IMPACT OF SEX AND AGING ON THE EXPRESSION OF ESTROGEN RECEPTORS IN CARDIOVASCULAR TISSUES USING DROPLET DIGITAL PCR

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Introduction

Estrogen has protective effects in the cardiovascular systems of both males and females (1), including vasodilation via release of nitric oxide and prevention of atherosclerosis and vascular injury (2). The incidence of cardiovascular disease is lower in premenopausal women relative to men of similar age but increases after menopause, suggesting estrogens as the primary agents for the observed cardioprotective effect (3). However, clinical trials assessing the impact of menopausal hormone therapy on cardiovascular risk have produced mixed results (4).

Estrogen signals through two major pathways. Activation of estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ), induces their translocation to the nucleus where they function as transcription factors to alter gene expression (5). Additionally, estrogen binds a cell surface receptor, G protein-coupled estrogen receptor (GPER), which induces rapid, nongenomic signaling that precedes the long-term effects of nuclear signaling (6).

Estrogen receptors are expressed in a variety of tissues in the body, including locations outside of the reproductive system such as the heart, kidney, uterus, spleen, and brain (7). Sex differences in estrogen receptor expression are frequently found as well as changes in response to aging. For example, females express more ERα in the medial preoptic area and medial basal hypothalamus than males (8). Additionally, expression of estrogen receptors may be altered in response to aging, and aging induces changes in the expression of ERα in mice kidneys (9).

Until now, comparison of the expression of the different estrogen receptors has been limited by technology. Previous methods for quantifying transcript expression such as quantitative real-time PCR (qPCR) and Reverse Transcription qPCR (RT-qPCR)
generate results relative to a generated standard curve and thus do not allow for direct comparison of different genes (10). Droplet Digital PCR (ddPCR) is a novel technique which determines absolute count of target DNA copies. Therefore, utilizing ddPCR allows for direct comparison of mRNA transcripts from discrete experiments in various tissues (11).

Differences in estrogen receptor expression due to sex and aging and amongst various tissues may provide insight into estrogen’s pleiotropic effects on the cardiovascular system. Knowledge of these differences may aid in the development of more specific hormone therapies to combat declines in cardiovascular health. Therefore, the present study was performed to determine the impact of sex and aging on the expression of ERα, ERβ, GPER, and aromatase in cardiovascular tissues using ddPCR for absolute transcript quantification.
**Methods**

**Animals**

Young (24 ± 3 weeks) and aging (52 ± 3 weeks) male and female C57BL/6 mice were maintained in a temperature-controlled vivarium with an alternating 12-hour dark and light schedule with free access to standard chow and drinking water. Animal treatment was in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved and monitored by the Tulane University Institutional Animal Care and Use Committee.

**Tissue Lysis and RNA extraction**

The aorta, heart, and kidney were removed and immediately stored in RNAlater solution (Sigma Aldrich, St. Louis, MO) per the manufacturer’s directions to preserve RNA integrity. The tissues were mechanically homogenized and processed for total RNA isolation using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Germany). The concentration and purity of extracted RNA was monitored by Nanodrop spectrophotometry. Samples with an absorbance ratio (260/280 nm) > 1.8 were used for analysis.

**Droplet Digital PCR**

Transcript levels were determined using Droplet Digital PCR (ddPCR) as described elsewhere (11)(PMID: 28754792) using reagents from the One-Step RT-ddPCR Advanced Kit (Bio-rad, Hercules, CA). For each sample, 1.1 µg of RNA was added to a mixture containing 5.5 µL Supermix, 2.2 µL Reverse Transcriptase, 1.1 µL DTT, and 1.1
µL of each primer with labeled probe. The following Bio-rad validated primers with dual-labeled fluorescent probes were used: GPER (GPER/GPR30, Assay ID: dMmuCPE5103031), ERα (ESR1, Assay ID: dMmuCPE5092740), ERβ (ESR2, Assay ID: dMmuCPE5092742), and aromatase (CYP19A1, Assay ID: dMmuCPE5097863). Droplets were generated for each sample as per manufacturer’s instructions. RT-PCR amplification was performed with the following parameters for 41 cycles: amplification–denaturation at 95°C for 60 seconds, annealing at 55°C for 60 seconds, and extension at 72°C for 60 seconds. All samples were analyzed using the Bio-Rad QX200 Droplet Digital PCR system according to the manufacturer’s instructions.

