Oxidative stress-induced alterations in PPAR-γ and associated mitochondrial destabilization contribute to kidney cell apoptosis

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Small DM, Morais C, Coombes JS, Bennett NC, Johnson DW, Gobe GC. Oxidative stress-induced alterations in PPAR-γ and associated mitochondrial destabilization contribute to kidney cell apoptosis. Am J Physiol Renal Physiol 307: F814–F822, 2014. First published August 13, 2014; doi:10.1152/ajprenal.00205.2014.—The mechanism(s) underlying renoprotection by peroxisome proliferator-activated receptor (PPAR)-γ agonists in diabetic and nondiabetic kidney disease are not well understood. Mitochondrial dysfunction and oxidative stress contribute to kidney disease. PPAR-γ upregulates proteins required for mitochondrial biogenesis. Our aim was to determine whether PPAR-γ has a role in protecting the kidney proximal tubular epithelium (PTE) against mitochondrial destabilisation and oxidative stress. HK-2 PTE cells were subjected to oxidative stress (0.2–1.0 mM H2O2) for 2 and 18 h and compared with untreated cells for apoptosis, mitosis (morphology/biomarkers), cell viability (MTT), superoxide (dithydroethidium), mitochondrial function (Mitotracker red and JC-1), ATP (luminescence), and mitochondrial ultrastructure. PPAR-γ, phospho-PPAR-γ, PPAR-γ coactivator (PGC)-1α, Parkin (Park2), p62, and light chain (LC)3β were investigated using Western blots. PPAR-γ was modulated using the agonists rosiglitazone, pioglitazone, and troglitazone. Mitochondrial destabilization increased with H2O2 concentration, ATP decreased (2 and 18 h; P < 0.05), Mitotracker red and JC-1 fluorescence indicated loss of mitochondrial membrane potential, and superoxide increased (18 h, P < 0.05). Electron microscopy indicated sparse mitochondria, with disrupted cristae. Mitophagy was evident at 2 h (Park2 and LC3β increased; p62 decreased). Impaired mitophagy was indicated by p62 accumulation at 18 h (P < 0.05). PPAR-γ expression decreased, phospho-PPAR-γ increased, and PGC-1α decreased (2 h), indicating aberrant PPAR-γ activation and reduced mitochondrial biogenesis. Cell viability decreased (2 and 18 h, P < 0.05). PPAR-γ agonists promoted further apoptosis. In summary, oxidative stress promoted mitochondrial destabilisation in kidney PTE, in association with increased PPAR-γ phosphorylation. PPAR-γ agonists failed to protect PTE. Despite positive effects in other tissues, PPAR-γ activation appears to be detrimental to kidney PTE health when oxidative stress induces damage.

peroxisome proliferator-activated receptor-γ; thiazolidinediones; mitochondria; oxidative stress

ALTERATIONS IN THE BIOENERGETIC CONTROLS of kidney cells can lead to a failure in oxidant handling, leading to a state of oxidative stress in kidney disease. The causes are multiple, from high glucose in diabetes to ischemia-reperfusion, toxins, and acute kidney injury to the long-term effects of aging and the development of chronic kidney disease (CKD). Kidney tubular atrophy, mainly due to tubular cell apoptosis of the proximal tubule, is a common characteristic of these diseases and therefore presents a crucial target to preserve kidney health (36, 37). Oxidative stress is a mediating factor in mitochondrial dysfunction and subsequent cell dysregulation and death during kidney disease (16, 41, 47). The importance of mitochondrial dysregulation comes from the large number of mitochondria in proximal tubular epithelial (PTE) cells and their heavy reliance on oxidative phosphorylation for ATP-dependent active solute transport in the nephron (19). Identifying oxidant-handling pathways that are perturbed in the kidney during oxidative stress is necessary for the development of reliable therapies.

