PROTEIN KINASE C MEDIATES REPAIR OF MITOCHONDRIAL AND TRANSPORT FUNCTIONS FOLLOWING TOXICANT-INDUCED INJURY IN RENAL CELLS.

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Role of PKC in the repair of renal cell functions

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Abbreviations: RPTC, renal proximal tubular cells; TBHP, *tert*-butylhydroperoxide; DCVC, S-(1,2-dichlorovinyl)-L-cysteine; ARF, acute renal failure; PKC, protein kinase C, PMA, phorbol-12-myristate-13-acetate; DMEM:F12, Dulbecco's Modified Eagle's Essential medium (DMEM) : Ham's F-12 nutrient mix; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; MBP, myelin basic protein; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis-(β-aminoethylether)-N,N,N',N'-tetraacetic acid; TBS, Tris-buffered saline; QO_2, oxygen consumption; FCCP, p-trifluoromethoxyphenyl-hydrazone; MGP, α-D-glucopyranoside.

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ABSTRACT

Previously, we have shown that renal proximal tubular cells (RPTC) recover physiological functions following injury induced by the oxidant, *tert*-butylhydroperoxide (TBHP), but not by the nephrotoxic cysteine conjugate dichlorovinyl-L-cysteine (DCVC). This study examined the role of protein kinase C (PKC) in the repair of RPTC functions following sublethal injury produced by these toxicants. Total PKC activity decreased 65% and 86% following TBHP and DCVC exposures, respectively, and recovered in TBHP-injured but not in DCVC-injured RPTC. Mitochondrial function, active Na^+ transport, and Na^+-dependent glucose uptake decreased following toxicant exposure and recovered in TBHP- but not in DCVC-injured RPTC. PKC inhibition decreased the repair of RPTC functions following TBHP injury. PKC activation promoted recovery of mitochondrial function and active Na^+ transport in TBHP- and DCVC-injured RPTC but had no effect on recovery of Na^+-dependent glucose uptake. We conclude that in RPTC: 1) total PKC activity decreases following TBHP and DCVC injury and recovers following TBHP but not after DCVC exposure, 2) recovery of PKC activity precedes the return of physiological functions after oxidant injury, 3) PKC inhibition decreases recovery of physiological functions, and 4) PKC activation promotes recovery of mitochondrial function and active Na^+ transport but not Na^+-dependent glucose uptake. These results suggest that the repair of renal functions is mediated through PKC-dependent mechanisms and that cysteine conjugates may inhibit renal repair, in part, through inhibition of PKC signaling.

Numerous toxicants can cause renal dysfunction through their ability to induce sublethal injury to renal proximal tubular cells (RPTC), which results in decreased normal cellular functions without producing cell death and loss. Mitochondrial dysfunction followed by ATP depletion, reduced metabolic functions, and decreases in Na^+-K^+-ATPase activity and active Na^+ transport are the major alterations in RPTC injured by different toxicants (Elfara et al., 1986; Lash and Anders, 1987; Schnellmann, 1988, Lash et al., 1995; Nowak et al., 1998; Nowak et al., 1999; Lash et al., 2001). Oxidative stress has been implicated in the mechanism of the renal dysfunction associated with ischemia/reperfusion and the nephrotoxicity of a number of drugs and environmental chemicals including halocarbons. *Tert*-butylhydroperoxide (TBHP) has been commonly used as a model oxidant in *in vitro* models of RPTC (Schnellmann, 1988; Kays and Schnellmann, 1995, Nowak and Schnellmann, 1997; Baliga et al., 1997, Nowak et al., 1998). Exposure of RPTC to TBHP results in the formation of reactive oxygen species, lipid peroxidation, mitochondrial dysfunction, and impaired transport functions (Borkan et al. 1989; Schnellmann, 1988; Nowak et al., 1998). Halogenated hydrocarbons represent a large group of environmental chemicals that are used as chemical intermediates, solvents, and pesticides and produce toxicity and acute renal failure (ARF) after their enzymatic conversion to reactive intermediates and the formation of nephrotoxic cysteine S-conjugates (Elfarra et al., 1986; Lash and Anders, 1987). Dichlorovinyl-L-cysteine (DCVC) is a model halocarbon nephrotoxicant that is selective for RPTC and produces injury through multiple mechanisms including mitochondrial dysfunction (Lash and Anders, 1987; Stevens et al., 1986; Vamvakas et al., 1992; van der Water et al., 1994; Chen et al., 2001).

The kidney has the potential for complete recovery from ARF (Toback, 1992). Using an *in vitro* model of primary cultures of RPTC, we have shown that RPTC proliferate and recover physiological functions following sublethal injury induced by the oxidant TBHP (Nowak et al., 1998). However, the repair of RPTC functions does not occur in sublethally injured RPTC after DCVC exposure (Nowak et al., 1999). DCVC also decreases synthesis of extracellular matrix proteins of the RPTC basement

membrane, including collagen IV, and disrupts localization of collagen binding integrins (Nowak et al., 2000; Nony et al. 2001; Nony and Schnellmann, 2002). Interestingly, EGF and pharmacological concentrations of ascorbic acid promote the repair of mitochondrial function and active Na^+ transport in DCVC-injured RPTC (Nowak et al., 1999; 2000; Nony et al., 2001). The promotion of RPTC repair by ascorbic acid is mediated, in part, by collagen IV and is associated with the re-localization of collagen-binding integrins to the basement membrane (Nony et al., 2001).

Protein kinase C (PKC), a family of serine/threonine protein kinases, controls 70% of the phosphorylating activity in renal proximal tubules (Kobryn et al., 1994) and regulates numerous physiological functions of renal epithelial cells including gluconeogenesis, Na^+/K^+-ATPase activity, and the transport of amino acids, glucose, sodium, potassium, chloride, phosphate, water, and organic anions and cations (Dempsey et al., 2000). Recent studies suggest that PKC is also involved in the regulation of cell survival and drug-induced cell injury (Dempsey et al., 2000). PKC is a target for some toxicants. Short-time exposure to oxidants activates PKC in some cell types (O'Brian et al., 1988; Palumbo et al., 1992; Abe et al., 1998). In contrast, longer exposures to oxidants inactivate PKC by modifying its catalytic domain (Gopalakrishna and Anderson, 1987).

PKC signaling regulates the regenerative processes in the liver following partial hepatectomy (Daller et al., 1994; Tessitore et al., 1995). PKC also has been implicated in the renal recovery following ARF (Alberti et al., 1993; LaPorta and Commoli, 1993) and in wound healing following mechanically-induced injury in renal tubular epithelial cells (Sponsel et al., 1995). Renal regeneration following toxicant exposure is associated with differential modulation of PKC isozymes. Activation of PKCδ, PKCε and PKCζ, but not PKCα or PKCβ occurs during compensatory renal hypertrophy induced by unilateral nephrectomy (Dong et al., 1993). Regeneration following folic acid- and DCVC-induced injury in the kidney is associated with down-regulation of PKCα and PKCζ (Dong et al., 1993; Zhang et al., 1993). Although PKC appears to be involved in renal recovery following toxic

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insult, it is not known what functions and processes are regulated by this kinase in RPTC. Therefore, the aim of this study was to determine whether PKC plays a role in the recovery of mitochondrial function, active Na^+ transport, and Na^+-coupled glucose uptake in RPTC following sublethal injury induced by two different toxicant, the oxidant, TBHP, and the model halocarbon, DCVC.

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MATERIALS AND METHODS

Animals. Female New Zealand White rabbits (2.0 – 2.5 kg) purchased from Myrtle's Rabbitry (Thompson Station, TN) were used in this study.

