Inhibition of Advanced Glycation End Products (AGEs) Accumulation by Pyridoxamine Modulates Glomerular and Mesangial Cell Estrogen Receptor α Expression in Aged Female Mice

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Abstract

Age-related increases in oxidant stress (OS) play a role in regulation of estrogen receptor (ER) expression in the kidneys. In this study, we establish that in vivo 17β-estradiol (E2) replacement can no longer upregulate glomerular ER expression by 21 months of age in female mice (anestrous). We hypothesized that advanced glycation end product (AGE) accumulation, an important source of oxidant stress, contributes to these glomerular ER expression alterations. We treated 19-month old ovariectomized female mice with pyridoxamine (Pyr), a potent AGE inhibitor, in the presence or absence of E2 replacement. Glomerular ERα mRNA expression was upregulated in mice treated with both Pyr and E2 replacement and TGFβ mRNA expression decreased compared to controls. Histological sections of kidneys demonstrated decreased type IV collagen deposition in mice receiving Pyr and E2 compared to placebo control mice. In addition, anti-AGE defenses Sirtuin1 (SIRT1) and advanced glycation receptor 1 (AGER1) were also upregulated in mice treated with both Pyr and E2 replacement and TGFβ mRNA expression decreased compared to controls. Mesangial cells isolated from all groups of mice demonstrated similar ERα, SIRT1, and AGER1 expression changes to those of whole glomeruli. To demonstrate that AGE accumulation contributes to the observed age-related changes in the glomeruli of aged female mice, we treated mesangial cells from young female mice with AGE-BSA and found similar downregulation of ERα, SIRT1, and AGER1 expression. These results suggest that inhibition of intracellular AGE accumulation with pyridoxamine may protect glomeruli against age-related oxidant stress by preventing an increase of TGFβ production and by regulation of the estrogen receptor.
Introduction

Normal aging is associated with an increase in oxidant stress in multiple organs including the kidneys [1, 2]. This effect is observed in both sexes, however, young men have higher levels of oxidant stress markers compared with pre-menopausal age-matched women [3, 4]. These parameters of oxidant stress increase in women after menopause [5]. We previously reported that an age-related increase in oxidant stress mediates a decrease in estrogen receptor alpha (ERα) expression and function in the kidneys [6]. However, the consequences of differences in oxidant stress in the kidneys between pre- and post-menopausal women have not been well-studied.

Advanced glycation end products (AGEs) are a well-known cause of chronic renal oxidant stress and inflammation [7]. Their source is thought to be the high-AGE modern diet [4, 7–9]. Circulating levels of AGEs correlate with the AGE content of common foods, especially those of animal origin [10]. Food AGEs are accumulated by routine methods of industrial and/or home food processing, especially dry heat [11–14]. The amount of orally-absorbed AGEs that interact with tissues is estimated to be 2 to 3-fold greater than the amount in the circulation, an amount that far exceeds the kidney’s excretion capacity [15–17]. Chronic ingestion of excess AGEs is associated with a marked down-regulation of important anti-oxidant defense mechanisms. These include Sirtuin 1 (SIRT1), an NAD+-dependent histone deacetylase, advanced glycation receptor 1 (AGER1), and other anti-oxidant systems such as nuclear factor erythroid 2-related factor 2 (Nrf2) [10, 18]. Reduction of renal SIRT1 results in multiple downstream effects including inhibition of ER signaling and reduction of mitochondrial biogenesis and function [19]. In addition, SIRT1 plays a role in preventing NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) activation, which may also regulate ER expression [20, 21].

