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Protection of Human Umbilical Vein Endothelial Cells by Glycine and Structurally Similar Amino Acids Against Calcium and Hydrogen Peroxide-induced Lethal Cell Injury

Joel M. Weinberg,* James Varani,† Kent J. Johnson,† Nancy F. Roeser,* Michael K. Dame,† Julie A. Davis,* and Manjeri A. Venkatachalam‡

From the Division of Nephrology, Department of Internal Medicine, and Department of Pathology,† University of Michigan and Veterans Affairs Medical Center, Ann Arbor, Michigan; and the Department of Pathology,‡ University of Texas Health Science Center, San Antonio, Texas*

Cultured human umbilical vein endothelial cells treated with either the calcium ionophore, ionomycin, or ionomycin plus cyanide-m-chlorophenylhydrazone had immediate severe depletion of adenosine triphosphate (ATP) and increases of cytosolic free calcium (Ca_f) and then sustained lethal cell injury as manifested by release of lactate dehydrogenase and failure to exclude vital dyes within 15 minutes. Inclusion of glycine in the experimental medium prevented the enzyme leakage for at least 60 minutes without altering the ATP depletion or increases of Ca_f. The physiologic glycine concentration of 0.25 mmol/l gave 50% protection, and protection was complete at 1 mmol/l. Several other small neutral amino acids, L- and D-alanine, β-alanine, 1-aminocyclopropane-1-carboxylate, α-aminoisobutyrate, and L-serine, had effects similar to glycine, but other amino acids and metabolic substrates did not. The endothelial cells were relatively resistant to damage from hydrogen peroxide, but sensitivity could be increased by preloading with Fe^{2+}. In both non-loaded and Fe^{2+}-loaded cells, hydrogen-peroxide–induced lactate dehydrogenase (LDH) release developing over 180 minutes was prevented by glycine in a fashion analogous to that seen with ionomycin damage. Mn^{2+} also partially protected against hydrogen peroxide injury but was not required for glycine's effects. These data demonstrate that striking modulatory effects of glycine and structurally similar amino acids that have previously been characterized in most detail using kidney tubule cells are strongly expressed in human umbilical vein endothelial cells and are involved in their response to Ca^{2+} and oxidant-mediated damage. These amino acid effects must be considered in the design of* in vitro *studies of endothelial cell injury and may contribute to endothelial cell pathophysiology* in vivo. *(Am J Pathol 1992, 140:457–471)

Studies of freshly isolated kidney tubule preparations have suggested a fundamental role for small neutral amino acids, specifically glycine and alanine, in the maintenance of cell structural integrity.[1–11] Biochemically the target of protection remains unknown; however, it appears to be an event distal to several of the major metabolic disturbances that occur during cell injury. These disturbances include depletion of adenosine triphosphate (ATP) and intracellular glutathione,[1–5] inhibition of the Na^+ pump,[10] and large, sustained increases of cytosolic free Ca^{2+} (Ca_f).[12]

There is little information on whether amino acid protective effects are expressed in other cell types or tissues. Oxidant processes do not play a major role in the kidney tubule cell injury models used thus far to characterize amino acid actions.[1,5] Glycine and other amino acids, however, have recently been shown to serve as cofactors with Mn^{2+} to metabolize hydrogen peroxide,[13–16] and protective effects of amino acids + Mn^{2+} have recently

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Address reprint requests to Dr. Joel M. Weinberg, Nephrology Research Labs, Room 1560, MSRB II, University of Michigan Medical Center, Ann Arbor, MI 48109-0676.

been described for endothelial cells *in vitro* and in a model of oxidant-induced lung injury *in vivo*.[16]

Endothelial cell involvement in the pathogenesis of inflammatory and ischemic damage to multiple organs is well recognized. Oxidant damage appears to play a significant role in the cellular pathophysiology of these processes,[17–19] and oxidant-induced disturbances of cellular Ca^{2+} homeostasis potentially contribute to both sublethal alterations of endothelial cell structure and function and lytic cell injury.[20–24] The studies in this report were designed to assess whether amino acid protective actions of the type seen in kidney tubule cells are expressed in endothelial cells and whether the protective actions involve antioxidant properties of glycine + Mn^{2+}. We detail successful development of a model of acute, ionomycin-induced, Ca^{2+}-dependent, lethal cell injury to human umbilical vein endothelial cells that allows demonstration of characteristic 'renal tubule cell type' protective effects specific for glycine and structurally similar amino acids. We also show, in the same system, that glycine also has Mn^{2+}-independent actions to protect against lethal cell injury induced by hydrogen peroxide.

Methods

Growth of Endothelial Cells

Human umbilical vein endothelial cells obtained according to the procedure of Jaffe[25,26] were grown for experiments for 72 hours on 35-mm plastic dishes in a complete endothelial cell growth medium consisting of medium 199 supplemented with 20% fetal bovine serum, 25 mg/liter endothelial cell growth supplement (Collaborative Research, Boston, MA), and 90 mg/liter bovine lung heparin. Cells, confirmed as endothelial by previously detailed criteria,[25] were used for up to five passages, but mostly on the first or second passage.

Basic Experimental Procedures

To start experiments, the growth medium was removed and plates were washed three times with phosphate-buffered saline (PBS). Then 1 ml of a medium consisting of (in mmol/l [millimolar]) 115 NaCl, 3.5 KCl, 25 $NaHCO_3$, 1.0 KH_2PO_4, 1.25 $CaCl_2$, and 1.0 $MgSO_4$ gassed with 5% CO_2 (medium A) was placed in each dish.

For studies with ionomycin and carbonyl cyanide-m-chlorophenylhydrazone (CCCP), test protective agents were added from 100- to 200-fold concentrated, neutral aqueous stock solutions, and plates were returned to the incubator for 60 minutes. At the end of the 60-minute preincubation, plates were removed and toxins were added. Fifteen micromolar CCCP and 5 μmol/l ionomycin were delivered from 100-fold concentrated stock solutions in ethanol. Plates then were returned immediately to the incubator for 5 to 60 minutes of experimental incubation before sampling for metabolic and structural studies.

For studies of hydrogen peroxide-induced injury, plates were preincubated for 30 minutes in medium A without further additions. Then plates received either 10 μmol/l 8-hydroxyquinoline from a 100-fold concentrated 2% ethanol stock solution, 10 μmol/l ferrous ammonium sulfate, a combination of the two agents, or a sham addition, and were incubated for an additional 30 minutes before being washed three times with PBS. One milliliter fresh medium A containing test protective agents was than added. Ten minutes later, hydrogen peroxide was added from a 100-fold concentrated stock solution to the desired concentration. Plates then were returned to the incubator for 180 minutes of experimental incubation before sampling.

Determination of Viability Using Fluorescein Diacetate-Propidium Iodide or Trypan Blue

For labeling with fluorescein and propidium iodide,[27] 0.25 ml medium was removed from paired plates for lactate dehydrogenase (LDH) determination and replaced with 0.25 ml PBS containing either fluorescein diacetate (Molecular Probes Inc., Eugene, OR) to produce a final concentration of 18.75 μg/ml or propidium iodide to produce a final concentration of 3 μmol/l. The plates were returned to the 37°C incubator for 3 minutes and then placed on ice. After no more than 15 minutes on ice, the medium was removed and the plates were observed and photographed using either the 20× or 100× objective of a Zeiss Axioskop fluorescent microscope (Carl Zeiss, Oberkochen, Germany). Fluorescein staining was visualized using a 450- to 490-nm excitation filter, 510-nm beam splitter, and 520-nm long pass barrier filter. Propidium iodide staining was viewed using a 546-nm exciter filter, a 580-nm beam splitter, and a 590-nm long pass barrier filter.

Measurement of Cytosolic Free Calcium in Fura-loaded Cells

For studies of Ca_i, cells were grown on 25-mm diameter #1½ glass coverslips (Nicholson Precision Instruments, Gaithersberg, MD). To load cells with fura-2 (Molecular Probes), the coverslips were washed three times with PBS and then placed in medium A containing 3 μmol/l fura-2/AM from a dimethylsulfoxide stock solution that had been vigorously premixed into the medium. In ex-

periments testing effects of glycine, it was also included in medium A. After 1 hour of incubation at 37°C, the coverslip was removed from the fura-containing medium, washed three times with PBS, and mounted in a Dvorak-Stotler culture chamber (Nicholson). Perfusion at 0.82 ml/minute with experimental medium from a 20- or 60-ml syringe using a Harvard Apparatus (South Natick, MA) Model 975 pump was started immediately.