Chemicals and reagents. S-(1,2-dichlorovinyl)-L-cysteine (DCVC) was synthesized according to the method of Moore and Green (1988). L-Ascorbic acid-2-phosphate magnesium salt was purchased from Wako BioProducts (Richmond, VA). Protein kinase C assay kit, phorbol-12-myristate-13-acetate (PMA), and cell culture media were obtained from Gibco Invitrogen Corporation (Grand Island, NY). Cell culture hormones, t*ert*-butyl hydroperoxide, and protease and phosphatase inhibitors were obtained from Sigma (St. Louis, MO). Calphostin C was purchased from Calbiochem (La Jolla, CA). Cell permeable PKC peptide inhibitor, myristoylated protein kinase C fragment (20-28) (myr-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln; pseudosubstrate sequence from PKC) was supplied by BIOMOL Research Laboratories (Plymouth Meeting, PA). [γ-^{32}P]ATP (S.A. 3000 Ci/mmol) and methyl α-D-glucopyranoside, [glucose-^{14}C(U)] (S.A. 282 mCi/mmol) were purchased from Amersham Biosciences (Piscataway, NJ) and DuPont New England Nuclear (Boston, MA), respectively. PKC-α, PKC-δ, PKC-ε, and PKC-ζ antibodies were obtained from BD Transduction Laboratories (San Diego, CA). Antibodies against phosphorylated forms of PKC-α and PKC-ε were supplied by Upstate (Lake Placid, NY). Anti-mouse IgG coupled to horseradish peroxidase was supplied by Kirkegaard & Perry Laboratories (Gaithersburg, MD) and Supersignal Chemiluminescent Substrate by Pierce (Rockford, IL). The sources of the other reagents have been described previously (Nowak and Schnellmann, 1996).

Isolation of proximal tubules and culture conditions. Rabbit renal proximal tubules were isolated by iron oxide perfusion method and grown in 35 mm culture dishes in improved conditions as described previously (Nowak and Schnellmann, 1996). The purity of the renal proximal tubular S_1 and S_2 segments isolated by this method is approximately 96%. The culture medium was a 50:50 mixture of Dulbecco's modified Eagle's essential medium (DMEM) and Ham's F-12 nutrient mix without phenol red, pyruvate,

and glucose, supplemented with 15 mM $NaHCO_3$, 15 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (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 (0.05 mM) were added to the medium immediately before daily media change (2 ml/dish).

Toxicant treatment of RPTC monolayer. RPTC cultures reached confluence within 6 days and were treated with toxicants on the seventh day of culture. RPTC were treated with 0.2 mM TBHP for 45-50 minutes or with 0.2 mM DCVC for 1.5 hr to obtain approximately 25% cell death and loss. Following toxicant exposure, the remaining cellular monolayer was washed with fresh culture medium and cultured for the following 4 days. PKC inhibitors (100 nM calphostin C and 20 μM myristoylated protein kinase C (20-28) peptide) were added daily starting with the media change following TBHP exposure. PKC activator, 100 nM PMA, was added 30 min prior to toxicant and was present in the media for 24 hours following toxicant exposure. Samples of RPTC were taken at various time points after toxicant exposure for measurements of cellular functions.

Isolation of cytosolic and membrane fractions. RPTC samples were harvested at various time points during recovery period following toxicant exposure. Monolayers were washed 4 times with ice-cold PBS (pH 7.4) to remove all non-viable cells, cells scraped from the dishes, suspended in 1 ml PBS, and pelleted by centrifugation. RPTC pellet was resuspended in ice-cold isolation buffer (20 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$, 80 mM β-glycerophosphate, 2 mM EGTA, 2 mM EDTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 μg/ml aprotinin, 5 μg/ml pepstatin, and 5 μg/ml leupeptin). The cells were briefly sonicated (3x5 sec) on ice and centrifuged at 1,000 x g for 5 min to remove cell debris and nuclei. The supernatant was spun down at 100,000xg for 30 min at 4°C and the supernatant resulting from this centrifugation represented cytosolic fraction. The pellet was resuspended in the isolation buffer containing 1% Triton X-100, incubated on ice for 30 min, and centrifuged at 100,000xg for 30 min at 4°C. The supernatant resulting from this spin represented the particulate fraction.

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Measurement of PKC activity. The activity of PKC was based on measurement of the phosphorylation of a synthetic peptide derived from the myelin basic protein (MBP) sequence (4-14) as described by Yasuda et al. (1990). The N-terminal glutamine of this peptide has been acetylated in order to maintain the peptide's stability (Ac-MBP(4-14)). Specificity of the phosphorylation of Ac-MBP(4-14) by PKC was confirmed by using the PKC pseudosubstrate inhibitor peptide PKC(19-36), which acts as a potent inhibitor for this substrate. PKC activity was measured in the reaction mixture that contained 20 mM Tris (pH 7.5), 20 mM $MgCl_2$, 1.0 mM $CaCl_2$, 0.28 mg/ml phosphatidyl-L-serine, 10 μM PMA, 1 mM dithiotreitol, pepstatin, leupeptin, aprotinin (25 μg/ml each), 1 mM PMSF, 40 mM β-glycerophosphate, 0.3% Triton X-100, 50 μM Ac-MBP(4-14), 20 μM [γ-^{32}P]ATP, and sample. Specificity of the phosphorylation of Ac-MBP(4-14) by PKC was measured in the reaction mixture containing all above components and 20 μM PKC(19-36) peptide inhibitor. The reaction was carried out for 5 min at 30^{o}C and terminated by spotting an aliquot of reaction mixture onto P-81 phosphocellulose discs. Following washing the discs, γ-^{32}P incorporation into substrate was determined by liquid scintillation spectrometry. Specific PKC activity was calculated as the PKC(19-36) inhibitor-sensitive activity. Non-specific activity (background) was determined in samples incubated in the absence of activators (phosphatidylserine, PMA, $CaCl_2$).

Immunoblotting - Immunoblot analysis was used for the measurement of protein levels of PKC–α, PKC–δ, PKC–ε, and PKC–ζ in the cytosolic and particulate fractions of RPTC and for assessment of levels of phosphorylated forms of PKC-α and PKC-ε in total RPTC homogenates. Samples of cytosolic and particulate fractions and cell homogenates were lysed and boiled for 10 min in Laemmli sample buffer (60 mM Tris-HCl, pH 6.8 containing 2% SDS, 10% glycerol, 100 mM β-mercaptoethanol, and 0.01 % bromophenol blue) (Laemmli, 1970). Proteins were separated using SDS-PAGE. Following electroblotting of the proteins to a nitrocellulose membrane, blots were blocked for 1 hr in Tris-buffered saline (TBS) buffer containing 0.5% casein and 0.1% Tween 20 (blocking buffer), and incubated

overnight at 4°C in the presence of primary antibodies diluted in the blocking buffer. Following washing with TBS containing 0.05% Tween-20, the membranes were incubated for 1 hr with anti-rabbit or anti-mouse IgG coupled to horseradish peroxidase and washed again. The supersignal chemiluminescent system was used for protein detection. The results were quantified using scanning densitometry.

Oxygen consumption. RPTC monolayers were washed with 37°C culture medium and gently detached from the dishes with a rubber policeman, suspended in warm (37°C) culture medium and transferred to the oxygen consumption (QO_2) measurement chamber. QO_2 was measured polarographically in RPTC suspended in the culture medium using Clark type electrode as described previously (Nowak and Schnellmann, 1996; Nowak et al., 1998; 1999; 2000). Basal QO_2 was used as a marker of entire mitochondrial function. Oligomycin-sensitive QO_2 was used as a maker of oxidative phosphorylation. Oligomycin-sensitive QO_2 was determined following addition of oligomycin (1 μg/ml) and was calculated as the difference between basal and oligomycin-insensitive QO2. Uncoupled QO_2 was used as a marker of integrity of the electron transport chain and was measured following the addition of carbonyl cyanide p-trifluoromethoxyphenyl-hydrazone (FCCP, 1.5 μM).

Active Na^+ transport. Active Na^+ transport was measured using the ouabain-sensitive QO_2 as a marker as described previously (Nowak and Schnellmann, 1996; Nowak et al., 1998; 1999; 2000). Ouabain-sensitive QO_2 was measured as the difference between basal QO_2 and QO_2 in the presence of 1 mM ouabain.

Na^+-coupled glucose uptake. Na^+-coupled glucose uptake was assessed using the non-metabolizable glucose analogue methyl α-D-glucopyranoside (MGP) as described previously (Nowak and Schnellmann, 1996). MGP uptake was measured in glucose-free medium used for RPTC culture and corrected for Na^+-independent (phlorizin-insensitive) and zero time uptakes.

Protein assay. Protein concentration in all samples was determined using bicinchoninic acid assay (BCA) with bovine serum albumin as the standard.

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Statistical analysis. Data are presented as means ± SE and were analyzed for significance using ANOVA. Multiple means were compared using Student-Newman-Keuls test. Statements of significance were based on $P<0.05$. Renal proximal tubules isolated from an individual rabbit represented a separate experiment ($n = 1$) consisting of data obtained from 2 plates.

