Effects of chloride channel inhibitors on H_{2}O_{2}-induced renal epithelial cell injury

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Meng, Xianmin, and W. Brian Reeves. Effects of chloride channel inhibitors on H_{2}O_{2}-induced renal epithelial cell injury. Am. J. Physiol. Renal Physiol. 278: F83–F90, 2000.—Oxidative stress contributes to renal epithelial cell injury in certain settings. Chloride influx has also been proposed as an important component of acute renal epithelial cell injury. The present studies examined the role of Cl\(^{-}\) in H_{2}O_{2}-induced injury to LLC-PK\(_{1}\) renal epithelial cells. Exposure of LLC-PK\(_{1}\) cells to 1 mM H_{2}O_{2} resulted in the following: depletion of intracellular ATP content; DNA damage; lipid peroxidation; and a loss of membrane integrity to both small molecules, e.g., trypan blue, and macromolecules, e.g., lactate dehydrogenase (LDH), and cell death. Substitution of Cl\(^{-}\) by isethionate or the inclusion of certain Cl\(^{-}\)-channel blockers, e.g., diphenylamine-2-carboxylate (DPC), 5-nitro-2-(3-phenylpropylamino)benzoate (NPPB), and niflumic acid, prevented the H_{2}O_{2}-induced loss of membrane integrity to LDH. In addition, the H_{2}O_{2}-induced loss of membrane integrity was prevented by raising the osmolality of the extracellular solutions, by depletion of cell ATP, and by inhibitors of volume-sensitive Cl\(^{-}\) channels. However, these maneuvers did not prevent the H_{2}O_{2}-induced permeability to small molecules or H_{2}O_{2}-induced ATP depletion, DNA damage, lipid peroxidation, or cell death. These results support the view that volume-sensitive Cl\(^{-}\) channels play a role in the progressive loss of cell membrane integrity during injury.

A CARDINAL FEATURE of the necrotic form of cell death is a loss of membrane integrity. Indeed, the development of membrane permeability to small dyes, such as trypan blue or propidium iodide, or to macromolecules, such as lactate dehydrogenase (LDH), has been considered a marker of irreversible necrotic cell death. The mechanisms that are responsible for the loss of membrane integrity are poorly understood. The maintenance of cellular ion homeostasis requires the precise balance of solute influx and efflux via both active and passive membrane transport pathways. It has been proposed that the uptake of solute, mainly sodium and chloride, with subsequent cell swelling contributes to membrane injury in renal epithelial cells (23) and other tissues (6, 12, 18). In support of this view are both in vivo (24) and in vitro studies (27, 35) that demonstrate an increase in cell chloride content and cell volume following ischemia or “chemical hypoxia.” However, the role of Cl\(^{-}\) influx in subsequent membrane injury and the pathways by which Cl\(^{-}\) enters the cell remain uncertain.

Cl\(^{-}\) uptake by isolated proximal tubules increases during hypoxia and chemical ATP depletion (27, 31, 43). That Cl\(^{-}\)-channels are involved in this Cl\(^{-}\)-uptake is suggested by the observation that certain classic Cl\(^{-}\)-channel blockers reduce injury in in vitro models of renal tubular injury (31, 41, 43). Miller and Schnellmann (28) have reported the presence of the β-subunit of the neuronal glycine receptor in the proximal tubule and speculated that the Cl\(^{-}\)-uptake during cell injury proceeded through these glycine-gated Cl\(^{-}\)-channels. In a similar vein, Venkatachalam et al. (41) and Waters et al. (42) showed that certain agonists and antagonists of GABA receptors (also a ligand-gated Cl\(^{-}\)-channel) were cytoprotective in MDCK cells and rabbit proximal tubule cells. However, despite these observations, the role of Cl\(^{-}\)-in necrotic cell death remains controversial. For example, no in vitro study has shown a protective effect of substituting Cl\(^{-}\) with other impermeant anions. Moreover, the fact that both agonists as well as antagonists of the glycine and GABA receptors are cytoprotective suggests that the cytoprotection may be independent of Cl\(^{-}\)-influx. Finally, Chen and Mandel (11) found that cell swelling and electrolyte fluxes were, in themselves, insufficient to account for anoxia-induced lysis of rabbit proximal tubule cells. Finally, in all of the aforementioned studies, the evaluation of chloride channel inhibitors has been limited to their short-term effects on membrane integrity (either LDH release or vital dye exclusion). No published study has evaluated the effects of chloride channel inhibitors on long-term cell survival.

