient than in wild-type mice. This suggests that ER β modulates oestrogen-induced vascular relaxation but is not responsible for relaxation *per se*. In the ER β deficient mice stimulation with oestrogen involves activation of ER α only and thus activation of this receptor is unopposed by ER β . However, based on our findings and those by Freay and co-workers (1997) showing that oestrogen causes relaxation in aorta from mice lacking functional ER α and that these preparations are equally sensitive to oestrogen as those from wild-type mice, vascular supersensitivity to oestrogen may

Figure 3 Effect of 17 β -oestradiol added to aortic rings from +/+ and -/- mice precontracted with 10 μ M noradrenaline. When a stable contraction to noradrenaline was achieved increasing concentrations of 17 β -oestradiol was added in a cumulative manner. Each concentration was present for 15 min. Concentration-response relationship was evaluated at 5, 10 and 15 min and normalized to that of control rings run in parallel. In this figure force responses at 5 min of exposure are shown. Values are means $\pm$ S.E.M. $n=4-6$. * $P<0.05$ when compared with female +/+ and § $P<0.05$ when compared with male +/+.

perhaps not be mediated through ER α but through another ER or another nonreceptor dependent mechanism. Another possibility is that ER α and ER β may have partially redundant functions and may substitute for one another when one of them is deleted.

Previously reported studies on vascular smooth muscle cells have shown that 17 β -oestradiol, in the same concentration-range as used in this study, interacts with plasma membrane ion-channel properties, e.g. inhibits Ca²⁺ channels and activates BK_{Ca} channels (Zhang *et al.* 1994, White *et al.* 1995), suggesting that vascular smooth muscle cells contain plasma-membrane ER. Absence of ER β as in ER β knock-out animals might cause upregulation and/or enhanced activity of another, yet unknown, ER that may modify membrane properties. Upregulation of such a receptor will thus lead to a more pronounced relaxation in response to oestrogen. It is also

possible that, normally, ER β dampens vasorelaxation induced by ER α so that this effect becomes more pronounced after deletion of ER β . Such an antagonistic effect of ER β on ER α has been seen in other contexts (J-Å Gustafsson, unpublished observations). A double knock-out animal that lacks both ER α and ER β will provide a definite answer as to which of the two explanatory models is most relevant.

Vascular wall structure was similar in sections of aorta from ER β knock-out and wild-type mice. Animals lacking ER β had unchanged medial wall thickness and capacity to produce force in response to noradrenaline and these quantitative measurements support that vascular wall structure is intact in ER β knock-out animals. ER β is thus probably not important for vascular development but is suggested to set sensitivity of vascular smooth muscle to high concentrations of oestrogen.

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