Peroxisome proliferator-activated receptor (PPAR)-γ is a member of the highly conserved nuclear hormone receptor superfamily of ligand-dependent transcription factors that play central roles in lipid metabolism, glucose homeostasis, cell proliferation, and inflammation (1). Ligand binding induces heterodimer formation with the retinoic acid receptor and complex binding to the peroxisome proliferator response element within the promoter region of target genes (18). Phosphorylation may modulate the activity of PPAR-γ through activating multiple kinase signaling pathways, including ERK and AMP-activated protein kinase (AMPK). However, PPAR-γ phosphorylation does not always indicate the activation of PPAR-γ (5, 23, 51). Its inhibition may also be important for the overall outcome, which largely depends on tissue type and the mode of PPAR-γ modulation.

The family of thiazolidinediones (TZDs) consists of potent pharmacological agonists of PPAR-γ, commonly used in the treatment of type 2 diabetes. The targeting of PPAR-γ, however, is not limited to diabetes and its effect or dependence on insulin. For example, PPAR-γ mRNA expression is increased in kidney biopsies of CKD patients of diverse etiologies (32) raising the possibility of mechanisms not dependent on insulin; the TZD rosiglitazone attenuated kidney structural and functional damage in a mouse model of diabetes, yet no change was observed in glucose, insulin, or lipid levels (4), and a reduction in proteinuria was achieved in patients with nondiabetic kidney disease of various etiologies after 4 mo of rosiglitazone treatment (27). A possible explanation for the diverse outcomes from PPAR-γ agonist success is that PPAR-γ also positively regulates mitochondrial biogenesis through the activation of PPAR-γ coactivator (PGC)-1α. Thus, PPAR-γ may influence mitochondrial function (6, 12). This is important given the vital...
role mitochondria play in regulating the intracellular redox environment (50). Healthy mitochondrial function relies on the degradation of defective mitochondria via the activation of selective autophagy pathways (mitophagy) and upregulation of mitochondrial biogenesis pathways to restore a healthy pool of functioning mitochondria (44). PPAR-α, an alternate iso-
form of PPAR, also plays a role in mitochondrial biogenesis, but is more widely expressed in the skeletal muscle and liver (17) than in the kidney.

Several molecular pathways may be involved for the influence of PPAR-γ on mitochondrial function. A major pathway for injury-induced mitochondrial degradation is the phosphate and tensin homology-induced kinase-Parkin 2 (Park2) path-
way. Park2 is an E3 ubiquitin ligase that translocates to mitochondria and mediates mitochondrial degradation (3, 35). The adaptor protein p62 facilitates selective autophagy into the light chain (LC)3A-regulated machinery for lysosomal degra-
dation (2, 13). p62 is not required for Park2 translocation to damaged mitochondria but is essential for final autophagic clearance (13). PGC-1α is a nuclear transcription factor that has been shown to mediate almost all aspects of mitochondrial biogenesis and is therefore considered a master regulator of biogenesis (21). Failure to restore mitochondrial homeostasis and function can lead to a progressive increase in ROS and ensuing oxidative stress. Therefore, PGC-1α activation pres-
ents a crucial target for mitochondrial preservation. Previous studies (22, 39) have demonstrated TZD induction of mito-
chondrial biogenesis.

This study sought to 1) investigate the role of PPAR-γ in oxidative stress and mitochondrial destabilization in damage and loss of human kidney proximal tubular cells and 2) determine whether PPAR-γ modulation can protect human kidney PTE cells from oxidative stress-induced damage by preserving mitochondrial function.

METHODS AND MATERIALS

Experimental design. HK-2 PTE cells were maintained in DMEM-
F-12 Ham’s medium containing 10% FBS and 1% penicillin-strepto-
mycin. Cells were grown at 37°C in a humidified atmosphere of 95% O2 and 5% CO2 in 25- or 75-cm2 flasks. Cells were treated with H2O2 (0.2–1.0 mM) for 2 and 18 h to induce oxidative stress. In some treatments, the PPAR-γ agonists troglitazone, rosiglitazone, and pi-
oglitazone were used as treatments for 2 h before and during H2O2 exposure. Troglitazone (T2573) and pioglitazone (E6910) were pur-
chased from Sigma-Aldrich (Castle Hill, NSW, Australia); rosigli-

tazone (No. 71740) was purchased from Cayman Chemical (Ann Arbor, MI). All agonists were dissolved as stock solutions in DMSO and stored frozen as small aliquots. A vehicle control was always used for these experiments.

