Corrections

For the following 18 articles, the authors note, in addition to the partial funding of this study from KaroBio AB, that J.-A.G. is co-founder, deputy board member, stockholder, and consultant of KaroBio AB.

(i) NEUROSCIENCE. “Liver X receptors in the central nervous system: From lipid homeostasis to neuronal degeneration,” by Ling Wang, Gertrud U. Schuster, Kjell Hultenby, Qinghong Zhang, Sandra Andersson, and Jan-Åke Gustafsson, which appeared in issue 21, October 15, 2002, of Proc. Natl. Acad. Sci. USA (99, 13878–13883; first published October 4, 2002; 10.1073/pnas.172510899);


(iv) NEUROSCIENCE. “Estrogen receptor (ER)β knockout mice reveal a role for ERβ in migration of cortical neurons in the developing brain,” by Ling Wang, Sandra Andersson, Margaret Warner, and Jan-Åke Gustafsson, which appeared in issue 2, January 21, 2003, of Proc. Natl. Acad. Sci. USA (100, 703–708; first published January 6, 2003; 10.1073/pnas.242735799);


(vi) MEDICAL SCIENCES. “Differential effects on bone of estrogen receptor α and androgen receptor activation in orchidectomized adult male mice,” by Sofia Movéreau, Katrien Venken, Anna-Lena Eriksson, Niklas Andersson, Stanko Skrtic, Jon Wergedal, Subburaman Mohan, Phil Salmon, Roger Bouillon, Jan-Åke Gustafsson, Dirk Vanderschuren, and Claes Ohlsson, which appeared in issue 23, November 11, 2003, of Proc. Natl. Acad. Sci. USA (100, 13573–13578; first published October 22, 2003; 10.1073/pnas.2233084100);


(xiii) DEVELOPMENTAL BIOLOGY. “Estrogen receptor α and imprinting of the neonatal mouse ventral prostate by estrogen,” by Yoko Omoto, Otakbeb Imamov, Margaret Warner, and Jan-Åke Gustafsson, which appeared in issue 5, February 1, 2005, of Proc. Natl. Acad. Sci. USA (102, 1484–1489; first published January 21, 2005; 10.1073/pnas.0409168102);

(xiv) DEVELOPMENTAL BIOLOGY. “Early onset of puberty and early ovarian failure in CYP7B1 knockout mice,” by Yoko Omoto, Richard Lathe, Margaret Warner, and Jan-Åke Gustafsson, which appeared in issue 8, February 22, 2005, of Proc. Natl. Acad. Sci. USA (102, 2814–2819; first published February 14, 2005; 10.1073/pnas.0500198102);


(xvi) NEUROSCIENCE. “Inactivation of liver X receptor β leads to adult-onset motor neuron degeneration in male mice,” by Sandra Andersson, Nina Gustafsson, Margaret Warner, and Jan-Åke Gustafsson, which appeared in issue 10, March 8, 2005, of Proc. Natl. Acad. Sci. USA (102, 3857–3862; first published February 28, 2005; 10.1073/pnas.0500634102);

(xvii) PHYSIOLOGY. “Muscle GLUT4 regulation by estrogen receptors ERα and ERβ,” by Rodrigo A. Barros, Úbiratan F. Machado, Margaret Warner, and Jan-Åke Gustafsson, which appeared in issue 5, January 31, 2006, of Proc. Natl. Acad. Sci. USA (103, 1605–1608; first published January 19, 2006; 10.1073/pnas.0510391103);

www.pnas.org/cgi/doi/10.1073/pnas.0602780103
Characterization of the ERβ−/− mouse heart

Carola Förster**, Silke Kietz§, Kjell Hultenby®, Margaret Warner#, and Jan-Ake Gustafsson*###

Departments of *Medical Nutrition and 1Biosciences and *Clinical Research Centre, Karolinska Institutet, Novum, S-141 86 Huddinge, Sweden