In this study, we investigated the potential role of AGEs as a mechanism of glomerular ER regulation. First, we determined the time course of age-related loss of 17β-estradiol (E2)-stimulated ER expression regulation in the glomerulus of aged female mice. This phenomenon was observed in 21-month old female mice that were ovariectomized at 19 months at the advent of their anestrous period and prolonged exposure to oxidant stress. We therefore selected 21-month old ovariectomized female mice and fed them a regular mouse diet (high in AGEs) with or without pyridoxamine (Pyr), which is a potent anti-AGE that is currently used in patients with kidney disease. Additional mice were administered E2 alone or E2 in addition to pyridoxamine. We found that in vivo treatment with Pyr and E2 increased glomerular ERα expression, while administration of E2 alone did not. The combination of Pyr and E2 also lowered the glomerular mRNA expression of transforming growth factor beta (TGFβ), a profibrotic cytokine. Moreover, this combination treatment prevented type IV collagen accumulation, which is associated with age-related glomerulosclerosis [22, 23]. SIRT1 and AGER1, important anti-AGE defenses, were upregulated in the Pyr and E2 group. Finally, we demonstrate a decrease in ERα and SIRT1 expression in response to AGEs in vitro using mesangial cells isolated from young female kidneys, suggesting that AGE accumulation is involved in oxidant stress-related changes in the aged kidney.

Materials and Methods

Mice

Female C57Bl/6 mice were obtained from the National Institute of Aging, National Institutes of Health (Bethesda, MD). Mice were ovariectomized at either 12 or 19 months of age using the previously described procedure that has been approved by the Institutional Animal Care and Use Committee at the University of Miami Miller School of Medicine (protocol 12–043).
The mice were divided into 2 groups and received either placebo or 17β-estradiol (E2) 90-day release pellets (Innovative Research of America, Sarasota FL) as previously described [25]. The 19-month group was further divided and were provided water with or without pyridoxamine (200 mg/kg per day in 10 ml H2O; Biostratum). Mice were euthanized by intraperitoneal injection of ketamine and xylazine as approved by protocol.

**Mouse Sacrifice.** Mice were housed under pathogen-free conditions with food and water ad libitum. Mice were sacrificed 2 months after treatment (at 14 or 21 months of age). Left kidneys were perfused with a buffered solution containing collagenase and RNase inhibitors for micro dissection of glomeruli, as previously described [25]. Right kidneys were perfused in situ with 6 ml of phosphate-buffered saline and 3 ml of 4% paraformaldehyde, post-fixed in 4% paraformaldehyde solution for at least 12 hours and embedded in methacrylate. 4 μm thick sections were stained with periodic acid–Schiff stain. Other kidney fragments were immediately frozen in OCT [26]. Glomeruli were microdissected to isolate mesangial cells from each group.

**Measurements of Urinary Albumin and Creatinine**

Spot urine samples were collected at the same hour on a weekly basis and at time of sacrifice. Urine albumin was measured by ELISA following manufacturer’s instructions (Bethyl, Houston, TX) and was corrected for the concentration of urine creatinine. This was expressed as the urinary albumin/creatinine excretion ratio (UAE).

**Kidney tissue histological analysis of type collagen IV**

Deparaffinized kidney sections (4 μm) were blocked for endogenous peroxidases. Sections were stained with either rabbit anti-mouse (Biodesign, Saco, ME) or rabbit anti-mouse collagen IV. After 1 h, the slides were washed and incubated for 30 min at room temperature with biotinylated-labeled goat anti-rabbit, followed by Vectastain ABC reagent (Vector Labs, Burlingame, CA) and 3,3’-diamino-benzidine chromogen solution (Sigma, St. Louis, MO). The sections were examined and graded on a scale of 0 to 4, as previously described [26], by a renal pathologist (GS) who was blinded to the treatment group.

