PGC-1α attenuates hydrogen peroxide-induced apoptotic cell death by upregulating Nrf-2 via GSK3β inactivation mediated by activated p38 in HK-2 Cells

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Ischemia/reperfusion injury triggers acute kidney injury (AKI) by aggravating oxidative stress mediated mitochondria dysfunction. The peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α) is a master player that regulates mitochondrial biogenesis and the antioxidant response. We postulated that PGC-1α functions as cytoprotective effector in renal cells and that its regulation mechanism is coordinated by nuclear factor erythroid 2-related factor 2 (Nrf-2). In this study, to understand the effect and molecular mechanisms of PGC-1α, we developed an empty vector or PGC-1α-overexpressing stable cell lines in HK-2 cells (Mock or PGC-1α stable cells). PGC-1α overexpression increased the viability of cells affected by H2O2 mediated injury, protected against H2O2-mediated apoptotic events and inhibited reactive oxygen species accumulation in the cytosol and mitochondria as compared to that in Mock cells. The cytoprotective effect of PGC-1α was related to Nrf-2 upregulation, which was counteracted by Nrf-2-specific knockdown. Using inhibitor of p38, we found that regulation of the p38/glycogen synthase kinase 3β (GSK3β)/Nrf-2 axis was involved in the protective effects of PGC-1α. Taken together, we suggest that PGC-1α protects human renal tubule cells from H2O2-mediated apoptotic injury by upregulating Nrf-2 via GSK3β inactivation mediated by activated p38.
injury is controversial. Some studies have shown that a PPARγ-dependent down-regulation of PGC-1α promotes cancer growth and progressions in several cancers. Further, transient expression of PGC-1α in mouse cardiac-derived H9c2 cells causes cell death after ischemia-reoxygenation injury. Although mitochondria dysfunction is a major characteristic of a diverse range of diseases, cell fate is differently determined by altered gene expression patterns in a cell type- or tissue type-specific manner.

Nuclear factor erythroid 2-related factor 2 (Nrf-2; NFE2L2) plays a central role not only in overall cellular redox homeostasis by regulating the coordinated induction of cytoprotective genes but also in enhancing the structural and functional integrity of mitochondria under stress conditions via relationship with various proteins. Bardoxolone methyl, a first-in-class oral Nrf-2 agonist, has been shown to improve kidney function in diabetic nephropathy patients with transcriptional expression of network genes (like as PGC-1α, nuclear respiratory factor-1) that are linked with mitochondrial function. Further, Nrf-2 null mice were found to be markedly sensitized to cisplatin-induced AKI. It could be assumed that PGC-1α and Nrf-2 may be part of the same signaling pathway involved in the maintenance of both cellular redox homeostasis and mitochondrial homeostasis. Recently, some studies reported that PGC-1α is involved in the regulation of the Nrf-2 expression and activity, and direct interaction between PGC-1α and Nrf-2 was also identified by protein-protein interaction. However, the molecular mechanisms that interconnect the functional relation between PGC-1α and Nrf-2 are not still completely understood.

In this study, we evaluated whether the expression of PGC-1α is involved in I/R induced kidney injury and H2O2-treated human proximal epithelial tubule (HK-2) cells. We also investigated whether PGC-1α overexpression has a beneficial or maladaptive effect against H2O2-mediated apoptosis and ROS accumulation by using stable PGC-1α-overexpressing HK-2 cells. We analyzed whether the PGC-1α/Nrf-2 has a cytoprotective effect on H2O2-treated HK-2 cells, including Nrf-2 mediated antioxidant response element (ARE) activation, reduction of apoptotic signal activation, and ROS accumulation. We hypothesized that activation of p38 and sequential inactivation of glycogen synthase kinase 3 (GSK3β), which is mediated by PGC-1α overexpression, would be one of molecular mechanisms for effective PGC-1α/Nrf-2 axis. Therefore, we assumed that PGC-1α-dependent Nrf-2 upregulation may be a crucial part for the protective effect against H2O2-mediated apoptosis in HK-2 cells.

