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Protection from hypoxic injury in cultured hepatocytes by glycine, alanine, and serine

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Summary. Isolated hepatocytes from rat liver in primary culture rapidly lost viability under hypoxic conditions. In the presence of glycine, L-alanine or L-serine loss of viability under hypoxic conditions was greatly retarded. Glycine and L-serine already showed significant protection from hypoxic injury at a concentration of 0.1 mM; at 10 mM, all three amino acids offered almost complete protection. Beside these standard amino acids, 1-aminocyclopropane-1-carboxylic acid (ACPC) and sarcosine significantly decreased hypoxic injury of the hepatocytes, although to a lesser extent. Other amino acids tested provided only slight protection or had no effect on hypoxic injury of the hepatocytes. In the presence of the protective amino acids neither the ATP content nor the lactate production of the hypoxic hepatocytes were significantly affected. The addition of glycine, L-alanine and L-serine led to marked membrane alterations (blebs). These alterations, however, occurred without loss of viability and were reversible upon reoxygenation after up to 4 h of hypoxia.

Keywords: Amino acids – Liver – Hepatocytes – Hypoxia – Reoxygenation

Abbreviations: LDH, lactate dehydrogenase; ACPC, 1-amino-cyclopropane-1-carboxylic acid; HEPES, 2-(4-(2-hydroxyethyl)-1-piperazinyl)-ethanesulfonic acid

Introduction

Hypoxic liver injury may occur in acute heart failure and in shock due to trauma, burns, hemorrhage, sepsis or peritonitis. Tissue damage due to hypoxia results from a loss of functional and regulatory processes in the cell (Hochachka, 1986; de Groot et al., 1988). An early and decisive event in hypoxic cell injury is a depletion of intracellular ATP. In cultured hepatocytes, in the first 5 min of the hypoxic incubation the ATP content decreased from 22 to 12 nmol/10^6 cells (Brecht et al., 1992). Consequently, fructose, which significantly diminished the

decrease in ATP, led to an improved survival of the hypoxic hepatocytes (Anundi et al., 1989; Anundi and de Groot, 1989). Calcium channel blockers and phospholipase A_2 inhibitors have also been shown to be protective against hypoxic cell injury (Chien et al., 1977; Yoshimi et al., 1988). Accordingly, an increase in the cytosolic free calcium concentration due to ATP depletion and thereby an activation of the calcium-dependent phospholipase A_2 has been implicated as a primary cause of cell death (Chien et al., 1978; Farber and Young, 1981; Snowdowne et al., 1985).

Recent experiments with isolated cultured hepatocytes have demonstrated marked protection against hypoxic cell injury by a protein-free extract of calf blood (Actovegin^R) (De Groot et al., 1990). Using fractions of this extract[1], evidence was provided that amino acids are probably responsible for this protection. These observations led us to study the effect of amino acids on the hypoxic cell injury of cultured hepatocytes in more detail.

Materials and methods

Chemicals

Collagenase (HEP plus), collagen (Type R), dexamethasone, gentamycin and trypan blue were purchased from Serva (Heidelberg, Germany). Hanks balanced salt solution (HBSS), Leibowitz L-15, fetal calf serum and glutamine were from Gibco (Eggenstein, Germany), sarcosine, lactate oxidase, L-valine, L-histidine, DL-aspartic acid, DL-glutamic acid, glycine, L-lysine and 1-aminocyclopropane-1-carboxylic acid (ACPC) from Sigma (Deisenhofen, Germany) and L-leucine, L-isoleucine, L-phenylalanine, L-tyrosine, L-tryptophan, L-proline, L-hydroxyproline, L-threonine, L-methionine, L-cysteine, L-arginine, L-ornithine, L-citrulline, L-asparagine, L-glutamine, L-alanine and L-serine from Merck (Darmstadt, Germany). Peroxidase was from Boehringer (Mannheim, Germany).

Isolation and culturing of hepatocytes

Hepatocytes were isolated from male Wistar rats (200–240 body wt.) by collagenase perfusion according to Seglen (1973) with some slight modifications. Animals were allowed free access to food and water. They were anaesthetized with pentobarbital (60 mg/kg i.p.). Freshly isolated hepatocytes excluded trypan blue to a level of about 90%. After isolation, hepatocytes were cultured in 25 cm^2 flasks (Falcon, Heidelberg, Germany) at 37°C in an atmosphere of 5% CO_2/95% air as previously described (De Groot and Brecht, 1991). Culture medium was Leibowitz L-15 completed by adding fetal calf serum (5%), L-glutamine (2 mM), glucose (8.3 mM), bovine serum albumine (0.1%), $NaHCO_3$ (14.3 mM), gentamycin (50 mg/l) and dexamethasone (1 μM).