**Real time PCR**

Amplification and measurement of target RNA was performed on the Step 1 Real Time-PCR System, as previously described [25]. The mRNA sequence was obtained from the National Center for Biotechnology Information (Bethesda, MD) to acquire the copy number for each ER subtype, as previously described [27]. The number of occurrences of each of the four nucleobases was counted and multiplied by its respective molecular weight. These four numbers were then summed together to obtain the mass of 1 mol of each subtype of the ER. The mass of the purified plasmid of each subtype and the unknown samples was calculated by the A260 method on a Molecular Devices SpectraMax PLUS (Ramsey, MI, USA) [27]. TGFβ, SIRT1 and AGER1 primers were purchased from Life Technologies (Carlsbad, CA). Specific primer sequences used were as previously described for ER [28], TGF-β [23], SIRT1 and AGER1 [8].

**Isolation of Mesangial Cells**

Mesangial cells were isolated from each group of mice treated with and without Pyr in the presence and absence of E2 pellets, as previously described [29]. Mesangial cells previously isolated from young female C57/B6 mice (3 months old) were treated with increasing concentrations of AGE-BSA (50–200 μg/ml) to determine effective dose for downregulating ERα protein.
expression [30]. Once the effective dose was established at 100 μg/ml of AGE-BSA, cells were treated with AGE-BSA for 24 hours. This treatment time frame was determined by exposing cells to increasing time intervals (2–48 hours) of AGE-BSA and determining its effect on ERα protein expression.

**Western Blot Analysis**

For protein analyses, cell lysates were extracted and protein quantity assessed using the Pierce BCA protein assay kit (Rockford, IL). Equal amounts of protein were applied to precast SDS polyacrylamide gels (Life Technologies, Grand Island, NY) and analyzed as previously described for ERα, AGER1, SIRT1, and β-actin [31]. In some experiments, cells were treated overnight with AGE-BSA (100 μg/ml for 18 hours). Western blots were also exposed to β-actin (Sigma Chemical, St. Louis MO.) to control for protein loading. Human recombinant ERα was used as a control (PanVera, Madison, WI). Immunoreactive bands were determined by exposing nitrocellulose blots to a chemiluminescent solution (Denville Scientific Inc., Metuchen, NJ) followed by exposure to Amersham Hyperfilm ECL (GE Healthcare Limited, Buckinghamshire, UK). Relative amounts of protein were determined by densitometry using ImageJ software version 1.48 (National Institutes of Health, Bethesda, MD).

**Statistical analysis**

All values are expressed as mean ± standard error of the mean (SEM). Significance of overall differences within experimental groups was determined by analysis of variance (ANOVA) in combination of Tukey’s multiple comparison test. Student’s t-test was used to determine differences between groups, using Welch’s correction as appropriate. P values < 0.05 were considered significant.

**Results**

**Glomerular ERα mRNA upregulation by 17β-estradiol replacement is lost by anestrous period (21 months of age)**

To determine the time course of age-related loss of 17β-estradiol (E2)-stimulated glomerular ER expression regulation, we replaced E2 for 2 months in 12-month old (pre-anestrous) and 19-month old (anestrous) female mice. All mice were ovariectomized two weeks prior to E2 administration to ensure equivalent replacement. E2 replacement was only effective in upregulating glomerular ERα mRNA expression in 12-month old mice prior to entering the anestrous period (at approximately 18 months of age), and thus correlating with a shorter exposure to endogenous oxidant stress (Fig 1). By 21 months of age (anestrous) E2 replacement failed to upregulate ERα mRNA expression (Fig 1).

**Effect of pyridoxamine and 17β-estradiol replacement on body, kidney, uterine weight and albumin/creatinine ratio**

Treatment of mice did not alter body weight, however, kidney weight increased in Pyr+E2 treatment compared to placebo and Pyr alone (p<0.05). Uterine weight as a marker of estrogen replacement was increased in all mice receiving E2 regardless of whether they were also receiving Pyr (Table 1). Urinary albumin excretion did not change between groups (Table 1).
Inhibition of AGE accumulation with pyridoxamine and 17β-estradiol increases glomerular ERα mRNA expression and reduces TGFβ mRNA expression

