Protein Kinase C-α Interaction with F₀F₁-ATPase Promotes F₀F₁-ATPase Activity and Reduces Energy Deficits in Injured Renal Cells*

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Background: The functional significance of phosphorylations of F₀F₁-ATPase and the kinases involved in them are largely unknown.

Results: Active protein kinase C-α (PKC-α) interacts with subunits of F₀F₁-ATPase, leading to increases in their phosphorylation and protein levels, and increases F₀F₁-ATPase activity.

Conclusion: PKC-α is a novel regulator of ATP synthase.

Significance: PKC-α activation and interaction with F₀F₁-ATPase improves F₀F₁-ATPase activity and ATP content in injured renal cells.

We showed previously that active PKC-α maintains F₀F₁-ATPase activity, whereas inactive PKC-α mutant (dnPKC-α) blocks recovery of F₀F₁-ATPase activity after injury in renal proximal tubules (RPTC). This study tested whether mitochondrial PKC-α interacts with and phosphorylates F₀F₁-ATPase. Wild-type PKC-α (wtPKC-α) and dnPKC-α were overexpressed in RPTC to increase their mitochondrial levels, and RPTC were exposed to oxidant or hypoxia. Mitochondrial levels of the γ-subunit, but not the α- and β-subunits, were decreased by injury, an event associated with 54% inhibition of F₀F₁-ATPase activity. Overexpressing wtPKC-α blocked decreases in γ-subunit levels, maintained F₀F₁-ATPase activity, and improved ATP levels after injury. Deletion of PKC-α decreased levels of α-, β-, and γ-subunits, decreased F₀F₁-ATPase activity, and hindered the recovery of ATP content after RPTC injury. Mitochondrial PKC-α co-immunoprecipitated with α-, β-, and γ-subunits of F₀F₁-ATPase. The association of PKC-α with these subunits decreased in injured RPTC overexpressing dnPKC-α. Immunocapture of F₀F₁-ATPase and immunoblotting with phospho(Ser) PKC substrate antibody identified phosphorylation of serine in the PKC consensus site on the α- or β- and γ-subunits. Overexpressing wtPKC-α increased phosphorylation and protein levels, whereas deletion of PKC-α decreased protein levels of α-, β-, and γ-subunits of F₀F₁-ATPase in RPTC. Phosphoproteomics revealed phosphorylation of Ser1166 on the γ-subunit in response to wtPKC-α overexpression. We concluded that active PKC-α 1) prevents injury-induced decreases in levels of γ subunit of F₀F₁-ATPase, 2) interacts with α-, β-, and γ-subunits leading to increases in their phosphorylation, and 3) promotes the recovery of F₀F₁-ATPase activity and ATP content after injury in RPTC.

The kidney consumes large amounts of ATP to support its reabsorptive work driven by the active transport of ions. The demand for ATP is particularly high in the renal cortex, which is almost totally dependent on mitochondrial generation of ATP by the ATP synthase (F₀F₁-ATPase). Conditions leading to decreases in the activity of ATP synthase in the kidney, such as renal ischemia and nephrotoxicity, result in energy deficits in the renal cortex, reduced solute reabsorption by the kidney, injury and/or death of renal cortical cells, and acute kidney injury. Therefore, alleviating decreases in ATP synthase-driven oxidative phosphorylation is essential for maintaining kidney function and recovery from kidney injury.

The mammalian ATP synthase consists of 16 subunits assembled in two nanomotor domains. The F₀ complex forms a proton channel in the inner mitochondrial membrane and is driven by a proton gradient generated by the electron transport chain. The F₁ domain, consisting of three α- and three β-subunits and single copies of the γ-, δ-, and ε-subunits, protrudes from the inner membrane into the mitochondrial matrix (1–3). The F₁ and F₀ domains are connected by a central rotating stalk composed of the γ-, δ-, and ε-subunits and a peripheral stator stalk (1). The rotation of F₀ domain depends on the proton gradient across the inner mitochondrial membrane and drives the counterclockwise rotation of the central stalk and the formation of ATP from ADP and inorganic phosphate by the F₁ domain.

ATP synthase activity is regulated by numerous mechanisms including posttranslational modifications of its subunits at multiple amino acid residues. These posttranslational modifications include oxidation (4, 5), carbamidomethylation (5), glutathionylation (6), tyrosine nitration, and S-nitrosation (7), and phosphorylation. These modifications appear to play a key role in regulating the activity of this enzyme and producing ATP in physiological and pathological conditions. Nitration of only

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PKC-α Interacts with and Regulates $F_0F_1$-ATPase in Renal Cells