Statistics

Data were analyzed with GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA) and SPSS (IBM, Armonk, NY). Unpaired t-tests were used to analyze data comparing two groups, with $P < 0.05$ considered significant. Two-way ANOVA was used to compare data with sex and age as parameters, with $P<0.05$ considered significant. Three-way ANOVA was used to compare data with sex, age, and tissue as parameters, with $P<0.05$ considered significant. Bonferroni post tests were used following ANOVA and considered significant at $P<0.05$. 
Results

ERα mRNA was significantly greater in young male kidneys when compared to young female kidneys (Figure 1). However, there was no difference when comparing aging kidneys. In males, the young kidneys show a higher level of ERα mRNA versus aging. This contrasts with the females which show less ERα mRNA in the young versus aging kidneys.

Unlike ERα, GPER expression was not significantly different in young male versus female kidneys. However, significantly more GPER mRNA was present in aging female kidneys.

![Figure 1. ERα and GPER expression in young and aging male and female kidneys.](image1)

![Figure 2. ERα and GPER expression in young and aging male and female aortas.](image2)
versus male kidneys. GPER mRNA decreased with aging in males but trended upward in females (P<0.07). This relationship is analogous to that seen with ERα mRNA levels. Unlike the kidneys, there was no impact of sex or aging on ERα and GPER mRNA in the aorta (Figure 2).

ERα mRNA was significantly higher in aging female versus male hearts, but there was no effect of aging in either sex (Figure 3). GPER expression was significantly higher in young male hearts when compared to aging male hearts. This pattern was similar to both ERα and GPER expression in the kidneys. However, unlike the kidneys no change was observed between young and aging female hearts. No significant differences in GPER mRNA were observed between male and female hearts.

ERα mRNA differed vastly between the aorta, heart, and kidney when comparing within each sex and age group (Figure 4). The aorta had ~20 fold higher levels of ERα mRNA than the heart and significantly more than the kidneys. The kidneys also had significantly more ERα when compared with the heart.
Figure 4. Global comparison of ERα expression in the aorta, heart, and kidney.

Figure 5. Global comparison of GPER expression in the aorta, heart, and kidney.
Like ERα, GPER expression was significantly higher in the aorta than in the heart and kidney (Figure 5). However, no significant difference was observed between GPER mRNA levels in the heart and kidney.

We also analyzed the data in terms of the ratio of ERα/GPER (Figure 6). This relationship was significantly higher in the kidney as opposed to the aorta and heart, which express similar amounts of both transcripts. In males, young and aging, there is ~10 times more ERα than GPER. This relationship is present in females as well but is less dramatic, with 3-4 times more ERα than GPER mRNA.

ERβ mRNA was not detectable in the aorta and heart. In the kidney no sex differences were observed. However, ERβ mRNA decreased with aging to a similar extent in both males and females. For males, this is analogous to the decreases observed.
in ERα mRNA and GPER mRNA in response to aging. For females this is the opposite effect seen relative to the increases of ERα mRNA to GPER mRNA observed in aging mice.

Figure 7. ERβ gene expression in young and aging, male and female hearts.
Discussion

This study showed that gene expression of the various estrogen receptors differs between tissues, sexes, and age groups. The differential expression of ERα, ERβ, and GPER may allow estrogen to produce effects that are different by age, sex, and tissue.

The molecular events underlying aging are still unknown, but DNA damage resultant from oxidative stress during aging promotes changes in gene expression (12). In the current study, aging did not alter ERα and GPER in the aorta or ERα in the heart. The kidney showed the greatest sensitivity to aging, but interestingly the impact of aging was at times opposite in males and females. We found that mRNA generally decreased in aging males and increased in aging females. This trend was observed for ERα and GPER in the kidney (Figure 1), but only ERα in male hearts, GPER in female hearts (Figure 3), and ERβ in male kidneys (Figure 7). Only ERβ in the kidneys was lower in the aging female than in the young female (Figure 7). Previously, a reduction in ERα mRNA and protein in the kidneys of aging males but an increase in aging females was found using semiquantitative RT-PCR and Western blotting, respectively (11). An increase in estrogen receptor expression may be in response to, and a way of combatting, an increase in vascular injury and decline in cardiovascular health. Even though mice in the current study were around the age of reproductive senescence, rodents become acyclic but do not experience the extremely low levels of circulating estradiol as seen in humans (13, 14). However, serum levels are significantly reduced when compared to peak estradiol in young rats, and therefore a lack of negative feedback may be a factor in the increased mRNA in aging females (15).
Overall a surprising lack of sexual dimorphism was observed when comparing the transcript levels of estrogen receptors between males and females. Other than the high levels of ERα found in aging females relative to aging males (Figure 3), no significant differences in mRNA levels were detected between sexes in the heart and aorta regardless of age and receptor. These findings are consistent with the lack of sex differences in ERα expression in the kidney. Differences in circulating estradiol between males and females may not regulate tissue mRNA levels, as females would be expected to have lower estrogen receptor mRNA due to the higher circulating levels.