Western blot analysis. Western blots were performed from whole cell lysates dissociated in lysis buffer (0.15M sodium chloride, 0.025M sodium fluoride, 0.5M EDTA, 0.1% SDS, and 1.0% Igepal in 50 mM Tris-Cl, pH 7.5) containing phosphatase and protease inhibi-
tors (10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM sodium or-
thovanadate, and 100 μg/ml phenylmethylsulfonyl fluoride). Protein was separated by SDS-PAGE on 10% acrylamide gel and electropho-
retically transferred onto polyvinylidenefluoride membranes. Mem-

branes were blotted using antibodies against PPAR-γ, phosphorylated (p)-PPAR-γ (phosphorylation site: Ser112), PPAR-α, PGC-1α, Park2, p62, LC3B, and GAPDH as a loading control. Ponceau S was also used as a dye on the polyvinylidenefluoride membrane to indicate even loading of protein. Appropriate secondary antibodies were used and visualised using chemiluminescent enhancer and X-ray film.

Scion software (Release Alpha 4.0.3.2) was used to quantify bands for densitometry, which was normalized to GAPDH.

Light and fluorescence microscopy. Cells were grown to ~70% confluence on 12-mm glass coverslips. Immunofluorescence and cy-
tochemistry were used to assess p62 cellular localization. The primary antibody for p62 (1:500, Santa Cruz Biotechnologies, Santa Cruz, CA) was used with a secondary anti-rabbit Alexa fluor green fluorescence conjugate (1:300, Cell Signaling Technologies, Danvers, MA). Apoptosis and mitosis were assessed using morphology (14) after hematoxylin and eosin staining and digital scanning using Aperio ImageScope histology software. Data were obtained by counting 100–400 frames (×200) of cells for each treatment and calculating the percent-
age of apoptotic or mitotic cells. The morphological characteristics for apoptosis were 1) shrunken eosinophilic cells with condensed, margined nuclear chromatin and an intact cell membrane; 2) discrete apoptotic bodies compromising large, dense, pyknotic nuclear frag-
ments surrounded by a narrow eosinophilic cytoplasm; and 3) clusters of small apoptotic bodies (assessed as a single apoptotic occurrence) (14). The morphological characteristics used to distinguish mitosis were 1) the formation of mitotic spindles occurring during metaphase and remaining visible in anaphase or 2) cells in the later stages of mitosis, telophase, or undergoing cytokinesis. Morphological verifi-

cation of quantification has been confirmed in a similar experimental protocol using molecular biomarkers for apoptosis (ApopTag) and mitosis (proliferating cell nuclear antigen) in parallel with morphol-

ogy (41).

Oxidative stress and mitochondrial function. Oxidative stress was determined and quantified using microfluorimetry detection of the oxidation of dihydroethidium (DHE) to ethidium. Superoxide selec-
tively oxidizes this reaction and is an essential precursor to harmful cellular oxidants, such as the hydroxyl radical and peroxynitrite. Cells were cultured in black 96-well plates and treated as previously described. The growth medium was then replaced with 5μM DHE in serum-free DMEM-F-12 and incubated for 30 min. Fluorescence intensity was measured at 536-nm excitation and 590-nm emission (Synergy Mx Multi-Mode Microplate reader, BioTek, Winooski, VT). Fluorescence values were normalized to protein content for the corres-
ponding wells and expressed as DHE fluorescence per microgram of protein. JC-1 is a cationic fluorophore that possesses a mitochon-
drial membrane-dependent accumulation in mitochondria and was quantified using the same technique. A decrease in red fluorescence (JC-1 aggregate uptake into healthy mitochondria) and increase in green fluorescence (JC-1 in the cytoplasm) indicates mitochondrial membrane depolarization. JC-1 (3 μM) was used posttreatment, and fluorescence intensity was measured at 525-nm excitation and 590-nm emission (red) and 490-nm excitation and 530-nm emission (green) and normalized to protein content. ATP production was measured using a luciferase-based luminescence assay (Promega). Cells were seeded into white opaque 96-well plates and treated as previously described. Chemiluminescent reagent was added, and cell lysis was induced before luminescence was measured at an integration time of 0.5 s using a TiterTek-Berthold Luminometer (TiterTek-Berthold De-
tection Systems, Pforzheim, Germany).

