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Protein kinase C ϵ targets respiratory chain and mitochondrial membrane potential but not F₀F₁-ATPase in renal cells injured by oxidant.

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Abstract

We have previously shown that protein kinase C ϵ (PKC ϵ) is involved in mitochondrial dysfunction in renal proximal tubular cells (RPTC). This study examined mitochondrial targets of active PKC ϵ in RPTC injured by the model oxidant *tert*-butyl hydroperoxide (TBHP). TBHP exposure augmented the levels of phosphorylated (active) PKC ϵ in mitochondria, which suggested translocation of PKC ϵ to mitochondria after oxidant exposure. Oxidant injury decreased state 3 respiration, ATP production, ATP content, and complex I activity. Further, TBHP exposure increased Ψ_m and production of reactive oxygen species (ROS), and induced mitochondrial fragmentation and RPTC death. PKC ϵ activation by overexpressing constitutively active PKC ϵ exacerbated decreases in state 3 respiration, complex I activity, ATP content, and augmented RPTC death. In contrast, inhibition of PKC ϵ by overexpressing inactive PKC ϵ mutant restored state 3 respiration, respiratory control ratio, complex I activity, Ψ_m , and ATP production and content, but did not prevent decreases in F₀F₁-ATPase activity. Inhibition of PKC ϵ prevented oxidant-induced production of ROS and mitochondrial fragmentation, and reduced RPTC death. We conclude that activation of PKC ϵ mediates: 1) oxidant-induced changes in Ψ_m , decreases in mitochondrial respiration, complex I activity and ATP content, 2) mitochondrial fragmentation, and 3) RPTC death. In contrast, oxidant-induced inhibition of F₀F₁-ATPase activity is not mediated by PKC ϵ . These results show that, in contrast to the protective effects of PKC ϵ in the heart, PKC ϵ activation is detrimental to mitochondrial function and viability in RPTC and mediates oxidant-induced injury.

Keywords

Mitochondria; Protein kinase C- ϵ ; Renal proximal tubular cells; Respiratory chain; ATP synthase; Oxidant injury; Reactive oxygen species; Mitochondrial membrane potential

Acute renal injury is a common result of ischemic or nephrotoxic kidney damage. Renal proximal tubular cells (RPTC) are a major target of ischemia and nephrotoxicants due to

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their dependence on oxidative metabolism and mitochondria for ATP production. Oxidative stress is one of major mechanisms mediating renal cell injury caused by different drugs, toxicants, hypoxia, and ischemia/reperfusion (Koyner et al., 2008; Cachofeiro et al., 2008). Disruption of mitochondrial electron transfer through the respiratory chain often leads to increased production of reactive oxygen species (ROS) and initiates oxidative stress (Orrenius et al., 2007). Activation of protein kinase C- ϵ (PKC ϵ) has been implicated in a variety of pathological processes including ischemia/reperfusion, hypoxia, and oxidative stress in cardiomyocytes and pulmonary artery smooth muscle cells (Gopalakrishna et al., 2000; Rathore et al., 2006; Rathore et al., 2008). Numerous reports have demonstrated that activation of PKC ϵ mediates protection from ischemia/reperfusion injury in the heart (Liu et al., 1999; Mochly-Rosen et al., 2000; Ping et al., 1999a, 1999b, 2001, 2002; Baines et al., 2002; Gray et al., 1997, 2004; Budas et al., 2010; Jaburek et al., 2006). In ventricular myocytes, active PKC ϵ is present in mitochondria and colocalizes with mitochondrial ATP-dependent K⁺ channels (mitoK_{ATP}), which leads to their opening and increases in K⁺ flux into mitochondria (Jaburek et al., 2006). PKC ϵ interacts with the mitochondrial permeability transition pore (MPTP), inhibits its opening, and stabilizes cardiac mitochondria by preventing the loss of potential across the inner mitochondrial membrane (Baines et al., 2003; Ogbi et al., 2004, 2006; Guo et al., 2007). Finally, PKC ϵ associates with the cytochrome oxidase and this interaction increases cytochrome oxidase activity (Baines et al., 2003; Ogbi et al., 2004, 2006; Guo et al., 2007).

In contrast, the role of PKC ϵ and its subcellular targets in renal injury are less known. It has been shown that PKC ϵ activation inhibits Na⁺ transport in the cortical collecting duct (DeCoy et al., 1995). We have demonstrated that, in contrast to the protective role of PKC α , PKC ϵ activation is involved in mitochondrial dysfunction and decreases in active Na⁺ transport induced by oxidants in RPTC (Nowak, 2002, 2003; Nowak et al., 2004, 2011). Further, sustained activation of PKC ϵ in non-injured RPTC mediates mitochondrial dysfunction, production of reactive oxygen species (ROS), and results in cell death (Nowak et al., 2011). The model oxidant *tert*-butyl hydroperoxide (TBHP) activates PKC ϵ in RPTC and the translocation of active PKC ϵ to mitochondria is followed by mitochondrial dysfunction and decreases in active Na⁺ transport and Na⁺/K⁺-ATPase activity (Nowak et al., 2004). Inhibiting PKC ϵ and preventing PKC ϵ translocation to mitochondria without blocking PKC ϵ activation reduces mitochondrial dysfunction and accelerates recovery of mitochondrial function and active Na⁺ transport in injured RPTC (Nowak et al., 2004). However, specific targets of PKC ϵ in mitochondria of injured RPTC are not known. The goals of this study were to: 1) examine whether selective activation and inhibition of PKC ϵ alters mitochondrial function, ATP production and levels, mitochondrial morphology, and RPTC viability, and 2) determine mitochondrial functions and complexes targeted by PKC ϵ in oxidant-injured RPTC.

