Mitochondrial dysfunction is a common consequence of ischemia-reperfusion and drug injuries. For example, sublethal injury of renal proximal tubular cells (RPTCs) with the model oxidant tert-butylhydroperoxide (TBHP) causes mitochondrial injury that recovers over the course of six days. Although regeneration of mitochondrial function is integral to cell repair and function, the signaling pathway of mitochondrial biogenesis following oxidant injury has not been examined. A 10-fold overexpression of the mitochondrial biogenesis regulator PPAR-γ cofactor-1α (PGC-1α) in control RPTCs resulted in a 52% increase in mitochondrial number, a 27% increase in respiratory capacity, and a 30% increase in mitochondrial protein markers, demonstrating that PGC-1α mediates mitochondrial biogenesis in RPTCs. RPTCs sublethally injured with TBHP exhibited a 50% decrease in mitochondrial function and increased mitochondrial autophagy. Compared with the controls, PGC-1α levels increased 12-fold on days 1, 2, and 3 post-injury and returned to base line on day 4 as mitochondrial function returned. Inhibition p38 MAPK blocked the up-regulation of PGC-1α following oxidant injury, whereas inhibition of calcium-calmodulin-dependent protein kinase, calcineurin A, nitric-oxide synthase, and phosphoinositol 3-kinase had no effect. The epidermal growth factor receptor (EGFR) was activated following TBHP exposure, and the EGFR inhibitor AG1478 blocked the up-regulation of PGC-1α. Additional inhibitor studies revealed that the sequential activation of Src, p38 MAPK, EGFR, and p38 MAPK regulate the expression of PGC-1α following oxidant injury. In contrast, although Akt was activated following oxidant injury, it did not play a role in PGC-1α expression. We suggest that mitochondrial biogenesis following oxidant injury is mediated by p38 and EGFR activation of PGC-1α.

Peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) is a 92-kDa protein that was first identified as a regulator of adaptive thermogenesis in brown adipose tissue (1). PGC-1α is highly expressed in tissues with high metabolic demands, such as heart, skeletal muscle, and kidney (1). Ectopic expression of PGC-1α was later shown to stimulate the biogenesis of mitochondria by increasing the expression of nuclear respiratory factors 1 and 2 and enhancing the transcriptional activity of nuclear respiratory factor 1 on the promoter for mitochondrial transcription factor A (1). In addition to cold exposure, expression of PGC-1α is responsive to various physiological stimuli, such as exercise, caloric restriction, and exposure to lipopolysaccharide (1–4), demonstrating the ability of PGC-1α to alter the metabolic state of the cell in response to changes in the cellular or extracellular environment. What has not been examined is the signaling pathway responsible for mitochondrial biogenesis following cellular and mitochondrial injury, particularly oxidative stress.

A variety of signaling mechanisms have been proposed to regulate the expression of PGC-1α and mitochondrial biogenesis including nitric oxide-soluble guanylate cyclase (5–12), β-adrenergic/cAMP (13), calcineurin A, calcium/calmodulin-dependent protein kinase (CaMK) (14, 15), and AMP kinase (15). p38 MAPK is also thought to regulate PGC-1α through phosphorylation of Thr16, Ser265, and Thr298 within the repressor domain of PGC-1α (1, 16). Phosphorylation of these residues displaces a p160 myb-binding protein, stabilizing the PGC-1α protein and increasing its ability to function as a coactivator (17).

Ischemia-reperfusion injury in the kidney and other organs is characterized by de-energization of mitochondria resulting in severe energy deficits within the proximal tubule, which present within 60 min of the onset of ischemia (18–20). Further, a large number of nephrotoxic xenobiotics target the mitochondria (21). These changes in renal proximal tubular cell (RPTC) energetics also are observed in cellular models subjected to diverse stresses (22–26). For example, sublethally injured RPTCs exhibit mitochondrial dysfunction, such as decreases in oxygen consumption and ATP levels, following exposure to the model oxidant tert-butylhydroperoxide that spontaneously recovering over the course of six days (27, 28). The recovery of mitochondrial function is temporally related to RPTC de-differentiation, migration, and proliferation, and complete recovery of mitochondrial function is associated with RPTC redifferentiation (28, 29). The recovery of mitochondrial function is central to the overall restoration of cell structure and function. EGFR expression is up-regulated following renal ischemia-reperfusion injury (30, 31), and the EGFR is required for recovery of function following mercuric chloride-induced renal failure in mice (32). Further, the EGFR is an important mediator of RPTC dedifferentiation, migration, and proliferation following...
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diverse forms of injury (33, 34). For example, Src-dependent phosphorylation of p38 MAPK results in the transactivation of the EGFR following exposure to hydrogen peroxide (33), suggesting that p38 MAPK transactivation of the EGFR may be important in the recovery of RPTC function following oxidant injury.