RESULTS

The effect of TBHP and DCVC exposures on PKC activity in the cytosolic and particulate fractions of RPTC. Total PKC activity decreased by 60% and 63% in the cytosolic and particulate fractions of sublethally-injured RPTC, respectively, during the 24 hr following TBHP exposure (Fig. 1A and B). PKC activity in the cytosol returned to control levels on day 2 of the recovery period (Fig. 1A). However, PKC activity in the particulate fraction of RPTC did not recover until day 4 following TBHP-induced injury (Fig. 1B).

DCVC exposure decreased PKC activity in both the cytosolic and particulate fractions of RPTC but the DCVC-induced decreases in PKC activity were slower that those induced by TBHP. At 24 hr following the DCVC treatment, PKC activity was decreased by 86% in both fractions (Fig. 2 A and B). In contrast to TBHP-induced injury, PKC activity in RPTC did not recover after DCVC-injury (Fig. 2 and 6).

PKC and the recovery of mitochondrial function. Basal, uncoupled, and oligomycin-sensitive QO_2s were used as markers of mitochondrial function in RPTC. Specifically, uncoupled QO_2 served as a marker of the electron transport rate whereas oligomycin-sensitive QO_2 was used as a marker of the oxidative phosphorylation. TBHP treatment decreased basal, uncoupled, and oligomycin-sensitive QO_2s by 41%, 52%, and 48%, respectively, at 4 hr following the exposure (Fig. 3 and 4). Respiratory functions of RPTC returned to control levels on day 4 of the recovery period (Fig. 3 and 4). The return of mitochondrial function followed the recovery of PKC activity in the cytosol and was accompanied by the recovery of PKC activity in the particulate fraction (Fig. 1, 3, and 4).

To determine whether PKC plays a role in the recovery of mitochondrial function following TBHP-induced injury RPTC were treated with a specific PKC inhibitor, calphostin C (100 nM), and the return of basal QO_2, examined during the recovery period. Calphostin C had no effect on basal QO_2 in control RPTC or on the decrease in QO_2 in TBHP-treated RPTC, but it inhibited the recovery of this

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function in sublethally-injured RPTC (Fig. 3). Likewise, the treatment of RPTC with another specific PKC inhibitor (PKC (20-28)), a peptide derived from pseudosubstrate sequence of PKC, prevented the recovery of basal QO_2 (Fig. 4A). Furthermore, PKC (20-28) inhibited the return of oligomycin-sensitive and uncoupled QO_2s in TBHP-injured RPTC (Fig. 4 B and C).

Experiments were also performed to determine whether activation of PKC accelerates the repair of mitochondrial function following TBHP injury. PMA (100 nM) was used to activate PKC in RPTC prior to toxicant-induced injury. Protein levels of PKCα and PKCε in the particulate fraction of RPTC increased 1.3-fold and 2.5-fold, respectively, within 2 to 5 minutes of PMA treatment (Fig. 5A). Protein levels of PKCα and PKCε in the cytosolic fraction of PMA-treated RPTC decreased concomitantly (Fig. 5A). The levels of phosphorylated forms of PKCα and PKCε were increased 2.5 and 4.4-fold, respectively, in RPTC treated for 5 minutes with 100 nM PMA whereas the total levels of PKCα and PKCε remained unaffected (Fig. 5B). These data show that PMA induces phosphorylation and translocation of PKCα and PKCε from the cytosol to the particulate fraction, which suggests that PMA activates these PKC isozymes. No phosphorylation and translocation of PKCδ and PKCζ occurred in the presence of PMA in RPTC (data not shown). PMA treatment prior to TBHP exposure did not protect against the decrease in mitochondrial function following the exposure (Fig. 5B). However, the recovery of mitochondrial function in PMA-treated RPTC occurred on day 2 following TBHP injury and was accelerated in comparison with the recovery of this function in RPTC pretreated with the vehicle (day 4) (Figs. 5B and 4A).

DCVC exposure decreased basal QO_2 in sublethally injured RPTC by 33% and 47% at 4 and 24 hr, respectively. Oligomycin-sensitive QO_2 decreased by 77% (21.7 ± 1.1 vs. 5.1 ± 3.2 nmol O_2/min/mg protein in control and DCVC-injured RPTC, respectively) and uncoupled QO_2 decreased 63% (82.3 ± 5.6 vs. 30.1 ± 3.7 nmol O_2/min/mg protein in control and DCVC-injured RPTC, respectively) at 4 hr

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following the exposure. No repair of basal QO_2 occurred following DCVC exposure (Fig. 5C). However, treatment with PMA prior to DCVC exposure resulted in the return of basal QO_2 within 2 days in injured RPTC (Fig. 5C).

These results demonstrate that inhibition of PKC prevents the repair of mitochondrial function following toxicant injury in RPTC and that activation of PKC prior to toxicant exposure promotes recovery of respiratory functions. Thus, these data suggest that PKC plays a role in the repair of mitochondrial function following toxic insult in RPTC.

PKC and the recovery of active Na^+ transport. Active Na^+ transport was used as a marker of the basolateral membrane function and was assessed by measurement of ouabain-sensitive QO_2. Active Na^+ transport is an ATP-consuming process as it is driven by Na^+/K^+-ATPase. The consumption of oxygen associated with production of ATP required for maintaining the Na^+/K^+-ATPase activity and active Na^+ transport accounts for approximately 50% of basal oxygen consumption in RPTC. Ouabain, a specific Na^+/K^+-ATPase inhibitor, decreases oxygen consumption by the amount of oxygen associated with the synthesis of ATP that is consumed by Na^+/K^+-ATPase and active Na^+ transport. Therefore, ouabain-sensitive portion of QO_2 is an indirect indicator of active Na^+ transport.

Ouabain-sensitive QO_2 decreased (51%) at 4 hr following TBHP exposure and returned on day 4 of the recovery period (Fig. 6A). Inhibition of PKC (using calphostin C or PKC (20-28)) did not affect decreases in ouabain-sensitive QO_2 at 4 hr following TBHP injury but prevented the return of this function on day 4 of the recovery period (Figs. 6A and 6B). In contrast, the return of ouabain-sensitive QO_2 in RPTC treated with PMA prior to TBHP exposure occurred on day 2 and was accelerated in comparison with RPTC exposed to TBHP only (Fig. 7A).

DCVC exposure in RPTC resulted in 40% decrease in ouabain-sensitive QO_2 at 4 hr of the recovery period (Fig. 7B). Ouabain-sensitive QO_2 did not recover over time (Fig. 7B). However,

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treatment with PMA prior to DCVC exposure resulted in the return of ouabain-sensitive QO_2 in RPTC within 2 days following the exposure (Fig. 7B).

These data show that PKC inhibition prevents the recovery of active Na^+ transport following oxidant injury in RPTC and that PKC activation promotes the repair of active Na^+ transport following toxicant exposure.

Interestingly, inhibition of PKC by PKC (20-28) peptide decreased ouabain-sensitive QO_2 at 4 hr, whereas calphostin C had no effect on this function in control RPTC (Fig. 6). This could be explained by dissimilar sensitivity of different PKC isozymes to these two inhibitors. PKC (20-28) is more specific and general PKC inhibitor whereas calphostin C is thought to inhibit primarily novel (Ca^{2+}-independent and PMA-sensitive) isozymes of PKC.

PKC and the recovery of Na^+-dependent glucose uptake. Na^+-dependent glucose uptake was used as marker of the brush-border membrane function in RPTC. TBHP and DCVC decreased Na^+-dependent glucose uptake by 66% and 63%, respectively, at 4 hr following the exposure (Fig. 8A and 8B). Na^+-dependent glucose uptake recovered following TBHP- but not following DCVC-induced injury (Fig. 8A and 8B). Inhibition of PKC with calphostin C prior to TBHP exposure inhibited the return of Na^+-dependent glucose uptake in regenerating RPTC (Fig. 8A). Furthermore, 4-day treatment of RPTC with calphostin C inhibited Na^+-dependent glucose uptake by 89% (Fig. 8A).

Activation of PKC using PMA did not accelerate the repair of Na^+-dependent glucose uptake in TBHP-injured RPTC (Fig. 8B). Likewise, treatment of RPTC with PMA prior to DCVC injury did not promote the recovery of Na^+-dependent glucose uptake (Fig. 8B).