MATERIALS AND METHODS

Animals and materials

Female New Zealand White rabbits (2.0–2.5 kg) were purchased from Myrtle's Rabbitry (Thompson Station, TN). All animal procedures were approved by the Institutional Animal

Care and Use Committee at the University of Arkansas for Medical Sciences. The cell culture media were purchased from MediaTech Cellgro (Herndon, VA). Phospho-PKC ϵ and PKC ϵ antibodies were purchased from Upstate Biotechnology (Lake Placid, NY) and BD Transduction Laboratory (San Diego, CA), respectively. The sources of the other reagents have been described previously (Nowak and Schnellmann, 1995, 1996; Nowak et al., 2004).

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 previously described (Nowak and Schnellmann, 1995, 1996). 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 NaHCO₃, 15 mM *N*-2-hydroethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), 6 mM lactate (pH 7.4, 295 mosmol/kg), human transferrin (5 μ g/ml), selenium (5 ng/ml), hydrocortisone (50 nM), bovine insulin (10 nM), and L-ascorbic acid-2-phosphate (50 μ M).

Adenoviral constructs and amplification

Adenoviral vector encoding dominant negative (inactive) PKC ϵ (dnPKC ϵ) was constructed by mutations at the ATP-binding site (replacement of lysine with arginine at position 436) and the pseudosubstrate domain (replacement of alanine with glutamate at position 159) as described previously (Ping et al., 1999). These mutations destroyed the construct's kinase activity without changing the active conformation of PKC- ϵ (Ping et al., 1999). An aliquot of the adenovirus encoding dnPKC ϵ was provided by Dr. Peipei Ping (UCLA, Los Angeles, CA). PKC ϵ was made constitutively active by deletion of residues 154–163 of its inhibitory pseudosubstrate domain (Wotton et al., 1993). The adenoviral vector encoding caPKC ϵ was constructed as described previously (Strait and Samarel., 2000) and provided by Dr. Allen Samarel (Loyola University Medical Center, Maywood, IL). Adenoviruses were amplified in AD293 and HEK293 cells and purified as described previously (Nowak et al., 2011; Shaik et al., 2007). The multiplicity of infection (MOI) was determined by a viral dilution assay in HEK-293 cells.

TBHP treatment of RPTC

Adenoviral infections of confluent, quiescent RPTC were carried out 48 h prior to TBHP exposure (350 μ M, 45 min). PKC ϵ was inhibited in RPTC by overexpressing dnPKC ϵ using adenoviral vector encoding dnPKC ϵ (MOI=50). PKC ϵ activation was initiated by overexpressing caPKC ϵ using adenoviral vector encoding caPKC ϵ mutant (MOI=25). Infection with adenovirus encoding an empty vector (MOI = 25) was used as a negative control. At 45 min after TBHP treatment of RPTC cultures, the oxidant exposure was terminated by washing the monolayer with fresh, warm (37°C) culture medium. Control RPTC cultures were treated with the diluent (dimethyl sulfoxide, 0.1%). RPTC samples were taken at 4 and 24 hours after TBHP exposure to assess mitochondrial functions, cell viability, and changes in protein levels using immunoblotting.

LDH release

Lactate dehydrogenase (LDH) release from RPTC into the culture medium was used as a marker of plasma membrane permeabilization (cell lysis). LDH activity was determined spectrophotometrically at 340 nm by measuring NADH (0.3 mM) oxidation in the presence of 1.8 mM pyruvate as a substrate as described previously (Nowak et al., 2008, 2011).

Apoptosis

Apoptosis was evaluated by measuring phosphatidylserine externalization on the plasma membrane using the annexin V/propidium iodide-binding assay as previously described (Nowak et al., 2003).

Production of Reactive Oxygen Species

The carboxy-derivative of fluorescein, 5-(and-6)-carboxy-2',7'-dichlorodihydro-fluorescein (carboxy-H₂DCFDA), was used to assess oxidant generation in RPTC as described previously.[26] RPTC were loaded with 5 μ M carboxy-H₂DCFDA followed by treatment with TBHP for 45 minutes. Following incubation, the fluorescence of RPTC monolayers was analyzed by fluorometry. Final results were calculated and presented as % of fluorescence in control RPTC.

State 3 respiration

Oxygen consumption was measured polarographically using a Clark-type electrode as described previously.[25, 44, 50] State 3 respiration in RPTC energized by electron donors to the respiratory complexes I (5 mM glutamate + 5 mM malate), II (10 mM succinate + 0.1 μ M rotenone), and IV (1 mM ascorbate + 1 mM N,N,N',N'-tetramethyl-p-phenylenediamine) was measured at 37°C in digitonin-permeabilized RPTC (0.01% final concentration) in an assay buffer resembling an intracellular electrolyte milieu as described previously.[26, 47, Shaik et al., 2008 50, 51] At the concentration of 0.01%, digitonin permeabilizes RPTC plasma membrane without affecting mitochondrial membranes.[Shaik et al., 2008] State 3 respiration was initiated by addition of 0.4 mM ADP. State 4 respiration was measured following addition of oligomycin (0.6 μ g/ml) to RPTC respiring at state 3. Uncoupled respiration was measured by adding FCCP (0.5 μ M final concentration) to RPTC respiring at state 3.

ATP production rate and intracellular content

ATP production rate was measured in digitonin-permeabilized RPTC (0.01% final concentration) respiring at state 3 using the method of Borkan et al. (1993), modified as described previously (Nowak et al., 2006). The assay was carried out using 5 mM glutamate + 5 mM malate or 10 mM succinate + 0.1 μ M rotenone as the energizing substrates. The reaction was terminated by adding an aliquot of ice-cold perchloric acid (3% final concentration) and the suspension was spun down (15,000 $\times$ g for 1 min). The supernatant was neutralized to pH 7.5 and analyzed for ATP content using the luciferase method and ATP Bioluminescence Assay Kit HS II (Roche, Mannheim, Germany).

Intracellular ATP content was measured in freshly prepared RPTC lysates using the luciferase method and ATP Bioluminescence Assay Kit HS II as described previously (Nowak et al., 2004).