Although the regenerative properties of the RPTC are known, the mechanisms of mitochondrial biogenesis following injury have not been examined nor has a central role of PGC-1α been determined in this process. In this study, we have elucidated a mechanism for mitochondrial biogenesis following oxidant injury.

EXPERIMENTAL PROCEDURES

Reagents—LY294002, AG1478, and SB203580 were obtained from Cell Signaling Technology (Beverly, MA), Biomol (Plymouth Meeting, PA), and Tocris (Ellisville, MO), respectively. Fenvalerate and BAY 41-2272 were purchased from Alexis Corporation (San Diego, CA). ODQ was purchased from Acros Organics (Geel, Belgium). All other chemicals were purchased from Sigma. Antibodies to phospho-EGFR (Tyr1068), phospho-Src (Tyr416), Src, phospho-p38, and p38 were obtained from Cell Signaling Technology. The antibodies to PGC-1α, COX III, and the EGFR were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-PGC-1α and anti-COX III was used at concentrations of 1:500 and 1:300, respectively, whereas all other antibodies were used at 1:1000.

Isolation and Culture of Renal Proximal Tubules—Female New Zealand White rabbits (2 kg) were purchased from Myrtle’s Rabbitry (Thompson Station, TN). RPTCs were isolated using the iron oxide perfusion method and grown in 35-mm tissue culture dishes under improved conditions as previously described (35). The culture medium was a 1:1 mixture of Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (without glucose, phenol red, or sodium pyruvate) supplemented with 15 mM HEPES buffer, 2.5 mM L-glutamine, 1 mM pyridoxine HCl, 15 mM sodium bicarbonate, and 6 mM lactate. Hydrocortisone (50 nM), selenium (5 ng/ml), human transferrin (5 μg/ml), bovine insulin (10 nM), and L-ascorbic acid-2-phosphate (50 μM) were added daily to fresh culture medium.

Confluent RPTCs were used for all experiments. RPTC monolayers were pretreated with various inhibitors for 1 h and exposed to 200 μM TBHP for 45 min, at which point the oxidant-containing media were removed, fresh media were added, and inhibitors replaced as indicated in the figure legends.

Replication-deficient Adenovirus Infection—Recombinant adenovirus-expressing PGC-1α and nuclear GFP control were generous gifts from Bruce Spiegelman (Harvard Medical School) (36). Viral titers were determined by plaque formation assay using HEK293 cells and expressed as plaque-forming units. RPTCs were infected with each virus at a multiplicity of infection of 30 plaque-forming units for 24 h at 37 °C in a humidified 5% CO2 incubator. Afterward, the cultures were placed in normal culture media for an additional 24 h, at which point all measurements were taken. At a multiplicity of infection of 30 plaque-forming units, 99% of the cells showed expression of the viral gene insert as indicated by flow cytometry (data not shown).

Basal Oxygen Consumption (QO2)—On days 1–6 after TBHP injury, RPTCs were gently detached from the culture dishes with a rubber policeman and transferred to a 37 °C O2 chamber. Basal and uncoupled (FCCP) RPTC QO2 was measured polarographically by using a Clark-type electrode as described previously (35).

Lipid-based Transfections—Transient transfections of RPTCs were performed using Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen).

Cell Number—Measurement of monolayer protein content over time was used to estimate cell number. On days 1–6 after TBHP injury, RPTC monolayers were washed with phosphate-buffered saline, solubilized in Triton buffer (0.05% Triton X-100, 100 mM Tris-base, 150 mM NaCl, pH 7.5), sonicated for 60 s, and protein concentrations determined by the bicinchoninic acid according to the manufacturer’s instructions (Pierce).

Confocal Microscopy—All microscopy was performed on a Zeiss LSM 510 confocal microscope. Cells for imaging were plated on 35-mm glass bottom 14-mm microwell Petri dishes from MatTek Corporation (Ashland, MA).