The present studies examined the role of extracellular Cl\(^{-}\)- and Cl\(^{-}\)-channels in oxidant-induced cell injury. LLC-PK\(_{1}\) cells, a model proximal tubule cell line, were exposed to exogenous hydrogen peroxide (H_{2}O_{2}). Exposure to H_{2}O_{2} produced ATP depletion, lipid peroxidation, DNA damage, and loss of membrane integrity to macromolecules. Replacement of Cl\(^{-}\)-with impermeant anions or addition of Cl\(^{-}\)-channel blockers prevented the H_{2}O_{2}-induced loss of membrane integrity without affecting the other manifestations of injury. In addition, Cl\(^{-}\)-channel blockers did not improve overall cell survival after oxidant injury. Maneuvers that inhibit volume-sensitive Cl\(^{-}\)-channels also reduced the H_{2}O_{2}-induced loss of membrane integrity. The results support
the view that Cl− influx via volume-sensitive Cl− channels contributes to the loss of membrane integrity during oxidant injury but that other cellular targets of oxidant injury determine the ultimate outcome.

MATERIALS AND METHODS

Cell culture. LLC-PK1 obtained from the American Type Culture Collection (CRL 1392; ATCC, Rockville, MD) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 2 mM glutamine (GIBCO, Life Technologies) at 37°C and aerated with 5% CO2-95% air. Cells were grown in either T-75 flasks (Costar) or 12-well plates and studied 1–2 days after reaching confluence.

Oxidant-induced injury. Monolayers were washed free of medium and incubated in a bicarbonate-buffered saline solution (KRB) containing (in mM) 115 NaCl, 25 NaHCO3, 3.5 KCl, 1 KH2PO4, 1.25 CaCl2, and 1 MgSO4 (pH 7.4 after bubbling with 95% O2-5% CO2). For ion-substitution studies, NaCl was replaced by either NaBr, NaNO3, sodium gluconate, or sodium isethionate. Where indicated, hydrogen peroxide was added to a final concentration of 1 mM from a 100 mM stock solution prepared fresh each day.

LDH release. At the indicated times, the incubation medium was removed and the cells were lysed in 0.2% Triton X-100. The activity of LDH in the incubation solution (supernatant) and in the detergent extract of cells was determined spectrophotometrically from the oxidation of NADH (7). The results are expressed as the percentage of the total LDH content of the well which appeared in the supernatant (LDHsuper): percent release = LDHsuper/(LDHsuper + LDHcell) × 100. None of the test agents or vehicles interfered with the assay for LDH.

Trypan blue staining. At the end of the incubation period, the incubation solution was removed and the cells were stained with 0.4% trypan blue in PBS for 5 min followed by two washes with PBS. The cells were then scraped from the dish into 500 µl of KRB. The number of stained and unstained cells was counted using a hemocytometer.

DNA damage. DNA damage was determined using the alkaline unwinding assay (8) as reported previously from this laboratory (33). In this assay, the rate of DNA unwinding under mild alkaline conditions is increased by the presence of either single- or double-strand DNA breaks. The amount of residual double-stranded DNA after alkaline treatment is expressed as a percentage of the total DNA in the sample.

ATP content. At the end of the incubation period, the incubation solution was removed quickly, and the cell monolayer was extracted into 400 µl of cold 2% perchloric acid. The perchloric acid extract was neutralized with KOH and then diluted 500-fold with 10 mM Tris (pH 7.5). The ATP content of the diluted sample was determined by mixing 20 µl of sample with 100 µl of a 10 mg/ml solution of luciferin-luciferase (Sigma Chemical) while measuring the luminescence in a Turner model TD-20e luminometer. The ATP content is expressed relative to the protein content of the well.

Lipid peroxidation. Lipid peroxidation was measured by the thiobarbituric acid (TBA) reaction (13). Cells were incubated for 1 h with or without H2O2 and the other test agents. At the end of the incubation, the supernatant was removed and mixed with an equal volume of 0.76% TBA in 0.25 M HCl and heated at 95°C for 30 min. The samples were cooled to room temperature, and the absorbance at 532 nm was measured in a spectrophotometer. A standard curve was constructed using tetramethoxypropane hydrolyzed by H2SO4 as the substrate for the TBA reaction. The results are expressed as nanomoles of TBA-reactive substances (TBARS) per milligram cell protein.