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DISCUSSION

Toxicant-induced renal dysfunction is often caused by chemically produced sublethal injury to RPTC, which decreases the physiological functions of renal proximal tubules without producing apparent cell death. It has been also proposed that some toxicants cause renal dysfunction not only by inducing injury but also by inhibiting RPTC regeneration (Counts et al., 1995). Our previous study showed that RPTC in primary culture recover their functions following sublethal injury induced by an oxidant (TBHP) and that this repair is mediated by autocrine mechanisms (Nowak et al., 1998). In contrast, physiological functions do not recover in RPTC following injury produced by the halocarbon nephrotoxicant DCVC (Nowak et al., 1999). This suggested that DCVC disrupts the autocrine mechanisms of RPTC repair. The repair of mitochondrial function and active Na^+ transport but not Na^+-dependent glucose transport in DCVC-injured RPTC are promoted by EGF, pharmacological concentrations of ascorbic acid, and collagen IV (Nowak et al., 1999; Nowak et al., 2000; Nony et al., 2001). Therefore, our previous data suggested that different toxicants affect the mechanisms of RPTC repair in a differential manner. The present study examined the role of one of the signaling molecules, PKC, in the recovery of RPTC functions following toxicant-induced injury.

The transmission of intracellular signals is mediated by a network of interacting proteins that regulate a variety of cellular processes and functions. PKC, a family of serine/threonine protein kinases, is a critical element of this network (Dempsey et al., 2000). Toxicants have been implicated in the alterations in PKC activity. Our results show that TBHP injury in RPTC is associated with decreases in total PKC activity. These data remain in contrast to early and transient activation of PKC in neuronal and endothelial cells in response to short-term exposure to an oxidant (O'Brian et al., 1988; Palumbo et al., 1992; Abe et al., 1998). In contrast, longer exposure to oxidative stress modifies the catalytic domain of PKC and inactivates PKC (Gopalakrishna and Anderson, 1987), which is consistent with our results in RPTC. DCVC-induced decreases in total PKC activity in our model are

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in agreement with the down-regulation of renal PKCα following an exposure to DCVC *in vivo* and with the inhibition of PKC activity in the kidney by exposure to folic acid *in vivo* (Dong et al., 1993).

PKC has also been implicated in regeneration of different tissues and organs. Liver regeneration following partial hepatectomy and carbon tetrachloride injury is associated with the activation of PKCα, PKCβ, PKCδ, and PKCζ (Daller et al., 1994; Tessitore et al., 1995; Sasaki et al., 1987). In the kidney, the activations of PKCδ, PKCε, and PKCζ, but not PKCα or PKCβ occur during compensatory renal hypertrophy induced by unilateral nephrectomy (Dong et al., 1993). PKC activation also plays a role in the acceleration of wound healing following mechanical injury in renal tubular epithelial cells (Sponsel et al., 1995). In our RPTC model, decreased PKC activity following TBHP- and DCVC-induced injury was accompanied by major decreases in the mitochondrial function, active Na^+ transport and Na^+-dependent glucose uptake. The repair of RPTC functions following TBHP-induced injury was preceded by the recovery of total PKC activity. On the other hand, the lack of repair of RPTC functions in DCVC-injured RPTC was associated with the lack of return of the PKC activity. Furthermore, inhibition of PKC prevents the return of mitochondrial function, active Na^+ transport and Na^+-dependent glucose uptake in TBHP-injured RPTC, which suggests that PKC plays a role in the repair of RPTC functions following the oxidant injury.

Two dissimilar PKC inhibitors have been used in this study, the chemical inhibitor calphostin C and a short peptide derived from PKCα and PKCβ pseudosubstrate sequence. PKC(20-28) inhibitor decreased whereas calphostin C had no effect on mitochondrial function and active Na^+ transport in control RPTC, which could be explained by higher specificity of PKC(20-28) towards PKCα and PKCβ. The fact that calphostin C, which is rather an inhibitor of novel PKC isozymes (δ, ε, η, μ, and θ), did not have any effect on these functions in control RPTC suggests that the maintenance of these functions is dependent on classical PKC isozymes.

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The lack of functional repair in RPTC treated with PKC inhibitors was not due to toxic effects produced by these compounds. These inhibitors did not potentiate decreases in mitochondrial function and active Na^+ transport in TBHP-injured RPTC. Furthermore, both inhibitors produced a similar degree of inhibition of the repair of mitochondrial function and active Na^+ transport. Interestingly, a 4-day treatment of control RPTC with calphostin C decreased Na^+ dependent glucose uptake in RPTC by 89%, which suggests that functional PKC is necessary to maintain Na^+-dependent glucose transport in control RPTC.

Because our data suggested that functional PKC is a critical for the recovery of RPTC functions following a toxic insult, we hypothesized that activation of PKC prior to toxicant exposure may prevent the decreases in RPTC functions or promote the repair of some or all RPTC functions. Activation of PKC is associated with redistribution from the cytosol to cellular membranes and organelles. This event is necessary for phosphorylation of various intracellular proteins targeted by PKC. Previous studies suggested that renal regeneration following toxicant exposure is associated with differential modulation of PKC isozymes, which may be associated with different functions of these isozymes in the process of RPTC regeneration (Dong et al., 1993). At the present, however, the function of individual PKC isozymes in the repair of these functions is unknown. In our RPTC model, among 4 major PKC isozymes present in RPTC (PKCα, PKCδ, PKCε and PKCζ), only PKCα and PKCε were phosphorylated and redistributed from the cytosol to the particulate fraction in response to PMA treatment. In contrast, PMA had no effect on the activation of ERK1/2 (data not shown). This suggested that only PKCα and PKCε were activated by PMA. PMA pretreatment had no effect on the decrease in mitochondrial function induced by TBHP or DCVC, which demonstrates that PKC activation does not protect against mitochondrial dysfunction caused by these nephrotoxicants. However, the recovery of mitochondrial function was accelerated in TBHP-injured RPTC pretreated with PMA. Furthermore, PKC activation abolished inhibition of the repair of mitochondrial function in

DCVC-injured RPTC. These results show that PKC plays an important role in the repair of mitochondrial function and suggest that PKCα and/or PKCε are involved in these processes.

Likewise, our data show that the repair of active Na^+ transport following toxicant injury is controlled by PKC. In contrast to the lack of repair of active Na^+ transport in DCVC-injured RPTC, the recovery of this function occurred in DCVC-injured RPTC pretreated with PMA. Moreover, PKC activation accelerated the repair of active Na^+ transport following TBHP injury, which was not due to the protection against TBHP- and DCVC-induced decreases in this function because the degree of injury was equivalent in RPTC treated with toxicants and RPTC treated with both PMA and toxicants.

Active Na^+-transport in RPTC is mediated through Na^+/K^+-ATPase, which is regulated through phosphorylation by PKC and PKA (Bertorello and Aperia, 1989; Feschenko and Sweadner, 1994; Chibalin et al., 1997). Phosphorylation of Na^+/K^+-ATPase leads to internalization of the pump into endosomes and decreased expression on the basolateral membrane (Chibalin et al., 1997). Our data suggest that this is also true in our model as the short-term exposure to PMA (which results in PKC activation) decreased ouabain-sensitive QO_2 in control RPTC. In contrast, long-term exposure to PMA (24 hr), which does not activate PKC (Fig. 5A), had no effect on ouabain-sensitive QO_2. PKC activation appears to be necessary for promoting the recovery of active Na^+ transport following toxicant exposure. Previously, it has been proposed that the endosomes may constitute reservoirs during Na^+/K^+ pump synthesis and degradation (Chibalin et al., 1997). It is likely that Na^+/K^+-ATPase can be either protected or repaired with higher efficiency in the endosomes and is, therefore, available for reassembly on the basolateral membrane sooner during the recovery process. It is also likely that the accelerated return of active Na^+ transport by PKC activation is due to the promotion of mitochondrial function and ATP availability. Na^+/K^+-ATPase activity requires constant supply of ATP. The lack of recovery of active Na^+ transport in DCVC-treated RPTC is associated with decreased ATP levels (Nowak et al., 1999). Promotion of the recovery of mitochondrial function in DCVC-injured

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RPTC treated with PMA may support the recovery of active Na^+ transport by supplying ATP for Na^+/K^+-ATPase function.

