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Protection by glycine against chemical ischemia produced by cyanide in cultured hepatocytes

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Abstract: The killing of cultured hepatocytes by cyanide accelerated phospholipid metabolism, with a reduction in cytoplasmic pH, but did not accelerate proteolysis. Alkalinization of the cytoplasm by monensin, a proton-sodium exchange ionophore, enhanced the loss of viability and acceleration of phospholipid metabolism caused by cyanide. Thus, acidification of the cytoplasm appears to protect against the toxic effects of cyanide. Glycine reduced the killing of hepatocytes, concomitant with reduced phospholipid metabolism. The protective effect of glycine neither enhanced the reduction in cytoplasmic pH nor prevented the depletion of adenosine triphosphate (ATP) by cyanide. The mechanism of the protection exerted by glycine against chemical ischemia can be attributed neither to changes in cytoplasmic pH nor to the prevention of ATP depletion, but appears to be due to other mechanisms that have yet to be identified.

Key words: ischemia, adenosine triphosphate (ATP), glycine, cyanide, intracellular pH

Introduction

It has been suggested that the depletion of adenosine triphosphate (ATP) is a critical event in the lethal cell injury produced by anoxia, oxygen radicals, and toxic chemicals.[1–4] In addition, it has been shown, in cultured neonatal rat myocardial cells,[5] as well as in intact animals,[6,7] treated with metabolic inhibitors as a model of anoxia-ischemia, that phospholipid metabolism was altered. This alteration was manifested by the release of arachidonic acid from phospholipids and by accompanying sarcolemmal membrane defects and loss of cell viability. The prevention of phospholipid degradation by a steroid diamine[5] protected against this anoxic myocardial cell injury, and the release of arachidonic acid, leading to cell death, was considered to be a direct consequence of the loss of ATP content in the cells.

However, in a model of the biological consequences of ischemia-anoxia, it was shown that ATP depletion alone was not sufficient to produce irreversible injury in cultured rat hepatocytes by anoxia.[8–10] It has been suggested that accelerated proteolysis causes some kind of toxic injury to hepatocytes and that protease inhibitors can protect against this kind of injury.[11] It has also been shown that acidosis has a protective effect not only on ischemic cell injury but also on oxygen radical-mediated cell injury.[12]

It has recently been shown that glycine exerts a significant cytoprotective effect in isolated renal tubules exposed to hypoxia[13] and in such preparations in which ATP depletion is evoked by mitochondrial inhibitors.[14] Thus, it is of great interest, particularly in regard to organ preservation for liver transplantation, to investigate whether glycine can also protect cultured hepatocytes against anoxia-ischemia.

In this study, we used cyanide to injure cultured hepatocytes as a model of chemical ischemia, and we investigated whether glycine exerted a protective effect, as it does in renal tubules, on phospholipid metabolism, cytoplasmic pH, phospholipid metabolism, proteolysis, acidosis, and ATP depletion. First, we showed that treatment of cultured hepatocytes with cyanide altered phospholipid metabolism in a manner similar to that seen in anoxia or hepatic ischemia in the intact animal, without alterations of proteolysis. Second, we demonstrated that this alteration in phospholipid metabolism did not parallel ATP depletion. Finally, we showed that the protective effects of glycine were not attributable to the depletion of ATP or to changes in cytoplasmic pH.

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Materials and methods

Animals and culture conditions

Male Sprague-Dawley rats (150–200 g) were obtained from Nippon SLC Co., Ltd. (Shizuoka Japan). All animals received feed ad libitum and were fasted for 24 h before use. Hepatocytes were isolated by collagenase (Sigma, St. Louis, Mo.) perfusion, as described by Seglen.[15] Yields of 3–5 $\times$ 10^8 cells/liver, of 90%–95% viability, determined by trypan blue exclusion, were routinely obtained. The hepatocytes were plated in 25-cm^2 flasks, at a density of 1.33 $\times$ 10^6 cells/flask, in 5 ml of Williams E medium (GIBCO BRL Inc., Gaithersburg, MD) containing penicillin (10 IU/ml), streptomycin (10 µg/ml), gentamicin (0.5 mg/ml), insulin (0.02 U/ml), and 10% heat-inactivated (55°C for 15 min) fetal calf serum (complete Williams E). After 2 h at 37°C in an atmosphere of 5% CO_2 and 95% air, cultures were washed twice with prewarmed N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES; Sigma) buffer at pH 7.4 (0.14 M NaCl, 6.7-mM KCl, 1.2 mM $CaCl_2$, and 2.4 mM HEPES) to remove unattached dead cells. Complete Williams E medium was replaced, and the cells were incubated for 16–24 h, then washed twice with prewarmed HEPES buffer and incubated in Krebs-Ringer bicarbonate buffer (120-mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 24 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose) with the agents described below (glycin, cyanide, leupeptin, antipain, monensin). Glycine and potasium cyanide (Sigma) were dissolved in 0.9% NaCl. Leupeptin and antipain (Sigma) were dissolved in water. Monensin (Sigma) was dissolved in dimethyl sulfoxide (Me_2SO).