Mitochondrial viability. MitoTracker red CMXRos (Invitrogen) and confocal fluorescence microscopy were used to determine mito-

Cell viability. Cell viability was measured using the assay based on the reduction 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma). Briefly, cells were treated as previously described in 96-well plates, and 100μL MTT solution was prepared in DMEM-F-12 solution (0.5 mg/ml), added to wells, and incubated for 90 min at 37°C. MTT culture media were removed, and formazan crystals that had formed during the assay were dissolved in 200 μl DMSO. Absorbance was measured in a microplate reader at 570 nm with background collection at 690 nm.

Statistical analysis. Values are reported as means ± SE. Data were analyzed using one-way ANOVA and Tukey’s post hoc analysis, two-way ANOVA and Bonferroni’s post hoc test, or Student’s t-test where appropriate. Significance was established at \( P < 0.05 \).

RESULTS

Oxidative stress decreased PPAR-γ expression and increased PPAR-γ Ser112 phosphorylation. HK-2 cells displayed a dose-dependent significant increase in apoptosis and dose-dependent decrease in mitosis after oxidative stress at both 2 and 18 h (Fig. 1, A and B). This was associated with a decrease in PPAR-γ protein levels (\( P < 0.001 \) starting at 0.4 mM H\(_2\)O\(_2\); Fig. 2A) and a dose-dependent increase in p-PPAR-γ levels (\( P < 0.001 \) starting at 0.4 mM H\(_2\)O\(_2\); Fig. 2A) after 2 h of exposure to H\(_2\)O\(_2\). After 18 h of H\(_2\)O\(_2\) exposure (Fig. 2B), PPAR-γ and p-PPAR-γ returned to basal levels, indicating that oxidative stress promotes the phosphorylation of PPAR-γ as an early response. Conversely, PPAR-α expression decreased in a dose-dependent manner after 2 h of exposure to H\(_2\)O\(_2\) (\( P < 0.05 \) starting at 0.4 mM H\(_2\)O\(_2\); Fig. 2A) and returned to control levels after 18 h of H\(_2\)O\(_2\) exposure (Fig. 2B).

Oxidative stress induced mitochondrial dysfunction. Mitochondrial function was significantly reduced after exposure to H\(_2\)O\(_2\) for both 2 and 18 h (Fig. 3). H\(_2\)O\(_2\) (0.6 mM) significantly increased DHE fluorescence, indicating enhanced levels of superoxide production and confirming an intracellular state of oxidative stress (\( P < 0.05 \); Fig. 3A). The mitochondrial membrane potential significantly decreased, as indicated by a decrease in JC-1 red fluorescence intensity (\( P < 0.01 \) starting at 0.4 mM H\(_2\)O\(_2\); Fig. 3B) and an increase in JC-1 green fluorescence intensity (\( P < 0.01 \) starting at 0.4 mM H\(_2\)O\(_2\); Fig. 3C). Confocal microscopy revealed that, compared with untreated HK-2 cells (Fig. 3H), uptake of MitoTracker red CMXRos fluorescence was impaired after H\(_2\)O\(_2\) exposure at both 2 h (Fig. 3I) and 18 h (Fig. 3J). Mitochondrial ultrastructure (Fig. 3, D–G) revealed abundant elongated mitochondria with clear cristae in untreated HK-2 cells (Fig. 3, D and E) compared with segmented and swollen mitochondria (Fig. 3F) and absent cristae (Fig. 3G) after H\(_2\)O\(_2\) exposure. These changes relate to a significant dose-dependent decrease in ATP production by mitochondria after H\(_2\)O\(_2\) exposure at both 2 h (\( P < 0.001 \) at 0.4 mM H\(_2\)O\(_2\); Fig. 4A) and 18 h (\( P < 0.001 \) at 0.4 mM H\(_2\)O\(_2\); Fig. 4B), indicating impaired oxidative phosphorylation.