Chromium-51 release. Confluent cells growing in 12-well tissue culture plates were loaded with 51Cr by overnight incubation with 0.5 µCi 51Cr/well in culture medium. Immediately prior to the experiment, the cells were washed four times with PBS to remove the extracellular 51Cr. The cells were then incubated with the test solutions for 1 h at 37°C. At the end of the incubation, the supernatant solution was removed and the cell monolayer was solubilized in 0.2% Triton X-100. The 51Cr content of the supernatant solution and of the cell extract were determined by scintillation counting. The release of 51Cr is expressed as the percentage of total 51Cr that appeared in the supernatant solution.

MTS assay. Cell survival was quantified using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) assay (CellTiter 96 Aqueous NonRadioactive Cell Proliferation Assay; Promega, Madison, WI) following the manufacturer’s instructions. For this assay, LLC-PK1 cells were grown in 96-well tissue culture plates until confluent. Cells were then treated for 30 min at 37°C in KRB or KRB containing 1 mM H2O2 in the presence or absence of chloride channel inhibitors. After the treatment, the KRB solution was replaced with culture medium, and the plates were returned to the incubator. Cell survival was measured 24 h after the initial treatment using the MTS assay. Briefly, 20 µl of the MTS solution were added to each well, and the plates were incubated for 2 h at 37°C. The absorbance at 490 nm was measured using a microplate reader (Vmax Kinetic Microplate Reader; Molecular Devices, Sunnyvale, CA) and corrected for background absorbance.

Statistical analysis. Values are presented as means ± SE, unless indicated otherwise. Comparisons between data were made using an unpaired t-test. P < 0.05 was considered significant.

RESULTS

Hydrogen peroxide was used to produce oxidant injury to LLC-PK1 cells. Figure 1 shows the time course of injury, as measured by the release of LDH, after the addition of 1 mM H2O2 to the medium. After 1 h, there was little detectable release of LDH. However, LDH

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Fig. 1. Time course of H2O2-induced lactate dehydrogenase (LDH) release from LLC-PK1 cells. Confluent LLC-PK1 cells were incubated in either Krebs-Ringer bicarbonate (KRB) or KRB containing 1 mM H2O2. At the indicated times, samples of supernatant solution were removed for measurement of LDH activity. Results are means ± SE for n = 4–20 experiments. *P < 0.0002 vs. control.
release increased progressively over the next 3 h. The 2-h time point was chosen for many of the subsequent studies.

To examine the role of extracellular \( \text{Cl}^- \) in oxidant injury, cells were incubated with \( \text{H}_2\text{O}_2 \) in a solution in which all but 2.5 mM of \( \text{Cl}^- \) was replaced by isethionate. As shown in Fig. 2, in cells incubated in the standard KRB solution, \( \text{H}_2\text{O}_2 \) increased the release of LDH from 46 to 376% (n = 4) whereas in cells incubated in the \( \text{Cl}^- \)-free KRB solution, \( \text{H}_2\text{O}_2 \) increased the LDH release from 56 to 143% (n = 4). Thus the \( \text{H}_2\text{O}_2 \)-induced release of LDH was significantly diminished in the absence of extracellular \( \text{Cl}^- \) (P < 0.02). Similar results were obtained when \( \text{Cl}^- \) was replaced by gluconate or methanesulfonate (not shown). Figure 3 shows the relation between the extracellular \( \text{Cl}^- \) concentration and \( \text{H}_2\text{O}_2 \)-induced LDH release. There was a clear monotonic increase in LDH release with increasing extracellular \( \text{Cl}^- \) concentrations.

We reported previously that certain \( \text{Cl}^- \) channel blockers reduced hypoxia-induced LDH release from freshly isolated proximal tubule suspensions (31). We examined the effects of \( \text{Cl}^- \) channel blockers on the oxidant-induced release of LDH from LLC-PK\(_1\) cells. As in hypoxic injury, oxidant-induced LDH release was significantly reduced by a number of \( \text{Cl}^- \) channel blockers (Fig. 4). The overall pattern of protection, i.e., protection by diphenylamine-2-carboxylate (DPC) and 5-nitro-2-(3-phenylpropylamino)benzoate (NPPB), but not by stilbenes, was also similar to that seen in hypoxic injury (31, 43).