Results

PGC-1α was downregulated in the I/R-injured kidney, as well as in H2O2-treated HK-2 cells. To investigate the involvement of PGC-1α in I/R-induced AKI, we analyzed the PGC-1α expression pattern in I/R induced mouse model. Groups that were subjected to renal I/R (n = 4) showed marked deterioration of renal functional parameters along with significant increase in the plasma creatinine level (sCr) and blood urea nitrogen (BUN), as compared to the finding for the controls (n = 4; Fig. 1A). We then performed a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay to determine the degree of renal tubular apoptosis in I/R-injured kidney. We found an increased number of tubular epithelial cells with TUNEL-positive nuclei in I/R-injured kidney (Fig. 1B). The levels of apoptotic proteins, for example, the Bax to Bcl2 ratio and the cleaved caspase 3 to caspase 3 ratio, also increased in the I/R group (Fig. 1C). The mRNA level and protein level of PGC-1α were lower in the I/R-injured kidney group as compared to those in the control group (Fig. 1D,E). We assessed the effect of PGC-1α in HK-2 cells under oxidative stress condition. To mimic I/R stress in HK-2 cells, we treated them with H2O2, which is the main culprit in the pathogenesis of I/R injury. The PGC-1α expression level in H2O2-treated HK-2 cells was gradually decreased under condition of concentrations (0.5 mM) and duration (3 h) of treatment with H2O2 (Fig. 2A,B). We further assessed whether H2O2-induced PGC-1α down-regulation was dependent on ROS level by H2O2 treatment and, if so, whether PGC-1α downregulation could be inhibited by removing ROS using the well-known chemical antioxidant N-acetyl-L-cysteine (NAC). The level of PGC-1α expression, which was downregulated by H2O2, was restored by 20 mM of NAC (Fig. 2C).

PGC-1α overexpression protected cells from H2O2-mediated injury. To examine the physiological effect of PGC-1α in proximal tubule cells, we stably transfected HK-2 cells with an empty vector (Mock) or a plasmid encoding human PGC-1α (PGC-1α) (Fig. 3A). Expression of c-terminal c-Myc tagged PGC-1α was assessed with anti-c-Myc antibody. Stable cells clone were selected via confirmation of expression of zeo-lysinate (Fig. 4A). The phosphorylation of p53 at Ser 15, which is involved in stabilization and mitochondrial accumulation of p53, were lesser in H2O2-treated PGC-1α stable cells than in Mock cells (Fig. 4A,C). Further, the level of activated caspase 3, assessed using the cleaved caspase 3 to caspase 3 ratio, markedly reduced in H2O2-treated PGC-1α stable cells (Fig. 4A,D). Release of mitochondrial cytochrome C to the cytosol, which resulted in activation of caspase 3, was also lesser in H2O2-treated PGC-1α stable cells than in Mock cells (Fig. 4A,E).

PGC-1α had an anti-apoptotic effect. To verify the protective effects of PGC-1α in injured proximal tubule cells, the expression and activation of pro-apoptotic proteins was evaluated in H2O2-treated Mock and PGC-1α stable cells (Fig. 4). Although PGC-1α expression was lower in H2O2-treated PGC-1α stable cells than in H2O2-untreated PGC-1α stable cells, PGC-1α expression was higher in PGC-1α stable cells than in Mock cells (Fig. 4A). The phosphorylation of p53 at Ser 15, which is involved in stabilization and mitochondrial accumulation of p53, were lesser in H2O2-treated PGC-1α stable cells than in Mock cells (Fig. 4A,C). Further, the level of activated caspase 3, assessed using the cleaved caspase 3 to caspase 3 ratio, markedly reduced in H2O2-treated PGC-1α stable cells (Fig. 4A,D). Release of mitochondrial cytochrome C to the cytosol, which resulted in activation of caspase 3, was also lesser in H2O2-treated PGC-1α stable cells than in Mock cells (Fig. 4A,E).