Mitochondrial membrane potential (Ψ_m)

Ψ_m was assessed by flow cytometry (FACSCalibur; BD Biosciences) as described previously using JC-1, a cationic dye that exhibits potential-dependent accumulation and formation of red fluorescent J-aggregates in mitochondria, which is indicated by the fluorescence shift from green (525 nm) to red (590 nm) (Nowak, 2002; Nowak et al., 2006). Fluorescence was measured using excitation by a 488-nm argon-ion laser. JC-1 monomer (green) and the mitochondrial J-aggregates (red) were detected separately in FL1 (emission, 525 nm) and FL2 (emission, 590 nm) channels, respectively. Ψ_m is presented as the ration of red to green fluorescence intensity.

Isolation of RPTC mitochondria

RPTC were homogenized in the ice-cold isolation buffer (10 mM Hepes, 225 mM mannitol, 75 mM sucrose, 2 mM EGTA, and 0.1% BSA, fatty acid free, pH 7.4). The homogenate was spun down at 1,000 g for 5 min at 4°C and the resulting supernatant was centrifuged at 15,000 g for 15 min at 4°C. The mitochondrial pellet was washed twice using the isolation buffer and centrifuged at 15,000 g for 10 min at 4°C. The final mitochondrial pellet was resuspended in the isolation buffer and used to measure state 3 respiration or in the assay buffer (10 mM KH_2PO_4 , 5 mM MgCl_2 , pH 7.2) used to determine the activities of respiratory complexes and F_0F_1 -ATPase.

Activity of respiratory complexes

Activities of complexes of the electron transport chain were measured in isolated mitochondria as described previously (Nowak et al., 2011). **Complex I** (NADH:Ubiquinone Oxidoreductase) activity was assayed spectrophotometrically at 30°C by following the oxidation of NADH (0.25 mM) at 340 nm in the assay buffer (10 mM KH_2PO_4 , 5 mM MgCl_2 , 0.25% BSA; pH 7.2) containing 62.5 μM ubiquinone, antimycin A (2 $\mu\text{g}/\text{ml}$), and mitochondria. The decrease in absorbance due to NADH oxidation was recorded for 3 min, rotenone (10 $\mu\text{g}/\text{ml}$) added, and the absorbance was recorded for another 2 min. Complex I activity was calculated as the rotenone-sensitive NADH:Ubiquinone Oxidoreductase activity. **Complex II** (Succinate:Ubiquinone Oxidoreductase) activity was measured at 30°C by following the reduction of dichlorophenolindophenol (0.25 mM) at 590 nm in the presence of 20 mM succinate, antimycin A (2 $\mu\text{g}/\text{ml}$), rotenone (10 $\mu\text{g}/\text{ml}$), 0.25% BSA, and ubiquinone (62.5 μM). **Complex III** (Ubiquinol:Cytochrome c Oxidoreductase) activity was assessed at 30°C, by following the reduction of cytochrome c (60 μM) at 550 nm in the assay buffer containing rotenone (10 $\mu\text{g}/\text{ml}$), decylubiquinol (50 μM), 0.25% BSA, and KCN (0.24 mM). The increase in absorbance due to the reduction of cytochrome c was recorded in the absence and presence of antimycin A (2 $\mu\text{g}/\text{ml}$). Complex III activity was calculated as the antimycin A-sensitive activity. **Complex IV** (cytochrome oxidase) activity was assessed by following the oxidation of reduced cytochrome c (90 μM) at 550 nm (30°C) in the assay buffer containing 10% BSA, antimycin A (2 $\mu\text{g}/\text{ml}$) in the presence and absence of

KCN (0.24 mM). Complex IV activity was calculated as KCN-sensitive cytochrome oxidase activity.

F₀F₁-ATPase activity

ATPase activity of the ATP synthase was determined in freshly isolated mitochondria by measuring the release of P_i from ATP by the method of Law et al.(1995) as described previously (Nowak et al., 2011). Each sample was run in the absence and presence of oligomycin (10 µg/ml) and the oligomycin-sensitive ATPase activity of F₀F₁-ATPase was calculated.

Mitochondrial morphology

RPTC monolayers were loaded with 100 nM MitoTracker Red 580 for 30 minutes at 37°C. The live monolayers were examined under a Zeiss fluorescent microscope (Axioscop) using a water immersion objective (63x) as described previously (Nowak et al., 2011). The images were captured using Zeiss AxioCam digital camera.

Immunoblotting

Phosphorylation and protein levels of PKC ϵ in mitochondria isolated from RPTC were assessed by immunoblot analysis as described previously (Nowak et al., 2004).

Protein concentration was determined using bicinchoninic acid assay with bovine serum albumin as the standard.

Statistical analysis

Data are presented as means $\pm$ S.E. and were analyzed for significance by the two-sided Student *t* test for independent samples or by ANOVA. Multiple means were compared using Fisher's protected least significance difference (LSD) test with a level of significance of $p < 0.05$. Renal proximal tubular cells isolated from an individual rabbit represented one experiment ($n=1$) consisting of data obtained from two to ten culture plates.

RESULTS

PKC ϵ translocates to mitochondria in oxidant-injured RPTC

Figure 1 shows that oxidant exposure in RPTC is associated with phosphorylation (indicative of activation) of PKC ϵ shortly after the exposure (Fig. 1A) and translocation of the phosphorylated PKC ϵ (p-PKC ϵ) to mitochondria (Fig. 1B). The levels of phosphorylated (active) PKC ϵ in RPTC decreased at 24 hours after oxidant exposure, but it remained at an elevated level in mitochondria of injured RPTC (Fig. 1A,B). Adenoviral delivery of the constitutively active PKC ϵ mutant (caPKC ϵ) to RPTC resulted in increased levels of total and phosphorylated PKC ϵ in mitochondria (Fig. 1C). TBHP exposure further increased the mitochondrial levels of active PKC ϵ suggesting increased translocation of caPKC ϵ to mitochondria in response to oxidant exposure (Fig. 1C). Overexpressing the inactive PKC ϵ mutant (dnPKC ϵ) in RPTC did not change the mitochondrial levels of PKC ϵ and blocked PKC ϵ translocation to mitochondria after TBHP treatment (Fig. 1C), which suggests that the non-phosphorylatable (permanently inactive) form of PKC ϵ does not

localize in mitochondria of RPTC. These data show that oxidant exposure induces activation of PKC ϵ in RPTC and that the active PKC ϵ localizes to mitochondria in oxidant-injured RPTC.