Preparation of Cell Lysates and Immunoblot Analysis—After treatment, RPTCs were washed twice with phosphate-buffered saline without Ca2+ and Mg2+ and harvested in cell lysis buffer from BioVision (Mountain View, CA). Cells were disrupted by sonication for 30 s. Equal amounts of cellular protein lysates were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. After treatment with 5% skim milk or bovine serum albumin at 4 °C overnight, membranes were incubated with various antibodies for 2 h and then incubated with an appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 h. Bound antibodies were visualized after chemiluminescence detection on an AlphaEaseFC stand alone imaging system (San Leandro, CA).

Statistical Analysis—Data are presented as means ± S.E. and were subjected to one- or two-way analysis of variance as appropriate. Multiple means were compared using Student-Newman-Keuls test. p < 0.05 was considered a statistically significant difference between mean values. Renal proximal tubules isolated from an individual rabbit represent a single experiment (n = 1). Experiments were performed with 2–5 plates of cells and repeated until an n value of at least 3 was reached.

RESULTS

Exposure of RPTC to the adenovirus that produced PGC-1α expression resulted in a 10-fold increase in PGC-1α protein expression at 48 h (Fig. 1A). The green fluorescent protein expressed on the C terminus of PGC-1α was localized to the RPTC nuclear compartment, consistent with its role as a nuclear receptor coactivator (Fig. 1B). The mitochondria of RPTC infected with the adenoviral PGC-1α and nuclear GFP controls were stained with the potentiometric dye MitoTracker red, and visualization of the mitochondria using confocal microscopy revealed an increase in red staining in RPTCs in the PGC-1α-treated group (Fig. 1B). Quantification of this change in staining intensity revealed a 51% increase in mitochondrial
number/mass in RPTCs overexpressing PGC-1α compared with GFP controls (Fig. 1C). These results reveal that PGC-1α increases mitochondrial biogenesis in confluent RPTCs under basal conditions.

To determine whether PGC-1α overexpression in RPTC results in changes in mitochondrial respiration, basal and uncoupled (FCCP) oxygen consumption (QO2) were measured. Increased PGC-1α resulted in a 32% increase in basal QO2 and a 26% increase in uncoupled QO2 compared with controls (Fig. 2), indicative of the biogenesis of functional mitochondria.

Examination of both nuclear and mitochondrial encoded protein constituents of the electron transport chain following adenovirus-mediated expression of PGC-1α revealed 2- and 1.5-fold increases of the nuclear encoded proteins ATP synthase β-subunit and cytochrome c and a 1.2-fold increase in COX III expression compared with controls (Fig. 3). These increases in mitochondrial number/mass, function, and protein expression demonstrate that PGC-1α is a primary regulator of mitochondrial biogenesis in the renal proximal tubule and a potential target for regulating cellular bioenergetics in the kidney following injury.

Control RPTCs transfected with a mitochondrially targeted GFP showed a normal branched morphology and did not associate with lysosomes stained with LysoTracker Red (Fig. 4). RPTCs affected by acute TBHP exposure (200 μM for 45 min) underwent significant mitochondrial autophagy 24 h following injury, as demonstrated by the colocalization of mitochondria and lysosomes (Fig. 4). These results are consistent with the loss of mitochondrial function triggering the selective autophagy of mitochondria (37, 38).

Although RPTCs expressed low levels of PGC-1α protein under basal conditions, following acute TBHP exposure, PGC-1α protein levels increased 12-fold 24 h after injury (Fig. 5). Levels of PGC-1α remained elevated for three days following injury and then returned to control levels four days after the oxidant insult. Basal QO2 increased on day 2 following oxidant exposure and returned to control levels on day 4 (Fig. 5). Uncoupled QO2 increased on day 3 and returned to control levels on day 4. Thus, following oxidant exposure, PGC-1α protein levels markedly increased within 24 h, were maintained while basal and uncoupled QO2 were increasing, and returned
to control levels when basal and uncoupled \( QO_2 \) returned to control levels.

