Protection of ATP-Depleted Cells by Impermeant Strychnine Derivatives

Implications for Glycine Cytoprotection

Zheng Dong,* Manjeri A. Venkatachalam,*
Joel M. Weinberg,[†] Pothana Saikumar,* and
Yogendra Patel*

From the Department of Pathology,* University of Texas Health Science Center, San Antonio, Texas; and the Department of Internal Medicine,[†] University of Michigan Medical Center, Ann Arbor, Michigan

Glycine and structurally related amino acids with activities at chloride channel receptors in the central nervous system also have robust protective effects against cell injury by ATP depletion. The glycine receptor antagonist strychnine shares this protective activity. An essential step toward identification of the molecular targets for these compounds is to determine whether they protect cells through interactions with intracellular targets or with molecules on the outer surface of plasma membranes. Here we report cytoprotection by a cell-impermeant derivative of strychnine. A strychnine-fluorescein conjugate (SF) was synthesized, and impermeability of plasma membranes to this compound was verified by fluorescence confocal microscopy. In an injury model of Madin-Darby canine kidney cells, ATP depletion led to lactate dehydrogenase release. SF prevented lactate dehydrogenase leakage without ameliorating ATP depletion. This was accompanied by preservation of cellular ultrastructure and exclusion of vital dyes. SF protection was also shown for ATP-depleted rat hepatocytes. On the other hand, when a key structural motif in the active site of strychnine was chemically blocked, the SF lost its protective effect, establishing strychnine-related specificity for SF protection. Cytoprotective effects of the cell-impermeant strychnine derivative provide compelling evidence suggesting that molecular targets on the outer surface of plasma membranes may mediate cytoprotection by strychnine and glycine. (*Am J Pathol* 2001, 158:1021–1028)

Originally identified in kidney proximal tubules, the cytoprotective effects of glycine, structurally related amino acids, and chloride channel modulators under situations of ATP depletion have now been documented in a variety of cell types by *in vitro* as well as *in vivo* studies.^{1–20} Glycine enables ATP-depleted cells to maintain structural

integrity despite complete disruption of ion homeostasis.²¹ Protected by glycine, ATP-depleted cells can sustain surprisingly high elevations of intracellular-free calcium, yet survive and subsequently proliferate.²² These actions may account at least in part for the *in vivo* beneficial effects of glycine in organ preservation, transplantation, and septic shock,^{13,15,18,23,24} although anti-inflammatory effects of glycine have been recognized by recent studies.^{25,26}

The molecular mechanisms underlying cytoprotection by glycine remain primarily unknown.²⁷ Glycine cytoprotection does not involve the generation or conservation of ATP, preservation of ion homeostasis, modification of intracellular pH, stabilization of the cytoskeleton, quenching of reactive oxygen species, or inhibition of phospholipid hydrolysis.^{28–32} Moreover, the cytoprotective effects do not rely on the metabolism of glycine.^{20,33} On the other hand, the protective activity is shared by a family of closely related amino acids, and several neural chloride channel modulators including the antagonist strychnine.^{3,8,11,16,19,34} Based on these observations, we have proposed that glycine and the related compounds may protect ATP-depleted cells by low affinity interactions with a multimeric channel protein, destabilization of which may otherwise lead to formation of pathological pores.³⁴ Such porous defects in plasma membranes of ATP-depleted cells have been characterized recently, showing definable exclusion limits for molecules of increasing sizes.²⁷ Glycine provided during ATP depletion blocked the development of membranous pores completely.²⁷ The actions of glycine could be mimicked by cross-linking of plasma membrane proteins with a cell-impermeant cross-linker, 3,3'-dithiobis-sulfosuccinimidylpropionate.²⁷ These observations are in support of our hypothesis that cytoprotection by glycine and the related compounds may depend on their interactions with a multimeric protein target in the plasma membranes. Recent studies showing glycine inhibition of ion fluxes in ATP-depleted hepatocytes are consis-

Supported by The American Heart Association (to Z. D.), the Texas Advanced Research Program (to Z. D.), and the National Institutes of Health (to M. A.V., P. S., and J. M. W.).

Accepted for publication November 16, 2000.