PKC ϵ activation mediates oxidant-induced cell death

TBHP exposure increased LDH release from RPTC ($19 \pm 3\%$ and $46 \pm 6\%$ of total LDH activity at 4 and 24 hours after TBHP exposure vs. $2.5 \pm 1\%$ in controls) (Fig. 2A). These data demonstrate the plasma membrane permeabilization in oxidant-injured RPTC indicative of cell death by oncosis. Expressing the constitutively active mutant of PKC ϵ (caPKC ϵ) increased LDH release to $16 \pm 1\%$ in non-injured RPTC and exacerbated LDH release to $78 \pm 2\%$ in TBHP-injured RPTC (Fig. 2A). Overexpressing the inactive mutant of PKC ϵ (dnPKC ϵ) and adenovirus carrying the empty vector had no effect on LDH release in non-injured RPTC ($5.7 \pm 1.1\%$) (Fig. 2A; data not shown). Overexpressing dnPKC ϵ blocked LDH release at 4h and reduced it at 24h following TBHP exposure (Fig. 2A). No apoptosis was observed in TBHP-injured RPTC regardless of PKC ϵ overexpression (Fig. 2B). These data show that PKC ϵ activation mediates oxidant-induced RPTC membrane permeabilization and lysis, and that inhibition of PKC ϵ activation offers protection against oxidant-induced cell death in RPTC.

Inhibition of PKC ϵ prevents oxidant generation

We demonstrated that TBHP exposure induces oxidative stress in RPTC (Nowak et al. 2008) and that sustained activation of PKC ϵ stimulates ROS production in non-injured RPTC (Nowak et al., 2011). Here, we tested whether PKC ϵ activation mediates TBHP-induced generation of ROS in RPTC. Overexpression of caPKC ϵ in non-injured RPTC increased ROS production 1.6-fold whereas TBHP exposure induced a 1.8-fold increase in ROS production in RPTC (Fig. 3). Overexpressing caPKC ϵ had no additional effect on TBHP-induced oxidant production. In contrast, inhibition of PKC ϵ activation by expressing dnPKC ϵ blocked ROS production in RPTC exposed to TBHP (Fig. 3). These data show that PKC ϵ activation mediates ROS generation and that blocking PKC ϵ activation prevents ROS production in oxidant-injured RPTC.

PKC ϵ activation disrupts integrity of the electron transport chain

Disruptions of mitochondrial electron transport chain are major contributors to the production of superoxide and reactive oxygen species derived from superoxide. We have previously shown that sustained activation of PKC ϵ in non-injured RPTC disrupts the integrity of the respiratory chain.[26] Here, we tested whether PKC ϵ activation is involved in oxidant-induced inhibition of the respiratory chain in injured RPTC. We also assessed which respiratory complexes are targeted by active PKC ϵ . Uncoupled respiration (used as a marker of integrity of the electron transport chain) in TBHP-injured RPTC decreased to 35% of controls following TBHP exposure whereas overexpressing caPKC ϵ in non-injured RPTC decreased uncoupled respiration to 57% of controls (Fig. 4A). Overexpressing caPKC ϵ exacerbated TBHP-induced decreases in uncoupled respiration (to 14% of controls) whereas expressing dnPKC ϵ blocked them (Fig. 4A). Expressing the empty vector had no effects on TBHP-induced decreases in uncoupled respiration (data not shown). These data show that PKC ϵ activation in RPTC decreases electron transport rate through the respiratory chain and

that PKC ϵ activation mediates TBHP-induced disruption of respiratory chain. In addition, these data suggest that PKC ϵ activation also mediates oxidant-independent decreases in the electron transfer through the respiratory chain.

To identify specific complexes of the respiratory chain that are targeted by PKC ϵ , we assessed state 3 respiration in the presence of different oxidative substrates that enter the respiratory chain through different complexes. TBHP exposure reduced (30% decrease in comparison with controls) state 3 respiration coupled to oxidation of substrates through complex I (Fig. 4B). Expressing caPKC ϵ in non-injured RPTC decreased complex I-coupled state 3 respiration by 32% in comparison with controls (Fig. 4B). Furthermore, expressing caPKC ϵ exacerbated (58% decrease in comparison with controls) whereas expressing dnPKC ϵ prevented TBHP-induced decreases in complex I-coupled state 3 respiration in injured RPTC (Fig. 4B). TBHP had no effect on state 3 respiration coupled to oxidation of substrates through complexes II and IV (Fig. 4C-D). However, activation of PKC ϵ by overexpressing caPKC ϵ decreased state 3 respiration coupled to complexes II and IV in both non-injured and TBHP-injured RPTC (Fig. 4C-D). Expressing the empty vector had no effect on state 3 respiration in non-injured and injured RPTC (data not shown).

State 4 respiration was not affected by TBHP exposure and/or PKC ϵ activation status (data not shown). TBHP exposure decreased the respiratory control ratio (the ratio of state 3/state 4 respiration; RCR) by 46% (2.4 ± 0.1 vs. 4.5 ± 0.3 in TBHP-treated and control RPTC, respectively). Inhibition of PKC ϵ activation restored RCR in TBHP injured RPTC (Fig. 6A).

