DIVERGENT ROLES OF A NOVEL PHOSPHOLIPASE A_2 IN CELL DEATH

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Phospholipase A_2 (s) are esterases that hydrolyze the *sn-2* ester bond in phospholipids, releasing a fatty acid and a lysophospholipid. We previously showed that most PLA_2 activity in rabbit renal proximal tubule cells (RPTC) was Ca^{2+}-independent, localized to the endoplasmic reticulum (ER-$iPLA_2$), and inhibited by the specific Ca^{2+}-independent PLA_2 inhibitor bromoenol lactone (BEL). Our recent molecular biology experiments suggest that ER-$iPLA_2$ is similar to a novel human $iPLA_2$ called $iPLA_2\gamma$. Numerous investigators have implicated phospholipase A_2 in cell injury and death. The goal of these studies was to determine the role ER-$iPLA_2$ in cell necrosis and apoptosis. Using primary cultures of RPTC, we examined the role of ER-$iPLA_2$ in oxidant-induced necrosis by inhibiting ER-$iPLA_2$ with BEL. The model oxidants *t*-butyl-hydroperoxide, cumene-hydroperoxide, menadione, duraquinone and cisplatin (a common chemotherapy drug) produced RPTC lipid peroxidation and necrosis. Inhibition of ER-$iPLA_2$ prior to oxidant exposure, potentiated oxidant-induced lipid peroxidation and necrosis. In contrast, necrosis produced by the non-oxidant antimycin A was not potentiated by ER-$iPLA_2$ inhibition. These results suggest that ER-$iPLA_2$ plays a protective in cell injury produced by diverse oxidants.

In a second series of studies we determined the role of ER-$iPLA_2$ in apoptosis. Low concentrations of cisplatin produce time- and concentration-dependent RPTC apoptosis, causing p53 nuclear translocation, caspase 3 activation, annexin V labeling, chromatin condensation and DNA hypoploidy. Using primary cultures of RPTC, we examined the role of ER-$iPLA_2$ in cisplatin-induced apoptosis by inhibiting ER-$iPLA_2$ with BEL. Inhibition of ER-iPLA2 decreased cisplatin-induced caspase 3 activation, annexin V labeling, chromatin condensation and DNA hypoploidy but not p53 nuclear translocation. Caspase 8 and 9 activities did not increase following cisplatin exposure. The contribution arachidonic acid metabolism downstream of ER-$iPLA_2$ in cisplatin-induced apoptosis was investigated using cyclooxygenase (indomethacin), 5-lipoxygenase (MK886), and 12-lipoxygenase (baicalien) inhibitors. MK886, but not indomethacin or baicalein, decreased cisplatin-induced apoptosis. These results suggest that ER-$iPLA_2$ is a mediator of apoptosis and acts subsequent to p53 nuclear translocation and prior to downstream markers of apoptosis. In addition, an arachidonic acid metabolite of 5-lipoxygenase may be the active molecule.

In summary, ER-$iPLA_2$ appears to play a complex, critical and divergent role in cell injury and death. Under oxidative stress leading to necrosis, ER-$iPLA_2$ is cytoprotective and may act as a phospholipid repair enzyme that removes oxidized arachidonic acid from damaged ER phospholipids. In contrast, ER-$iPLA_2$ signals apoptosis downstream of p53 and prior to caspase 3 activation.

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Progressive Disruption of the Plasma Membrane during Renal Proximal Tubule Cellular Injury

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The goal of this study was to examine the progression of plasma membrane disruption during cell injury using rabbit renal proximal tubules (RPT). The results demonstrated that the plasma membrane became permeable to larger and larger molecules as anoxia proceeded. At least three distinctive phases of membrane disruption were differentiated during anoxia. In phases 1, 2, and 3, plasma membranes became permeable to propidium iodide (PI, molecular weight = 668), 3 kDa dextrans, and 70 kDa dextrans or lactate dehydrogenase (LDH, molecular weight = 140 kDa), respectively. Phase 1 was reversible by reoxygenation but not prevented by the glycine. Phase 2 was inhibited by glycine. Phase 3 was inhibited by several membrane-permeable homobifunctional crosslinkers, dimethyl-pimelimidate (DMP), ethylene-glycolbis(succinimidylsuccinate), and dithiobis(succinimidylpropionate), but not by the membrane-impermeable crosslinker dithiobis(sulfosuccinimidylpropionate). In addition, DMP decreased RPT LDH release produced by mitochondrial inhibition (antimycin A), an oxidant (*t*-butylhydroperoxide) and a nephrotoxicant that is metabolized to an electrophile (tetrafluoroethyl-L-cysteine). These results identify (1) different phases of plasma membrane damage with increasing permeability during cell injury, (2) the reversibility of phase 1, (3) the relative site of action of the cytoprotectant glycine (prevents phase 2), and (4) the protective effects of chemical crosslinkers in RPT cell death produced by different toxicants. © 2001 Academic Press

Key Words: glycine; crosslinker; propidium iodide; lactate dehydrogenase; cell injury and death; plasma membrane disruption; plasma membrane permeability; oxidant; mitochondria; nephrotoxicant.

Plasma membrane permeability to various substances is strictly regulated under physiological conditions. After anoxia or toxicant exposure, plasma membrane permeability increases, allowing the release of intracellular proteins into the extracellular space. At this point, it is generally thought that cell injury becomes irreversible cell death. Better understanding of the mechanisms that results in increased membrane permeability would greatly enhance the ability to prevent or minimize cell injury and to predict the reversibility or irreversibility of pathological events.

Various alterations in membrane lipids and proteins during anoxia/hypoxia and toxicant injury have been observed. While changes in phospholipids occur following ischemia and/or ischemia/reperfusion injury in the rat kidney and in mouse, rabbit, and rat proximal tubular cells *in vitro*, the exact role of these phospholipids changes in cell death is unclear (Matthys *et al.*, 1984; Finkelstein *et al.*, 1985; Humes *et al.*, 1989; Portilla *et al.*, 1994; Weinberg *et al.*, 1995). The best evidence that phospholipid changes play a role in renal cell death is in oxidant-induced injuries and/or injuries in which phospholipase A_2 inhibitors decrease renal cell death (Bunnachak *et al.*, 1994; Portilla *et al.*, 1994; Schnellmann *et al.*, 1994; Zager *et al.*, 1999). However, the lack of complete cytoprotection with phospholipase A_2 inhibitors suggests that phospholipid changes may play a contributing role and accelerate the rate of cell death.

In contrast to phospholipids, membrane proteins are likely to play a critical role in plasma membrane disruption. Under physiological conditions, membrane proteins mediate most of the transmembrane movement of small hydrophilic molecules and ions. In numerous types of cell injury K^+ efflux and Na^+ influx through plasma membrane ion channels occur early in the process, subsequent to ATP depletion. In the late phase of cell injury, Ca^{2+} and Cl^- influx occur and lead to terminal cell death/lysis, using the release of cytoplasmic protein lactate dehydrogenase (LDH) as a marker (Kribben *et al.*, 1994; Miller and Schnellmann, 1993, 1995; Waters and Schnellmann, 1996; Waters *et al.*, 1997; Weinberg *et al.*, 1991, 1997). With large molecules such as proteins, evidence suggests that protein translocation across membranes may be mediated by protein channels (Simon and Blobel, 1991; Simon, 1995). The opening and closing of these membrane channels or transporters are strictly regulated under normal conditions. The malfunctioning of these membrane proteins during cell injury could cause

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TABLE 1
Summary of Properties of the Crosslinkers Used in the Present Studies

Crosslinker	Reactivity	Target	Space arm length (A)	Membrane permeability	Prevent anoxia-induced LDH release
DMP	Homobifunctional imidoester	Primary amine	9.2	Yes	Yes
EGS	Homobifunctional NHS-ester	Primary amine	16.1	Yes	Yes
DSP	Homobifunctional NHS-ester	Primary amine	12.0	Yes	Yes
DTSSP	Homobifunctional NHS-ester	Primary amine	12.0	No	No

these channels to open and result in the loss of the plasma membrane permeability barrier.

In a recent paper Dong *et al.* (1998) described the development of membrane pores in plasma membranes of Madin–Darby canine kidney (MDCK) cells treated with an uncoupler of oxidative phosphorylation (carbonyl cyanide-m-chlorophenylhydrazone (CCCP)) in the presence of a Ca^{2+} ionophore with low extracellular Ca^{2+} (100 nM) and in the absence of glucose. They observed the appearance of membrane pores that increased in size over time. One goal of the present study was to examine the progressive disruption of the plasma membrane in response to anoxia using a more differentiated cellular model, freshly isolated suspension of rabbit renal proximal tubules, in the presence of physiological concentrations of Ca^{2+}. Further, we explored the ability of protein cross-linking agents to prevent disruption of the plasma membrane damage secondary to anoxia and toxicant exposure. Finally, we examined the reversibility of the membrane damage.

METHODS

Renal Proximal Tubule Isolation and Incubation

Rabbit renal proximal tubules (RPT) were isolated and purified as described previously and suspended in Dulbecco's modified Eagle's medium (DMEM) at 2 mg protein/ml or an incubation buffer consisting of 1 mM alanine, 4 mM dextrose, 2 mM heptanoate, 4 mM lactate, 5 mM malate, 115 mM NaCl, 15 mM $NaHCO_3$, 5 mM KCl, 2 mM NaH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, and 10 mM HEPES (pH 7.4, 295 mOsm/kg) at approximately 1 mg protein/ml (Dickman and Mandel, 1989; Rodeheaver *et al.*, 1990). RPT cells in DMEM were gassed with 100% O_2 and preincubated at 37°C for 20 min. Subsequently, RPT cells were subjected to normoxia or anoxia by gassing with 100% O_2 or 100% N_2, respectively. RPT cells in incubation buffer were gassed with 95% air/5% CO_2 and preincubated at 37°C for 15 min.

Entry of Marker Compounds of Increasing Size during RPT Anoxia

Initial experiments were designed to differentiate between anoxia-induced membrane permeabilization to propidium iodide (PI, MW = 668) and BODIPY FL phallacidin (PHA, MW, = 1125). After a 20 min normoxia preincubation, RPT cells were put on ice and gassed with 100% N_2 for 5 min to stop endocytosis. PI (20 μM) and a 1:50 dilution of PHA were added to the RPT and anoxia continued at 37°C for 20 min. PI (568 nm excitation/590 nm emission) and PHA (488 nm ex./515 nm em.) fluorescence was immediately examined with a Zeiss confocal microscope. To confirm that the concentrations of PI and PHA were appropriate for staining the nuclei and F-actin, the anoxic RPT cells were permeabilized subsequently with 0.4% Triton X-100 (final concentration) and PI and PHA fluorescence examined.

In other experiments, after a 30-min anoxic period 20 μM PI and 0.1% 70 kDa fluorescein dextran or 0.1% 3 kDa tetramethylrhodamine dextran (568 nm ex./590 nm em.) and 0.1% 70 kDa fluorescein dextran (488 nm ex./515 nm em.) were added to RPT. Plasma membrane permeability to PI and the fluorescent dextrans was measured immediately with a confocal microscope.

Time-Dependent PI Entry and LDH Release during RPT Anoxia

After a 20-min normoxia preincubation, RPT cells were gassed with 100% N_2 in DMEM containing 20 μM PI for 0, 5, 10, 15, 20, 25, and 30 min and PI entry and LDH release determined at each time point. For counting PI-stained cells, extracellular PI was removed by washing the tubules with fresh DMEM twice immediately after the anoxic incubation. PI-stained cells were counted under a fluorescent microscope as expressed as the percentage of the total cells examined. LDH release was measured as described previously (Schnellmann and Mandel, 1986; Moran and Schnellmann, 1996). RPT were separated from the extracellular medium by centrifugation. RPT suspensions were permeabilized with Triton X-100 with the matched extracellular medium sample receiving the same quantity of Triton X-100 (final concentration 0.4%).

