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PROTEASE INHIBITORS DO NOT PREVENT THE KILLING OF CULTURED HEPATOCYTES BY CYSTAMINE

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A study was made of the conditions of the killing of cultured hepatocytes by the reactive disulfide cystamine. Six to 12 mM cystamine killed up to 60% of the hepatocytes within 3 hours. The cytosolic calcium ion concentration rose prior to the loss of viability. Treatment with EGTA in a Ca^{2+}-free medium lowered the initial Ca^{2+} concentration and prevented the rise in response to cystamine. However, there was no change in the number of dead cells. Furthermore, the sensitivity of cultured hepatocytes to cystamine was unaffected by the concentration of calcium in the culture medium. Addition to the culture medium of 3 protease inhibitors, leupeptin, antipain, or chymostatin, did not reduce the extent of cell killing by cystamine despite an inhibition of protein degradation. These data do not support the hypothesis that the toxicity of cystamine is necessarily mediated by proteases activated by a rise in the cytosolic calcium ion concentration.

A depletion of cellular protein thiols and the accompanying alterations in intracellular calcium homeostasis have been suggested to be causative events in the toxicity of an oxidative stress (1-4). Recently, cystamine has been used in an attempt to study the effects of a selective inhibition of Ca^{2+} extrusion by hepatocytes on intracellular calcium homeostasis and cell viability. In isolated hepatocytes cystamine was reported to form protein mixed disulfides with a consequent inhibition of the plasma membrane Ca^{2+}-ATPase activity and a decreased rate of Ca^{2+} efflux from the cells (5). The resulting intracellular accumulation of Ca^{2+} was attributed to these changes and was followed in turn by a stimulation of both phospholipid hydrolysis and proteolysis. Interestingly, pretreatment of the cells with the protease inhibitors leupeptin and antipain protected the hepatocytes from the toxicity of cystamine (5). In a subsequent report, the same protease inhibitors were reported to prevent the cell killing that accompanies

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the altered intracellular calcium homeostais produced by extracellular ATP and the ionophore A23187 (6). However, protection by leupeptin against the toxicity of A23187 could not be confirmed (7).

The killing of cultured hepatocytes by hydrogen peroxide can be dissociated from alterations in intracellular calcium homeostasis (8). Similarly, the toxicity of tert-butyl hydroperoxide has been dissociated from the rise in the intracellular Ca^{2+} ion concentration that occurs as a consequence of the metabolism of this toxin by glutathione peroxidase (9).

In the present report, we have examined the role of alterations in calcium homeostasis and proteolysis in the killing of cultured hepatocytes by cystamine. The data presented below do not confirm the previous conclusion with fresh suspensions of isolated hepatocytes (5) that there is a causal relationship between alterations of intracellular calcium homeostasis and the cell death produced by cystamine. In addition, three different protease inhibitors had no protective effect against the toxicity of cystamine under the present conditions. The possible bases of the differing results of the present and the previous studies (5,6) are discussed.

MATERIALS AND METHODS

Hepatocytes were isolated (10) and cultured (5% CO_2-95% air, 37°C) as previously reported (8). The cells were incubated in a modified Krebs-Ringer bicarbonate buffer (120 mM NaCl, 4.8 mM KCl, 1 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 24 mM $NaHCO_3$, 10 mM HEPES, 10 mM dextrose, MEM amino acids and 2 mM glutamine, pH 7.3) and treated with cystamine (Sigma, dissolved in deionized water). Cell death was assessed by the release of LDH into the medium as described previously (11). LDH release agreed with the uptake of trypan blue. To evaluate the effect of extracellular calcium, the cells were exposed to cystamine in the buffer with 0 to 5 mM Ca^{2+} or 1 mM EGTA. Cytosolic Ca^{2+} concentration was evaluated by phosphorylase a activity measured by incorporation of [^{14}C]-glucose-1-phosphate into glycogen according to Gilboe et al. (12). To deplete cellular calcium, the hepatocytes were preincubated in the Ca^{2+}-free buffer containing 1 mM EGTA for 45 minutes and then treated with cystamine for the times indicated in the text.

Several protease inhibitors were employed to assess the role of proteases in cystamine toxicity. Leupeptin (Sigma, dissolved in water), antipain (Sigma, dissolved in water) and chymostatin (Sigma, dissolved in dimethyl sulfoxide) were added at a final concentration of 200 ug/ml, 500 uM and 200 ug/ml, respectively, 15 minutes before the addition of cystamine. The extent of cell killing was determined after 3 hours. The hepatocytes were not killed by the protease inhibitors alone.