Incubation procedure

After culturing for 20 h, experiments were performed as previously described (De Groot and Brecht, 1991). Hypoxic conditions were initiated by addition of 5 ml nitrogen-saturated (95% N_2/5% CO_2) Krebs-Henseleit hydrogen carbonate buffer, pH 7.4, containing Hepes (20 mM) and trypan blue (83 μM). To maintain pH and hypoxic conditions during the whole incubation period, flasks were flushed with 95% N_2/5% CO_2 for 3 min every 2 h during the course of the experiments. The amino acids were dissolved in Krebs-Henseleit hydrogen

[1] Kindly provided by Dr. F. Machicao and Dr. B. Sixt, Hormon-Chemie, München.

carbonate buffer and added just before starting the experiments. For control, aerobic incubations were treated in a similar manner except that the gas used was 21% O_2/5% CO_2/74% N_2. In some experiments cultures were reoxygenated after 1 to 6 h of hypoxia by flushing the culture flasks with 21% O_2/5% CO_2/74% N_2 for 5 min.

Assays

Cell injury was determined by the uptake of the vital dye trypan blue and by measuring the leakage of cytosolic lactate dehydrogenase (LDH). The morphology of the hepatocytes was observed every hour with an inverted microscope (Zeiss IM 35, Oberkochen, Germany). ATP was determined by HPLC as described by Dwyer and Brown (1988). Lactate was assayed in neutralized extracts after deproteinization with 1 mM perchloric acid using lactate oxidase in according to the determination of glucose with glucose oxidase (Gutmann and Wahlefeld, 1974).

Statistics

Each experiment was performed with duplicate cultures. Values presented are means ± S.E.M. from 3 to 12 experiments with different hepatocyte cultures. Student's *t*-test was used to calculate statistical significance and a *p* value of 0.5 or less taken to indicate significance.

Results

Viability

Under hypoxic conditions cultured hepatocytes rapidly lost their viability. As indicated by lactate dehydrogenase (LDH) leakage (Fig. 1) and trypan blue exclusion (data not shown) nearly 50% of the cells had lost their viability after 2 h of hypoxia and after 6 h about 80% of the cells were damaged. Under normoxic conditions only a slight loss of viability was detectable during 6 h of incubation. The addition of the amino acids L-valine, L-leucine, L-isoleucine, L-phenylalanine, L-tyrosine, L-tryptophan, L-proline, L-hydroxyproline, L-threonine, L-methionine, L-cysteine, L-histidine, L-arginine, L-ornithine, L-citrulline, L-lysine, L-asparagine, L-glutamine, each at a concentration of 1 mM, had little or no effect on the release of LDH either under hypoxic or under normoxic conditions (Table 1). In the presence of 1 mM DL-aspartic acid, DL-glutamic acid or L-alanine small but reproducible decreases in LDH release, in a time window between 1 and 4 h of hypoxia, were detectable. Further increases in the concentration of DL-aspartic acid and DL-glutamic acid did not have any additional protective effect on the LDH release of the hypoxic hepatocytes. Only in the case of L-alanine was the protective effect further markedly increased at higher concentration; at 10 mM L-alanine only 15% of the hepatocytes were damaged after 6 h of hypoxia (Fig. 1). Pronounced protection against hypoxic cell injury was produced by the addition of glycine and L-serine (Fig. 1). Protection was already significant at a concentration of 0.1 mM glycine; after 2 h of hypoxia only 20% and after 4 h 50% of the hepatocytes were damaged. In the presence of 1 mM glycine after 2 h hypoxia 10% and after 4 h less than 30% of the cells were injured. Almost optimum protection occurred in the presence of 10 mM glycine. After 6 h of hypoxia only a slight loss of viability ($< 10\%$) was detectable. Similar although somewhat less protective effects were observed in the presence of L-serine.