Address reprint requests to Zheng Dong, Ph.D., Department of Pathology, University of Texas Health Science Center, 7703 Floyd Curl Dr., San Antonio, TX 78229. E-mail: dong@uthscsa.edu.

tent with the membranous pore mechanism.^{19,35} However, glycine may also have actions within cells. For example, at cytoprotective concentrations, glycine can inhibit calpain, a calcium-dependent protease involved in cell injury during ATP depletion.³⁶ Moreover, the evidence for a plasma membrane surface-located binding site for glycine remains circumstantial. Therefore, localization of the targeting sites for glycine and related compounds remains a critical step toward identification of the molecular mechanisms underlying cytoprotection.

To directly examine whether glycine, structurally related amino acids, and chloride channel modulators can protect ATP-depleted cells by interactions with plasma membranes, we synthesized a strychnine-fluorescein conjugate. This conjugate was cell-impermeant, and yet retained the protective activity against injury induced by ATP depletion in Madin-Darby canine kidney (MDCK) cells and rat hepatocytes. On the other hand, when a key structural motif in the active site of strychnine was chemically blocked, the SF lost its protective effects. These results have provided compelling evidence that the protective actions of strychnine and glycine may be mediated by interactions with putative target molecules on the outer surface of the plasma membranes.

Materials and Methods

Materials

The SF and its control compound, N(22)-methyl-strychnine-fluorescein (MSF), were synthesized by RANN Research Laboratory (San Antonio, TX). Purity of the compounds was established by thin-layer chromatographic analysis, and structural identity was verified by electrospray ionization-mass spectrometry (not shown).

Cells

MDCK cells were cultured as described.³⁴ The cells were plated at 400,000/well on Corning 12-well plates and used for experiments after overnight growth. Rat hepatocytes were isolated from adult male Sprague-Dawley rats by collagenase perfusion of the livers.³⁷ The hepatocytes were plated at 100,000/well on collagen-coated Corning 24-well plates in Williams' medium E supplemented with 10% fetal bovine serum, 7 $\mu\text{g}/\text{ml}$ insulin, 2 mmol/L glutamine, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin. The rat hepatocytes were used for experiments within 48 hours of culture.

ATP Depletion

For rat hepatocytes, ATP depletion was initiated by incubation in glucose-free Krebs-Ringer bicarbonate solution containing 15 $\mu\text{mol}/\text{L}$ carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP), a mitochondrial uncoupler. For MDCK cells, ATP depletion was performed as described in our previous studies.^{22,27,34} Briefly, cells were incubated in glucose-free Krebs-Ringer bicarbonate solution containing 15 $\mu\text{mol}/\text{L}$ CCCP. Free Ca^{2+} in this solution

was buffered to 100 nmol/L by adding 2.25 mmol/L EGTA. Ionomycin, a Ca^{2+} ionophore, was included at 5 $\mu\text{mol}/\text{L}$ in the buffer so that cells were permeable to Ca^{2+} and intracellular-free Ca^{2+} was clamped at extracellular levels (ie, 100 nmol/L). Clamping of intracellular Ca^{2+} at 100 nmol/L during ATP depletion avoided Ca^{2+} -dependent injury, and facilitated examination of the injury that is sensitive to protection by glycine and the related compounds.²⁷

Examination of Cell Permeability of SF by Fluorescence Confocal Microscopy

MDCK cells were plated at 400,000/35-mm dish on glass coverslips. To ensure adequate sample sizes of optical sections, the cells were pre-incubated for 30 minutes at 37°C in Ca^{2+} , Mg^{2+} -free phosphate-buffered saline to partially detach the cells from the substratum. The pre-incubation led to a spheroidal cell shape. The cells were subsequently incubated with 1 mmol/L SF in glucose-free Krebs-Ringer bicarbonate solution as control, or incubated with 1 mmol/L SF in glucose-free Krebs-Ringer bicarbonate solution with 100 nmol/L Ca^{2+} containing 15 $\mu\text{mol}/\text{L}$ CCCP and 5 $\mu\text{mol}/\text{L}$ ionomycin for ATP depletion. Confocal microscopy was performed as described previously.²⁷

Measurement of Lactate Dehydrogenase (LDH) and ATP

Leakage of intracellular LDH, an index of plasma membrane damage, was measured enzymatically by described methods.³⁴ Parallel dishes of cells were lysed with 0.1% Triton X-100 to determine total LDH activity. The LDH activities obtained from cell incubation medium was divided by the total LDH activity to calculate the percentage of LDH release. To measure ATP, cells were extracted with trichloroacetic acid. ATP in cell extracts was measured by luminometry of the luciferin firefly luciferase reaction.³⁸ ATP values were expressed as nmol per mg cell protein. Protein was quantitated with the bicinchoninic acid reagent purchased from Pierce Chemical Company, Rockford, IL.