To determine the signaling mechanisms responsible for the up-regulation of PGC-1\( \alpha \) following oxidant injury, RPTCs were pretreated with a variety of inhibitors of signaling molecules known to regulate PGC-1\( \alpha \), nitric-oxide synthase, soluble guanylyl cyclase, CaMK, calcineurin A, and p38 and exposed to the inhibitors for 24 h after injury. The nitric-oxide synthase inhibitor L-NAME (3 mM) had no effect on the expression of PGC-1\( \alpha \) after injury nor did treatment with the soluble guanylyl cyclase inhibitor ODQ (1 \( \mu M \)) or the soluble guanylyl cyclase agonist BAY41–2271 (1 \( \mu M \)), revealing that PGC-1\( \alpha \) is not being regulated by a nitric oxide-dependent pathway (Fig. 6). Treatment with inhibitors of CaMK (KN-62, 1 \( \mu M \)) and calcineurin A (fenvalerate, 1 \( \mu M \)) also had no effect on the expression of PGC-1\( \alpha \) compared with cells exposed to TBHP alone (Fig. 6). In contrast, inhibition of PGC-1\( \alpha \) expression following...
injury was observed with the addition of the p38 inhibitor SB203580 (10 μM) (Fig. 6).

We previously reported (33, 34) that p38 is activated in RPTCs in response to H2O2, and interacts with Src, phosphatidylinositol 3-kinase, and the EGFR. Similarly, exposure to TBHP also results in the phosphorylation of Src, p38, and the EGFR (Fig. 7). PP1 (10 μM) completely abolished the up-regulation of PGC-1α as did an inhibitor of the EGFR (AG1478, 20 μM) (Fig. 7). In contrast, inhibition of phosphatidylinositol 3-kinase with LY202190 (10 μM) had no effect on PGC-1α expression (Fig. 7).

Having established that Src, p38, and the EGFR play a role in PGC-1α expression following oxidant injury, we confirmed that these kinases are activated following oxidant exposure and sought to place these signaling molecules into a mitochondrial biogenesis pathway. Both Src and p38 were maximally phosphorylated within 5 min of TBHP exposure, whereas the EGFR was not maximally phosphorylated until 20 min after exposure to TBHP (Fig. 8).

RPTCs were preincubated with PP1, SB203580, or AG1478 and then exposed to TBHP or a vehicle control for 30 min, and samples were taken for immunoblot analysis. Pretreatment with PP1 prevented the phosphorylation of Src, p38 MAPK, and the EGFR, suggesting Src is upstream of p38 and the EGFR (Fig. 8). Pretreatment with SB203580 prevented the phosphorylation of p38 and the EGFR but not Src, suggesting that p38 is downstream of Src and is responsible for the transactivation of the EGFR (Fig. 8). Inhibition of EGFR activation with AG1478 prevented the phosphorylation of the EGFR but did not prevent the phosphorylation of either Src or p38, suggesting that the EGFR is indeed downstream of Src and p38 (Fig. 8) immediately following TBHP exposure.

Phosphorylation of both p38 and the EGFR is still present 60 min after TBHP exposure (Fig. 9). Pretreatment of RPTCs with SB203580 prevents the phosphorylation of p38 and the EGFR at 60 min; however, when SB203580 is added 30 min after exposure to TBHP, it no longer inhibits EGFR phosphorylation, suggesting that, although p38 is responsible for the initial transactivation of the EGFR, the sustained phosphorylation of the EGFR occurs through another mechanism (Fig. 9). As stated above, pretreatment with AG1478 does not inhibit p38 phosphorylation, placing p38 upstream of the EGFR during initiation of the signaling cascade (Fig. 9). Interestingly, when AG1478 is added after 30 min of TBHP exposure, p38 phosphorylation is abolished, suggesting that the EGFR mediates the late stage phosphorylation of p38 MAPK (Fig. 9).

FIGURE 6. Inhibition of p38 MAPK (but not nitric-oxide synthase), CaMK, or calcineurin A prevents up-regulation of PGC-1α following TBHP exposure. RPTCs were pretreated with various inhibitors for 1 h and then exposed to TBHP for 45 min, at which point the medium was changed and the inhibitors replaced. Immobots for PGC-1α were performed 24 h after exposure to TBHP. RPTCs were treated with L-NAME (A), ODQ (B), BAY-41-2272 (C), KN-62 (D), fenvalerate (E), or the p38 MAPK inhibitor SB203580 (F). *, p < 0.05, significantly different from controls.
DISCUSSION

PGC-1α is generally thought to be the master regulator of mitochondrial biogenesis in adipose tissue, heart, and liver (39). We have demonstrated that PGC-1α has the ability to induce mitochondrial biogenesis in control RPTCs, suggesting that PGC-1α also plays a central role in regulating the biogenesis in RPTCs. PGC-1α is known to up-regulate gluconeogenesis (36, 40), a process that may require increasing concentrations of ATP and thus also explain the increase in the basal respiration that is observed with the ectopic expression of PGC-1α.