The ATP content of cells was measured after 1 h of exposure to 1 mM \( \text{H}_2\text{O}_2 \), a time point prior to the release of significant amounts of LDH (Fig. 1). As noted by other investigators (1, 2), \( \text{H}_2\text{O}_2 \) resulted in marked intracellular ATP depletion (28.7 ± 5.8 vs. 9.8 ± 2 nmol ATP/mg protein, P = 0.02, n = 4). However, as shown in Fig. 5, treatment with either 100 \( \mu \text{M} \) NPPB, 1 mM DPC, or 100 \( \mu \text{M} \) niflumic acid did not protect cells against the \( \text{H}_2\text{O}_2 \)-induced ATP depletion. In fact, the ATP levels in NPPB-treated cells (3.4 ± 0.3 nmol/mg protein, n = 3, P = 0.04 vs. \( \text{H}_2\text{O}_2 \)) were lower than in cells treated with \( \text{H}_2\text{O}_2 \) alone.

Oxidant stress results in damage to DNA (1, 38). We determined the effects of \( \text{H}_2\text{O}_2 \) and certain chloride channel blockers on DNA damage using the alkaline unwinding assay (Fig. 6). Cells were exposed to 1 mM \( \text{H}_2\text{O}_2 \) for 10 min in the presence or absence of either 100 \( \mu \text{M} \) NPPB or 1 mM DPC. Even this brief exposure to \( \text{H}_2\text{O}_2 \) resulted in a significant degree of DNA damage (77 ± 5 vs. 40 ± 3% residual double-strand DNA, n = 6, P < 0.001). However, neither NPPB nor DPC attenuated the DNA damage.

**Fig. 2. Effects of \( \text{Cl}^- \) substitution on \( \text{H}_2\text{O}_2 \)-induced LDH release.** LLC-PK\(_1\) cells were incubated in either standard KRB (left) or low-\( \text{Cl}^- \) KRB (right) in presence or absence of 1 mM \( \text{H}_2\text{O}_2 \). Here, as in Figs. 4–11, open bars represent cells incubated in KRB only, and solid bars represent cells incubated in KRB containing 1 mM \( \text{H}_2\text{O}_2 \). LDH release was measured after 2 h. Results are means ± SE for 4 experiments. *P < 0.02 vs. high-\( \text{Cl}^- \) \( \text{H}_2\text{O}_2 \).

**Fig. 3. \( \text{Cl}^- \) dependence of \( \text{H}_2\text{O}_2 \)-induced LDH release.** LLC-PK\(_1\) cells were incubated with 1 mM \( \text{H}_2\text{O}_2 \) for 2 h in solutions in which \( \text{Cl}^- \) concentration was varied by substitution with isethionate. Results are normalized to LDH released into standard KRB solution (containing 120 mM \( \text{Cl}^- \)); n = 3. *P < 0.05 vs. 120 mM \( \text{Cl}^- \).

**Fig. 4. Effects of \( \text{Cl}^- \) channel blockers on \( \text{H}_2\text{O}_2 \)-induced LDH release.** LLC-PK\(_1\) cells. LDH release was measured after 2-h incubation in KRB (open bar) or KRB containing 1 mM \( \text{H}_2\text{O}_2 \) (solid bars) in presence of indicated agents. Concentrations of 5-nitro-2-(3-phenylpropylamino)benzoate (NPPB), indanyloxyacetic acid (IAA, 94/95), and dinitrostilbene disulfonic acid (DNDS) were 100 \( \mu \text{M} \). Concentration of niflumic acid (NFA) was 400 \( \mu \text{M} \). Concentration of diphenylamine-2-carboxylate (DPC) was 1 mM, and concentration of glycine was 5 mM; n = 31 for control and \( \text{H}_2\text{O}_2 \), and n = 3–10 for each test agent. *P < 0.03 vs. \( \text{H}_2\text{O}_2 \) alone.
ated the H$_2$O$_2$-induced DNA damage (41 ± 9 and 37 ± 5% residual double-strand DNA, respectively, n = 7).

Hydrogen peroxide exposure results in peroxidation of membrane lipids (1, 13). To determine whether chloride channel blockers may have direct antioxidant effects, lipid peroxidation was measured in cells exposed to H$_2$O$_2$ in the presence or absence of the channel blockers. As shown in Fig. 7, treatment of cells for 1 h with 1 mM H$_2$O$_2$ resulted in a large increase in lipid peroxidation products measured as TBARS (0.2 ± 0.2 vs. 1.9 ± 0.1 nmol TBARS/mg protein, $P$ < 0.0001, n = 3). However, the H$_2$O$_2$-induced lipid peroxidation was not significantly altered by any of the agents tested.