Double Staining of Cells with Ethidium Homodimer and Calcein-AM

At the end of incubation, cells were exposed for 5 minutes to 5 $\mu\text{mol}/\text{L}$ ethidium homodimer in a physiological solution buffered with 25 mmol/L HEPES, pH 6.9. Calcein-AM at 2 $\mu\text{mol}/\text{L}$ was subsequently added to the medium, and incubation continued for another 5 minutes. Cells were finally washed twice with the HEPES-buffered solution, and examined by fluorescence microscopy. Fluorescence of ethidium homodimer and calcein was viewed simultaneously using 420- to 490-nm excitation/520-nm long-pass emission.

A.

Strychnine-fluorescein conjugate (SF)

B.

N(22)-methyl-strychnine-fluorescein (MSF)

Figure 1. Structure of the synthesized strychnine derivatives. **A:** SF. **B:** MSF. The fluorescein moieties in both compounds are mixed isomers with covalent linkage to a carboxyl at the 5 or 6 positions, as indicated by the arrows.

Electron Microscopy

At the end of incubation, medium was saved to measure LDH release, and cells were fixed with 2% glutaralde-

hyde in 0.1 mol/L Na cacodylate buffer, pH 7.4, and subsequently processed for electron microscopy.²²

Results and Discussion

Structure and Synthesis of Strychnine Derivatives

To determine whether glycine protects ATP-depleted cells by its interaction with moieties exposed on the outer surface of plasma membranes, it would be ideal to have a cell-impermeant derivative of this small amino acid. However, our past experience shows that modification of glycine could lead to drastic decreases in its protective capacity.³ On the other hand, cytoprotective activity of glycine is shared by a family of structurally related amino acids and by several modulators of neural chloride channels.^{3,11,16,19,34} After study of the structure of these compounds, we decided to synthesize a derivative of strychnine. Strychnine is an antagonist of the glycinergic chloride-channel receptor in the central nervous system, and has been shown to be cytoprotective in several models of ATP depletion injury.^{11,19,34} Moreover, potential sites for modification are available in the structure of strychnine and its analog 2-amino-strychnine.

The strychnine derivative was designed to satisfy three main criteria. First, the key structure of strychnine that determines its cytoprotective activity should be preserved. Second, the conjugated group should be readily detectable with high sensitivity, and preferably be chromatic or fluorescent. This would make it easier to examine the cellular permeability of the synthesized compound by noninvasive methods such as confocal microscopy. Finally, the strychnine derivative should carry polar groups and be highly hydrophilic, and as a result, be cell-impermeant. With these considerations, we synthesized a SF. In the structure of SF (Figure 1A), the fluorescent fluorescein ligand was anchored onto the least active center of strychnine analogs, ie, the amino group at position-2 of

Figure 2. Cell permeability of the SF. MDCK cells were incubated with 1 mmol/L SF for 2 hours in glucose-free Krebs-Ringer bicarbonate solution as control (**a**), or incubated with 1 mmol/L SF for 0.5, 1, or 2 hours in glucose-free Krebs-Ringer bicarbonate solution with 100 nmol/L Ca^{2+} containing CCCP and ionomycin for ATP depletion (**b–d**). At the end of incubation, cells were examined in the SF-containing buffers by fluorescence confocal microscopy. Dark images represent cells that were impermeable to SF and did not have SF fluorescence signals inside. Strong fluorescence in the space between cells indicates SF in the medium. A few cells became SF permeable after 2 hours of ATP depletion (arrows), because of the loss of plasma membrane integrity (see Figure 4).