PGC-1α had an anti-oxidative effect. Many earlier studies showed that PGC-1α suppresses ROS production through the induction of ROS-detoxifying enzymes. Therefore, we confirmed whether intracellular ROS in cytosol or in mitochondria could be regulated by PGC-1α overexpression in our system using a selective...
ROS probe, CM-H$_2$DCF-DA or MitoTracker Red CM-H$_2$XRos, respectively. In PGC-1α stable cells, ROS levels were noticeably lower in the mitochondria as well as the cytosol, as compared to levels in Mock cells (Fig. 5).

Upregulation of Nrf-2 expression by PGC-1α was involved in the cytoprotective effects of PGC-1α. To further analyze the protective mechanism of the PGC-1α-associated anti-apoptotic effect and anti-oxidative effect, we studied the expression of Nrf-2, which is involved in the coordinated induction of genes that encode many stress-responsive and cytoprotective enzymes and related proteins. The Nrf-2 mRNA levels increased in H$_2$O$_2$-treated PGC-1α cells (Fig. 6A,B). The Nrf-2 protein level also increased following PGC-1α overexpression both in the cytosol and the nucleus (Fig. 6C). Moreover, the expression of heme oxygenase-1 (HO-1), known as a downstream target molecules of Nrf-2, also increased in consistent to Nrf-2 upregulation in PGC-1α stable cells, its expression in PGC-1α stable cells decreased on Nrf-2 specific siRNA knockdown in PGC-1α stable cells (Fig. 6D).

We examined whether the anti-apoptotic and anti-oxidative effects on H$_2$O$_2$-treated PGC-1α cells were dependent on the Nrf-2 level. The level of activated caspase 3, which was reduced by PGC-1α overexpression,
was partly restored by Nrf-2-specific reduction on H$_2$O$_2$-treated PGC-1$\alpha$ cells (Fig. 7A). In addition, the ROS level, which was reduced by PGC-1$\alpha$ overexpression, was also partly recovered by Nrf-2-specific reduction in H$_2$O$_2$-treated PGC-1$\alpha$ cells (Fig. 7B).

Regulation of the p38/GSK3$\beta$/Nrf-2 axis by PGC-1$\alpha$ was involved in the cytoprotective effects of PGC-1$\alpha$. Three ubiquitin ligase systems that act as negative regulator of Nrf-2 have been reported, those are, the Keap1-, GSK3$\beta$-, and Hrd1-mediated systems$^{24,33-35}$. Nrf-2 binds with these negative regulators, and hence maintains Nrf-2 to basal level. Keap1 and GSK3$\beta$ are present in cytoplasm, and Hrd1 in the endoplasmic reticulum (ER). We analyzed whether the molecular mechanisms underlying Nrf-2 upregulation in PGC-1$\alpha$ cells are Keap1-dependent or GSK3$\beta$-dependent or Hrd1-dependent. In cytosolic negative regulators of Nrf-2, The phosphor form of GSK3$\beta$ at Ser9 was considerably increased in H$_2$O$_2$ treated PGC-1$\alpha$ cells at 1, 3, and 6 h as compared to the levels in Mock cells, whereas the Keap1 levels did not show a significant difference between the two groups (Fig. 8A). Hrd1, as a negative regulator of Nrf-2 in the endoplasmic reticulum, was also reduced in H$_2$O$_2$ treated PGC-1$\alpha$ cells as compared to the levels in Mock cells, although the difference in Hrd1 was lesser than that of GSK3$\beta$.

We investigated whether any of the other upstream molecules were involved in the GSK3$\beta$-inactivated Nrf-2 upregulation. Interestingly, p38, an upstream signaling molecule affected by GSK3$\beta$ inactivation$^{36}$, was specifically...
activated in H$_2$O$_2$-treated PGC-1$\alpha$ cells (Fig. 8B). p38 inactivation by treatment with a p38 inhibitor (SB203580, 5$\mu$M) in H$_2$O$_2$-treated PGC-1$\alpha$ stable cells led to decreases in GSK3$\beta$ inactivation, followed by decrease in Nrf2/HO-1 expression (Fig. 8C) and cell viability (Fig. 8D). In contrast, use of an ERK inhibitor (PD98059, 50$\mu$M), used as a non-effective control on PGC-1$\alpha$ effect, did not lead to significant difference.