Our previous study showed an oxidant stress-related glomerular ERα downregulation associated with aging [6]. In this study, in vivo inhibition of AGEs, a source of oxidant stress, with Pyr and E2 administration increased ERα mRNA expression (Fig 2A) in 21 month-old ovariectomized female mice. TGFβ, a profibrotic cytokine, was decreased in an inverse manner to ERα mRNA expression in the group receiving Pyr and E2 (Fig 2B). There was no significant

Table 1. Effect of pyridoxamine and 17β-estradiol replacement on body, kidney, uterine weight and albumin/creatinine ratio.

|                | Pla (n = 10) | E2 (n = 12) | Pyr (n = 10) | Pyr+E2 (n = 6) |
|----------------|--------------|-------------|--------------|----------------|
| Body weight (g)| 31 ±1.3      | 30±0.9      | 31±1.5       | 30±1.4         |
| Kidney weight (g)| 0.28±0.01   | 0.29±0.01   | 0.27±0.009   | 0.34±0.2a      |
| Uterine weight (g)| 0.02±0.00b  | 0.14±0.02c  | 0.02±0.001d  | 0.15±0.02     |
| Albumin/Creatinine ratio | 0.43±0.32   | 0.26±0.06   | 0.34±0.06    | 0.25±0.05     |

a *p<0.05 compared to placebo (pla) and pyridoxamine (Pyr)
b***p<0.005 compared to 17β-estradiol (E2) and Pyr+E2
c***p<0.005 compared to Pyr
d ***p<0.005 compared to Pyr+E2.

doi:10.1371/journal.pone.0159666.t001
difference in ERα or TGFβ mRNA expression between placebo group and mice receiving either Pyr or E2 alone.

Type IV collagen deposition decreases with pyridoxamine and 17β-estradiol treatment in aged estrogen-deficient female mice

Type IV collagen, one of the hallmarks of glomerulosclerosis, increased in placebo-treated glomeruli of ovariectomized aged female mice as expected (3+ staining; Fig 3A). Treatment with the antioxidant pyridoxamine decreased the accumulation of type IV collagen in glomeruli and tubules (1 and 2+ staining; Fig 3C). E2 replacement, with or without pyridoxamine, also prevented accumulation of type IV collagen in estrogen-deficient (ovariectomized) aged female mice (1 and 2+ staining; Fig 3B–3D).

Prevention of AGE accumulation with pyridoxamine in the presence of E2 replacement increases glomerular SIRT1 and AGER1 mRNA

AGE accumulation down-regulates anti-oxidant stress defenses such as SIRT1 and AGER1 [8, 18]. Therefore, we measured glomerular SIRT1 and AGER1 mRNA expression in our 4 groups of ovariectomized 21-month old female mice. Glomerular expression of SIRT1 mRNA was increased in mice treated with pyridoxamine and E2 replacement compared to all other groups (Fig 4A, *p < 0.05). Similarly, AGER1 mRNA expression was increased in the glomeruli of mice receiving pyridoxamine and E2 replacement compared to placebo or E2 alone groups (Fig 4B, #p < 0.05). AGER1 expression also increased in mice treated with pyridoxamine alone (Pyr) versus placebo or E2 alone (Fig 4B, #p < 0.05).
Mesangial cells isolated from aged female mice treated with Pyr + E2 maintain a phenotypic switch with increased ERα, SIRT1 and AGER1 mRNA expression.

At the time of sacrifice, glomeruli were isolated and cells propagated from the four groups of mice described above. ERα mRNA copy number and protein expression was increased only in mesangial cells isolated from mice that were treated with both Pyr and E2 (Fig 5A and 5B). Similarly, we found an increase in SIRT1 and AGER1 protein expression in cells derived from mice treated with Pyr + E2 (Fig 5C).

AGEs reduce glomerular ERα protein expression in vitro

To further confirm that AGEs reduce glomerular ERα expression, mesangial cells isolated from young female mice were treated with AGEs in vitro. ERα protein expression was decreased after treatment with AGE-BSA (Fig 6A). There was also a decrease in SIRT1 and AGER1 protein expression in these cells (Fig 6B).

