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 ERβ antibody we have now, using immunocytochemistry, visualized ERβ 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 ERβ was observed. In these vessels endothelial cells also expressed ERβ. Vascular expression of the ERα subtype was lower than that of ERβ. In aorta and tail artery, no immunoreactivity towards ERα was observed, while in uterine vessels occasional medial smooth muscle and endothelial cells expressed this ER subtype. ERβ 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 ERα, as determined by immunocytochemistry, was supported by measurements of ERα levels by enzyme immunoassay. In the uterine artery, the level of ERα 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 ERβ was observed in the vascular wall of several parts of the vascular tree, while ERα predominantly was expressed in uterine vessels, suggesting that ERβ and α may have different roles in vascular function.


Introduction

Oestrogen receptors (ER) are divided into subtypes α and β (Green et al. 1986, Greene et al. 1986, Kuiper et al. 1996). ERβ cDNA shows some highly homologous domains with ERα cDNA. The overall homology between ERα and β is approximately 50%. Prominent expression of ERβ 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). ERβ has been visualized in many of these tissues by immunocytochemistry (Saunders et al. 1997, Pelletier et al. 2000). Cell-specific localization of ERα 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 ERα as well as ERβ are expressed in the nuclei of glandular epithelial cells of the rat mammary gland. ERα 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). ERα 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 ERα, suggesting that this receptor is regionally distributed within the vascular system. Human vascular smooth muscle cells have also been shown to contain ERα (Karas et al. 1994).

Vascular expression of mRNA for ERβ has been shown in rat aorta and carotid artery and in mouse aorta (Iafri et al. 1997, Lindner et al. 1998, Mäkelä et al. 1999). After vascular injury, the expression of ERβ is increased (Lindner et al. 1998, Mäkelä et al. 1999). The purpose of the present study was to visualize, by
immunocytochemistry, vascular ERβ using a specific antibody. Interestingly, we have observed an abundant vascular expression of ERβ, while ERα 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 dissection 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, 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 µm 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 ERα antibody (monoclonal mouse ERα antibody, ER1D5; DAKO A/S, Glostrup, Denmark) at a dilution of 1:100 (Petterson et al. 1997) and a specific antibody against ERβ (ERβ 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 Biotek, Tuscon, AZ, USA) with DAKO ChemMate Detection Kit peroxidase/3–3’ dianinobenzidine. For ERβ, 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 ERβ antibodies were obtained by incubating the antibody with the ERβ protein (50-fold excess of the protein) employed as antigen at 4 °C for 12 h. For negative controls, the primary antibodies (anti-ERα 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 (ERα or β).

Determination of ERα using enzyme immunoassay

For quantitative determination of ERα 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 ERα 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 ERα but not ERβ. Uterine tissue, which has been reported to have high expression of ERα (Kuiper et al. 1997), was used as a positive control. The sensitivity of the Abbott ERα enzyme immunoassay monoclonal system is approximately 1-5 fmol ERα/ml and values below this were considered to be below the detection limit. ERα content was expressed as fmol ERα/mg protein. Protein was determined as described by Lowry et al. (1951), using bovine serum albumin as standard.

Statistics

Values are presented as means ± 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 ERβ 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 ERβ. In the vessels, ERβ 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 ERβ was observed in glandular epithelial cells (Fig. 1D). These cells also showed immunoreactive nuclear staining towards ERα (Fig. 4H). The specificity of the ERβ antibody was investigated by inactivating antiserum with the antigen. No ERβ 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 ERβ antibody (ERβ 503 antibody, 1:1000). In (D) a section of breast tissue stained with ERβ 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 ERβ-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 μm (for A–D).
the inactivated antiserum (Fig. 1A - Dabs). In Fig. 2, a section of tail artery stained with ERβ antibody in the absence of nuclear counterstaining is shown. By omitting counterstaining, ERβ immunoreactive material within the nucleus of smooth muscle and endothelial cells was more prominent. No immunoreactivity was observed after staining with inactivated antiserum (right-hand side).

In aorta and tail artery, no immunoreactivity to ERα 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 ERα antibody supported the regional distribution of ERα 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 ERα 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 ERα 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 ERα than the uterine artery (112±18 vs 10±1±7 fmol/mg protein).

