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Increased estrogen receptor alpha in experimental aortic aneurysms in females compared with males

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ARTICLE INFO

Article history:
Received 3 April 2013
Received in revised form
24 July 2013
Accepted 25 July 2013
Available online 18 August 2013

Keywords:
Aortic aneurysm
Estrogen
Gender difference

ABSTRACT

Background: Estrogen receptor alpha (ERα) has been identified in the vessel wall, offering vasoprotective effects when upregulated. Estrogens are known to mediate the inflammatory milieu, and inflammation has long been associated with abdominal aortic aneurysm (AAA) formation. Therefore, it is theorized that increased estrogen receptor in females contributes to their relative resistance to AAAs. The objective of this study was to determine gender differences in ERα levels during experimental AAA formation.

Methods: Infrarenal aortas of male and female C57 mice ($n = 18$ and $n = 16$, respectively) were infused with 0.4% elastase. Diameters were measured at days 0 and 14. Aortic messenger RNA expression of ERα was determined on day 3 by reverse transcription–polymerase chain reaction, whereas ERα protein levels were measured via Western blot. Immunohistochemistry using rabbit antibody for ERα was performed on day 14 samples and quantified. Zymography was done for matrix metalloproteinases (MMP)2 and 9 activity levels. Samples of human AAAs were collected and Western blot performed. Data were compared for significance using a student *t*-test.

Results: Infrarenal aortic diameter increased in elastase-perfused males (ME) by 80% at 14 days after perfusion, whereas females (FE) increased by only 35% ($P = 0.0012$). FE had ×10 greater ERα messenger RNA expression compared with ME at day 3 ($P = 0.003$). Similarly, ERα protein levels were 100% higher in FE compared with those in ME on day 14 ($P = 0.035$). ERα protein levels were 80% higher in female human patients with AAA than those in their male counterparts ($P = 0.029$). ERα visualized via immunohistochemistry was 1.5 fold higher in FE than ME ($P = 0.029$). MMP2 and 9 activity levels were decreased in female compared with male aortas.

Conclusions: This study demonstrates an increase in aortic wall ERα in females compared with males that correlates inversely with MMP activity and AAA formation. These findings, coupled with observations that exogenous estrogen inhibits AAA formation in males, further suggest that estrogen supplementation may be important to prevent AAA formation and growth.

Presented at the American College of Surgeons, Washington DC, October 6, 2010.

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http://dx.doi.org/10.1016/j.jss.2013.07.050

1. Introduction

Abdominal aortic aneurysm (AAA) formation is known to be an inflammatory process involving infiltration of macrophages and lymphocytes, release of proinflammatory cytokines, and eventual activation of matrix metalloproteinases (MMPs), which degrade the extracellular matrix. In humans, AAA disease affects men four times as often as women. Investigational studies from our laboratories and others [1–3] suggest that this is in part because of a protective role of estrogen.

The biochemistry of sex hormones and their role in AAA formation is made more complex by the multiple and varied hormone receptors throughout the vasculature. A g-protein–related estrogen receptor (GPER), located in the endoplasmic reticulum, mediates rapid responses to changes in vascular tissues. In contrast, estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) are classic nuclear receptors in the cardiovascular system. Specifically, ERα mediates endothelial responses after vascular injury, whereas ERβ mediates arterial tone and blood pressure. ERα has also been identified as offering vasoprotective effects when upregulated in the vessel wall. This is likely due to decreased inflammation suggesting a possible role for ERα during AAA formation and that it perhaps is at at least partially responsible for the gender differences in AAA formation. The objective of this study was to examine the role of ERα during experimental AAA formation in a murine model.