Cross-Linking Cellular Membrane Proteins during RPT Anoxia

These experiments were designed to test the hypothesis that stabilization of membrane proteins by cross-linking may prevent anoxia- and toxicant-induced membrane disruption. Four homobifunctional imidoester or NHS-ester crosslinkers with different space arm lengths and membrane permeability were used in the study (Table 1). Dimethyl-pimelimidate (DMP; Brew *et al.*, 1975; Coco-Martin *et al.*, 1992), ethylene-glycolbis(succinimidylsuccinate) (EGS; Baskin and Yang, 1980; Browning and Ribolini, 1989), dithiobis(succinimidylpropionate) (DSP; Carlsson *et al.*, 1978; de Pont *et al.*, 1980; Lomant and Fairbanks, 1976), and dithiobis(sulfosuccinimidylpropionate) (DTSSP; Jung and Morol, 1983; Staros, 1982) have space arm lengths of 9.2, 16.1, 12, and 12 A, respectively. DMP, EGS, and DSP are membrane permeable while DTSSP is the membrane-impermeable analog of DSP. All the crosslinkers react with primary amine groups in proteins or amine-containing phospholipids to form covalent bonds at the two ends of the crosslinkers.

After a 20-min normoxia preincubation in DMEM, RPT cells were resuspended in an amine-free buffer containing 125 mM NaCl, 5 mM KH_2PO_4, 2 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM HEPES, 20 mM glucose, and heptanoic acid (0.03%; pH 7.4). The tubules were gassed with 100% N_2 for 30 min at 37°C in the absence or presence of each of the homobifunctional imdioester or NHS-ester crosslinkers, DMP (10 mM), EGS (3 mM), or DSP (3 mM). After the anoxic period, LDH release was determined as described above using Triton X-100. Since DTSSP is not membrane permeable, the presence of DTSSP in the extracellular medium may decrease LDH activity released from the RPT, leading to the misinterpretation of the effect of DTSSP on membrane permeability to LDH. To avoid this possibility, RPT were incubated with 3 mM DTSSP under normoxia for 20 min to allow the crosslinking reactions to occur. The RPT were washed with fresh amine-free buffer to remove extracellular DTSSP, subjected to anoxia for 30 min, and LDH release determined.

In some experiments, after a 15-min preincubation period RPT were resuspended in amine-free incubation buffer in the presence and absence of DMP

DIFFERENTIAL PHASES OF MEMBRANE DISRUPTION

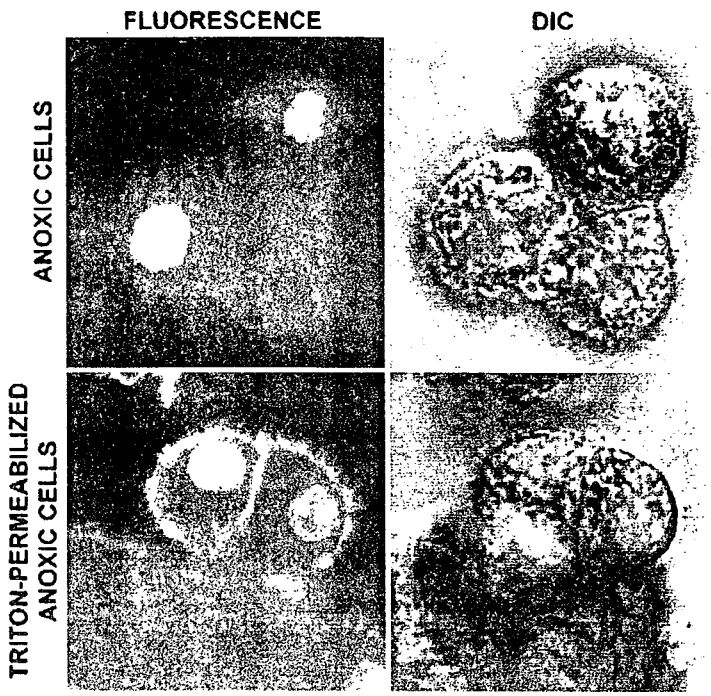

FIG. 1. Differentiation of anoxia-induced permeability of the plasma membrane to PI and PHA. Upper panels: RPT were incubated under anoxia in the presence of 20 μM PI (MW = 668) and 1:50 diluted PHA (MW = 1125) for 20 min. The nuclei of two cells were stained by PI while microvilla F-actin was not stained by PHA, indicating a differential permeability of the plasma membrane to PI and PHA during anoxia. Lower panels: After th anoxic period, the plasma membranes of cells in the same set as described above were permeabilized with 0.4% Triton X-100. PHA entered these cell and stained microvillar F-actin. The fluorescent (left column) and differential interference contrast (DIC, right column) microscopic images were take using a confocal microscope.

(10 mM) and incubated for an additional 30 min. RPT were washed, resuspended in incubation buffer, and incubated with antimycin A (10 μM for 2 h), tetrafluoroethyl-L-cysteine (25 μM for 3 h), *t*-butylhydroperoxide (0.5 mM for 3 h), or diluent. Previous results from our laboratory have shown that these concentrations of the toxicants and times of exposure result in a significant degree of LDH release (Waters *et al.*, 1997).

In addition to LDH release, we examined the plasma membrane permeability of crosslinker-treated anoxic RPT cells to 3 and 70 kDa dextrans. After 30 min of anoxia in the presence or absence of each of the crosslinkers, RPT cells were washed free of extracellular crosslinkers and resuspended in 0.1% 3 kDa tetramethylrhodamine dextrans and 0.1% 70 kDa fluorescein dextrans in DMEM. The plasma membrane permeability to these dextrans was examined using confocal microscopy. To confirm that the dextrans would stain crosslinker-treated RPT if plasma membrane permeability increased, 0.4% Triton X-100 was added to RPT and dextran staining examined as described above.

Detection of Mitochondrial Membrane Potential and Trypan Blue Uptake following Reoxygenation in PI Permeable RPT

This experiment was designed to determine whether mitochondrial function (using mitochondrial membrane potential as a marker) is recovered following anoxia/reoxygenation in which RPT are permeable to PI. RPT cells were incubated in DMEM containing 20 μM PI under anoxia for 20 min, washed

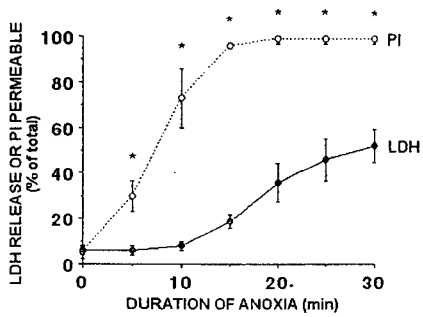

FIG. 2. Differentiation of anoxia-induced permeability of plasma membranes to PI and LDH. RPT were incubated under anoxia for 30 min in th presence of PI. At various time points of anoxic incubation, RPT LDH releas and PI staining were determined. $^*p < 0.05$, compared to LDH at th corresponding time point.

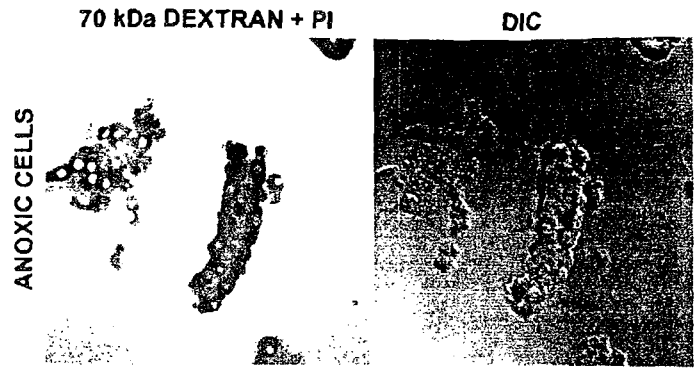

FIG. 3. Differentiation of anoxia-induced permeability of plasma membranes to PI and 70 kDa dextran. RPT were incubated under anoxia for 30 min in a medium containing 20 μM PI. RPT were then resuspended in a medium containing 0.1% 70 kDa fluorescein dextran. Fluorescent (left panel) and DIC (right panel) images were taken using a confocal microscope. Extracellular space in the left panel was bright due to the presence of the fluorescent dextran. Dark areas in the intracellular space indicate cells whose plasma membranes are impermeable to the dextran. Many cells were stained by PI, but only a few of these were permeable to the 70 kDa dextran.

free of extracellular PI, and reoxygenated for 25 min in DMEM containing 50 μM JC-1. JC-1 is a fluorescent mitochondrial potential indicator (Macho *et al.*, 1996; Reers *et al.*, 1995; Smiley *et al.*, 1991). PI and JC-1 (488 nm ex./515 nm em.) fluorescence were visualized using confocal microscopy immediately after the reoxygenation period.

Trypan blue (TB; MW = 961) has been used widely as a cell death marker (Gaudio *et al.*, 1989; Singh *et al.*, 1985; Walum *et al.*, 1985). Since it is impossible to test plasma membrane permeability to PI once the nuclei have been stained by PI, we used TB to determine if the plasma membrane integrity of RPT which had been permeable to PI during anoxia could be recovered by reoxygenation. RPT were incubated in DMEM containing 20 μM PI under anoxia for 20 min, washed free of extracellular PI, and reoxygenated for 25 min in DMEM. RPT were then exposed to 0.2% TB in phosphate-buffered saline (PBS) at 4°C for 15 min and examined with visible light and fluorescent confocal microscopy.

To exclude the possibility that PI-stained nuclei cannot be stained by TB, normoxic RPT cells were permeabilized with 0.1% Triton X-100. The permeabilized normoxic cells were incubated with 20 μM PI in DMEM at 4°C for 15 min. Nuclear-bound PI was visualized using confocal microscopy. Non-bound PI was removed by washing with fresh DMEM and RPT exposed to 0.2% TB in PBS at 4°C for 15 min. Nuclear-bound PI and TB were examined with visible light and fluorescent confocal microscopy.

Chemicals

Fluorescent dextrans, BODIPY FL phallacidin, and JC-1 were purchased from Molecular Probes (Eugene, OR). Chemical crosslinkers were purchased from Pierce (Rockford, IL). Tetrafluoroethyl-L-cysteine was a gift from Dr. Edward A. Lock (Zeneca, Cheshire, UK). Unless noted in the text, all other chemicals were purchased from Sigma Chemical Company (St. Louis, MO).

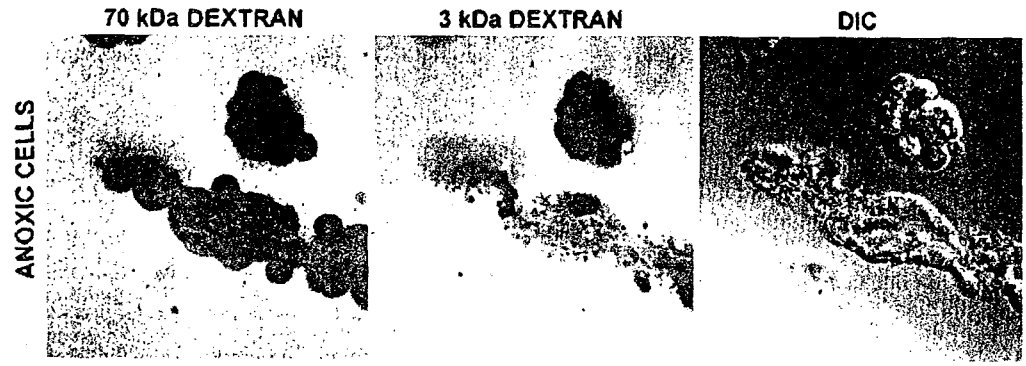

FIG. 4. Differentiation of anoxia-induced permeability of plasma membranes to 3 and 70 kDa dextran. RPT were suspended in a medium containing 0.1% 70 kDa fluorescein dextran and 0.1% 3 kDa tetramethylrhodamine dextran after 30 min of anoxia. Fluorescent and DIC images were taken using confocal microscopy. Extracellular space in the left and middle panels was bright due to the presence of fluorescent dextrans. Dark areas in the intracellular space indicate cells whose plasma membranes are impermeable to the dextran. Some cells were permeable to the 3 kDa dextran (middle panel), but not to 70 kDa dextran (left panel).