Figure 3. Inhibition of LDH release during ATP depletion of MDCK cells by the SF. **A:** Inhibition of LDH release from ATP-depleted cells by SF but not MSF. MDCK cells (Con, control) were subjected to 2 hours of ATP depletion with CCCP and ionomycin, in the absence (–) or presence of 1 mmol/L glycine, strychnine, SF, or MSF. At the end of incubation, LDH released into the incubation medium was measured and expressed as percentage of total cell LDH. Values, mean $\pm$ SD, were from four separate experiments. **B:** Comparison of the cytoprotective potency of glycine, strychnine, and SF. MDCK cells were subjected to 2 hours of ATP depletion in the presence of 0.1 to 1 mmol/L of glycine, strychnine, or SF. LDH release was measured at the end of incubation. Values were averages of three separate experiments; errors bars are not shown for clarity.

strychnine moiety, while maintaining a safe and steric distance of a 7-atom chain. To enhance the water solubility of this conjugate, a hydrophilic chain was chosen as the spacer between the strychnine and fluorescein moieties. In addition, a carboxylic group was added to the spacer structure.

To control for specificity of the bio-activity of SF, we also synthesized MSF. In the structure of MSF (Figure 1B), the nitrogen atom at position 22 was shielded by a

methyl group. Because the structure adjacent to this nitrogen atom is shown by molecular modeling to be critical for strychnine ligand-receptor interaction,^{39,40} we reasoned that blockage of this site by a methyl group would lead to the loss of its bio-activities.

Cell Permeability of SFs

We examined the permeability of SF to cells under situations of control incubation or ATP depletion (Figure 2). To this end, MDCK cells were incubated in a medium containing 1 mmol/L SF and monitored by confocal laser scanning microscopy. To deplete cells of ATP, the mitochondrial uncoupler CCCP and the Ca^{2+} ionophore ionomycin were included in the incubation medium in the absence of metabolic substrates. Examination of thin optical sections through equatorial planes of the cells revealed whether fluorescent SF had entered the cytoplasm. Impermeability was indicated by lack of fluorescence signal in the cell interior, and consequently a dark cytoplasm, contrasting sharply with bright fluorescence in the surrounding medium. On the other hand, permeability of plasma membranes to SF led to entry of the fluorescent conjugate into cells, revealing the presence of signal inside, and therefore, a lighter cytoplasm. The results are shown in Figure 2. Control cells were impermeable to SF, and all displayed dark images after 2 hours of SF exposure (Figure 2a). Complete exclusion of SF was also shown for cells after 30 or 60 minutes of ATP depletion (Figure 2, b and c). For cells with 2 hours of ATP depletion, the majority excluded SF and showed dark images, whereas a few others became permeable to SF, exhibiting faint or moderate fluorescein staining (Figure 2d). As we will show in Figure 4, even in the presence of SF, a small fraction of cells finally lost their plasma membrane integrity during ATP depletion and became permeable to vital dyes. Thus, SF staining of the few cells shown in Figure 2d was only a secondary event, caused by loss of plasma membrane integrity and associated with non-specific increase of membrane permeability. Taken together, the results indicate clearly that the synthesized SF

Figure 4. Double staining of cells with ethidium homodimer and calcein-AM. MDCK cells (**A**, control) were subjected to 2 hours of ATP depletion in the absence (**B**) or presence of 1 mmol/L glycine (**C**), strychnine (**D**), SF (**E**), or MSF (**F**). At the end of incubation, cells were stained with ethidium homodimer and calcein-AM as described in Materials and Methods. Fluorescence of ethidium homodimer (red) and calcein (green) was recorded simultaneously by a fluorescence microscope. Green and red images represent viable and dead cells, respectively.

Figure 5. Electron microscopy of MDCK cells. MDCK cells (**a**, control) were subjected to 2 hours of ATP depletion in the absence (**b**) or presence of 1 mmol/L glycine (**c**), strychnine (**d**), SF (**e**), or MSF (**f**), and fixed with glutaraldehyde for electron microscopy as described.²⁷ Percent LDH release was 2, 76, 2, 4, 15, and 72 for **a**, **b**, **c**, **d**, **e**, and **f**, respectively. Original magnification, ~5,000.

cannot permeate plasma membranes of cells that are structurally intact.