Contributed by Jan-Ake Gustafsson, August 6, 2004

Although the heart responds to estrogen, it is not clear whether estrogen acts directly on heart muscle or indirectly by means of the vascular, immune, or nervous system. No role for estrogen receptor (ER) β in the heart has been established, but ERβ−/− mice are hypertensive, and as they age, their hearts become enlarged. Histological and ultrastructural analysis of the heart revealed a disarray of myocytes, a disruption of intercalated discs, an increase in the number and size of gap junctions, and a profound alteration in nuclear structure, concomitantly with a loss of expression of lamin A/C from the nuclear envelope. In the lungs of ERβ−/− mice, lamin A/C was located in the nuclear membrane, indicating that lamin A/C is not an ERβ-regulated gene. Immunohistochemical studies with ERβ antibodies failed to detect ERβ in the myocardium. We conclude that abnormalities in heart morphology in ERβ−/− mice are likely due to stress on the nuclear envelope as a result of the chronic sustained systolic and diastolic hypertension observed in ERβ−/− mice. Because neither ERα nor ERβ could be detected in heart muscle, the effects of estrogen on the myocardium seem to be indirect.

Estrogen regulates heart rate by increasing vagal tone and decreasing sympathetic tone to the heart (3) and has cardiovascular protective effects through its modulation of endogenous vasoconstrictors such as angiotensin II and vasodilators such as nitric oxide in blood vessels (4).

Histology. After paraffin embedding, sections (4 μm) were mounted on organosilane-coated slides. Slides were stained with hematoxylin/eosin for histological evaluation under the light microscope. Transversal histological sections were prepared at equal distance from the heart apex (3 mm) and viewed under a Zeiss Axioplan 2 microscope.

Immunohistochemistry. ERβ IgY was prepared in this laboratory and characterized previously (20). It was raised against the whole human ERβ protein. For Western blotting, two ERβ antibodies were raised in rabbits, one against the ligand binding domain of human ERβ and one against the sequence of amino acids 1–50 of the human ERβ. N-cadherin (sc-1502, Santa Cruz Biotechnology, 1:100) and Cx43 (Zymed, 1:100) were detected in paraffin sections by immunofluorescence procedures. Briefly, sections were incubated with primary antibodies [in 3% (wt/vol) BSA in PBS] overnight at 4°C. PBS was used in place of primary antibodies in negative controls. Slides were washed with PBS and incubated with the respective FITC-conjugated donkey secondary antibodies (Jackson ImmunoResearch) for 45 min at 37°C. Nuclei were counterstained with propidium iodide in the case of Cx43 stains.

For visualization of f-actin, the sections were dewaxed and washed in PBS, and the cells were permeabilized by the addition of 0.1% Triton X-100 in PBS for 20 min. The sections were then stained with FITC-phalloidin (Molecular Probes) diluted 1:200 according to the manufacturer’s instructions.

For lamin A/C (sc-7293, Santa Cruz Biotechnology, 1:100) and lamin B (sc-6217, Santa Cruz Biotechnology, 1:100) staining, frozen, 9-μm sections were air-dried for 30 min. After washing with ice-cold methanol and acetone, each for 3 min, sections were fixed for 10 min at room temperature in 4% paraformaldehyde, followed first by 0.1% Triton X-100 in PBS for 5 min, next by 10% normal donkey serum in PBS for 30 min at 25°C.

Materials and Methods

Animals and Tissue Collection. ERβ−/− mice and WT littermates were generated from heterozygous breeding as described (18). All animals were housed in the animal-care facility with a 12-h light/12-h dark photoperiod with 50% humidity and given free access to tap water and rodent chow. Genotyping by PCR was performed as described in ref. 19. Mice were killed by CO2 asphyxiation. Hearts and lungs were excised, frozen immediately, and stored at −80°C until used for immunohistochemistry and Western blotting, or fixed in 4% paraformaldehyde overnight and routinely embedded in paraffin wax for immunohistochemistry.

Abbreviations: ID, intercalated disc; GJ, gap junction; ER, estrogen receptor; Cx43, connexin 43.

*Present address: Institute of Anatomy and Cell Biology, University of Würzburg, D-97070 Würzburg, Germany.
1Present address: Department of Pediatrics, Martin Luther University Halle-Wittenberg, 06099 Halle, Germany.
2To whom correspondence should be addressed. E-mail: jan-ake.gustafsson@mednet.ki.se.

