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The Effects of Estrogen and Testosterone on Gene Expression in the Rat Mesenteric Arteries

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Abstract

A dramatic difference exists in the timing of development of cardiovascular disease in men vs. women. The primary candidates underlying the cause of this gender difference are the sex steroids, estrogen and testosterone. The vasculature is considered to be a site of action of these steroids. In spite of these concepts there is little data on the direct effects of estrogen and testosterone on gene expression in the vasculature. In this study, ovariectomized Sprague Dawley rats were treated for 4 days with vehicle (sesame oil), estradiol benzoate (0.15 mg/kg/day), or testosterone (1 mg/kg/day). The mesenteric arteries were obtained, total RNA was extracted, and CodeLink Uniset Rat I DNA microarrays were used to identify differential gene expression. Seven genes were identified as differentially expressed from the DNA microarray data and confirmed by real time RT-PCR. The expression of D site albumin promoter binding protein and fatty acid synthase were increased in response to both estrogen and testosterone. 3 alpha-hydroxysteroid dehydrogenase, interleukin 4 receptor, JunB and c-Fos expression were increased by estrogen but not by testosterone. Aryl hydrocarbon nuclear translocator-like gene was reduced by testosterone. These data identify genes not previously known to be responsive to estrogen and testosterone in the vasculature.

Keywords

vasculature; gender; steroid; DNA microarray; differential expression

1. Introduction

It is generally accepted that cardiovascular function exhibits sexual dimorphism. Prior to the menopause, women have a much lower rate of cardiovascular diseases such as hypertension and atherosclerosis than do men (Calhoun and Oparil, 1999; Mendelsohn, 2000). Similarly, sex differences in cardiovascular and vascular function have been reported in animal models (Dubey et al., 2002; Reckelhoff, 2005). Nevertheless, the relative role of androgens and estrogens in these sex differences remain to be fully delineated. Since the rate of cardiovascular disease in postmenopausal women rises to approach, or exceed, that of men, it was long assumed that estrogen was protective to the cardiovascular system (Mendelsohn, 2000). There is considerable data in the literature that support a protective effect of estrogen in the cardiovascular system (Dubey et al., 2002; Oparil, 1999; Mendelsohn, 2000); however, other

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data suggest that estrogens may also have deleterious effects. For example, the recent large Women's Health Initiative (WHI) trials of estrogen and estrogen plus progesterone did not support a protective effect of estrogen in the cardiovascular system (Speroff, 2004; Writing Group for WHI, 2002).

The data concerning androgenic influences on cardiovascular function are also conflicting. For example, several lines of evidence support the argument that androgens enhance the development of cardiovascular diseases such as hypertension in both animals and humans (Reckelhoff, 2005; Reckelhoff et al., 1999). The postmenopausal ovary continues to secrete androgens (Bulun and Adashi, 2003) which may contribute to the postmenopausal development of cardiovascular disease (Reckelhoff, 2005). Nevertheless other data, such as the inverse correlation between testosterone concentrations and coronary artery disease (Rosano et al., 2005), are consistent with a cardiovascular protective effect of androgens. Thus, while it is clear that sex steroids influence cardiovascular function, their precise effects remain controversial. Some of this controversy may reside in the fact that many studies assessed aggregate variables representing the summation of a number of individual influences. In order to understand these complexities, it may be necessary to initially focus on a single variable.

It is clear that the vasculature is a target for sex steroids. Vascular smooth muscle was reported to express the estrogen receptors (Karas et al., 1994) and the androgen receptor (Fujimoto et al., 1994). These receptors are important functionally. For example, estrogen treatment has been reported to modulate vascular function when given chronically (Colucci et al., 1982; Farhat et al., 1996) or acutely (Darkow et al., 1997). Estrogen treatment of human endothelial cells for 24 hours also influenced vascular adhesion molecule expression (Piercy et al., 2002) suggesting that estrogen directly affects vascular tissue. Similarly, while not as extensively investigated, current data suggest that androgens affect vascular function in both the acute (Ding and Stallone, 2001) and chronic settings (Song et al., 2006a). Physiological levels of testosterone were also reported to influence endothelial control of vascular tone (Gonzales et al., 2004) while chronic exposure of human endothelial cells in culture to testosterone increased synthesis of biglycan, a proteoglycan, and enhanced lipoprotein binding (Hashimura et al., 2005). Our previous work is consistent with an effect of sex steroids on both vascular function and structure. We observed that estrogen reduced vascular expression of structural components such as biglycan (Rodrigo et al., 2003a) and functional components such as endothelin converting enzyme (Rodrigo et al., 2003b) whereas testosterone increased Rho kinase expression in vascular tissue (Song et al., 2006b). Collectively these data suggest that the vasculature is a target for sex steroid modulation of cardiovascular function. Nevertheless, comprehensive assessment of sex steroid influences on vascular gene expression are sparse. Accordingly, the current study chose to focus on the effects of sex steroids on vascular gene expression by utilizing a DNA microarray driven gene expression profiling approach. The goal of the current study was to identify new targets of estrogen and testosterone in the blood vessels using DNA microarray technology.

2. Materials and Methods

2.1. Animal treatments

All protocols for animal handling and treatment were approved by the Institutional Animal Care and Use Committee of the University of South Dakota and conform to the NIH Guide for the Care and Use of Laboratory Animals. In order to establish a common baseline from which to study the vascular genomic effects of estrogen and testosterone, prepubertal (28 day old) female Sprague-Dawley rats were ovariectomized under isoflurane anesthesia and allowed a 2 week recovery period. (It is important to note that at this age the animals have not yet experienced the dramatic increase in ovarian steroidogenesis that occurs with the initiation of ovarian cyclicity at puberty (Ojeda and Urbanski, 1988).) The rats were then injected

intraperitoneally daily for 4 days with vehicle (sesame oil), estradiol benzoate (0.15 mg/kg/day) or testosterone (1 mg/kg/day). After the injection on the fourth day the animals were euthanized. The uterus was removed from each animal and weighed, blood was collected in heparinized containers, and the mesenteric vascular tree was dissected from the gastrointestinal tract and placed into RNAlater (Ambion, Austin, TX). Blood samples were also collected from day 21 pregnant rats for comparison of estradiol concentration in treated rats to that of natural pregnancy. The mesenteric arteries were dissected free from the surrounding fat and connective tissues while immersed under RNAlater as previously described (Rodrigo et al., 2002). The mesenteric vasculature was chosen for these studies because the it can be separated from surrounding fat and connective tissue with relative ease (Rodrigo et al., 2002) and because the mesenteric vessels comprise a reactive vascular bed that is intimately involved in the regulation of blood pressure (Christensen and Mulvaney, 1993).