SFs Prevent LDH Release from ATP-Depleted MDCK Cells

We first examined the effects of SF on cell injury during ATP depletion of MDCK cells. A model of MDCK cell injury by ATP depletion has been characterized in our previous studies.^{27,34} In this model, ATP depletion is induced in the absence of intracellular Ca^{2+} alterations. Clamping of intracellular Ca^{2+} is achieved by inclusion of ionomycin in the incubation medium containing 100 nmol/L Ca^{2+} . Advantages of this model include the removal of Ca^{2+} -dependent damage while studying the injury processes that are sensitive to glycine and related

compounds.²⁷ In the first series of experiments, we monitored leakage of LDH, an index of the loss of plasma membrane integrity. As shown in Figure 3A, 2 hours of ATP depletion led to 71% LDH release, indicating breakdown of the plasma membranes in the majority of cells. Provision of 1 mmol/L of glycine or strychnine prevented the leakage of LDH completely. Like glycine and strychnine, the cell-impermeant SF strikingly inhibited LDH release. Only 13% LDH release was shown in the cells with SF, at the end of 2 hours of ATP depletion. On the contrary, MSF, the control analogue of SF in which the nitrogen atom at position 22 was shielded by a methyl group, was ineffective for cytoprotection and showed 66% LDH release. We subsequently compared the protective potency of glycine, strychnine, and SF (Figure 3B). Glycine has the highest activity, followed by strychnine.

Figure 6. Protection of rat hepatocytes during ATP depletion by the SF. Rat hepatocytes (Con, control) were incubated for 3 hours with CCCP in a physiological buffer without metabolic substrates, in the absence (–) or presence of 1 mmol/L glycine (Gly), strychnine (Stry), SF (SF), or MSF (MSF). LDH release was measured at the end of incubation. Values, mean $\pm$ SD, were from three separate experiments performed on two hepatocyte preparations.

nine, and then SF. The protective potency of 0.75 mmol/L SF was comparable to that of 0.5 mmol/L strychnine or 0.25 mmol/L glycine.

Effects of SFs on Vital Dye Exclusion during ATP Depletion

To further substantiate the effects of SF on membrane integrity as measured by LDH release, we stained the cells with vital dyes: ethidium homodimer and calcein-

Figure 7. SF does not ameliorate ATP depletion during CCCP treatment. MDCK cells were incubated for 0 to 120 minutes with CCCP and ionomycin in the absence or presence of 1 mmol/L glycine, strychnine, or SF. Cell ATP was determined by luminometry of the luciferin firefly luciferase reaction. Values were averages of three separate experiments; error bars (standard deviations) were not shown for clarity.

AM. Ethidium homodimer with a molecular weight of 857 Daltons is cell-impermeant and only stains cells in which plasma membranes are damaged and have lost the integrity. On the contrary, calcein-AM enters intact cells and is de-esterified to calcein that is retained only by cells with membrane barrier function. Double staining of cells with ethidium homodimer and calcein-AM is shown in Figure 4. Control cells showed exclusively calcein staining (green fluorescence; Figure 4A). After 2 hours of ATP depletion, 70 to 80% cells lost the ability to retain calcein, and gained red fluorescent staining of ethidium homodimer, indicating loss of plasma membrane integrity (Figure 4B). In the presence of glycine or strychnine, plasma membrane integrity was well preserved (Figure 4, C and D). SF prevented plasma membrane damage as well, whereas MSF was without effect (Figure 4, E and F). These results indicate a specific cytoprotective property shared by glycine, strychnine, and the cell-impermeant strychnine derivative SF.

Ultrastructural Effects of the SFs

We examined cellular ultrastructure by electron microscopy. A control cell is shown in Figure 5a. After 2 hours of ATP depletion in the absence of protective agents, cells became swollen and totally disrupted, showing fragmented organelles and empty cytosol (Figure 5b). In sharp contrast, cells protected by glycine, strychnine, or SF showed reasonable preservation of internal structures (Figure 5; c, d, and e). Although these cells were swollen to various degrees, the integrity of their organelles was primarily preserved and cytosol was retained, as indicated by the presence of electron-dense materials. Relative to control cells, a noticeable alteration that took place in protected cells was swelling of mitochondria. Mitochondrial swelling was not specific for SF-protected cells, and was also shown for the cells protected by glycine and strychnine (Figure 5; c, d, and e). In contrast to SF, its control analog MSF failed to preserve cellular integrity during ATP depletion (Figure 5f).