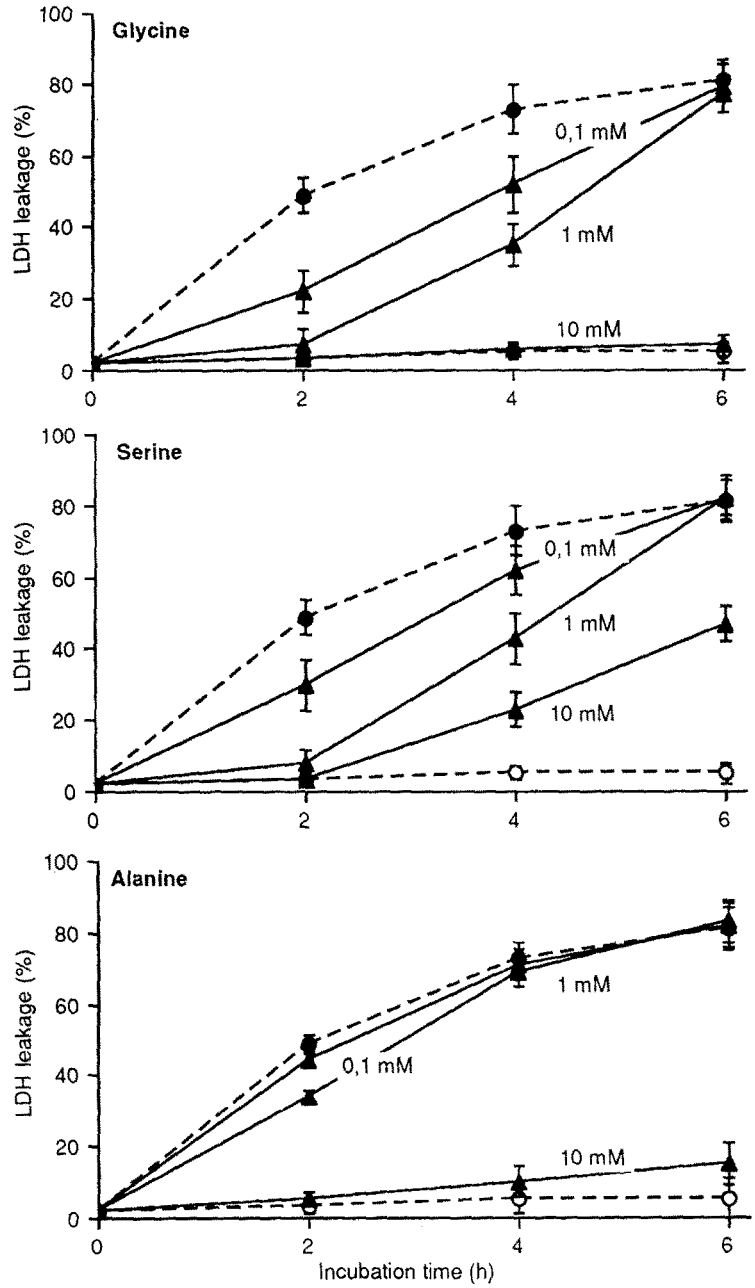

Fig. 1. Effects of varying concentrations of glycine, L-alanine and L-serine on hypoxic injury of hepatocytes. Normoxic (open circles) and hypoxic (closed circles) control incubations (dotted lines) were maintained in an atmosphere of oxygen (5% CO_2/21% O_2/74% N_2) and nitrogen (5% CO_2/95% N_2), respectively, over the whole incubation period. Amino acids were added just before starting the experiments under hypoxic conditions. Data presented are means ± S.E.M. of 10–14 separate incubations of 12 preparations

In addition to the standard amino acids ACPC and sarcosine were tested for their effects on hypoxic injury of the hepatocytes (Fig. 2). In the presence of 10 mM ACPC pronounced protection occurred; after 6 h of hypoxia less than

Table 1. Effects of amino acids on hypoxic injury of hepatocytes

Amino acid	LDH leakage (%)
Hypoxic Control	49 ± 5
L-Leucine	46 ± 6
L-Isoleucine	48 ± 5
L-Phenylalanine	52 ± 7
L-Tyrosine	51 ± 6
L-Tryptophan	48 ± 4
L-Proline	48 ± 4
L-Hydroxproline	50 ± 5
L-Threonine	47 ± 7
L-Methionine	46 ± 6
L-Cysteine	48 ± 5
L-Histidine	49 ± 3
L-Arginine	46 ± 6
L-Ornithine	46 ± 5
L-Citrulline	48 ± 4
L-Lysine	48 ± 5
L-Valine	48 ± 4
DL-Aspartic Acid	48 ± 5
DL-Glutamic Acid	48 ± 5
L-Asparagine	37 ± 4
L-Glutamine	36 ± 6
L-Alanine	36 ± 7
L-Serine	8 ± 4
Glycine	7 ± 4
1-Aminocyclopropane-1-carboxylic acid (ACPC)	40 ± 5
Sarcosine	40 ± 5