2.2. RNA extraction

Total RNA was extracted from the mesenteric arteries using a modification of our previously published technique (Rodrigo et al., 2002). The mesenteric arteries were homogenized in TRI reagent (MRC, Cincinnati, OH) using a Polytron (Brinkmann). The RNA-containing layer was removed and purified on an RNeasy extraction column (Qiagen, Valencia, CA). The samples were treated with an on-column DNase treatment (RNase-free DNase, Qiagen) to remove any potentially contaminating DNA from the RNA samples. The purity and quantity of the RNA was evaluated by spectrophotometry and agarose gel electrophoresis.

2.3. DNA microarray

CodeLink Uniset Rat I DNA microarrays (Ramakrishnan et al., 2002) were obtained from GE Healthcare (Piscataway, NJ). These microarrays contained 10,000 single-stranded 30-mer oligonucleotide probes for rat genes/transcribed sequences. The total RNA samples from the mesenteric arteries of 4 individual animals per group were used in the microarray analysis; no samples were pooled. Each individual sample was analyzed with duplicate microarrays. First-strand cDNA was reverse transcribed from the mRNA in the total RNA sample and second-strand cDNA was synthesized from the first strand. Complementary RNA was synthesized from the cDNA; this reaction incorporated biotin-11-UTP into the cRNA. The cDNA and cRNA synthesis reactions used an in vitro translation kit (Codelink Expression Assay Reagent Kit, GE Healthcare). Purified biotinylated cRNA was hybridized with the glass slide microarrays for 18 hours at 37°C. The hybridized slides were washed, incubated with streptavidin-Alexa Fluor 647 (Molecular Probes) to label the biotinylated cRNA hybridized to the slides, and washed again (Ramakrishnan et al., 2002). The slides were scanned with an Axon GenePix Scanner and analyzed with GenePix Pro (Axon), CodeLink (GE Healthcare) and GeneSpring (Agilent) software. The GenePix Pro software aligns and acquires the microarray image. Positive and negative bacterial control genes are spotted on the CodeLink arrays, and specific bacterial mRNA is used to spike the cDNA synthesis reaction. The bacterial gene spikes are used to assess the background and signal threshold and provide an additional means of normalizing gene expression in CodeLink software. Background correction was performed by the CodeLink software. GeneSpring software normalized the expression of each gene to the median and each slide to the 50th percentile. The DNA microarray data presented herein have been deposited at the NCBI Gene Expression Omnibus (GEO, <www.ncbi.nlm.nih.gov/geo>) as recommended by MIAME standards (Brazma et al., 2001) and can be accessed through GEO Series accession number GSE4782.

2.4. Real time RT-PCR

Real time reverse transcription-polymerase chain reaction (RT-PCR) (n=5) was used to confirm the differential expression of genes identified by DNA microarray. Real time RT-PCR

used TaqMan Gold RT-PCR reagents (Applied Biosystems, Foster City, CA). MultiScribe reverse transcriptase was used for the first strand cDNA synthesis and thermal stable AmpliTaq Gold DNA polymerase was used for DNA amplification. Primers and minor groove binding probes were obtained from Assays on Demand (Applied Biosystems) (D site albumin promoter binding protein: Rn00497539_m1, 3α-hydroxysteroid dehydrogenase: Rn00593618_m1; fatty acid synthase: Rn00569117_m1; Interleukin 4 receptor: Rn00591282_m1; JunB: Rn00572994_s1; c-Fos: Rn02105433_s1; ARNT-like: Rn00577590_m1). Cyclophilin was used as the housekeeping gene for each sample Changes in expression of the genes of interest were calculated relative to the cyclophilin control. Real time RT-PCR data were analyzed with qBase software (Hellemans, et al., 2007). This program uses a delta-Ct (threshold cycle) relative quantitation model with PCR efficiency correction and multiple reference gene normalization.

2.5. Enzyme-linked immunosorbent assays (EIA)

Estradiol and testosterone levels were measured in plasma samples. An estradiol EIA kit was obtained from Alpco Diagnostics (Salem, NH). The estradiol EIA was specific for estradiol and did not crossreact with estriol or estrone. The sensitivity of the assay was 10 pg/ml. A testosterone EIA kit was obtained from Biosource International (Camarillo, CA). The testosterone EIA was specific for testosterone and did not crossreact with other androgens or steroid molecules. The sensitivity of the testosterone EIA was 0.05 ng/ml.

2.6. Statistical Analysis

Because of the need for RT-PCR confirmation of the microarray data, we applied strict criteria to limit the number of genes targeted for follow up. GeneSpring software was used to apply Welch's one-way analysis of variance (parametric test, variances not assumed to be equal) to the DNA microarray data; $p<0.05$ represented statistically significant difference. Multiple testing correction for this test used the Benjamini and Hochberg False Discovery Rate; approximately 5% of the identified genes would be expected to pass this restriction by chance. The 7 genes that met all criteria for statistical significance were analyzed by real time RT-PCR. The RT-PCR data derived from the qBase analysis were analyzed by one-way ANOVA followed by Newman-Keuls post hoc test to test for differences among groups (GraphPad Prism 4, GraphPad Software, San Diego, CA).

3. Results

3.1. Microarray and real time RT-PCR results

Of the 10,000 genes on the DNA microarrays, statistical analysis by GeneSpring identified a list of 759 genes for which either the estrogen- or the testosterone-treated group was significantly different from control with a p value less than 0.05. 449 of the genes in the list were transcribed sequences with no name and no known function; these genes were not further analyzed. Genes for which the average expression values for control, estrogen-, and testosterone-treated values were all less than 0.2 were also excluded from further comparison as those values were considered to be at the limits of detection of the microarray. This exclusion criterion removed 221 genes from further analysis. An order statistic was applied to the remaining 89 genes on the list. The order statistic analyzed the individual values within each group average to search for overlap. Genes were eliminated from further study if individual values in the control group overlapped with individual values in the statistically different estrogen-treated or testosterone-treated group. Only 7 of the 89 genes passed the order statistic. Confirmatory studies for these 7 genes were performed using the independent and more quantitative technique of real time RT-PCR.

The first differentially expressed gene was the transcription factor D site albumin promoter binding protein (*Dbp*). Testosterone stimulated the expression of *Dbp* by DNA microarray data (Figure 1A) (control 3.27 ± 0.2, estrogen 5.70 ± 0.8, testosterone 11.34 ± 0.6). (Data from the DNA microarrays are expressed as the signal strength (pixel intensity) of the fluorescent dye attached to the cRNA probe that hybridized to the microarray slide.) The more quantitative technique of real time RT-PCR confirmed significant stimulation of the expression of *Dbp* by testosterone and indicated a significant increase in expression of *Dbp* in response to estrogen as well (Figure 1B) (control (C) 1.0 ± 0.15, estrogen (E) 3.74 ± 0.58, ($p<0.01$ C vs. E) testosterone (T) 9.41 ± 0.90 ($p<0.001$ C vs. T)). The effect of testosterone was significantly greater than that of estrogen ($p<0.001$ E vs. T).

