Cytochrome P450-2E1 is involved in aging-related kidney damage in mice through increased nitroxidative stress

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Abstract

The aim of this study was to investigate the role of cytochrome P450-2E1 (CYP2E1) in aging-dependent kidney damage since it is poorly understood. Young (7 weeks) and aged female (16–17 months old) wild-type (WT) and *Cyp2e*1-null mice were used. Kidney histology showed that aged WT mice exhibited typical signs of kidney aging such as cell vacuolation, inflammatory cell infiltration, cellular apoptosis, glomerulonephropathy, and fibrosis, along with significantly elevated levels of renal TNF-α and serum creatinine than all other groups. Furthermore, the highest levels of renal hydrogen peroxide, protein carbonylation and nitration were observed in aged WT mice. These increases in the aged WT mice were accompanied by increased levels of iNOS and mitochondrial nitroxidative stress through altered amounts and activities of the mitochondrial complex proteins and significantly reduced levels of the antioxidant glutathione (GSH). In contrast, the aged *Cyp2e1*-null mice exhibited significantly higher antioxidant capacity with elevated heme oxygenase-1 and catalase activities compared to all other groups, while maintaining normal GSH levels with significantly less mitochondrial nitroxidative stress compared to the aged WT mice. Thus, CYP2E1 is important in causing aging-related kidney damage most likely through increasing nitroxidative stress and that CYP2E1 could be a potential target in preventing aging-related kidney diseases.

Keywords

Kidney; aging; CYP2E1; apoptosis; fibrosis; oxidative stress

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Conflict of interest statements

The authors declared no conflicts of interest relevant to this article.

1. Introduction

Aging is an inevitable gradual physiological process which affects all the organs of the body. It is characterized by a slow, but progressive, deterioration of the body ability to maintain normal homeostasis or to respond efficiently to various stimuli and stressors, including environmental agents, eventually leading to organ failure with increased morbidity and mortality (Lopez-Otin et al., 2013). A mounting number of theories have been proposed to explain the normal aging process (Lopez-Otin et al., 2013; Medvedev, 1990; Sergiev et al., 2015). The widely accepted theory for the aging process is "the free radical theory of aging" (Harman, 1956). This theory states that the highly reactive oxidative radicals may increase damage in aged tissues by the reaction of the free radicals with cellular macromolecules, leading to alteration and/or deterioration of normal cellular functions, resulting in organ damage. Constantly, reactive oxygen species (ROS) and reactive nitrogen species (RNS) were reported to increase progressively with aging leading to the accumulation of oxidized and/or nitrated proteins in aged organs (Chakravarti and Chakravarti, 2017; Reeg and Grune, 2015).

Kidneys are one of the most affected organs by aging, and aged kidneys have been reported in humans and many other species such as rats, mice, and hamsters. Aging causes significant changes both to the (sub-)organelle structures and physiological functions of the kidneys (Martin and Sheaff, 2007; Percy et al., 2008). Moreover, kidney aging and the deterioration of its functions may lead to the development of many other age-related diseases in various tissues (Martin and Sheaff, 2007). The aged kidneys undergo many significant changes during the aging process such as inflammation, glomerulonephropathy, glomerulosclerosis, cell apoptosis, and renal fibrosis which indirectly reflects kidney aging (Hodgin et al., 2015; Martin and Sheaff, 2007; Percy et al., 2008). Interestingly, and in agreement with the "free radical theory of aging" (Harman, 1956), oxidative stress along with oxidative protein modifications have been correlated with kidney toxicities, acute and chronic kidney diseases, and aged kidney with deteriorating functions (Hodgin et al., 2015; Hosohata, 2016; Martin and Sheaff, 2007; Pedraza-Chaverri et al., 2016; Percy et al., 2005; Percy et al., 2008; Poulianiti et al., 2016; Small et al., 2012).

The cytochrome P450-2E1 (CYP2E1) is an enzyme, constitutively expressed in hepatic and extrahepatic tissues, including the kidney (Guengerich et al., 1991). CYP2E1 is involved in the metabolism of many small molecule substrates such as alcohol (ethanol), drugs, solvents, procarcinogens, and fatty acids while its expression can be induced by many of its substrate compounds. In addition, various pathophysiological conditions such as obesity and diabetes as well as many hormones, gender, and nutrition factors may regulate CYP2E1 levels and activities. Interestingly, CYP2E1-related catalytic activity may produce the superoxide anion even in the absence of its substrates (Caro and Cederbaum, 2004;Novak and Woodcroft, 2000; Song et al., 1996). The superoxide anion can become a potent oxidative radical that can cause severe tissue damage through interaction with other free radicals such as nitric oxide (NO) to produce a more potently toxic peroxynitrite and may also produce toxic hydroxyl radical in the presence of metals (Abdelmegeed et al., 2015; Abdelmegeed and Song, 2014; Song et al., 2014).

We have recently reported a role of CYP2E1 in liver aging-related liver fibrosis and we showed that oxidative stress played a significant role in this process (Abdelmegeed et al., 2016). However, the potential role of CYP2E1 in the process of aging-dependent kidney damage with the development cellular apoptosis, glomerulonephropathy, and fibrosis, was never been studied. Thus, this study was aimed to investigate the role of CYP2E1 in age-related renal changes by comparing the histological, biochemical and functional markers in young and aged wild-type (WT) and *Cyp2e1*-null mice.

2. Materials and methods

2.1. Materials

All chemicals used in this study were from Sigma Chemical (St. Louis, MO, USA), unless indicated otherwise. Specific antibodies against CYP2E1, inducible nitric oxide synthase (iNOS), 3-nitrotyrosine (3-NT), nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), myeloperoxidase, total mitochondrial electron transport chain proteins (OXPHOS), and β-actin were from Abcam Inc. (Cambridge, MA, USA). Antibodies against glutamate-cysteine ligase catalytic subunit (GCLC) and glutamate-cysteine ligase modifier subunit (GCLM) were from Aviva Systems Biology Corp. (San Diego, CA, USA). Rabbit polyclonal antibodies against CYP1A, 2B, or 4A were generous gifts from Dr. James Hardwick, Northeastern Ohio University College of Medicine, Rootstown, OH, USA. Respective antibodies against heme oxygenase-1 (HO-1), collagen 1A1, ATP synthase subunit beta (ATP5B), and secondary antibodies conjugated with alkaline phosphatase were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Animals and tissue collection for histology analyses

Young (approximately 7 weeks) and old female (approximately 16–17 months) inbred WT and *Cyp2e*1-null mice on a 129/Svj background, used in this study, were obtained from Dr. Frank Gonzalez, National Cancer Institute, Bethesda, MD (Lee et al., 1996). Mice were divided into four groups (n=10/group): (1) young WT; (2) old WT; (3) young *Cyp2e1*-null; and (4) old *Cyp2e1*-null. All mice were fed with the NIH31 rodent diet (ContD, 7017 NIH-31 open formula mouse/rat sterilizable diet (Choi et al., 2017). Parts from the kidneys were fixed in 10% neutral formalin for histological analyses and the rest of the kidneys from each mouse was snap-frozen for further biochemical analyses and trunk blood was collected immediately after killing by decapitation of mice briefly sedated by CO_2 exposure. The histological and fibrosis scoring evaluation were performed blindly by a board-certified pathologist, using hematoxylin and eosin (H&E)-, periodic acid-Schiff (PAS)-, and Sirius red-stained slides. Each section was evaluated for glomerulonephropathy, inflammatory cell foci, tubular cytoplasmic vacuolation, tubular degeneration and/or regeneration, and kidney fibrosis (Table. 1). The image was captured by Olympus BX-51 microscope equipped with Digital Camera DP-70 (Olympus, Japan). For the characterization of the magnitude of fibrosis in all four groups, fibrotic and total areas were determined and the percentage of fibrotic area was calculated using Image J software (National Institutes of Health, Bethesda, MD, USA) from various image fields per slide. Animal experiments were performed in accordance with the National Institutes of Health guidelines for small animals and approved by the NIAAA Institutional Animal Care and Use Committee.

2.3. Measurements of serum creatinine, total renal tumor necrosis factor-α (TNF-α), and glutathione (GSH) levels in young and aged mice

Intra-renal GSH in 50 mg kidney extracts were determined by using commercially available kits (Cayman Chemical, Ann Arbor, Michigan, USA). TNF-α levels were evaluated by a specific ELISA kit (Abcam Inc., Cambridge, MA, USA). The amounts of serum creatinine for each mouse of the four groups were determined using a detection kit from Cayman Inc., (Ann Arbor, MI). The manufacturer's protocols were followed for all commercial kits.