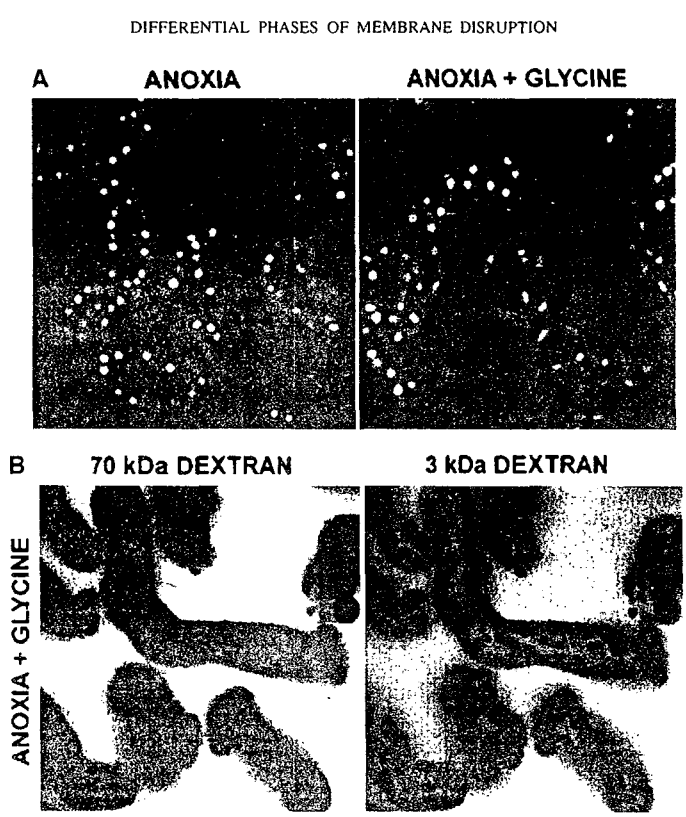

FIG. 5. Glycine prevents anoxia-induced plasma membrane permeability to 3 and 70 kDa dextrans but not to PI. (A) RPT were incubated under anoxia in the presence of 20 μM PI for 30 min in the absence (left) and presence of 4 mM glycine (right). Fluorescent images were taken using a confocal microscope. Glycine did not prevent anoxia-induced membrane permeabilization to PI. (B) RPT which had been subject to 30 min of anoxia in the presence of 4 mM glycine were resuspended in a medium containing 0.1% 70 kDa fluorescein dextran and 0.1% 3 kDa tetramethylrhodamine dextran. Extracellular space was bright due to the presence of fluorescent dextrans. Dark areas in the intracellular space indicate cells whose plasma membranes are impermeable to the dextrans.

Data Analysis

Data are shown as means ± SEM from at least four preparations. Unless noted in the figure legend, analysis of variance (ANOVA) was used to compare the means of various experimental groups and multiple mean values compared using Fisher's protected least significance difference test and $p < 0.05$.

RESULTS

Differentiation of Anoxia-Induced Plasma Membrane Permeability

RPT were incubated under anoxia in the presence of PI and PHA for 20 min. Unlike control cells, most of the cells subjected to anoxia were stained by PI (Fig. 1, upper left panel). Although the cells were permeable to PI, when visualized with differential interference contrast (DIC) microscopy, many of the cells maintained visible microvilli (Fig. 1, upper and lower right panels). However, no microvillar F-actin in any of these anoxic cells was stained in the presence of extracellular PHA (Fig. 1, upper left panel). To confirm that PHA would stain anoxic cells if the plasma membranes were permeable to PHA, Triton X-100 (0.4% final concentration) was added to the cells to permeabilize the plasma membrane. Triton X-100 addition resulted in PHA uptake and staining of the microvilli (Fig. 1, lower left panel). These results demonstrate that anoxia increases the permeability of the plasma membrane to the lower molecular weight PI (MW = 668) but not PHA (MW = 1125).

RPT were subjected to anoxia for various periods of time to determine time-dependent PI staining and LDH (MW = 140 kDa) release. PI staining increased within 5 min of anoxia exposure (approximately 30% of the cells being stained) and stained all cells after 15 min of anoxia (Fig. 2). In contrast, LDH release did not begin until 15 min of anoxia and reached approximately 50% after 30 min of anoxia. These results demonstrate that anoxia increases the permeability of the

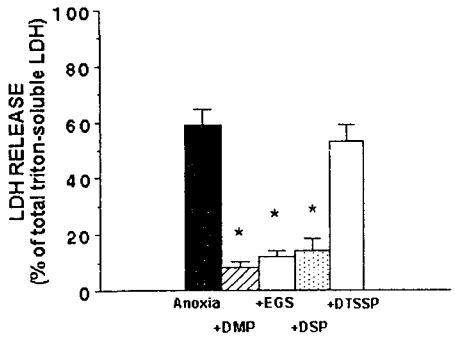

FIG. 6. LDH release from crosslinker-treated RPT after 30 min of anoxia. RPT were subjected to 30 min of anoxia in the absence or presence of the homobifunctional imidoester and NHS-ester crosslinkers. DMP (10 mM), EGS (3 mM), DSP (3 mM), or DTSSP (3 mM). The membrane-permeable crosslinkers are DMP, DSP, and EGS, while DTSSP is the membrane-impermeable analog of DSP. LDH release was expressed as percent of total Triton X-100 soluble LDH activity. $*p < 0.05$, compared to anoxia.

plasma membrane in a time- and size-dependent manner from smaller to larger molecules.

RPT were subjected to 30 min of anoxia, then resuspended in a medium containing 0.1% 70 kDa fluorescein dextran and PI or 0.1% 3 kDa tetramethylrhodamine dextran and 0.1% 70 kDa fluorescein dextran. The anoxic cells exhibited differential permeability to PI, 3 and 70 kDa dextran. While many of the anoxic cells were stained by PI, only a few of these were permeable to the 70 kDa dextran (Fig. 3). Comparing the permeability of anoxic cells to 3 and 70 kDa dextrans revealed that numerous cells were permeable to the 3 kDa dextran but not to the 70 kDa dextran (Fig. 4). These results demonstrate a "step-wise" increase in plasma membrane permeability from 0.7 to 3 kDa to 70 and 140 kDa.

Effect of Glycine on Anoxia-Induced Plasma Membrane Permeability

RPT were subjected to anoxia for 30 min in DMEM containing PI in the absence and presence of 4 mM glycine. This concentration of glycine is known to be cytoprotective in this model (Mandel *et al.*, 1990). Glycine did not prevent the entry of PI during anoxia (Fig. 5A). After the anoxic period, the cells were resuspended in DMEM containing 3 kDa tetramethylrhodamine dextran or 70 kDa fluorescein dextran. Neither 3 or 70 kDa dextrans entered the glycine-treated anoxic cells (Fig. 5B), demonstrating separate mechanisms of increased plasma membrane permeability to PI versus 3 and 70 kDa dextrans during anoxia.

Effect of Chemical Crosslinkers on Anoxia-Induced Plasma Membrane Permeability

RPT cells were subjected to 30 min of anoxia in the absence or presence of the homobifunctional imidoester and NHS-ester crosslinkers, DMP (10 mM), EGS (3 mM), DSP (3 mM), or DTSSP (3 mM). DMP, EGS, and DSP are membrane permeable, while DTSSP is the membrane-impermeable analog of DSP (Jung and Morol, 1983; Staros, 1982). All the crosslinkers except the membrane-impermeable DTSSP prevented LDH release from RPT subjected to anoxia (Fig. 6). The decrease in LDH activity was not due to crosslinking between LDH molecules or between LDH and other intracellular proteins since Triton X-100 solubilization released the LDH activity (data not shown). Likewise, the release of LDH activity into the extracellular medium by Triton X-100 indicates that plasma membrane lipids were not covalently crosslinked together.

To determine whether the chemical crosslinkers were effective in other types of toxic insults, RPT cells were exposed to a mitochondrial inhibitor (10 μM antimycin A), an oxidant (0.5 mM *t*-butylhydroperoxide), or a nephrotoxic halocarbon that is metabolized to a reactive electrophile (25 μM tetrafluoroethyl-L-cysteine) in the presence and absence of DMP. All three toxicants caused extensive LDH release (40–60%) that was decreased in the presence of DMP (Fig. 7). These results suggest that although these toxicants have different mechanisms, there is a commonality in the injury pathways that is sensitive to chemical crosslinkers.

Consistent with the LDH (140 kDa) release data (Fig. 6), the plasma membranes of crosslinker-treated anoxic cells were impermeable to 70 kDa dextran (Fig. 8, left column). However, the anoxia-induced increase in plasma membrane permeability to 3 kDa dextran (Fig. 8, middle column) and PI (data not shown) was not prevented by the crosslinkers, indicating separate mechanisms for the differential permeability of the plasma membrane to 3 and 70–140 kDa molecules. To confirm that the 70 kDa dextran would stain crosslinker-treated RPT if plasma membrane permeability increased, Triton X-100 was added and 70 kDa dextrans entered the anoxic cells (data not shown).

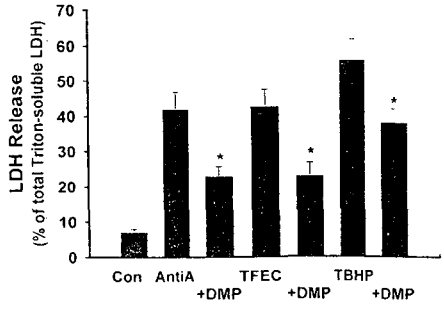

FIG. 7. LDH release from DMP-treated RPT after toxicant exposure. RPT were incubated with antimycin A (AntiA, 10 μM for 2 h), tetrafluoroethyl-L-cysteine (TFEC, 25 μM for 3 h) or *t*-butylhydroperoxide (TBHP, 0.5 mM for 3 h) in the absence or presence of DMP (10 mM). LDH release was expressed as percent of total Triton-X-100 soluble LDH activity. $*p < 0.05$, compared to toxicant alone.

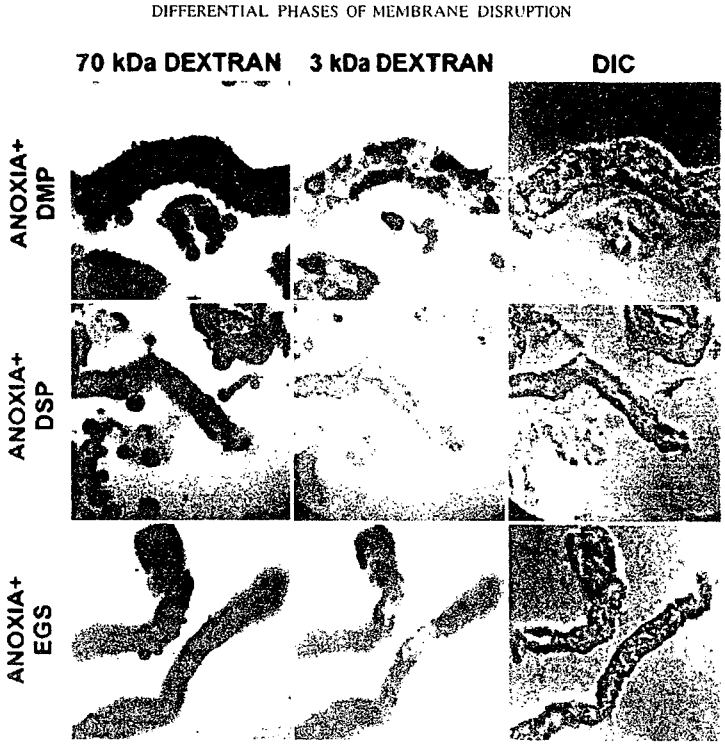

FIG. 8. Chemical crosslinkers prevent anoxia-induced plasma membrane permeability to 70 kDa dextran but not to 3 kDa dextran. RPT were incubated with 10 mM DMP (upper panels), 3 mM DSP (middle panels), or 3 mM EGS (lower panels) under anoxia for 30 min. RPT were resuspended, in the absence of crosslinkers, in DMEM containing 0.1% 70 kDa fluorescein dextran or 0.1% 3 kDa tetramethylrhodamine dextran. Fluorescent and DIC images were taken using confocal microscopy. Extracellular space in the left and middle panels was bright due to the presence of fluorescent dextrans. Dark areas in the intracellular space indicate cells whose plasma membranes are impermeable to the dextran. Some cells were permeable to the 3 kDa dextran (middle panel), but not to the 70 kDa dextran (left panel).