one of the two tyrosine residues (Tyr$^{345}$ and Tyr$^{368}$) is sufficient to decrease the activity of ATP synthase and reduce the production of ATP (7). Phosphorylations of the tyrosine, serine, and threonine residues of the ATP synthase subunits are extensive and highly conserved across mammalian species (8–16). However, the role of different phosphorylations in the regulation of ATP synthase activity and kinase/phosphatase systems responsible for these phosphorylations is relatively unknown. Phosphorylations of the catalytic β-subunit of the $F_1$ domain are the best known. Højlund et al. (10) have implicated phosphorylation of the β-subunit in the changes in ATP synthase in diabetic muscle and suggest that phosphorylation of the β-subunit contributes to the pathogenesis of type 2 diabetes. Arrell et al. (13) implicate the phosphorylation of the β-subunit of cardiac $F_0F_1$-ATPase in the preconditioning and cardioprotection offered by adenosine. Recently, Kane et al. (15) have demonstrated in a yeast model system that mutations mimicking phosphorylations of specific serine and threonine residues of the β-subunit of $F_0F_1$-ATPase have implications for the function, structure, and assembly of the ATP synthase complex. Some phosphorylations of the β-subunit block the ATPase function, whereas others change the stability of the $F_1$ domain of ATP synthase or induce dimerization of $F_0F_1$ complex (15). Likewise, phosphorylation of the γ-subunit has been linked to the formation of $F_1$ dimers (17, 18). Several reports document the phosphorylation of the α- and γ-subunits (14, 19, 20), whereas Zhang et al. (8) report phosphorylation of tyrosine 75 on the δ-subunit of the $F_1$ domain of $F_0F_1$-ATPase in response to platelet-derived growth factor (PDGF) stimulation in cortical neurons. PDGF-mediated tyrosine phosphorylation of the δ-subunit of ATP synthase has also been reported in other cell types (9). $F_0F_1$-ATPase associates and interacts with protein kinase C δ (PKC-δ) in cardiac myocytes and with PKC-β in the hippocampus after transient ischemia (21, 22). In vitro incubation of $F_0F_1$-ATPase with the recombinant PKC-δ inhibits $F_0F_1$-ATPase activity (21). Our previous report documents phosphorylation of the isolated β-subunit of renal $F_0F_1$-ATPase by recombinant PKC-α in vitro (23). Incubation of the catalytic β-subunit of $F_0F_1$-ATPase with recombinant PKC-α in vitro reduces the ATPase activity of this subunit (23). However, it is not known whether PKC-α interacts with ATP synthase complex in situ in mitochondria and which subunits of this complex may associate with PKC-α. The goal of our current study was to examine whether PKC-α interacts with $F_0F_1$-ATPase in the mitochondria of renal proximal tubules, to identify subunits involved in this interaction, and to determine whether they serve as PKC-α substrates. A second goal of this study was to examine whether the association of PKC-α with $F_0F_1$-ATPase plays a role in improved recovery of $F_0F_1$-ATPase activity and ATP levels after RPTC$^{2}$ injury.

**EXPERIMENTAL PROCEDURES**

**Animals and Materials**—Female New Zealand White rabbits (2.5–3.0 kg) were purchased from Harlan Laboratories (Oxford, MI). All animal procedures involved in this study were approved by the Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences. The cell culture media, a 50:50 mixture of Dulbecco’s modified Eagle’s medium (DMEM) and Ham’s F-12 nutrient mix without phenol red, pyruvate, and glucose, were purchased from MediaTech Cellgro (Herndon, VA). Phospho-PKC-α and PKC-α antibodies were purchased from Upstate Biotechnology (Lake Placid, NY) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Phospho(Ser) PKC substrate and phosphoserine/threonine Akt antibodies were supplied by Cell Signaling Technology (Beverly, MA). Antibodies against the α- and γ-subunits of $F_0F_1$-ATPase and the ATP synthase immunocapture kit were purchased from Abcam (Cambridge, MA). Antibodies against the β-subunit of $F_0F_1$-ATPase were supplied by Life Technologies. The sources of other reagents have been described previously (24–26).

**PKC-α-deficient Mice**—Heterozygous mice carrying a deletion of the PKC-α gene (B6;129-Prkca$^{tm1Jmk/J}$) were obtained from The Jackson Laboratory (Bar Harbor, ME). The homologous recombination used to replace the exon encoding the ATP-binding cassette of PKC-α with a neomycin resistance gene was made by Dr. Jeffery Molkentin (Cincinnati Children’s Hospital, Cincinnati, OH). The heterozygous mice were bred to obtain homozygous PKC-α-deficient animals (PKC-α KO). PKC-α-deficient mice were selected from the offspring of heterozygous matings based on the results of polymerase chain reaction of the tail DNA. Wild-type homozygous littermates were used as controls.

**Isolation and Culture of Renal Proximal Tubular Cells**—Renal proximal tubules were isolated from rabbit kidneys by the iron oxide perfusion method and cultured in 35-mm culture dishes in improved conditions as described previously (24, 25). Isolation of PKC-α-deficient renal proximal tubules was carried out using kidneys harvested from 3 month-old-female mice as described earlier (41). The culture medium was a 50:50 mixture of DMEM and Ham’s F-12 nutrient mix without phenol red, pyruvate, and glucose, supplemented with 15 mM NaHCO$_3$, 15 mM Hepes, and 6 mM lactate (pH 7.4, 295 mosmol/kg). Human transferrin (5 μg/ml), selenium (5 ng/ml), hydrocortisone (50 nM), bovine insulin (10 nM), and L-ascorbic acid-2-phosphate (50 μM) were added to the media immediately before daily media change.

**Adenoviral Constructs and Amplification**—The dominant negative (kinase dead, catalytically inactive) PKC-α mutant (dnPKC-α) was constructed by replacing lysine with arginine at position 368 of the ATP-binding site (27). An adenoviral vector encoding the dnPKC-α was constructed, and an aliquot was provided by Dr. Trevor Biden (Garvan Institute of Medical Research, St. Vincent’s Hospital, Sydney, Australia). An adenoviral vector encoding the wild-type PKC-α (wtPKC-α) was constructed as described previously (28). Aliquots of both adenoviruses were generously provided by Dr. Alan Samarel (Loyola University Medical Center, Chicago). Adenoviruses were amplified in AD293 and HEK293 cells as we described previously (29, 30). Adenoviral particles were isolated and purified from HEK293 lysates by centrifugation in a CsCl density gradient (7.5 and 8.3 g/ml) at 175,500 × g for 1 h. The multiplicity of
**PKC-α Interacts with and Regulates F$_{0}$F$_{1}$-ATPase in Renal Cells**

Infection (m.o.i.) was determined by a viral dilution assay in HEK293 cells grown in 96-well plates.

**Overexpression of PKC**—All transfections were carried out in confluent quiescent cultures of rabbit RPTC as described previously (30, 31). Selective overexpression of wild-type and inactive PKC-α was achieved by infecting rabbit RPTC using adenoviral vectors encoding wtPKC-α (m.o.i. = 75) and dnPKC-α (m.o.i. = 50), respectively. Hypoxia and oxidant injury were induced at 48 h after infections.

**Oxidant Treatment of RPTC Monolayer**—Confluent monolayers of RPTC were treated with a model oxidant, tert-butyl hydroperoxide (TBHP, 0.35 mmol/liter), for 45 min as described previously (30). Controls were treated with the diluent dimethyl sulfoxide (0.1%).