Protection of ATP-Depleted Rat Hepatocytes by SFs

To extend our observations of SF protection to other experimental models, we isolated hepatocytes from Sprague-Dawley rats. When rat hepatocytes were incubated for 3 hours with CCCP in a physiological buffer without metabolic substrates, 76% LDH was released from cells (Figure 6). As shown for MDCK cells, like glycine and strychnine, SF exhibited a strong protective effect, reducing LDH release to 21% (Figure 6). Again, no protection was detected for the control compound MSF, which showed 80% LDH release (Figure 6). The results indicate that protection by the cell-impermeant strychnine derivative SF is not cell-type or experimental model-specific.

SFs Do Not Prevent ATP Declines during CCCP Treatment

Because SF protected MDCK cells and rat hepatocytes without entering the cells, we considered it unlikely that this compound preserved cellular ATP during CCCP incubation. The inference was confirmed by ATP measurements. As shown in Figure 7, neither SF nor MSF had significant effects on cell ATP during the course of CCCP treatment. Glycine and strychnine did not affect declines of ATP in CCCP-treated cells either.

Conclusions

Despite the robust protective effects of glycine, structurally related amino acids, and chloride channel modulators against cell injury by ATP depletion, the underlying mechanisms remain unclear. The ultimate goal of this line of research would be to identify the key molecules or molecular processes that are targeted by these protective compounds. The working hypothesis underlying the body of work on glycine cytoprotection in our laboratory is that porous defects develop in plasma membranes of ATP-depleted cells as a consequence of molecular perturbation of a multimeric channel protein leading to pathological expansion of channel size.^{27,34} According to this model, glycine and strychnine may exert their effects by molecular interactions that preserve the integrity of the multimeric membrane protein and prevent its breakdown. That the channel protein hypothesis has a valid base was suggested by our experiments with 3,3'-dithiobis-sulfosuccinimidylpropionate, a bivalent cell-impermeant protein cross-linker that protected ATP-depleted cells.²⁷ If the postulated similarity of the channel protein to members of the glycine-GABA chloride channel receptors of the central nervous system has a rational foundation, it seemed probable that protective ligands must bind the protein at the extracellular domains of plasma membranes as in the case of the central nervous system receptors.⁴¹ These considerations directly led to the experimental design that provided the framework for the experiments that are reported herein. Our strategy was simple and straightforward. A cell-impermeant SF was synthesized and its effects on cell injury during ATP depletion were examined. Our results with two experimental models demonstrated remarkable cytoprotective effects of the SF. On the other hand, the control compound made with inactive strychnine, MSF, was without effect. The results therefore, have provided strong evidence for molecular interactions of strychnine with surface-oriented extracellular domains of a plasma membrane protein that result in cytoprotection by the ligand. By extrapolation, interactions with target molecules exposed on the outside surface of plasma membranes may also underlie the cytoprotection afforded by glycine.

Acknowledgments

We thank J. G. Maresh and N. L. Criscimagna from the Department of Biochemistry of the University of Texas Health Science Center at San Antonio for their assistance in isolation of rat hepatocytes and confocal microscopy; and J. Wang from the Department of Pathology for technical assistance.

