Immunocytochemical demonstration of oestrogen receptor β in blood vessels of the female rat

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Abstract

The role of oestrogen receptor (ER) β in vascular function remains unclear. With the use of a specific ER β antibody we have now, using immunocytochemistry, visualized ER β in different parts of the vascular tree. In about 70% of medial smooth muscle cells of female rat aorta, tail artery and uterine artery, nuclear immunoreactivity to ER β was observed. In these vessels endothelial cells also expressed ER β . Vascular expression of the ER α subtype was lower than that of ER β . In aorta and tail artery, no immunoreactivity towards ER α was observed, while in uterine vessels occasional medial smooth muscle and endothelial cells expressed this ER subtype. ER β and α expression in uterine vessels was independent of the stage of the oestrous cycle, suggesting that variations in uterine blood flow

occurring during the cycle are independent of ER density. The regional distribution of ER α , as determined by immunocytochemistry, was supported by measurements of ER α levels by enzyme immunoassay. In the uterine artery, the level of ER α was several times higher ($P < 0.001$) than that of aorta and tail artery (10.1 ± 1.7 fmol/mg protein in the uterine artery vs 3.3 ± 1.0 and 0.5 ± 0.5 fmol/mg protein in aorta and tail artery respectively). Thus, a prominent nuclear expression of ER β was observed in the vascular wall of several parts of the vascular tree, while ER α predominantly was expressed in uterine vessels, suggesting that ER β and α may have different roles in vascular function.

Journal of Endocrinology (2001) **169**, 241–247

Introduction

Oestrogen receptors (ER) are divided into subtypes α and β (Green *et al.* 1986, Greene *et al.* 1986, Kuiper *et al.* 1996). ER β cDNA shows some highly homologous domains with ER α cDNA. The overall homology between ER α and β is approximately 50%. Prominent expression of ER β mRNA, as determined by RT-PCR, has been shown in rat prostate, ovary, epididymis, testis, bladder, uterus, lung, colon, small intestine and brain (Kuiper *et al.* 1996, 1997). ER β has been visualized in many of these tissues by immunocytochemistry (Saunders *et al.* 1997, Pelletier *et al.* 2000). Cell-specific localization of ER α and β in the reproductive tract of male and female rats has been reported by Pelletier *et al.* (2000). In a recent paper, Saji *et al.* (2000) showed, using immunocytochemistry, that ER α as well as ER β are expressed in the nuclei of glandular epithelial cells of the rat mammary gland. ER α expression varies substantially during breast development from as low as 5% of cell nuclei at day 14 of pregnancy to 70% at day 21 of lactation, while the

expression of β was constant (60–70% of cell nuclei) during all stages of breast development.

Binding sites for oestrogen have been shown in different vascular preparations using radioligand binding (Farhat *et al.* 1996, Freay *et al.* 1997). ER α has been visualized, using immunocytochemistry, in rat aortic smooth muscle cells (Orimo *et al.* 1993) and in guinea pig, rabbit and human uterine arteries (Perrot-Applanat *et al.* 1988, Leiberman *et al.* 1990). In some other vascular segments, i.e. carotid artery and aorta of the guinea pig, Leiberman *et al.* (1990), however, observed no immunoreactivity towards ER α , suggesting that this receptor is regionally distributed within the vascular system. Human vascular smooth muscle cells have also been shown to contain ER α (Karas *et al.* 1994).

Vascular expression of mRNA for ER β has been shown in rat aorta and carotid artery and in mouse aorta (Iafrafi *et al.* 1997, Lindner *et al.* 1998, Mäkelä *et al.* 1999). After vascular injury, the expression of ER β is increased (Lindner *et al.* 1998, Mäkelä *et al.* 1999). The purpose of the present study was to visualize, by

immunocytochemistry, vascular ER β using a specific antibody. Interestingly, we have observed an abundant vascular expression of ER β , while ER α was predominantly expressed in uterine vessels.

Materials and Methods

Animals and tissues

Adult (3-month-old) female Sprague–Dawley rats, weighing 200 g, were used. The animals were kept on a standard laboratory animal diet with water available *ad libitum* and under constant light (0700–2100 h). The rats were killed by cervical dislocation. The animal handling was approved by the Animal Ethics Committee at Lund University. Thoracic aorta, tail artery, uterine artery and uterus were dissected free from fat and connective tissue under a dissecting microscope in Ca²⁺-free Krebs solution of the following composition (in mM): NaCl 122, KCl 4.7, NaHCO₃ 15.5, KH₂PO₄ 1.2, CaCl₂ 2.5, MgCl₂ 1.2 and glucose 11.5. After dissection the tissues were carefully washed in buffer.