**Hypoxia Exposure**—Hypoxia was induced in RPTC by aspirating culture media, overlaying cells with fresh and warm (37 °C) media pregassed with 95% N$_{2}$, 5% CO$_{2}$, and placing the dishes in an airtight hypoxia chamber (equipped with the ProOx oxygen controller, BioSpherix, Lacona, NY), which was gassed with 95% N$_{2}$, 5% CO$_{2}$, and kept in the atmosphere of 94% N$_{2}$, 5% CO$_{2}$, 1% O$_{2}$ and housed in a regular cell culture incubator at 37 °C. Hypoxia was terminated by returning cultures to air, 5% CO$_{2}$ atmosphere (reoxygenation).

**Isolation of RPTC Mitochondria**—RPTC were homogenized and mitochondrial and mitochondria isolated as described previously (29, 32). Mitochondria resulting from this isolation were free of lysosomal and endoplasmic reticular contaminations and contained small amounts of peroxisomes (29, 32). The final mitochondrial pellet was resuspended in the assay buffers used for different analyses.

**Immunoprecipitation**—To identify mitochondrial proteins interacting with PKC-α, freshly isolated RPTC mitochondria were solubilized in radioimmunoprecipitation assay buffer (RIPA), and the lysates were processed as described previously (31). Equal amounts of protein (500 µg) from each sample were used for immunoprecipitation, which was carried out as described previously (31). The supernatants containing eluted proteins were mixed with the Laemmli sample buffer, boiled, and used for immunoblotting.

**Immunocapture of ATP Synthase Complex**—F$_{0}$F$_{1}$-ATPase complex was immunocaptured from mitochondrial lysates and eluted from the immunocomplexes using an antibody irreversibly cross-linked to protein G-agarose beads. Immunocaptured proteins were resolved using SDS-PAGE and analyzed by phosphoproteomics or used for immunoblotting. Protein bands identities were confirmed by mass spectrometry.

**Proteomic and Phosphoproteomics Analyses**—To identify proteins interacting with PKC-α, the bead-immunoprecipitate complexes obtained from PKC-α immunoprecipitation were washed with PBS and double deionized water. The proteins were eluted from bead-immunoprecipitate complexes using a buffer containing 2 M thiourea, 7 M urea, 4% CHAPS, and 30 mM Tris-HCl, pH 8.8. The eluates were used for proteomic analysis using two-dimensional differential in-gel electrophoresis performed at Applied Biosomics (Hayward, CA) as described previously (31). In brief, digested tryptic peptides were extracted from the gel and desalted by using a C-18 Zip-tip (Millipore, MA). Samples were analyzed by MALDI-TOF, and MS spectra were obtained using an Applied Biosystems proteomic analyzer. Ten to 20 of the most abundant peptides were further subjected to fragmentation (MS/MS), and both MS and MS/MS spectra were subjected to a database search using GPS Explorer equipped with the MASCOT search engine, which uses the National Center for Biotechnology Information (NCBI) protein database. Phosphoproteomic analysis was carried out at the Proteomics Core Facility at the University of Arkansas for Medical Sciences. Protein gel bands were subjected to in-gel trypsin digestion at 37 °C for 12–16 h using 100 ng of trypsin in 100 mM ammonium bicarbonate. The digested tryptic peptides were then acidified in 0.1% formic acid and separated by reverse-phase Jupiter Proteo resin (Phenomenex) on a 100 × 0.075-mm column using a nanoACQUITY UPLC system (Waters). Eluted peptides were ionized by electrospray (1.9 kV) followed by MS/MS analysis using collision-induced dissociation on an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific). MS/MS data were acquired for the top 15 peaks from each MS scan using the ion trap analyzer in centroid mode and normal mass range with a normalized collision energy of 35.0. Proteins and phosphorylation sites were identified by a database search using MASCOT.

**Immunoblotting**—Protein levels in cellular and mitochondrial lysates were assessed by immunoblot analysis as described previously (26).

**Enrichment of Mitochondrial Phosphoproteins**—Enrichment of mitochondrial phosphoproteins was carried out using a Pierce phosphoprotein enrichment kit (Thermo Fisher Scientific) according to the manufacturer’s protocol. In brief, 4 mg of mitochondrial protein was diluted to 0.5 mg/ml using the lysis buffer provided by the manufacturer, applied to a resin column equilibrated previously with the lysis buffer containing 0.25% CHAPS, and incubated with rocking for 30 min at 4 °C. The column was centrifuged at 1,000 × g for 1 min at 4 °C, washed with 5 ml of lysis buffer containing 0.25% CHAPS, and centrifuged again. Following two additional washing steps, 1 ml of the elution buffer was applied to the column and incubated at room temperature with agitation for 3 min. The column was centrifuged at 1,000 × g for 1 min, and the elution of proteins was repeated four times. All eluted fractions were pooled and subjected to concentration by centrifuging them at 2600 × g for 30 min at 4 °C until the sample volume reached 0.15–0.20 ml.

**F$_{0}$F$_{1}$-ATPase Activity**—The ATPase activity of the ATP synthase was determined in freshly isolated mitochondria by measuring the release of inorganic phosphate from ATP by the method of Law et al. (33) as described previously (23, 26). Each sample was run in triplicate in the absence and presence of oligomycin (10 µg/ml), and the oligomycin-sensitive ATPase activity of F$_{0}$F$_{1}$-ATPase was calculated.

**Intracellular ATP Content**—Intracellular ATP content in RPTC was measured by the luciferase method in freshly prepared RPTC lysates using an ATP bioluminescence assay kit HS II (Hoffmann-La Roche) and the manufacturer’s protocol as...
PKC-α Interacts with and Regulates F_0F_1-ATPase in Renal Cells

**RESULTS**

**Hypoxia Exposure and Oxidant Injury Lead to Decreases in ATP Content in RPTC**—ATP content was used to evaluate the energy status of non-injured and injured RPTC. ATP content in RPTC decreased to 17% of controls following hypoxia (4 h) and remained decreased after 24 h of reoxygenation following hypoxia (Fig. 1A). Energy deficits in hypoxia-injured RPTC were associated with 31 ± 4.1% necrosis in RPTC. Injury induced by oxidant (TBHP) reduced the ATP content to 36 and 40% of controls at 4 and 24 h, respectively, after the exposure (Fig. 1B). Energy deficits in oxidant-injured RPTC were associated with 41 ± 3.8% necrosis in RPTC. ATP content recovered at 96 h following TBHP exposure (data not shown). These data demonstrate that injury caused by hypoxia and oxidative stress leads to major energy deficits and necrosis in RPTC.