**Discussion**

This study showed that PGC-1$\alpha$ is physiologically involved for the cytoprotective effects and one of its regulation mechanisms is the regulation of the p38/GSK3$\beta$/Nrf-2 axis by PGC-1$\alpha$ overexpression.

In the current study, we found decreased PGC-1$\alpha$ expression in I/R-induced AKI, which is associated with impaired renal function. The S3 segment of the proximal tubule and the thick ascending limb of Henle are highly susceptible to AKI, such as ischemic injury$^{37}$. Moreover, an earlier study involving *in situ* hybridization for PGC-1$\alpha$ mRNA showed that PGC-1$\alpha$ is mainly expressed in proximal tubules and the thick ascending limb of Henle$^{38}$. In addition, the PGC-1$\alpha$ protein level in H$_2$O$_2$-treated HK-2 cells was gradually decreased at high H$_2$O$_2$ concentrations or following longer exposures to H$_2$O$_2$. These findings are consistent with previous observations$^{39,39}$. And also, H$_2$O$_2$-induced PGC-1$\alpha$ downregulation was inhibited by NAC pre-treatment in H$_2$O$_2$-treated HK-2 cells. It has been recently reported that NAC plays a role as a mitochondrial enhancer as well as an antioxidant precursor.
to glutathione (GSH)\textsuperscript{40}. In psychiatry and related neurodegenerative diseases, NAC used to increase mitochondrial resilience and prevent allostatic load by inhibiting mechanism of oxidative stress and inflammation\textsuperscript{41, 42}. Given the prominent role of PGC-1\textalpha in mitochondrial biology, it is not surprising that PGC-1\textalpha is involved in the cellular response to ischemia. These findings suggest that PGC-1\textalpha could be a potential target to improve renal recovery following I/R-induced kidney injury. In stable cells, PGC-1\textalpha overexpression attenuated H\textsubscript{2}O\textsubscript{2}-induced cellular toxicity via anti-apoptotic and anti-oxidative effects. Mitochondria are the central executive of apoptosis\textsuperscript{43}, and ROS generation has been suggested to be a major inducer of mitochondrial dysfunction and to play an important role in apoptosis regulation\textsuperscript{44}. Our results suggest that a defect in PGC-1\textalpha is one of the major causes of H\textsubscript{2}O\textsubscript{2}-induced renal tubule cell apoptosis, and provides a novel strategy for preventing ROS-induced kidney tubule injury.

Nrf-2 serves as a master player of mitochondrial redox homeostasis by regulating the expression of diverse cytoprotective proteins that allow for cellular adaptation and survival under stress conditions\textsuperscript{45, 46}. The findings of the current study suggest that the PGC-1\textalpha upregulated Nrf-2 expression and sequentially induced phase 2 detoxifying enzymes and related proteins, such as HO-1, leading to a cytoprotective effect against ROS-mediated injury. Consistent with our data, it has recently been reported that Nrf-2 knockout cells have higher mitochondrial ROS levels than wild-type cells, suggesting that Nrf-2 regulates mitochondrial ROS production\textsuperscript{47}. In addition, HO-1-knockout mice were found to be markedly sensitized to diverse forms of AKI\textsuperscript{48}. In this study, we didn't check whether or not Nrf-2 mediated HO-1 expression in PGC-1\textalpha stable cells is specific in mitochondria. The several papers were reported about its mitochondrial function\textsuperscript{49}. HO-1 overexpression appears to protect the heart from oxidative injury by regulating mitochondrial quality control\textsuperscript{50}. In addition to HO-1, it has been reported that PGC-1\textalpha regulates the mitochondrial antioxidants, such as MnSOD, Prx5, and Prx3 in vascular endothelial cells\textsuperscript{51}. In agreement with previous data, we also identified upregulation of their expression in PGC-1\textalpha stable cells (data not shown). But, whether upregulation of their expression in PGC-1\textalpha stable cells was dependent on the expression of Nrf-2 would be further elucidated.