These results demonstrate that 1) PKC ϵ activation decreases oxidation of substrates coupled to respiratory complexes I, II, and IV, 2) PKC ϵ activation mediates oxidant-induced decreases in oxidation of substrates through the respiratory complex I, and 3) activation of PKC ϵ exacerbates inhibitory effects of oxidant-injury on mitochondrial respiration coupled to complex I.

PKC ϵ activation mediates inhibition of complexes I and IV and oxidant-induced decreases in activity of complex I.

TBHP exposure decreased the activity of complex I by 40%, but had no effect on activities of other complexes (Fig. 5). Overexpressing caPKC ϵ exacerbated (81% decrease) and expressing dnPKC ϵ blocked TBHP-induced decreases in the activity of complex I (Fig. 5A). Furthermore, sustained activation of PKC ϵ by overexpressing caPKC ϵ decreased the activity of complex IV in non-injured and TBHP-injured RPTC (49% and 44% decreases, respectively) (Fig. 5D). Expressing caPKC ϵ had no effect on activities of complexes II and III (Fig. 5B-C). Overexpressing dnPKC ϵ had no effect on activities of complexes of the respiratory chain (Fig. 5A-D). These results demonstrate that transient activation of PKC ϵ mediates oxidant-induced decreases in activity of complex I and that sustained activation of PKC ϵ exacerbates TBHP effects on activities of complexes I and IV.

PKC ϵ activation mediates oxidant-induced changes in mitochondrial membrane potential (Ψ_m)

J-aggregate/JC-1 monomer ratio in RPTC was used as a marker of Ψ_m . Active PKC ϵ levels in mitochondria of TBHP-injured RPTC were the highest at 4h after injury (Fig. 1).

This event was accompanied by 1.8-fold increase in J-aggregate/JC-1 monomer ratio, indicative of mitochondrial hyperpolarization, and decreased RCR (Fig. 6A, B). Mitochondrial hyperpolarization was followed by decreases in Ψ_m and RCR at 24h after TBHP exposure (Fig. 6B, data not shown). Blocking PKC ϵ activation prevented TBHP-induced increases in Ψ_m at 4h and maintained Ψ_m at control levels at 24h after injury (Fig. 6B). This protective effect was associated with improved state 3 respiration, complex I activity, and viability in TBHP-injured RPTC (Figs. 2, 4 and 5). In contrast, persistent activation of PKC ϵ induced hyperpolarization of the mitochondrial membrane in non-injured RPTC (Fig. 6B). Thus, PKC ϵ activation and translocation to mitochondria mediates the hyperpolarization of the mitochondrial membrane and Ψ_m increases whereas PKC ϵ inhibition blocks these changes after oxidant exposure in RPTC.

PKC ϵ activation mediates oxidant-induced decreases in ATP production and content

Because the data show that PKC ϵ activation mediates mitochondrial dysfunction and cell death in RPTC, we tested whether PKC ϵ is also involved in the decreases in ATP production and content in oxidant-injured RPTC. TBHP exposure decreased ATP production to 60% of controls whereas sustained activation of PKC ϵ decreased ATP production to 55% of controls (Fig. 7A). Blocking PKC ϵ activation by expressing dnPKC ϵ had no effect on ATP production in non-injured RPTC, but it ameliorated the decreases in ATP production in TBHP-injured RPTC (Fig. 7A). In contrast, ATP production remained decreased (64% of controls) at 24h after TBHP exposure in RPTC overexpressing caPKC ϵ , whereas it returned to control levels in RPTC expressing the empty vector (data not shown). Thus, these data show that the active PKC- ϵ is involved in oxidant-induced decreases in ATP production and blocks recovery of ATP production in oxidant-injured RPTC.

TBHP exposure decreased intracellular ATP content to 50% and 41% of controls at 4h and 24h, respectively, after TBHP exposure (Fig. 7B, data not shown). Overexpressing caPKC ϵ decreased ATP content in non-injured RPTC and exacerbated the decreases in ATP content in TBHP-injured cells (Fig. 7B). In contrast, blocking PKC ϵ activation improved ATP content at 4h (78% of controls) after oxidant exposure and restored it at 24h after injury (Fig. 7B, data not shown). These results demonstrate that PKC ϵ activation mediates decreases in ATP content in oxidant-injured RPTC.

Subsequently, we tested whether F₀F₁-ATPase is a target of PKC ϵ and if the decreases in ATP production and content are mediated by PKC ϵ -dependent reduction in F₀F₁-ATPase activity in oxidant-injured RPTC. F₀F₁-ATPase activity in oxidant-injured RPTC declined to 55% of controls whereas activation of PKC ϵ reduced F₀F₁-ATPase activity to 72% of controls (Fig. 7C). Neither inhibition nor activation of PKC ϵ had any effect on TBHP-induced decreases in F₀F₁-ATPase activity (Fig. 7C). These data demonstrate that PKC ϵ does not mediate decreases in F₀F₁-ATPase activity in oxidant-injured RPTC. Together, our data show that PKC ϵ mediates mitochondrial dysfunction and decreases in ATP production in oxidant-injured RPTC through a mechanism independent of changes in F₀F₁-ATPase activity.

PKC ϵ activation mediates oxidant-induced mitochondrial fragmentation

Morphological examination of control RPTC revealed a network of elongated mitochondria distributed throughout the cytoplasm with a higher density of mitochondria around the nucleus (Fig. 8A). Exposure to TBHP resulted in fragmentation of mitochondria (Fig. 8B). Expressing caPKC ϵ in non-injured RPTC produced short, punctuated, and brightly stained mitochondria that often concentrated around the nucleus (Fig. 8C). These mitochondria had similar morphology as those in TBHP-injured RPTC (Fig. 8D). Expressing caPKC ϵ exacerbated TBHP-induced fragmentation of mitochondria, disrupted their network and subcellular distribution, and produced irregular clusters of fragmented mitochondria (Fig. 8D). In contrast, expressing dnPKC ϵ did not affect mitochondrial morphology in non-injured RPTC (Fig. 8E) and prevented TBHP-induced mitochondrial fragmentation and maintained the mitochondrial network similar to that observed in controls (Fig. 8F). These data demonstrate that PKC ϵ activation mediates mitochondrial fragmentation induced by oxidant exposure in RPTC.