The results shown in Figs. 2–4 indicate that Cl$^-$ channel blockers and Cl$^-$ substitution decrease LDH release from oxidatively injured cells. LDH is a large

cytosolic protein (mol wt = 136,000). As will be discussed later, there is evidence that during injury cells develop size-selective defects in membrane integrity. Therefore, it was relevant to determine whether Cl$^-$ channel blockers maintained the membrane integrity for small molecules as they did for LDH. Figure 8 shows that hydrogen peroxide increased the number of cells that could not exclude the vital dye trypan blue (mol wt = 961). Notably, neither 100 µM NPPB nor 5 mM glycine, both of which reduced oxidant-induced LDH release (Fig. 4), reduced oxidant-induced trypan blue uptake. Similarly, hydrogen peroxide increased the release of $^{51}$Cr from LLC-PK$_1$ cells (10 ± 1% vs. 40 ± 4%, $P$ < 0.001) but this increased Cr release was not prevented by NPPB or dinitrostilbene disulfonic acid (DNDS) (Fig. 9). Thus Cl$^-$ channel blockers prevent the
development of membrane permeability to large, but not small, molecules following oxidant injury.

The effects of Cl\textsuperscript{-} channel blockers on cell survival after oxidant injury were determined by the reduction of MTS, a measure of mitochondrial function. Figure 10 shows the results of experiments in which LLC-PK\textsubscript{1} cells were treated with 1 mM H\textsubscript{2}O\textsubscript{2} for 30 min in the presence or absence of Cl\textsuperscript{-} channel blockers or glycine and then incubated in serum-containing culture medium for 24 h. Cell viability at the end of the 24-h incubation was dramatically reduced by the H\textsubscript{2}O\textsubscript{2} exposure. None of the Cl\textsuperscript{-} channel blockers enhanced cell viability. Likewise, 5 mM glycine, which reduced LDH release by 50% (Fig. 4) had no effect on cell viability.

The data in Figs. 2–4 are consistent with the view that Cl\textsuperscript{-} entry, probably via a Cl\textsuperscript{-} channel, is required to manifest oxidant-induced membrane injury. A variety of Cl\textsuperscript{-} channels are present in the plasma mem-

Volume-sensitive anion channels are present in many tissues, including renal epithelial cells (34, 36). These channels are activated by cell swelling and are permeable to Cl\textsuperscript{-} and small organic anions. Figure 11 shows the results of experiments in which the extracellular osmolality was increased by the addition of relatively impermeant solutes. Mannitol, raffinose, and maltose all significantly reduced the H\textsubscript{2}O\textsubscript{2}-induced release of LDH. Volume-sensitive anion channels are also blocked by certain nonclassic Cl\textsuperscript{-} channel blockers, such as ketoconazole and tamoxifen (25, 46), and also require intracellular ATP for channel activation (21). Figure 12 shows that 50 µM ketoconazole, 20 µM tamoxifen, and depletion of ATP with 10 µM antimycin A all significantly reduced H\textsubscript{2}O\textsubscript{2}-induced release of LDH.

DISCUSSION

The present studies examined the role of chloride in oxidative injury to renal epithelial cells. The generation of hydrogen peroxide has been implicated in the pathogenesis of several forms of acute tubular cell injury (3). Whereas several lines of evidence support the view that Cl\textsuperscript{-}, or Cl\textsuperscript{-} transport, may contribute to cell injury induced by hypoxia (31, 43) or ATP depletion (41), the role of Cl\textsuperscript{-} in oxidant-induced injury has not been determined. Moreover, even in hypoxia or ATP deple-

Fig. 9. Effects of NPPB and DNDS on H\textsubscript{2}O\textsubscript{2}-induced \textsuperscript{51}Cr release. LLC-PK\textsubscript{1} cells were loaded with \textsuperscript{51}Cr and then incubated for 2 h in KRB (open bar) or KRB containing 1 mM H\textsubscript{2}O\textsubscript{2} (solid bars) and either 100 µM NPPB or 100 µM DNDS. *P < 0.001 vs. control; n = 6.