In this study, we first demonstrated that regulation of the p38/GSK3\textbeta/Nrf-2 axis by PGC-1\textalpha is one of mechanisms protecting renal tubule cells against ROS-mediated cellular toxicity. Under basal condition, Keap1 is inactivated by oxidation of the reactive cysteine residue or down-regulated by epigenetic silencing\textsuperscript{52}; then, GSK3\textbeta is inactivated by Ser9 phosphorylation. Nrf-2/Keap1 or Nrf-2/GSK3\textbeta complex is disrupted by conformational change. Consequently, Nrf-2 stabilizes and is then translocated to the nucleus for Nrf-2...
mediated gene expression. Among cytosolic negative regulators of Nrf-2, we showed that GSK3β, in particular, changed to the inactive form on phosphorylation at Ser9 in PGC-1α cells at the increased time point of H2O2 treatment, while Keap1 did not. Consistent with our data, mice with renal-proximal-tubule-specific GSK-3β knockout and chemical inhibition of GSK3 were found to have better survival and renal function than wild-type mice in a previous study. In our study, the protein expression of Nrf-2 was increased both in cytosol and in nucleus by the overexpression of PGC-1α. The degree of expression was greater in cytosolic fractionation than in nuclear fraction. It is speculated that Nrf-2 is more regulated by cytosolic events, such as inactivation of GSK3β. However, detailed mechanisms of Hrd1-dependent Nrf-2 upregulation would be further elucidated. Furthermore, our data showed that p38 was specifically activated in PGC-1α stable cells and that p38-specific inactivation in PGC-1α cells inversely affected the sequential activation and expression of GSK3β/Nrf-2/HO-1 as its downstream targets. 15d-PGJ(2), a potent endogenous ligand for peroxisome proliferators-activated receptor gamma, induces HO-1 expression using p38 MAP kinase and Nrf-2 pathway in ROS-mediated VSMCs; an inhibitor of p38 MAP kinase was found to abolish 15d-PGJ(2)-induced HO-1 expression. Although we didn't check the regulation mechanism of p38/GSK3β/Nrf-2 axis by PGC-1α in I/R-injured kidney, several studies reported that the reduction of Nrf-2 and its downstream molecule, HO-1, in I/R-injured kidney resulted in I/R-induced ROS generation and inflammation, followed by acute kidney injury. Renal ischemia-reperfusion injury (IRI) is a major cause of AKI, which has common pathophysiological features including renal tubular apoptosis/necrosis, ROS generation, mitochondria dysfunction and inflammatory cell infiltration. Therefore, it is reasonable to explore and to understand the therapeutic mechanism to treat common pathophysiology features of IRI. Our studies imply that PGC-1α may be one of therapeutic targets against AKI.

Figure 5. Reduction of intracellular ROS by PGC-1α. To check the efficiency of PGC-1α overexpression in reducing ROS, stable cells were plated onto four well-cell culture slides (5 × 10⁴/well) and treated with 0.5 mM H2O2 for 30 min. Cytosolic (magnification at ×200, Bar = 100 μm) (A) and mitochondrial ROS (magnification at ×400, Bar = 50 μm) (B) were labeled using CM-H2DCFDA or CM-H2XROS probes, respectively. Images were immediately visualized using confocal microscopy on a laser scanning microscope (LSM 510; Carl Zeiss), and analyzed using a LMS 5 browser imaging software. Error bars denote the mean ± S.D. of triplicate samples. *p < 0.05 H2O2-untreated vs. H2O2-treated in Mock; #p < 0.05; H2O2-treated Mock vs. PGC-1α.
In conclusion, PGC-1α protects human renal tubule cells from H₂O₂-mediated apoptotic injury by upregulating Nrf-2 via GSK3β inactivation mediated by activated p38. Regulation of the p38/GSK3β/Nrf-2 axis by PGC-1α could be a viable target for ameliorating mitochondrial dysfunction following AKI.