**Hypoxia and Oxidant Injury Decrease Activity of F_0F_1-ATPase in RPTC**—ATP synthesis is dependent on the ability of mitochondria to generate proton-motive force and on the activity of ATP synthase (F_0F_1-ATPase). Our goal was to determine whether hypoxia- and oxidant-induced injury affect the protein levels and/or catalytic activity of F_0F_1-ATPase. Previously, we had shown that exposure of RPTC to 2-h hypoxia does not change activity of F_0F_1-ATPase (34). Here, we showed that 4-h hypoxia decreased the activity of F_0F_1-ATPase to 66% of controls and that F_0F_1-ATPase activity did not recover within 24 h of reoxygenation following hypoxia (Fig. 2A). The F_1 domain of the F_0F_1-ATPase is composed of three α- and three β-subunits and single copies of the δ-, ε-, and γ-subunits. The protein levels of the α- and β-catalytic subunits of F_0F_1-ATPase did not change significantly during the course of hypoxia and reoxygenation (Fig. 2B), whereas the levels of the γ-subunit were decreased by hypoxia and did not fully recover until 24 h of reoxygenation (Fig. 2B). These decreases coincided with dephosphorylation of mitochondrial PKC-α (Fig. 3C). The recovery of active PKC-α during reoxygenation was associated with the return of protein levels of the γ-subunit (Fig. 2B and data not shown).

Oxidant injury decreased the activity of F_0F_1-ATPase to 50 and 46% of controls at 4 and 24 h, respectively, after the exposure. Oxidant injury decreased the protein levels of the γ-subunit but had no effect on the levels of the α- and β-subunits of ATP synthase (Fig. 2, C and D). These data show that hypoxia and oxidative stress reduce the catalytic activity of F_0F_1-ATPase and produce ATP deficits. The results also show that injury leads to the degradation of the γ-subunit of the F_1 complex.

**Hypoxia and Oxidant Injury Induce Dephosphorylation of Mitochondrial PKC-α**—Pretreatment of RPTC with phorbol 12-myristate 13-acetate prior to hypoxia prevented decreases in F_0F_1-ATPase activity in RPTC (2.53 ± 0.05 μmol PO_4/ min/mg protein following hypoxia versus 3.32 ± 0.05 μmol PO_4/ min/mg protein in RPTC pretreated with phorbol 12-myristate 13-acetate prior to hypoxia and 3.25 ± 0.051 μmol PO_4/ min/mg protein during normoxia). Because phorbol esters mimic the action of diacylglycerol, the physiological activator of PKC, these observations suggested the involvement of PKC in the regulation of F_0F_1-ATPase activity in RPTC. Previously, we had shown that PKC-α is a major PKC isozyme in RPTC mitochondria and promotes recovery of mitochondrial function in oxidant-injured RPTC (30). Mitochondrial levels of phosphorylated (active) PKC-α were maintained during 2 h of hypoxia, but longer exposure of RPTC to hypoxia resulted in dephosphorylation of mitochondrial PKC-α (Fig. 3, A and B). Oxidant increased phosphorylation of mitochondrial PKC-α at 1 h following exposure, but this increase was transient and was followed by PKC-α dephosphorylation between 2 and 8 h after TBHP exposure (Fig. 3, A and B). These data show that hypoxia and oxidant exposure result in PKC-α dephosphorylation and decreases in active PKC-α levels in RPTC mitochondria.

**PKC-α Activation Maintains F_0F_1-ATPase Activity in Injured RPTC**—To determine whether mitochondrial PKC-α plays a role in the regulation of F_0F_1-ATPase activity in RPTC, the
PKC-α Interacts with and Regulates F₀F₁-ATPase in Renal Cells

levels of active PKC-α were increased by overexpressing wtPKC-α or decreased by overexpressing dnPKC-α, and then F₀F₁-ATPase activity and protein levels were examined following injury. Overexpressing wtPKC-α increased the mitochondrial levels of phosphorylated (active) PKC-α, reduced dephosphorylation of PKC-α during hypoxia, and prevented oxidant-induced dephosphorylation of PKC-α in the mitochondria of oxidant-injured RPTC (Fig. 3). Overexpressing wtPKC-α prevented hypoxia- and oxidant-induced decreases in F₀F₁-ATPase activity in RPTC (Fig. 4, A and C), increased protein levels of α and β-subunits, and maintained the levels of the γ-subunit of F₀F₁-ATPase in injured RPTC (Fig. 4, A and B). In contrast, overexpressing the inactive mutant of PKC-α (dnPKC-α) and blocking PKC-α activation resulted in the loss of phosphorylated PKC-α from the mitochondria of injured RPTC (Fig. 3) and blocked the recovery of F₀F₁-ATPase activity following hypoxia and oxidant exposure (Fig. 4, A and C). Overexpressing dnPKC-α decreased protein levels of α-, β-, and γ-subunits of F₀F₁-ATPase in oxidant-injured RPTC (Fig. 4D). These results demonstrate that active PKC-α promotes activity and maintains the protein levels of the subunits of F₀F₁-ATPase in injured RPTC.