2. Methods

2.1. Animal surgery

Mice were obtained from Jackson Laboratories (Bar Harbor, ME). Infrarenal aortas of 8- to 12-wk-old male and female C57BL/6 mice ($n = 18$ and $n = 16$, respectively) were infused with 0.4% pancreatic porcine elastase. Animals were harvested at days 0, 1, 3, and 14. The day 0 was nonperfused animals for baseline control polymerase chain reaction (PCR). Day 1 and 3 samples were for PCR, and day 3 samples were also processed for zymography. Day 14 samples were prepared for Western blot and immunohistochemistry. Aortic diameters were measured midaorta before perfusion and then at postoperative days 3, 7, and 14. This was done using a video micrometer and NIS Elements software on a computer attached to the microscope (Nikon, Melville, NY). The baseline (day 0) measurement was subtracted from the subsequent measurements to determine the percent increase in diameter. All experiments were approved by the University of Michigan Universal Committee on the Use and Care of Animals (UCUCA No.09679).

2.2. Messenger RNA extraction, reverse transcription–PCR, and real time-PCR

Aortic messenger RNA (mRNA) expression of ERα was determined on days 1 and 3 by PCR. Later time points have not produced rigorous PCR data in our laboratory previously. Established techniques using TRIzol reagent (Invitrogen, Carlsbad, CA) were used to extract mRNA for reverse transcription (RT)–PCR. In brief, fresh explanted aortic tissue was added to 1.5 mL of TRIzol reagent and homogenized for 45 s. Samples were frozen at this point at −70°C. Chloroform (+99%) was then added to the homogenized tissue, vortexed, and centrifuged. The clear supernatant was pipetted into Eppendorf tubes, whereas the RNA precipitation was performed with isopropanol (+99%) and 7.5 μg of glycogen. The resulting solution was centrifuged, and the supernatant was again poured off. The remaining mRNA pellet was then washed by adding 70% ethanol in diethylpyrocarbonate water and centrifuged. The supernatant was aspirated off once more, and the pellet was dried at room temperature. The pellet was redissolved in diethylpyrocarbonate water at 58°C. The RNA concentration was then measured on the Nanodrop 1000 Spectrophotometer (Thermo Scientific, Pittsburgh, PA). Appropriate dilutions were made to produce 5 μg/μL of RNA. RT reaction with standard reagents in a GeneAmp 2400 PCR System (Perkin Elmer-Applied Biosystems, Norwalk, CT) was then performed. The concentration of the resulting complementary DNA was measured using the Nanodrop 1000 Spectrophotometer. Dilutions were made to calculate the amount needed to obtain 22 ng/μL of complementary DNA for the real-time PCR. The primers and SYBR Green Master Mix-PCR were obtained from SABiosciences (Qiagen, Frederick, MD). The RotorGene 6000 Series Software 1.7 (Corbett Research; Qiagen) was used with the following program: 95°C, 10 min; 40 cycles of (95°C, 15 s; 60°C, 60 s). Target mRNA was therefore amplified and the take-off values and melt curves were obtained for analysis. mRNA expression of estrogen receptors (ERs) 1 (α) and 2 (β) was compared with that of β-actin, a housekeeping gene.

2.3. Western blot

ERα protein levels were measured via Western blot from aortic tissue harvested on day 14 and human aortic tissue. For Western blot analysis, the frozen tissues were thawed and then lysed by homogenization and sonication before overnight incubation in ice-cold radio-immunoprecipitation assay buffer (Sigma, St. Louis, MO) containing protease and phosphatase inhibitors (Roche, Basel, Switzerland). Protein concentration in the lysate was determined with the BCA protein assay kit (Pierce, Rockford, IL). Equal amounts of protein were loaded into each well and resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with 10% gels (Novex; Invitrogen). They were then electroblotted onto polyvinylidene membranes (Immobilon-P; Millipore, Billerica, MA) by semidry transfer blot (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. The membranes were incubated in StartBlock TBS (Pierce) for 1 h and then with total ERα or ERβ in StartBlock solution at 1:500 dilution overnight with gentle shaking. The membranes were washed in 25 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.4 (TBST) for 1 h at room temperature. They were then incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h and again washed in TBST. For normalization of proteins on the Western blots, the