The chamber was mounted on the stage of a Nikon inverted microscope connected to a SPEX (Edison, New Jersey) CM-2 spectrofluorometer system using the DM 3000 computer and software. The chamber and microscope stage were kept at 37°C using an Air Stream Incubator (Nicholson). The monolayer was viewed using a CF FLUOR 40X lens (Nikon, Garden City, NY) and was illuminated alternately with 340- and 380-nm excitation signals for 0.33-second intervals through 0.4-mm (1.44-nm bandpass) slits ahead of the chopper, an NDO.3 neutral density filter (Omega Optical, Brattleboro, VT), and a DM 400 dichroic mirror (Nikon). Emitted light was collected through the dichroic mirror and then transmitted through a narrow-band 510-nm filter (510DF40, Omega) in the microscope and a 500FS40 filter (SPEX) in the photomultiplier assembly to the fluorometer photomultiplier.

Mounting the chamber on the microscope stage, locating and positioning an area to study using visible light from the microscope, and making final adjustments to perfusion syringes and experimental parameters on the computer generally required 5 to 7 minutes, during which the coverslip was perfused with medium A at 37°C. An additional period of at least 100 seconds' perfusion then was recorded before starting experimental maneuvers.

Experimental agents were introduced by replacing the perfusion syringe with one containing a solution of the desired composition. At the end of the last desired experimental maneuver, coverslips either were subjected to a calibration procedure or were stained with trypan blue. The calibration procedure consisted of perfusion with a nominally Ca^{2+}-free form of medium A that also contained 2.5 mmol/l ethylene glycol tetra-acetic acid (EGTA) and 5 μmol/l ionomycin (Calbiochem, La Jolla, CA), followed by medium supplemented with Ca^{2+} to a concentration of 3.5 mmol/l, and 5 μmol/l ionomycin, then by medium A containing 200 μmol/l $MnCl_2$. Each of the calibration solutions was continued long enough to achieve a stable response. In some experiments, Trypan blue exclusion was assessed by perfusion with a solution consisting of a 50:50 mixture of 0.4% Trypan blue in normal saline (Gibco, Grand Island, NY) and Hank's balanced salt solution.

To determine Ca_f, the values of the 340- and 380-nm signals at the end of the $MnCl_2$ perfusion were subtracted to correct for intrinsic autofluorescence and other Ca^{2+}-independent fluorescence in the system.[28] Cytosolic free calcium was calculated from the 340/380 ratios using the equation:

$$[Ca^{2+}]_f = (K_d \times S_{f2}/S_{b2}) \times (R - R_{min})/(R_{max} - R),$$

where R is the ratio at any point; R_{min} is the ratio achieved during perfusion with EGTA and ionomycin in Ca^{2+}-free solution C; R_{max} is the ratio achieved during perfusion with ionomycin in 3.5 mmol/l Ca^{2+} solution C; Sf_2 is the absolute value of the corrected 380 signal at R_{min}; Sb_2 is the absolute value of the corrected 380 signal at R_{max}; K_d is 224 nm.[29] In coverslips that were stained with trypan blue instead of being calibrated, R_{min}, R_{max}, and autofluorescence values obtained from a fully calibrated, control coverslip on the same day were used to quantify Ca_f.

Although fura-2 has been shown to become compartmentalized in bovine aortic endothelial cells,[30] this was not a problem with the human umbilical vein cells used here. Fura fluorescence was observed to be diffusely distributed in the cytosol and was largely released by mild digitonin treatment.

Analytical Methods

To assess LDH release, a 0.25-ml sample of medium was removed from the plate and rapidly centrifuged at 13,000*g* to pellet any nonadherent cells. The supernatant was removed for LDH assay by a fluorometric method as previously described.[2] Total cell layer LDH was measured on control plates by adding Triton X-100 to a final concentration of 0.1% to the medium and swirling the plates several times during 2 to 3 minutes before sampling. This resulted in complete LDH release. Percent free LDH values were calculated as 100× activity in medium/total cell + medium activity of a paired control plate.

To measure ATP, the entire 1.0 ml medium and any nonadherent cells were removed from the plate and immediately spun at 13,000*g* to pellet the cells. In the meantime, 0.75 ml 6% trichloroacetic acid (TCA) was added to the plate. A 0.25-ml aliquot of medium was removed from the centrifuged sample for LDH determination, and the rest of the medium was added to an equal volume of 12% TCA. The 6% TCA from the plate then was transferred to the pelleted cells to extract them. The TCA extracts of cells and medium were neutralized with trioctylamine-freon as previously described[1] and assayed for ATP by high-pressure liquid chromatography (HPLC).[1] Protein was measured by the method of Lowry et al.[31] Hydrogen peroxide was assayed spectrophotometrically using horseradish peroxidase.[32]

Reagents

Reagents were obtained from Sigma (St. Louis, MO) unless otherwise indicated. 1799, 2,6-dihydroxy-

1,1,1,7,7,7-hexafluoro-2,6-bis(trifluoromethyl)heptan-4-one[bis(hexafluoroacetonyl)]acetone, a nonfluorescent uncoupler of mitochondrial oxidative phosphorylation,[33] was provided by Dr. Peter Heytler (DuPont, Wilmington, DE). One hunmdred nanomolar Ca^{2+} media were prepared by addition of EGTA to medium A based on calculations using software developed by Fabiato[34] and checked by measuring fluorescence of fura pentapotassium salt added to medium in the absence of cells.

Statistics

Data from multigroup experiments were analyzed statistically by analysis of variance for repeated measure designs. Individual group comparisons then were made using the Neuman-Keuls test for multiple comparisons (PC ANOVA, Human Systems Designs, Northridge, CA). Two group studies were assessed using paired or unpaired *t*-tests as appropriate. $P < 0.05$, two-tailed, was considered to be statistically significant.

Results

Responses to Treatment with Ionomycin

Treatment of the endothelial cells with ionomycin alone and in combination with the mitochondrial uncoupler, CCCP, produced rapid loss of plasma membrane integrity and LDH leakage (Figure 1). After a lag of about 7.5 minutes, free LDH values of ionomycin and ionomycin + CCCP-treated cells rapidly increased to > 80%. The effect of ionomycin + CCCP was no greater than the effect of ionomycin alone. Glycine virtually completely prevented LDH release for the full 60 minutes of study (Figure 1). Endothelial cells treated with CCCP alone did not sustain damage leading to LDH release for up to 180 minutes of incubation (not shown).

Adenosine triphosphate levels dropped to 20% of control values during the first 7.5 minutes in all groups and then dropped further to less than 2% of control values. Adenosine triphosphate depletion was just as severe in cells treated with ionomycin alone as in cells treated with ionomycin + CCCP (Figure 2). Glycine did not affect either the rate or the extent of ATP depletion (Figure 2).

Control cells had the appearance of typical monolayers of endothelial cells (Figures 3 and 4). In the presence and absence of glycine, ionomycin and ionomycin + CCCP–treated groups had severe morphologic changes consisting of retraction, rounding up, extensive membrane blebbing, and widespread detachment (Figure 3). Cells exposed to glycine were severely damaged, but most of them maintained a distinctly denser cytosol and tended to remain more adherent to the dish than cells not treated with glycine. The morphologic concomitant of LDH retention was more clearly evident in plates stained with fluorescein-diacetate and propidium iodide (Figure 4). The majority of cells on ionomycin + CCCP plates not treated with glycine showed little or no retention of cytosolic fluorescein and had bright red propidium iodide staining of their nuclei. Glycine-treated preparations had extensive retention of fluorescein in both intact cells and free blebs. Most glycine-treated cells excluded propidium iodide, although the glycine-treated plates also had occasional cells of an intermediate type with very weak propidium iodide nuclear staining. This type of cell was not seen in either control plates or ionomycin + CCCP plates without glycine. Plates treated with ionomycin alone and ionomycin + glycine behaved similarly to the corresponding ionomycin + CCCP plates with regard to fluorescein diacetate and propidium iodide staining (not shown).

Concentrations of ionomycin down to 1 μmol/l produced effects similar to the 5-μmol/l concentration (Figure 5). Toxicity of ionomycin was dependent on the presence of calcium in the medium (Figure 6). Glycine had a substantial protective effect at the lowest concentration tested, 0.25 mmol/l, and was fully protective at 1 mmol/l and higher (Figure 5).