Fig. 10. Effects of Cl\textsuperscript{-} channel blockers on cell viability. Cell viability was determined by the MTS assay as described in METHODS. The value of the absorbance at 490 nm (A\textsubscript{490}) is proportional to the number of viable cells. LLC-PK\textsubscript{1} cells were treated for 30 min in KRB (open bar) or KRB containing 1 mM H\textsubscript{2}O\textsubscript{2} (solid bars) in absence or presence of either 100 µM NPPB, 1 mM DPC, 100 µM NFA, or 5 mM glycine. *P < 0.001 vs. control. Data are means ± SD of n = 6 wells for each agent. Results are representative of 3 experiments.

Fig. 11. Effects of extracellular hypertonicity on H\textsubscript{2}O\textsubscript{2}-induced LDH release. LDH release was measured after 2-h incubation in KRB (open bar) or KRB containing 1 mM H\textsubscript{2}O\textsubscript{2} (solid bars) and 200 mM of either mannitol, raffinose, or maltose. *P < 0.03 vs. H\textsubscript{2}O\textsubscript{2} alone. Data are means ± SE for n = 4–9 experiments.
Cl– CHANNEL BLOCKERS AND MEMBRANE INJURY

The mechanism whereby Cl– or Cl– entry contributes to cell injury is not certain. The studies of Schnellmann et al. (27, 43) and our previous study of hypoxic injury in rat proximal tubules (31) were consistent with the view that Cl– entry occurred at a rather late stage in cell injury and that the effects of Cl– entry, and of Cl– channel blockers, were limited mainly to the cell membrane. The present results provide additional support for that view. Specifically, Cl– channel blockers, while reducing oxidant-induced LDH release, did not protect cells against oxidant-induced ATP depletion (Fig. 5), oxidant-induced DNA damage (Fig. 6), or oxidant-induced lipid peroxidation (Fig. 7). These observations also indicate that the Cl– channel blockers were not acting simply as antioxidants or free radical scavengers to reduce LDH release.

Chen and Mandel (10) have shown that in the course of hypoxic injury to proximal tubules, cell membranes become permeable to progressively larger molecules. That is, membranes first become permeable to small molecules and then to macromolecules. Dong et al. (15) have also examined the size selectivity of the plasma membrane during chemical ATP depletion in MDCK cells using fluoresceinated dextrans of graded sizes. After 2 h of ATP depletion, cell membranes were freely permeable to dextran of 4 kDa, partly permeable to 70-kDa dextran, and only slightly permeable to dextrans larger than 145 kDa. Our results are also consistent with the presence of size-selective defects during oxidant-induced injury. Namely, using Cl– channel blockers or Cl– substitution (not shown), we were able to dissociate the increase in permeability to small molecules (mol wt < 1,000), i.e., trypan blue (Fig. 8) or chromium (Fig. 9), from the release of the macromolecules (Figs. 2–4). Chen and Mandel (10) found that cross-linking of membrane proteins with homobifunctional reagents also could reduce the anoxia-induced permeability to LDH but not to propidium iodide (mol wt = 668). Thus the effects of Cl– channel blockers in the present study were similar to the effects of cross-linking reagents in that study. It has been proposed that the permeability to macromolecules during injury results from a rearrangement of membrane proteins to form large water-filled pores (10). If so, then Cl– channel blockers, either directly or indirectly, may prevent the formation of these pores. Of interest, Chen and Mandel (10) found that the permeability to small molecules was a potentially reversible event upon reoxygenation. Thus the use of small "vital" dyes, such as trypan blue, calcine, or chromium, as indicators of cell death may be misleading under certain circumstances.

The mechanism whereby Cl– substitution and Cl– channel blockers inhibit membrane permeability to macromolecules remains unknown. An increase in cell volume due to Cl– influx does not appear to be sufficient, in itself, to account for macromolecular leak. For example, cell swelling induced by osmotic gradients