Although mitochondrial biogenesis has been studied in response to different stimuli in a few tissues (1–4), the mechanisms of mitochondrial biogenesis following cell injury and regeneration has not been examined. We previously demonstrated that oxidant exposure (i.e., TBHP) decreases mitochondrial function and that mitochondrial function returns over a period of days to control levels (27, 28). In this paper, we have shown that the loss of mitochondrial function is associated with mitochondrial damage and autophagy.

One day following oxidant injury, basal respiration increased, whereas uncoupled respiration did not. This response is consistent with the idea that the cells utilize the residual capacity of the mitochondria to survive and initiate repair. On day two following injury, both basal and uncoupled respiration increased and returned to control values on day 4. Because ectopic expression of PGC-1α in RPTCs resulted in robust biogenesis of mitochondria, we hypothesized that up-regulation of endogenous PGC-1α was responsible for the restoration of mitochondrial function by regulating the production of new mitochondria. The up-regulation of PGC-1α on days 1–3 post-injury is consistent with the initiation of the recovery of mitochondrial function, whereas the decrease in PGC-1α on day four is consistent with a negative feedback mechanism preventing the formation of additional mitochondria as mitochondrial function is restored.

Of the various signaling mechanisms that have been implicated in the regulation of PGC-1α expression, including nitric oxide (5–12), β-adrenergic receptors, CaMK, and AMP kinase
(1, 13–15), only p38 MAPK appears to be important in the regulation of PGC-1α expression following oxidant injury in our model. Our results are consistent with reports that phosphorylation of p38 MAPK increases PGC-1α expression by stabilizing existing protein (1, 16) or promoting the transcription of PGC-1α through an activating transcription factor-2-dependent mechanism (41). Interestingly, following oxidant injury, p38 MAPK appears to play two roles in mitochondrial biogenesis upstream and downstream of the EGFR (see below).

Following injury, p38 MAPK is phosphorylated by Src and then transactivates the EGFR. This signaling pathway appears to be a common response of RPTCs to oxidant injury, as the same signaling cascade was observed following acute exposure of RPTCs to H2O2 (33). However, the mechanism of EGFR transactivation by p38 MAPK has yet to be determined. Our experiments also provide evidence that p38 MAPK is an EGFR substrate in this model system. The addition of the EGFR inhibitor AG1478 30 min post-oxidant addition blocked the phosphorylation of p38 MAPK and decreased PGC-1α expression. Our results are consistent with reports that phosphorylation of p38 MAPK increases PGC-1α expression dependent upon the transactivation of the EGFR.

This is the first report that the EGFR is a mediator of mitochondrial biogenesis. We previously demonstrated that oxidant-induced injury results in the dedifferentiation, migration, and proliferation through the EGFR (33, 34). EGFR initiation of mitochondrial biogenesis, in concert with dedifferentiation, migration, and proliferation, may represent an integrated pathway to ensure sufficient energy for repair, regeneration, and ultimately, the return of normal differentiation function. The EGFR is capable of activating a series of signaling pathways that may be involved in regulating PGC-1α expression, including phosphatidylinositol 3-kinase (34, 42). However, inhibition of phosphatidylinositol 3-kinase/Akt had no effect on the expression of PGC-1α following oxidant exposure. Yet, phosphatidylinositol 3-kinase/Akt is activated following oxidant injury in this model and is required for cellular proliferation (34). Thus, the pathways may diverge at this point. However, phosphatidylinositol 3-kinase/Akt may contribute to the biogenesis of mitochondria downstream of PGC-1α by directly phosphorylating nuclear respiratory factor 1 and inducing mitochondrial transcription factor-A (2). This pathway may also be involved in regulating the shift from glycolgen storage to glucose utilization through the Akt-dependent phosphorylation and inactivation of glycogen-synthase kinase 3 (43).

We cannot disregard a possible role for the activation of AMP kinase in regulating PGC-1α in our model. AMP kinase is activated in response to cell stressors causing perturbations in cAMP/ATP ratios (44), can participate in the activation of the p38MAPK-signaling cascade (4, 45), and has been implicated in regulating the expression of PGC-1α in muscle in response to a variety of stimuli (36, 46–49). Because rapid decreases in cellular ATP are observed in TBHP-treated RPTCs (28) and AMP kinase is known to be activated under conditions of renal ische-

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