The expression of 3α-hydroxysteroid dehydrogenase 1C (*3α-Hsd 1C*) was increased in response to estrogen treatment by DNA microarray (Figure 2A) (control 1.37 ± 0.2, estrogen 4.01 ± 0.3, testosterone 1.79 ± 0.1). Significant stimulation of *3α-Hsd 1C* by estrogen was confirmed by real time RT-PCR (Figure 2B) (control 1.0 ± 0.16, estrogen 2.67 ± 0.20 ($p<0.001$ C vs. E), testosterone 1.26 ± 0.10 ($p>0.05$ C vs. T)).

Microarray data indicated that fatty acid synthase (*Fas*) expression was also differentially expressed in response to estrogen (Figure 3A) (control 18.52 ± 2.1, estrogen 30.92 ± 1.9, testosterone 21.80 ± 1.7). The more quantitative methodology of real time RT-PCR data indicated a significant increase in *Fas* expression in the mesenteric arteries in response to both estrogen and testosterone (Figure 3B) (control 1.0 ± 0.30, estrogen 5.37 ± 0.54 ($p<0.001$ C vs. E), testosterone 3.05 ± 0.60 ($p<0.05$ C vs. T)). The effect of estrogen on *Fas* expression was significantly greater than that of testosterone ($p<0.01$ E vs. T).

The DNA microarray data indicated that interleukin 4 receptor (*Il4r*) was differentially expressed in response to estrogen (Figure 4A) (control 2.09 ± 0.2, estrogen 3.90 ± 0.3, testosterone 1.58 ± 0.07). Real time RT-PCR data confirmed the stimulation of *Il4r* in response to estrogen (control 1.0 ± 0.09, estrogen 3.58 ± 0.34 ($p<0.001$ C vs. E), but not testosterone (testosterone 1.30 ± 0.10 ($p>0.05$ C vs. T)) (Figure 4B).

The microarray data indicated that the expression of JunB was increased in the mesenteric arteries in response to both estrogen and testosterone (Figure 5A) (control 0.18 ± 0.01, estrogen 0.49 ± 0.07, testosterone 0.39 ± 0.05). However, the more quantitative method of real time RT-PCR analysis demonstrated that estrogen, but not testosterone, significantly stimulated JunB expression (Figure 5B) (control 1.0 ± 0.10, estrogen 2.13 ± 0.25 ($p<0.01$ C vs. E), testosterone 1.38 ± 0.19 ($p>0.05$ C vs. T)). The expression of the related gene, c-Fos, also changed in response to estrogen (Figure 6A) (control 3.21 ± 0.40, estrogen 9.25 ± 2.74, testosterone 6.61 ± 0.62). Real time RT-PCR analysis confirmed the significance of this differential expression in response to estrogen but not to testosterone (Figure 6B) (control 1.0 ± 0.19, estrogen 3.92 ± 0.78 ($p<0.01$ C vs. E), testosterone 2.01 ± 0.45 ($p>0.05$ C vs. T).

The microarray data indicated that the expression of the aryl hydrocarbon receptor nuclear translocator-like gene (*Arnt-like*) was reduced in response to estrogen and testosterone (Figure 7A) (control 3.74 ± 0.16, estrogen 2.86 ± 0.20, testosterone 1.67 ± 0.17). However, real time RT-PCR confirmed the inhibition of *Arnt-like* expression in the mesenteric arteries by testosterone (Figure 7B) (control 2.25 ± 0.22, testosterone 1.00 ± 0.12 ($p<0.01$ C vs. T), but not estrogen (estrogen 1.88 ± 0.25 ($p>0.05$ C vs. E)).

3.2. EIA and uterine weight results

The uterine weight of ovariectomized vehicle-treated animals was 43.85 ± 5.2 mg. The uterine weight of ovariectomized estradiol benzoate-treated animals was 144.01 ± 20.3 mg, whereas the uterine weight of ovariectomized testosterone-treated animals was 25.58 ± 3.1 mg.

The concentration of estradiol in estradiol benzoate-treated rats was 212.12 ± 46.4 pg/ml, which is similar to the levels of natural estradiol achieved in late pregnancy in the rat (232.5 ± 66.6 pg/ml). The concentration of testosterone achieved in the testosterone-treated ovariectomized rats was 0.14 ± 0.03 ng/ml. This level of testosterone is similar to normal levels of testosterone in the female rat (i.e., approximately 10% of adult male concentrations).

4. Discussion

Relatively few studies have assessed the effect of sex steroids on gene expression in mesenteric arteries (Hemmings et al., 2004; Nagar et al., 2005; Yallampalli et al., 2004; Ross et al., 2006). The majority of previous work was highly focused on select gene targets. In previous studies we showed that estrogen reduced the expression of biglycan (Rodrigo et al., 2003a) and endothelin converting enzyme-1 (Rodrigo et al., 2003b) in the mesenteric arteries. These data indicate that estrogen can affect the expression of both structural and enzymatic system genes in the vessels and suggest a wide ranging effect on gene expression in these vessels. Accordingly, the current project was designed to more comprehensively assess the effect of estrogen and testosterone on gene expression in the mesenteric arteries using concentrations of estradiol and testosterone similar to the natural levels of these steroids in the adult female.

While we observed that expression of approximately 759 genes was modulated by the sex steroid treatments, we used stringent selection criteria to identify 7 genes that had not previously been known to respond to steroid hormones in the mesenteric arteries. These genes are not part of the classical vascular control system architecture and thus represent novel targets for further investigation in the vasculature. These seven genes code for proteins that belong to diverse classes: transcription factors, energy and hormone metabolism, and cytokine signaling, suggesting that steroid hormones may have wide ranging effects on vascular function. In keeping with our goal of identifying new avenues for the study of vascular function, this discussion will focus on linking these novel targets to vascular biology and will serve as the starting point for the development of new hypotheses for future studies.

3α-hydroxysteroid dehydrogenase 1C (*3α-Hsd*, aldo-ketoreductase 1C) was significantly increased in the mesenteric arteries in response to estrogen in agreement with work of others in the liver (Penning, 1997). In contrast, testosterone treatment did not significantly affect expression of 3α-HSD. 3α-HSD converts 5α-dihydrotestosterone, a potent androgen, to 3α-androstanediol, a weak androgen (Penning, 1997; Dufort et al., 2001). If the hypothesis that androgens have a deleterious effect in the cardiovascular system is correct (Reckelhoff et al., 1998; Reckelhoff et al., 1999; Reckelhoff, 2005), then the potential for estrogen to stimulate the conversion of a strong androgen to a weak androgen could be a powerful mechanism for the protective effect of estrogen in the vasculature. Since androgens can be converted to estrogens, these data suggest the intriguing possibility of a paracrine negative feedback loop in vascular tissue. Thus the first differentially expressed gene of interest in the mesenteric arteries is consistent with a potential protective effect of estrogen.