© 2004 by The National Academy of Sciences of the USA

www.pnas.org/cgi/doi/10.1073/pnas.0405571101
and finally by incubation with primary antibodies and FITC-conjugated secondary antibodies. Nuclei were counterstained with propidium iodide. Sections were mounted in VECTASHIELD antifading medium (Vector Laboratories) and examined at comparable depths on a Leica TCSSP confocal microscope. At a ×63 magnification used to evaluate connexin expression, each fluorescent spot indicates a gap junction (GJ) plaque (21).

For ERβ staining, sections were incubated for 1 h at 4°C with normal goat serum diluted at 1:10 in PBS. ERβ IgY was diluted 1:200 in PBS containing 3% BSA. Sections were incubated with antibodies overnight at 4°C. For negative controls, the primary antibody was replaced with PBS alone or with primary antibody after absorption with ERβ protein. Before addition of the secondary antibody, sections were rinsed in PBS. Sections were then incubated in biotinylated goat anti-chicken Ig (1:200 dilution) for 2 h at room temperature, followed by washing with PBS and incubation in avidin-biotin-horseradish peroxidase for 1 h. After thorough washing in PBS, slides were developed with 3,3'-diaminobenzidine tetrahydrochloride, slightly counterstained with Mayer’s hematoxylin, and dehydrated through an ethanol series, followed by exposure to xylene and mounting.

Construction of Figures. Each single confocal image in a z-series captures the distribution of antibody-labeled protein in a 0.5-μm section of the tissue. The z-series were then projected to give a single image showing the distribution of the visualized protein in a tissue depth of up to 15 μm (see ref. 21 for methods). Quantitative analysis of GJ plaque size and numerical density was performed by using the Leica TCSSP image processing tool.

Transmission Electron Microscopy. Hearts were dissected and small pieces were cut and fixed in 2% glutaraldehyde plus 0.5% paraformaldehyde in 0.1 M sodium cacodylate buffer containing 3 mM CaCl₂ (pH 7.4) at room temperature for 30 min followed by 24 h at 4°C. Specimens were rinsed in 0.15 M sodium cacodylate buffer containing 3 mM CaCl₂ (pH 7.4) and postfixed in 2% osmium tetroxide in 0.07 M sodium cacodylate buffer containing 1.5 mM CaCl₂ (pH 7.4) for 2 h, dehydrated in ethanol followed by acetone, and embedded in LX-112 (Ladd Research Laboratories, Burlington, VT). Semi-thin sections were cut and stained with toluidine blue and used for light-microscopic analysis. Ultrathin sections (~40–50 nm) were cut and contrasted with uranyl acetate followed by lead citrate and examined in a Tecnai 10 (Fei, Eindhoven, The Netherlands) transmission electron microscope at 80 kV.

Calculation of Intercalated Disc (ID) Ratio. Ten randomly selected IDs in left ventricles from WT and ERβ−/− mice were analyzed. The total length of the ID was divided by the width of the disk, giving a ratio.

Western Blotting. Nuclear extracts, total cell extracts, and mitochondrial fractions were analyzed by Western blotting. Frozen tissues were homogenized with a Polytron PT3100 (Kinematica, Lucerne, Switzerland). Two tablets of mixture protease inhibitors (Boehringer Mannheim) were added per 50 ml of PBS before use. To prepare nuclear extracts, crude nuclear fractions were sedimented from tissue homogenates at 600 × g for 10 min. Nuclei were sedimented through a cushion of 25 ml of 30% sucrose at 100,000 × g for 1 h. Mitochondrial fractions were sedimented from the postnuclear supernatant by centrifugation at 10,000 × g for 20 min. Protein was extracted by incubating the pellets in 0.5% deoxycholate/0.1% Nonidet P-40 in PBS. Solubilized protein was separated from debris by centrifugation. Solubilized protein or total homogenates were dissolved in SDS sample buffer, and aliquots were used for Western blotting. Protein content was measured by the Bio-Rad protein assay with BSA as the standard. Aliquots of 30 μg of protein were loaded onto each lane of an 8% polyacrylamide gel. Western blotting was done according to the protocol described in ref. 22. Antibody dilutions were 1:1,000 for lamin A/C and lamin B, 1:3,000 for ERβ ligand binding domain antibody, and 1:5,000 for the peroxidase-conjugated goat anti-rabbit; anti-mouse and anti-chicken IgGs were used at 1:5,000. Signals were detected with ECL (Amersham Pharmacia).