Cell killing was assessed by the release of lactate dehydrogenase (LDH) into the culture medium, as described previously.[16] Cellular ATP content was assessed by the luciferin-luciferase method, as described previously.[16] Protein was measured by the method of Lowry et al.[17]

Experimental design

Cultured hepatocytes were treated with 0.75 mM cyanide with or without simultaneous administration of 2 mM glycine. Cell killing (LDH release), ATP content, phospholipid degradation and protein turnover were measured 90 min after the addition of cyanide with or without the various agents described below.

Assay of phospholipid degradation

Phospholipid degradation was determined by the release of ^3H-labeled arachidonic acid, as described previously.[16] After 2-h incubation, hepatocytes were replaced with complete Williams E medium containing 1-µCi [^3H]arachidonic acid (135 Ci/mmol; Amersham, London, UK), then, 24 h later, the cells were washed three times with HEPES buffer. The medium for the cultured hepatocytes was changed to Krebs-Ringer bicarbonate buffer and cells were incubated for 90 min. At the end of incubation with cyanide, leupeptin, antipain or glycin, the medium was immediately transferred to microcentrifuge tubes on ice and centrifuged at 10000 g for 10 min at 4°C. The radioactivity of the supernatant was measured by liquid scintillation counting. The radioactivity released into the culture medium reflects the degradation of prelabeled phospholipids and represents free arachidonic acid (70%) plus metabolites.[18] Radioactivity released from the cultured hepatocytes was expressed as a percentage of the total radioactivity in the hepatocytes at the beginning of the assay. There was no change in the total radioactivity present as free fatty acids in the hepatocytes under any of the conditions studied.

Measurement of protein degradation

Protein turnover was measured by a modification of the methods of Seglen et al.[19] and Hopgood et al.,[20] described previously.[21,22] Two hours after the cells had been plated, hepatocyte proteins were labeled for 24 h with 0.5 µCi/culture of [^{14}C]valine (285 mCi/mmole, Amersham). The cells were washed twice and incubated in Williams E with 2 mM valine for 1 h. The cells were washed again and incubated in modified Krebs-Ringer bicarbonate buffer for 90 min with or without a protease inhibitor and glycine. Triton X-100 was then added, to a final concentration of 0.5%. Hepatocyte protein was precipitated by the addition of perchloric acid, to a final concentration of 10%. After 15 min at 0°C and centrifugation for 10 min at 5000 rpm, the radioactivity in the acid-soluble and acid-insoluble fractions was determined by liquid scintillation counting.

Measurement of cytoplasmic pH

The cytoplasmic pH of cultured hepatocytes was measured by digital imaging fluorescence microscopy with a pH-sensitive fluorescent probe, 2′7′-bis-carboxyethyl-5,6-carboxyfluorescein (BCECF; Calbiochem, Behring Diagnostics, La Jolla, CA, USA).

For this measurement, hepatocytes were cultured in complete Williams E medium on a coverslip (VWR Scientific, La Jolla, CA, USA) coated with poly-D-lysine hydrobromide (Sigma) for 24 h. The hepatocytes were washed with prewarmed HEPES buffer twice and then placed in a modified Krebs-Ringer bicarbonate buffer (120 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.0 mM $CaCl_2$, 5 mM $NaHCO_3$, and 10 mM glu-

cose, and 2% FCS at pH 7.4) with 10 μM BCECF/AM in 0.005% Pluronic F-127 (Molecular Probes, Eugene, Or.) at 37°C for 30 min. After this loading, the cells were washed three times with HEPES buffer. One coverslip was mounted on a Zeiss IM-35 inverted microscope equipped with a thermostatically controlled unit stage (37°C) in a chamber with 1 ml of bicarbonate-free modified Krebs-Ringer buffer (no FCS).