Glucose, pyruvate, glutamate, and glutamine, potential ATP-generating substrates, had no effect on injury produced by ionomycin + CCCP (Table 1). In contrast, a number of compounds structurally similar to glycine were highly protective. L-Alanine, D-alanine, β-alanine, 1-aminocyclopropane-1-carboxylate acid, and α-aminoisobutyrate were all just as protective as glycine. L-Serine was moderately protective. L-Aminobutyrate, DL-β-aminobutyrate, L-valine, and L-leucine were not consistently protective (Tables 1 and 2).

Manganese had a moderate, dose-dependent effect to reduce LDH release when used alone in the ionomycin model. Combining 0.5 mmol/l Mn^{2+} with glycine did not alter the protection provided by glycine alone. Combining 0.5-mmol/l Mn^{2+} with leucine, L-aminobutyrate, or DL-β-aminobutyrate resulted in a moderate reduction of LDH release similar to that produced by 0.5 mmol/l Mn^{2+} alone (Table 2).

Effects of 1799, Ionomycin, and Glycine on Ca_f

For studies of Ca_f, CCCP was replaced with 20 μmol/l 1799, a nonfluorescent uncoupler[12,33] that had similar effects on cell ATP and viability as CCCP (not shown). 1799 alone induced a small, inconstant Ca_f transient (Fig

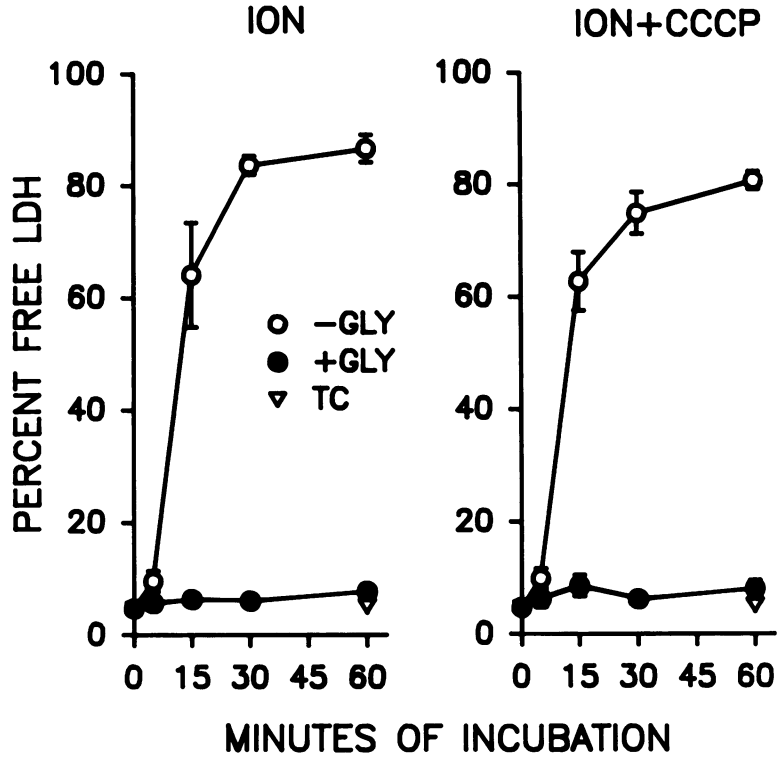


Figure 1. *LDH leakage from endothelial cells treated for up to 60 minutes with either 5 μM ionomycin (ION), or 5 μM ionomycin plus 15 μM CCCP (ION + CCCP) in the absence (−GLY) or the presence of 5 mM glycine (+GLY). Time control (TC) preparations received no additions. Values are means ± SE of four to six experiments. Values for ION and ION + CCCP groups treated with glycine were significantly different from corresponding groups not treated with glycine at 15, 30, and 60 min.*

ure 7). Ionomycin alone and ionomycin + 1799 induced a large sustained increase of Ca_f to micromolar levels that saturated the fluorescent Ca_f indicator, fura-2. The changes of Ca_f were not modified by glycine. The apparent decrease of Ca_f after the peak response to ionomycin + 1799 was variable from experiment to experiment and coincided with a marked change in physical conformation of the cells when they began to lift off the plates and out of the initial plane of focus of the microscope.

Hydrogen Peroxide-induced Injury

Most of the primary cultures tested were relatively resistant to the toxic effects of H_2O_2. As shown in Figure 8,

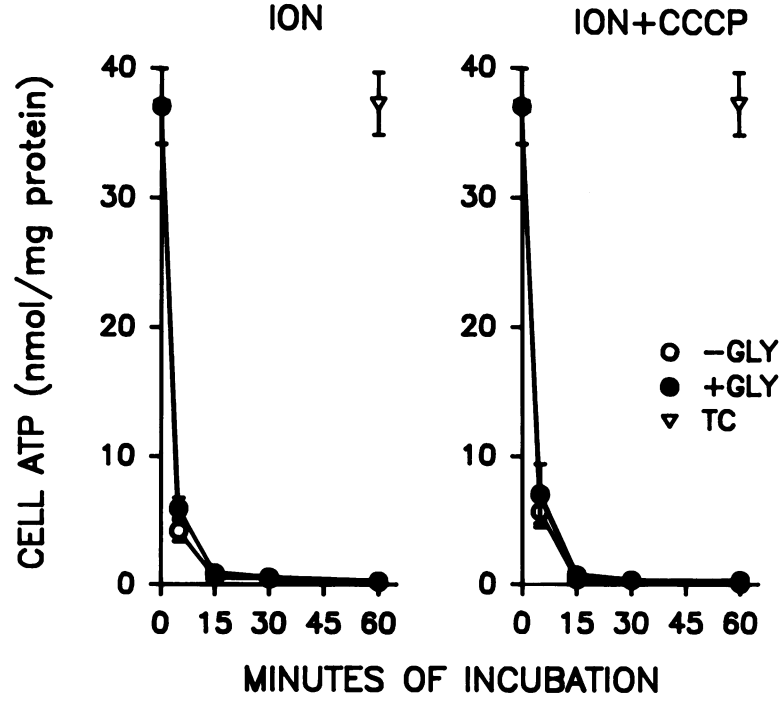


Figure 2. *ATP levels for the same experiments as in Figure 1. Labeling is the same as Figure 1. ION, and ION + CCCP-treated cells had significantly less ATP than the zero and 60-minute time controls at all durations of exposure. ATP levels were not significantly affected by the presence of glycine.*