**Materials and Methods**

**Materials.** The selective antibiotics, zeocin was purchased from Invitrogen (CA, USA). 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) and MitoTracker were purchased from Molecular Probes (Invitrogen, CA, USA). Antibodies against PGC-1α, Keap1, and Nrf-2, were purchased from Santa Cruz (Dallas, Texas, USA). Antibody against Hrd1 was purchased from Novus Biologicals (Littleton, CO, USA). Antibodies against caspase 3, cleaved caspase 3, Bax, Bcl2, phosphor-p53, total-p53, phosphor-GSK3β, total-GSK3β, phosphor-p38, phosphor-ERK1/2, phosphor-JNK, total-p38, total-ERK1/2, total-JNK, HO-1 and c-myc were all purchased from Cell Signaling Technology (Danvers, MA, USA). SB203580 and PD98059 were purchased from Calbiochem (Cat#559398 for SB203580 and Cat#513001 for PD98059, Darmstadt, Germany). N-acetyl-L-cysteine (NAC, Cat#A7250) and Thiazolyl Blue Tetrazolium (Cat#M2128) for MTT assay was purchased from sigma-aldrich. For over-expression of human PGC-1α in HK-2 cells, human PGC-1α/pCDNA4
plasmid was purchased from Addgene (Cat#10974, Cambridge, USA). Nrf-2 specific siRNA (Cat# sc-37030) and control siRNA (Cat# sc-37007) were purchased from Santa Cruz (Dallas, Texas, USA). DhamaFECT 1 Transfection reagent was purchased from GE Healthcare (Cat# T-2001-02, USA).

Animals. All methods were carried out in accordance with relevant guidelines and regulations. All experimental protocols were approved by the Animal Care Regulations (ACR) Committee of Chonnam National University Medical School (CNU IACUC-H-2016-26). Eight-week-old male C57BL6 mice were purchased by Samtako (Korea). Mice were anesthetized with 2% isofurane and 100% oxygen and placed on a temperature-regulated table (38.5 °C) to maintain body temperature. Renal ischemia was induced by clamping both renal pedicles with micro clamp (ROBOZ, Gaithersburg, USA) for 30 min. I/R group (n = 4) was sacrificed after 1 day of reperfusion. Control group (n = 4) underwent the same procedure, except that the clamp was not applied. Blood samples were then collected from the heart, and the left kidney was rapidly removed and processed for western blotting or fixed in 4% paraformaldehyde solution for immunohistochemistry (IHC). The right kidney was frozen at −80 °C for real-time PCR.

Cell culture. HK-2 cells (ATCC, Manassas, VA), were cultured in complete DMEM-F12 media (WelGene, Daegu, Korea) supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin at 37°C under a temperature-regulated table (38.5 °C) to maintain body temperature. Renal ischemia was induced by clamping both renal pedicles with micro clamp (ROBOZ, Gaithersburg, USA) for 30 min. I/R group (n = 4) was sacrificed after 1 day of reperfusion. Control group (n = 4) underwent the same procedure, except that the clamp was not applied. Blood samples were then collected from the heart, and the left kidney was rapidly removed and processed for western blotting or fixed in 4% paraformaldehyde solution for immunohistochemistry (IHC). The right kidney was frozen at −80 °C for real-time PCR.

Stable cell lines. HK-2 cells were then transfected with 2 μg of empty vector (Mock) or PGC-1α DNA using 6 μl of Fusion HD reagent (Promega, Madison, WI, USA) in antibiotic-free DMEM-F12. Beginning 1 day after transfection, transfectants were selected in DMEM-F12 containing 200 μg/ml zeocin, which was refreshed every 3 days for 2 weeks. Colonies surviving in the selection medium were collected and sequentially plated in 48, 12, 6-well plates, and then 60 and 100 mm dishes. Cells stably overexpressing human PGC-1α were identified by immunoblotting with anti c-myc and anti β-actin antibodies or by PCR analysis using zeocin primers.