Discussion

We have previously shown that E2 upregulates glomerular ERα mRNA and protein expression in young mice [28], but during aging there is a steady decline in both [6]. In the present study, we demonstrate that timing of estrogen replacement in relation to reproductive age is critical for regulation of glomerular ER expression. E2 replacement at 14 months (before anestrus) was effective in upregulating ERα. This effect, however, was lost by 21 months of age coinciding with the anestrus period and prolonged exposure to oxidant stress. These data derived in
Experimental animals may provide insight into the findings of the Women’s Health Initiative (WHI) and Heart and Estrogen/Progestin Replacement Study (HERS). In those trials, women that received estrogen replacement up to 10 years after menopause exhibited some adverse clinical outcomes. The KEEPs trial, on the other hand, studied women not more than three years after menopause and found benefits in terms of cardiovascular outcomes. Inhibition of AGEs reduces oxidant stress and modulates glomerular estrogen receptor α expression.

Fig 4. Glomerular AGER1 and SIRT-1 mRNA are upregulated by reduction of AGEs in vivo. Glomeruli were isolated from 4 groups of mice; placebo (pla), 17β-estradiol (E2), pyridoxamine (Pyr) or E2+Pyr. SIRT1, AGER1 and 18s were measured by RT-PCR as described in Methods. Data are graphed as mean ± SEM of ratio of SIRT1/18s (*p<0.05 compared to all groups) or AGER1/18s (#p<0.05 compared to placebo and E2 treatments). n = 5/group.

doi:10.1371/journal.pone.0159666.g004
years post menopause and suggested that this window of time for initiation of hormone replacement may lead to a beneficial effect for disease prevention [32]. Our previous data showed that increased oxidant stress is associated with reduced ERα expression in the kidney of aging mice [6]. Therefore, it is possible that administration of estrogen during this time of increased age-related oxidant stress leading to decrease in ER expression and action may exacerbate downstream deleterious events.

Based on our previous findings, we designed the current study to further investigate the role of oxidant stress and regulation of glomerular ERα expression in vivo. We examined the effect on glomerular ERα expression of pyridoxamine, a derivative of vitamin B6, that prevents intracellular accumulation of AGEs and scavenges reactive oxygen species [33]. Pyridoxamine treatment coupled with E2 replacement increased glomerular ERα expression, while E2 replacement alone did not. Furthermore, ERα expression in mesangial cells isolated from in vivo treated
mice followed a similar expression pattern as in the glomeruli. This was expected, as we have previously reported that a phenotypic switch in glomerular ERα expression occurring in vivo is maintained in vitro [25, 34].

Aged female mice (24 months of age and older) have increased urinary albumin excretion and collagen types I and IV deposition leading to glomerulosclerosis [23]. This increase in glomerulosclerosis markers associated with age can be observed in experimental models and humans [23] [35, 36]. Although baseline urinary albumin excretion was higher in our aged female mice compared to young female mice (data not shown), this was not affected by treatment with pyridoxamine and/or E2 in aged ovariectomized female mice. It is possible that prolonged treatment period and sacrifice at an older age may have revealed an effect. In contrast, all treatment combinations prevented glomerular type IV collagen deposition in aged females. Of note, despite the effectiveness of oral pyridoxamine in preserving kidney function in type 1 and 2 diabetic rat and mouse models [37–39], recent clinical trials in patients with type 1 and type 2 diabetes produced mixed results [40, 41]. Williams et al. [40] showed a reduction of baseline serum creatinine without a change in urine albumin excretion. A larger study failed to
show any change in renal function after 1 year, although the authors suggested that patients with less severe renal damage may respond to the drug [41].