Oxidative stress-induced PPAR-γ dysregulation impaired mitochondrial autophagy (mitophagy) and biogenesis. After oxidative stress and the initial insult affecting mitochondrial function (Figs. 3 and 4), the cellular autophagy machinery needed for selective mitochondrial clearance was upregulated. These results are shown in Fig. 5. Two hours of H\(_2\)O\(_2\) exposure significantly increased Park2 expression (\( P < 0.01 \) at 0.4 mM H\(_2\)O\(_2\); Fig. 5A), indicating effective tagging of defective/damaged mitochondria for autophagic clearance. Simultaneously, expression of the adapter protein p62 was significantly downregulated (\( P < 0.05 \) at 0.4 mM H\(_2\)O\(_2\); Fig. 5B), and a significant increase was seen in LC3β protein levels (\( P < 0.01 \) at 0.6 mM H\(_2\)O\(_2\); Fig. 5C), demonstrating early selective autophagy of mitochondria (mitophagy). Park2 expression was not significantly altered after extended (18 h) H\(_2\)O\(_2\) exposure (Fig. 5D), yet there was a significant increase in p62 and LC3β protein levels, indicating impaired p62 degradation with intracellular accumulation. Altered p62 expression, as seen by Western immunoblot analysis and assumed intracellular accumulation, was verified using confocal microscopy (Fig. 5, G–I). There was a decrease in the PPAR-γ coactivator and mitochondrial biogenesis marker PGC-1α after 2 h of H\(_2\)O\(_2\) exposure (\( P < 0.05 \) at 0.4 mM H\(_2\)O\(_2\); Fig. 6A) that corresponded with the changes in Park2, p62, and LC3β. The cumulative results demonstrate impaired PPAR-γ-dependent mitochondrial biogenesis.

PPAR-γ activation does not protect kidney proximal tubular cells from oxidative stress-induced damage. PPAR-γ expression and phosphorylation changes with selective PPAR-γ TZD agonists were tested to determine whether this protected cells...
from oxidative stress. The TZD agonists were delivered 2 h before H2O2 (0.6 mM) exposure and during the 2- and 18-h H2O2 treatment periods. Concentrations of the TZD agonists (rosiglitazone and troglitazone at 10 μM and pioglitazone at 1 μM) were chosen from our preliminary data (not shown) and were consistent with concentrations used in similar previous studies (6, 11, 22, 33, 34). Expression of p-PPAR-γ was calculated as a ratio of PPAR, and the fold change compared with control cells was then compared for each of the TZDs at 2 h (Fig. 7, A–C) and 18 h (Fig. 7, G–I). H2O2 exposure alone caused a significant increase in Ser 112 phosphorylation of PPAR-γ at both 2 and 18 h (P < 0.05, Fig. 7, A–C; and P < 0.001, Fig. 7, G–I). This occurred in conjunction with a significant decrease in cell viability compared with control cells at 2 and 18 h (P < 0.001, Fig. 7, D–F; and P < 0.001, Fig. 7, J–L). Cell viability was not improved above H2O2 levels when each of the PPAR-γ agonists was used in combination with H2O2 for 2 h (Fig. 7, D–F). Thus, these results demonstrate a lack of protection by TZDs against oxidative stress-induced loss of viability at a time when the p-PPAR-γ-to-PPAR ratio was significantly increased. After extended exposure to PPAR-γ agonists and H2O2 (18 h), cell viability levels remained significantly low (P < 0.001; Fig. 7, J–L), with the cell viability of the dual TZD and H2O2 treatment showing a further significant reduction with rosiglitazone and troglitazone (P < 0.05; Fig. 7, J and K). Expression of the p-PPAR-γ-to