Primary cultures of isolated hepatocytes from rat livers were incubated at 37°C in an atmosphere of nitrogen (5% CO_2/95% N_2). The amino acids (each at a concentration of 1 mM) were added just before starting the experiments. Cell injury after 2 h of hypoxia was estimated by LDH leakage. Values presented are means ± S.E.M. of 4 separate incubations of 4 preparations.

20% of the cells were injured. The protection offered by sarcosine was rather less pronounced; after 6 h under hypoxic conditions 60% of the hepatocytes were damaged. At concentrations below 10 mM both ACPC and sarcosine rapidly lost their protective effects.

ATP content and lactate formation

The initial ATP content of 22 nmol/10^6 cells decreased to 18 nmol/10^6 cells after 1 h of incubation under normoxic conditions but then remained almost constant (Fig. 3). In hypoxic hepatocytes the ATP content decreased to 12 nmol/10^6 cells within 5 min of incubation. Within the next 2 h it further decreased to 8 nmol/10^6 cells and then stayed constant until 6 h of hypoxia. The addition of 10 mM glycine, 10 mM L-serine or 10 mM L-alanine had no significant effect on the ATP content under either hypoxic or under normoxic conditions (data not shown).

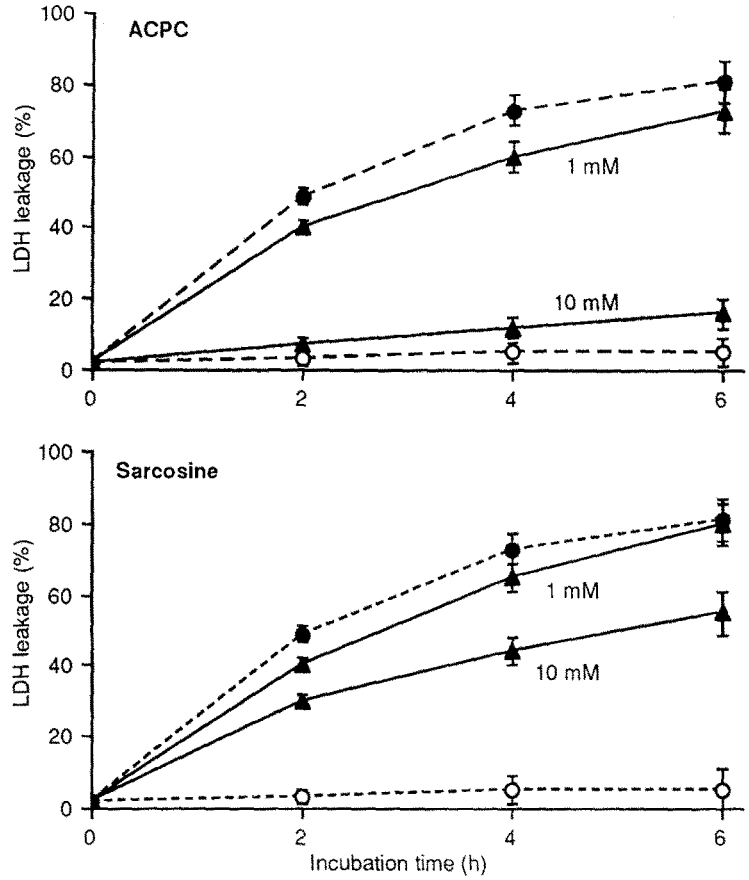

Fig. 2. Effects of ACPC and of sarcosine on hypoxic injury of hepatocytes. Experimental details are the same as in Fig. 1. Data presented are means ± S.E.M. of 4–7 separate incubations of 4–6 preparations