Fatty acid synthase (*Fas*) was also shown to be up-regulated in the mesenteric arteries in response to both estrogen and testosterone treatments. FAS is an important enzyme in the synthesis of fatty acids from malonyl CoA (Semenkovich, 1997). Up-regulation of *Fas* in response to estrogen has previously been reported in the chicken oviduct and liver (Aprahamian et al., 1979; Manning et al., 1989), and androgens have been reported to stimulate the expression of lipogenic genes including *Fas* (Heemers et al., 2003). FAS may affect cardiovascular function via multiple mechanisms. By virtue of an increase in free fatty acids (FFA), induction of FAS may directly affect vascular tone. Previous work has suggested that FFA can potentiate adrenergic constriction (Stepniakowski et al., 1996) and inhibit endothelial dilator mechanisms (Shankar and Steinberg, 2005). Thus, the increased FAS expression in

response to estrogen and testosterone is consistent with the ability of these steroid hormones to increase adrenergic vascular constrictor activity (Colucci et al., 1982; Bedran-de-Castro and Bedran-de-Castro, 1991; Song and Martin, 2006).

Our data demonstrate a significant increase in the expression of the IL4 receptor (*Il4r*) in the mesenteric arteries in response to estrogen but not to testosterone. Interleukins play an important role in the immune system but their actions are now known to extend beyond the immune system (Jiang et al., 2000; Nelms et al., 1998). Consistent with a role in smooth muscle control, estrogen has been previously shown to up-regulate *Il4r* in rat uterine smooth muscle (Rivera-Gonzalez et al., 1998). Activation of the IL4 receptor by either IL4 or IL13 (Jiang et al., 2000) leads to increased expression of the peroxisome proliferator activated receptor γ (PPARγ) (Huang et al., 1999) as well as the 12/15 lipoxygenase (Huang et al., 1999). 12/15 lipoxygenases convert linoleic acid to hydroxyoctadecadienoic acid (HODE) and arachidonic acid to hydroxyeicosatetraenoic acid (HETE); HODE and HETE, in turn, act as ligands at PPARγ (Chinetti et al., 2001; Huang et al., 1999; Poff and Balazy, 2004). Thus, increasing the sensitivity of this pathway by up-regulating the IL4 receptor can increase both PPARγ expression as well as ligands for PPARγ. Activation of PPARγ increases insulin sensitivity and regulates carbohydrate and fat metabolism (Lee et al., 2003). Fatty acids also act as agonists at PPARγ (Kleiwer et al., 1997; Sauma et al., 2006). Thus estrogen may affect this interactive pathway through both the IL4 receptor and through the increase in FFA that results from an up-regulation of FAS, and may thereby modulate cardiovascular function via effects on insulin sensitivity and carbohydrate and fat metabolism through PPARγ. Indeed, estrogen has been shown to increase insulin sensitivity in ovariectomized rats (Alonso et al., 2006; Song et al., 2005).

D site albumin promoter binding protein (*Dbp*) codes for a transcription factor whose expression is increased in the mesenteric arteries by both estrogen and testosterone. The transcription of the genes that are regulated by DBP will not automatically be up-regulated when the transcription factor is up-regulated, but the cells will be more sensitive to the pathway that activates the transcription factor. In the liver the expression of DBP follows a circadian rhythm with the nadir of expression in the morning and peak expression in the evening (Wuarin and Schibler, 1990). This is the first report of an increase in *Dbp* transcript in the vasculature in response to steroid hormones; it is unknown whether the steroid regulation of the *Dbp* gene transcript is imposed on a circadian rhythm of *Dbp* expression in the mesenteric arteries or whether *Dbp* expression in the mesenteric arteries is independent of circadian rhythms.

The expression of both *JunB* and *c-Fos* increased in the mesenteric arteries in response to estrogen treatment; the Jun-Fos dimer is also a transcription factor. The up-regulation of *JunB* (Cicatiello et al., 1992; Ing and Zhang, 2004) and *c-Fos* (Okada et al., 2004) in response to estrogen have been reported previously in uterine smooth muscle and oviduct. Jun-Fos dimers bind and transactivate DNA transcription at AP-1 sites (Chinenov and Kerppola, 2001; Mechta-Grigoriou, 2001; Shaulian and Karin, 2002); the specific family member of each partner (JunB, c-Jun, JunD, and c-Fos, FosB, Fra-1 or Fra-2) in the dimer as well as additional binding partners determines the specificity of binding at AP-1 sites (Mechta-Grigoriou, 2001). The coincident up-regulation of *JunB* and *c-Fos* by estrogen is consistent with an enhancement of the expression of genes that are regulated specifically by the Jun-Fos heterodimer. The estrogen-bound estrogen receptor is one of the many proteins shown to bind to Jun-Fos dimers which then bind to DNA at AP-1 sites (Kushner et al., 2000; Paech et al., 1997). Thus the estrogen-receptor complex has the ability to regulate one set of genes through estrogen response element binding sites and another set of genes through interactions with Jun-Fos at AP-1 response element binding sites. It is not known whether the specificity of the AP-1 site binding may be modulated by its concurrent interaction with the estrogen-receptor complex since the specific Jun and Fos family members involved in the interaction of Jun-Fos dimers

with the estrogen receptor were not identified (Kushner et al., 2000; Paech et al., 1997); perhaps the up-regulation of *JunB* and *c-Fos* by estrogen enhances the ability of estrogen to regulate gene transcription in the mesenteric arteries through the AP-1 site.

The expression of *Arnt-like*, also called *Tic*, in the mesenteric arteries was not affected by estrogen but was reduced by testosterone treatment. ARNT-like is a member of the bHLH (basic helix-loop-helix) and PAS (Per-Arnt-SIM homology) (Kewley et al., 2004) domain-containing family of transcription factors. The PAS domain allows dimerization with other proteins containing a PAS domain (Kewley et al., 2004). The literature has no information about *Arnt-like* beyond the initial cloning of the gene (Wolting and McGlade, 1998). However, ARNT, for which ARNT-like is named, is the nuclear binding partner for the aryl hydrocarbon xenobiotic receptor (AhR). The ARNT-AhR dimer binds to DNA and activates the transcription of genes that are involved in the metabolism of xenobiotics (Kewley et al., 2004). Activation of AhR has been shown to interfere with the estrogen transactivation of genes in breast cancer cells (Safe et al., 2000) and in the female reproductive tract (Pocar et al., 2005). However, it is unknown whether ARNT-like also dimerizes with AhR or whether an ARNT-like-AhR transcription factor dimer is also capable of interfering with testosterone activation of gene transcription as ARNT-AhR does with estrogen.