2.4. Immunoblot analysis for evaluating the levels of target proteins, oxidized proteins, and immunoprecipitated proteins

Total renal homogenates from four different mouse groups were prepared in ice-cold extraction buffer (50 mM Tris–Cl, pH 7.5, 1 mM EDTA, and 1% CHAPS), and mitochondrial fractions were prepared by differential centrifugation, as described previously (Abdelmegeed et al., 2016; Choi et al., 2016, 2017). All extraction buffers were pre-equilibrated with nitrogen gas to remove the dissolved oxygen. Protein concentrations for total protein lysates and mitochondrial lysates were determined using the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL, USA). Equal amounts of lysates were resolved on 7 or 12% SDS-PAGE gels and electrophoretically transferred to nitrocellulose membranes (Hercules, CA, USA). Membranes were blocked with 5% (w/v) nonfat milk proteins, and then incubated with a specific primary antibody to each target protein in 3.5% (w/v) bovine serum albumin (BSA) overnight at 4°C. Following separate steps of washing to remove unbound antibodies, membranes were probed with a specific secondary antibody for 1 h. Image was detected by ECL SuperSignal West Pico Kit (Thermo Fisher scientific, Waltham, MA 02451). β-actin and ATP-5A were used as loading controls for whole kidney extracts and mitochondrial proteins, respectively. For protein carbonylation, an aliquot of the total renal lysates was derivatized with dinitrophenylhydrazine (DNPH) under acid denaturing conditions using Oxyblot protein oxidation detection kit (Millipore, Billerica, MA) by following the manufacturer's instructions. A separate aliquot of the renal lysates, that had been acid-denatured as well but not treated with DNPH, were used as a negative control. For the immunoprecipitation experiment, two pooled samples were prepared from each of the four groups. Each sample with a total 500 μg of renal cell lysates was pooled equally from 5 mice/sample (n=10/group). Then, the specific antibody (5 μg) to collagen 1A1 was used for immunoprecipitation. Pierce™ protein A/G magnetic beads from Life Technologies (Grand Island, NY, USA) were used by following the manufacturer's protocol. The immunoprecipitated proteins were subsequently analyzed by immunoblot analysis using the specific antibody against collagen 1A1. Relative levels of target proteins were normalized to the levels of the loading controls, β-actin and ATP-5B, for total cell lysates and mitochondria, respectively, and collagen 1A1 for IP followed by immunoblot. Values for the young WT mice group were set at 100%.

2.5. Immunohistochemistry and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) analyses

Formalin-fixed kidney samples were processed and 5-μm thick paraffin sections were used for immunohistochemistry (IHC). In brief, deparaffinized kidney sections were subjected to

3% hydrogen peroxide followed by antigen retrieval. The sections were blocked with 2% (w/v) non-fat skim milk solution, and incubated with the primary antibody against myeloperoxidase, oxidized proteins, nitrated proteins detected by anti-3-NT antibody, or collagen type 1A1 at 4 °C overnight. After subsequent washing steps to remove the unbound antibody, the attached primary antibody was then linked to the dextran polymer per manufacturer's protocol (Envision kit, Dako, Carpinteria, CA, USA). The final reaction was performed by immersing the sections in a solution of 3,3′-diaminobenzidine (DAB). The sections were then counterstained with hematoxylin. For detection of DNA strand breaks to characterize apoptotic cell death in kidney tissues, the ApopTag Peroxidase in situ apoptosis detection kit (cat. # S7100) (Millipore, Billerica, MA) was used. TUNEL-positive kidney cells were counted in 10 high-power microscope fields for all four groups and tabulated.

2.6. Measurements of renal hydrogen peroxide (H_2O_2) levels, mitochondrial complexes activities, ATP levels, CYP2E1, and anti-oxidant enzyme activities

Hydrogen peroxide (H_2O_2) levels were measured using the hydrogen peroxide assay kit (Abcam Inc., Cambridge, MA, USA) following the colorimetric assay protocol and as previously described (Abdelmegeed et al., 2016). Mitochondrial complexes I, II, III, and IV activities were evaluated using mitochondrial proteins by commercial kits from Cayman Chemical Inc. (Ann Arbor, MI, USA) per manufacturer's protocol. ATP levels in snap-frozen liver tissues were evaluated by using the ATP Assay Kit (Abcam Inc., Cambridge, MA, USA) following the manufacturer's protocol. CYP2E1 activity was measured by the rate of p-nitrophenol oxidation to p-nitrocatechol with 1 mg of renal cytosol/sample (Reinke and Moyer, 1985). The catalytic activities of total renal superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (Gpx) in the total renal lysates were determined by using the kits from Cayman (Ann Arbor, MI, USA), by following the manufacturer's instruction.

2.7. Data evaluation

Data presented are mean ± SEM, n=8–10, unless indicated otherwise. All statistical analyses were performed using Prism7 software. Two-way ANOVA was performed to assess the differences in all the results. Only, when two-way ANOVA revealed the presence of age-genotype interaction, one-way ANOVA followed by post hoc Tukey's test at a significance level of 0.05 was performed to determine the differences between the four groups as previously described (Abdelmegeed et al., 2017; Abdelmegeed et al., 2016; Abdelmegeed et al., 2011).

3. Results

3.1. Increased renal histopathological changes in aged WT mice

We first compared the respective kidney tissues of young (7-weeks) and aged (16-months) WT and *Cyp2e1*-null groups (n=10/group) to evaluate the age-related histological kidney changes. The kidneys of the aged WT mice appeared normal but slightly pale. We observed elevated inflammatory cell foci (often lymphocytes and neutrophils), tubular cytoplasmic vacuolation, tubular degeneration/regeneration with occasionally thickened basement membranes, glomerulonephropathy with hyaline material expanding the mesangium (PAS-

positive), and enlarged glomerular tufts in aged WT mice. Additionally, the levels of renal fibrosis were markedly increased in aged WT compared to the corresponding *Cyp2e1*-null mice and young WT (Fig. 1A and B and Table 1). These histological results were supported by the significantly increased levels of neutrophil marker myeloperoxidase IHC (Fig. 1C), renal TNF-α contents (Fig. 1D), and serum creatinine levels (Fig. 1E) in aged WT mice compared to all other groups. The renal TNF-α levels were significantly affected by the genotype (i.e. gene effect), and there was a significant age-genotype interaction (Fig. 1D). One-way ANOVA revealed that TNF-α levels were highest in aged WT mice compared to all other groups (Fig. 1D). Serum creatinine, an indirect indicator of renal functions and which inversely related to the health of the kidneys, was significantly higher in aged mice (i.e., age effect), and a significant age-genotype interaction was monitored (Fig. 1E). The levels of serum creatinine in aged WT were significantly higher than all other groups, although aged *Cyp2e1*-null mice exhibited significantly higher levels than young mice (Fig. 1E). None of these structural or histopathological changes were detected in the young mice of both genotypes.

3.2. Increased apoptosis of kidney cells and kidney fibrosis in aged WT mice

To evaluate any difference in the levels of cell apoptosis of the kidneys in all four groups, the TUNEL assay was performed. TUNEL-positive apoptotic kidney cells were remarkably elevated in the aged WT (Fig. 2A). Significant differences were observed in the levels of apoptotic kidney cells resulting from age effects (Fig. 2B). In addition, a significant age-genotype interaction was monitored, and further analysis revealed that aged WT mice showed the highest levels of apoptotic cells (Fig. 2B). In addition, kidney fibrosis, as evidenced by Sirius red staining, was significantly increased in aged WT, compared to all other groups (Fig. 3A and B, and Table 1). Two-way ANOVA revealed both significant age and genotype effects on kidney fibrosis (Fig. 3B). The accumulation of collagen fiber and development of renal fibrosis in aged WT mice were further confirmed by IHC for increased collagen formation and distribution determined with the specific anti-collagen 1A1 antibody (Fig. 3C), and by immunoprecipitation of two different samples (pooled from 5 mice in each group/sample) by using the specific collagen 1A1 followed by immunoblotting with the same antibody (Fig. 3D). Collectively, these results (Fig. 1–3) clearly demonstrate that the WT mice undergo more progressive aging-dependent renal histopathological and structural changes. In contrast, the corresponding aged *Cyp2e1*-null mice seem to exhibit far less histopathological changes and were resistant to aging-related kidney damage, suggesting an important role of CYP2E1 in regulating the kidney functions.

3.3. Elevation of oxidative stress and decreased anti-oxidant capacity in aged WT

We further determined whether oxidative stress levels were increased in aged WT and correlated with the signs of aging-related renal damage. We first evaluated the levels of nitroxidative protein modification, a foot print of increased oxidative/nitrative stress. IHC evaluation showed remarkably higher levels of oxidized and nitrated proteins in the aged WT compared to those of all other groups (Fig. 4A and D, respectively). It is noteworthy to mention that the specificity of the oxidized proteins was confirmed by disappearance of the bands when they were not treated with DNPH (Fig. 4B). Oxyblot and immunoblot using anti-3-NT antibody followed by two-way ANOVA showed that the levels of renal oxidized

(Fig. 4B and C) and nitrated proteins (Fig.4E and F) were significantly elevated in aged WT mice than young mice (i.e., age and genotype effects). In addition, a significant age-genotype interaction was observed with both oxidized and nitrated protein levels both of which were significantly higher in aged WT mice, compared to all other groups, although both of these covalently modified proteins were significantly higher in aged *Cyp2e1*-null mice compared to young groups (Fig. 4C and F).

Indeed, the levels of H_2O_2 were higher in aged mice than young mice (i.e., age effect) and there was a significant age-genotype interaction (Fig. 5A). One-way ANOVA revealed that aged WT mice exhibited highest H_2O_2 levels, while aged *Cyp2e1*-null mice showed lowest levels compared to all other groups. Since oxidative stress may result from the imbalance between oxidative radical production and their removal by the antioxidant defense system, we evaluated potential alterations of some nitroxidative marker proteins and the mitochondrial electron transport chain proteins as well as antioxidant molecule GSH and other antioxidant enzymes. We first investigated some potential sources of the nitroxidative stress. As expected, there was a significant difference in the CYP2E1 levels in WT mice compared to *Cyp2e1*-null mice (i.e., genotype effect) while the amounts were similar in young and aged WT mice (Fig. 5B and C), and cytosolic CYP2E1 activity exhibited similar pattern (data not shown). The iNOS levels were significantly higher in aged WT mice compared to all other groups (Fig. 5B and D). Interestingly, NADPH oxidase protein exhibited a significant age-genotype interaction, and further statistical analysis showed that the levels of NADPH oxidase in aged *Cyp2e1*-null mice were significantly higher than their corresponding young controls and aged WT mice, although the levels were not higher than young WT mice (Fig. 5B and E). Xanthine oxidase, however, did not exhibit significant changes in aged versus young groups (data not shown).