RNA Extraction and cDNA Synthesis. Extraction of total RNA from frozen left ventricle tissue, DNase digestion, and cDNA synthesis were carried out as described in ref. 23.

Quantitative Real-Time RT-PCR. The primers and probes for the target genes were determined with the assistance of the computer program PRIMER EXPRESS (Applied Biosystems). The primers and probes used in this study are as follows: ANF forward 5'-ATTCTCAAGAACCTGTAGACACCCT-3' (200 nM) and reverse 5'-CAGTCTGCTACCTCAGGGGCC-3' (200 nM), probe FAM-5'-GTGAAAGGGCATGAGAAAGGAC-3' (300 nM) and reverse 5'-AGGCTTTCACCTTCGTC-3' (300 nM), and probe FAM-5'-CACCTACCCAGAAGAGAGACAG-ACCTAC-3'-TAMRA (300 nM). The real-time PCR reactions were performed as described in ref. 24, and the amplified products were sequenced. Standard curves were generated by using serially diluted solutions of standard cDNA derived from the left ventricles of WT mice. Real-time PCR was done in triplicate for each sample. The 18S rRNA (PDAR, PRIMER EXPRESS, Applied Biosystems) was used as a reference gene. The target gene transcripts in each sample were normalized on the basis of their 18S rRNA transcript content. Each experimental group (WT and ERβ−/−) contained samples of three different animals (n = 3).

Statistical Analysis. The differences in mRNA expression levels of the target genes between the experimental groups were evaluated by using Student’s t test.

Results

Altered Cardiac Morphology in ERβ−/− Mice. After 8 months of age, ERβ−/− mouse hearts (Fig. 1B) were larger than those of WT littermates (Fig. 1A). Cross sections from hearts of ERβ−/− and WT mice documenting hypertrophy and dilation of ventricles are shown in Fig. 1 C and D. Histologically, there was an increase in myocyte diameter and an increase in the distance between myocytes (Fig. 1 E and F). In addition, there was an up-regulation of β-myosin heavy chain in hearts of ERβ−/− mice (Fig. 1 G and H), a clear indication of pressure and volume overload (25).

Expression of ERs in WT Mice. There was no detectable expression of ERα or ERβ in the myocardium. Immunohistochemical studies with ERα-specific MC20 antibodies (data not shown) failed to detect ERα in the myocardium, but it was well expressed in the epithelial cells lining the large arteries. ERβ-specific IgY also did not detect ERβ in the myocardium. ERβ was well expressed in the nucleus of cells in the alveoli and in the epithelial cells of the bronchioles (Fig. 2A). Western blots with mitochondrial fractions and nuclear extracts prepared from hearts of WT mice were probed with specific ERβ ligand binding domain and N-terminal antibodies. As shown previously (26), with the ligand binding domain antibody, ERβ was easily detected on Western blots with nuclear extracts from the lungs of WT mice. No specific signals of the correct size were found in mitochondrial fractions (Fig. 2B) or in nuclear extracts of hearts (Fig. 2C) when blots were probed with the N-terminal antibody.
However, in mitochondrial fractions, a band that migrated at a molecular mass of 70 kDa was recognized by this antibody. This band was present in fractions prepared from hearts of ERβ/H9252/H11002 mice and is thus unlikely to be related to ERβ/H9252/H11002. To confirm equal loading in the lanes, the blot was probed for a specific mitochondrial marker, cytochrome oxidase-2. The antibody was a gift from Nils-Göran Larsson (Karolinska Institutet).