Protein degradation was measured by modification of the methods of Seglen et al. (13) and Hopgood et al. (14). Two hours after plating the cells, hepatocyte proteins were labeled for 24 hours with 0.5 uCi/culture of [^{14}C]valine (285 mCi/mmole, Amersham). After washing the cells twice, they were incubated in Williams E with 2 mM valine for 1 hour. The cells were washed again and incubated in the modified Krebs-Ringer bicarbonate buffer for 3 hours with or without a protease inhibitor. Triton X-100 was then added to a final concentration of 0.5%. Hepatocyte proteins were precipitated by the addition of perchloric acid to a final concentration of 10%. After 15 minutes at 0° C and then centrifugation for 10 minutes at 5,000 rpm (Dupont, RC5C), the radioactivity in the acid-soluble and -insoluble fractions was determined by liquid scintillation counting.

RESULTS

Isolated hepatocytes in culture for 24 hours were washed and placed in a modified Krebs-Ringer bicarbonate buffer, a medium similar to that used to study the killing of

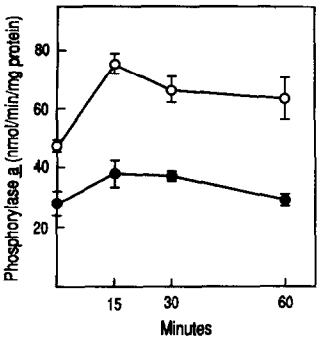

Figure 1. Response of phosphorylase a activity to cystamine. Cultured hepatocytes were washed, placed in a modified Krebs-Ringer bicarbonate buffer, and after 45 minutes treated with 10 mM cystamine (open circles). The phosphorylase a activity in whole cell homogenates was determined at the times indicated. Alternatively, the washed cells were placed in the modified Krebs-Ringer bicarbonate buffer that contained 1 mM EGTA and no $CaCl_2$. After 45 minutes the cells were treated with 10 mM cystamine, and the activity of phosphorylase a measured at the times indicated (closed circles). The results are the mean ± SD of the determinations on 3 separate cultures. After 2 hours 30% of the cells were dead in the cultures treated with EGTA; 25% of the cells were dead in the cultures treated with cystamine in the presence of extracellular calcium.

suspensions of freshly isolated hepatocytes by cystamine (5). The addition of amino acids to this buffer reduced by a factor of two both the rate and extent of the killing of the cultured hepatocytes by cystamine. With 12 mM cystamine, 50% of the hepatocytes were killed within 3 hours. There was no loss of viability for at least 1 hour, after which time the number of dead cells steadily increased.

In liver cells, changes in cytosolic Ca^{2+} concentrations are reflected by changes in the activity of glycogen phosphorylase a. Figure 1 (open circles) shows that the activity of phosphorylase a increased only modestly during the first 15 minutes of exposure of the cultured hepatocytes to 10 mM cystamine. The absence of a greater rise cannot be attributed to an inhibition of the activity of the enzyme by cystamine. In addition, the presence of cystamine had no effect on the rise in phosphorylase a activity produced by the ionophore A23187 (data not shown).

More importantly, the rise in cytosolic calcium could be prevented without modifying the toxicity of cystamine. Cultured hepatocytes can be depleted of much of their cell-associated calcium by treating them with 1 mM EGTA. Figure 1 (closed circles) shows that the basal level of phosphorylase a activity in such hepatocytes depleted of calcium was half that of the controls. In addition, the rise in phosphorylase a activity in response to 10 mM cystamine was much reduced in the EGTA-treated cells. Over the 1 hr course of the experiment, the phosphorylase a activity never attained the control level of untreated cells. Nevertheless, the calcium-depleted hepatocytes did not differ in their sensitivity to the toxicity of cystamine. The extent of cell killing was the same in both situations.

The sensitivity of the cultured hepatocytes to cystamine was also unaffected by the concentration of calcium in the extracellular medium. There was no difference in the extent of the killing of cultured hepatocytes by 10 mM cystamine when the calcium

TABLE I

Protease Inhibitors Do Not Prevent Cystamine Toxicity

Treatment	Protein Turnover (%/hr)	Cell Death (%)
Control	3.52 ± 0.07	3 ± 1
Cystamine (12 mM)	3.14 ± 0.27	54 ± 5
A23187 (5 uM)	3.18 ± 0.36	50 ± 4
Leupeptin (200 ug/ml)	2.04 ± 0.17	5 ± 1
Chymostatin (200 ug/ml)	1.95 ± 0.29	5 ± 1
Antipain (500 uM)	2.04 ± 0.14	4 ± 1
Cystamine + leupeptin	1.78 ± 0.19	59 ± 4
Cystamine + chymostatin	1.73 ± 0.24	60 ± 5
Cystamine + antipain	1.85 ± 0.26	55 ± 6
A23187 + leupeptin	1.75 ± 0.27	54 ± 4
A23187 + chymostatin	1.63 ± 0.11	56 ± 5
A23187 + antipain	1.85 ± 0.23	55 ± 5

The results are the mean ± SD of the determinations on 3 separate cultures.

concentration of the culture buffer was varied from zero to 5 mM $CaCl_2$ (data not shown).