Fig. 12. Effects of inhibitors of volume-sensitive anion channels on H2O2-induced LDH release. LDH release was measured after 2-h incubation in KRB (open bar) or KRB containing 1 mM H2O2 (solid bars) in presence of indicated agents; ketocon., ketoconazole. *P < 0.005 vs. H2O2 alone. Data are means ± SE for n = 3–6 experiments.
produced LDH release only at extremes of cell volume (11). In addition, glycine prevented LDH release from ATP-depleted MDCK cells without preventing cell swelling (15). However, a change in the intracellular Cl⁻ content or concentration could affect steps proximal to membrane injury, e.g., by facilitating changes in the cytoskeleton (9, 14) or activation of intracellular proteases. The cellular K⁺ content, for example, appears to be a critical factor in the activation of proteases during apoptosis (19). The dependence of enzymes such as calpain and caspasas, phospholipases, and endonucleases, all of which are activated during hypoxic injury and are proposed to contribute to cell death (16, 17, 22, 30, 40), on Cl⁻ has not been determined. Alternatively, the effects of Cl⁻ channel blockers could be related to their effects on the transport of substances other than Cl⁻. In this regard, increases in cell volume, which occur during many forms of cell injury, activate volume-sensitive anion channels (36). These channels are permeable to a variety of organic osmolytes, including glycine (37). By reducing activation of these volume-sensitive anion channels, Cl⁻ channel blockers and Cl⁻ substitution could prevent the efflux of potentially protective substances. The present results provide several lines of support, albeit indirect, for the view that volume-sensitive anion channels may be activated and contribute to cell injury. First, the anion dependence of H₂O₂-induced LDH release followed the same pattern as the selectivity sequence of volume-sensitive outward-rectifying Cl⁻ channels (29, 36). Second, certain volume-sensitive channels require intracellular ATP for activation (5, 20), and we found that severe depletion of ATP with antimycin A markedly reduced oxidant-induced LDH release (Fig. 12). We note that, although H₂O₂ itself led to an approximate 60% decrease in ATP levels, the addition of antimycin A reduced ATP levels by over 95% (not shown). It is possible that some of the protection afforded by NPPB on LDH release (Fig. 4) could be related to its effect on cell ATP levels (Fig. 5). Third, volume-sensitive Cl⁻ channels are inhibited by a number of nonclassic Cl⁻ channel blockers, including ketoconazole and tamoxifen (25, 46). We found that these agents also markedly reduced oxidant-induced LDH release (Fig. 12). Finally, the addition of impermeant solutes (200 mM mannitol, maltose, or raffinose) to the extracellular solution to prevent cell swelling markedly reduced oxidant-induced LDH release (Fig. 11). Direct electrophysiological measurement will be required to confirm that the effects of these maneuvers are mediated through inhibition of volume-sensitive channels.

Finally, since the effects of Cl⁻ channel blockers appeared to be limited to the plasma membrane, while oxidant-induced ATP depletion (Fig. 5), DNA damage (Fig. 6), and lipid peroxidation (Fig. 7) were unaffected, it was relevant to determine whether these agents actually provided any long-term benefit against oxidant injury. In this regard, previous studies employing freshly isolated proximal tubules (31, 43) could not address the effects of Cl⁻ channel blockers on long-term survival due to the limited viability of tubules in suspension, and the study of Venkatachalam et al. (41) in MDCK cells examined only the end point of LDH release after 6 h of ATP depletion. Our results indicated that neither Cl⁻ channel blockers nor glycine, an extensively studied cytoprotective agent (15, 26, 27, 41, 44, 45), enhanced cell survival after oxidant injury. These results are consistent with the view, mentioned above, that the effects of Cl⁻ channel blockers are primarily on the plasma membrane, whereas other targets of oxidant stress are not protected. It is not known whether the failure of Cl⁻ channel blockers to enhance survival in oxidant injury can be extrapolated to other forms of acute cell injury, such as that induced by ischemia or toxins. Further studies will be required to address that issue. However, these results should prompt caution in interpreting the effects of any maneuver, i.e., acidosis (43, 45), glycine (44), or enzyme inhibition (16, 17, 38), based solely on short-term in vitro studies or end points such as LDH release.

In summary, substitution of Cl⁻ by impermeant anions in the extracellular solution or the addition of Cl⁻ channel blockers prevented oxidant-induced permeabilization of the cell membrane to macromolecules. These maneuvers did not prevent oxidant-induced DNA damage, ATP depletion, lipid peroxidation, or membrane permeability to small molecules. Likewise, Cl⁻ channel blockers did not improve cell survival, as judged by mitochondrial function 24 h following oxidant injury. These results are consistent with the view that Cl⁻ channels may play a role in membrane damage during cell injury. Further studies are required to determine the electrophysiological features of Cl⁻ channels in injured cells and to define the mechanism whereby Cl⁻ channels influence membrane integrity.

This work was supported by awards from the Extramural Grant Program of Baxter Healthcare and the Veterans Affairs Research Service. W. B. Reeves is an Established Investigator of the American Heart Association.

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Received 2 October 1998; accepted in final form 17 August 1999.

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