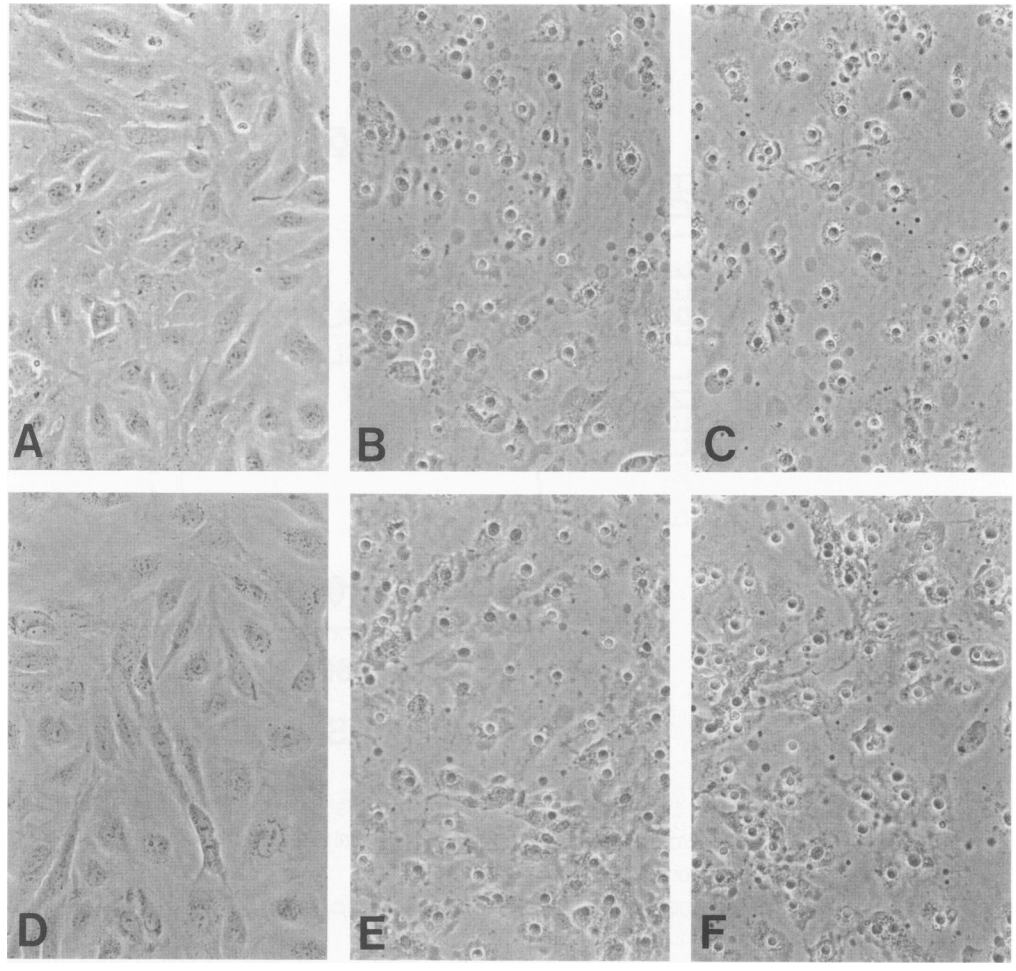


Figure 3. *Phase contrast micrographs of control endothelial cell monolayers without* (A) *and with* (D) *5 mM glycine, monolayers exposed to 5 μM ionomycin for 30 minutes without* (B) *and with* (E) *5 mM glycine, and monolayers exposed to ionomycin + 15 μM CCCP for 30 minutes without* (C) *and with* (F) *5 mM glycine. Magnification, ×100.*

even after 3 hours' exposure to 5 mmol/l H_2O_2, LDH release averaged less than 30%. Glycine and ACPC completely prevented this LDH release. L-Aminobutyrate, DL-β-aminobutyrate, and leucine did not. Mn^{2+} (0.1 mmol/l) had a partial effect to reduce cytotoxicity in the absence of amino acids and in the presence of the three amino acids that were not protective by themselves (Figure 8). At 0.5 mmol/l, Mn^{2+} was toxic (data not shown). Catalase was completely protective (Figure 8).

To produce a more severe insult against which to assess amino acid protection, we used iron loading as recently reported for bovine aortic endothelial cells.[35] Figure 9 shows that 30 minutes' preloading with Fe^{2+} in the presence of 8-hydroxyquinoline significantly increased cytotoxicity of H_2O_2, but considerable variability remained. When amino acid and Mn^{2+} manipulation studies were done in an additional group of experiments, however, even more toxicity and a higher degree of uniformity were found (Figure 10). As was seen in the cells not preloaded with Fe^{2+}, glycine and ACPC provided complete protection. L-Aminobutyrate, DL-β-aminobutyrate, and leucine were without effect. Mn^{2+} had a small but significant effect both in the absence of amino acid supplementation and when given with the otherwise nonprotective amino acids. Catalase completely prevented the toxicity of H_2O_2 in both the normal and Fe^{2+}-loaded cells (Figures 8 and 10). 8-Hydroxyquinoline plus Fe^{2+} had minimal toxicity in the absence of H_2O_2.

Glycine and leucine in combination with Mn^{2+} had a mild effect to promote metabolism of H_2O_2 in medium incubated at 37°C without cells. Neither Mn^{2+} nor glycine nor leucine alone affected medium H_2O_2 levels (Figure 11).

Adenosine triphosphate levels were reduced to about 2% of control values during 180 minutes' exposure to H_2O_2 in both Fe^{2+}-preloaded cells and cells that were not pretreated (Table 3). Neither of the protective amino acids, glycine and 1-aminocyclopropane-1-carboxylate,

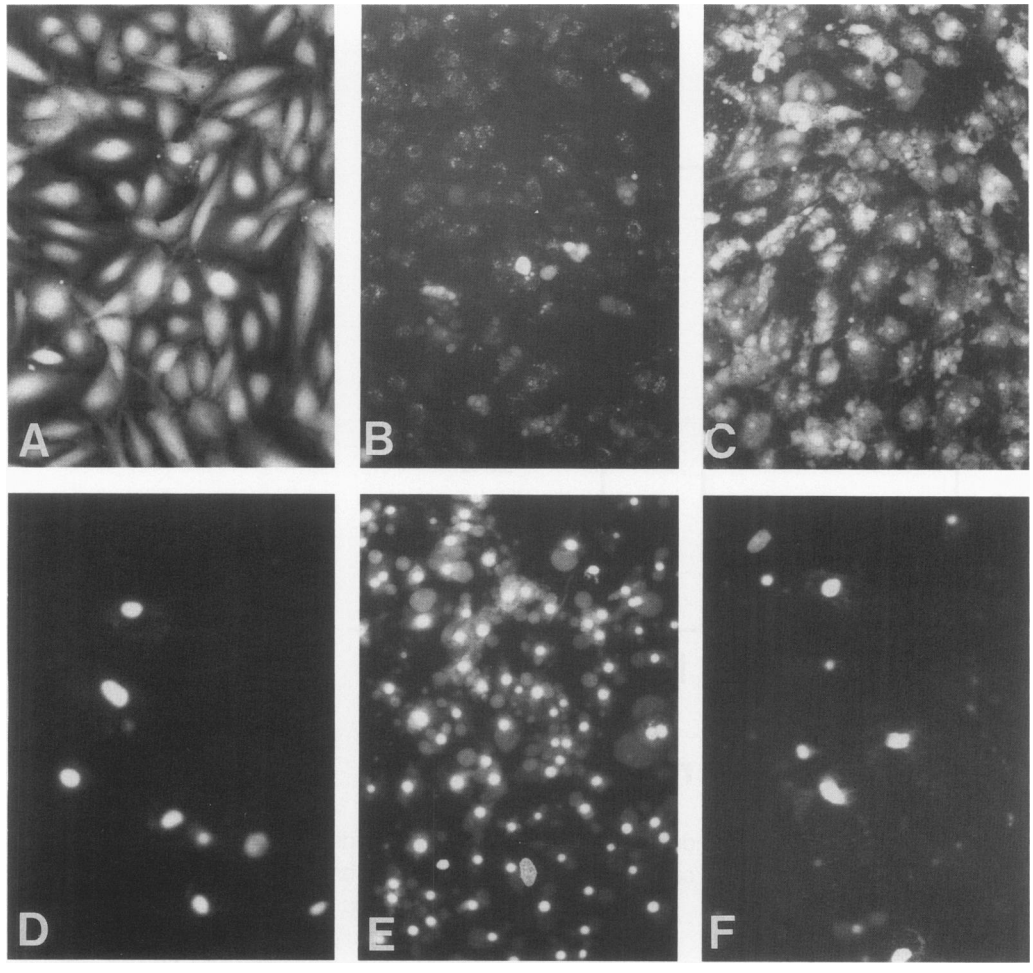
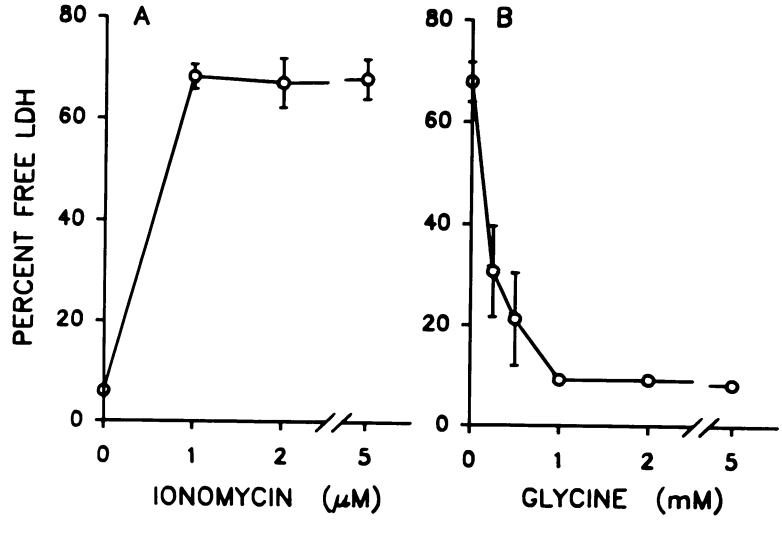


Figure 4. *Fluorescent micrographs of fluorescein diacetate and propidium iodide-stained control endothelial cell monolayers* (A, D), *30-minute ionomycin + CCCP-treated monolayers* (B, E), *and 30 minute ionomycin + CCCP + 5 mM glycine-treated monolayers* (C, F). (A, B, C) *are fluorescein diacetate-treated plates viewed with the 450–490 nm excitation and 520 nm long pass barrier emission filters;* (D, E, F) *are propidium iodide-treated plates viewed using the 546 nm excitation and 590 nm long pass barrier emission filter set. Magnification, ×100.*