Since alteration of mitochondria functions might be another major source of elevated oxidative stress and the iNOS levels in the old WT mice were highest, we first evaluated whether mitochondrial proteins were differentially nitrated in aged WT mice compared to all other groups (Fig. 6A and B). Immunoblot analysis revealed that there was a significant increase in nitrated proteins in aged WT mice (i.e., age and gene effects), and there was a significant age-genotype interaction. Further one-way ANOVA showed that the levels of nitrated proteins were significantly higher in aged WT mice than all other groups, even though the levels observed in aged *Cyp2e1*-null mice were significantly higher than the young groups (Fig. 6A and B). Since CYP2E1 is also present in mitochondria, we evaluated its protein levels and activities in the kidneys of all groups. However, we only monitored a significant gene effect without any age effect for both levels and activities (Fig. 6C and D). We then analyzed the levels of mitochondrial complex proteins using the total OXPHOS rodent WB antibody cocktail, which contains 5 mouse monoclonal antibodies against CI subunit NDUFB8, CII-SDHB, CIII-Core protein 2 (UQCR2), CIV subunit 1 (MTCO1) and CV alpha subunit (ATP-5A) as well as complexes activities. Immunoblot and statistical analyses showed no significant changes in the levels of the subunits of CI, CIII, and CV (Fig. 7A, B, D, and F). In contrast, significant age effect and age-genotype interaction was observed for CII (SDHB) subunit protein levels (Fig. 7A and C). One-way ANOVA revealed that the amount of CII (SDHB) subunit was significantly lower in aged WT than all other groups and that such alteration was absent in *Cyp2e1*-null mice regardless of their age (Fig.

7A and C). Further, the levels of CIV (MTCO1) subunit in aged mice were lower than young counterparts (i.e., age effect) (Fig. 7A and E). Since immunoblot measurement of the functional proteins mainly detects one subunit per complex which might not necessarily represent the complex state, we therefore evaluated the activities of mitochondrial complexes. Complex I activity measurement revealed lower activity levels in aged mice than young mice (i.e., age effect) regardless of the gene effect (Fig. 7G). Significant age and gene effects and age-genotype interaction for CII activities were monitored (Fig. 7H). One-way ANOVA revealed that the CII complex activity levels were lowest in the aged WT mice among all other groups despite the fact that CII complex activity levels were significantly lower in aged *Cyp2e1*-null mice compared to their young counterparts and young WT mice (Fig. 7H). Complex III activity exhibited no significant changes related to age or gene (Fig. 7I). Significant age and age-genotype interactions for CIV activities were recorded (Fig. 7J). One-way ANOVA revealed that the CIV complex activity levels were the lowest in the aged WT mice among all other groups (Fig. 7J). We did not, however, observe any significant alteration in ATP levels in the aged mice groups compared to the young groups (Fig. 7K). Taken together (Fig. 1, Fig. 5, Fig. 6, and Fig. 7), these data suggest that elevated inflammation, iNOS, and mitochondrial dysfunction via alteration of the mitochondrial electron transport chain proteins and activities are likely involved in the increased nitroxidative stress during the kidney aging process and that this combination is more prominent in the aged WT than in the aged *Cyp2e1*-null mice.

Following the evaluation of various sources of oxygen radical production, we next evaluated some cellular defense systems against oxygen radicals. We observed significant age effects and age-genotype interaction for both GSH levels and HO-1 protein levels (Fig. 8A and D). One-way ANOVA showed that GSH levels were lowest in aged WT mice (Fig. 8A). The evaluation of GCLC revealed no differences among the groups, although there was a tendency for higher levels in the KO mouse groups (Fig. 8B). GCLM levels revealed that there was a significant age, genotype, and age-genotype interaction (Fig. 8C). Further analysis of GCLM showed that aged WT exhibited the highest levels of GCLM (Fig. 8C). This data suggests that the decreased levels of GSH in aged WT mice was probably due to increased levels of oxidation and conjugation and that the increase of GCLM levels might be adaptive response to decreased GSH levels. In addition HO-1 levels were highest in aged *Cyp2e1*-null (Fig. 8D), compared to all other groups. Catalase activity was significantly affected by age and genotype and there was a significant age-genotype interaction (Fig. 8E). Catalase activity was highest in aged *Cyp2e1*-null mice, compared to all other groups (Fig. 8E). Both SOD and Gpx activities were significantly affected by genotype, however, a significant age-genotype interaction was only monitored for SOD activity (Fig. 8F and G). Further analysis of SOD activity showed that its levels were similarly significantly lower in aged mice than young mice (Fig. 8F).

We finally determined the levels of few other renal cytochrome P450 proteins to evaluate whether these proteins could be altered during the aging or contribute to aging-related damage, since CYP4A is also known to produce ROS, as previously reviewed (Hardwick et al., 2009). We did not monitor any significant changes in the protein levels of CYP1A and 2B among the groups (Fig. 9A, B and C). CYP4A, however, exhibited a significant difference depending on both age and genotype. Aged WT mice exhibited highest CYP4A

levels among all the groups (Fig. 9A and D), suggesting a potential differential regulation of some cytochrome P450 proteins upon CYP2E1 deletion and during aging process.

Collectively, these results (Fig. 1–9) suggest that oxidative stress is increased in parallel with the kidney aging process in WT mice. Furthermore, since all these changes were not as prominent in aged *Cyp2e1*-null mice, it is reasonable to assume that CYP2E1 plays at least a partial role in the aging-related kidney disease such as inflammation, apoptosis, glomerulonephropathy, and fibrosis.

4. Discussion

Mice exhibited many similarities to humans suffering from age-related kidney changes such as inflammation, apoptosis, glomerulonephropathy, and fibrosis (Hodgin et al., 2015; Martin and Sheaff, 2007; Percy et al., 2005; Percy et al., 2008; Sataranatarajan et al., 2012). In addition, mice have a short life span and thus they are suitable models to investigate the aging process and potential underlying mechanism(s). Interestingly, women seem to be less prone to the decreased kidney functions until reaching menopause when aging accelerates, probably due to the reduced effects of estrogen and lower estrogen receptor expression (Gross et al., 2005). This notion was supported by the exacerbation of declining kidney functions and increased glomerulosclerosis in aged female rats (Fortepiani et al., 2003). The constitutive expression of CYP2E1, an established source ROS production, has been suggested to be significantly higher in female than male mice (Konstandi et al., 2013), although it has been suggested otherwise previously (Chanas et al., 2003). In addition, physiological implications of our rodent results would need to be evaluated in senior (female) people who may use CYP2E1 substrate or inducer drugs such as acetaminophen (APAP), chlorzoxazone, and isoniazid since a multivariate logistic regression report showed that some females, who used high doses of APAP as a pain killer, exhibited increased risks of decreased renal function (Curhan et al., 2004). Thus, we decided to evaluate the effect of CYP2E1 using female mice to determine the aging-related kidney damage. However, it is not very well-established whether males or females are more sensitive to kidney damage in response to different drugs and chemicals particularly during aging state. Thus, it is still important to evaluate the role of CYP2E1 in promoting aging-related kidney damage in male mice to compare the effects observed with females to determine potential sex-dependent difference(s) in this process.

CYP2E1 is a well-established producer of high levels of ROS (Caro and Cederbaum, 2004; Novak and Woodcroft, 2000; Song et al., 1996) and is involved in promoting various post-translational modifications such as protein oxidation, nitration, phosphorylation, adduct formations, among others. (Abdelmegeed et al., 2015; Abdelmegeed and Song, 2014; Song et al., 2013, 2014). CYP2E1 has been suggested in mediating kidney injury and toxicity in response to acetaminophen (Ko et al., 2017), myoglobinuric acute kidney injury, which resembles rhabdomyolysis (Wang et al., 2014), acute rejection in kidney transplantation recipients (Kim et al., 2014), chronic ethanol ingestion (Latchoumycandane et al., 2014), chloroform (Sasso et al., 2013), and cisplatin (Liu and Baliga, 2003), to name a few, mainly via nitroxidative stress-mediated events. All these reports support the hypothesis that CYP2E1 in mice is likely to play an important role in promoting aging-related kidney

disorders, possibly via increased levels of nitroxidative stress. In this study, we have evaluated age-related changes in histochemical features and biochemical parameters and these outcomes were compared between aged WT mice and other groups, including aged *Cyp2e1*-null mice, to further characterize the involvement of CYP2E1 in aging-related kidney damage.