Fig. 2. Peroxisome proliferator-activated receptor (PPAR)-γ and PPAR-α respond early (2 h) after oxidative stress. HK-2 cells were exposed to a range of H2O2 concentrations (0.2–1.0 mM) for 2 and 18 h. A: representative Western blots. There was a dose-dependent significant increase in PPAR-γ phosphorylation (phosphorylated (p-)PPAR-γ) after 2 h of H2O2 exposure that corresponded to a decrease in PPAR-γ expression. PPAR-α significantly increased in a dose-dependent manner after 2 h of H2O2 exposure. B: results for 18 h. H2O2 exposure for 18 h did not significantly alter PPAR-γ phosphorylation or PPAR-α expression. p-PPAR-γ and PPAR-γ are expressed as a ratio, and PPAR-α protein expression was normalized to GAPDH expression. Results are expressed as means ± SE. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with controls.

Fig. 3. Oxidative stress induces mitochondrial dysfunction. HK-2 cells were exposed to a range of H2O2 concentrations (0.2–0.6 mM) for 2 and 18 h. Dihydroethidium (DHE) and JC-1 fluorescence was quantified by fluorimetry at 18 h. A: DHE fluorescence significantly increased after 0.6 mM H2O2 exposure, demonstrating ROS generation at 18 h. B and C: JC-1 red fluorescence significantly decreased in a dose-dependent manner (B) while increasing in green fluorescence (C), indicating mitochondrial membrane depolarization at 18 h. D–G: transmission electron microscopy revealed elongated morphologically healthy mitochondria with cristae in untreated HK-2 cells (D and E) and segmented, swollen mitochondria (F) often with absent cristae (G) in 0.6 mM H2O2-treated HK-2 cells at 18 h (arrow heads). H–J: MitoTracker red CMXRos uptake was visualized with confocal microscopy and shown to decline after both 2 h (I) and 18 h (J) of 0.6 mM H2O2 exposure compared with untreated HK-2 cells (H). Results are expressed as means ± SE. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with controls.
PPAR-γ ratio remained significantly increased at 18 h for rosiglitazone combined with H₂O₂ (P < 0.001; Fig. 7G), whereas the ratio returned to that observed in untreated controls after troglitazone co-treatment (Fig. 7H) and was lower than untreated controls with pioglitazone co-treatment (P < 0.01; Fig. 7I). Extended exposure of TZD agonists for 18 h before H₂O₂ exposure also demonstrated no protection to cell viability (data not shown).

Fig. 4. Oxidative stress impaired cellular ATP production. HK-2 cells were exposed to a range of H₂O₂ concentrations (0.2–1.0 mM) for 2 and 18 h. An ATP assay was performed based on luminescence. ATP levels significantly decreased in a dose-dependent manner after H₂O₂ exposure for both 2 h (A) and 18 h (B). Results are expressed as means ± SE. ***P < 0.001 compared with controls.

Fig. 5. Oxidative stress promotes selective mitochondrial autophagy (mitophagy). HK-2 cells were exposed to a range of H₂O₂ concentrations (0.2–1.0 mM) for 2 and 18 h. Cells were lysed, and Western blot analysis was performed. Immunofluorescence of p62 was performed (anti-p62 is green fluorescence and nuclear 4',6-diamidino-2-phenylindole is blue). Protein levels of Parkin (Park2; A) and p62 (B) significantly increased and decreased, respectively, in a dose-dependent manner, whereas light chain (LC3)β (C) significantly increased after 2 h of H₂O₂ exposure. Park2 protein levels returned to control levels after 18 h of H₂O₂ (D), whereas p62 (E) and LC3β (F) expression significantly increased in a dose-dependent manner. Immunofluorescence revealed decreased cytoplasmic localisation of p62 after 2 h of H₂O₂ (H) compared with untreated HK-2 cells (G), which increased after 18 h of H₂O₂ (I) to show a punctate perinuclear cytoplasmic pattern (white arrowhead). Protein expression was normalized to GAPDH expression. Results are expressed as means ± SE. *P < 0.05, **P < 0.01, and ***P < 0.001, compared with controls.
DISCUSSION