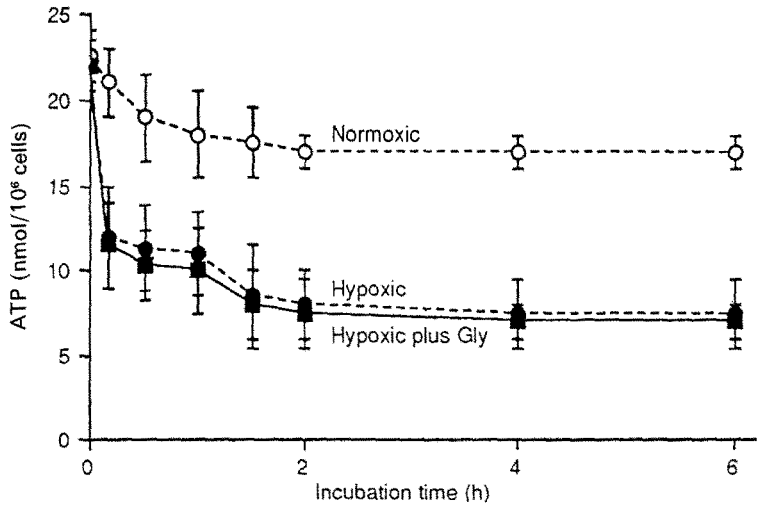

Fig. 3. Effect of glycine on the ATP content of hypoxic hepatocytes. Experimental details are the same as in Fig. 1. Data presented are means ± S.E.M. of 3–6 separate incubations of 3–6 preparations

Under normoxic conditions 0.65 ± 0.09 μmol/10^6 cells lactate were released after 6 h hypoxia. Under hypoxic conditions lactate formation increased to 1.15 ± 0.18 μmol/10^6 cells × 6 h. The amino acids glycine (10 mM), L-serine (10 mM) and L-alanine (10 mM) did not have significant effects on the normoxic and the hypoxic rate of lactate formation.

Morphological alterations

In the presence of glycine, L-alanine and L-serine marked morphological alterations developed under hypoxic conditions (Fig. 4). After 1 h of hypoxic incubation in the presence of 10 mM of these amino acids, in almost all of the cells a great number of cell membrane protrusions, blebs, were visible. Under continuous hypoxic conditions the size and number of blebs increased. After

