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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.

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

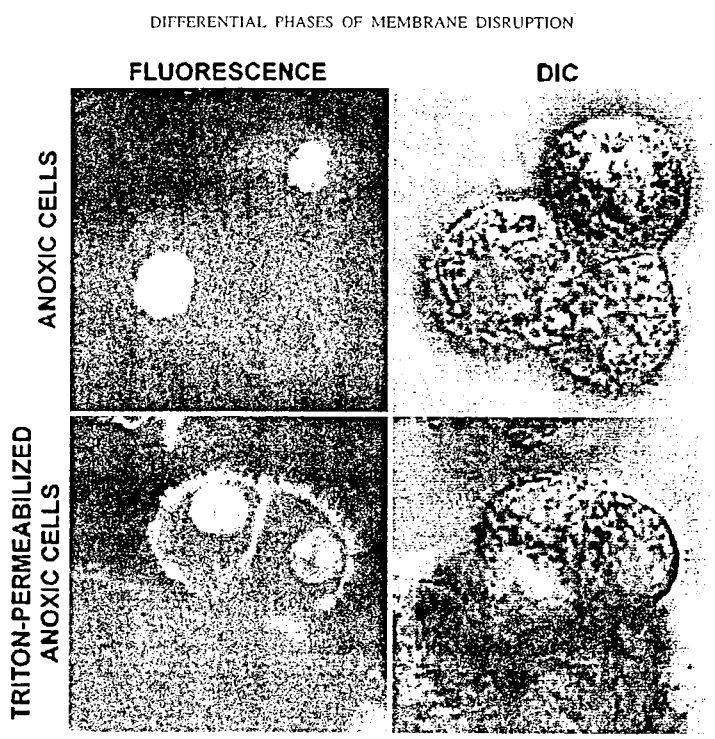

FIG. 1. Differentiation of anoxia-induced permeability of the plasma membrane to PI and PHA. Upper panels: RPT were incubated under anoxia in the presence of 20 μM PI (MW = 668) and 1:50 diluted PHA (MW = 1125) for 20 min. The nuclei of two cells were stained by PI while microvillar F-actin was not stained by PHA, indicating a differential permeability of the plasma membrane to PI and PHA during anoxia. Lower panels: After the 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 cells and stained microvillar F-actin. The fluorescent (left column) and differential interference contrast (DIC, right column) microscopic images were taken 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

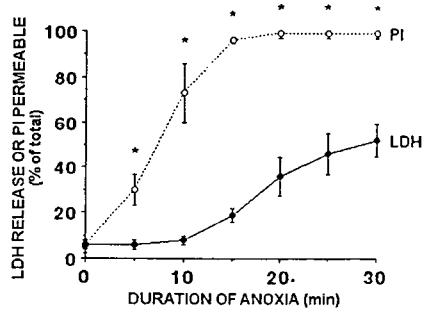

FIG. 2. Differentiation of anoxia-induced permeability of plasma membranes to PI and LDH. RPT were incubated under anoxia for 30 min in the presence of PI. At various time points of anoxic incubation, RPT LDH release and PI staining were determined. $*p < 0.05$, compared to LDH at the corresponding time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

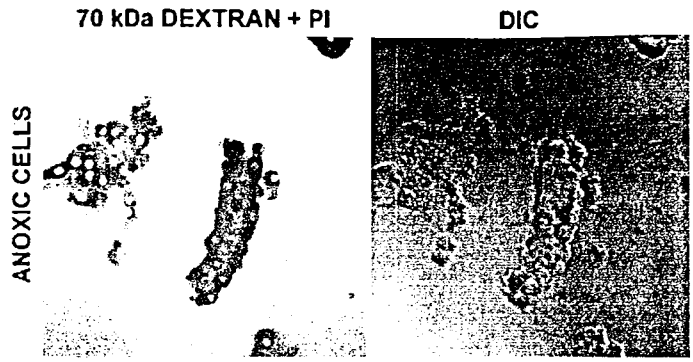

FIG. 3. Differentiation of anoxia-induced permeability of plasma membranes to PI and 70 kDa dextran. RPT were incubated under anoxia for 30 min in a medium containing 20 μM PI. RPT were then resuspended in a medium containing 0.1% 70 kDa fluorescein dextran. Fluorescent (left panel) and DIC (right panel) 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).