Reversibility of Anoxia-Induced PI Permeability of RPT Cells

Since the plasma membranes of RPT were permeable to PI at a very early stage of anoxia (Fig. 2), these cells may not be irreversibly damaged but recoverable. Two approaches were taken to determine the reversibility of PI-stained cells: (1) testing the mitochondrial membrane potential in these cells after reoxygenation, and (2) testing plasma membrane permeability of these cells after reoxygenation using another indicator of plasma membrane permeability (TB).

RPT were subjected to normoxia or anoxia for 20 min in DMEM containing PI. Extracellular PI was removed by washing with fresh oxygenated DMEM and the cells allowed to recover under normoxia for 25 min. The dye JC-1 was used to measure mitochondrial membrane potential. Control RPT exhibited punctate staining throughout the cytoplasm with no PI staining in the nucleus (Fig. 9). In RPT subjected to anoxia and reoxygenation, the nuclei were PI stained and many of the cells contained punctate JC-1 staining, representing functional mitochondria. These results suggest that PI staining does not represent irreversible cell death but reversible cell injury.

RPT cells were subjected to anoxia for 20 min in DMEM containing PI. Extracellular PI was removed by washing with fresh oxygenated DMEM and the cells allowed to recover for 25 min. TB (MW = 961) was added following the recovery period. Cells subjected to anoxia/reoxygenation exhibited PI staining (Fig. 10, upper left panel), but were impermeable to TB (Fig. 10, upper right panel). These results further suggest that PI staining does not represent irreversible cell death but reversible cell injury. To exclude the possibility that PI-stained nuclei cannot be stained by TB, RPT containing PI-stained nuclei were permeabilized with Triton X-100 and exposed to TB. The nuclei were stained blue and the fluorescence of PI in the nuclei became invisible at the excitation/emission wavelengths of 488/515 nm (Fig. 10, lower panels).

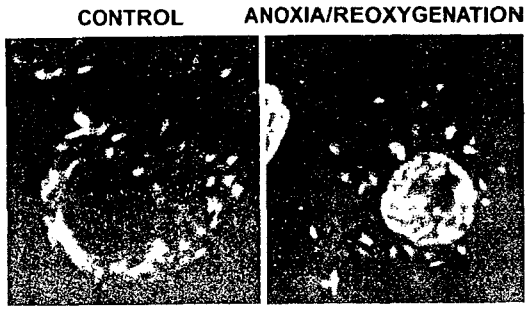

FIG. 9. Functioning mitochondria in RPT permeabilized to PI during anoxia/reoxygenation. RPT were subjected to anoxia/reoxygenation in the presence of 20 μM PI for 20 min. RPT were resuspended in the absence of PI and subjected to reoxygenation for 25 min in the presence of a mitochondrial membrane potential indicator. JC-1. Fluorescent images were taken using confocal microscopy. Control cells do not exhibit PI staining but exhibit punctate JC-1 staining (left panel). consistent with mitochondrial labeling. In contrast, cells subjected to anoxia/reoxygenation exhibited PI staining and punctate JC-1 staining after 25 min of recovery (right panel). The results suggest that mitochondrial membrane potential can be recovered following anoxia-induced injury even if the membrane had been permeabilized to PI.

DISCUSSION

The results obtained in the present studies demonstrate that plasma membrane disruption is a gradual process during anoxia. The plasma membrane became permeable to larger and larger molecules as anoxia prolonged. This is in contrast to what is commonly thought concerning oncosis or necrotic cell death, that increased plasma membrane permeability is an "all or none" process with cell lysis or gross membrane rupture occurring (Herman *et al.*. 1988; Lemasters *et al.*, 1987). Three distinct phases of membrane disruption could be differentiated during anoxia. In phase 1, the plasma membrane became permeable to PI (0.7 kDa). In phase 2, the plasma membrane became permeable to 3 kDa dextrans, and in phase 3, the plasma membrane became permeable to 70 kDa dextrans and LDH (140 kDa).

In phase 1, a few minutes after the onset of anoxia, plasma membranes became permeable to PI (MW = 668). However, the results suggest that the increase in plasma membrane permeability is restrictive since a slightly larger probe (MW = 1125) was impermeable. It is possible that the uptake of PI or PHA could occur through endocytosis. However, any potential endocytosis of PI was minimized by leaving the RPT suspension on ice and depleting oxygen in the suspension 5 min before the addition of PI. Further, the membrane impermeability to PHA further demonstrated minimal endocytosis.

The cytoprotectant glycine did not prevent membrane permeabilization to PI during anoxia. Previous studies in this model showed that glycine prevents LDH release and promotes the recovery of mitochondrial function and ion transport following anoxia (Moran and Schnellmann, 1997). The current studies support the previous studies by demonstrating that a mitochondrial membrane potential was observed in RPT exposed to anoxia and reoxygenation in the presence of glycine. In addition, RPT exposed to anoxia and reoxygenation were impermeable to TB (MW = 961) when trypan blue was added after the reoxygenation period. Consequently, increased membrane PI permeability, phase 1, is reversible by reoxygenation and does not represent irreversible cell death but reversible cell injury. In addition, these findings raise the question of the reliability of PI permeability as a marker of cell death.

Phase 2 is represented by plasma membrane permeabilization to 3 kDa dextrans during anoxia and occurs subsequent to increased membrane permeability to PI but prior to increased membrane permeability to 70 kDa dextrans and LDH (140 kDa). This phase is distinguished from phase 1 by the fact that glycine prevented anoxia-induced membrane permeability to 3 kDa dextrans. Little information is available concerning the reversibility/irreversibility of phase 2; however, we did not observe the presence of functioning mitochondria in RPT during phase 2 using JC-1 (data not shown).

Phase 3 is represented by plasma membrane permeabilization to 70 kDa dextrans and LDH during anoxia and is distinguished from phase 2 based on the effects of the chemical crosslinkers. All the membrane-permeable homobifunctional imidoester and NHS ester crosslinkers, DMP, DSP. and EGS. prevented LDH release and membrane permeability to 70 kDa dextrans but not to 3 kDa dextrans. indicating different mechanisms for membrane permeabilization to 3 kDa dextrans versus 70 kDa dextrans and LDH. Extracellular modification of membrane proteins and phospholipids are not likely to be involved in the protective effects of the crosslinkers since the membrane-impermeable crosslinker DTSSP was ineffective, while its membrane-permeable analog, DSP, prevented LDH release. In addition, the effect of chemical crosslinkers extends beyond anoxia-induced plasma membrane changes to cell injury and death produced by the mitochondrial inhibitor antimycin A, the model oxidant *t*-butylhydroperoxide, and the nephrotoxic cysteine conjugate tetrafluoroethyl-L-cysteine. These results suggest that phase 3 is common to cell injuries produced by diverse toxicants. However, DMP was less efficacious in the presence of toxicants compared to anoxia. This may reflect multiple actions of the toxicants on a number of cellular functions.

Since both ends of these crosslinkers can react with primary amine groups in proteins or phospholipids to form covalent bonds, the protective effect of the crosslinkers could be mediated either by intra- and intermolecule crosslinking or by simply modifying the amine groups in proteins or amine-containing phospholipids. Though the space arm lengths differ among the crosslinkers (Table 1), they exhibited similar pro-

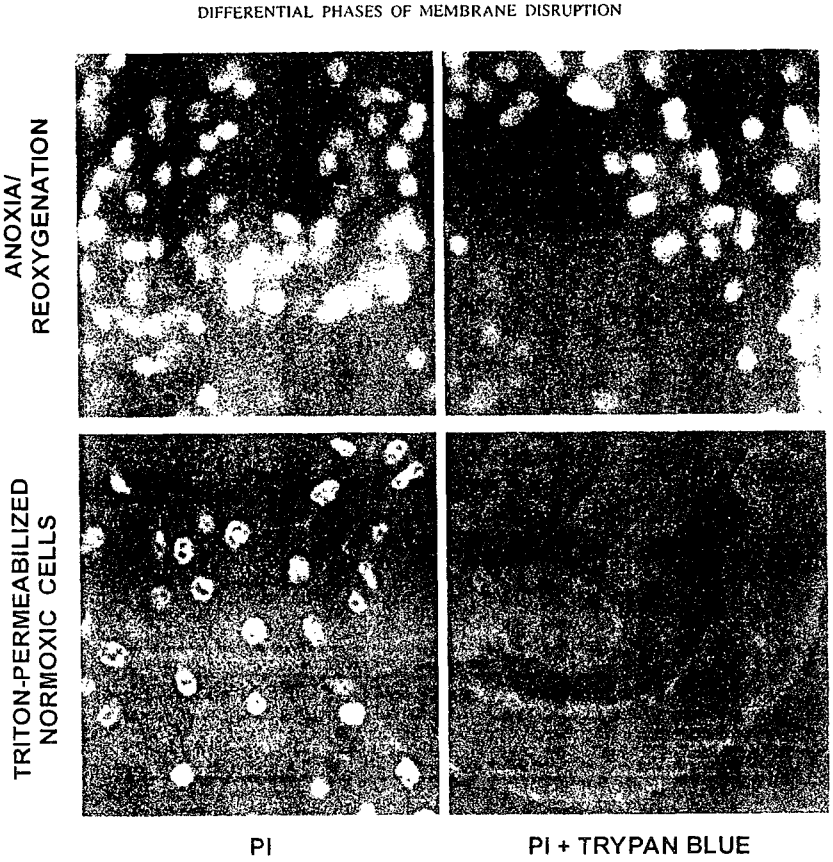

FIG. 10. Lack of trypan blue (TB) staining in RPT permeabilized to PI during anoxia/reoxygenation. RPT were subjected to anoxia in the presence of 20 μM PI for 20 min. RPT were resuspended in the absence of PI and subjected to reoxygenation for 25 min. RPT were then exposed to 0.2% TB in PBS and examined with visible light and fluorescent confocal microscopy. RPT subjected to anoxia/reoxygenation exhibited PI staining (upper left panel) but no TB staining (upper right panel). Normoxic RPT were permeabilized with 0.1% Triton X-100 and incubated with 20 μM PI at 4°C for 15 min (lower left panel). RPT were resuspended in the absence of PI, in PBS with 0.2% TB for 15 min (lower right panel). The results suggest that the ability to exclude large molecules can be recovered following anoxia-induced injury even if the membrane had been permeabilized to PI.

tective effects during anoxia. These results suggest that modification of amine groups may play a more important role than intra- or intermolecule crosslinking. For example, modification of the amine groups of membrane proteins and/or phospholipids may disrupt enzyme activities and substrate access and thereby prevent further disruption of the plasma membrane during anoxia.

In summary, our results demonstrate three time-dependent phases of plasma membrane damage during anoxia in RPT incubated in physiological concentrations of Ca^{2+}. Phase 1 is reversible and is characterized by increased plasma membrane permeability to small molecules (0.7 kDa). Phase 2 is characterized by increased plasma membrane permeability allowing 3 kDa dextrans to pass and is inhibited by the cytoprotectant glycine. Phase 3 during anoxia and other toxicant exposures is characterized by increased plasma membrane permeability to larger molecules (70 and 140 kDa) and is inhibited by intracellular crosslinking agents. Finally, our results question the use of PI as a marker of cell death.