DISCUSSION

It has been well established that PKC ϵ activation is involved in the mechanisms of cardioprotection and neuroprotection against injury caused by ischemia-reperfusion and hypoxia (Liu et al., 1999; Ping et al., 1999a; Gray et al., 1997; Mackay and Mochly-Rosen, 2001; Budas and Mochly-Rosen, 2007; Di-Capua et al., 2003). However, PKC ϵ activation is not protective in all cell types. In contrast to the protective actions of PKC ϵ in cardiac, neuronal, and cancer cells, activation of PKC ϵ mediates injury in epithelial cells that carry out trans-epithelial transport functions. This includes injury induced by nitric oxide to intestinal epithelial mucosal cells, vasopressin-stimulated decreases in active Na⁺ transport in renal cortical collecting ducts, and injury to RPTC (DeCoy et al., 1995; Nowak et al., 2004; Tepperman et al., 1999). Thus, the effect of PKC ϵ activation on specific cellular functions and cell survival appears to be cell type-dependent. Previously, we have shown that sustained activation of PKC ϵ in RPTC induces accumulation of PKC ϵ in mitochondria, mitochondrial dysfunction and fragmentation, energy deficits, and cell death (Nowak et al., 2011). We have also demonstrated that PKC regulates RPTC injury and recovery after injury and that PKC ϵ activation is involved in oxidant-induced changes in cellular respiration, active Na⁺-transport, and Na⁺/K⁺-ATPase activity in RPTC (Nowak et al., 2004). Inhibition of PKC ϵ activation accelerates recovery of active Na⁺-transport and Na⁺/K⁺-ATPase activity following oxidant injury in RPTC (Nowak et al., 2004). Those studies utilized pharmacological inhibitors and activators of PKC ϵ that are also known to affect other protein kinases and signaling pathways. The present study utilized constitutively active and inactive mutants of PKC ϵ to test whether oxidant-induced mitochondrial dysfunction and fragmentation, energy deficits, and RPTC death are mediated by PKC ϵ .

Active PKC ϵ associates with RPTC mitochondria. Activation and association of PKC ϵ with mitochondria in TBHP-injured RPTC is transient, subsides within 24 h after oxidant exposure, and is followed by the recovery of cellular respiration and ATP production (Nowak et al., 1998, 2004). Therefore, we tested whether the electron transport chain, Ψ_m , and/or F₀F₁-ATPase (three major components required for oxidative phosphorylation of

ADP) are the targets of PKC ϵ . Our previous report shows that the respiratory chain is a target of active PKC ϵ in non-injured RPTC (Nowak et al., 2011). This study shows that TBHP-induced decreases in uncoupled respiration (a marker of the respiratory chain's integrity) are mediated by PKC ϵ and that specific inhibition of PKC ϵ improves respiratory chain's integrity and mitochondrial maximum respiration in injured RPTC. Transient activation of PKC ϵ mediates TBHP-induced decreases in oxidation of substrates through complex I, but not oxidation through other complexes. The decreases in respiration are due to the inhibitory effect of active PKC ϵ on the activity of complex I. They are abrogated by inhibition of PKC ϵ and exacerbated by activation of PKC ϵ (overexpression of caPKC ϵ). PKC ϵ -mediated decrease in complex I activity is not due to reduced protein levels of complex I in the mitochondria because sustained activation of PKC ϵ does not change the levels of crucial subunits of responsible for the assembly and function of complex I (Nowak et al., 2011). It is still unknown whether PKC ϵ phosphorylates complex I or has an indirect effect on complex I, for example, by decreasing substrate availability for complex I. Proteomic analysis did not reveal any evidence of phosphorylation of complex I by PKC ϵ (data not shown). However, the large number of peptides making up complex I (46 peptides) and instability of phosphorylation residues of certain sites during sample processing and proteomic analysis makes this analysis difficult and the negative results of the analysis do not preclude an existence of such phosphorylation(s) in the living cell. Nevertheless, this is the first report demonstrating functional data documenting that PKC ϵ mediates oxidant-induced decreases in complex I activity.

In contrast to transient activation of PKC ϵ that reduces activity of complex I, sustained activation of the kinase by overexpressing its active mutant (caPKC ϵ) reduces substrate oxidation through complexes I, II and IV, and decreases activities of complexes I and IV (cytochrome oxidase). Furthermore, active PKC ϵ prevents recovery of mitochondrial function after oxidant injury. Thus, we conclude that transient activation of PKC ϵ mediates disruption of the respiratory chain's integrity in TBHP-injured RPTC through decreasing the activity of complex I and reducing oxidation of substrates through complex I. In contrast, sustained activation of PKC ϵ leads to the inhibition of complex IV in addition to inhibition of complex IV and prevents recovery of their activity after oxidant injury.

Translocation of protons from the mitochondrial matrix into the intermembrane space by respiratory complexes generates Ψ_m and the proton-motive force, both of which serve as the driving force for F₀F₁-ATPase rotation and ATP synthesis from ADP. Oxidant-induced PKC ϵ activation decreased the activity of F₀F₁-ATPase, ATP production, and ATP content in RPTC. Inhibition of F₀F₁-ATPase reduces utilization of the proton-motive force and leads to proton accumulation in the intermembrane space and increases in Ψ_m . Electrochemical gradient becomes too great for the complexes to pump protons against their concentration gradient and activities of complexes, electron flow, and respiration decrease. This is often followed by superoxide and ROS generation and subsequent collapse of Ψ_m . Dysfunction of complex I has particularly profound effects on Ψ_m as complex I provides approximately 40% of the proton-motive force for ATP synthesis (Brandt, 2006). Our results show decreases in F₀F₁-ATPase activity, mitochondrial hyperpolarization, and ROS generation at 4h followed by decreases in Ψ_m at 24h after TBHP exposure. TBHP-induced hyperpolarization of mitochondria was accompanied by decreases in complex I activity, state