Pairs of images were obtained every 20s over a 30-min period, and analyzed with ARGUS-100/CA (Hamamatsu Photonics, Hamamatsu, Japan). The mean fluorescence intensity was calculated over the same area for successive images. Excitation wavelengths were 470 nm and 430 nm, and the emission wavelength was 500 nm.

After subtracting the backgrounds obtained from the experiments using BCECF/AM-unloaded cells with the same reagents, we calculated the fluorescence ratio (intensity at 470 nm divided by the value at 430 nm). For calibration of the relationship between intracellular pH and the BCECF fluorescence ratio, we employed the $[K^+]$-nigericin technique.[23] To determine the cytoplasmic pH of hepatocytes treated with cyanide alone and cyanide plus glycine, we compared the minimum cytoplasmic pH values.

Statistical analysis

Results were expressed as means ± SD and the data obtained were evaluated by one-way or two-way analysis of variance (ANOVA). The level of significance was set at 5% for each analysis.

Results

Time course of cell death and accelerated phospholipid degradation by cyanide

Hepatocytes cultured for 24 h were treated with 0.75 mM cyanide. Fig. 1A shows that 0.75 mM cyanide killed more than 70% of cultured hepatocytes within 90 min compared with untreated cells. A higher dose of cyanide (1.5 mM) did not increase the loss of viability (data not shown), i.e., the toxic effect of cyanide was saturated at a dose of 0.75 mM.

Hepatocytes were labeled with [^{3}H]arachidonic acid during the first 24 h in culture. The cells were then washed and incubated in a Krebs-Ringer bicarbonate buffer with 0.75 mM cyanide. Phospholipid degradation was measured by the release of [^{3}H]arachidonic acid into the culture medium and expressed as a percentage of the total [^{3}H]arachidonic acid incorporated into phospholipids. Figure 1B shows that untreated cultured hepatocytes released about 3% of total [^{3}H]arachidonic acid incorporated into phospholipids within 90 min. In contrast, treatment with 0.75 mM cyanide increased this release about twofold in parallel with cell death.

Prevention of cyanide toxicity by glycine

Table 1 shows that 2 mM glycine markedly prevented the loss of viability due to cyanide. Treatment with glycine also reduced the accelerated phospholipid degradation, to the baseline level in untreated cultured hepatocytes.

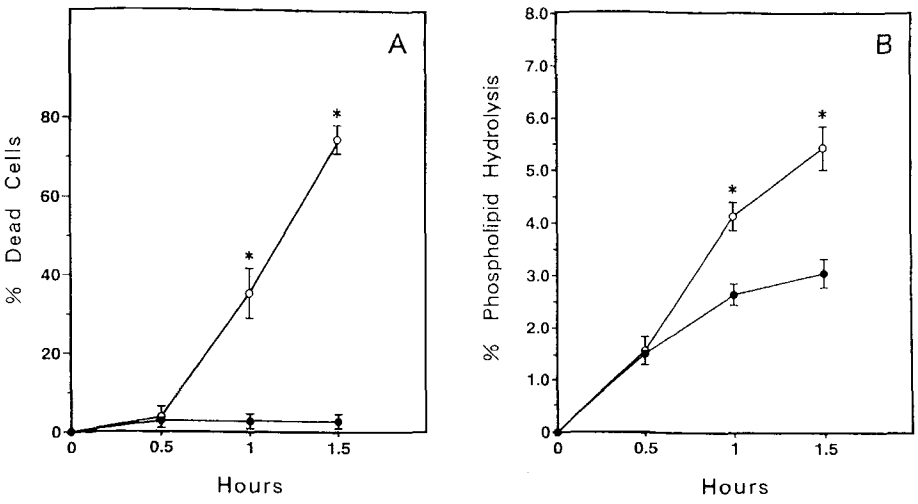

Fig. 1A,B. Time course of **A** cell death and **B** phospholipid degradation with and without 0.75 mM cyanide. Cultured hepatocytes were incubated with cyanide (*open circles*) or with no treatment (*closed circles*). Loss of viability and phospholipid degradation, assessed by the release of [3H]arachidonic acid, as described in Materials and Methods, were measured at the time points indicated. The data presented (mean ± SD) are representative of the results of determinations in three separate cultures in three different experiments. Statistical analysis was performed by one-way ANOVA. *$P < 0.01$ compared with no treatment