PKC-α Deficiency Lowers F₀F₁-ATPase Activity in RPTC—To determine whether PKC-α deficiency alters F₀F₁-ATPase activity, confluent primary cultures of RPTC isolated from PKC-α-deficient mice were subjected to 4-h hypoxia followed by reoxygenation. F₀F₁-ATPase activity in normoxic RPTC derived from PKC-α-deficient mice was decreased 40% in comparison with F₀F₁-ATPase activity in normoxic RPTC from wild-type mice (Fig. 5A). This decrease was comparable to the reduction in F₀F₁-ATPase activity caused by hypoxia in RPTC from wild-type mice (Fig. 5A). Hypoxia had no further effect on F₀F₁-ATPase activity in PKC-α-deficient RPTC (Fig. 5A). Protein levels of α-, β-, and γ-subunits of F₀F₁-ATPase in PKC-α-deficient RPTC were decreased in comparison with wild-type RPTC (Fig. 5B).

PKC-α Activation Improves, whereas PKC-α Deletion Reduces, ATP Content in Injured RPTC—To test whether PKC-α-mediated increases in F₀F₁-ATPase activity result in improvements in ATP content in injured RPTC, wtPKC-α and dnPKC-α were overexpressed in RPTC, and ATP content was examined following hypoxia and oxidant exposure. Fig. 1A shows that hypoxia decreased the ATP content to 17% of controls (normoxia), but the decrease was partially abrogated in hypoxic RPTC overexpressing wtPKC-α (55% of normoxia) and was associated with decreased necrosis (8.0 ± 1.2% versus 31 ± 4.1% in the presence and absence of wtPKC-α, respectively). In contrast, deletion of PKC-α reduced the ATP content by 36% in non-injured RPTC, exacerbated hypoxia-induced decreases in ATP levels, and blocked recovery of ATP during reoxygenation (Figs. 1A and 5C).

Overexpression of wtPKC-α also promoted recovery of ATP content (Fig. 1B) and decreased RPTC necrosis after oxidant injury (21.8 ± 3.3% versus 41 ± 3.8% in the presence and absence of wtPKC-α, respectively). In contrast, overexpressing dnPKC-α blocked recovery of ATP levels in oxidant-injured RPTC (Fig. 1B). These results demonstrate that PKC-α-mediated improvements in F₀F₁-ATPase activity are associated with increased ATP levels and decreased necrosis in RPTC after hypoxia and oxidant injury.

PKC-α Associates with F₀F₁-ATPase in RPTC—To test whether PKC-α associates with mitochondrial proteins of oxidative phos-
PKC-α Interacts with and Regulates F_{0}F_{1}-ATPase in Renal Cells

FIGURE 3. Hypoxia and oxidant exposure decrease the levels of phosphorylated (active) PKC-α. Mitochondria were isolated from confluent RPTC exposed to different times of hypoxia (A and B, left panels) or at different time points after TBHP (0.35 mmol/liter for 45 min) exposure (A and B, right panels) and protein levels of phosphorylated PKC-α (p-PKC-α) and total PKC-α (PKC-α) were assessed by immunoblotting. C, effects of overexpressing wtPKC-α and inactive dnPKC-α on PKC-α phosphorylation at 0.5 h after hypoxia (left panel) and 4 and 24 h after TBHP exposure (right panel). Immunoblots are representative of three independent experiments (RPTC isolations).

Overexpression of PKC-α Increases Phosphorylation of F_{0}F_{1}-ATPase—We hypothesized that the association of PKC-α with F_{0}F_{1}-ATPase leads to changes in the phosphorylation of F_{0}F_{1}-ATPase subunit(s) to regulate catalytic function of this complex. To test this hypothesis, we determined: 1) the identity of mitochondrial phosphoproteins that alter their phosphorylation status in response to increased levels of active (phosphorylated) PKC-α and 2) the phosphorylation status of serine residues on the immunocaptured subunits of F_{0}F_{1}-ATPase, specifically serines at the PKC consensus sites. Isolation and enrichment of mitochondrial phosphoproteins from controls and RPTC overexpressing wtPKC-α followed by proteomic analysis revealed a 40–50% increase in the levels of phosphorylated subunits α, β, and γ in response to overexpression of wtPKC-α. These results demonstrated that α-, β-, and γ-subunits are phosphorylated in response to PKC-α activation and suggested that PKC-α is involved directly or indirectly in their phosphorylation. To determine whether these subunits serve as direct substrates for PKC, F_{0}F_{1}-ATPase complex was immunocaptured and subjected to SDS-PAGE to resolve individual subunits (peptides). This was followed by immunoblot analysis using antibody recognizing phosphorylated serine residues and antibody recognizing phosphoserine present in the PKC consensus site (phospho(Ser) PKC substrate antibody).

The latter antibody is more specific for serines phosphorylated by classic PKC isozymes (PKC-α, PKC-β, and PKC-γ) when phosphorylated at serine residues surrounded by arginine or lysine at phosphorylation. PKC-α was immunoprecipitated from RPTC mitochondria, and major proteins present in PKC-α immunocomplexes were identified. Proteomic and immunoblot analyses identified several proteins of oxidative phosphorylation associated with PKC-α including three subunits of the F_{1} domain of ATP synthase: 1) ATP5A1 (α-subunit), 2) ATP5B (β, the catalytic subunit), and 3) ATP5C1 (γ-subunit) (Fig. 6, A and B, and Table 1). Injury and overexpressing wtPKC-α and dnPKC-α had no effect on the association of the F_{0}F_{1}-ATPase subunits with PKC-α (Fig. 6B). Association of the β-subunit with PKC-α was unaltered by injury but decreased in injured cells overexpressing dnPKC-α (Fig. 6B). In contrast, association of the γ-subunit with PKC-α was decreased by injury and by overexpressing dnPKC-α in injured cells (Fig. 6, B and C). This decrease was noted despite large amounts of PKC-α protein present in the immunoprecipitates from injured cells overexpressing dnPKC-α (Fig. 6B).