These data demonstrate that estrogen and testosterone have direct effects on gene expression in the mesenteric arteries. However, we were surprised that the responses of mesenteric artery gene expression to estrogen and testosterone treatment were not more dramatic. The relatively subtle effects of these steroids on gene expression may explain why these differentially expressed genes have not been identified previously and highlight the difficulties in identifying the sources of gender differences in the vasculature. For these studies, we used female animals that had been ovariectomized prior to puberty so their vessels had not yet been exposed to the increase of sex steroids that occurs at puberty. The vascular response to steroids may be different in aged animals that have been exposed to multiple cycles of ovarian hormones or multiple pregnancies. We anticipate that there are other estrogen and testosterone modulated genes that play important roles in the blood vessels that were not identified through these microarrays; only 10,000 rat genes were included on these arrays and strict criteria were used to limit the number of genes followed up by RT-PCR. The 4-day treatment period was chosen to induce measurable steroid mediated gene expression but at the same time avoid induction of vascular remodeling or changes in other factors (e.g. lipid profile) that may occur with more prolonged treatment and could trigger secondary changes in vascular gene expression that would complicate data interpretation. The differences in vascular function occur in normally cycling female rats versus normally functioning male rats (Toba et al., 1991) and in females across the estrous cycle (Dalle Lucca et al., 2000; Neves et al., 2004); thus a 4-day treatment period was considered to be sufficient to mimic normal physiological effects of steroids on gene expression in the vasculature but avoid long term complicating effects such as vascular remodeling.

There are some limitations to this study. Since estrogen and testosterone were administered systemically, some of the differential expression of genes that we observed in the mesenteric arteries may have occurred through indirect rather than direct effects. That is, direct effects of estrogen and testosterone on other tissues may have elicited the synthesis of factors that then directly modified gene expression in the mesenteric arteries. We attempted to limit these potential secondary effects by using relatively brief sex steroid treatment duration. Another potential concern is the level of sex steroids achieved by the treatment regimens. We measured circulating estrogen and testosterone. The levels of estrogen were found to approximate those observed in pregnant rats. Similarly, the circulating level of testosterone was found to be in the some order of magnitude as those of normally cycling female rats (Shaw et al., 2001). Accordingly, our treatment regimens produced circulating levels of sex steroid that were within

the physiological range. In addition to direct measurement of circulating sex steroid concentrations, we used the uterus as a bioassay for the effectiveness of steroids treatment (Owens and Ashby, 2002). As one might expect, estrogen treatment was associated with uterine hypertrophy compared to ovariectomized rats, whereas testosterone treatment failed to induce this effect. Accordingly, two independent measures indicate that our steroid treatment was effective and physiologically relevant. Finally, our goal was to identify novel gene expression patterns modulated by sex steroids. While we achieved this goal, it is important to recognize that future studies will be necessary to further refine these observations at both the cellular protein and organ and systems function levels.

5. Conclusions

In summary, these data have identified genes not previously associated with steroid hormone actions in the vasculature and provide interesting new targets for future investigation. We propose that 3α-HSD may play a protective role in the vasculature by metabolizing a potent androgen to a weak androgen. Moreover, potentially protective effects of estrogen on the vasculature have been identified through effects on fat metabolism through FAS and the interleukin 4 receptor. The proteins encoded by the remaining four genes, *c-Fos, JunB, Dbp,* and *Arnt-like* act as transcription factors. The up-regulation (*c-Fos, JunB, Dbp*) or down-regulation (*Arnt-like*) of these transcription factors in response to sex steroids will modulate the ability of the mesenteric artery cells to respond to the pathways that activate those transcription factors. The role of each of these genes in the normal function of the blood vessels deserves further examination to determine their physiological and pathophysiological functions.

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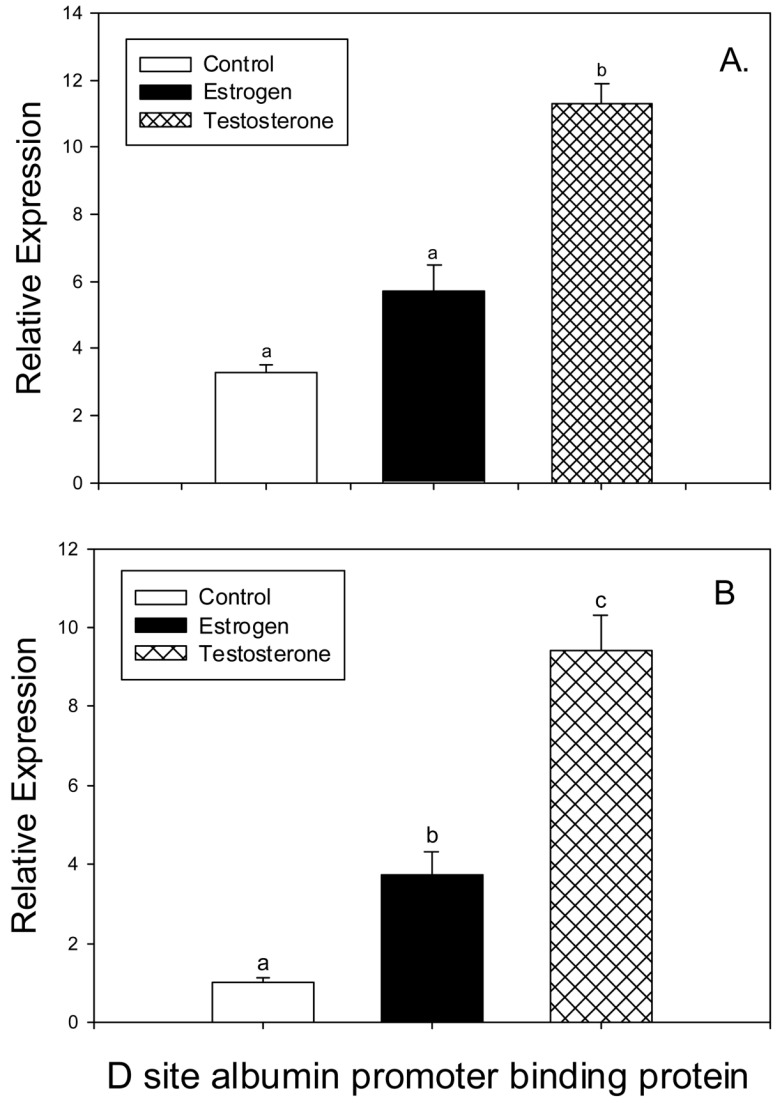

Figure 1.