Figure 5. *Dose dependence of killing by ionomycin and protection by glycine. In* (A) *cells were treated for 30 minutes with 15 μM CCCP plus the indicated concentration of ionomycin. In* (B), *cells were treated with 15 μM CCCP plus 5 μM ionomycin plus the indicated concentration of glycine for 30 minutes. Values are means + SE of results from three experiments. Effects of all ionomycin and glycine concentrations shown were statistically significant.*



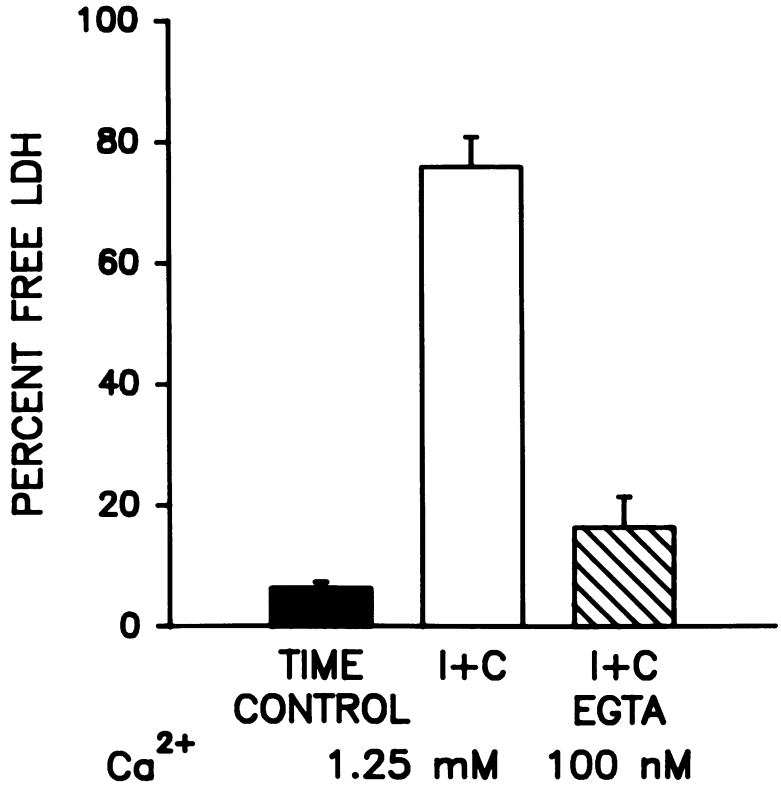


Figure 6. *Effects of reduced Ca^{2+} on killing. Cells were treated with 5 μM ionomycin plus 15 μM CCCP (I + C) in medium with either the normal concentration of 1.25 mM Ca^{2+} or with 100 nM Ca^{2+} produced by adding EGTA. Values are means + SE for three experiments. Both I + C groups had significantly greater free LDH than the time control but the 100 nM Ca^{2+} group was significantly lower than the 1.25 mM Ca^{2+} group.*

nor Mn^{2+}, nor the nonprotective amino acids consistently affected this ATP depletion. In contrast, catalase completely prevented the ATP depletion.

Discussion

These studies demonstrate potent effects of amino acids to modify acute lethal cell injury to cultured human umbilical vein endothelial cells. Glycine and structurally similar amino acids have a specific action to prevent loss of plasma membrane integrity manifested by leakage of a large cytosolic enzyme and failure to exclude vital dyes during a rapidly evolving form of injury secondary to ionophore-induced increases of Ca_f as well as during a slower-developing process consequent to hydrogen per-

Table 1. *Effects of Metabolic Substrates and Highly Protective Amino Acids on Endothelial Cell Injury Produced by Ionomycin + CCCP*

	Percent free LDH
Time control (30 min)	5.9 ± 0.6
Ionomycin + CCCP (I + C)	63.2 ± 3.9†
I + C, 5 mM glucose	64.4 ± 4.6†
I + C, 5 mM pyruvate	59.4 ± 4.2†
I + C, 5 mM glutamate	65.0 ± 5.0†
I + C, 5 mM glutamine	60.4 ± 2.4†
I + C, 5 mM glycine	9.7 ± 1.6*
I + C, 5 mM L-alanine	7.0 ± 1.0*
I + C, 5 mM D-alanine	9.7 ± 1.0*
I + C, 5 mM β-alanine	8.0 ± 1.3*
I + C, 5 mM 1-aminocyclopropane-1-carboxylate	6.8 ± 1.1*
I + C, 5 mM α-aminoisobutyrate	9.4 ± 2.2*
I + C, 5 mM L-serine	26.0 ± 4.7*†

* Significantly different from ionomycin + CCCP alone.
† Significantly different from ionomycin + CCCP, 5 mM glycine.
Values are means ± SE from 3–5 experiments.

Table 2. *Effects of Poorly Protective Amino Acids and Mn^{2+} on Endothelial Cell Injury Produced by Ionomycin + CCCP*

	Percent free LDH
Time control (30 min)	7.3 ± 0.8
Ionomycin + CCCP (I + C)	86.2 ± 4.6†
I + C, 0.1 mM Mn^{2+}	74.8 ± 10.7†
I + C, 0.2 mM Mn^{2+}	64.3 ± 6.5*†
I + C, 0.5 mM Mn^{2+}	57.3 ± 6.3*†
I + C, 1.0 mM Mn^{2+}	48.9 ± 5.6*†
I + C, 5 mM glycine	8.7 ± 1.4*
I + C, 5 mM glycine, 0.5 mM Mn^{2+}	6.6 ± 2.3*
I + C, 5 mM L-aminobutyrate	65.6 ± 7.2*†
I + C, 5 mM L-aminobutyrate, 0.5 mM Mn^{2+}	43.8 ± 2.3*†
I + C, 5 mM DL-β-aminobutyrate	80.1 ± 4.8†
I + C, 5 mM DL-β-aminobutyrate, 0.5 mM Mn^{2+}	43.3 ± 1.3*†
I + C, 5 mM L-leucine	67.7 ± 4.5*†
I + C, 5 mM L-leucine, 0.5 mM Mn^{2+}	54.5 ± 1.4*†
I + C, 5 mM L-valine	67.6 ± 4.0*†

* Significantly different from ionomycin + CCCP alone.
† Significantly different from ionomycin + CCCP, 5 mM glycine.
Values are means ± SE from 3–5 experiments.