Oxidative stress and mitochondrial dysfunction have been implicated in the pathogenesis of kidney disease of diverse causes, including diabetes and CKD, two diseases of increasing incidence (8, 15, 16). However, the molecular mechanisms are still unclear. Here, oxidative stress-induced PPAR-γ alterations were associated with mitochondrial destabilization and a failure to restore mitochondrial health due to defective activation of PPAR-γ-dependent biogenesis pathways. Furthermore, selective PPAR-γ activation enhanced oxidative stress-mediated tubular cell damage that was associated with PPAR-γ Ser112 phosphorylation. The results suggest that, despite positive benefits in other tissues, PPAR-γ activation may be detrimental to kidney PTE cells in the scenario of oxidative stress-induced injury.

Our results demonstrate that PPAR-γ is present in kidney PTE cells and is responsive early in oxidative stress-induced injury. The associated increase in Ser112-phosphorylation of PPAR-γ (p-PPAR-γ) was not beneficial to cellular outcome in these cells. The increase in the p-PPAR-γ-to-PPAR-γ ratio may be injurious to cells via the development of mitochondrial dysfunction. However, at 18 h of exposure to oxidative stress, the p-PPAR-γ-to-PPAR-γ ratio was not elevated, perhaps demonstrating that early stress-induced PPAR-γ dysregulation stimulates a series of persistent negative cellular events, primarily targeting mitochondrial homeostasis.

Oxidative stress-induced posttranslational modifications to PPAR-γ, including phosphorylation, have had little study in kidney PTE cells. In adipocytes, MAPK-induced phosphorylation of PPAR-γ at Ser112 was linked to decreased transcriptional activity of PPAR-γ by inhibition of ligand binding and cofactor recruitment (5, 23, 51). In addition, PPAR-γ phosphorylation at Ser87 by AMP kinase in baby hamster kidney cells repressed the transactivating function of the receptor (31). Our data, demonstrating a decrease in PPAR-γ transcriptional activity along with a failure to upregulate PGC-1α and mitochondrial biogenesis, at a time of increased Ser112 PPAR-γ phosphorylation in human kidney PTE cells, agree with those reports. The decrease in total functional PPAR-γ is likely due to phosphorylation. Similar results have been shown in kidney mesangial cells exposed to a high-glucose environment (45), a mechanism of kidney injury involving increased oxidative stress (40, 42).

PTE cells of the kidney have a high density of mitochondria (20). It is likely, therefore, that the relationship between PPAR-γ and mitochondrial function will be especially important. Yang and colleagues (48) have previously shown, in an in vivo rat model of aging, that PPAR-γ activation attenuates age-related mitochondrial injury in the kidney by maintaining the integrity of the mitochondrial membrane potential. However, these results represent an outcome from the heterogeneous renal cell population and not specifically mitochondria from PTE cells. The majority of in vitro studies that have demonstrated protective actions of PPAR-γ have done so in glomerular cell types, namely, podocytes and mesangial cells (26, 34, 49). Our results demonstrate oxidative stress-induced PTE cell mitochondrial dysfunction that is associated with a decreased p-PPAR-γ-to-PPAR-γ ratio. This alteration is persistent, indicating that PPAR-γ induces negative downstream effects. We hypothesized that PPAR-γ phosphorylation induced disruptions to the mitochondrial homeostatic machinery and was responsible for this persistent cumulative dysfunction.