Our results showed that the deletion of *Cyp2e1* gene significantly prevented and/or decreased the prominent features associated with kidney aging (Martin and Sheaff, 2007; Percy et al., 2005) such as inflammation, apoptosis, and fibrosis, as observed in aged WT compared to all other groups. It is well-recognized that aging process, including kidney aging, is correlated and/or facilitated with increased levels of oxidative radicals, particularly in chronic basis (Percy et al., 2005). The increased oxidative stress levels might be originated from normal metabolism of endogenous and exogenous compounds, leading to oxidative damage (Gregg et al., 2012) to cellular macromolecules such as proteins and lipids. Indeed, nitroxidative post-translational protein modifications are considered a well-established marker for oxidative stress during the aging process, which may negatively affect the functions of the modified target proteins prior to the observation of a full-blown organ damage (Abdelmegeed et al., 2013; Chakravarti and Chakravarti, 2017; Moon et al., 2008; Ortuno-Sahagun et al., 2014; Percy et al., 2005). Consistent with these reports, we found that the levels of renal H_2O_2, protein oxidation, and nitration, were significantly higher in aged WT compared to the aged *Cyp2e1*-null mice and young mouse groups.

The development of oxidative stress-mediated cell/organ damage, including the kidneys, is related to the imbalance between the production of oxidants and the defense capacity by the anti-oxidant system (Small et al., 2012; Song et al., 2014). There are many endogenous sources of ROS and RNS such as CYP2E1, iNOS, NADPH-oxidase, xanthine oxidase, and the mitochondrial electron transport chain, inflammatory cells, among others (Abdelmegeed and Song, 2014; Small et al., 2012; Song et al., 2014). Indeed, we monitored significantly higher levels of inflammation and oxidative stress, and lower antioxidant capacity in aged WT mice compared to other groups. The differential regulations of many proteins in NADPH oxidase, HO-1, catalase, and GSH were observed between aged WT and aged *Cyp2e1*-null mice. The deletions of one of the genes, particularly an important gene like CYP2E1, would possibly alter the expression of many other genes and proteins, either to compensate for the effect of the deleted gene or as an expected result if that gene's regulation is affected by the deleted gene. Some of the genes might induce ROS to compensate for CYP2E1 deletion as certain basal levels of ROS is required for normal signaling and organs' functions. Thus, it is possible that the upregulation of NADPH oxidase in the aged *Cyp2e1*-null mice, might be partly due to the deletion of CYP2E1 as an alternative source of oxidative stress as monitored in the aged *Cyp2e1*-null mice. In contrast, the decrease of NADPH oxidase in aged WT mice might be a defense mechanism by the cells to minimize the production of ROS. Similarly, the upregulation of catalase, HO-1, and the persistence of GSH levels in the aged KO mice might be due to the relatively more intact adaptive mechanisms in the aged KO mice than the aged WT mice. It is also reasonable to assume that there might be differential temporal efficiency in the adaptation mechanism between WT and *Cyp2e1*-null mice, and to assume that the WT mice have been exposed to the higher levels of ROS much earlier than the aged *Cyp2e1*-null mice, leading to earlier

consumption of adaptive mechanisms' capacity in the aged WT mice. However, eventually similar effects will take place in the aged *Cyp2e1*-null mice, but at later time point. The temporal regulation of different renal oxidative stress-inducing proteins antioxidant adaptive mechanisms will require further investigation.

We also observed changes in the levels of the mitochondrial electron transport chain protein complexes levels and activities. Despite the most significant changes in CII-SDHB protein levels in the aged WT mice, activity measurements revealed that complexes I, II, and IV were also affected. The discrepancy between immunoblot data and activity measurements might be attributed to (1) immunoblot data only evaluate one of the subunits of the each complex, and (2) enzyme activity might be inhibited by oxidative stress even when protein levels are similar (Moon et al., 2006; Moon et al., 2008),. This data suggests a potential role of mitochondrial impairment with increased production of ROS such as superoxide anion and H_2O_2, further leading to increased levels of mitochondrial protein nitration in aged WT to a higher degree compared with aged *Cyp2e1*-null mice. This is not surprising since it was previously reported that mitochondria isolated from old animals released higher levels of oxidative radicals than young animals (Sohal and Sohal, 1991). In addition, there was an inverse relation between the increased ROS production by mitochondria and the life span (Lambert et al., 2007). Furthermore, our previous reports showed causal roles of mitochondrial dysfunction in promoting full-blown tissue injury usually observed at later time points following exposure to CYP2E1 substrates such as APAP (Abdelmegeed et al., 2013; Abdelmegeed et al., 2010) and carbon tetrachloride (Jang et al., 2015) or under pathological conditions (Moon et al., 2006; Moon et al., 2008). The potential role of mitochondria in mediating the aging-related renal damage in WT mice warrant further investigation with treatment agents targeting mitochondria, such as antioxidant mito-Q (Akbar et al., 2016; Song et al., 2014), to establish their importance in the aging process and disease manifestation. Interestingly, despite the fact that mitochondrial respiration is essential for the production of ATP and that oxidatively-damaged mitochondria may produce less amounts of ATP, in our study, the levels of ATP in aged mice were not significantly changed, suggesting an adaptive mechanism (Anantharaju et al., 2002; Abdelmegeed et al., 2016). The intracellular GSH constitutes a major antioxidant in many organs, including the kidney (Small et al., 2012). In addition, there is a systematic elimination of many oxygen radicals by the coordinated action of many anti-oxidant enzymes such as SOD, CAT, and GPx (Small et al., 2012). Any disruption or suppression of the activities of one or more of these enzymes presumably would contribute to the increased oxidative stress. Furthermore, decreased expression of other anti-oxidant defense systems such as HO-1, might also lead to increased oxidative stress. Indeed, it has been shown that the induction of HO-1 was protective against in some models of nephrotoxicity (Liu and Baliga, 2003; Shiraishi et al., 2000). Our data showed that the overall antioxidant adaptive response to increased oxidative stress levels seems to be significantly stronger in the aged *Cyp2e1*-null mice than the aged WT group. The increase of antioxidant defense in aged *Cyp2e1*-null mice might explain the lower levels of accumulated oxidatively modified proteins along with the decreased levels of oxidative radicals compared to aged WT mice.

The amounts of several other hepatic cytochrome P450 proteins such as CYP1A and CYP2B exhibited no significant changes among the groups, although CYP4A were significantly

higher in the aged WT mice compared to all other groups. These results suggest that there is a differential regulation in the levels of some other P450 proteins between the aged WT and the corresponding *Cyp2e1*-null mice. Since P450-mediated metabolisms produce ROS and the importance of P450 isoforms in the development of kidney toxicities has been reported (Carroll et al., 1997; Fan et al., 2015; Hirt and Jacobson, 1991), it becomes important to evaluate the implications of these differential regulations on the aging-related kidney diseases in future studies.

Taken together, our data clearly showed that a significant difference exists in the cellular redox balances between the aged WT and the corresponding *Cyp2e1*-null mice. The differences in oxidative stress levels could probably result from increased ROS/RNS production from inflammation, iNOS, and through the altered mitochondrial electron transport chain function, along with the decreased antioxidant capacity consisting of GSH, HO-1 and catalase activity in aged WT mice compared to aged *Cyp2e1*-null mice. Even though there was no additional increase in the CYP2E1 levels in aged WT mice, the increased levels of oxidative stress in WT mice could have been produced through CYP2E1-mediated metabolism of endogenous substrates throughout the life span. This is not surprising since CYP2E1 is known to increase the levels of ROS and RNS even in the absence of its substrates (Caro and Cederbaum, 2004; Song et al., 1996). It is also possible that the presence of CYP2E1, regardless of its levels, might exert its permissive action for other proteins, for example iNOS and CYP4A. It is possible that CYP2E1 was upregulated at certain time points during the ~17 month duration and then was returned to control levels at the time of tissue collection. Thus, the increased levels of CYP2E1-related inflammation and nitroxidative stress might play a significant role in the advancement of aging-related kidney damage such as apoptosis, glomerulonephropathy and fibrosis.

5. Conclusion

Our results revealed that CYP2E1 appears to be involved in the development of aging-related kidney disorders. This can be explained by the fact that elevated iNOS, altered mitochondrial electron transport chain, and permissive CYP2E1 increase the production of ROS/RNS and elevated nitrated proteins presumably via peroxynitrite in aged WT mice due to the redox imbalance resulting from increased oxidative stress along with decreased adaptive antioxidant responses. These changes likely increase inflammation and nitroxidative protein modifications, which can negatively affect the vital functions of the kidneys, leading to the development of the pathological features observed in the aged kidneys. Our results demonstrate for the first time the novel role of CYP2E1 in the kidney aging process in rodents, rendering CYP2E1 a potential target for translational research in preventing aging-related kidney damage as well as other kidney diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

APAP	acetaminophen
ATP5B	ATP synthase subunit Beta
CAT	catalase
Cytochrome P450	CYP
DNPH	dinitrophenylhydrazine
GCLC	glutamate-cysteine ligase catalytic subunit
GCLM	glutamate-cysteine ligase modifier subunit
GSH	glutathione
Gpx	glutathione peroxidase
HO-1	heme oxygenase-1
H_2O_2	hydrogen peroxide
IHC	immunohistochemistry
iNOS	inducible nitric oxide synthase
NADPH oxidase	nicotinamide adenine dinucleotide phosphate oxidase
NO	nitric oxide
3-NT	3-nitrotyrosine
PAS	periodic acid-Schiff
RNS	reactive nitrogen species
ROS	reactive oxygen species
SOD	superoxide dismutase
TNFα	tumor necrosis factor-α
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
WT	wild-type.

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Highlights

* The role of CYP2E1 in aging-related kidney damage is largely unknown.

* Young and aged female wild-type (WT) and *Cyp2e1*-null mice were evaluated.