Structure of the Heart of ERβ−/− Mice. By light microscopy, it was evident that the orientation of myofibrils was irregular and they were of heterogeneous length in ERβ−/− mice. However, as shown by phalloidin stain, which stains f-actin in the I-bands,  

were stained for f-actin with FITC-phalloidin, no alterations in the f-actin cross striations were observed in ERβ−/− hearts; however, the individual myofibrils do not run as strictly parallel in the ERβ−/− cardiomyocytes as they do in WT and are of heterogeneous size. (Scale bar, 20 μm.) (I and J) Levels of ANF (proatrial natriuretic factor) and β-MHC (β-myosin heavy chain) genes were measured by RT-PCR. The data are expressed as mean ± SEM. *** P < 0.001. The expression of β-MHC mRNA was increased 5-fold in ERβ−/− mice.
there was no gross alteration of sarcomere striation (Fig. 1 G and H).

By transmission electron microscopy, the IDs were more convoluted in ERβ−/− mouse hearts (Fig. 3 B and C) than in hearts of WT littermates (Fig. 3A). Measurements of ID length showed an increase of 110 ± 15% (SD) in membrane length in ERβ−/− mice. The ratio of membrane length to width was 1.62 ± 0.26 for WT and 3.03 ± 0.62 for ERβ−/− hearts, confirming a higher degree of convolution of the IDs in ERβ−/− mice.

N-cadherin was organized in a parallel pattern along the ID region of the plasma membrane in WT cardiomyocytes (Fig. 3D), but in ERβ−/− hearts, N-cadherin was dispersed (Fig. 3E). Often, regions of the ID were ruptured, and the distance between the myofibrils was increased (Fig. 3F).

By confocal microscopy (Fig. 4A, Cx43 signals in WT mice were concentrated at points of intercellular apposition in a pattern consistent with the distribution of GJs and IDs. In sections of hearts from ERβ−/− mice, there was an increase in the amount of Cx43 signal at points of intercellular apposition, and the staining was more disperse (Fig. 4B). Such a distribution is consistent with the long and convoluted disks. Digital image processing confirmed that the proportion of total myocardial tissue area occupied by a strong Cx43 immunoreactive signal was higher in ERβ−/− than in WT mouse hearts. The mean plaque size was 0.65 ± 0.05 μm² in WT mice and 1.62 ± 0.36 μm² in ERβ−/− mice (Fig. 4C). The area densities in WT and ERβ−/− mice were 8.5 ± 1.3 and 12.1 ± 1.7 plaques per 1,000 μm², respectively (Fig. 4D).

Nuclear Envelope Architecture. Ultrastructural and immunofluorescence analysis of nuclear lamina components in cardiomyocytes of 6-month-old WT mice (Fig. 5) showed a normal smooth nuclear envelope (Fig. 5A). The cardiomyocyte nuclei in ERβ−/− mice were of bizarre shapes with folded nuclear envelope (Fig. 5B) and convoluted membranous structures close to the nuclei (Fig. 5C). Immunohistochemical analysis revealed that lamin A/C was associated with the nuclear envelope in myocytes from WT mice (Fig. 5D), but in ERβ−/− mice, there was sparse patchy
Discussion

ERβ⁻/⁻ mice develop sustained systolic and diastolic hypertension as they age (1). In the present study, we have shown that there are abnormalities in the hearts of these mice and have investigated these abnormalities to determine whether they are simply normal responses to hypertension or whether the loss of ERβ from the heart has contributed to the morphological abnormalities.

We found that the hearts of ERβ⁻/⁻ mice responded to the stress of chronic hypertension in a predictable fashion. One indicator of this stress was the increase in the expression of β-MHC, the fetal form of myosin in the mouse (27, 28). This is an energy conservation switch as β-MHC has a lower ATPase activity. In addition, there were changes in the IDs, a disarray in GJs, and an increase in the major ventricular GJ protein Cx43, all typical adjustments of the heart to hypertension (29–37).

There was also rupture of the myocyte fibrillar apparatus, a condition that has been described in mice lacking muscle LIM protein (38) or plakoglobin (39).