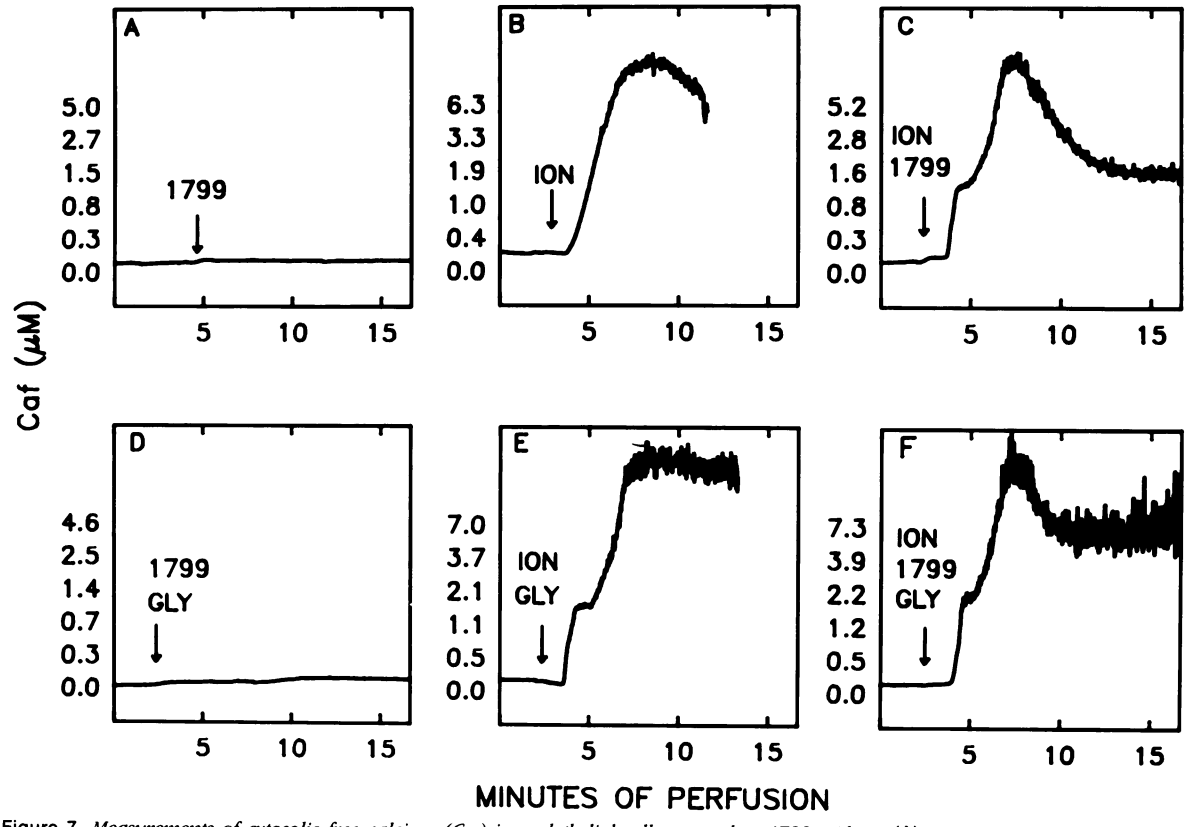


Figure 7. *Measurements of cytosolic free calcium (Ca_f) in endothelial cells exposed to 1799 without* (A), *and with 5 mM glycine* (D), *ionomycin without* (B) *and with* (E) *5 mM glycine, and ionomycin + 1799 without* (C) *and with* (F) *5 mM glycine. Each tracing is representative of results seen in three to five experiments. The Ca_f axis is labeled only up to the low μM range because fura estimates of Ca_f are not accurate at higher levels.*[59]

oxide exposure. This effect is distinct from the actions of amino acids plus Mn^{2+} to ameliorate oxidant injury by promoting degradation of H_2O_2.[13–16]

Protective effects of glycine and related compounds against ionomycin-induced injury were very similar to those previously seen in fresh kidney proximal tubules. In the proximal tubules, protective effects were found during injury produced by hypoxia, chemical inhibitors of oxidative phosphorylation, ouabain, and ionomycin.[1–5,10,12] For hypoxia and removable inhibitors of oxidative phosphorylation, protection during the insult by amino acids allowed essentially full recovery afterward,[1–3,5] indicating that protection was a real change in the biology of injury and not simply a transient effect to alter the measured injury parameters during the insult.

As in prior studies of cultured cell systems,[36–38] the endothelial cells were relatively resistant to lethal cell injury induced by inhibition of oxidative phosphorylation despite efficacy of the inhibitor, CCCP, to markedly reduce ATP levels under the glucose-free experimental conditions that had been chosen to favor ATP depletion. Studies with CCCP alone, therefore, did not allow for a test of amino acid protection and have not been detailed in the results. Rapidly increasing Ca_f with ionomycin, however, produced extensive lethal cell injury over an accelerated time frame, similar to that seen in fresh kidney tubules[12] and other cell types.[39] As in the kidney tubules, amino acids provided potent protection, even though they did not prevent the increases of Ca_f to micromolar levels or the profound ATP depletion associated with the injury. The patterns of both injury and protection were similar in cells treated with ionomycin and ionomycin + CCCP.

For studies comparing protective effects of glycine with those of other amino acids, ionomycin + CCCP was used to produce a maximally damaging insult. The ATP measurements, however, which were not available until later, indicate that ionomycin alone would have been sufficient. Protective effects of compounds structurally similar to glycine were somewhat broader than reported for hypoxic injury to rabbit kidney tubules.[9] β-Alanine, 1-aminocyclopropane-1-carboxylate, and D-alanine, which had weaker effects than glycine and alanine in the kidney tubules, were all fully effective in the endothelial cells. α-Aminoisobutyrate and L-serine, which were ineffective for the tubules,[9] were active for the endothelial cells. As in the kidney cells, however, several other amino acids and potential substrates for energy metabolism were without effect. The overall similarity of the nature of protection and the compounds affording protection strongly suggests

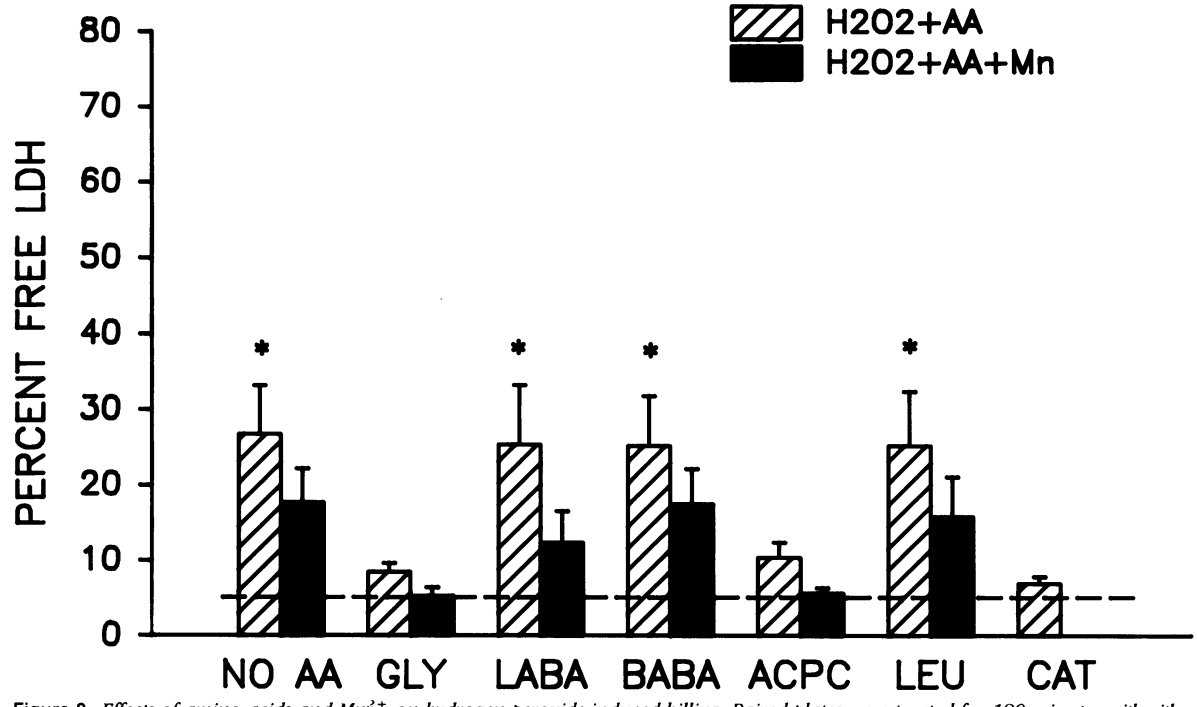


Figure 8. *Effects of amino acids and Mn^{2+} on hydrogen peroxide-induced killing. Paired plates were treated for 180 minutes with either H_2O_2 alone, H_2O_2 plus 5 mM of the indicated amino acid, or H_2O_2 plus 5 mM amino acid plus 0.1 mM Mn^{2+}. No amino acid = NO AA, glycine = GLY, L-aminobutyrate = LABA, DL-β-aminobutyrate = BABA, 1-aminocyclopropane-1-carboxylate = ACPC, leucine = LEU, CAT = 1800 U/ml catalase. Time control LDH release shown by the interrupted line was 5.3 ± 0.7. Values are means + SE for seven experiments. *Significantly different from time control value. None of the individual Mn^{2}-treated groups significantly differed from either the time control or the corresponding group studied without Mn^{2+}, but the effect of Mn^{2+} to decrease injury was significant when the data for the NO AA, LABA, BABA, and LEU groups were pooled.*