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Interactions between Collagen IV and Collagen-Binding Integrins in Renal Cell Repair after Sublethal Injury

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ABSTRACT

Recent studies demonstrate that collagen IV selectively promotes the repair of physiological processes in sublethally injured renal proximal tubular cells (RPTC). We sought to further define the mechanisms of cell repair by measuring the effects of toxicant injury and stimulation of repair by L-ascorbic acid-2-phosphate (AscP), exogenous collagen IV, or function-stimulating integrin antibodies on the expression and subcellular localization of collagen-binding integrins (CBI) in RPTC. Expression of CBI subunits α_1, α_2, and β_1 in RPTC was not altered on day 1 after sublethal injury by *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC). On day 6, expression of α_1 and β_1 subunits remained unchanged, whereas a 2.2-fold increase in α_2 expression was evident in injured RPTC. CBI localization in control RPTC was limited exclusively to the basal membrane. On day 1 after injury, RPTC exhibited a marked inhibition of active Na^+ transport and a loss of cell polarity characterized by a decrease in basal CBI localization and the appearance of CBI on the apical membrane. On day 6 after injury, RPTC still exhibited marked inhibition of active Na^+ transport and localization of CBI to the apical membrane. However, DCVC-injured RPTC cultured in pharmacological concentrations of AscP (500 μM) or exogenous collagen IV (50 μg/ml) exhibited an increase in active Na^+ transport, relocalization of CBI to the basal membrane, and the disappearance of CBI from the apical membrane on day 6. Function-stimulating antibodies to CBI β_1 did not promote basal relocalization of CBI despite stimulating the repair of Na^+/K^+-ATPase activity on day 6 after injury. These data demonstrate that DCVC disrupts integrin localization and that physiological repair stimulated by AscP or collagen IV is associated with the basal relocalization of CBI in DCVC-injured RPTC. These data also suggest that CBI-mediated repair of physiological functions may occur independently of integrin relocalization.

Cellular integrins are heterodimeric transmembrane receptors that provide a means for anchorage to extracellular substrates as well as two-way communication between the intracellular and the extracellular environment (Ruoslahti and Engvall, 1997; Molitoris and Marrs, 1999; Schoenwaelder and Burridge, 1999). Activation and clustering of integrins upon binding to extracellular matrix (ECM) proteins initiate focal adhesion formation and the activation of cytoskeletal signaling cascades involved in cell growth, proliferation, migration, differentiation, and gene expression (Molitoris and Marrs, 1999; Schoenwaelder and Burridge, 1999, Zuk et al., 1998). In addition to binding to ECM substrates and mediating cytoskeletal signaling, integrins also are known to influence the formation and composition of the ECM (Riikonen et al., 1995; Gotwals et al., 1996). In renal proximal tubular cells (RPTC), integrins and other proteins, such as Na^+/K^+-ATPases, are localized to the basal membrane where cells interact with the ECM as well as neighboring cells. This is in contrast to the apical membrane, where distinct physiological processes, such as Na^+-dependent glucose and amino acid transport, occur. The cellular polarity derived from the distinct functions carried out at separate membrane regions supports and is critical for proper renal tubular function (Bush et al., 2000).

The renal tubular basement membrane (BM) is composed mainly of collagens, laminins, and heparan sulfate proteoglycans (Furness, 1996, Miner, 1999). The most abundant type of collagen in the BM of the glomerulus and renal tubules is collagen IV, a globular, nonfibrillar protein (Furness, 1996). The combination of α and β integrin subunits that form the

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ABBREVIATIONS: ECM, extracellular matrix; RPTC; renal proximal tubular cells; BM, basement membrane; DCVC, *S*-(1,2-dichlorovinyl)-L-cysteine; AscP, L-ascorbic acid-2-phosphate; CBI, collagen-binding integrins; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; PBS, phosphate-buffered saline; ERK, extracellular-signal regulated kinase.

functional heterodimer largely determine the binding of integrins to collagens and other ECM proteins. At least eight β-subunits and 17 α-subunits have been identified so far, and they associate noncovalently to form more than 20 heterodimers with various signaling and substrate binding properties (Kreidberg and Symons, 2000). Cells most often use the integrin heterodimers $\alpha_1\beta_1$ and $\alpha_2\beta_1$ to bind collagen IV, and the importance of signals derived from collagen-binding integrins (CBI) in normal cellular activities has been studied (Kuhn and Ebel, 1994; Gardner et al., 1996; Knight et al., 1998).

In cases of acute renal failure resulting from chemical exposure or ischemia, tubular epithelial cells may lose polarity as characterized by decreased localization of integrins in the basal membrane and their redistribution throughout the plasma membrane (Goligorsky and DiBona, 1993; Lieberthal et al., 1997; Zuk et al., 1998; Molitoris and Marrs, 1999). The result is cellular disorientation, decreased renal tubular function, and cell death and/or detachment from the tubular BM (Goligorsky and DiBona, 1993; Frisch and Ruoslahti, 1997; Tang et al., 1998; Molitoris and Marrs, 1999). Sublethally injured cells that do not die or become detached from the BM are thought to repair and/or dedifferentiate, proliferate, migrate to denuded areas of the tubule, differentiate, and promote the return of normal renal function (Abbate and Remuzzi, 1996; Molitoris and Marrs, 1999). The effects of cell injury on integrin localization and renal cell polarity have been investigated, but their importance in tubular regeneration after injury is not well understood (Goligorsky and DiBona, 1993; Lieberthal et al., 1997; Zuk et al., 1998; Molitoris and Marrs, 1999; Kreidberg and Symons, 2000).

Previous studies from this laboratory focused on determining the mechanisms of renal tubular cell regeneration using the model nephrotoxicant *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC) to produce sublethal injury in primary cultures of rabbit RPTCs. RPTC exposure to DCVC produced approximately 50% cell death and loss caused the irreversible inhibition of key physiological functions, including mitochondrial function, active Na^+ transport, and Na^+/K^+-ATPase activity, in the remaining sublethally injured RPTC (Nowak et al., 1999). However, addition to the culture media of L-ascorbic acid-2-phosphate (AscP) at pharmacological concentrations promoted proliferation and repair of physiological functions in DCVC-injured RPTC (Nowak et al., 2000). The regeneration of DCVC-injured RPTC was associated with the stimulation of collagen IV deposition by AscP, suggesting that collagen IV deposition plays a key role in the ability of RPTC to recover from sublethal toxicant injury (Nony et al., 2001). Furthermore, the addition of exogenous collagen IV to the culture media of injured RPTC promoted the repair of physiological functions (Nony et al., 2001). Based on these findings, we hypothesized that AscP and exogenous collagen IV act to promote RPTC regeneration through the restoration of interactions between collagen IV and CBI. The specific goals of this study were 1) to determine the fate of CBI after sublethal RPTC injury with regard to expression and subcellular localization and 2) to examine the effect of AscP, exogenous collagen IV, and function-stimulating CBI antibodies on CBI expression and/or localization after sublethal injury in relation to the repair of physiological functions.

Experimental Procedures

Materials. Female New Zealand White rabbits (1.5–2.0 kg) were purchased from Myrtle's Rabbitry (Thompson Station, TN). DCVC was a generous gift from Dr. T. W. Petry (Pharmacia Upjohn, Kalamazoo, MI) and was synthesized as described previously (Moore and Green, 1988). L-Ascorbic acid-2-phosphate (magnesium salt) was purchased from Wako Chemicals USA, Inc. (Richmond, VA). Ouabain was obtained from RBI/Sigma (Natick, MA). FITC-conjugated goat anti-mouse IgG and mouse monoclonal antibodies directed against human integrin subunits α_1 (clone FB12), α_2 (clone JBS2), and β_1 (clone B3B11) were purchased from Chemicon International, Inc. (Temecula, CA). All other materials were purchased from Sigma Chemical Co. (St. Louis, MO).

Isolation of Proximal Tubules and Culture Conditions. Rabbit renal proximal tubules were isolated using the iron oxide perfusion method and grown in 35-mm tissue culture dishes or 48-well cell culture clusters under improved conditions as described previously (Nowak and Schnellmann, 1995, 1996; Nony et al., 2001). The cell culture medium was a 1:1 mixture of Dulbecco's modified Eagle's medium/Ham's F-12 (without D-glucose, phenol red, or sodium pyruvate) supplemented with 15 mM HEPES buffer, 2.5 mM L-glutamine, 1 μM pyridoxine HCl, 15 mM sodium bicarbonate, and 6 mM lactate. Hydrocortisone (50 nM), selenium (5 ng/ml), human transferrin (5 μg/ml), bovine insulin (10 nM), and L-ascorbic acid-2-phosphate (50 or 500 μM) were added to the culture medium immediately before daily media change. AscP was used because L-ascorbic acid is unstable in culture media. AscP is stable in culture media and, after intracellular dephosphorylation, has the same effect on cultured cells as L-ascorbic acid (Hata and Senoo, 1989).

Sublethal Injury of RPTC. Confluent monolayers of RPTC (day 6 after seeding) were exposed to 200 μM DCVC (dissolved in water) for 1.75 h followed by toxicant removal and addition of fresh culture media. This method produces approximately 50% cell death and loss 24 h after the exposure. In some experiments, exogenous collagen IV (50 μg/ml), collagen I (50 μg/ml), or function-stimulating antibodies to CBI subunits α_2 or β_1 (5 μg/ml) were added daily to the culture media of DCVC-injured RPTC cultured in the absence of pharmacological concentrations of AscP. Function-stimulating antibodies to CBI α_1 are not commercially available at this time. On days 1 and 6 after DCVC exposure, active Na^+ transport or Na^+/K^+-ATPase activity, and CBI expression and/or localization in the remaining sublethally injured RPTC was determined as described below.

Active Na^+ Transport. RPTC were gently detached from culture dishes with a rubber policeman and transferred to a 37°C oxygen consumption (QO_2) chamber. QO_2 in RPTC was measured polarographically in the absence (basal QO_2) or presence of ouabain (100 μM) (ouabain-insensitive QO_2) using a Clark-type electrode as described previously (Nowak and Schnellmann, 1995). Active Na^+ transport (ouabain-sensitive QO_2) was calculated by subtracting ouabain-insensitive QO_2 from basal QO_2. Protein concentrations were determined using the bicinchoninic acid microassay according to the manufacturer's instructions (Pierce, Rockford, IL).

Na^+/K^+-ATPase Activity. Total ATPase activity was measured as previously described (Nony et al., 2001). Briefly, RPTC cultured in 48-well cell culture clusters were scraped, solubilized in dissociation buffer (5 mM HEPES, pH 7.4, 25 mM imidazole, 1% BSA, 0.065% SDS) for 10 min at room temperature, and combined with fresh ATPase assay buffer (2.54 mM $MgCl_2$, 100 mM NaCl, 10 mM KCl, 5 mM HEPES, 10 U/ml lactate dehydrogenase, 7 U/ml pyruvate kinase, 2.54 mM Na_2ATP, 2.54 mM phospho(enol) pyruvate, and 0.5 mM β-NADH). ATPase activity was measured under linear conditions spectrophotometrically (340 nm) as the oxidation of β-NADH to NAD^+ at 37°C in the absence or presence of ouabain (0.1 mM). Na^+/K^+-ATPase activity was calculated as total ATPase activity minus ouabain-insensitive ATPase activity.

Expression of CBI. CBI expression was measured by flow cytometry of RPTC immunostained with monoclonal antibodies to in-

tegrin subunits α_1, α_2, and β_1. RPTC monolayers were washed three times with ice-cold PBS, gently scraped from culture dishes into cell culture media containing 5% BSA (BSA/media), and transferred to microcentrifuge tubes. RPTC were dissociated by pipetting, incubated on ice with moderate shaking for 20 min, centrifuged, and resuspended in 1% BSA/media containing 2 μg/ml of a specific anti-integrin antibody or nonspecific IgG on ice with moderate shaking for 1 h. After three washes with 1% BSA/media, RPTC were incubated for 30 min in the dark with a goat-anti mouse FITC-conjugated IgG diluted 1:100 in 1% BSA/media, followed by three washes with ice-cold PBS. Membrane expression of CBI was determined immediately by flow cytometry using a FACSCalibur four-color cell sorter/analyzer (Becton Dickinson, San Jose, CA) with a blue argon laser for detection of FITC. Specific binding was calculated as total fluorescence minus that in IgG controls.