Immunocytochemistry

Tissue specimens were fixed in 4% buffered formalin solution, dehydrated and then embedded in paraffin. The preparations were cut in 4 μ m sections transversely and longitudinally, deparaffinized and treated for 15 min with citrate buffer (pH 6.0) in a microwave oven before the immunostaining (Shi *et al.* 1991). The primary antibodies used were a specific ER α antibody (monoclonal mouse ER α antibody, ER1D5; DAKO A/S, Glostrup, Denmark) at a dilution of 1:100 (Pettersson *et al.* 1997) and a specific antibody against ER β (ER β 503 antibody), raised in chickens at dilutions of 1:750, 1:1000 or 1:1500 as indicated (Saji *et al.* 2000). In preliminary experiments, the dilutions of the primary antibodies were titrated in each tissue to achieve optimal nuclear staining with minimal unspecific 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). Counterstaining to delineate nuclei was performed by dipping the slides in Mayer's haematoxylin for 20 s. At each staining a positive control (breast tissue), expressing both ER subtypes (Saji *et al.* 2000), was run in parallel. Preabsorbed ER β antibodies were obtained by incubating the antibody with the ER β protein (50-fold excess of the protein) employed as antigen at 4 °C for 12 h. For negative controls, the primary antibodies (anti-ER α and β) were omitted. In these different types of control stainings no immunoreactivity

was observed. Tissues for immunocytochemistry were obtained from eleven rats and at least two staining slides were analyzed for each individual tissue and protein (ER α or β).

Determination of ER α using enzyme immunoassay

For quantitative determination of ER α samples (at least 15 mg) of aorta, tail artery and uterine artery consisting of all three layers (tunica intima, tunica media and tunica adventitia) and uterus from 14 rats were homogenized with a microdismembrator, after which the powder was dissolved in buffer (10 mM Tris, 1.5 mM EDTA, 5.0 mM Na₂MoO₄, 1.0 mM monothioglycerol, pH 7.4). In order to increase the amount of tissue for accurate measurements, tail artery and uterine arteries from two rats were pooled. The homogenate was then centrifuged at 100 000 g for 60 min at 0 °C, whereafter the content of ER α in the supernatant was measured with an enzyme immunoassay according to the kit instructions (Abbott Laboratories, Diagnostic Division, Chicago, IL, USA). The Abbott antibody is a rat monoclonal antibody (Greene *et al.* 1980) which recognizes ER α but not ER β . Uterine tissue, which has been reported to have high expression of ER α (Kuiper *et al.* 1997), was used as a positive control. The sensitivity of the Abbott ER α enzyme immunoassay monoclonal system is approximately 1.5 fmol ER α /ml and values below this were considered to be below the detection limit. ER α content was expressed as fmol ER α /mg protein. Protein was determined as described by Lowry *et al.* (1951), using bovine serum albumin as standard.

Statistics

Values are presented as means $\pm$ s.e.m. ANOVA and Student's two-tailed *t*-test for unpaired comparisons were used to determine statistical significance. *P* values less than 0.05 were regarded as denoting statistical significance.

Results

Nuclear immunoreactivity to ER β was observed in numerous medial smooth muscle cells in aorta, tail artery and the uterine artery (Fig. 1A–C). In these three vessels, about 70% of the medial cells expressed ER β . In the vessels, ER β immunoreactivity was also observed in endothelial cells. Breast tissue was used as a positive control and processed in parallel. In sections of breast tissue, nuclear staining with ER β was observed in glandular epithelial cells (Fig. 1D). These cells also showed immunoreactive nuclear staining towards ER α (Fig. 4H). The specificity of the ER β antibody was investigated by inactivating antiserum with the antigen. No ER β immunoreactivity was observed in sections stained with

Figure 1 Immunocytochemical staining of longitudinal sections of aorta (A), tail artery (B) and uterine artery (C) with ER β antibody (ER β 503 antibody, 1:1000). In (D) a section of breast tissue stained with ER β antibody as a positive control is shown. On the right-hand side, stainings of consecutive tissue sections with preabsorbed antibody (A_{abs}–D_{abs}) are shown. Specific nuclear immunostaining was observed in the medial smooth muscle cells of the vascular preparations and in the glandular epithelium of the breast tissue. (A–C) Arrowheads show examples of positive nuclei of medial smooth muscle cells. As shown by the arrow in (B) occasional ER β -positive endothelial cells could be observed. Endothelial cells were sometimes lost during preparation of tissue sections and staining. This occurred independently of the type of vessel. Bar in (A)=25 μ m (for A–D).