To confirm the association of PKC-α with F_{0}F_{1}-ATPase, reverse immunoprecipitation was carried out using an antibody that captures the entire ATP synthase complex. Fig. 7 shows the presence of PKC-α, including phosphorylated (active) PKC-α in RPTC overexpressing wtPKC-α, in the complex that was immunocaptured using F_{0}F_{1}-ATPase antibody. Thus, the reverse immunoprecipitation confirmed the association of PKC-α with ATP synthase complex. These data also show that the association of the β- and γ-subunits of F_{0}F_{1}-ATPase with PKC-α decreases in injured cells when PKC-α is inactive.
the -2 and +2 positions with a hydrophobic residue at the +1 position. The antibody does not cross-react with phosphothe-reonine in the same motif or with phosphoserine in other motifs. SDS-PAGE and immunoblot analysis identified two major bands (50 and 33 kDa) containing phosphorylated serines. Mass spectrometry determined that the 50-kDa protein band was composed of the α- and β-subunits (ATP5A and ATP5B, respectively) and the 33-kDa protein was the γ-subunit (ATP5C1) of ATP synthase.

The increase in serine phosphorylation of the γ-subunit upon PKC-α activation was much more pronounced than the serine phosphorylation of the α/β-subunits (Fig. 8A). Overexpression of wtPKC-α also increased phosphorylation of serines in the PKC consensus site(s) of α/β-subunits (Fig. 8A). Phosphoproteomic analysis of peptides present in both bands identified phosphorylation of the Ser 146 residue (RGILHRTHS146DQFLVTFK) of the γ-subunit (Fig. 8B and C) but was unable to find phosphorylated serines on the peptide fragments derived from the α- and β-subunits. However, proteomic analysis of α- and β-subunits yielded only 80% sequence coverage of both subunits. Six MS/MS spectra of the phosphorylated peptide fragment (RGILHRTHS146DQFLVTFK) of the γ-subunit matched the phosphopeptide with 95% confidence or better. Because the levels of serine phosphorylation on the γ-subunit increased in response to overexpression of wtPKC-α and not...
the inactive dnPKC-α, these data suggest that PKC-α activation is involved in the phosphorylation of the γ-subunit of ATP synthase (Fig. 8C). Probing the immunocaptured F$_0$F$_1$-ATPase subunits using phospho(Ser/Thr) Akt substrate antibody demonstrated two phosphorylated proteins of apparent molecular mass of 49–50 kDa corresponding to α/β-subunits, but no phosphorylated 33-kDa protein (Fig. 9), suggesting that the γ-subunit of ATP synthase is not phosphorylated by Akt.

**Injury Decreases and PKC-α Overexpression Increases F$_0$F$_1$-ATPase Subunit Phosphorylation**—Exposure of RPTC to hypoxia resulted in decreases in serine phosphorylation in the PKC consensus site on three proteins of the apparent molecular mass of 52–53 and 32–33 kDa (Fig. 9). Overexpression of wtPKC-α, but
PKC-α Interacts with and Regulates F_{0}F_{1}-ATPase in Renal Cells

FIGURE 7. F_{0}F_{1}-ATPase associates with PKC-α. wtPKC-α and dnPKC-α were overexpressed in confluent RPTC. Mitochondria were isolated from non-transfected and wtPKC-α- and dnPKC-α-transfected RPTC, lysed using RIPA buffer containing 1% lauryl maltoside, and subjected to immunocapture using F_{0}F_{1}-ATPase antibody irreversibly cross-linked to protein G-agarose beads. Immunocaptured proteins were resolved using SDS-PAGE following immunoblotting using antibody that recognizes different subunits of F_{0}F_{1}-ATPase, phoso-PKCa, and PKCa. Immunoblots are representative of three independent experiments (RPTC isolations).

FIGURE 8. F_{0}F_{1}-ATPase subunits are substrates for PKC-α, wtPKC-α and dnPKC-α were overexpressed in confluent RPTC. Mitochondria were isolated from non-transfected and wtPKC-α and dnPKC-α-transfected RPTC, lysed using RIPA buffer containing 1% lauryl maltoside, and subjected to immunocapture using F_{0}F_{1}-ATPase antibody irreversibly cross-linked to protein G-agarose beads. Immunocaptured proteins were resolved using SDS-PAGE following immunoblotting using antibody that recognizes different subunits of F_{0}F_{1}-ATPase, phoso-PKCa, and PKCa. Immunoblots are representative of three independent experiments (RPTC isolations).

DISCUSSION

Phosphorylation of tyrosine and serine/threonine residues is a ubiquitous mechanism for the regulation of protein function at the posttranslational level. Yet, the functional significance of specific phosphorylations of mitochondrial proteins is known only for a small number of them. ATP synthase (F_{0}F_{1}-ATPase) is subject to phosphorylation on tyrosine, serine, and threonine residues, but the functional role of these modifications is unclear. Phosphorylation may have differential effects on the conformation, catalytic activity, subunit assembly, dimer formation, and stability of the F_{0}F_{1}-ATPase. In addition, phosphorylation or dephosphorylation may provide a signal for dissociation and/or degradation of this complex. The kinases and phosphatases involved in phosphorylation and dephosphorylation of individual subunits of F_{0}F_{1}-ATPase are largely unknown. Because only a few protein kinases have been identified in mitochondria, it has been proposed that mitochondrial complexes contain an autophosphorylation mechanism, which regulates their functions (35). With the exception of the F_{0} subunits A6L and α, which are synthesized in mitochondria, all other subunits of ATP synthase are synthesized in the cytoplasm and can be phosphorylated there before they are imported into the mitochondria. Recent screening of protein kinase inhibitors identified four protein kinases (protein kinase A, PKC-δ, calcium/calmodulin-dependent protein kinase 2, and smooth muscle type myosin light chain kinase) that regulate ATP synthase activity in HeLa cells (36). However, no evidence has been shown that these kinases phosphorylate ATP synthase. It has been suggested that protein kinase A regulates ATP synthase by phosphorylating the cAMP response element (CREB) and activating the expression of the PGC-1α gene, implying that protein kinase A regulates ATP synthase at the transcriptional rather than the posttranslational level (37). Nevertheless, the presence of numerous phosphorylation motifs on ATP synthase subunits suggests that this complex can be regulated through phosphorylation by several kinases. Akt/protein kinase B has been implicated in the phosphorylation of the α- and β-subunits of ATP synthase in response to insulin growth factor-1 stimulation, but the site(s) and outcomes of this phosphorylation are unknown (38). PKC-δ associates with the δ-subunit of cardiac F_{0}F_{1}-ATPase and decreases the activity of F_{0}F_{1}-ATPase, but no specific phosphorylation of ATP synthase by PKC-δ has been identified (21). We have shown that PKC-α can phosphorylate a serine residue on the β-subunit of isolated renal cortical F_{0}F_{1}-ATPase in vitro (23), but it is not known whether such phosphorylation occurs in mitochondria on a fully assembled F_{0}F_{1}-ATPase complex and what the implications of this phosphorylation may be.