3 respiration, ATP production, and ATP content. Thus, decreases in F_0F_1 -ATPase activity could have been the initiating factor leading to dysfunction of the respiratory chain. However, increases in ROS production and Ψ_m and decreases in respiration, complex I activity, and ATP production were alleviated by PKC ϵ inhibition whereas the decreases in F_0F_1 -ATPase activity were independent of PKC ϵ . These facts suggest that F_0F_1 -ATPase inhibition is not the initiating mechanism of TBHP- and PKC ϵ -mediated mitochondrial dysfunction and that the mitochondrial permeability pore, voltage-dependent anion channel (VDAC) mediating the transport of oxidative substrates, and/or the respiratory chain are the targets of active PKC ϵ in TBHP-injured RPTC. It has been shown that PKC ϵ associates with the cardiac mitochondrial permeability transition pore (MPTP) and inhibits pore opening (Baines et al., 2003).

Our data show that TBHP injury and PKC ϵ activation are associated with mitochondrial fragmentation. In contrast, PKC ϵ inhibition prevents this event suggesting that PKC ϵ activation mediates mitochondrial fragmentation in TBHP-injured RPTC. Recently, we have shown that PKC ϵ activation induces accumulation of the dynamin-related protein DRP1 in RPTC mitochondria and that this event precedes mitochondrial fragmentation (Nowak et al., 2011). DRP1 localization to mitochondria is required for mitochondrial fission and occurs after stress associated with apoptosis (Smirnova et al., 2001; Van der Bliek, 2009). Interestingly, TBHP exposure in RPTC caused oncosis and not apoptosis, which demonstrates that PKC ϵ -dependent mitochondrial fission occurs during oncosis as well. Thus, PKC ϵ regulates mitochondrial morphology and dynamics in oxidant-injured RPTC.

TBHP exposure produces RPTC oncosis, which is mediated by PKC ϵ activation. Blocking PKC ϵ activation by expressing dnPKC ϵ prevents PKC ϵ association with mitochondria, reduces mitochondrial dysfunction and fragmentation, and decreases RPTC death. In contrast, overexpressing caPKC ϵ exacerbates mitochondrial dysfunction and RPTC death. Interestingly, overexpressing caPKC ϵ using the same adenoviral vector protects endothelial cells against apoptosis (Steinberg et al., 2007). The role of PKC ϵ activation in mitochondrial dysfunction and RPTC injury remains in contrast with the protective function of PKC ϵ in cardiac tissue (Rathore et al., 2006; Liu et al., 1999; Ping et al., 1999a, 2001, 2002; Jaburek et al., 2006; Korzik et al., 2007; Liu et al., 2002; McCarthy et al. 2005). Several targets of PKC ϵ have been identified in cardiomyocytes to explain PKC ϵ -mediated cardioprotection, including cardiac Ca^{2+} and Na^{2+} channels (Hu et al., 2000; Xiao et al., 2001), $mitoK_{ATP}$ channels (Jaburek et al., 2006; Liu et al., 2002; Zhang et al., 2005; Ohnuma et al., 2002), adenine nucleotide translocase, VDAC, cytochrome oxidase and MPTP (Baines et al., 2003; Ogbi et al., 2004, 2006; Guo et al., 2007; McCarthy et al., 2005). Cardiomyocytes originate from mesodermal cells and are excitable cells. RPTC are epithelial and non-excitable cells whose main function is trans-epithelial transport. We speculate that PKC ϵ targets different mitochondrial proteins in RPTC or the phosphorylation of the same targets by PKC ϵ has different outcomes in RPTC mitochondria. We hypothesize that VDAC is one of the likely targets for PKC ϵ in RPTC mitochondria. Phosphorylation of this channel could be carried out during a transient translocation and association of PKC ϵ with mitochondria, such as observed after the exposure of RPTC to TBHP, and would not require importing PKC ϵ into the mitochondrial matrix. Phosphorylation induces VDAC closure, which leads to reduced import of substrates oxidized by the citric acid cycle and the complexes of the electron

transport chain. Our unpublished data show that the deletion of VDAC1 reduces the electron transport rate, complex I activity, and ATP content in renal tubular cells (data not shown). We speculate that overexpression of caPKCe results in persistent association of active PKCe with mitochondria, possibly and import of caPKCe into this organelle, which leads to decreases in activities of complex I and complex IV. This further limits the ability of mitochondria to maintain ATP production and exacerbates mitochondrial dysfunction and RPTC injury.

In conclusion, our study demonstrates that PKCe activation in RPTC mediates mitochondrial dysfunction, ROS production, energy deficits, mitochondrial fragmentation, and oxidant-induced cell death. The respiratory chain, specifically, complex I and Ψ_m are major mitochondrial targets of PKCe and their disruption leads to decreases in ATP production and content in oxidant-injured RPTC. Persistent activation of PKCe and its association with mitochondria exacerbates dysfunction of the electron transport chain by inhibiting complexes I and IV of the chain. In contrast to cardiomyocytes, PKCe activation in RPTC exacerbates injury and PKCe inhibition offers protection against RPTC injury by improving mitochondrial function, decreasing energy deficits, and increasing cell survival.

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Fig. 1.

Protein levels of phosphorylated PKCε (p-PKCε) and total PKCε (PKCε) in homogenates (upper panel) and mitochondria (middle panel) isolated from RPTC at different time points after *tert*-butyl hydroperoxide (TBHP; 0.35 mM, 45 min) exposure. Lower panel: Protein levels of phosphorylated PKCε (p-PKCε) and total PKCε (PKCε) in mitochondria of non-injured and TBHP-injured RPTC expressing the constitutively active (caPKCε) or inactive (dnPKCε) mutants of PKCε at 4 hours after TBHP exposure. The blots are representative of 3 independent experiments.