Figure 7. Nrf-2 specific-protective effects by PGC-1α. To prove the dependence of Nrf-2 in anti-apoptotic and anti-oxidative effect of PGC-1α, the level of activated caspase 3. Full-length blots of each tested protein are reported in Supplementary Figure 56 (A) and the level of DCF fluorescence (B) were assessed in Nrf-2 suppressed PGC-1α cells under H2O2 treatment, as earlier mentioned. Magnification at x200, Bar = 100 μm. Relative protein level and ROS level were expressed as fold normalized to the untreated Mock cells. β-actin levels were analyzed as internal controls. Error bars denote the mean ± S.D. of triplicate samples. *p < 0.05 H2O2-untreated Mock vs. PGC-1α; †p < 0.05 H2O2-untreated vs. H2O2-treated Mock; ‡p < 0.05; H2O2-treated Mock vs. PGC-1α; §p < 0.05; H2O2-treated siCon vs. siNrf-2 in PGC-1α stable cells; †p < 0.05; H2O2-untreated siCon vs. siNrf-2 in PGC-1α cells.
Figure 8. The involvement of p38/GSK3β/Nrf-2 axis for cytoprotective effects of PGC-1α. To understand the molecular mechanism for cytoprotective effects of PGC-1α, the activation of Keap1-, GSK3β- or Hrd1 (A) and MAPKs (B), as an upstream signal molecules of Nrf-2, were compared to Mock and PGC-1α cells for indicated time points after H2O2 treatment. To further elucidate the specificity of p38-mediated signal cascade for cytoprotective effects of PGC-1α cells, p38 specific effects were analyzed by western blotting (C) and MTT assay (D) in PGC-1α cells treated with H2O2 in the presence or absence of p38 inhibitor (SB203580) for 2 h or 4 h, respectively. Inhibitor of ERK1/2 (PD98059) was used as non-effective control on PGC-1α effect. Activation of p38 or inactivation of GSK3β was checked with phosphor specific antibody at Thr180/Tyr182 residue or at Ser9 residue, respectively. Full-length blots of each tested protein are reported in Supplementary Figure S7. Error bars denote the mean ± S.D. of triplicate samples. MTT assay was performed to n = 7. *p < 0.05 H2O2-untreated Mock vs. PGC-1α at indicated time points; †p < 0.05; p38 inhibitor treated vs. untreated; †p < 0.05; ERK1/2 inhibitor treated vs. untreated.

5′-ATGGCCAAGTTGACCAGTGCCGTT-3′ (forward) and 5′-GTCCTGGTCCTCGGCCACGAAGTG-3′ (reverse)45. A loading control was analyzed by using GAPDH primers 5′-ACCACAGTCCATGCCATCAC-3′ (forward) and 5′-TCCACCACCTGTGTGTGT-3′ (reverse).
Tunel staining in kidney tissue. Apoptosis was determined using the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon International; Temecula, CA, USA) according to the manufacturer’s protocol. The sections were counterstained with hematoxylin and examined by light microscopy. Image was magnified at x100.

siRNA knockdown. RNA interference of Nrf-2 was performed using an Nrf-2-specific siRNA from Santa Cruz (Cat# sc-37030). Briefly, cells were transfected with indicated concentration of siRNA (30 nM and 50 nM) using DhamaFECT 1 transfection reagent according to the manufacturer’s protocol. Cells transfected with control siRNA (Santa Cruz, Cat# sc-37007) were used as controls for direct comparison.

RT-PCR & real-time PCR. To quantify mRNA levels, total RNA was extracted from frozen mouse kidney or HK-2 cells using TRIzol reagent (Invitrogen). cDNA was then reverse transcribed from 1 μg samples of total RNA using QuantiTect Reverse Transcription kit (Qiagen Science, Maryland, USA). Real-time PCR was performed using QuantiTect SYBR Green PCR master mix (Qiagen Science, Maryland, USA) and a Rotor-Gene TM 3000 Detector System (Corbett research, Mortlake, New South Wales, Australia). RT-PCR primer sequences were as follows: for mouse β-actin, 5′-ATATGCGTGCACGCTCGTC-3′ (F) and 5′-GATGGGCAACATGTGGGTTGA-3′ (R); for mouse PGC-1α, 5′-AATGCACGCGCTTCTAGACT-3′ (F) and 5′-TTCTGTGGGTTGTTGTTGA-3′ (R). The primer sequences used for real time-PCR were as follows: for human GAPDH, 5′-GACATAAGAAGGTGGTGAA-3′ (F) and 5′-TGTCACTACAGGAATGAGC-3′; for human PGC-1α, 5′-CTCTAGTACAGGACACCATGGA-3′ (F) and 5′-GCTCCATGATTCTCTAGTTCAAAC-3′ (R); for Nrf-2, 5′-GAGAGCGAGTCTCTATTGC-3′ (F) and 5′-TGCTCAATGCTCTGTGCTAT-3′ (R). Data from the reaction were collected and analyzed with the appropriate software package from Corbett Research.