Differential expression of D site albumin promoter binding protein (*Dbp*) in the mesenteric arteries in response to estrogen and testosterone. Panel A. Data from DNA microarray analysis (n=4). Differential expression of DNA microarray data was determined by Welch's one-way analysis of variance using GeneSpring software ($p<0.05$). Panel B. Data from real time RT-PCR (n=5). Differences among the three groups were identified by one-way ANOVA followed by Newman-Keuls post hoc test ($p<0.05$) of data from real time RT-PCR. Bars with different letters are significantly different from each other.

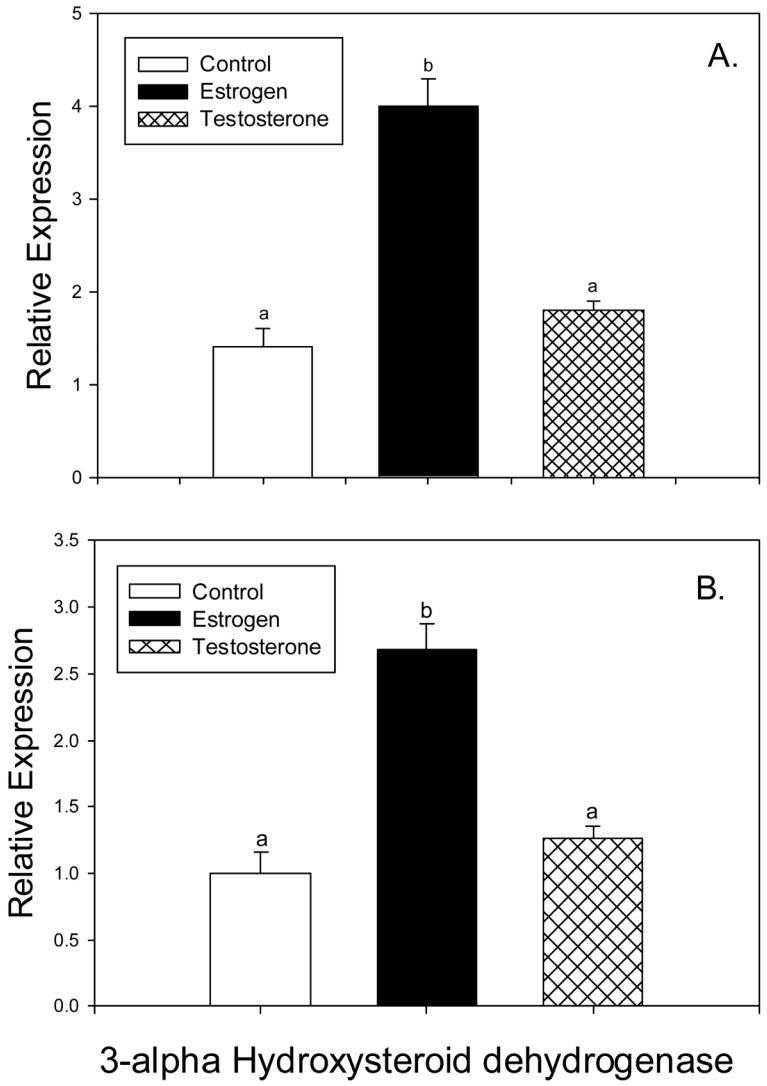

Figure 2.

Differential expression of 3α-hydroxysteroid dehydrogenase (*3α-Hsd)* in the mesenteric arteries in response to estrogen and testosterone. Panel A. Data from DNA microarray analysis (n=4). Differential expression of DNA microarray data was determined by Welch's one-way analysis of variance using GeneSpring software ($p<0.05$). Panel B. Data from real time RT-PCR (n=5). Differences among the three groups were identified by one-way ANOVA followed by Newman-Keuls post hoc test ($p<0.05$) of data from real time RT-PCR. Bars with different letters are significantly different from each other.

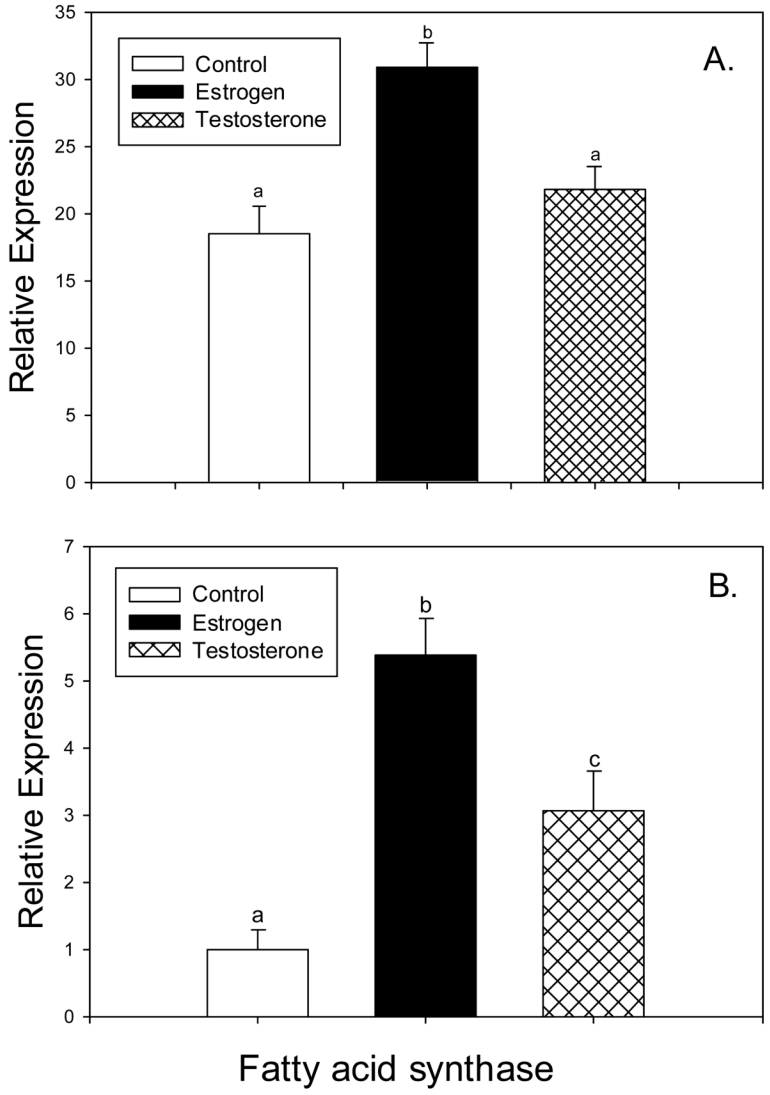

Figure 3.

Differential expression of fatty acid synthase (*Fas*) in the mesenteric arteries in response to estrogen and testosterone. Panel A. Data from DNA microarray analysis (n=4). Differential expression of DNA microarray data was determined by Welch's one-way analysis of variance using GeneSpring software ($p<0.05$). Panel B. Data from real time RT-PCR (n=5). Differences among the three groups were identified by one-way ANOVA followed by Newman-Keuls post hoc test ($p<0.05$) of data from real time RT-PCR. Bars with different letters are significantly different from each other.