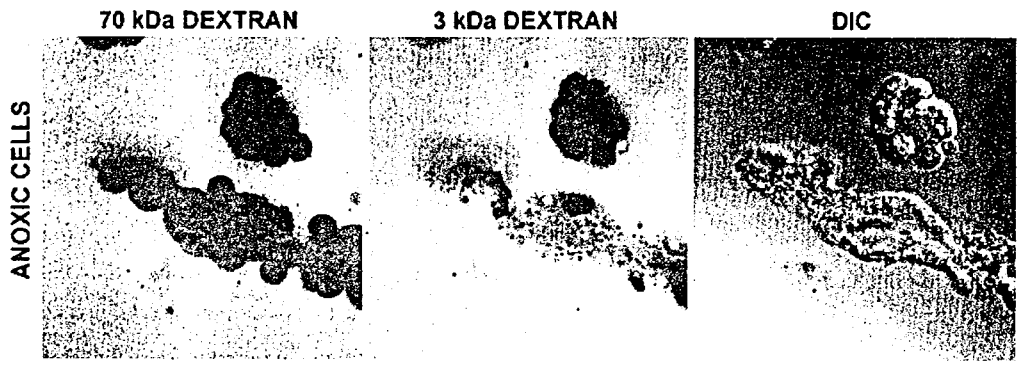

FIG. 4. Differentiation of anoxia-induced permeability of plasma membranes to 3 and 70 kDa dextran. RPT were suspended in a medium containing 0.1% 70 kDa fluorescein dextran and 0.1% 3 kDa tetramethylrhodamine dextran after 30 mm of anoxia. Fluorescent and DIC images were taken using confocal microscopy. 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).

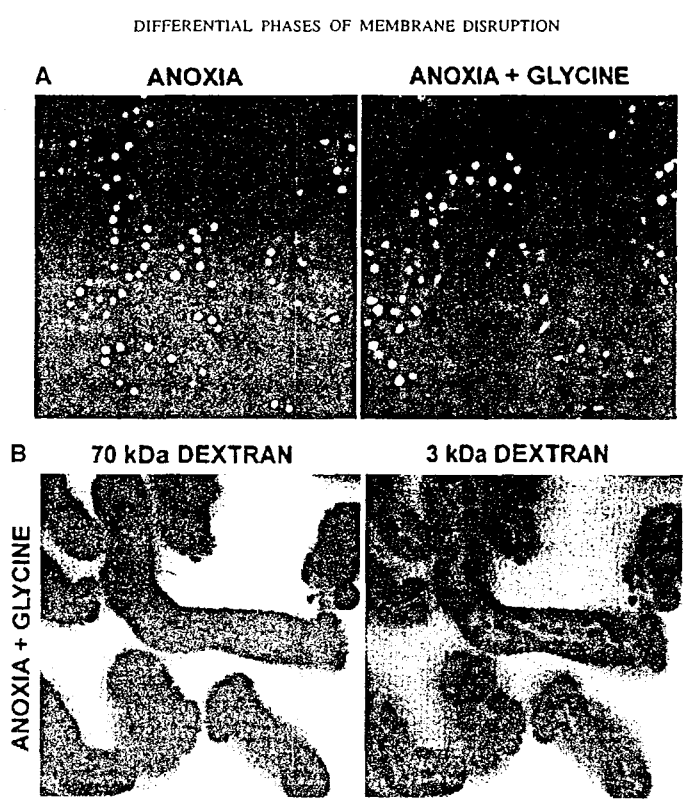

FIG. 5. Glycine prevents anoxia-induced plasma membrane permeability to 3 and 70 kDa dextrans but not to PI. (A) RPT were incubated under anoxia in the presence of 20 μM PI for 30 min in the absence (left) and presence of 4 mM glycine (right). Fluorescent images were taken using a confocal microscope. 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