References

1. Weinberg JM, Davis JA, Abarzua M, Rajan T: Cytoprotective effects of glycine and glutathione against hypoxic injury to renal tubules. *J Clin Invest* 1987, 80:1446–1454
2. Garza-Quintero R, Ortega-Lopez J, Stein JH, Venkatachalam MA: Alanine protects rabbit proximal tubules against anoxic injury in vitro. *Am J Physiol* 1990, 258:F1075–F1083
3. Weinberg JM, Venkatachalam MA, Garza-Quintero R, Roeser NF, Davis JA: Structural requirements for protection by small amino acids against hypoxic injury in kidney proximal tubules. *FASEB J* 1990, 4:3347–3354
4. Marsh DC, Hjelmhaug JA, Vreugdenhil PK, Belzer FO, Southard JH: Glycine prevention of cold ischemic injury in isolated hepatocytes. *Cryobiology* 1991, 28:105–109
5. Mangino MJ, Murphy MK, Grabau GG, Anderson CB: Protective effects of glycine during hypothermic renal ischemia-reperfusion injury. *Am J Physiol* 1991, 261:F841–F848
6. Mandel LJ, Schnellmann RG, Jacobs WR: Intracellular glutathione in the protection from anoxic injury in renal proximal tubules. *J Clin Invest* 1990, 85:316–324
7. Dickson RC, Bronk SF, Gores GJ: Glycine cytoprotection during lethal hepatocellular injury from adenosine triphosphate depletion. *Gastroenterology* 1992, 102:2098–2107
8. Heyman S, Spokes K, Rosen S, Epstein FH: Mechanism of glycine protection in hypoxic injury: analogies with glycine receptor. *Kidney Int* 1992, 42:41–45
9. den Butter G, Schilling MK, Lindell SL, Gandolf D, Southard JH, Belzer FO: Effect of glutathione and glycine in kidney preservation. *Transplant Proc* 1993, 25:1633–1634
10. Wetzels JF, Wang X, Gengaro PE, Nemenoff RA, Burke TJ, Schrier RW: Glycine protection against hypoxic but not phospholipase A2-induced injury in rat proximal tubules. *Am J Physiol* 1993, 264:F94–F99
11. Miller GW, Schnellmann RG: Cytoprotection by inhibition of chloride channels: the mechanism of action of glycine and strychnine. *Life Sci* 1993, 53:1211–1215
12. Nissim I, Weinberg JM: Glycine attenuates Fanconi syndrome induced by maleate or ifosfamide in rats. *Kidney Int* 1996, 49:684–695
13. Bachmann S, Peng XX, Currin RT, Thurman RG, Lemasters JJ: Glycine in Carolina rinse solution reduces reperfusion injury, improves graft function, and increases graft survival after rat liver transplantation. *Transplant Proc* 1995, 27:741–742
14. Zhong Z, Jones S, Thurman RG: Glycine minimizes reperfusion injury in a low-flow, reflow liver perfusion model in the rat. *Am J Physiol* 1996, 270:G332–G338
15. Currin RT, Caldwell-Kenkel JC, Lichtman SN, Bachmann S, Takei Y, Kawano S, Thurman RG, Lemasters JJ: Protection by Carolina rinse solution, acidotic pH, and glycine against lethal reperfusion injury to sinusoidal endothelial cells of rat livers stored for transplantation. *Transplantation* 1996, 62:1549–1558
16. Reeves WB: Effects of chloride channel blockers on hypoxic injury in rat proximal tubules. *Kidney Int* 1997, 51:1529–1534
17. Qian T, Nieminen AL, Herman B, Lemasters JJ: Mitochondrial permeability transition in pH-dependent reperfusion injury to rat hepatocytes. *Am J Physiol* 1997, 273:C1783–C1792
18. Arora AS, Nichols JC, DeBernardi M, Steers JL, Krom RA, Gores GJ: Glycine rinse protects against liver injury during transplantation. *Transplant Proc* 1999, 31:505–506
19. Frank A, Rauer U, de Groot H: Protection by glycine against hypoxic injury of rat hepatocytes: inhibition of ion fluxes through nonspecific leaks. *J Hepatol* 2000, 32:58–66