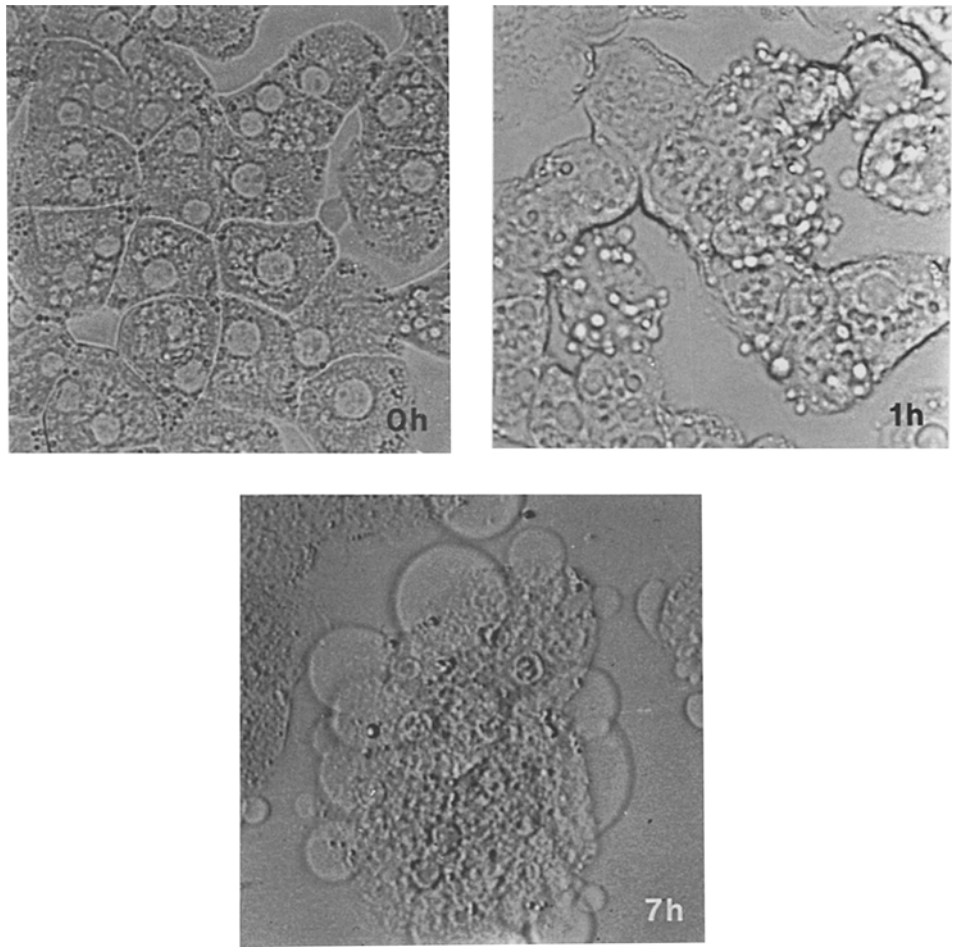

Fig. 4. Bleb formation in hypoxic hepatocytes in the presence of glycine. Cultured hepatocytes were exposed to hypoxia in the presence of 10 mM glycine for 0, 1 and 7 h. Photographs were produced by phase-contrast microscopy (× 100). Experimental details are the same as in Fig. 1

7 h of hypoxia (Fig. 4) the blebs had the appearance of large bubbles caused by the expansion and fusion of small blebs. Under phase-contrast blebs appeared phase-lucent without containing large cell organelles. Without addition of these amino acids, in hypoxic controls, bleb formation also occurred, but always to a much lesser extent. Under these conditions only some of the cells (< 10%) showed 1 or 2 small blebs within the entire incubation period.

Reoxygenation

After 1 h of hypoxic incubation and subsequent reintroduction of oxygen, there was only a slight loss of cell viability during the following 10 h of aerobic incubation (Fig. 5A). Upon reoxygenation at time points later than 2 h of hypoxic incubation, however, hepatocytes continuously lost their viability; 6 h after reintroduction of oxygen only a few cells (< 10%) were still viable. In the presence

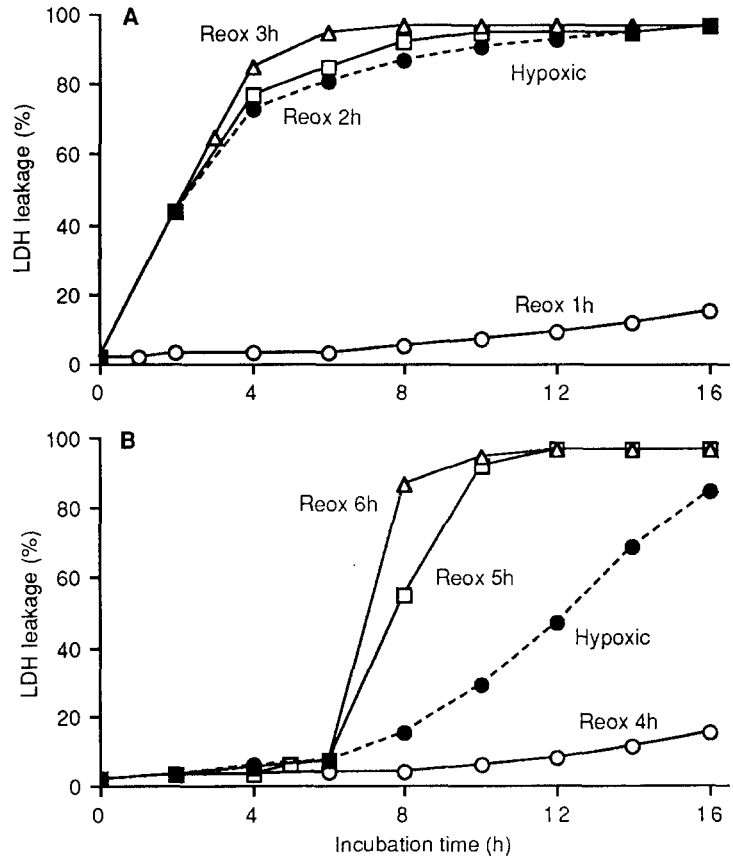

Fig. 5. Effect of glycin on the viability of hepatocytes upon hypoxia followed by reoxygenation. Cultured hepatocytes were first incubated under hypoxic conditions in the absence (**A**) and in the presence of 10 mM glycine (**B**). They were reoxygenated (*Reox*) by flushing with oxygen (5% CO_2/21% O_2/74% N_2) at (**A**) 1 h (open circles), 2 h (open squares) and 3 h (open triangles), and (**B**) at 4 h (open circles), 5 h (open squares) and 6 h (open triangles). Data presented are means of 3–4 separate incubations of 3–4 preparations. S.E.M. were less than 8% at each point. Further experimental details are the same as in Fig. 1

of either 10 mM glycine, 10 mM L-serine or 10 mM L-alanine, reoxygenation after up to 4 h of hypoxia led to almost complete survival of the hepatocytes (Fig. 5B) and the pronounced alterations of cell structure reversed. Upon reoxygenation at a time later than 5 h, however, all of the hepatocytes rapidly lost their viability under these conditions too.