Autophagy is a highly conserved protective response to eliminate damaged cellular components. Mitochondrial homeostasis relies on the degradation of defective mitochondria and upregulation of mitochondrial biogenesis to restore a healthy pool of functioning mitochondria (9). Our results demonstrate significant perturbation to these pathways during oxidative stress. The induction of selective mitophagy mediated by the Park2-p62-LC3β pathway is evident after the initial (2 h) response to oxidative stress. However, long-term (18 h) oxidative stress facilitates significant impairments to this system, as indicated by p62 aggregation. Park2 upregulation after 2 h of oxidative stress demonstrates labeling and translocation of the E3 ligase Parkin to depolarized mitochondria and the initiation of polyubiquination to signal p62 binding (13). p62 degradation has been shown to be a consistent marker of selective autophagy due to its clearance within the autophagosome (25, 28, 29, 43), and our results further confirm this with enhanced LC3β expression. Mitophagy has received little study in the kidney, with Parkin-mediated mitophagy previously shown to be dependent upon p62 in HeLa and neuroblastoma cell lines (13). Recently, Ishikawa and colleagues (24) used rat acute kidney injury models to demonstrate that increased mitophagy in the kidney was highly influenced by ROS. The results of the present investigation show an increase in p62 after 18 h of oxidative stress, demonstrating impaired selective turnover and accumulation of p62. A previous study (30) has demonstrated liver injury accompanied by the formation of p62-positive inclusions and that the removal of p62...
attenuated this injury. Impaired selective mitochondrial autophagy caused by persistent ROS production and ensuing oxidative damage results in p62 accumulation, which further contributes to cellular dysfunction. This result was compounded by the failure of PPAR-γ/H9253 to activate PGC-1α/H9251 to stimulate mitochondrial biogenesis and restore the cellular redox environment. This change would have provided a continuation of negatively regulating intracellular signaling, with persistent oxidative stress and reduced mitochondria-dependent ATP production. This demonstrates early, persistent, and indirect mitochondrial dysfunction resulting from a failure of PGC-1α activity after oxidative stress.

PPAR-γ agonist treatment has previously been shown to protect against mitochondrial dysfunction in other cell types, including neuronal cells (10, 46) and endothelial cells (11). In the kidney, disparate results have been previously described and largely depend on the cell type and insult used (26, 45, 46). Important to note is that PPAR-γ Ser112 phosphorylation has been shown to modify PPAR-γ activity rather than act as a marker of PPAR-γ activation. PPAR-γ agonists can inhibit
obesity-linked Ser\textsuperscript{273} phosphorylation of PPAR-\textgamma by cyclin-dependent kinase 5 in adipose tissue (7). Our results show an inability of rosiglitazone to inhibit PPAR-\textgamma Ser\textsuperscript{112} phosphorylation during oxidative stress, therefore preventing classical downstream actions of PPAR-\textgamma, primarily the upregulation of PGC-1\textalpha and maintenance of mitochondrial homeostasis (21, 39). This finding appears to be unique to kidney tubular cells. Of note is that PPAR-\alpha is an alternate inducer of PGC-1\textalpha-dependent mitochondrial biogenesis (38, 44), and its down-regulation in response to oxidative stress further impairs the restoration of mitochondrial homeostasis. The inability of rosiglitazone and troglitazone to decrease PTE cell viability after extended oxidative stress is a novel finding and may result from the inability to prevent PPAR-\gamma phosphorylation, not only at Ser\textsuperscript{112} but on other sites of phosphorylation, including Ser\textsuperscript{273} and Ser\textsuperscript{382}, that were not studied here.

In summary, our findings describe oxidative stress-induced PPAR-\gamma alterations and resulting mitochondrial destabilization and a failure to restore mitochondrial health due to defective activation of PPAR-\gamma-dependent biogenesis pathways. These changes may contribute to the pathogenesis of diverse kidney disease, including CKD. A novel finding is that activation of PPAR-\gamma by TZD enhances oxidative stress-mediated tubular cell damage that is associated with PPAR-\gamma Ser\textsuperscript{112} phosphorylation. This may account for the tubular damage and a lack of therapeutic benefit of TZDs seen in some human trials.

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AUTHOR CONTRIBUTIONS

Author contributions: D.M.S., J.S.C., and G.C.G. conception and design of experiments; D.M.S. prepared figures; D.M.S. drafted manuscript; D.M.S., J.S.C., and N.C.B. performed experiments; D.M.S. and G.C.G. performed transmission electron microscopy.

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