Unlike the heart and kidney, the expression of estrogen receptors in the aorta was stable across sex and age (Figure 4). Interestingly, Post et al. reported markedly low levels of estrogen receptor DNA methylation in the aorta in aging mice when compared with the heart and other tissues, indicating protection from this aging pathology (16, 17). The high levels of ERα and GPER mRNA in the aorta (Figure 5) coupled with the lack of change in response to aging may also indicate a conserved and necessary function for these receptors in the aorta. Additionally, the relatively high level of GPER mRNA may indicate that the aorta is a primary target for estrogen’s nongenomic and rapid signaling effects such as vasodilation (18).

Our finding of more ERα mRNA in the kidney than the heart is consistent with results found using RT-qPCR (19). The ratio of ERα to GPER mRNA was 1:1 in the heart and aorta, while the kidneys expressed a greater proportion of ERα in relation to GPER. This divergence was significantly more apparent in the males than in the females, reaching an approximate 10:1 ratio. This higher proportion of ERα may indicate an important role for ERα in the kidneys relative to the role of GPER. Indeed, mice lacking ERα have
significant alterations in kidney size at baseline as well as in response to unilateral nephrectomy (20).

Undetectable ERβ mRNA in the heart and aorta combined with the relatively low levels of ERβ mRNA in the kidney (Figure 7) indicate ERα and GPER as the primary receptors mediating estrogen’s effects in the cardiovascular system. Previous studies also failed to detect ERβ mRNA in mouse kidneys using RT-qPCR (9). The very little detected in this study may be attributed to the increased sensitivity of ddPCR. However, real-time PCR of bovine kidneys detected three-fold more ERβ than ERα (7), and ERβ was detected in human fetal kidneys (21), indicating that receptor expression may differ by species. Consistent with our results in the heart, Pugach et al. detected ERα but not ERβ mRNA in cardiac tissue from rats and mouse of both sexes (22). Additionally, aromatase mRNA was not found in any of the analyzed tissues indicating that estrogen synthesis does not occur within the aorta, heart, or kidney. The presence of aromatase activity and protein in the rat kidney (23) as well as mRNA in human fetal kidney, heart, and brain (24) is therefore inconsistent with our findings.

Utilizing whole tissue homogenates for RNA extraction produces a heterogeneous mixture from various differentiated cells. Use of this methodology in the current study does not allow for the assessment of receptor variations between cell types. For example, our aortic samples included mRNA from smooth muscle cells, endothelial cells, as well as fibroblasts. Future studies should analyze differences in the expression of estrogen receptors in specific cell types in each tissue. Another caveat of our study was not controlling for estrous cycle, which is known to influence gene expression (25). Future
studies should assess the impact of estrous status as well as estrogen removal via ovariectomy on hormone receptor expression.

The use of ddPCR provided increased sensitivity and the ability to perform absolute quantification. Using this method, we had the ability to quantify ER\(\beta\) in mice kidneys despite that fact that mRNA for this receptor was previously undetected using RT-qPCR (9). Furthermore, a study comparing PCR methods found that ddPCR was 30-fold more sensitive than RT-qPCR when quantifying strains of flu virus (26). The discrepancies between this study and previous research regarding estrogen receptor expression suggest future research should be performed utilizing ddPCR for its increased accuracy.

The results of this study provide a framework for future research on estrogen’s pleiotropic effects on the cardiovascular system and the underlying mechanisms.
References


Biography

Rakesh Gurrala graduated from the University of Massachusetts Amherst in 2016 with a B.S. in Biochemistry and Molecular Biology. After graduating, he worked in the Dominican Republic for an NGO. In 2017 he came to Tulane University to pursue a Master’s degree in Pharmacology.