* Aged WT mice exhibited highest levels of inflammation, nitroxidative stress, apoptosis, and fibrosis.

* *Cyp2e1*-null mice were resistant to aging-related kidney damage partly due to significantly high levels of antioxidant capacity.

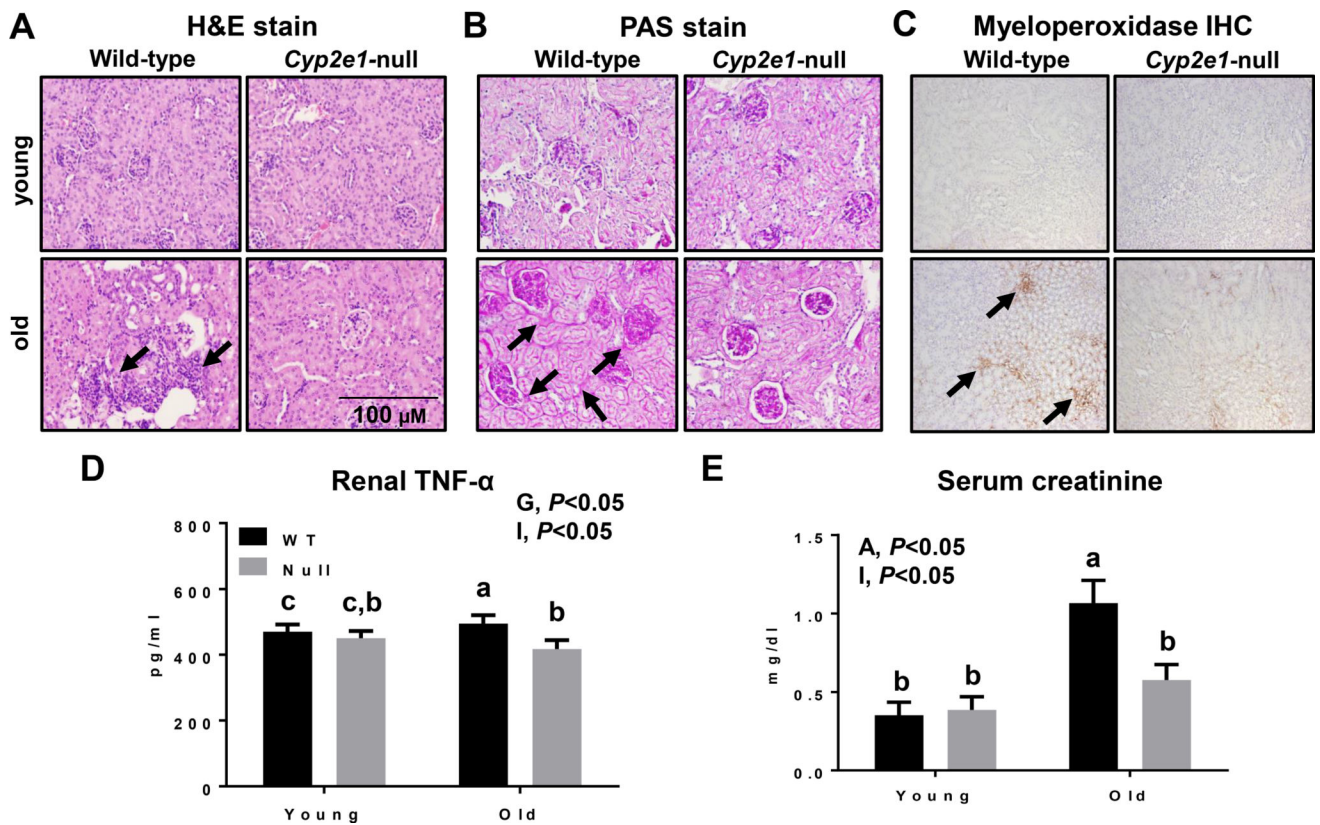* CYP2E1 is important in mediating aging-related kidney damage.