Cell viability. The MTT assay was applied to test cell viability. In brief, stable HK-2 cells (Mock or PGC-1α) were seeded into plates at 1 × 10⁴ cells per 96 wells. After 1 day, cells were incubated in 100 μl of 0.5 mM H₂O₂ diluted in HBSS for the indicated time at 37 °C and with 5% CO₂. Subsequently, 10 μl of MTT reagent (5 mg/ml) was added to yield a final concentration of 0.5 mg/ml. After 2 h of incubation, all solution was removed, and 100 μl of DMSO was directly added to the cells to dissolve water-insoluble MTT-formazan. Absorption at 590 nm was determined with an ELISA reader (BioTek, Winooski, VT, USA).

Apoptosis assay. The number of apoptotic cells was quantified using the Ezway Annexin V-FITC Apoptosis Detection kit (KOMA BIOTECH, Seoul, Korea) according to the manufacturer’s protocol. Cells were sequentially probed with Annexin V-FITC and propidium iodide (PI) dye. Fluorescent intensity was measured by a FACSCalibur™ flow cytometry (BD Biosciences, San Jones, CA, USA).

DAPI staining for apoptosis analysis. The apoptotic effect was analyzed by using fluorescent nuclear dye 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). The cells were seeded onto four well-cell culture slides (5 × 10⁴/well) and treated as mentioned previous. Cells were then washed with PBS and fixed in 4% paraformaldehyde for 10 min. Subsequently the cells were permeabilized with equilibration buffer (1% BSA and 0.5% Triton X-100 in PBS) and stained with DAPI dye. After staining, the images were captured using a confocal microscope (LSM 510; Carl Zeiss). Image was magnified at x800, Bar = 20 μm.

Measurement of ROS generation. Level of intracellular and mitochondrial ROS 58, 59 were assessed using 5,6-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA; Invitrogen, Carlsbad, CA, USA) or Rosamine-based MitoTracker probes (MitoTracker Red CM-H₂XROS, Invitrogen, Carlsbad, CA, USA), respectively. Labeling with both probes was conducted on live cells, but not fixed cells. After cells were treated with 0.5 mM H₂O₂ for 30 min, and then were loaded with 10 μM CM-H₂DCFDA or 0.2 μM CM-H₂XROS for 30 min at 37 °C. Images were immediately visualized using confocal microscopy on a laser scanning microscope (LSM 510; Carl Zeiss), and analyzed using imageJ software. Image was magnified at x200 or x400.

Preparation of nuclear and cytoplasmic extracts. HK-2 cells were lysed using NE-PER® nuclear extraction reagent (NER) (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer’s protocol. Briefly, 100 μl of ice-cold cytoplasmic extraction reagent (CER) I was added to the harvested cells. After incubated on ice for 10 min, ice-cold CER II was added to the tube. The tube was centrifuged at 16,000 × g for 5 min and the supernatant fraction was saved as cytosolic protein. The remained pellet was suspended in 50 μl of ice-cold NER. After centrifuging the tube at 16,000 × g for 10 min, the supernatant (nuclear protein) fraction was transferred to a clean tube.

Statistical analysis. All experiments were conducted in triplicate. The results were expressed as mean ± standard deviation (S.D). We used Student’s t test for the comparison between two groups, and used One-way ANOVA when we compared more than two groups. Differences with values of p < 0.05 were considered significant.

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Author Contributions
Hoon-In Choi and Soo Wan Kim designed by the study and wrote the manuscript and revised manuscript. Hoon-In Choi, Hye-Jin Kim, and In-Jin Kim performed the study. Jung-Sun Park, Eun Hui Bae, Seong Kwon Ma helped revise the manuscript. All authors reviewed the manuscript.

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