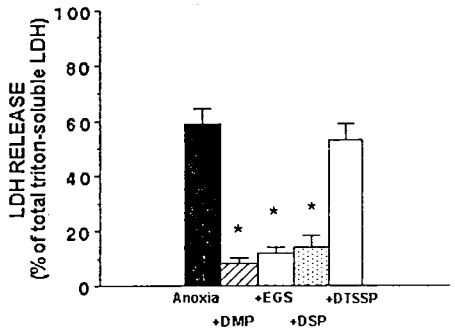

FIG. 6. LDH release from crosslinker-treated RPT after 30 min of anoxia. RPT were subjected to 30 min of anoxia in the absence or presence of the homobifunctional imidoester and NHS-ester crosslinkers. DMP (10 mM), EGS (3 mM), DSP (3 mM), or DTSSP (3 mM). The membrane-permeable crosslinkers are DMP, 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).

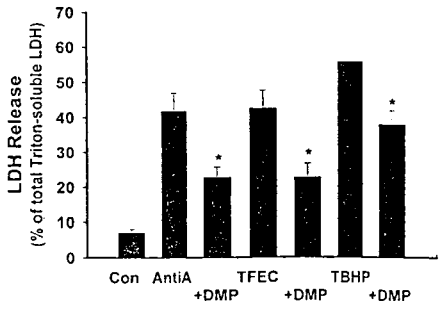

FIG. 7. LDH release from DMP-treated RPT after toxicant exposure. RPT were incubated with antimycin A (AntiA, 10 μM for 2 h), tetrafluoroethyl-L-cysteine (TFEC, 25 μM for 3 h) or *t*-butylhydroperoxide (TBHP, 0.5 mM for 3 h) in the absence or presence of DMP (10 mM). LDH release was expressed as percent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

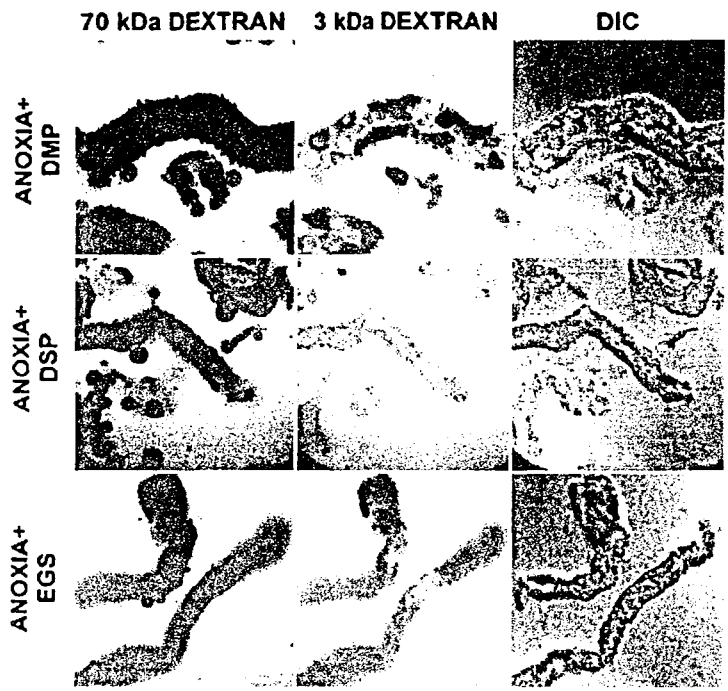

FIG. 8. Chemical crosslinkers prevent anoxia-induced plasma membrane permeability to 70 kDa dextran but not to 3 kDa dextran. RPT were incubated with 10 mM DMP (upper panels), 3 mM DSP (middle panels), or 3 mM EGS (lower panels) under anoxia for 30 min. RPT were resuspended, in the absence of crosslinkers, 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).