In contrast, activation of PKC did not promote the repair of Na^+-dependent glucose uptake in DCVC-injured RPTC. These results suggest that the disruption of Na^+ homeostasis following DCVC injury is not the exclusive mechanism responsible for the inhibition of repair of Na^+-dependent glucose uptake. Furthermore, our present data suggest that the recovery of this function also involves PKC-independent mechanisms.

In conclusion, our results show that toxicant-induced injury in RPTC causes decreases in mitochondrial function, active Na^+ transport and Na^+-dependent glucose uptake and that these events are associated with decreases in PKC activity. PKC inhibition blocks whereas PKC activation promotes the repair of mitochondrial function and active Na^+ transport. In contrast, activation of PKC does not stimulate the recovery of Na^+-dependent glucose uptake. Thus, our data show that PKC mediates the repair of mitochondrial function and active Na^+ transport following toxicant injury and suggest that PKCα and/or PKCε are involved in this regulation.

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Footnotes

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FIGURE LEGENDS

Figure 1. Time-dependent changes in total PKC activity in cytosolic (A) and particulate (B) fractions of RPTC following TBHP-induced injury (0.2 mM; 45 min). Samples of RPTC were collected at different time points after TBHP exposure and PKC activity measured in cytosolic and particulate fractions as described in the Methods. Data are presented as means $\pm$ SE, n = 4 separate RPTC cultures. * $P<0.05$, significantly different from controls.

Figure 2. Time-dependent changes in total PKC activity in cytosolic (A) and particulate (B) fractions of RPTC following DCVC-induced injury (0.2 mM; 90 min). Samples of RPTC were collected at different time points after DCVC exposure and PKC activity measured in cytosolic and particulate fractions as described in the Methods. Data are presented as means $\pm$ SE, n = 5 separate RPTC cultures. * $P<0.05$, significantly different from controls.

Figure 3. The effect of PKC inhibition (Calphostin C, 100 nM) on the recovery of overall mitochondrial function in RPTC following TBHP-induced injury (0.2 mM; 45 min). Data are presented as means $\pm$ SE, n = 3 separate RPTC cultures. Values with dissimilar superscripts on a given day are significantly different ($P<0.05$) from each other.

Figure 4. The effect of PKC inhibition (myristoylated protein kinase C (20-28) peptide derived from pseudosubstrate sequence of PKC, 20 μM) on the recovery of overall mitochondrial function (A), oxidative phosphorylation (A), and electron transport rate (C) in RPTC following TBHP-induced injury (0.2 mM; 45 min). Data are presented as means $\pm$ SE, n = 3 separate RPTC cultures. Values with dissimilar superscripts on a given day are significantly different ($P<0.05$) from each other.

Figure 5. The effect of PKC activation (PMA, 100 nM) on protein levels of PKCα and PKCε in cytosolic and particulate fractions of RPTC (A) and phosphorylated and total protein levels of PKCα and PKCε in total cell homogenates following 5 minute exposure of RPTC to 100 nM PMA (B). C – controls, E – RPTC treated with diluent (0.1% ethanol), P – RPTC treated with 100 nM PMA for 5

minutes. C. The effect of PKC activation (PMA, 100 nM) on the recovery of overall mitochondrial function in RPTC following TBHP-induced injury. D. The effect of PKC activation (PMA, 100 nM) on the recovery of overall mitochondrial function in RPTC following DCVC-induced injury. Data are presented as means ± SE, n = 3 separate RPTC cultures. Values with dissimilar superscripts on a given day are significantly different ($P<0.05$) from each other.

Figure 6. Decrease in the recovery of active Na^+ transport by inhibition of PKC in TBHP-injured RPTC. Two dissimilar inhibitors of PKC were used in these studies: A. Myristoylated protein kinase C (20-28) peptide, 20 μM and B. Calphostin C, 100 nM. Data are presented as means ± SE, n = 3 separate RPTC cultures. Values with dissimilar superscripts on a given day are significantly different ($P<0.05$) from each other.

Figure 7. Promotion of the recovery of active Na^+ transport by PKC activation (PMA, 100 nM) in TBHP-injured (A) and DCVC-injured (B) RPTC. Data are presented as means ± SE, n = 3 separate RPTC cultures. Values with dissimilar superscripts on a given day are significantly different ($P<0.05$) from each other.

Figure 8. Decrease in the recovery of Na^+-dependent glucose uptake by inhibition of PKC (calphostin c, 100 nM) in TBHP-injured RPTC (A). Lack of an effect of PKC activation (PMA, 100 nM) on the recovery of Na^+-dependent glucose uptake in TBHP- and DCVC-injured RPTC (B). Data are presented as means ± SE, n = 3 separate RPTC cultures. Values with dissimilar superscripts on a given day are significantly different ($P<0.05$) from each other.

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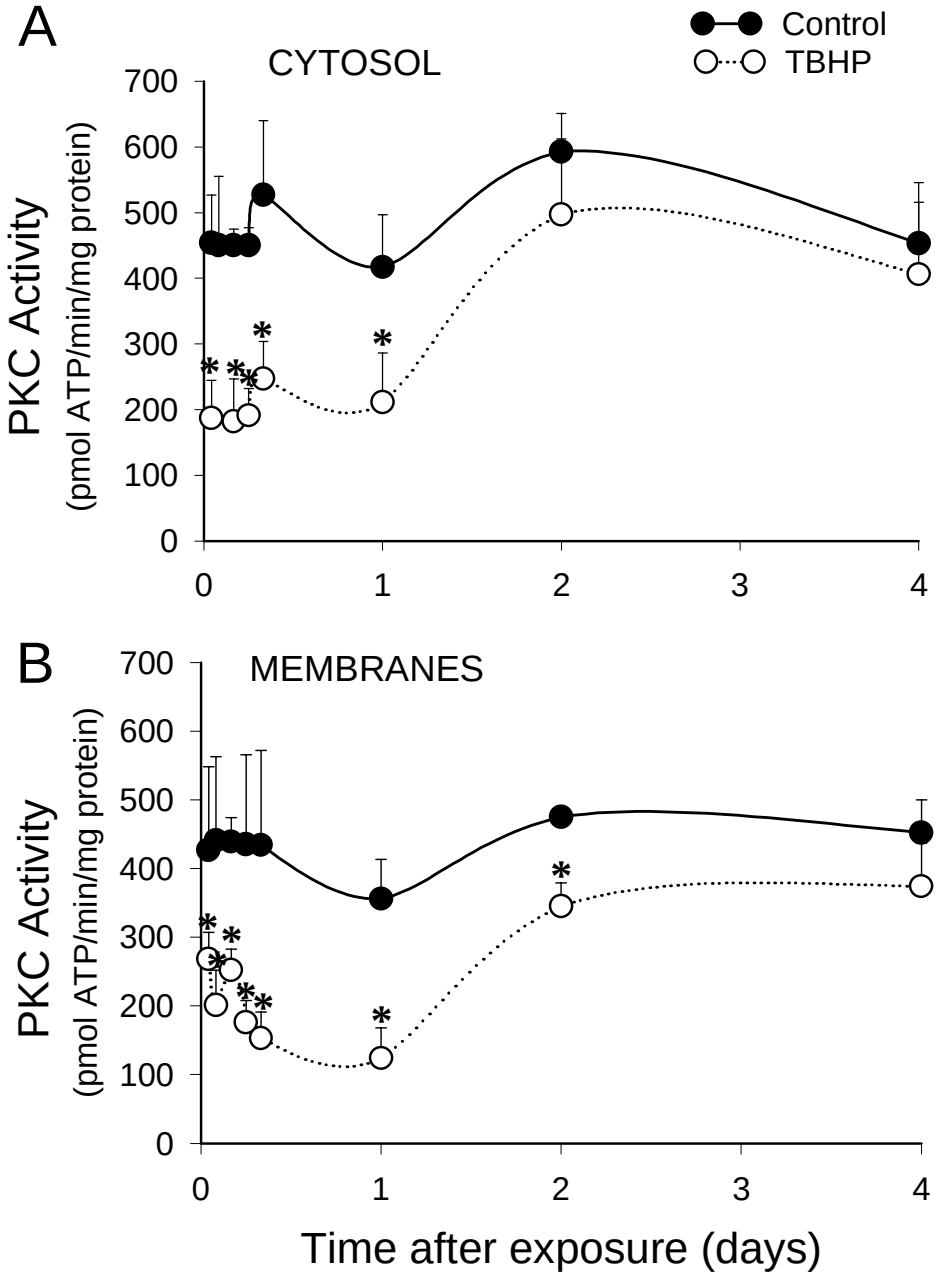