Figure 1.
Histological and biochemical changes in aged WT compared to other groups. Aging induced profound histopathological changes in kidneys, as evidenced by increased inflammatory cell foci, tubular cytoplasmic vacuolation, tubular degeneration, glomerulonephropathy, and kidney fibrosis in old WT mice, which were significantly decreased in aged *Cyp2e1*-null mice, as shown with: (A) H&E staining, (B) PAS staining, and (C) myeloperoxidase IHC (*n*=7/group). Black arrows represent (A) inflammatory foci, (B) glomerulonephropathy and tubular degeneration/regeneration, and (C) myeloperoxidase. The levels of (D) renal TNF-α, and (E) serum creatinine are shown (*n*=6–8/group). A, age; G, genotype; I, age-genotype interaction. Labeled means, which do not share a common letter, represent significant difference from each other. *P* < 0.05.
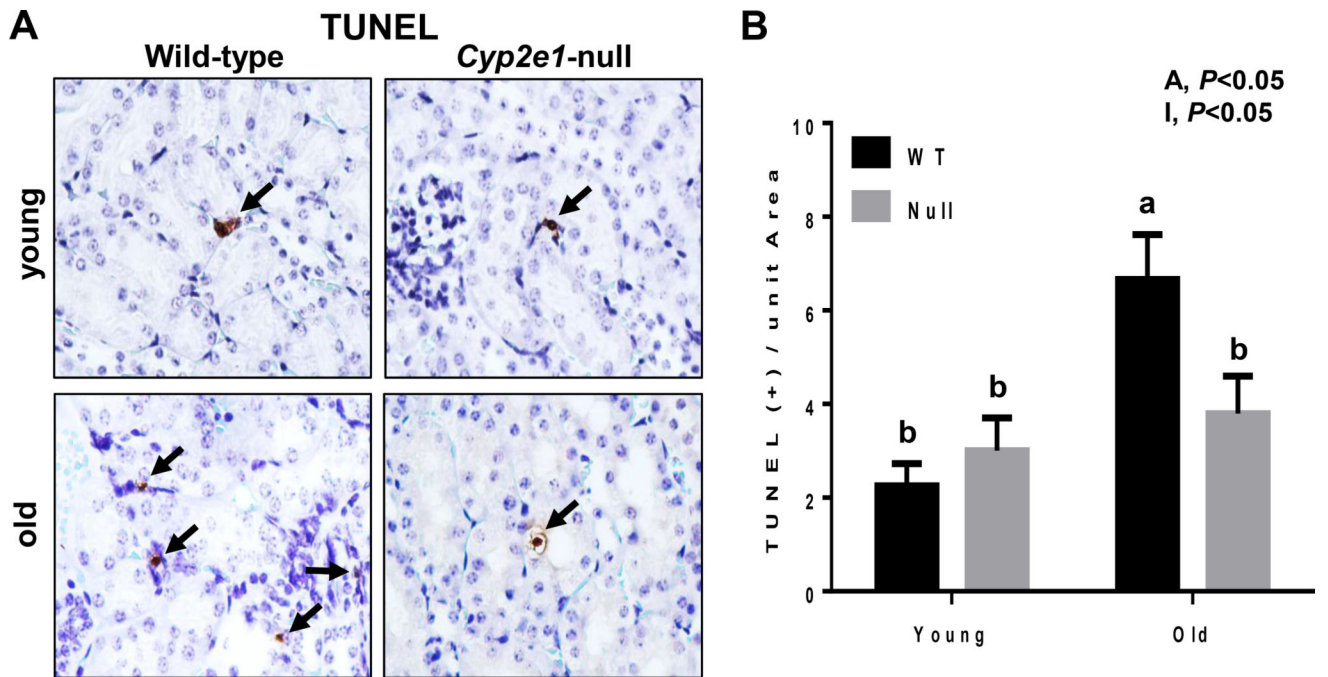
Figure 2.
Increased levels of apoptotic cells in kidneys of aged WT mice. (A) TUNEL-positive hepatocytes were marked with black arrows and (B) quantified in 10 high-power fields (n=7/ group). A, age; I, age-genotype interaction. Labeled means, which do not share a common letter, indicate significant difference from each other. $P < 0.05$.
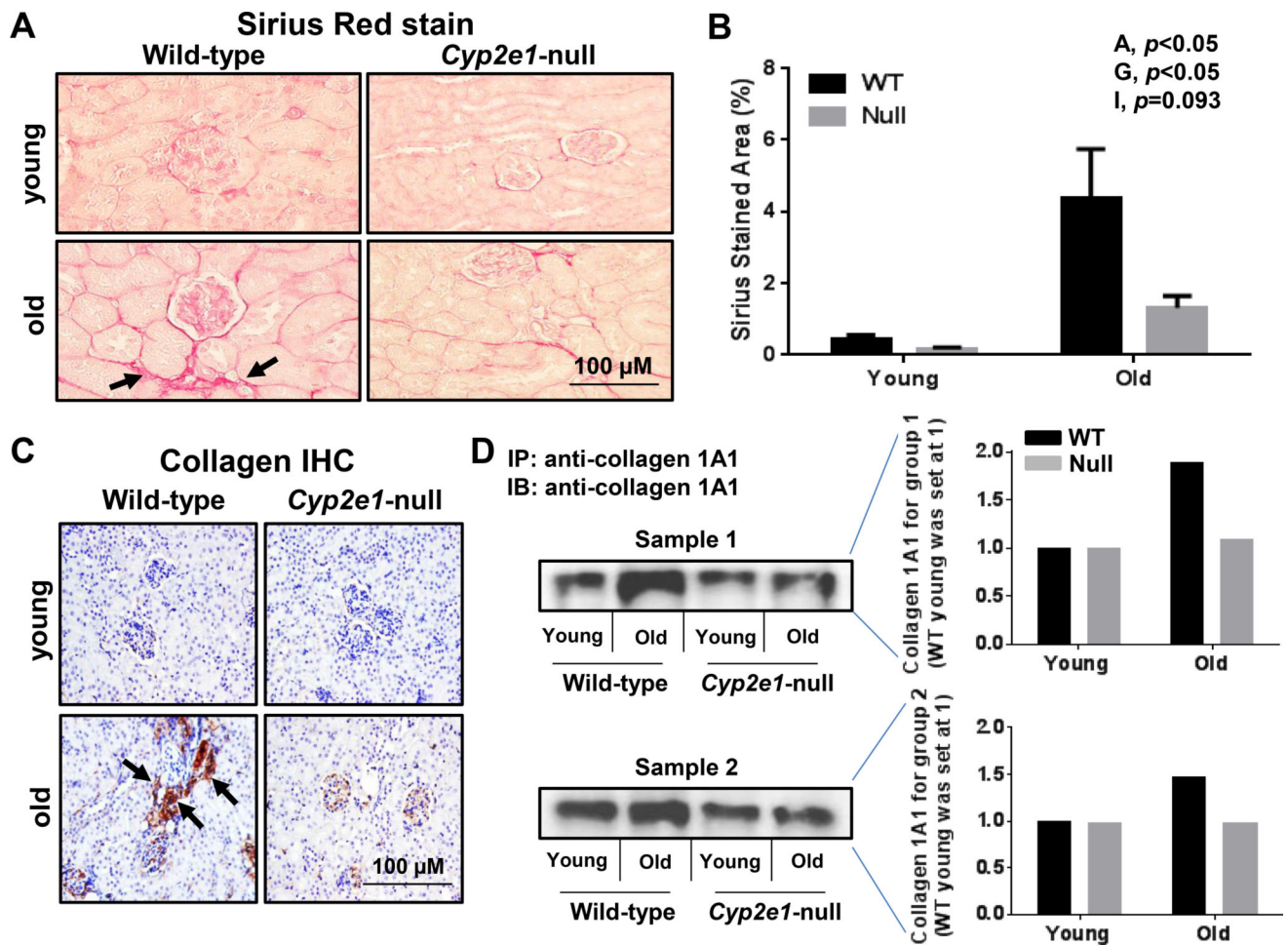
Figure 3.
Increased kidney fibrosis in aged WT mice. (A and B) Representative histological analysis of kidney sections (*n*=7/group), which show Sirius red stained collagen and percentage of fibrotic areas, respectively. The increased levels of collagen in aged WT mice were further proven by (C) collagen IHC analysis, and (D) immunoprecipitation followed by immunoblot analyses of two pooled samples (pooled from 5 mice) per each mouse group (*n*=10/group) and densitometric analysis, respectively. Specific anti-collagen 1A1 antibody was used for both C and D. A, age; G, genotype; I, age-genotype interaction. Labeled means, which do not share a common letter, reflect significant difference from each other. *P* < 0.05.
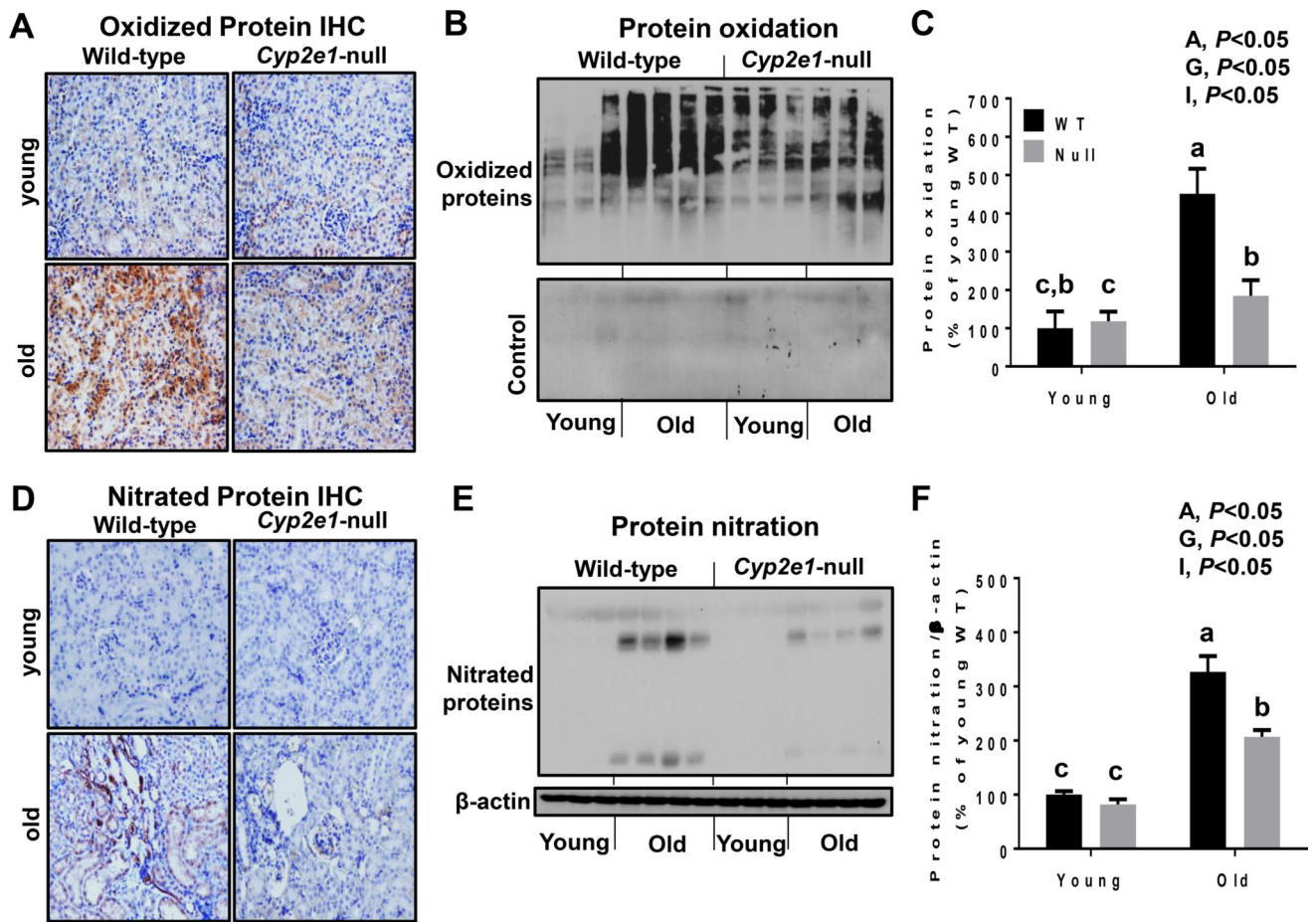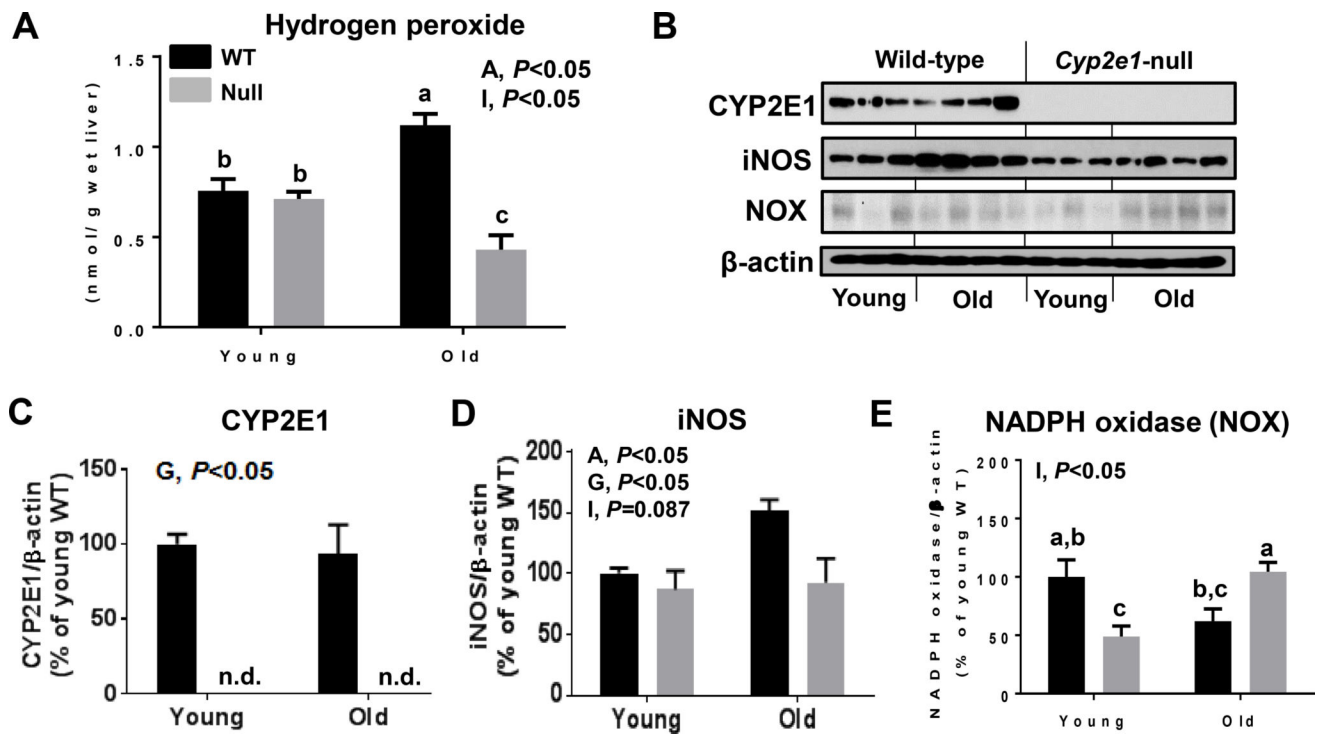
Figure 4.
Increased levels of oxidized and nitrated proteins in aged WT mice. (A, D) Representative results of IHC (*n*=5–6/group) and (B–F) immunoblot and densitometric analyses (*n*=8–10/group) for (A, B) oxidized proteins and (D, E) nitrated proteins, as indicated. A, age; G, genotype; I, age-genotype interaction. Labeled means, which do not share a common letter, represent significant difference from each other. *P* < 0.05.
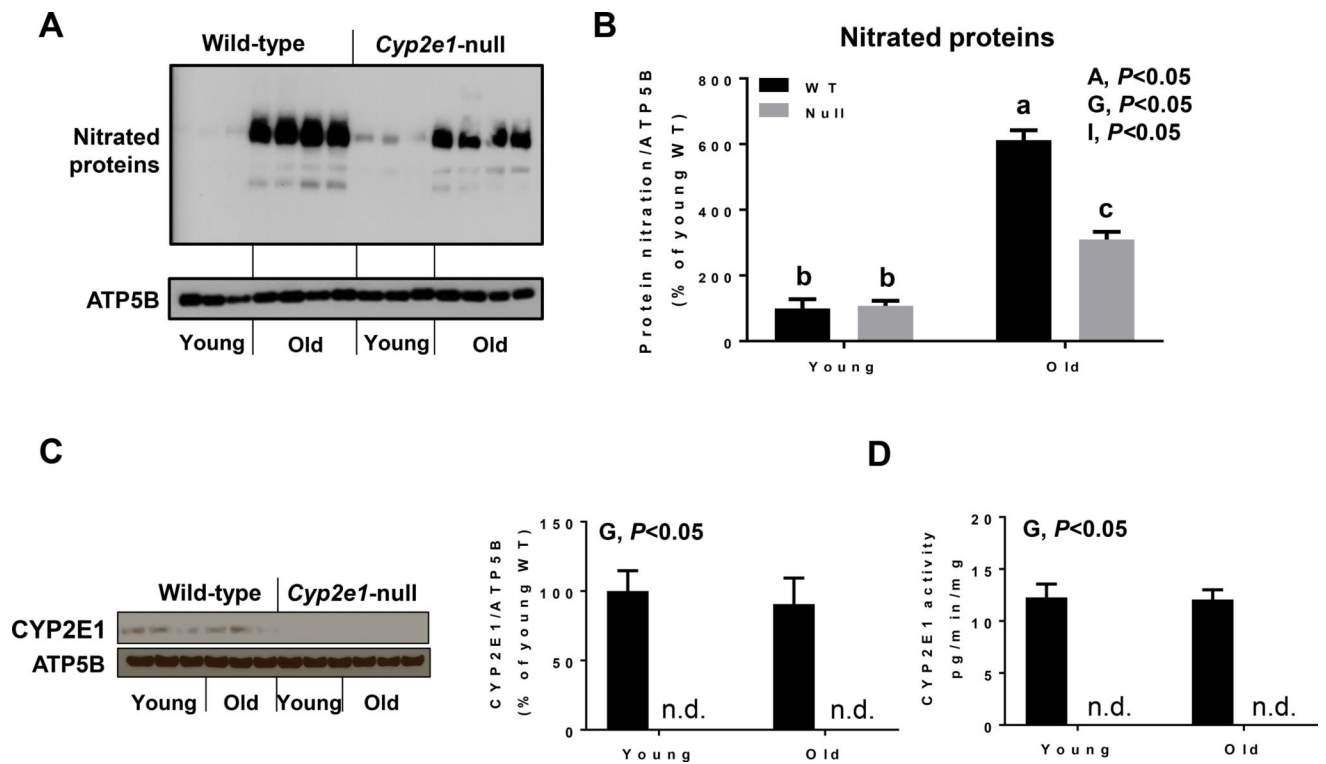
Figure 5.
Increased levels of H₂O₂ and some nitroxidative marker proteins in aged WT mice. Equal amounts of whole renal lysate proteins were (*n*=8–10/group) analyzed to determine the levels of: (A) H₂O₂, and (B–E) other proteins, as indicated by immunoblot and densitometric analyses of (B, C) CYP2E1, (B, D) iNOS, and (B, E) NADPH oxidase. A, age; G, genotype; I, age-genotype interaction. Labeled means, which do not share a common letter, indicate significant difference from each other. *P* < 0.05.