Subcellular Localization of CBI. CBI localization was determined using confocal microscopy of RPTC monolayers stained with monoclonal antibodies to integrin subunits α_1, α_2, and β_1. RPTC monolayers were washed three times with PBS and fixed with 10% buffered formalin (4% formaldehyde) for 20 min at room temperature. After three washes with PBS, monolayers were permeabilized in PTB buffer (PBS, 0.3% Triton X-100, 0.1% BSA) for 10 min at room temperature. Monolayers were washed three times with 0.1% BSA in PBS and incubated with 8% BSA in PBS for 30 min at room temperature. BSA (1%) in PBS containing 5 μg/ml of specific integrin antibodies or nonspecific IgG was added to RPTC monolayers and incubated overnight at 4°C with moderate shaking. After three washes with PTB buffer, monolayers were incubated for 2 h in the dark at room temperature with 1% BSA in PBS containing a 1:100 dilution of a FITC-conjugated goat anti-mouse IgG. Monolayers were washed three times with PTB and glass coverslips applied after the addition of two to three drops of mounting media. Confocal microscopy was performed using a Zeiss confocal laser scanning microscope (model 410; Carl Zeiss, Inc., Thornwood, NY). Basal and apical membrane locations were determined visually in the Z-plane using light field microscopy. Two to three photomicrographs per monolayer at the basal and apical membranes were then scanned with an omnichrome laser filtered at 488 nm to detect FITC.

Statistical Analysis. RPTC isolated from one rabbit represent one experiment (n = 1) that consisted of data collected from one to two plates of cells. Experiments were repeated until an n of 3 to 6 was reached. Data are presented as means ± S.E.M. Significant differences between treatment groups ($p < 0.05$) were determined using SigmaStat one-way analysis of variance and Student-Newman-Keuls *post hoc* test for the comparison of multiple means (Jandel Scientific, San Rafael, CA).

Results

Effect of AscP and Exogenous Collagen IV on Active Na^+ Transport in DCVC-Injured RPTC. Exposure of uninjured RPTC to pharmacological concentrations of AscP or exogenous collagen IV had no effect on active Na^+ transport on days 1 or 6 (data not shown). On day 1 after injury, active Na^+ transport was decreased approximately 80% in injured RPTC grown in the absence or presence of pharmacological concentrations of AscP or collagen IV (Fig. 1A). On day 6, DCVC-injured RPTC cultured in the presence of exogenous collagen IV exhibited a concentration-dependent improvement in active Na^+ transport, similar to that seen in injured RPTC cultured in the presence of pharmacological concentrations of AscP (Fig. 1B).

Effects of Sublethal Injury and Exogenous Collagen IV on Total CBI Expression in RPTC. Monoclonal antibodies to the CBI subunits α_1, α_2, and β_1 and flow cytometry were used to measure total plasma membrane expression of CBI on days 1 and 6 after DCVC exposure. Figure 2 demonstrates the fluorescence-shift observed in response to incubation of rabbit RPTC with the anti-integrin antibodies for individual CBI subunits. Exposure of uninjured RPTC to pharmacological concentrations of AscP or exogenous collagen IV did not affect CBI α_1, α_2, and β_1 expression (data not shown). After exposure to DCVC, levels of expression of CBI subunits α_1, α_2, and β_1 in injured RPTC were unchanged on day 1 compared with control (Fig. 3, a, c, and e). On day 6 after injury, expression of CBI subunits α_1 and β_1 was unchanged in DCVC-injured RPTC compared with control (Fig. 3, b and f). However, membrane expression of CBI subunit α_2 was increased approximately 2.2-fold in DCVC-injured RPTC (Fig. 3d). The presence of pharmacological concentrations of AscP or exogenous collagen IV did not affect the expression of CBI subunits α_1, α_2, or β_1 in sublethally injured RPTC.

Effects of Sublethal Injury and Exogenous Collagen IV on the Subcellular Localization of CBI in RPTC. On day 1 after DCVC exposure, the intensity of CBI α_1, α_2, and β_1 fluorescent staining at the basal membrane was decreased compared with control (A–D in Figs. 4, 6, and 8). For comparison with injured RPTC on day 6, uninjured, subconfluent (80%) RPTC cultures were used as controls for basal localization of CBI. As opposed to uninjured RPTC in day 12 of culture (6 days of growth to confluence plus the 6 experimen-

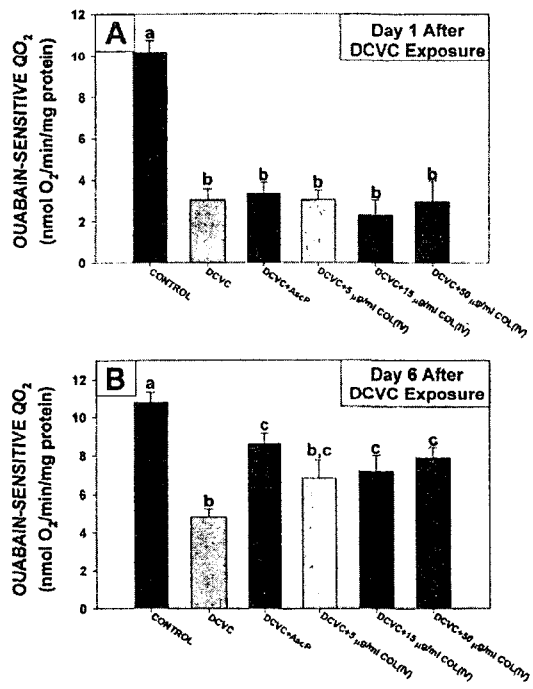

Fig. 1. Active Na^+ transport measured as ouabain-sensitive QO_2 in RPTC sublethally injured by DCVC and cultured in the absence or presence of exogenous collagen IV (0, 5, 15, and 50 μg/ml). Ouabain sensitive QO_2 was measured on days 1 and 6 after DCVC injury. Data are presented as means ± S.E.M., n = 4 to 5 separate experiments. Bars labeled with different letter symbols are significantly different from each other ($P < 0.05$).

tation days), subconfluent RPTC cultures exhibit morphology and cell density more like that of sublethally injured RPTC cultures. On day 6 after injury, basal localization of CBI subunits α_1, α_2, and β_1 in RPTC cultured in the absence of AscP or exogenous collagen IV was still decreased compared with subconfluent controls (F in Figs. 4, 6, and 8). In contrast, sublethally injured RPTC cultured in the presence of either pharmacological concentrations of AscP or exogenous collagen IV exhibited a return to control levels of basal localiza-

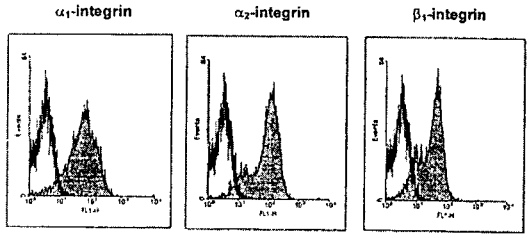

Fig. 2. Binding of monoclonal antibodies to CBI subunits. Untreated rabbit RPTC were gently scraped from culture dishes and incubated with nonspecific IgG or primary monoclonal antibodies to CBI subunits α_1, α_2, or β_1. Fluorescence intensity of FITC-conjugated goat anti-mouse secondary antibodies was measured by flow cytometry. FL1-H (x-axis) displays fluorescence intensity on a logarithmic scale. Shaded histograms represent fluorescence intensity of RPTC labeled with primary antibodies to CBI subunits. Open histograms represent RPTC incubated with a nonspecific IgG.

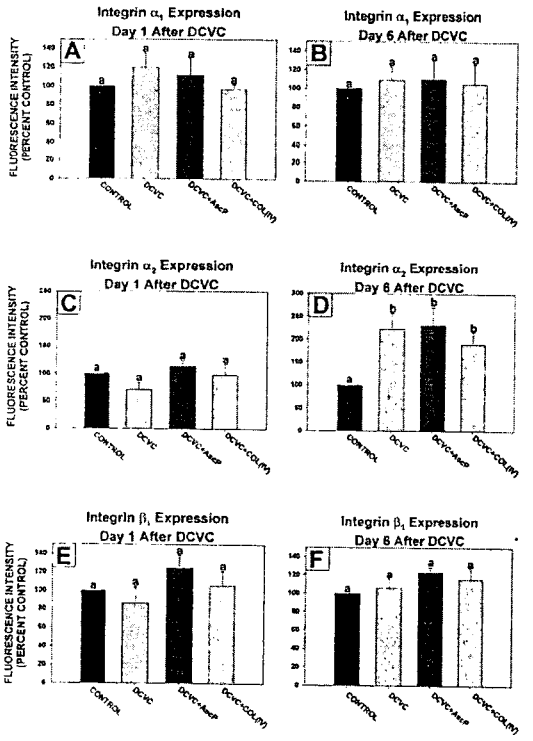

Fig. 3. Expression of CBI subunits α_1, α_2, and β_1 in RPTC sublethally injured by DCVC and cultured in the absence or presence of pharmacological concentrations of AscP (500 μM) or exogenous collagen IV (50 μg/ml). On days 1 (A, C, and E) and 6 (B, D, and F) after DCVC injury, plasma membrane expression of CBI was analyzed by flow cytometry. Fluorescence intensity is expressed as a percent of control ± S.E.M. after subtraction of fluorescence attributed to nonspecific IgG binding (n = 4–6). Bars labeled with different letter symbols are significantly different from each other ($P < 0.05$).

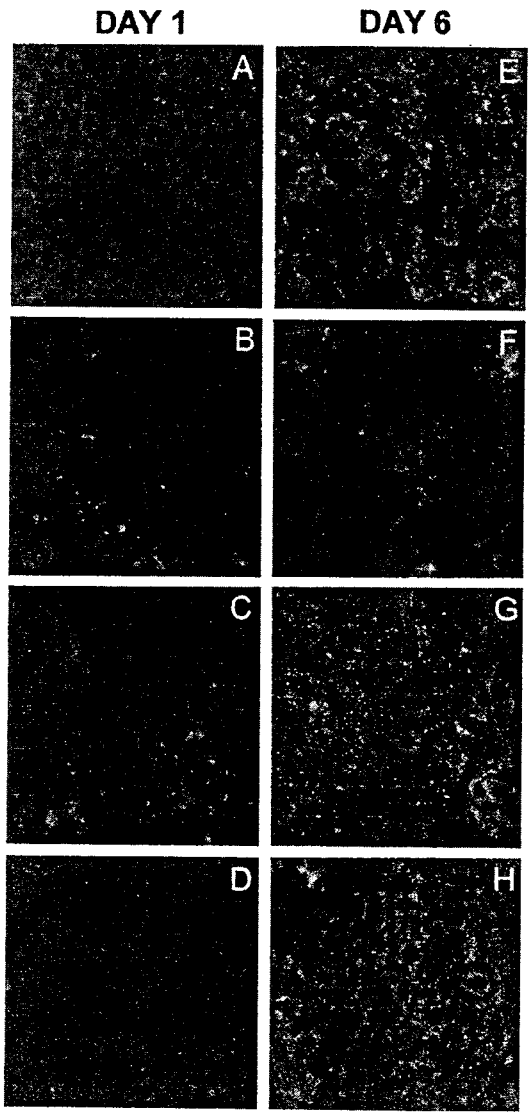

Fig. 4. Basal membrane localization of CBI subunit α_1 in RPTC on day 1 (left) and day 6 (right) after DCVC exposure. A, control RPTC; B and F, DCVC-injured RPTC; C and G, DCVC-injured RPTC cultured in the presence of pharmacological concentrations of AscP; D and H, DCVC-injured RPTC cultured in the presence of exogenous collagen IV; E, subconfluent control RPTC. Shown are representative confocal photomicrographs from three to four separate experiments (magnification, 400×).

tion of CBI subunits α_1, α_2, and β_1 (G and H in Figs. 4, 6, and 8). With respect to the apical membrane, uninjured control animals showed no CBI staining, whereas CBI subunits α_1, α_2, and β_1 were partially redistributed to the apical membrane in sublethally injured RPTC on day 1 after injury (D in Figs. 5, 7, and 9). On day 6, sublethally injured RPTC continued to exhibit CBI distributed to the apical membrane (F in Figs. 5, 7, and 9). However, injured RPTC cultured in the presence of pharmacological concentrations of AscP or exogenous collagen IV revealed a complete disappearance of CBI from the apical membrane by day 6 (G and H in Figs. 5, 7, and 9). These data show that sustained redistribution of CBI characterized by decreased basal localization and the appear-