Figure 1

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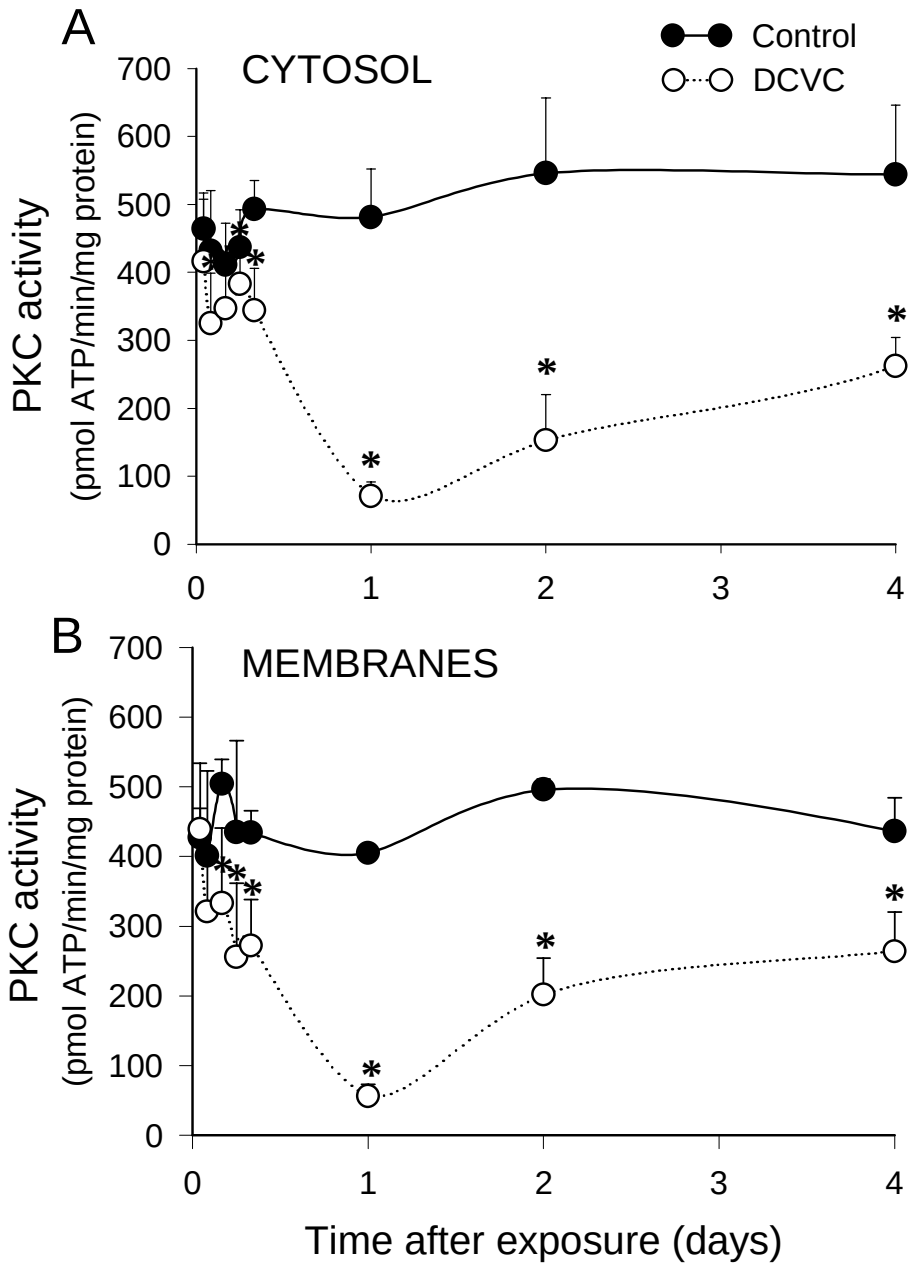

Figure 2

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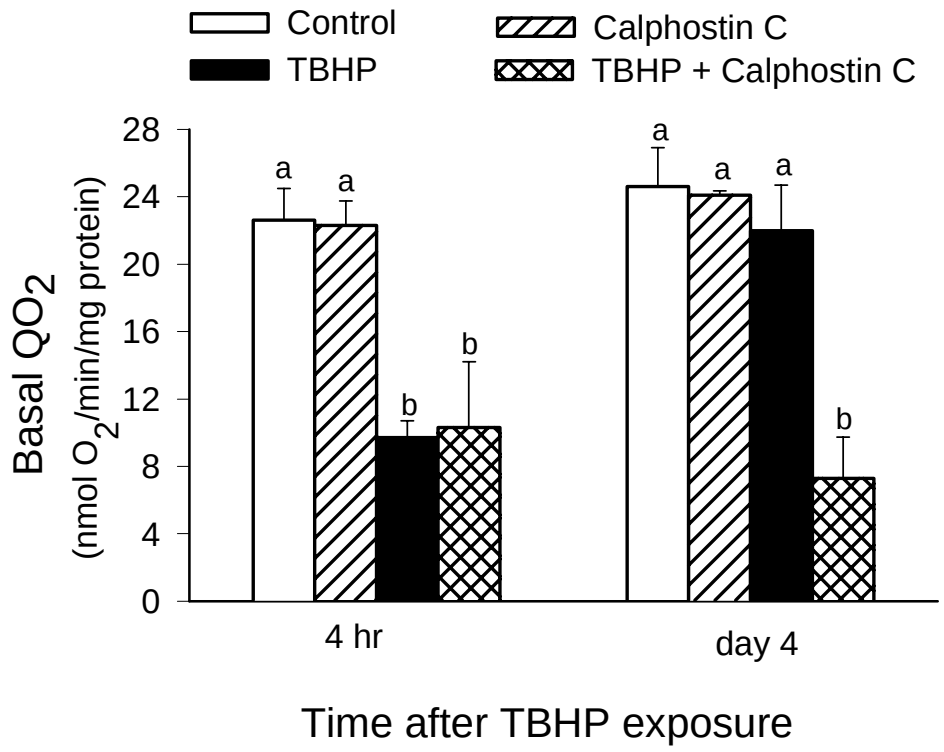

Figure 3

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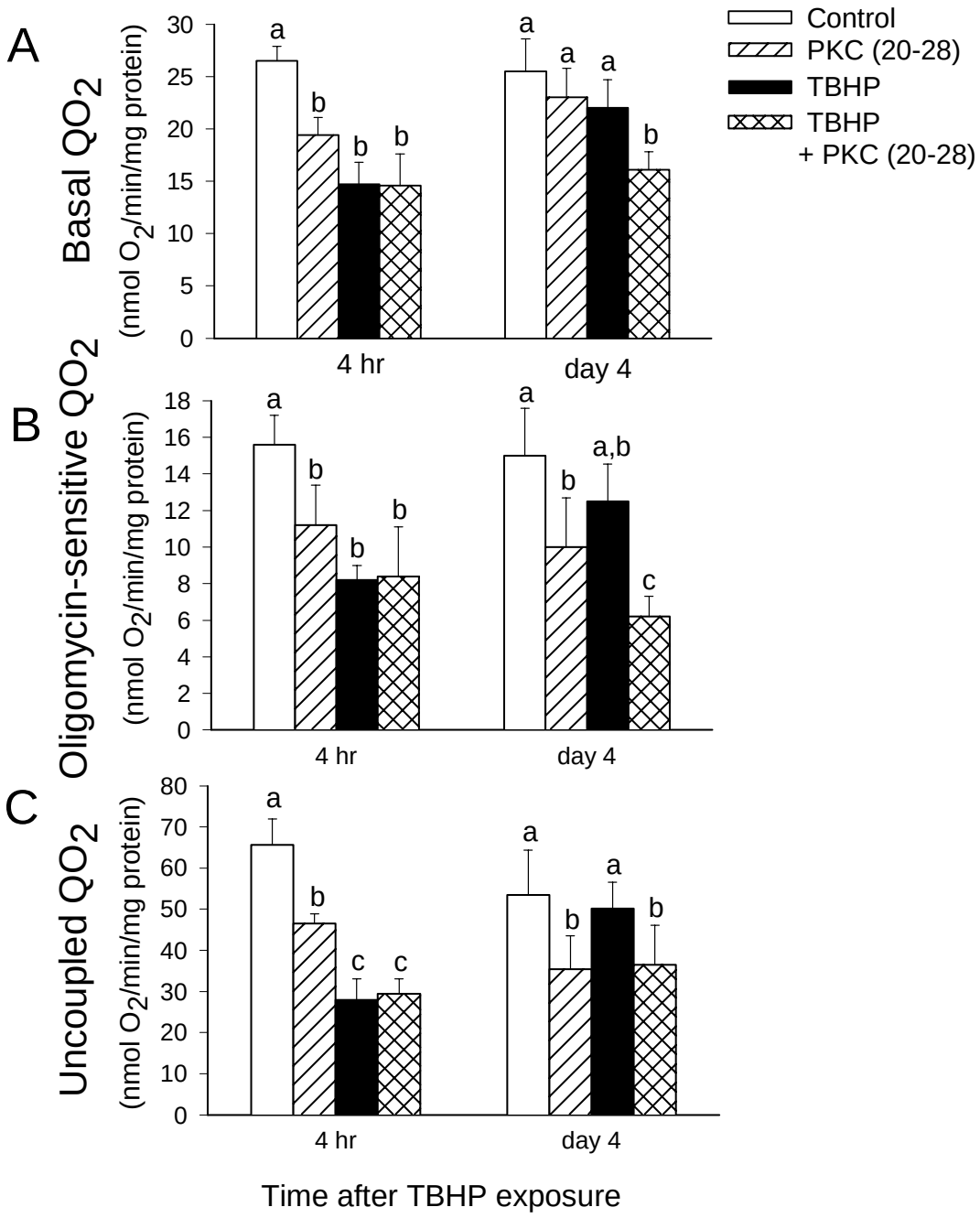