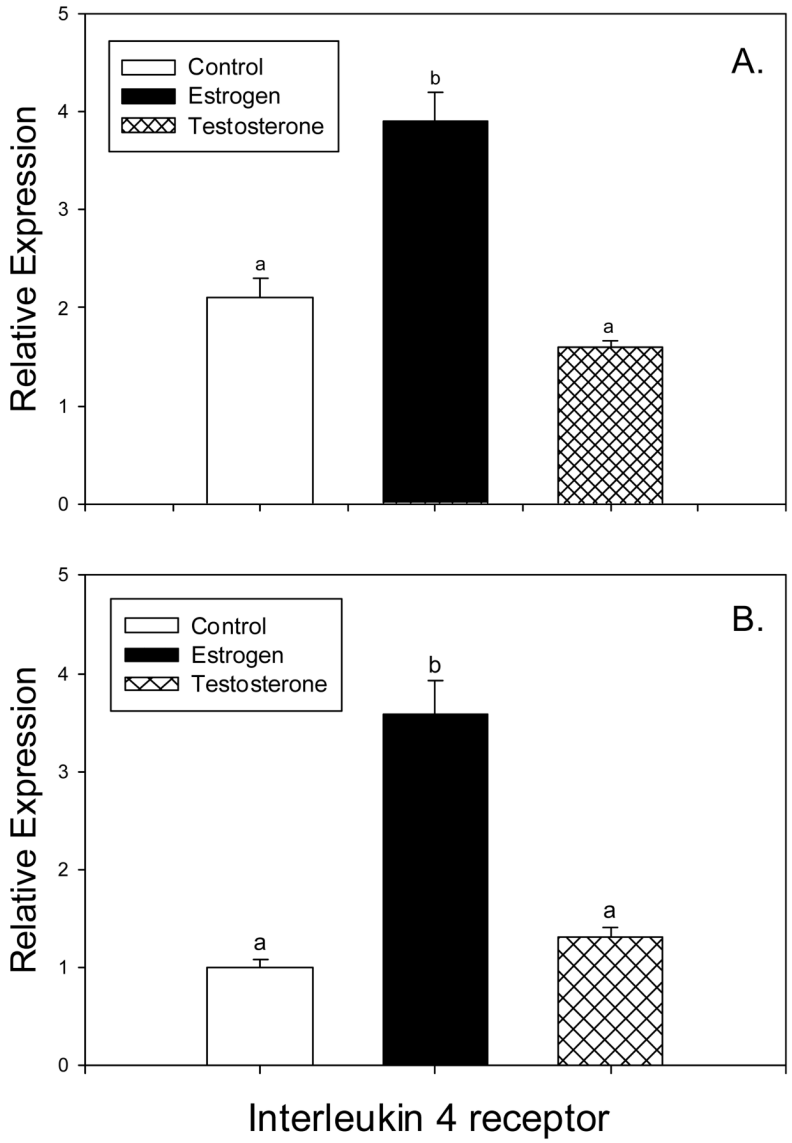

Figure 4.

Differential expression of interleukin 4 receptor (*Il4r*)in the mesenteric arteries in response to estrogen and testosterone. Panel A. Data from DNA microarray analysis (n=4). Differential expression of DNA microarray data was determined by Welch's one-way analysis of variance using GeneSpring software ($p<0.05$). Panel B. Data from real time RT-PCR (n=5). Differences among the three groups were identified by one-way ANOVA followed by Newman-Keuls post hoc test ($p<0.05$) of data from real time RT-PCR. Bars with different letters are significantly different from each other.

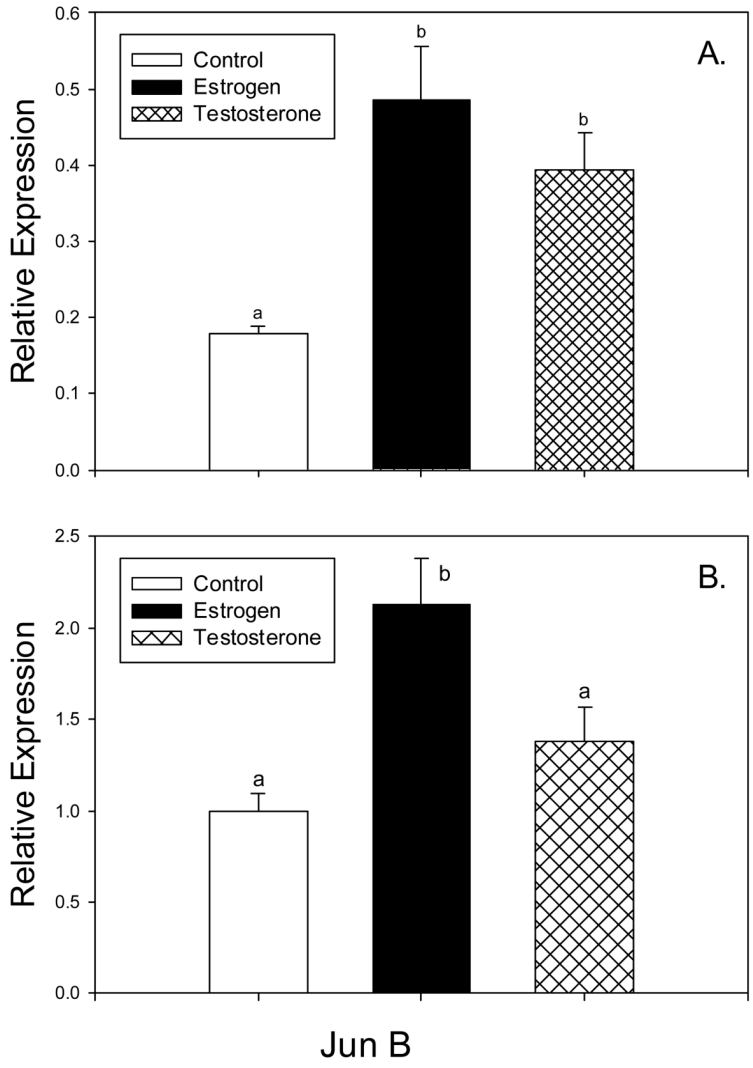

Figure 5.

Differential expression of *JunB* in the mesenteric arteries in response to estrogen and testosterone. Panel A. Data from DNA microarray analysis (n=4). Differential expression of DNA microarray data was determined by Welch's one-way analysis of variance using GeneSpring software ($p<0.05$). Panel B. Data from real time RT-PCR (n=5). Differences among the three groups were identified by one-way ANOVA followed by Newman-Keuls post hoc test ($p<0.05$) of data from real time RT-PCR. Bars with different letters are significantly different from each other.