Table 1. Relation between cell death, phospholipid hydrolysis, and ATP depletion

	Cell death (%)	Phospholipid hydrolysis (%)	ATP (nmol/mg protein)
No additions	3 ± 1	2.58 ± 0.26	10.7 ± 0.25
Glycine alone	2 ± 1	2.53 ± 0.31	10.5 ± 0.23
Monensin alone	5 ± 3	2.66 ± 0.39	10.1 ± 0.21
Monensin/glycine	6 ± 3	2.52 ± 0.31	10.0 ± 0.26
Cyanide alone	78 ± 8*	5.05 ± 0.57*	0.55 ± 0.32*
Cyanide/glycine	5 ± 3**	2.76 ± 0.28**	0.58 ± 0.25*
Cyanide/monensin	95 ± 3*,***	7.09 ± 0.12*,***	0.53 ± 0.31*
Cyanide/monensin/glycine	5 ± 3**	2.65 ± 0.33**	0.48 ± 0.35*

* $P < 0.01$ vs no addition; ** $P < 0.01$; *** $P < 0.05$ vs cyanide alone
Cultured hepatocytes were treated with 0.75 mM cyanide in the presence or absence of 2 mM glycine or 10 μM monensin. Cellular ATP content, phospholipid hydrolysis, and cell viability were determined after 90 min. The data presented are representative results (mean ± SD) of determinations for three separate cultures in three different experiments
Statistical analysis was performed by two-way ANOVA

Table 2. Protease inhibitors do not protect against cyanide toxicity

	Protein turnover (%/h)	Cell death (%)
Cyanide alone	3.75 ± 0.38	78 ± 7
+ Leupeptin 100 μg/ml	2.06 ± 0.27*	72 ± 8
+ Antipain 200 μM	2.15 ± 0.23*	77 ± 6
+ Gly 2 mM	3.95 ± 0.18	8 ± 2*
No addition	4.08 ± 0.15	5 ± 1
Leupeptin 100 μg/ml	2.12 ± 0.24*	6 ± 1
Antipain 200 μM	2.36 ± 0.21*	5 ± 1
Gly 2 mM	4.11 ± 0.17	3 ± 1

* $P < 0.01$ vs cyanide alone
Cultured hepatocytes were treated with 0.75 mM cyanide alone or with the additions indicated. Protein turnover was measured as the net release of 24-h prelabeled [^{14}C] valine into the acid-soluble fraction during a 90-min period and expressed as a percentage of the initial protein radioactivity in the cell sample. Protein turnover and cell viability were determined after 90 min. The data presented are representative results (mean ± SD) of determinations for three separate cultures in three different experiments
Statistical analysis was performed by two-way ANOVA

Interestingly, this protective effect of glycine was not due to prevention of the ATP depletion that occurs in the presence of cyanide, an inhibitor of cytochrome oxidase, since there was no difference of ATP content between cyanide alone and cyanide plus glycine as shown in Table 1.

Protease inhibitors did not prevent cyanide toxicity

Table 2 shows that cyanide did not accelerate protein turnover. Leupeptin and antipain, protease inhibitors, inhibited protein turnover without preventing the loss of cell viability caused by cyanide.

Glycine (2 mM) prevented the killing of hepatocytes by cyanide but did not have any effect on protein turnover, indicating that the protective effect of glycine is not related to protein turnover.

Potentiation of cyanide toxicity by increasing intracellular pH

Monensin, an ionophore, increases cytoplasmic pH by exchanging hydrogen for sodium. As shown in Table 1, monensin enhanced the extent of cell death and phospholipid degradation. Figure 2 shows the typical response of cytoplasmic pH (pHi) to the successive addition of 0.75 mM cyanide and 10 μM monensin. The addition of cyanide reduced the pHi from the baseline level within 5 min.

However, monensin rapidly reversed the intracellular acidification produced by cyanide to the baseline level. The increased cytoplasmic pH produced by monensin potentiated the toxicity of cyanide, with accelerated