Figure 2 Immunocytochemical staining of a longitudinal section of tail artery with ER β antibody (1:750) in the absence of nuclear counterstaining with Mayer's haematoxylin. Numerous positively stained smooth muscle and endothelial cells can be seen. No immunoreactivity was observed after staining with inactivated antiserum (right-hand side). Bar=25 μ m.

the inactivated antiserum (Fig. 1A_{abs}–D_{abs}). In Fig. 2, a section of tail artery stained with ER β antibody in the absence of nuclear counterstaining is shown. By omitting counterstaining, ER β immunoreactive material within the nucleus of smooth muscle and endothelial cells was more prominent. No immunoreactivity was observed after staining with inactivated antiserum (Fig. 2, right-hand side).

In aorta and tail artery, no immunoreactivity to ER α was observed, while in the uterine vessels occasional medial smooth muscle and endothelial cells expressed the receptor (see below). Results obtained by enzyme immunoassay using a specific ER α antibody supported the regional distribution of ER α observed by immunocytochemistry (Fig. 3). The enzyme immunoassay was performed on homogenates of aorta, tail artery and uterine artery. The vascular preparations consisted of all three vessel wall layers, i.e. tunica intima, tunica media and tunica adventitia, and the amount of ER α was normalized to total protein content. As shown in Fig. 3, the uterine artery contained 3 and 20 times higher ($P<0.001$) levels of ER α compared with aorta and tail artery respectively. Homogenate of rat uterus was used as a positive control. The uterine tissue contained about 10 times higher ($P<0.001$) levels of ER α than the uterine artery (112 ± 18 vs 10.1 ± 1.7 fmol/mg protein).

In order to determine ER β and α in uterine vessels, sections of uterus were prepared and immunostained for ER subtypes. In Fig. 4 immunostainings made on tissues collected at dioestrus are presented. The stage of cycle was monitored cytologically by vaginal smears. After two regular cycles, animals were killed at pro-oestrus, oestrus, metoestrus and dioestrus and tissues collected. Positive nuclear immunoreactive staining for ER β and α was observed in vascular smooth muscle cells of arterioles (Fig. 4A and E). Occasional endothelial cells showed positive staining for ER β and α . In uterine vessels, no

differences in ER β expression with the stage of the oestrous cycle were observed. Vascular ER α expression was also independent of the stage of the cycle.

Immunoreactivity towards ER α was observed in 80% of the nuclei of glandular epithelial cells (Fig. 4F). In contrast to the strong ER α expression, ER β was only weakly

Figure 3 Amount of ER α (fmol/mg protein) in tail artery, aorta and uterine artery determined by enzyme immunoassay. The numbers of observations are given in brackets.

Figure 4 Immunocytochemical staining of consecutive transverse sections of uterus at dioestrus with ER β (A and B, 1:1500) and ER α (E and F, 1:100) antibodies. In (A and E) segments with uterine vessels are shown. Note ER β - and ER α -positive vascular smooth muscle (arrowheads) and endothelial cells (arrows). A majority of myometrial smooth muscle cells (m) show immunoreactive staining towards ER β and α . In (B and F) segments in the endometrium with uterine glands are shown. A majority of the glandular epithelial cells contained nuclear immunoreactivity towards ER α (F), while only a fraction of these cells contained nuclear ER β immunoreactive material (B). Immunoreactivities towards ER β as well as ER α were observed in the stroma cells of the endometrium (e). In (C and D) consecutive sections of uterine vessels and uterine glands stained with preabsorbed ER β antibody are shown. No immunostaining was observed after preabsorption of the ER β antibody. No immunoreactivities were observed either in vessels (left-hand side) or glands (right-hand side) if the primary ER α antibody was omitted (G). (H) Breast tissue stained with ER α as a positive control. Bar in (A)=25 μ m (for A–H).

expressed (<10% of the cells) in these cells (Fig. 4B). In the smooth muscle cells of the myometrium, about 70% of the cells expressed nuclear ER β and ER α (Fig. 4A and E). About the same number of endometrial stroma cells showed nuclear immunoreactivity towards ER β and α (Fig. 4B and F). Glandular epithelial, myometrial smooth muscle and endometrial stroma cells expressed ER β and α at all stages of the oestrous cycle. No immunostaining was observed if the ER β antibody was preabsorbed with antigen (Fig. 4C and D) or if the ER α antibody was omitted (Fig. 4G).

Discussion

In this study, we have visualized ER β protein in vascular smooth muscle cells from three different parts of the vascular tree in the female rat. Almost 70% of the medial cells independent of vessel type expressed nuclear ER β , indicating that ER β mediates the transcriptional effects of oestrogen in the whole vascular tree and not only in specific segments.