20. Weinberg JM, Nissim I, Roeser NF, Davis JA, Schultz S: Relationships between intracellular amino acid levels and protection against injury to isolated proximal tubules. *Am J Physiol* 1991, 260:F410–F419
21. Weinberg JM, Davis JA, Venkatachalam MA: Cytosolic-free calcium increases to greater than 100 micromolar in ATP-depleted proximal tubules. *J Clin Invest* 1997, 100:713–722
22. Dong Z, Saikumar P, Griess GA, Weinberg JM, Venkatachalam MA: Intracellular Ca^{2+} thresholds that determine survival or death of energy-deprived cells. *Am J Pathol* 1998, 152:231–240
23. den Butter G, Lindell SL, Sumimoto R, Schilling MK, Southard JH, Belzer FO: Effect of glycine in dog and rat liver transplantation. *Transplantation* 1993, 56:817–822
24. Schemmer P, Bradford BU, Rose ML, Bunzendahl H, Raleigh JA, Lemasters JJ, Thurman RG: Intravenous glycine improves survival in rat liver transplantation. *Am J Physiol* 1999, 276:G924–G932
25. Ikejima K, Qu W, Stachlewitz RF, Thurman RG: Kupffer cells contain a glycine-gated chloride channel. *Am J Physiol* 1997, 272:G1581–G1586
26. Wheeler M, Stachlewitz RF, Yamashina S, Ikejima K, Morrow AL, Thurman RG: Glycine-gated chloride channels in neutrophils attenuate calcium influx and superoxide production. *FASEB J* 2000, 14: 476–484
27. Dong Z, Patel Y, Saikumar P, Weinberg JM, Venkatachalam MA: Development of porous defects in plasma membranes of adenosine triphosphate-depleted Madin-Darby canine kidney cells and its inhibition by glycine. *Lab Invest* 1998, 78:657–668
28. Weinberg JM, Davis JA, Roeser NF, Venkatachalam MA: Role of increased cytosolic free calcium in the pathogenesis of rabbit proximal tubule cell injury and protection by glycine or acidosis. *J Clin Invest* 1991, 87:581–590
29. Weinberg JM, Davis JA, Roeser NF, Venkatachalam MA: Role of intracellular pH during cytoprotection of proximal tubule cells by glycine or acidosis. *J Am Soc Nephrol* 1994, 5:1314–1323
30. Weinberg JM, Venkatachalam MA, Goldberg H, Roeser NF, Davis JA: Modulation by Gly, Ca, and acidosis of injury-associated unesterified fatty acid accumulation in proximal tubule cells. *Am J Physiol* 1995, 268:F110–F121
31. Nurko S, Sogabe K, Davis JA, Roeser NF, Defrain M, Chien A, Hinshaw D, Athey B, Meixner W, Venkatachalam MA, Weinberg JM: Contribution of actin cytoskeletal alterations to ATP depletion and calcium-induced proximal tubule cell injury. *Am J Physiol* 1996, 270: F39–F52
32. Venkatachalam MA, Weinberg JM, Patel Y, Hussong U, Davis JA: Effects of Ca^{++} and glycine on lipid breakdown and death of ATP-depleted MDCK cells. *Kidney Int* 1995, 48:118–128
33. Weinberg JM, Buchanan DN, Davis JA, Abarzua M: Metabolic aspects of protection by glycine against hypoxic injury to isolated proximal tubules. *J Am Soc Nephrol* 1991, 1:949–958
34. Venkatachalam MA, Weinberg JM, Patel Y, Saikumar P, Dong Z: Cytoprotection of kidney epithelial cells by compounds that target amino acid gated chloride channels. *Kidney Int* 1996, 49:449–460
35. Carini R, De Cesaris MG, Splendore R, Bagnati M, Bellomo G, Albano E: Glycine protects against hepatocyte killing by KCN or hypoxia by preventing intracellular Na^{+} overload in the rat. *Hepatology* 1997, 26:107–112
36. Nichols JC, Bronk SF, Mellgren RL, Gores GJ: Inhibition of nonlysosomal calcium-dependent proteolysis by glycine during anoxic injury of rat hepatocytes. *Gastroenterology* 1994, 106:168–176
37. Seglen PO: Preparation of isolated rat liver cells. *Methods Cell Biol* 1976, 13:29–83
38. Garza-Quintero R, Weinberg JM, Ortega-Lopez J, Davis JA, Venkatachalam MA: Conservation of structure in ATP-depleted proximal tubules: role of calcium, polyphosphoinositides, and glycine. *Am J Physiol* 1993, 265:F605–F623
39. Aprison MH, Galvez-Ruano E, Lipkowitz KB: On a molecular comparison of strong and weak antagonists at the glycinergic receptor. *J Neurosci Res* 1995, 41:259–269
40. Aprison MH, Galvez-Ruano E, Lipkowitz KB: Identification of a second glycine-like fragment on the strychnine molecule. *J Neurosci Res* 1995, 40:396–400
41. Betz H, Kuhse J, Schmieden V, Laube B, Kirsch J, Harvey RJ: Structure and functions of inhibitory and excitatory glycine receptors. *Ann NY Acad Sci* 1999, 868:667–676