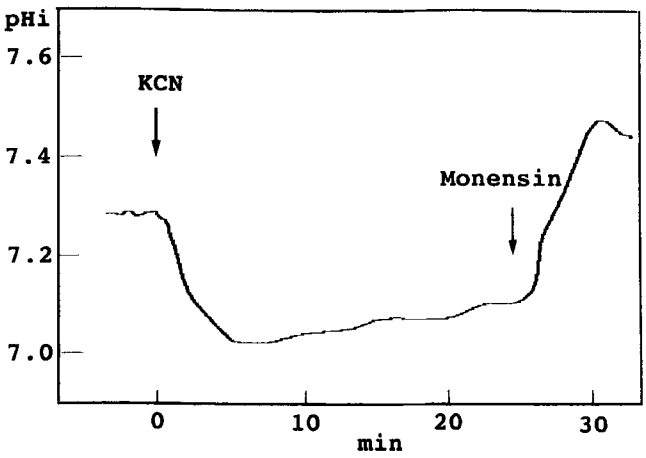

Fig. 2. Effects of cyanide and monensin on the cytoplasmic pH (pHi) of cultured hepatocytes. Cultured hepatocytes were loaded with BCECF/AM, and the cells were then washed; cytoplasmic pH changes in response to 0.75 mM cyanide (adjusted to pH 7.4) and 10 μM monensin were recorded. Representative change in the cytoplasmic pH of one hepatocyte is shown

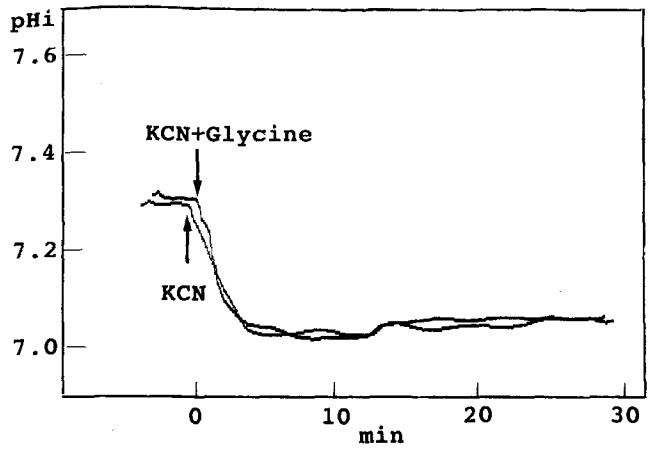

Fig. 3. Effect of glycine on the cytoplasmic pH of cultured hepatocytes. Changes in cytoplasmic pH were measured with 0.75 mM cyanide alone or cyanide plus 2 mM glycine. Representative changes in the cytoplasmic pH with cyanide alone or cyanide plus 2-mM glycine are shown

Table 3. Effect of glycine on cytoplasmic pH of cyanide-treated hepatocytes

	Cytoplasmic pH
Cyanide alone (0.75 mM)	7.01 ± 0.21*
+ Glycine (2 mM)	7.03 ± 0.25*,**
No addition	7.32 ± 0.18
Glycine alone (2 mM)	7.30 ± 0.17

* $P < 0.01$ vs no addition; ** NS vs cyanide alone
Cytoplasmic pH was measured during the 30-min period after addition of the above agents. The minimum pH during the 30-min period was recorded as the cytoplasmic pH. Results are the mean ± SD of determinations for 20 separate cells. The data presented are representative of three different experiments carried out in different cell preparations. Statistical analysis was performed by one-way ANOVA

phospholipid metabolism being shown (Table 1). Acidification of cytoplasmic pH after the addition of cyanide thus appears to have a protective effect.

Again, glycine prevented the loss of cell viability caused by cyanide plus monensin, without preventing the depletion of ATP.

Glycine did not change intracellular pH

Figure 3 shows the change in pHi after the addition of 0.75 mM cyanide plus 2 mM glycine. During the 30-min period after the addition of cyanide, glycine did not have a significant effect on the change in cytoplasmic pH (Table 3). Glycine alone had no effect on pHi in cultured hepatocytes (data not shown). These results suggest that the protective effect of glycine in hepatocytes is not attributable to enhanced acidification of the cytoplasm.

Discussion

Cyanide is an inhibitor of cytochrome oxidase and prevents oxidative phosphorylation and electron transport, thus preventing ATP synthesis; ATP stores are then rapidly depleted.