Our results suggest that ER α , in contrast to ER β , is predominantly expressed in certain vascular beds, e.g. the uterine vessels. In the tail artery and in the aorta, low levels of ER α were observed compared with the uterine artery. In endothelial cells, physiological concentrations (nM) of oestrogen enhance the production of nitric oxide within minutes and further cause a translocation of endothelial nitric oxide synthase (eNOS) from the plasma membrane to intracellular sites close to the nucleus (Lantin-Hermoso *et al.* 1997, Chen *et al.* 1999, Goetz *et al.* 1999). The rapid induction of eNOS is ER dependent since it is blockable by ER antagonists and it has been reported to be dependent on ER α activation (Chen *et al.* 1999). Thus, one could speculate that this mechanism is more pronounced in regions of the vascular tree rich in ER α , e.g. in the uterine artery, than in regions with low ER α expression.

The available evidence suggests that oestrogens mediate the increase in uterine blood flow occurring in pregnancy and during the oestrous cycle (Markee 1932, Greiss & Anderson 1970, Killam *et al.* 1973, Rosenfeld 1989). The mechanism(s) responsible for oestrogen-induced elevated blood flow remain, despite much effort, unclear (Mendelsohn & Karas 1999). Increases in uterine blood flow by 17 β -oestradiol are prevented by the NOS inhibitor nitro-L-arginine methyl ester, suggesting that nitric oxide is involved (Van Buren *et al.* 1992, Rosenfeld *et al.* 1996). However, other vasoactive substances, such as prostanoids and vasoactive polypeptides have also been implicated (Killam *et al.* 1973, Clark *et al.* 1981a,b). Here, we report that both ER β and α are expressed in uterine vessels and thus both receptors may be involved in the regulation of uterine blood flow by oestrogens. No changes in uterine vessel ER β and α expression were observed during the oestrous cycle, suggesting that mechanisms other than ER density are important.

In the present study, we report a cell-specific nuclear distribution of ER α and β in uterus. Both subtypes are expressed in myometrial smooth muscle cells, endometrial stroma cells and in the vascular wall of uterine vessels. In glandular epithelial cells, on the other hand, intense immunoreactivity towards ER α was observed in a majority of cells, while ER β immunoreactivity was much weaker and only occurred in a few cells. This suggests that oestrogen exerts its effect on uterine glandular function mainly through activation of ER α . In a recent study, Taylor & Al-Azzawi (2000) have reported a similar cellular distribution pattern of ER subtypes in human uterus. The isoflavone phyto-oestrogen genistein, which has 20 times higher binding affinity for ER β than for ER α has been shown to exert a vasculoprotective effect after vascular injury but not a uterotrophic effect, while an unselective ER agonist like 17 β -oestradiol has both a vasculoprotective and a uterotrophic effect (Mäkelä *et al.* 1999). After balloon injury, expression of ER β mRNA in male rat aorta and carotid artery is increased in endothelial as well as smooth muscle cells, while the expression of ER α mRNA remains at a low level (Lindner *et al.* 1998, Mäkelä *et al.* 1999). The tissue selective effect of genistein may thus be explained by differences in ER β expression.

In breast glandular epithelial cell nuclei from the rat, ER α expression is decreased at puberty and at pregnancy compared with at prepuberty (Saji *et al.* 2000). During lactation, a large induction of ER α is observed with 70% of cell nuclei showing positive immunostaining. This suggests that ER α expression is not governed by the elevated level of oestrogen occurring during puberty and pregnancy but through other hormonal mechanisms. ER β expression seems to be independent of hormonal status since it stays constant (60–70% of glandular cell nuclei) during all phases of breast development. It is interesting to note that expression of ER β in breast glandular cells is quantitatively similar to vascular smooth muscle cell expression of ER β , suggesting that this ER subtype is important in both of these cell types.

The most striking finding in the present study was the abundant vascular expression of ER β . The role of ER β in vascular function has been addressed in a few studies but has not been explained. In the ER β knock-out mice (BERKO), vascular morphology and force responses to noradrenaline are unaltered, suggesting that ER β is not crucial for vascular development (Nilsson *et al.* 2000). Oestrogen inhibits the response to vascular injury in wild-type, ER α knock-out (ERKO) as well as in BERKO mice, suggesting that ER α and ER β may both mediate vascular protection by oestrogen in a redundant manner or that a third mechanism exists for oestrogen-dependent vascular protection (Iafrafi *et al.* 1997, Karas *et al.* 1999). The challenge for the future must be to investigate the importance of vascular ER β further.

Acknowledgements

This study was supported by the Swedish Medical Research Council (grant no. K99-04X-13017-01A), the Swedish Heart and Lung Foundation and the foundations of Åke Wiberg and Magnus Bergvall as well as by the Swedish Cancer Fund and KaroBio AB.

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Received in final form 11 December 2000

Accepted 12 January 2001