In order to determine ERβ 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 ERβ and α was observed in vascular smooth muscle cells of arterioles (Fig. 4A and E). Occasional endothelial cells showed positive staining for ERβ and α. In uterine vessels, no differences in ERβ expression with the stage of the oestrous cycle were observed. Vascular ERα expression was also independent of the stage of the cycle. Immunoreactivity towards ERα was observed in 80% of the nuclei of glandular epithelial cells (Fig. 4F). In contrast to the strong ERα expression, ERβ was only weakly

**Figure 2** Immunocytochemical staining of a longitudinal section of tail artery with ERβ 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 μm.

**Figure 3** Amount of ERα (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 ERβ (A and B, 1:1500) and ERα (E and F, 1:100) antibodies. In (A and E) segments with uterine vessels are shown. Note ERβ- and ERα-positive vascular smooth muscle (arrowheads) and endothelial cells (arrows). A majority of myometrial smooth muscle cells (m) show immunoreactive staining towards ERβ 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 ERα (F), while only a fraction of these cells contained nuclear ERβ immunoreactive material (B). Immunoreactivities towards ERβ as well as ERα 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 ERβ antibody are shown. No immunostaining was observed after preabsorption of the ERβ antibody. No immunoreactivities were observed either in vessels (left-hand side) or glands (right-hand side) if the primary ERα antibody was omitted (G). (H) Breast tissue stained with ERα as a positive control. Bar in (A) = 25 μm (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 ERβ and ERα (Fig. 4A and E). About the same number of endometrial stroma cells showed nuclear immunoreactivity towards ERβ and α (Fig. 4B and F). Glandular epithelial, myometrial smooth muscle and endometrial stroma cells expressed ERβ and α at all stages of the oestrous cycle. No immunostaining was observed if the ERβ antibody was preabsorbed with antigen (Fig. 4C and D) or if the ERα antibody was omitted (Fig. 4G).

**Discussion**

In this study, we have visualized ERβ 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 ERβ, indicating that ERβ mediates the transcriptional effects of oestrogen in the whole vascular tree and not only in specific segments.

Our results suggest that ERα, in contrast to ERβ, is predominantly expressed in certain vascular beds, e.g. the uterine vessels. In the tail artery and in the aorta, low levels of ERα 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 ERα dependent since it is blockable by ER antagonists and it has been reported to be dependent on ERα activation (Chen et al. 1999). Thus, one could speculate that this mechanism is more pronounced in regions of the vascular tree rich in ERα, e.g. in the uterine artery, than in regions with low ERα 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 17β-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 ERβ 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 ERβ 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 ERα 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 ERα was observed in a majority of cells, while ERβ 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 ERα. 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 ERβ than for ERα has been shown to exert a vasculoprotective effect after vascular injury but not a uterotrophic effect, while an unselective ER agonist like 17β-oestradiol has both a vasculo-protective and a uterotrophic effect (Mäkelä et al. 1999). After balloon injury, expression of ERβ mRNA in male rat aorta and carotid artery is increased in endothelial as well as smooth muscle cells, while the expression of ERα 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 ERβ expression.

In breast glandular epithelial cell nuclei from the rat, ERα expression is decreased at puberty and at pregnancy compared with at prepuberty (Saji et al. 2000). During lactation, a large induction of ERα is observed with 70% of cell nuclei showing positive immunostaining. This suggests that ERα expression is not governed by the elevated level of oestrogen occurring during puberty and pregnancy but through other hormonal mechanisms. ERβ 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 ERβ in breast glandular cells is quantitatively similar to vascular smooth muscle cell expression of ERβ, 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 ERβ. The role of ERβ in vascular function has been addressed in a few studies but has not been explained. In the ERβ knock-out mice (BERKO), vascular morphology and force responses to noradrenaline are unaltered, suggesting that ERβ is not crucial for vascular development (Nilsson et al. 2000). Oestrogen inhibits the response to vascular injury in wild-type, ERα knock-out (ERKO) as well as in BERKO mice, suggesting that ERα and ERβ may both mediate vascular protection by oestrogen in a redundant manner or that a third mechanism exists for oestrogen-dependent vascular protection (Iafriati et al. 1997, Karas et al. 1999). The challenge for the future must be to investigate the importance of vascular ERβ further.
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References


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