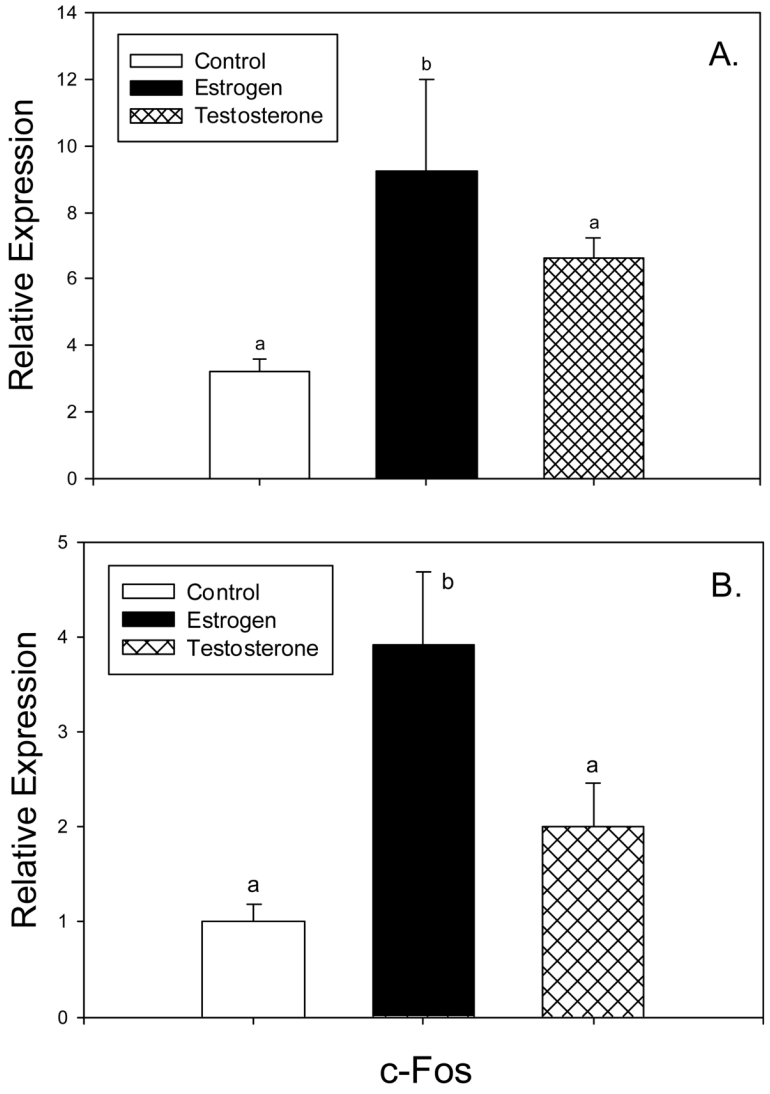

Figure 6.

Differential expression of *c-Fos* in the mesenteric arteries in response to estrogen and testosterone. Panel A. Data from DNA microarray analysis (n=4). Differential expression of DNA microarray data was determined by Welch's one-way analysis of variance using GeneSpring software ($p<0.05$). Panel B. Data from real time RT-PCR (n=5). Differences among the three groups were identified by one-way ANOVA followed by Newman-Keuls post hoc test ($p<0.05$) of data from real time RT-PCR. Bars with different letters are significantly different from each other.

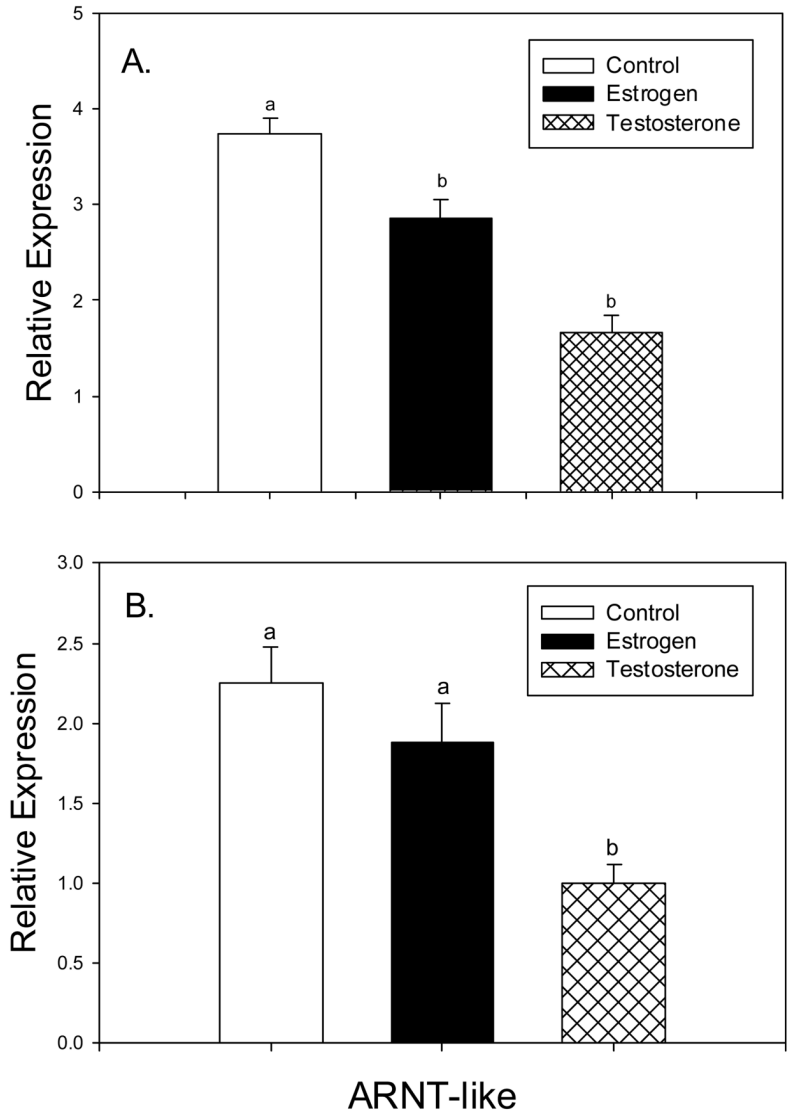

Figure 7.

Differential expression of aryl hydrocarbon receptor nuclear translocator-like (*Arnt-like*) in the mesenteric arteries in response to estrogen and testosterone. Panel A. Data from DNA microarray analysis (n=4). Differential expression of DNA microarray data was determined by Welch's one-way analysis of variance using GeneSpring software ($p<0.05$). Panel B. Data from real time RT-PCR (n=5). Differences among the three groups were identified by one-way ANOVA followed by Newman-Keuls post hoc test ($p<0.05$) of data from real time RT-PCR. Bars with different letters are significantly different from each other.