In the present study, in vivo pyridoxamine treatment along with E₂ replacement decreased TGFβ mRNA expression in kidneys of aged ovariectomized female mice. Accumulation of gene expression of growth factors and cytokines such as TGFβ and vascular endothelial growth factor (VEGF) are associated with the formation of AGEs [42]. We and others have shown that kidney disease in mice and humans is often associated with increased TGFβ expression [26, 43–45]. In fact, TGFβ signaling can be initiated by reactive oxygen species, which could ultimately increase extracellular matrix protein (ECM) accumulation through direct upregulation of collagen synthesis and/or decreased matrix metalloproteinase activity. In addition, TGF-β1 contributes to glomerulosclerosis by stimulating podocyte apoptosis [44, 46]. Finally, TGF-β receptor 2 is increased in isolated mesangial cells and in glomeruli of diabetic mice, suggesting an increased sensitivity due to the effects of endogenous TGF-β1 [47, 48]. Interestingly, there was an inverse relationship in our study between the NAD+-dependent deacetylase SIRT1 and TGFβ expression. Negative cross-talk between TGFβ signaling and SIRT has been previously demonstrated in the kidney, liver, and lung [49–51]. SIRTs have been shown to downregulate TGFβ either by degradation or inhibition of transcriptional activity and further studies are ongoing in our laboratory to understand these findings.

SIRT1 and ERα expression were positively correlated in both glomeruli and mesangial cells. We postulate that SIRT may have a direct effect on ER regulation. Estrogen receptors are dynamically modulated by post-translational modification, i.e. phosphorylation, methylation, acetylation, ubiquitination, or sumoylation [52]. For instance, hyperactivation of ERK/MAPK (Extracellular-signal-regulated kinases/Mitogen-activated protein kinases) causes functional repression of ER transcription through NFκB activation [53, 54], which we have shown to be increased in 28-month old female mice [22]. In contrast, SIRT1 prevents undue activation of NFκB [55, 56]. AGEs promote NFκB activation [57] but suppress SIRT1 and its deacetylase activity on NFκB-p65 [58]. This could influence ER transcription, given that decreased SIRT1 expression can disrupt the basal transcription factor complex of ERα promoter in some cells [20]. These studies are currently under investigation.

The concentration of AGEs and their cross-linked products increases with aging and leads to higher basal levels of oxidant stress [10, 59]. Importantly, levels of AGEs are elevated in post-menopausal women compared to healthy young women. This increase is more pronounced in diabetic post-menopausal women [3–5]. These data correlate with the higher female to male ratio in patients with diabetic end-stage renal disease, which increases sharply in the postmenopausal age groups [60]. To confirm our in vivo data suggesting an important role for AGEs in regulation of glomerular ERα expression in aged females, we examined the direct effects of AGEs in vitro. Mesangial cells isolated from young (estrogen replete) female mice were treated with increasing concentrations of AGEs. We observed a dose- and time-dependent reduction in ERα expression in response to AGEs. Similarly, levels of the major cellular anti-AGE/oxidant stress defenses, anti-AGE receptor AGER1 and SIRT1 protein expression were decreased in response to AGE. This correlates with the inverse relationship between SIRT1/AGER1 and AGEs both in the current study and other experimental and human studies [2, 8, 61].

In summary, the ability of pyridoxamine to reverse a fibrotic marker of glomerulosclerosis (TGFβ) and ERα expression in aged female mice (21 months old) suggests that oxidant stress-related damage in the aging kidney is reversible. Furthermore, it is possible that reduced antioxidant defenses, such as SIRT1 and AGER1, in postmenopausal women could impair glomerular E₂/ER activity.
Acknowledgments

The authors would like to thank Dr. Helen Vlassara for her guidance and support in this study. All authors have no financial conflicts of interest to disclose.

Author Contributions

Conceived and designed the experiments: SJE GES. Performed the experiments: SP XX WC RC. Analyzed the data: SP GR SJE. Contributed reagents/materials/analysis tools: SJE GES. Wrote the paper: SJE GR.

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