Figure 6.
Increased levels of nitrated mitochondrial proteins and altered levels of mitochondrial CYP2E1 levels and activities in the kidneys of aged WT mice. Equal amounts of renal mitochondrial proteins were analyzed to determine the levels of (A, B) nitrated mitochondrial proteins, (C,D) CYP2E1 protein levels, and (E) CYP2E1 activity (n=8–10/ group). A, age; G, genotype; I, age-genotype interaction. Labeled means, which do not share a common letter, reflect significant difference from each other. P< 0.05.
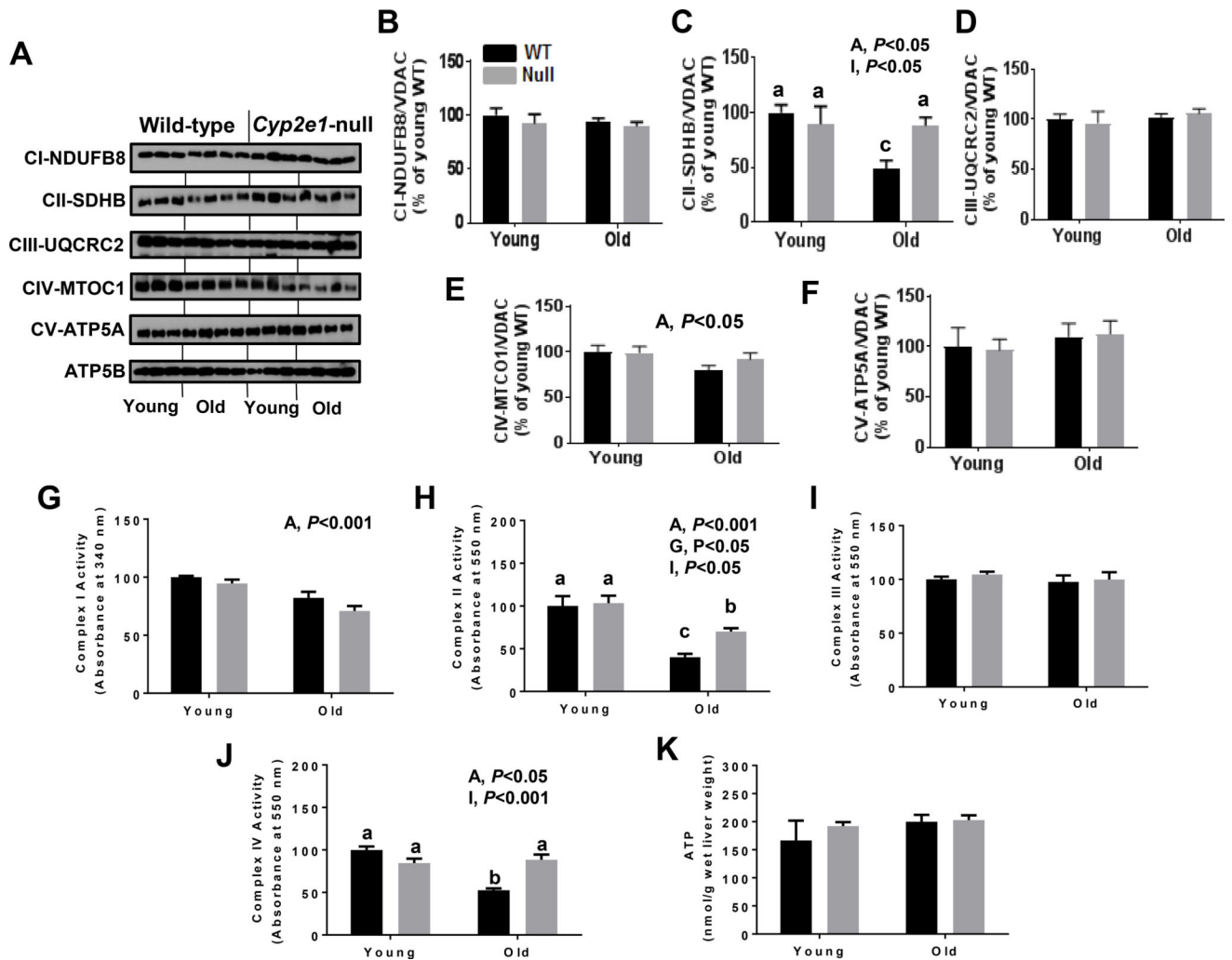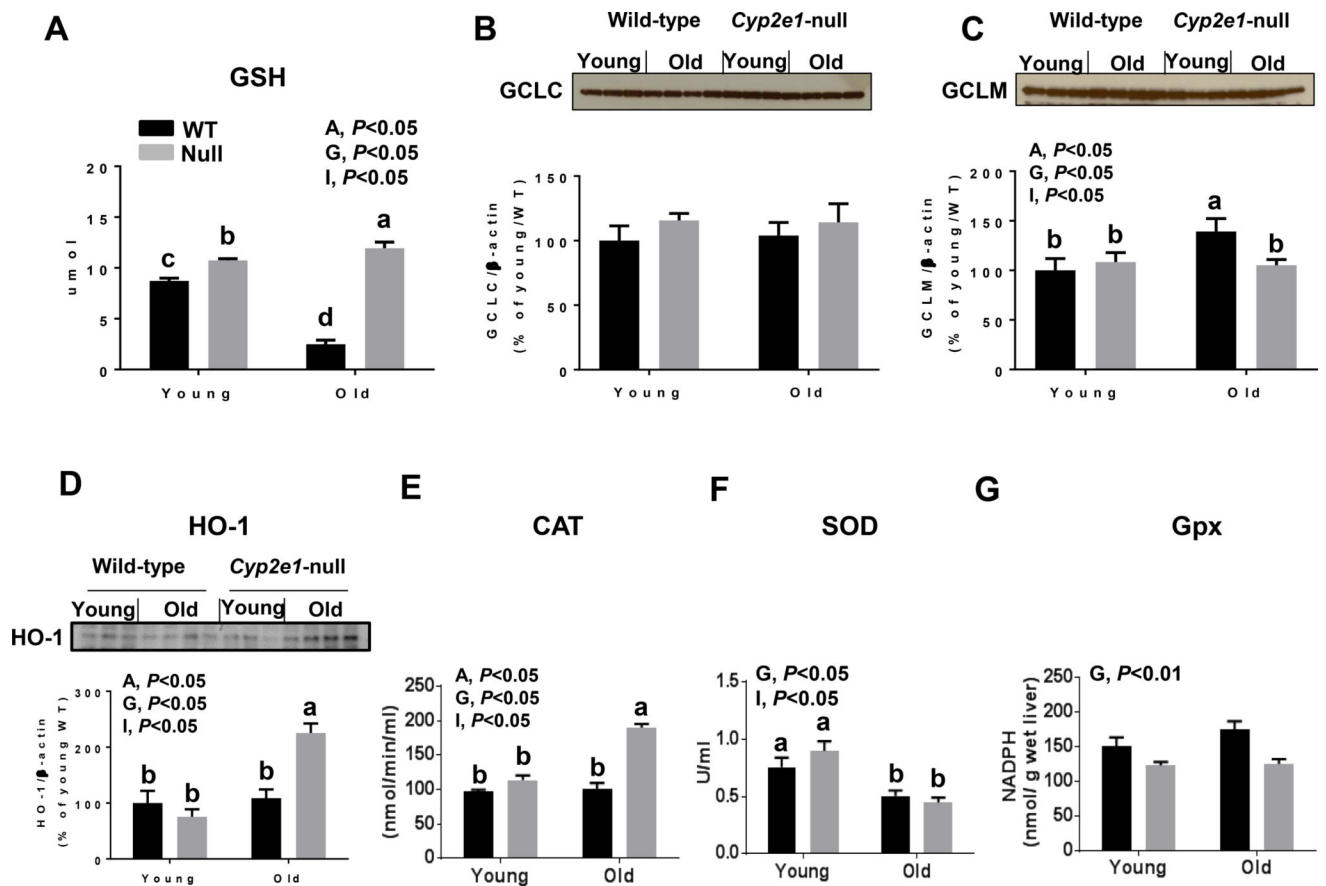
Figure 7.
More prominent alteration in the protein levels and activities of mitochondrial electron transport chain complex proteins in the kidneys of aged WT mice. Equal amounts of renal mitochondrial proteins were analyzed to determine the protein levels (A–F) and mitochondrial electron transport chain complex activities (G–K) as indicated.. A, age; G, genotype; I, age-genotype interaction. Labeled means, which do not share a common letter, reflect significant difference from each other. $P < 0.05$.