Figure 4

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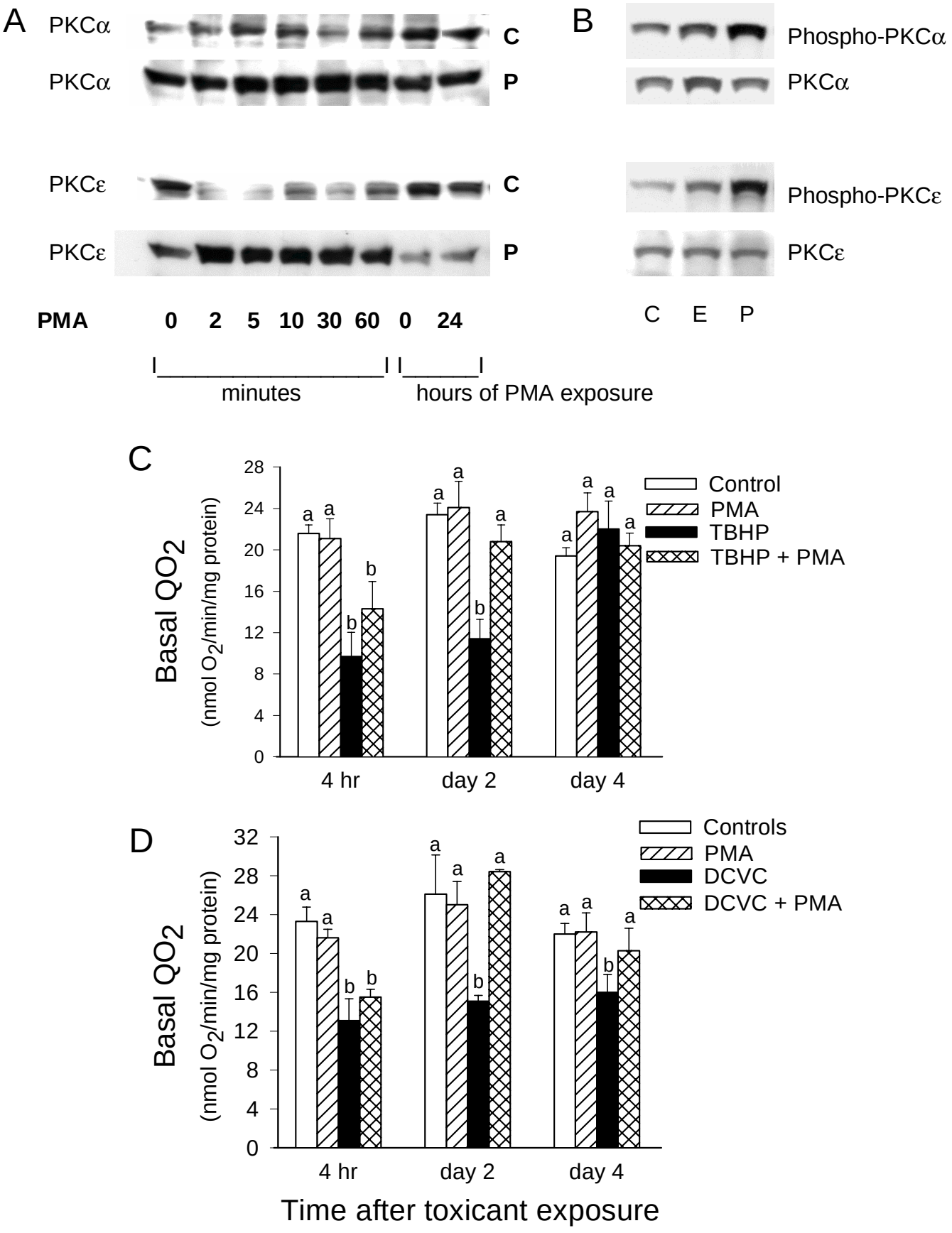

Figure 5

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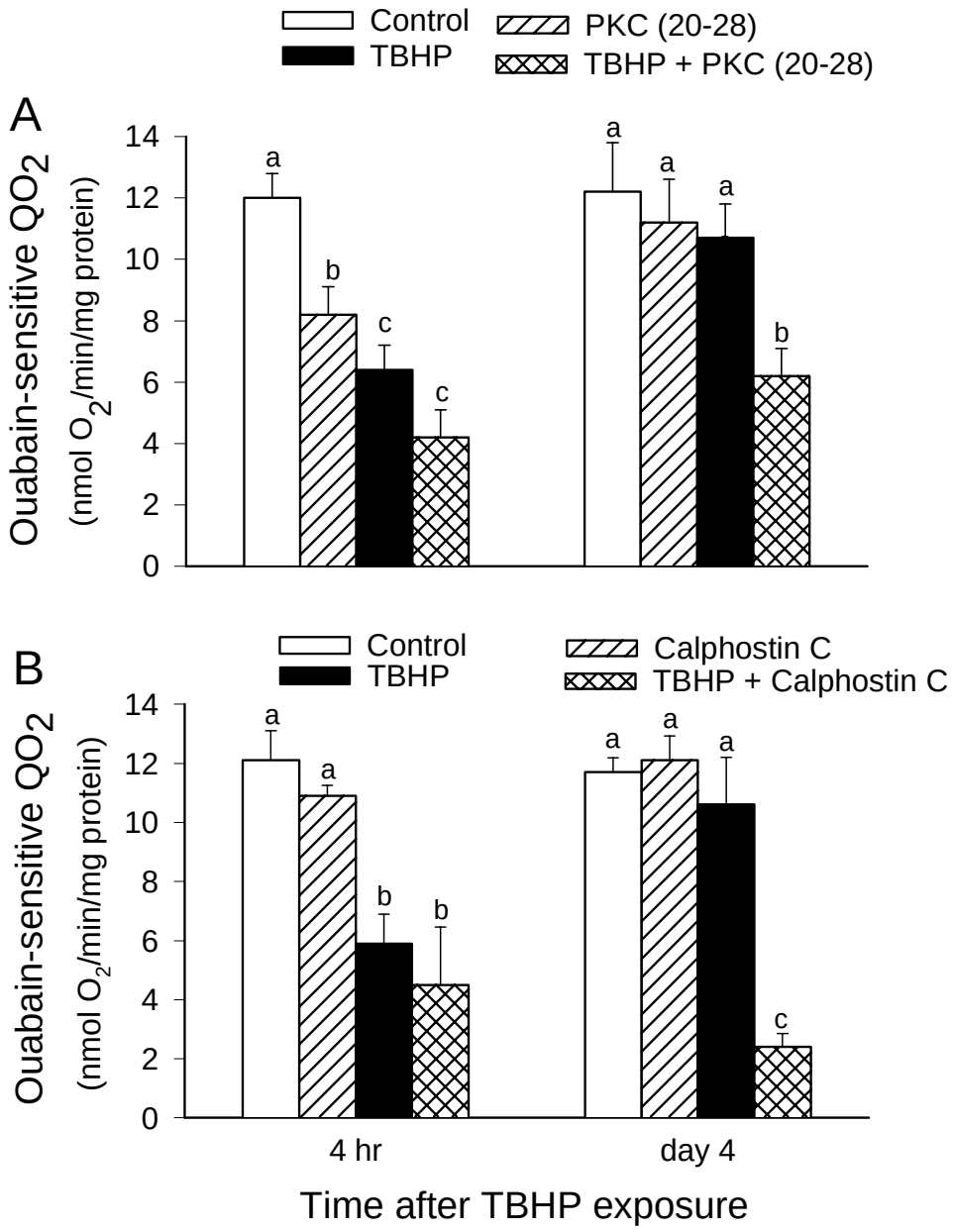