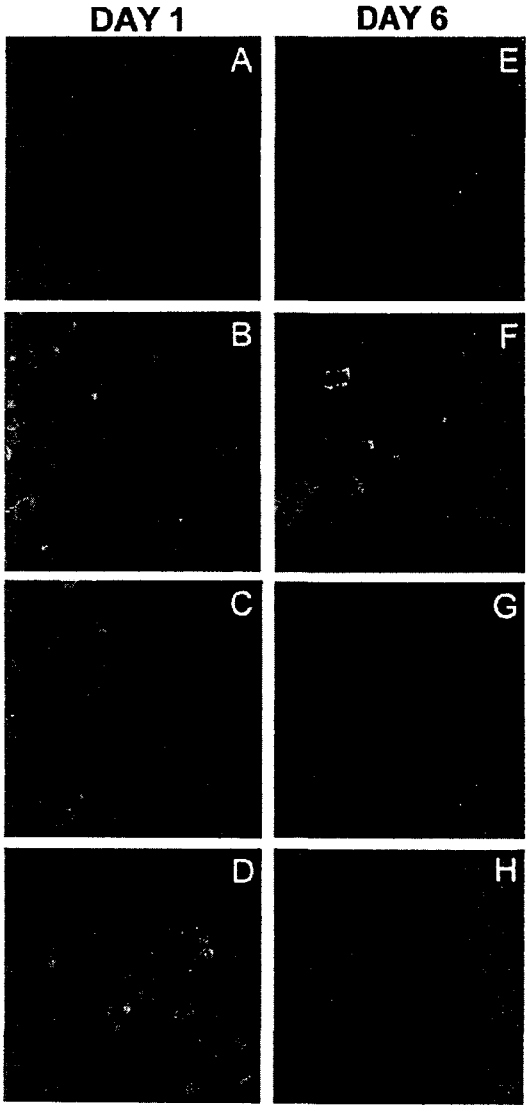

Fig. 5. Apical membrane localization of CBI subunit α_1 in RPTC on day 1 (left) and day 6 (right) after DCVC exposure. A, control RPTC; B and F, DCVC-injured RPTC; C and G, DCVC-injured RPTC cultured in the presence of pharmacological concentrations of AscP; D and H, DCVC-injured RPTC cultured in the presence of exogenous collagen IV; E, subconfluent control RPTC. Shown are representative photomicrographs from three to four separate experiments (magnification, 400×).

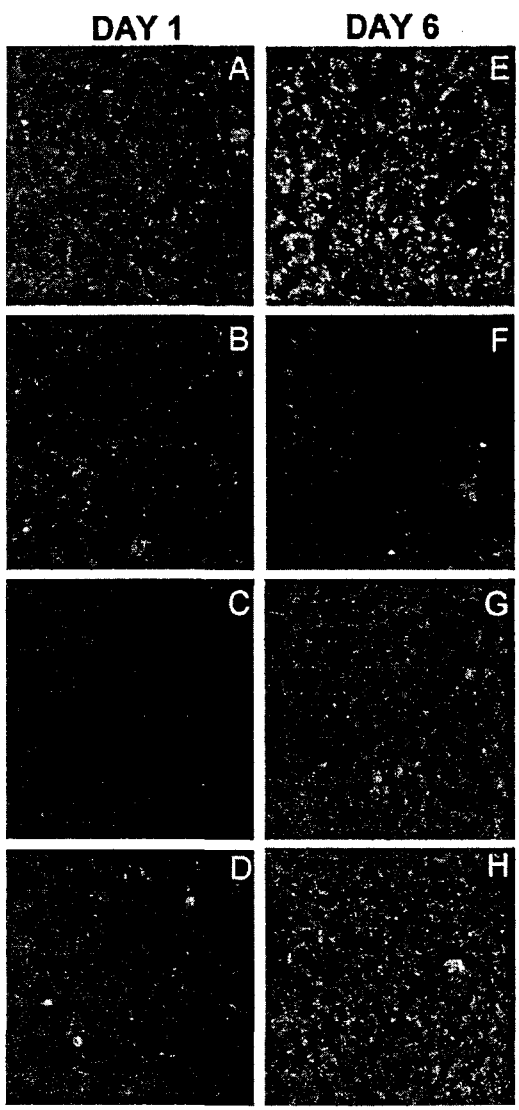

Fig. 6. Basal membrane localization of CBI subunit α_2 in RPTC on day 1 (left) and day 6 (right) after DCVC exposure. A, control RPTC; B and F, DCVC-injured RPTC; C and G, DCVC-injured RPTC cultured in the presence of pharmacological concentrations of AscP; D and H, DCVC-injured RPTC cultured in the presence of exogenous collagen IV; E, subconfluent control RPTC. Shown are representative confocal photomicrographs from three to four separate experiments (magnification, 400×).

ance of CBI on the apical membrane of RPTC is a consequence of sublethal injury by DCVC. In addition, sublethally injured RPTC cultured in the presence of pharmacological concentrations of AscP or exogenous collagen IV are able to reorient CBI to the basal membrane.

Effect of Function-Stimulating Antibodies to CBI on Na^+/K^+-ATPase Activity and the Subcellular Localization of CBI in RPTC. Function-stimulating antibodies to CBI subunits α_2 and β_1 were added to the culture media of DCVC-injured RPTC. The function-stimulating antibodies to CBI subunits α_2 and β_1 did not affect the degree of DCVC-induced RPTC injury on day 1 after exposure (data not shown). On day 6 after injury, the CBI β_1-stimulating anti-

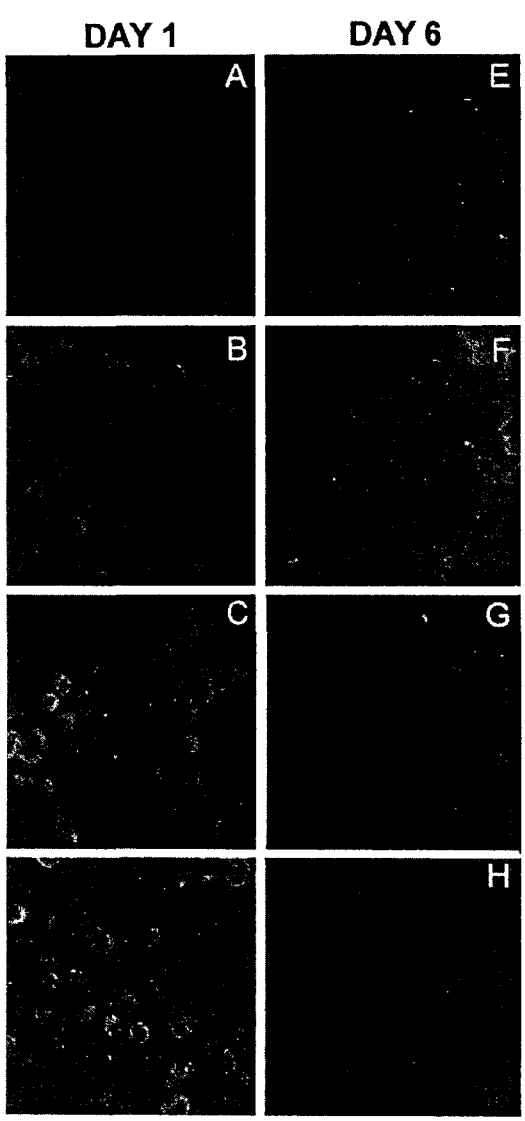

Fig. 7. Apical membrane localization of CBI subunit α_2 in RPTC on day 1 (left) and day 6 (right) after DCVC exposure. A, control RPTC; B and F, DCVC-injured RPTC; C and G, DCVC-injured RPTC cultured in the presence of pharmacological concentrations of AscP; D and H, DCVC-injured RPTC cultured in the presence of exogenous collagen IV; E, subconfluent control RPTC. Shown are representative photomicrographs from three to four separate experiments (magnification, 400×).

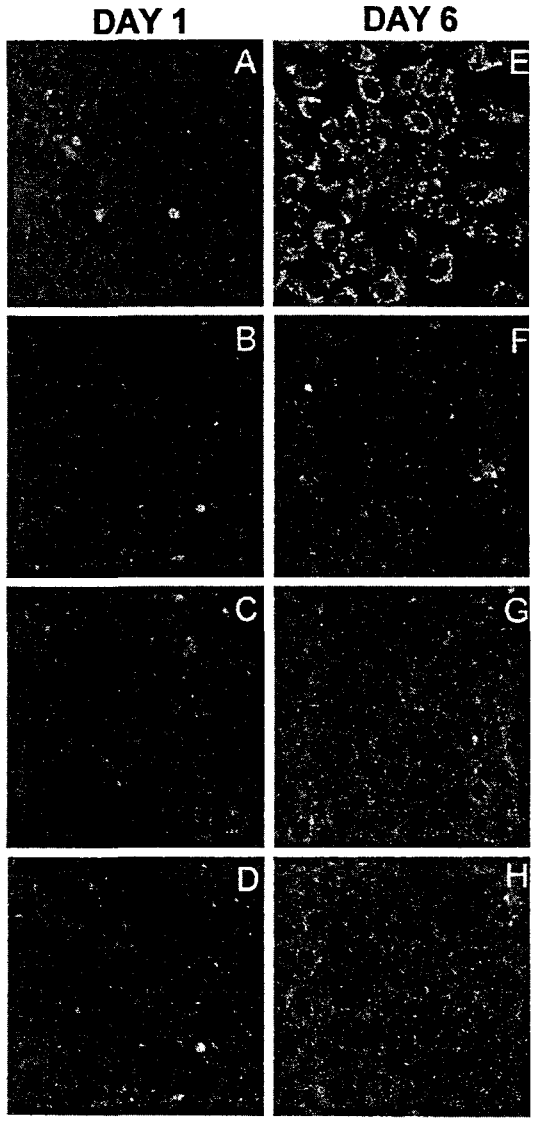

Fig. 8. Basal membrane localization of CBI subunit β_1 in RPTC on day 1 (left) and day 6 (right) after DCVC exposure. A, control RPTC; B and F, DCVC-injured RPTC; C and G, DCVC-injured RPTC cultured in the presence of pharmacological concentrations of AscP; D and H, DCVC-injured RPTC cultured in the presence of exogenous collagen IV; E, subconfluent control RPTC. Shown are representative confocal photomicrographs from three to four separate experiments (magnification, 400×).

body, but not the CBI α_2-stimulating antibody, promoted the repair of Na^+/K^+-ATPase activity in DCVC-injured RPTC (Fig. 10). The addition to the culture media of function-stimulating antibodies to CBI subunits α_2 or β_1 did not prevent basal delocalization or partial apical redistribution of CBI α_2 or β_1 on day 1 after DCVC exposure (data not shown). Despite the repair of Na^+/K^+-ATPase activity on day 6, CBI subunit β_1 remained delocalized and partially redistributed to the apical membrane in RPTC cultured in the presence of the β_1-stimulating antibody (Fig. 11).