Fig. 2.

Lactate dehydrogenase (LDH) release (**A**) and annexin V binding (**B**) non-injured and TBHP-injured RPTC expressing the constitutively active (caPKCε) or inactive (dnPKCε) mutants of PKCε at 4 and 24 hours following TBHP (0.35 mM, 45 min) exposure. RPTC were subjected to adenoviral infection for 24 hours, the adenovirus was removed by aspirating media, and RPTC were incubated for subsequent 24 hours in fresh media prior to the exposure to TBHP. Cells positive for annexin V and negative for propidium iodide were considered apoptotic. Cells positive for propidium iodide and negative for annexin V were considered necrotic. Results are the average $\pm$ S. E. of 6–8 independent experiments (RPTC isolations). Values with superscripts are significantly different ($P < 0.05$) from their respective controls at 4 and 24 hours. Values with dissimilar superscripts at a given time point are significantly different from each other.

Fig. 3.

Reactive oxygen species (ROS) production in non-injured and TBHP-injured RPTC expressing the constitutively active (caPKC ϵ) or inactive (dnPKC ϵ) mutants of PKC ϵ . RPTC were subjected to adenoviral infection for 24 hours, the adenovirus was removed by aspirating media, and RPTC were incubated in fresh media for subsequent 24 hours prior to the exposure to TBHP. Results are the average $\pm$ S.E. of 5 independent experiments (RPTC isolations). * significantly different ($p < 0.05$) from controls.

Fig. 4.

A. Uncoupled respiration in non-injured and TBHP-injured RPTC expressing the constitutively active (caPKC ϵ) or inactive (dnPKC ϵ) mutants of PKC ϵ . **B-D:** State 3 respiration coupled to the oxidation of electron donors to complex I (**B**), complex II (**C**), and complex IV (**D**) of the electron transport chain in non-injured and TBHP-injured RPTC expressing the constitutively active (caPKC ϵ) or inactive (dnPKC ϵ) mutants of PKC ϵ . State 3 was measured in digitonin-permeabilized RPTC energized with 5 mM glutamate + 5 mM malate (**B**), 10 mM succinate + 0.1 μ M rotenone (**C**), and 1 mM ascorbate + 1 mM N,N,N',N'-tetramethyl-p-phenylenediamine (**D**) at 4 hours after TBHP (0.35 mM) exposure (45 min). Results are the average $\pm$ S.E. of 3–7 independent experiments (RPTC isolations). Values with dissimilar superscripts at a given time point are significantly different ($p < 0.05$) from controls and from each other.

Fig.5.

Activities of NADH:ubiquinone oxidoreductase (complex I, **A**), succinate:ubiquinone oxidoreductase (complex II, **B**), ubiquinol:cytochrome c oxidoreductase (complex III, **C**) and cytochrome oxidase (complex IV, **D**) in mitochondria isolated from control and TBHP-injured RPTC expressing the constitutively active (caPKCε) or inactive (dnPKCε) mutants of PKC-ε at 4 hours after TBHP (0.35 mM) exposure (45 min). RPTC were subjected to adenoviral infection for 24 hours, the adenovirus was removed by aspirating media, and RPTC were incubated in fresh media for subsequent 24 hours prior to the exposure to TBHP. Results are the average ± S.E. of 3–9 independent experiments (mitochondrial isolations). Values with dissimilar superscripts are significantly different ($p < 0.05$) from controls and from each other.

Fig. 6.

Respiratory control ratio (**A**) and mitochondrial membrane potential (Ψ_m , **B**) in non-injured and TBHP-injured RPTC expressing caPKCε or dnPKCε mutants of PKCε at 4 hours (**A**) and 4 and 24 hours following TBHP-induced injury. Ψ_m is expressed as the ratio of J-aggregate (red fluorescence) to monomeric form of JC-1 (green fluorescence). RPTC were subjected to adenoviral infection for 24 hours, the adenovirus was removed by aspirating media, and RPTC were incubated in fresh media for subsequent 24 hours prior to the exposure to TBHP. Results are the average $\pm$ S.E. of 6–9 independent experiments (RPTC isolations). Values with dissimilar superscripts are significantly different ($p < 0.05$) from their respective controls and from each other.

Fig. 7.

ATP production rate (**A**), intracellular ATP content (**B**) and F₀F₁-ATPase activity (**C**) in non-injured and TBHP-injured RPTC expressing the constitutively active (caPKCε) or inactive (dnPKCε) mutants of PKCε at 4 hours after TBHP (0.35 mM) exposure (45 min). RPTC were subjected to adenoviral infection for 24 hours, the adenovirus was removed by aspirating media, and RPTC were incubated in fresh media for subsequent 24 hours prior to the exposure to TBHP. ATP production was measured in the presence of 2 mM ADP in digitonin-permeabilized RPTC energized with 5 mM glutamate and 5 mM malate. Results are the average ± S.E. of 5–7 independent experiments (RPTC isolations). Values with dissimilar superscripts on a given day are significantly different ($P < 0.05$) from each other.

Fig. 8. Mitochondrial morphology in non-injured and TBHP-injured RPTC expressing the constitutively active (caPKC ϵ) or inactive (dnPKC ϵ) mutants of PKC ϵ at 4 hours after TBHP (0.35 mM) exposure (45 min). **A:** Controls. **B:** TBHP-injured RPTC. **C:** non-injured RPTC expressing caPKC ϵ . **D:** TBHP-injured RPTC expressing caPKC ϵ . **E:** non-injured RPTC expressing dnPKC ϵ . **F:** TBHP-injured RPTC expressing dnPKC ϵ . RPTC were subjected to adenoviral infection for 24 hours, the adenovirus was removed by aspirating media, and RPTC were incubated in fresh media for subsequent 24 hours prior to the exposure to TBHP. Representative images for each experimental group are shown. Original magnification, x630.