Previously, we demonstrated that active PKC-α is critical for maintaining oxidative phosphorylation, F_{0}F_{1}-ATPase activity, and ATP levels during RPTC recovery after oxidant injury (30). We hypothesized that: 1) interaction between PKC-α and ATP...
PKC-α Interacts with and Regulates F₀F₁-ATPase in Renal Cells

Immunocapture: F₀F₁-ATPase Ab

| Immuno blot: P-Ser PKC substrate | P-Ser/Thr-Akt substrate |
|----------------------------------|------------------------|
| F₁ subunit α                     |                         |
| F₁ subunit γ                     |                         |
| F₁ subunit β                     |                         |

49 kDa
30 kDa

Normoxia +
Hypoxia -
wtPKC-α -
 dnPKC-α -

FIGURE 9. PKC-α restores hypoxia-decreased phosphorylation of α-, β-, and γ-subunits of F₀F₁-ATPase. wtPKC-α and dnPKC-α were overexpressed in confluent RPTC, and monolayers were exposed to normoxia or 4 h of hypoxia. Mitochondria were isolated from non-transfected and wtPKC-α- and dnPKC-α-transfected RPTC at 0.5 h of reoxygenation, lysed using RIPA buffer containing 1% lauryl maltoside, and subjected to immunocapture using F₀F₁-ATPase antibody irreversibly cross-linked to protein G-agarose beads. Immunocaptured proteins were resolved using SDS-PAGE followed by immunoblotting using antibodies recognizing phospho(Ser) PKC substrate, phosphoserine/threonine Akt substrate, and different subunits of F₀F₁-ATPase. Immunoblots are representative of three independent experiments (RPTC isolations).

synthase complex is necessary to sustain ATP synthase function in injured cells. 2) PKC-α phosphorylates ATP synthase or activates a downstream kinase that phosphorylates it, and 3) this phosphorylation ameliorates the decreases in catalytic activity of ATP synthase. The first step in addressing our hypothesis was to test whether mitochondrial PKC-α associates with F₀F₁-ATPase. Proteomic analyses of PKC-α immunocomplexes from mitochondrial lysates demonstrated that the major subunits (α, β, and γ) of the F₁ domain associate with the active PKC-α. This interaction was confirmed by identifying PKC-α among proteins immunocaptured by F₀F₁-ATPase antibody. Binding of PKC-α to the F₁ domain projecting into the mitochondrial matrix suggests that mitochondrial PKC-α is localized to the mitoplast. Interestingly, no subunits of the F₀ domain, which is embedded in the inner membrane, were among the proteins that interact with PKC-α. Interaction of mitochondrial PKC-α with subunits of ATP synthase suggests the involvement of PKC-α in the regulation of F₀F₁-ATPase function in injured RPTC. One mechanism accounting for this regulation could be through stimulating F₀F₁-ATPase synthesis to maintain its protein levels after injury. Small reductions in the protein levels of the α- and β-subunits of F₀F₁-ATPase in injured RPTC do not explain major decreases in F₀F₁-ATPase activity in injured RPTC. Thus, degradation of these two major subunits does not account for the decreases in catalytic activity of F₀F₁-ATPase in injured RPTC. However, the persistent lack of active PKC-α, such as in PKC-α-deficient RPTC, results in significant reductions in the levels of α-, β-, and γ-subunits. Likewise, the protein levels of the γ-subunit are markedly decreased by injury, and this decrease is abrogated by the active PKC-α.

The next step in addressing our hypothesis was to test whether phosphorylation of α-, β-, and/or γ-subunits changes in response to increased levels of active PKC-α in mitochondria. Proteomic analysis of enriched mitochondrial phosphoproteins identified α-, β-, and γ-subunits of F₀F₁-ATPase among those proteins. The levels of phosphorylated α-, β-, and γ-subunits increased in response to PKC-α activation, suggesting PKC-α involvement in phosphorylation of F₀F₁-ATPase. Immunoblot analysis of phosphoserine residues present in immunocaptured F₀F₁-ATPase demonstrated two bands representing α- and/or β-subunits and the γ-subunit. Phosphorylation of these peptides, particularly the γ-subunit, was increased in the mitochondria of RPTC overexpressing wtPKC-α. Further, these peptides contained a phosphoserine residue present in the consensus motif of classic PKC isozymes. Because PKC-β and PKC-γ have not been detected in RPTC mitochondria (data not shown), our data suggested that PKC-α is involved in the phosphorylation of subunits of the F₁ domain. Increased levels of phosphorylated serine in the PKC consensus motif on α- and/or β- and γ-subunits in RPTC overexpressing wtPKC-α demonstrated that PKC-α phosphorylates these subunits. Because the molecular mass of the α- and β-subunits are very similar, these two proteins migrated together as one protein band of an apparent molecular mass of 50 kDa and could not be separated by SDS-PAGE. Therefore, we could not determine with high confidence which of these subunits was phosphorylated in response to PKC-α activation. The α-, β-, and γ-subunits of the F₁ domain have numerous consensus motifs containing serines and threonines that could be phosphorylated. The search for protein kinases predicted to phosphorylate α- and β-subunits of human and mouse ATP synthase revealed that classic PKCs can potentially phosphorylate serine (Ser353) and threonine (Thr487) on the β-subunit, but no serine-containing consensus motifs for classic PKCs are present on the α-subunit of ATP synthase (Scansite). According to the Scan site analysis, novel and atypical PKC-δ, -ε, -ζ, and -μ could phosphorylate subunit α, whereas classic PKC isozymes are predicted to phosphorylate subunit β. Phosphorylation of the β-subunit is consistent with our previous report (23). Therefore, it is most likely that the identified phosphorylated serine in the PKC consensus site was present on the β- but not the α-subunit of ATP synthase. Phosphoproteomics was unsuccessful in identifying phosphorylated serine or threonine residues on the α- and β-subunits, despite the positive results obtained by immunoblotting. The proteomic techniques are capable of detecting only the most stable posttranslational protein modifications, and detection of phosphorylation is limited by stoichiometry of phosphorylation as well as ionization efficiency and stability of phosphorylated peptides. Finally, peptides obtained by trypsin digestion of the α- and β-subunits provided
PKC-α Interacts with and Regulates $F_{0}F_{1}$-ATPase in Renal Cells

80% sequence coverage, and it is possible that phosphorylated residues were present in the missing 20% sequence coverage.