The nuclei of ERβ⁻/⁻ mouse hearts were of irregular shape with distended herniations of the nuclear membrane and presence of convoluted membranous structures close to the nucleus. These types of changes are observed in cardiomyocytes where lamin A/C gene is abnormal (40, 41). Nuclear lamins give the nucleus its shape and are involved in chromatin organization, DNA replication, gene expression, and transmission of mechanical signals from the cell surface to the nucleus (42–46). We found in the present study that although there was no reduction in the cellular content of lamin A/C, there was loss of lamin A/C from the nuclear envelope in ERβ⁻/⁻ mice. Mislocation of lamins is the most likely cause of the observed abnormalities in nuclear shape.

Unlike what has been reported in previous studies from other laboratories (12–17), we could not detect ERβ in the nuclei of cardiac myocytes. The antibodies used in this study have been tested extensively for specificity for ERβ in immunochemical studies in the mammary gland, testes, salivary glands, prostate, and uterus. ERα was also undetectable. The levels of nuclear receptors in tissues are usually very low. For ERs, it is in the fmol/mg protein range. In breast cancer, the range is 10–1,000

nuclear staining and intense cytoplasmic staining (Fig. 5E). In the lungs of WT (Fig. 5F) and ERβ⁻/⁻ (Fig. 5G) mice, lamin A/C was localized as a clear ring surrounding the nucleus. On Western blots with nuclear fractions, lamin A/C was well expressed in WT mice and absent from ERβ⁻/⁻ mouse hearts. Levels of lamin A/C in the total cell extract were similar in WT and ERβ⁻/⁻ mice (Fig. 5H).

Fig. 5. Altered nuclear structure in ERβ⁻/⁻ cardiomyocytes. (A) WT nuclei display a normal elongated morphology with a smooth nuclear envelope. (B) Nuclei in ERβ⁻/⁻ mice display an irregular, folded nuclear envelope. (C) Occasionally, close to the nuclear envelope, stacks of membrane are found (arrowheads). N, nucleus. (Scale bar: 2 μm (A and B), 0.2 μm (C), and 20 μm (D–G).) Immunohistochemical analysis of lamin A/C in cardiomyocytes and alveolar epithelial cells from WT (D and F) and ERβ⁻/⁻ (E and G) mice. (E) In the ERβ⁻/⁻ muscle, the signal intensity of lamin A/C around the nuclei appears either increased or weaker, but commonly delocalized and not confined to the nuclear envelope. These immunolabeled structures in the cytoplasm possibly constitute the counterparts of the excess membranous material observed by transmission electron microscopy. Nuclei are counterstained with propidium iodide. In the lungs of WT (Fig. 5F) and ERβ⁻/⁻ (G) mice, lamin A/C is localized as a clear ring surrounding the nucleus. (F) Western blot analysis of lamin A/C protein expression levels in total cell extracts and nuclear extracts from WT (lanes 3–5) and ERβ⁻/⁻ mice (lanes 1 and 2). Levels of lamin A/C in total cell extracts are similar in WT and ERβ⁻/⁻ mice. Note the total lack of lamin A/C expression in nuclear extracts from ERβ⁻/⁻ mice.
In some cancers, ERα levels can reach as high as 2–3 pmol/mg cytosolic protein (120–180 ng/mg protein), but this is very rare (47). The recent study (17) showing the content of ERα and ERβ in MCF-7 cell homogenates at 1 μg/mg protein (16 pmol/mg protein) seems to be unrealistic. It should be routine that standard amounts of ER proteins be used on Western blots, along with the samples being tested. An aliquot containing 30 μg of protein from tissue homogenates should not contain more than 60–90 fmol of ER.

As discussed above, there is no consensus on whether ERβ is present in the myocardium and little evidence for direct effects of estrogen in the myocardium. When estrogen is shown to be present in the myocardium and little evidence for direct effects of estrogen on cellular localization of lamins. This research was supported by grants from the Swedish Cancer Fund and KaroBio AB and by EC Marie Curie Individual Postdoctoral Fellowship MCFI-2000-01758 (to C.F.).