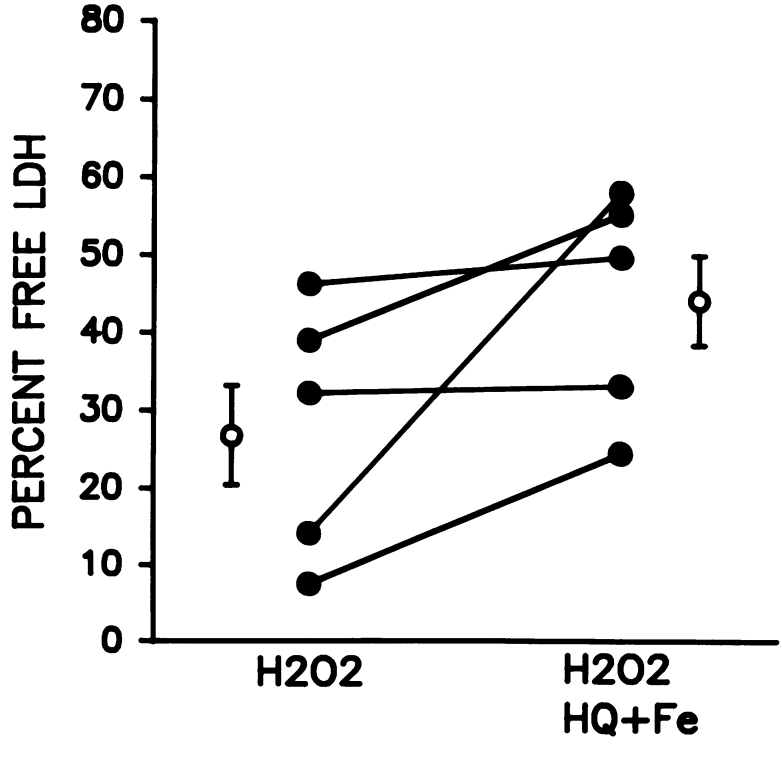


Figure 9. *Effect of 8-hydroxyquinoline (HQ) and Fe^{2+} on H_2O_2-induced injury. Plates were preincubated for 60 minutes. During the last 30 minutes HQ + Fe plates were treated with 10 μM HQ + 10 μM Fe^{2+}. All plates were then washed three times with PBS and returned to the incubator in the standard experimental medium (medium A) for 10 min. prior to addition of 5 mM H_2O_2. Plates were sampled for LDH after 180 minute exposure to H_2O_2. Open circles are the group means ± SE. Filled circles show results for each set of paired plates from separate cultures studied. HQ + Fe-treated cells had significantly greater LDH release.*

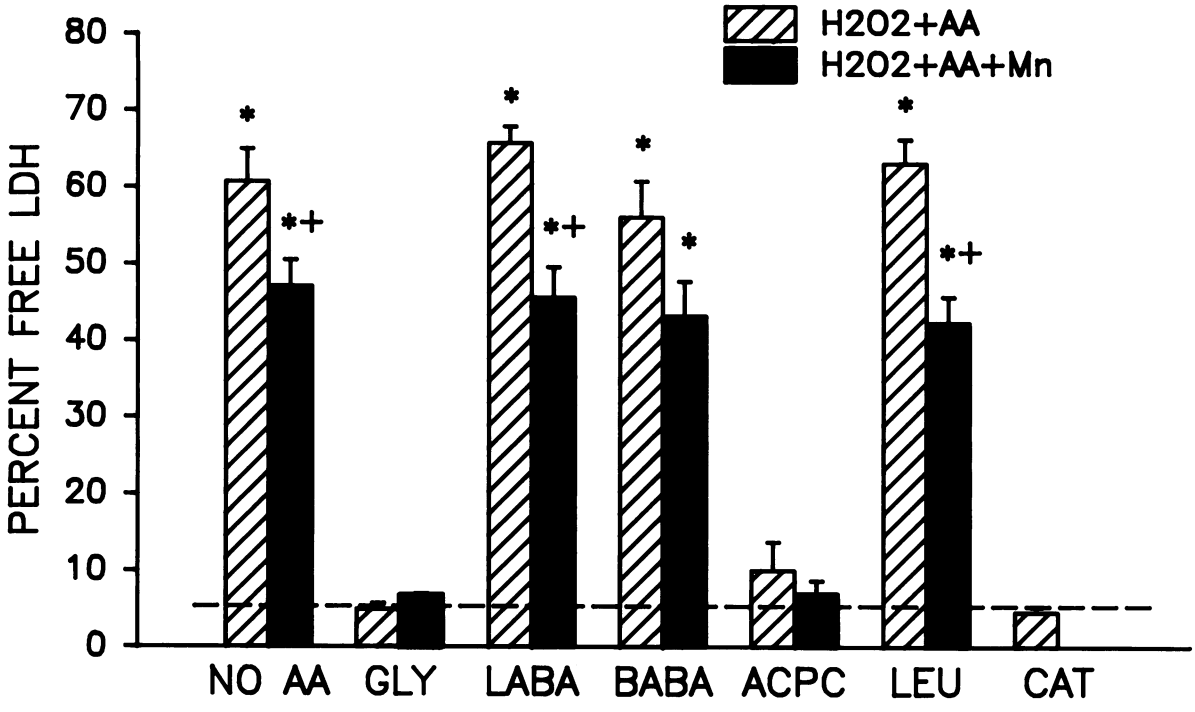


Figure 10. *Effects of amino acids and Mn^{2+} on H_2O_2, induced killing in cells pretreated with HQ + Fe. After 30 minutes pretreatment with HQ + Fe (as described with Figure 9), paired plates were treated for 180 minutes with either H_2O_2 alone, H_2O_2 plus 5 mM of the indicated amino acid, or H_2O_2 plus 5 mM amino acid plus 0.1 mM Mn^{2+}. No amino acid = NO AA, glycine = GLY, L-aminobutyrate = LABA, DL-β-aminobutyrate = BABA, 1-aminocyclopropane-1-carboxylate = ACPC, leucine = LEU, CAT = 1800 U/ml catalase. Time control LDH release by cells not treated with HQ + Fe was 1.8 ± 0.3. Time control LDH release by HQ + Fe pretreated cells, shown as the interrupted line, was 5.6 ± 1.0 (significantly different from nonpretreated time control). Values are means ± SE for three experiments. *Significantly different from time control value. †Significantly different from corresponding group without Mn^{2+}*

that the same phenomenon is being studied. It is of interest, in this regard, that protective effects of amino acids against hypoxic injury to rat thick ascending limb tubules that are strongly expressed for the same core compounds, glycine, alanine, β-alanine, and 1-aminocyclopropane-1-carboxylate, also extend to D-serine but not to L-serine.[40] Whether the species and cell type differences in specificity of this effect reflect differences in interaction with the target molecule or in access to the active site will require further studies and better definition of the cellular target for protection.

The concentration dependence of protection by glycine is well within the physiologic range of circulating levels of the amino acid in the human, which range from 0.2 to 0.4 mmol/l.[41,42] Strong but partial protection was evident at 0.25 mmol/l; protection was complete by 1.0 mmol/l. This is a somewhat more potent effect than in the rabbit tubules, where protection was just evident at 0.25 mmol/l and was not maximal until 2 mmol/l. Circulating glycine levels in the rabbit are 1 to 1.5 mmol/l.[43,44] Thus protection was maximal in both species at levels approximating the high end of normal serum values. This suggests that variations of glycine within the physiologic range could modify expression of endothelial cell injury *in vivo*.

Recent reports using cell-free systems have shown that Mn^{2+} by itself induces disproportion of hydrogen peroxide,[13] and it promotes the effects of amino acids to oxidize H_2O_2 with formation of amino acid carbonyl derivatives.[14,15] We have provided support for the biologic relevance of this process by demonstrating protective effects of amino acids + Mn^{2+} both *in vitro* and *in vivo*.[16] In the current studies, Mn^{2+} was partially protective against hydrogen peroxide damage, irrespective of amino acid addition. Glycine and ACPC, however, were completely protective without addition of Mn^{2+}. In contrast, leucine, L-aminobutyrate, and DL-β-aminobutyrate were without protective effects in the absence of Mn^{2+}. Although amino acids + Mn^{2+} did promote metabolism of H_2O_2 in the medium, this effect was not specific for glycine and was not produced by glycine alone. These observations suggest that most of the effect of glycine to strongly suppress hydrogen peroxide-induced lethal cell injury was due to an action similar to that seen with ionomycin-induced injury rather than to modulation of the availability of H_2O_2 or reactive oxygen metabolites derived from it. The moderate protective effect of Mn^{2+} in the ionomycin model could be related to direct interactions of Mn^{2+} with ionomycin to alter its effects on Ca_i. The quenching actions of Mn^{2+} on fura[12,28] prevented us from assessing this issue directly.