Our results also showed that PKC-α is involved in the phosphorylation of the γ-subunit. Phosphomapping of the γ-subunit identified phosphorylation of serine Ser146. Several spectra from phosphoproteomic analysis matched this phosphopeptide with a confidence of 95% or better. However, it is possible that more than one phosphorylated serine (or threonine) residue, in addition to Ser146, was present on the γ-subunit, as the missing sequence of the N terminus had three predicted consensus motifs for classical PKCs. Also, due to phosphate instability, the nature and length of tryptic digests, and 70% coverage sequence of this protein, some phosphorylations, including PKC-α-mediated phosphorylation, could have been missed. Scansite analysis predicted classic PKC isoforms to phosphorylate Thr27, Ser37, and Ser47 localized to the terminal α-helix of the γ-subunit, but phosphoproteomic analysis did not recover terminal peptides containing these amino acids. However, this terminal α-helix is present in the vertical shaft surrounded by α- and β-subunits, making the access of a kinase to the peptide rather difficult. The sequence containing Ser146 is localized at the core of the γ-subunit and within the consensus motif of basophilic serine/threonine kinases, to which PKC belongs. The Scansite search favored Akt as the predicted kinase phosphorylating Ser146. However, no phosphorylation of the γ-subunit by Akt was identified by phosphoserine/threonine Akt substrate antibody (Fig. 9), which demonstrated that Ser146 is not phosphorylated by Akt in RPTC mitochondria. Our results suggest rather that Akt phosphorylates the α- and/or β-subunit of ATP synthase in RPTC mitochondria. These results would be consistent with the recent report that Akt1 activation and translocation to the mitochondria is associated with increased activity of ATP synthase in normal myocardium in vivo and restored activity of this complex in diabetic myocardium (39). Among the three subunits that associate with PKC-α, the γ-subunit levels underwent the most pronounced decreases in response to injury (hypoxia) and decreases in serine phosphorylation in the PKC consensus motif. As the γ-subunit constitutes the central shaft inside the α(3)/β(3) hexamer and connects the catalytic $F_{1}$ domain with the rotor $F_{0}$ domain, the conformation of this subunit is essential for the assembly of the whole ATP synthase complex and its catalytic activity. We speculate that phosphorylation of the γ-subunit on different amino acid residues can stabilize or destabilize the structure of the $F_{1}$ domain as well as the whole ATP synthase complex, depending on where phosphorylation occurs (Fig. 10). Adding negative charges to the N terminus of an α-helix by phosphorylation of serines adjacent to the N terminus leads to protein stabilization, whereas phosphorylation of serines at the C terminus destabilizes the protein in terms of protein solvation and favorable interactions with other proteins (40). Cellular stresses that decrease the levels of active PKC-α in mitochondria reduce the phosphorylation and protein levels of the γ-subunit and diminish the activity of $F_{0}F_{1}$-ATPase. The levels of the γ-subunit are decreased by hypoxia in whole cells (containing functional and damaged mitochondria undergoing mitophagy; data not shown) and in isolated mitochondria. Because these decreases are blocked by active PKC-α, which improves phosphorylation of the γ-subunit and increases recovery of $F_{0}F_{1}$-ATPase activity, our data suggest that phosphorylation of the γ-subunit protects this critical peptide from degradation and prevents disassembly of the $F_{1}$ domain. Levels of the β-subunit remain unchanged during hypoxia in isolated mitochondria but increase in whole cell lysates due to the accumulation of fragmented mitochondria that undergo mitophagy (Figs. 4B and 5B and data not shown). Active PKC-α blocks the accumulation of the β-subunit in hypoxic RPTC, whereas inactive PKC-α increases it in normoxic and hypoxic RPTC (Fig. 4B and data not shown). In contrast, hypoxia and PKC-α activity status have no effect on the cellular and mitochondrial levels of the α-subunit (Fig. 4B and data not shown). This shows that the cellular ratio of β- to α-subunits increases in response to hypoxia or inhibition of PKC-α and suggests that injury leads to dissociation of αβ3 hexamers into individual subunits with differential rates of disposal or new protein synthesis. Proteomic analysis of injured mitochondria showed fragmented α- but not β-subunit peptides. These data suggest that there is a pool of free α- and β-subunits that do not remain associated in injured mitochondria.

In conclusion, we have presented here the first evidence for the interaction of active PKC-α with α-, β-, and γ-subunits of $F_{0}F_{1}$-ATPase and modulation of $F_{0}F_{1}$-ATPase activity by PKC-α. This interaction occurs in situ in RPTC mitochondria, increases upon activation of PKC-α, and decreases when PKC-α is inactive. Our data suggest that this interaction leads to the phosphorylation of α- and/or β- and γ-subunits on serine residue(s) in the PKC consensus motif. PKC-α deletion decreases the abundance of $F_{1}$ domain and $F_{0}F_{1}$-ATPase activity. Inactivation of mitochondrial PKC-α during hypoxia or oxidative stress leads to dephosphorylation of the γ-subunit and its loss from mitochondria. In contrast, PKC-α activation protects the γ-subunit from degradation and improves $F_{0}F_{1}$-ATPase activity and cellular ATP levels during and following injury.

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