The killing of cultured hepatocytes by cystamine was not accompanied by an accelerated degradation of protein (Table I). Despite the death of more than 50% of the cells with 12 mM cystamine, the turnover of protein was the same as in the control cultures. Furthermore, the addition of 3 protease inhibitors reduced by 40-50% the rate of protein turnover in the cystamine-intoxicated cells without effect on the extent of cell killing (Table I).

The protease inhibitors also had no effect under conditions where alterations in calcium homeostasis can be directly related to cell killing. Cultured hepatocytes are made sensitive to extracellular calcium ions by incubating them in a calcium-free medium containing the calcium ionophore A23187 (8). Under such conditions and in the absence of added extracellular calcium ions, the cells are stable for hours. By contrast, the presence of calcium ions in the culture medium was associated with significant cell killing without an accelerated degradation of protein (Table I). Furthermore, the extent of cell killing was not affected by pretreatment of the cells with leupeptin, antipain, or chymostatin, a result that occurred despite a 40-50% inhibition of the turnover of protein.

DISCUSSION

The present study was prompted by the recent reports of the ability of protease inhibitors to prevent the killing of suspensions of freshly isolated hepatocytes by

cystamine (5,6). Cystamine is an electrophilic disulfide that reacts with cellular thiols to form mixed disulfides. With suspensions of freshly isolated hepatocytes, cystamine inactivates the plasma membrane enzyme system responsible for active efflux of calcium from the cells (5). A consequent rise in the cytosolic free calcium ion concentration was held to mediate the resulting cell killing by a mechanism that depended on the activation of non-lysosomal proteases (5).

The conditions affecting the killing of cultured hepatocytes reported here are quite different from those observed with suspensions of freshly isolated cells (5). Whereas the cytosolic Ca^{2+} concentration increased only modestly, such a change was readily dissociated from the toxicity of cystamine. With the depletion of cellular stores of calcium with EGTA, the rise in cytosolic Ca^{2+} was prevented (Figure 1) without effect on the rate or extent of the cell killing by cystamine. Furthermore, the removal of extracellular Ca^{2+} had no protective effect against the toxicity of cystamine,

With fresh suspensions of isolated hepatocytes, the altered Ca^{2+} homeostasis induced by cystamine was accompanied by the activation of proteolysis, and protease inhibitors protected the cells (5). By contrast, no accelerated degradation of protein occurred in cultured hepatocytes that were intoxicated with cystamine, and the protease inhibitors did not reduce the extent of the cell killing by cystamine-treated hepatocytes (Table I). Despite this negative result, the protease inhibitors reduced the rate of protein degradation by 50% in the cystamine-intoxicated hepatocytes.

The role of accelerated proteolysis was also examined in a situation where an altered intracellular calcium homeostasis clearly mediates cell killing. The killing of cultured hepatocytes by A23187 is a calcium-dependent phenomenon (8). Addition of Ca^{2+} to the medium of hepatocytes that have been treated with A23187 results in a rapid rise in cytosolic calcium and the death of the cells (8). Protease inhibitors prevent the killing by A23187 of fresh suspensions of isolated hepatocytes (6). The present study was unable to document a similar ability of protease inhibitors to protect cultured hepatocytes from such calcium-dependent cell killing (Table I). Again, such a negative result was obtained despite the inhibition of protein turnover by the protease inhibitors in A23187-intoxicated cells (Table I). Interestingly, under the conditions of the present study, the killing of cultured hepatocytes by A23187 is accompanied by an accelerated degradation of phospholipids, and inhibition of phospholipid degradation prevents the cell killing.[4]

The most obvious explanation of the difference between the conditions of the killing of fresh suspensions (5,6) as opposed to cultured rat hepatocytes (Figure 1 and Table I) is a greater sensitivity of the former to extracellular calcium ions. Freshly suspended hepatocytes exposed to an atmosphere of 95% O_2-5% CO_2 are more fragile than cells in culture for 24 hours with room air (20% O_2). Whereas available evidence suggests that freshly isolated and suspended hepatocytes react to a variety of toxic

[4] Sakaida, I., and Farber, J.L. Unpublished data.

injuries in a manner similar to that of cultured hepatocytes, the fragility of the former renders them more readily permeable to extracellular calcium ions, an effect that does not occur with cultured hepatocytes. The differences in the response of suspended versus cultured hepatocytes to tert-butyl hydroperoxide was previously attributed to such an enhanced sensitivity to extracellular calcium (9). Importantly, such exaggerated changes in calcium homeostasis are very likely an artefact of the preparation and immediate suspension of the hepatocytes, rather than a manifestation of the reactivity of native liver cells in the intact animal. Culturing the cells in a 5% CO_2-95% air atmosphere seems to avoid such an artefact.

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