Figure 6

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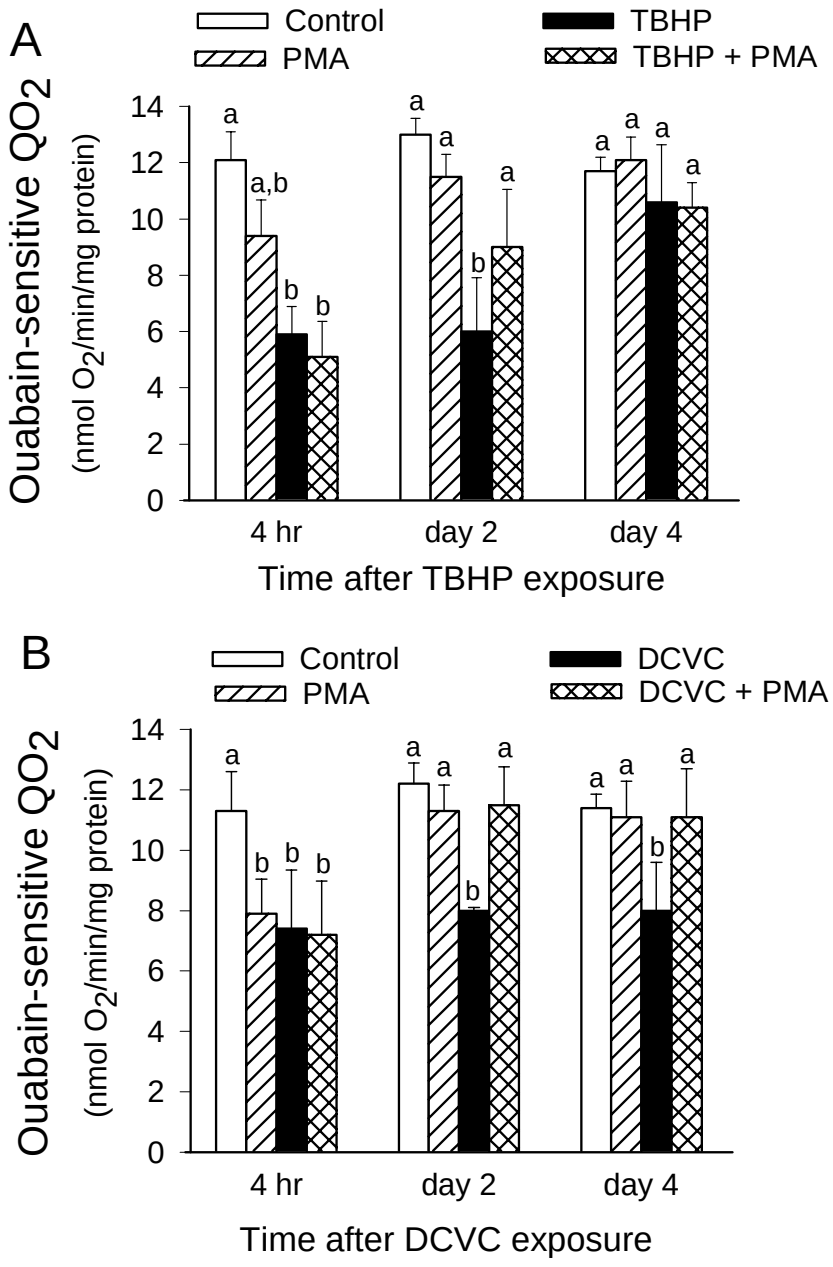

Figure 7

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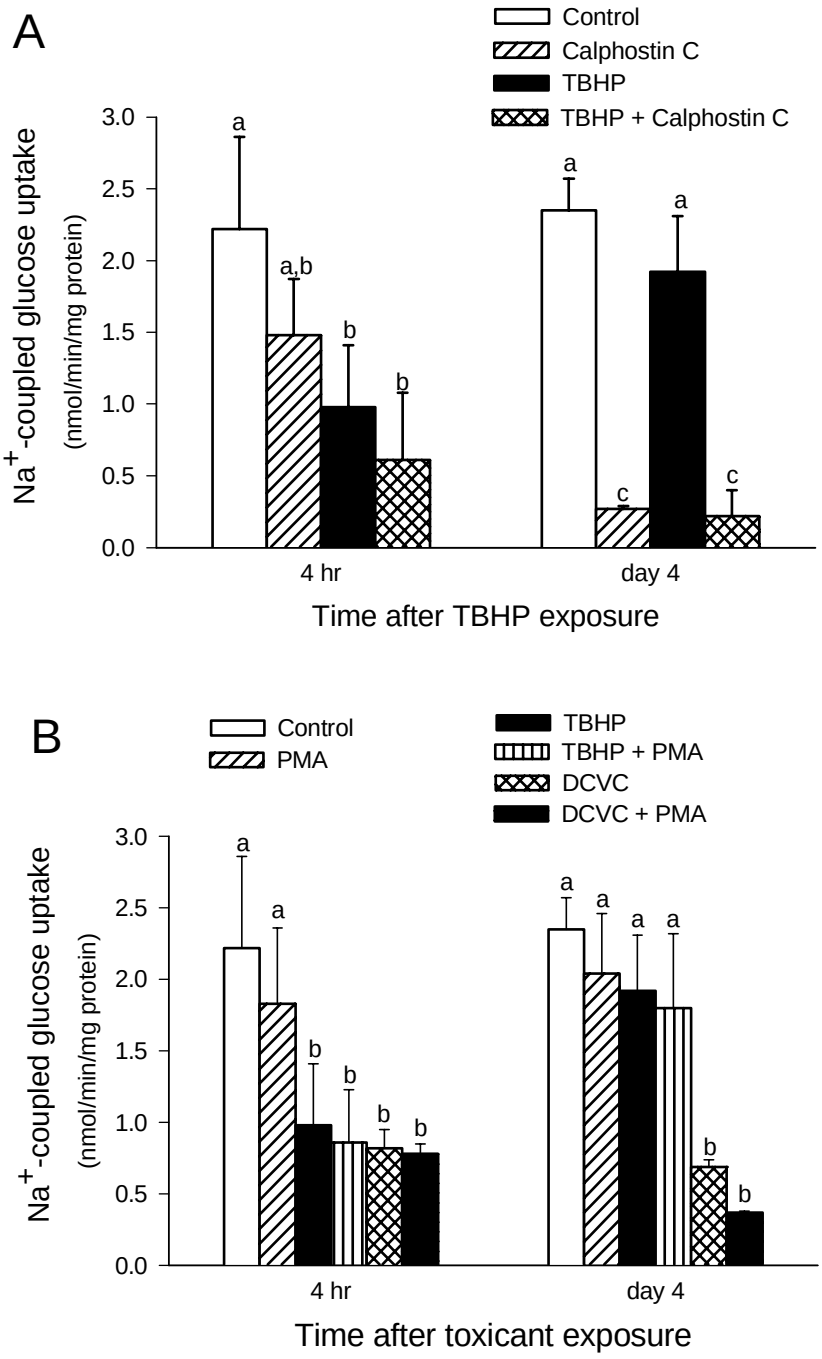

Figure 8