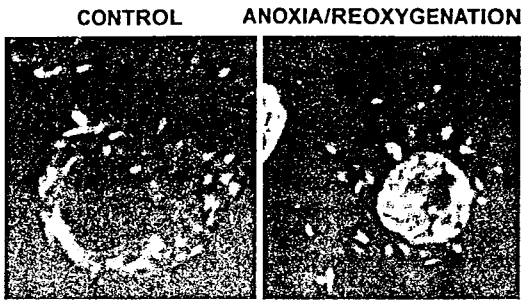

FIG. 9. Functioning mitochondria in RPT permeabilized to PI during anoxia/reoxygenation. RPT were subjected to anoxia/reoxygenation in the presence of 20 μM PI for 20 min. RPT were resuspended in the absence of PI and subjected to reoxygenation for 25 min in the presence of a mitochondrial membrane potential 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-

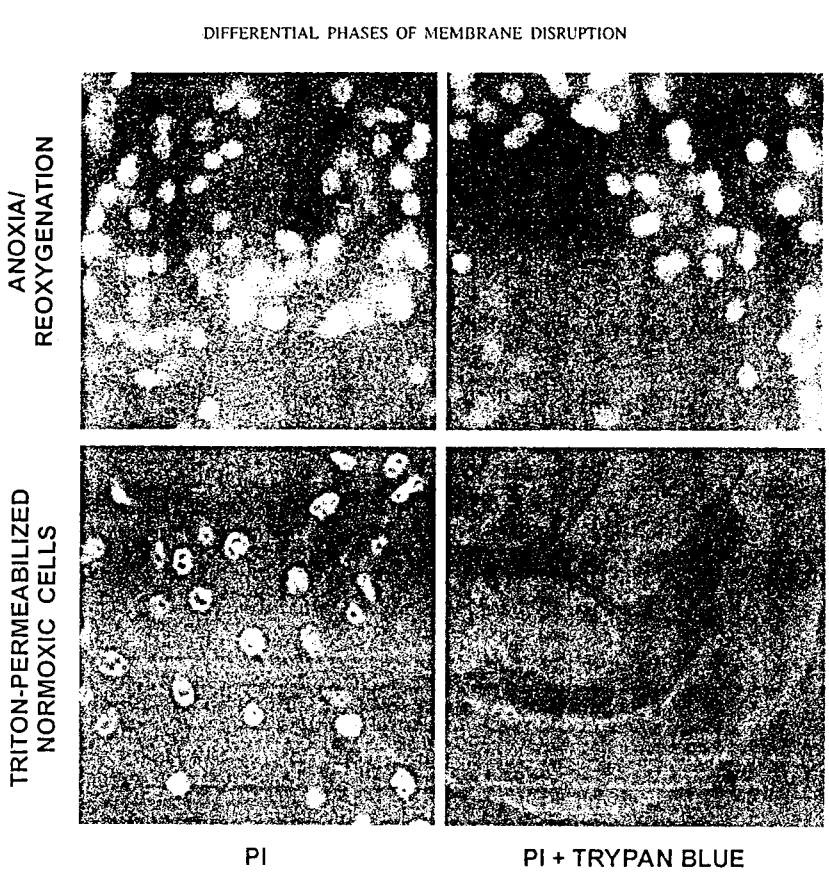

FIG. 10. Lack of trypan blue (TB) staining in RPT permeabilized to PI during anoxia/reoxygenation. RPT were subjected to anoxia in the presence of 20 μM PI for 20 min. RPT were resuspended in the absence of PI and subjected to reoxygenation for 25 min. RPT were then exposed to 0.2% TB in PBS and examined 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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