Figure 8.

High levels of renal antioxidant capacity in aged *Cyp2e1*-null mice. (A–G) Equal amounts of whole kidney lysates were used to measure the relative levels and densitometric analyses of (A) GSH, (B) GCLC, (C) GCLM, (D) HO-1, and the renal activities of (E) catalase, (F) superoxide dismutase, and (G) Gpx in different mouse groups, as indicated. A, age; G, genotype; I, age-genotype interaction. Labeled means, which do not share a common letter, represent significant difference from each other. $P < 0.05$.
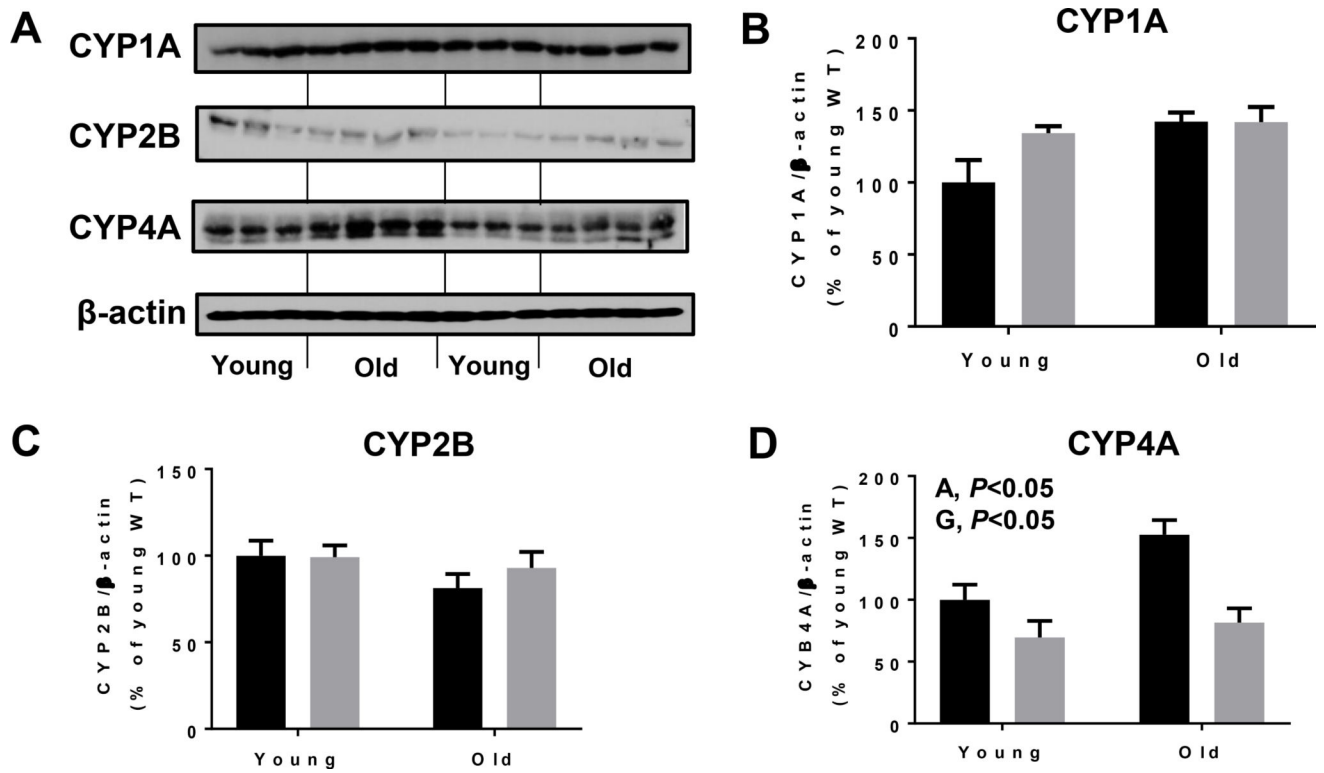
Figure 9.
Changes in the levels of some cytochrome P450 proteins in young and aged WT and *Cyp2e1*-null mice. Equal amounts of whole kidney lysate proteins were used for immunoblot analyses. Representative results of immunoblot analysis and densitometric analysis results (n=8–10/group) are presented for the levels of (A, B) CYP1A, (A, C), CYP2B and (A, D) CYP4A. A, age; G, genotype.

Table 1

Summary of histopathological evaluation of kidneys in young or aged WT and *Cyp2e1*-null mice.

	Wild type young	Wild type old	*Cyp2e1*-null young	*Cyp2e1*-null old
Number of animals examined	7	7	7	7
Glomerulonephropathy	0	7	0	4
Slight		5		4
Moderate		2		
Inflammatory cell foci	1	7	1	4
Slight	1	2	1	1
Moderate		2		2
Severe		3		1
Tubular cytoplasmic vacuolation	0	4	0	2
Slight		4		2
Tubular degeneration/regeneration	0	6	0	2
Slight		4		1
Moderate		2		1
Fibrosis	0	5	0	2
Slight		2		1
Moderate		2		1
Severe		1		

Grading System: slight, few glomeruli or tubules or surrounding cells affected; moderate, multiple glomeruli or tubules or surrounding cells affected; severe, diffuse tissue infiltration and/or damage.