Oxidation of reduced glutathione catalyzed by gluta-

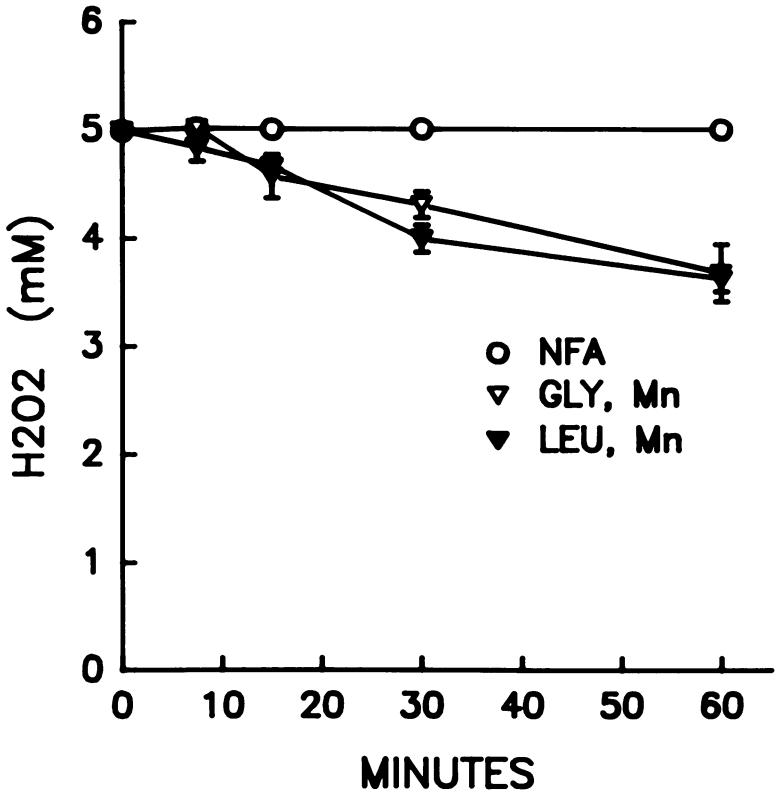


Figure 11. *Catabolism of H_2O_2 in medium without cells. 5 mM H_2O_2 was added to medium A at time zero and samples for H_2O_2 determination were taken for up to 60 minutes of incubation at 37°C. No further additions = NFA. Mn^{2+} was 0.1 mM, other agents were 5 mM. Glycine = GLY, leucine = LEU. Values are means ± SE for three experiments. Addition of glycine, leucine, or Mn^{2+} alone resulted in essentially zero H_2O_2 consumption and would overlap with the NFA line (not shown). Incubation of H_2O_2 in water rather than medium also did not result in H_2O_2 consumption (not shown).*

thione peroxidase importantly contributes to detoxification of hydrogen peroxide. Reduced glutathione is depleted if the hexose monophosphate shunt and glutathione reductase cannot compensate.[45,46] Decreasing glutathione promotes hydrogen-peroxide–induced endothelial cell injury; augmenting glutathione retards injury.[47,48] Although glycine is a component of glutathione, and protection by glycine in some of the renal tubule cell models of injury is associated with preservation of glutathione, it has been shown through the use of glutathione-depleting maneuvers that glycine protection in that system is not mediated by glutathione.[2,4,5] Glutathione metabolism was not assessed in the present study; however, the protective effects of other small neutral amino acids such as D-alanine, 1-aminocyclopropane-1-carboxylate, and α-aminoisobutyrate, which do not enter into pathways related to glutathione synthesis, strongly argue that protection is not related to preservation of cellular glutathione.

Hydrogen-peroxide–induced damage to endothelial cells is associated with early depletion of cell ATP and loss of purine metabolites to the medium. These changes are prevented by agents such as catalase that protect by promoting catabolism of hydrogen peroxide.[49,50] We found similar protection against hydrogen-peroxide–induced adenine nucleotide depletion by catalase in our system, but protection by glycine and ACPC was not accompanied by preservation of ATP. This further argues against an action of glycine and related amino acids to primarily detoxify hydrogen peroxide. It is also similar to the protection by glycine in ionomycin and metabolic inhibitor-induced injury, which also occurs in spite of ATP depletion equivalent to that seen in unprotected cells.

The ultimate lytic cell lesion in hydrogen-peroxide–induced and related forms of oxidant damage has been shown to be strongly Fe^{2+} dependent in multiple systems, and most evidence indicates that this is due to Fe^{2+}-catalyzed formation of hydroxyl radical, a more potent oxidant than H_2O_2.[35,51,52] Our data provide support for the role of Fe^{2+} in human umbilical vein endothelial cells and confirm the efficacy of a recently reported method for rapidly preloading endothelial cells with Fe^{2+} by using 8-hydroxyquinoline.[35] That glycine retained its full protective effects despite enhancement of injury by Fe^{2+} loading suggests actions against hydroxyl radical-induced damage.

Increases of Ca_i have been implicated in hydrogen-peroxide–induced and related forms of oxidant cell injury,[22–24,53,54] although their role in the development of lethal cell injury has been questioned.[55,56] The potent protection by glycine against ionomycin-induced lytic cell damage raises the possibility that protection by glycine during hydrogen peroxide injury is also against a Ca^{2+}-mediated component; however further studies will be required to assess this issue. Glycine protection is not, however, limited to injury associated with increases of

Table 3. *ATP Levels of Hydrogen Peroxide and Amino Acid-treated Endothelial Cells*

	ATP (nmol/mg cell protein)	
	No pretreatment	HQ + Fe pretreatment
Zero time control	25.3 ± 1.16*	37.42
180 min time control	19.23 ± 0.68	27.84
180 min HQ + Fe		24.83
H_2O_2	0.35 ± 0.03*	0.30
H_2O_2 + Mn^{2+}	0.36 ± 0.04*	0.15
H_2O_2 + Gly	0.34 ± 0.11*	0.10
H_2O_2 + Mn^{2+} + Gly	0.42 ± 0.08*	0.18
H_2O_2 + LABA	0.48 ± 0.13*	0.10
H_2O_2 + Mn^{2+} + LABA	0.41 ± 0.08*	0.10
H_2O_2 + BABA	0.39 ± 0.09*	0.15
H_2O_2 + Mn^{2+} + BABA	0.38 ± 0.09*	0.50
H_2O_2 + ACPC	0.22 ± 0.06*	0.15
H_2O_2 + Mn^{2+} + ACPC	0.85 ± 0.12*	0.45
H_2O_2 + LEU	0.48 ± 0.09*	0.10
H_2O_2 + Mn^{2+} + LEU	0.28 ± 0.07*	0
H_2O_2 + CAT	20.43 ± 0.65	26.14

* Significantly different from 180-minute time control.

Values are means ± SE from four experiments (No pretreatment) and two experiments (HQ + Fe pretreatment, means only). These experiments were part of the groups which provided the LDH values in Figures 8 and 10. HQ + Fe indicates 30-minute exposure to 10 μM hydroxyquinoline + 10 μM ferrous ammonium sulfate before addition of H_2O_2 and the indicated test compounds for 180 minutes. Other abbreviations are as in Figures 8 and 10.

Ca_f. In kidney tubules, we have found that glycine also protects against damage induced by Ca^{2+} depletion.[57]

It is notable that glycine did not protect the ionomycin-treated cells against the typical retraction and severe blebbing that are common to calcium ionophore and hydrogen-peroxide–induced damage and that, in both cases, are probably due to effects of increased Ca_f on the cytoskeleton and membrane phospholipases.[20,22,53,58] The occurrence of these changes in glycine-treated cells supports the conclusions from the fura studies that glycine was not altering ionomycin-induced increases of Ca_f. We suspect that protection by glycine against lytic plasma membrane damage was delaying that process far beyond otherwise irreversible damage to intracellular structures that would have prevented recovery of the cells after reversal of ionomycin effects. We have not yet been able to assess this directly because of the hydrophobic nature of the compound.

The full applicability of these protective effects to more physiologically relevant types of endothelial cell injury will have to be determined by future studies. Expression of protection independent of Mn^{2+} in the somewhat milder lesion produced by hydrogen peroxide, however, suggests that protection will be evident in other settings. Furthermore the very similar protective effects in kidney tubules have already been shown to allow sustained recovery from more physiologic, reversible insults.[1,2] The fact that such protective effects are present in human-derived endothelial cells suggests that these findings may prove applicable to human disease processes in several organs *in vivo* and merit further investigation in this regard. At a minimum, these data indicate that content of specific amino acids independent of their potential for metabolism by traditional pathways must be explicitly considered in the design of *in vitro* studies of endothelial cell injury.

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