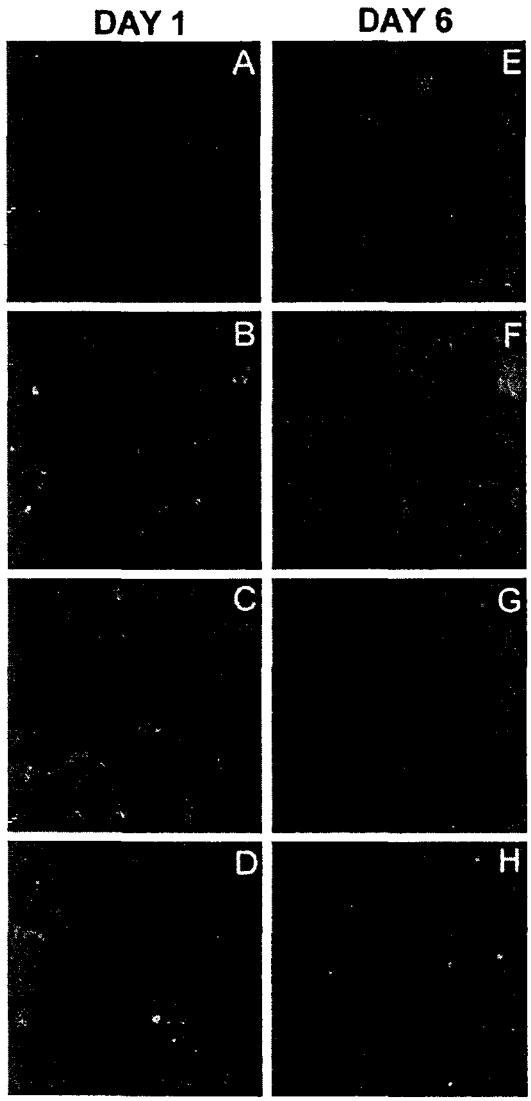

Fig. 9. Apical membrane localization of CBI subunit β_1 in RPTC on day 1 (left) and day 6 (right) after DCVC exposure. A, control RPTC; B and F, DCVC-injured RPTC; C and G, DCVC-injured RPTC cultured in the presence of pharmacological concentrations of AscP; D and H, DCVC-injured RPTC cultured in the presence of exogenous collagen IV; E, subconfluent control RPTC. Shown are representative photomicrographs from three to four separate experiments (magnification, 400×).

Discussion

Anchorage-dependent cell growth, proliferation, migration, and differentiation depend on the ability of the cell to recognize anchoring substrates in the ECM. Localization of ECM-binding integrins to the point of contact provides a strong but dynamic interaction that supports not only cellular attachment but also communication between the cell and the ECM. Given the importance of these interactions and the maintenance of cell polarity, the loss of integrin-ECM interactions and cell polarity plays an essential role in cell injury. Likewise, the restoration of integrin-ECM interactions and cell polarity probably plays an equally important role in the return of normal cell function after injury.

Loss of renal epithelial cell polarity caused by partial integrin redistribution throughout the plasma membrane has been shown to be a key event in renal dysfunction after acute chemical exposure or ischemia (Goligorsky and DiBona, 1993; Lieberthal et al., 1997; Zuk et al., 1998; Molitoris and Marrs, 1999). The resulting cellular disorientation and dysfunction with cell death and/or detachment from the BM leads to decreased renal tubular function (Goligorsky and DiBona, 1993; Frisch and Ruoslahti, 1997; Tang et al., 1998; Molitoris and Marrs, 1999). Despite evidence demonstrating the loss of integrin polarity during renal cell injury, the importance of the restoration of integrin localization and cell polarity in tubular regeneration after injury is not well understood (Goligorsky and DiBona, 1993; Lieberthal et al., 1997; Kreidberg and Symons, 2000; Molitoris and Marrs, 1999; Zuk et al., 1998). Previous studies in our laboratory demonstrated that the ability of injured RPTC to deposit collagen IV is associated with the repair of inhibited physiological functions after DCVC injury (Nony et al., 2001). Furthermore, exogenous collagen IV added to the culture media

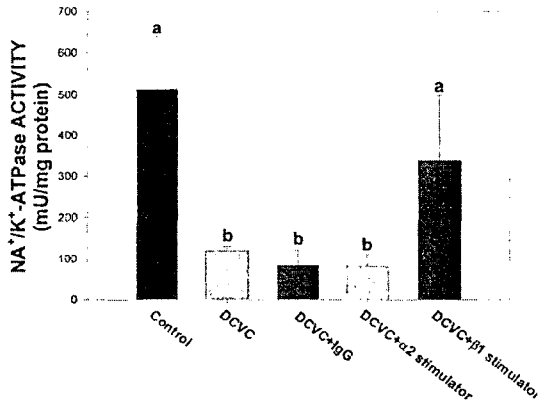

Fig. 10. Na^+/K^+-ATPase activity in RPTC sublethally injured by DCVC and cultured in the absence or presence of function-stimulating antibodies to CBI subunits α_2 or β_1 (5 μg/ml). Na^+/K^+-ATPase activity was measured on days 1 and 6 after injury. Data are presented as means ± S.E.M., $n = 3$ to 5 separate experiments. Bars labeled with different letter symbols are significantly different from each other ($P < 0.05$).

of injured RPTC promoted repair of physiological functions, providing the first evidence that a key ECM protein in the renal proximal tubule is involved in cell repair after injury (Nony et al., 2001). In contrast, collagen I, laminin, and fibronectin did not promote cell repair after injury. Because renal epithelial cells interact with collagen IV through CBI, our data suggested that physiological repair in injured RPTC may involve an effect of collagen IV on the expression, localization, and/or function of CBI. As mentioned above, sublethally injured cells may experience a loss in cell polarity because of decreased localization of certain proteins to specific areas of the plasma membrane. Two potential reasons for a decrease in basal membrane protein localization include a decrease in membrane expression of those proteins because of translational effects or receptor internalization or the redistribution of those proteins to other areas of the plasma membrane. After DCVC-induced sublethal injury to RPTC, no changes in total membrane expression of CBI were evident on day 1. However, confocal microscopy showed that CBI localization to the basal membrane was decreased and accompanied by the appearance of CBI on the apical membrane. These observations show that sublethal toxicant injury produces a decrease in basal CBI localization because of redistribution of CBI as opposed to decreased overall expression of CBI on the plasma membrane. On day 6 after injury, confocal microscopy demonstrated that all CBI were still redistributed to the apical membrane, suggesting that DCVC-injured RPTCs remain disoriented with lost cellular polarity. These novel findings associate sustained integrin redistribution with the lack of repair of physiological functions after DCVC injury.

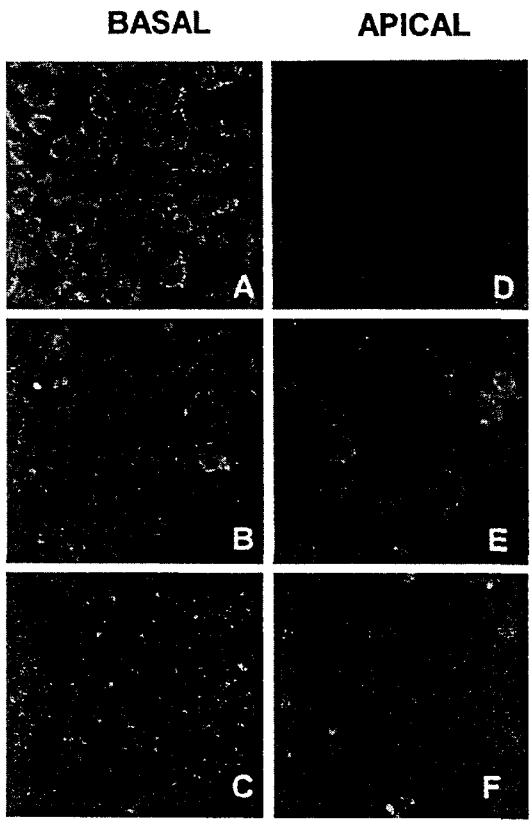

Fig. 11. Basal (left) and apical (right) localization of CBI subunit β_1 on day 6 after DCVC exposure in RPTC cultured in the absence or presence of CBI subunit β_1-stimulating antibodies. A and D, control RPTC; B and E, DCVC-injured RPTC; C and F, DCVC-injured RPTC+β_1-stimulating antibody. Shown are representative confocal photomicrographs from two separate experiments (magnification, 400×).

Because pharmacological concentrations of AscP and exogenous collagen IV stimulated repair of physiological functions after DCVC exposure, we determined the effects of AscP and exogenous collagen IV on the expression and localization of CBI. Addition of pharmacological concentrations of AscP or exogenous collagen IV to culture media of injured RPTC produced no changes in total membrane expression of CBI after sublethal injury. However, exposure to AscP and exogenous collagen IV resulted in the return of basal membrane localization of CBI with the loss of CBI from the apical membrane. These observations suggest that extracellular collagen IV, whether stimulated by AscP or added exogenously, promotes the return of cellular polarity characterized by the basal re-orientation of CBI and Na^+/K^+-ATPase (Nowak et al., 2000), and the return of Na^+/K^+-ATPase activity. Concerning cell repair, these data suggest that an important step in the repair of physiological functions stimulated by extracellular collagen IV is the reorientation of CBI to the basal membrane and the restoration of cellular polarity.

On day 6 after DCVC-injury, RPTC exhibited a significant increase in the membrane expression of the CBI subunit α_2 in the absence or presence of pharmacological concentrations of AscP or exogenous collagen IV. However, the basal membrane localization of CBI subunit α_2 on day 6 after injury correlated well with that of CBI subunits α_1 and β_1, although membrane expression of those subunits did not change. The qualitative assessment of CBI localization used in this study allows for the comparison of spatially distinct membrane regions with regard to the absence or presence of CBI. Our data do not permit an accurate quantification of CBI density at the observed regions of the plasma membrane, nor does it account for CBI localized to lateral membrane regions between the apical and basal membranes. Therefore, the physiological or pathological relevance of an increase in membrane expression of CBI subunit α_2 in this study is not clear.

Because interactions between collagen IV and CBI seemed to be associated with the promotion of physiological repair, we hypothesized that repair could be stimulated by activating CBI in the absence of collagen IV or AscP. To test this, injured RPTC were cultured in the presence of function-stimulating antibodies to CBI subunits α_2 or β_1. Indeed, the CBI subunit β_1 antibody, but not the subunit α_2 antibody, promoted the return of Na^+/K^+-ATPase activity in injured RPTC, suggesting that signaling through the β_1 integrin is linked to the repair of physiological functions. However, it is not known which α subunit is associated with β_1 under these conditions. Although it seems that the α_2 subunit is not associated with β_1, it cannot be excluded that the α_2 antibody can bind to α_2 but does not act as a stimulating antibody in this model. It is also possible that α_1 or another unknown

subunit is associated with β_1. Although an α_1-stimulating antibody was not available, the development of additional function stimulating or blocking antibodies would advance this line of research.

Despite the stimulation of repair, decreased CBI localization in the basal membrane and partial apical redistribution in injured RPTC were not reversed in response to the α_2 and β_1-stimulating integrin antibodies. This result suggests that activation of β_1 integrins promotes repair through a mechanism that is independent of CBI relocalization after injury. Furthermore, the repair and relocalization effects of collagen IV may be through two different CBI. Alternatively, the difference observed between the β_1 antibody response and collagen IV may occur downstream of the CBI binding. For example, outside-in signaling cascades mediated by integrins have been shown to stimulate the activation of extracellular-signal regulated kinases (ERKs) (Schlaepfer et al., 1994; Boudreau and Jones, 1999). In addition, integrin-mediated ERK activation has been shown to proceed through distinct pathways depending upon what type of integrin is involved (Lin et al., 1997; Barberis et al., 2000; Tian et al., 2000). At this time, the role of ERK activation in RPTC repair is unknown. Therefore, linking repair and the activation of integrin β_1 independent of integrin-ECM interactions in RPTC requires further study.

In conclusion, our data show that the total expression of CBI in sublethally injured RPTC is not altered on day 1 after injury, but that CBI are decreased in the basal membrane and partially redistributed to the apical membrane. On day 6 after injury, DCVC-treated RPTC that do not repair physiological functions still exhibit decreased CBI and redistribution. In contrast, the presence of pharmacological concentrations of AscP or exogenous collagen IV in the culture media of DCVC-injured RPTC promotes the disappearance of CBI from the apical membrane and basal membrane reorientation of CBI by day 6 after injury. This study demonstrates for the first time that AscP- or collagen IV-mediated relocalization of CBI is related to the repair of physiological functions. In addition, antibody addition experiments suggest that integrin-mediated repair of physiological functions may proceed through multiple pathways. These novel findings suggest that there is a specific role for collagen IV to promote physiological repair, in part, through the restoration of CBI localization and cellular polarity, shedding new light on the mechanisms of renal cell repair after chemical-induced injury.

Acknowledgments

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