These initial effects of cyanide are followed by the activation of degradative hydrolases (i.e., phospholipase and protease), by intracellular acidification, and by loss of plasma membrane permeability and cell death.[24] The depletion of ATP leads to the equilibration of lysosomal pH with the cytosolic pH as a result of the inhibition of lysosomal H^+-ATPase.[25] This increase in lysosomal pH decreases the activity of acidic lysosomal proteases. In contrast, an increase in nonlysosomal protease activity has been reported during the depletion of ATP.[26] Intracellular acidification is also a salient feature of ATP depletion. The liberation of protons from the net hydrolysis of nucleoside phosphates (i.e., ATP and adenosine diposphate), the generation of lactic acid from anaerobic glycolysis, and the efflux of protons from acidic vesicles, in addition to the partial inhibition of protective mechanisms (e.g., plasma membrane Na^+/H^+ exchange) all contribute the development of intracellular acidosis, a phenomenon which could be an adaptive mechanism by the cell to prevent necrosis.[24] Against this background, we investigated these events in cyanide-intoxicated hepatocytes with and without added glycine.

The conditions we used to reduce the extent of cell death by cyanide may possibly modify the ATP stores in hepatocytes. However, with 2 mM glycine, ATP was depleted, despite a reduction in cell killing by cyanide. Thus, the protective action of glycine must be independent of the loss of the mitochondrial ATP-generating system.

The killing of hepatocytes by cyanide was accompanied by an accelerated release of [^{3}H]arachidonic acid from cellular phospholipid. Glycine reduced this release, concomitant with a reduction in the cell death induced by cyanide. These findings suggest that degradation of plasma membrane phospholipid is related to the loss of membrane integrity, resulting in cell death.

These events are independent of the intracellular Ca^{2+} concentration, as previously reported.[16] In addition, it has been suggested that the toxic injury of hepatocytes induces altered Ca^{2+} homeostasis accompanied by accelerated proteolysis, and that protease inhititors protect the hepatocytes.[11] However, as shown in Table 2, protease inhibitors did not prevent the killing of hepatocytes by cyanide. Despite this finding, we did find that protease inhibitors actually reduced protein turnover, indicating that the proteolysis in the cultured hepatocytes was not attributable to the toxicity of cyanide. Gores and coworkers showed that nonlysosomal

proteolysis was accelerated in cyanide intoxicated hepatocytes and anoxic hepatocytes. Omission of calcium from the medium reduced the nonlysosomal proteolysis to the level of that of glycine-treated anoxic cells. They concluded that calcium-dependent nonlysosomal proteolysis was related to the loss of viability in rat hepatocytes produced by cyanide and anoxia.[27,28] However, our previous study[16,29] indicated that such omission of calcium from the medium did not prevent the cell death in cultured hepatocytes induced by cyanide.

The most obvious explanation of the difference between the conditions in fresh suspensions[29,30] as opposed to cultured hepatocytes (Table 2) is the greater sensitivity of the former to extracellular calcium ions, as has previously been described with cystamine-intoxicated hepatocytes[22] and hepatocytes treated with *tert*-butyl hydroperoxide.[29,30]

The effect of changing the cytoplasmic pH (pHi) was examined with monensin, a proton-sodium exchange ionophore. This ionophore has been widely used to alkalinize various cellular compartments, including the acidic endosome, lysosomes, and the cytosol itself.[31–33]

Cyanide lowered cytoplasmic pH in cultured hepatocytes. The addition of 10 μM monensin raised the pHi from 7.07 to more than 7.4 within 5 min. This alkalinization of the cytosol resulted in the potentiation of cell killing by cyanide, with accelerated phospholipid degradation, since an alkaline pH is optimal for phospholipase A activity. Thus, acidification of the cytoplasm would seem to protect hepatocytes against the toxicity of cyanide, presumably by reducing the activity of phospholipase A. Favoring such an interpretation, we found that acidification of the medium also reduced cell killing by cyanide in parallel with the reduction of phospholipid degradation in the plasma membrane, without sparing ATP depletion.[16]

These findings indicate that phospholipid degradation may be involved in the cell death caused by cyanide.

Thus, the protective effect exerted by glycine against the toxicity of cyanide could be the result of the stronger acidification of the cytoplasm, resulting in the inhibition of phospholipase A activity, compared with the effect of cyanide alone.

However, our results indicate that glycine did not affect the cytoplasmic pH. The protective effect of glycine against the toxicity of cyanide can be attributed neither to preventing the depletion of ATP nor to reducing the cytoplasmic pH. The leakage of BCECF from hepatocytes prevented the measurement of cytoplasmic pH after 30-min incubation. The late phase of cytoplasmic pH should be measured in future studies.

Recent findings suggest that glycine may act as a chloride channel inhibitor in renal cell injury.[34] We are now investigating whether glycine protects against ischemic injury to hepatocytes by inhibiting chloride influx.

Our findings may be help to improve organ preservation for liver transplantation.

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