Discussion

Of the 23 standard amino acids tested, only glycine, L-alanine and L-serine provided significant protection from hypoxic injury of the cultured hepatocytes (Fig. 1). The other amino acids either had no (L-valine, L-leucine, L-isoleucine, L-phenylalanine, L-tyrosine, L-tryptophan, L-proline, L-hydroxyproline, L-threonine, L-methionine, L-cysteine, L-histidine, L-arginine, L-ornithine, L-citrulline, L-lysine, L-asparagine, L-glutamine) or only slight protective effects (DL-glutamic acid, DL-aspartic acid, Table 1).

The protection afforded by glycine and L-serine was already significant at a concentration of 0.1 mM (Fig. 1), which is lower than their physiological concentrations of 0.24 mM and 0.14 mM, respectively (Iapichino et al., 1985). Increases in the concentration of glycine and L-serine further enhanced their protective effects. At concentrations of 10 mM glycine provided almost complete protection and L-serine markedly protected from hypoxic injury. L-alanine showed pronounced protection only at a concentration of 10 mM. At this concentration glycine was more efficient than L-alanine but L-alanine more efficient than L-serine.

The protective effects of the amino acids glycine, L-alanine and L-serine could have resulted from increased energy production, possibly due to their metabolism to pyruvate and subsequently to lactate. However, neither was the ATP content of hypoxic hepatocytes affected nor the lactate production increased by the presence of glycine, L-alanine or L-serine (Fig. 3). Thus, their marked protective effects obviously did not result from increased energy production, as has been shown for fructose (Anundi et al., 1989; Anundi and de Groot, 1989).

Protection from hypoxic cell injury by amino acids has already been described for the kidney. In isolated kidney cells (Weinberg et al., 1987; Garza-Quintero et al., 1990) as well as in the perfused kidney (Baines et al., 1990) hypoxic injury was markedly decreased by glycine and L-alanine. Among 45 amino acids and analogs examined in isolated hypoxic proximal tubule cells, only glycine, L-alanine, D-alanine, β-alanine and ACPC provided significant protection (Weinberg et al., 1990). As in the case of hepatocytes, in the kidney cells the protective effects were not energy dependent and presumably did not require metabolism of these compounds. In contrast to the kidney cells, L-serine and sarcosine were protective in hepatocytes in addition to glycine, L-alanine and ACPC. Thus, despite the similarities, differences in the protective mechanism of short amino acids against hypoxic cell injury may exist between the kidney and the liver.

The addition of either glycine, L-alanine or L-serine to hypoxic hepatocytes led, contrary to proximal tubule cells, to marked blebbing of the plasma

membrane (Fig. 4). As has been described for hepatocellular injury induced by KCN and iodoacetate (chemical hypoxia) (Lemasters et al., 1987; Herman et al., 1988) blebs appeared as bubble-like phase-lucent projections extending from the cell surface. The formation of blebs is considered as a prominent feature of liver cell injury (Reynold, 1963; Walker et al., 1983). Its mechanism is not clear, but is thought to result from a breakdown of cytoskeleton-membrane interactions between microvillar core structures and the plasma membrane (Gores et al., 1990). Despite the advanced formation of blebs under hypoxic conditions in the presence of either glycine, L-alanine or L-serine these alterations were reversible (until 4 h of hypoxia) by reintroduction of oxygen (Fig. 5). Bleb formation in the hypoxic hepatocytes in the presence of glycine, L-alanine or L-serine may be enhanced by interactions of these amino acids with parts of the membrane. Interestingly, such interactions may also be responsible for their protective effects as has been proposed for proximal tubule cells (Garza-Quintero et al., 1990; Weinberg et al., 1990).

The marked protective potency of glycine is also indicated by experiments using organ preservation solutions (Marsh et al., 1991; Southard et al., 1991). Upon cold storage of isolated hepatocytes as well as of the intact liver in University of Wisconsin (UW) solution, significant improvement in preservation was achieved by the addition of glycine.

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