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EXTRACELLULAR CALCIUM PROTECTS ISOLATED RAT HEPATOCYTES FROM INJURY

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The incubation of isolated rat hepatocytes in calcium-free medium resulted in a pronounced increase in lipid peroxidation, mitochondrial and cytoplasmic glutathione depletion, glutathione disulfide formation and efflux of reduced gluathione as compared with hepatocytes incubated in calcium containing medium. These data suggest that extracellular calcium ions serve a protective role in isolated rat hepatocytes against cell injury.

The absence of extracellular Ca^{2+} potentiates the hepatotoxicity of a variety of compounds whose proposed mechanisms of toxicity are quite different. Smith and co-workers [1] have shown in freshly isolated hepatocytes that three different liver cell toxins, CCl_4[1], bromobenzene and EMS, are far more toxic to hepatocytes in the absence of extracellular Ca^{2+} than in its presence. In addition, Acosta and Sorenson [2] have demonstrated that the toxicity of $CdCl_2$ is accelerated in cultured hepatocytes incubated with Ca^{2+} free medium. We have also observed an accelerated rate of cell death in isolated hepatocytes incubated with Ca^{2+} free medium and exposed to a combination of ADR and BCNU or the calcium ionophore A23187 [3]. In this previous study [3], we reported that ADR/BCNU treated hepatocytes incubated without extracellular Ca^{2+} had an accelerated loss of intracellular GSH as compared with treated cells incubated in Ca^{2+} containing medium. These results suggest that the incubation of hepatocytes in Ca^{2+} free medium increases the susceptibility of these cells to chemical-induced cell death by altering

[1]Abbreviations: CCl_4, carbon tetrachloride; EMS, ethylmethanesulfonate; ADR, adriamycin; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; LDH, lactate dehydrogenase; TBA, thiobarbituric acid; GSH, reduced glutathione; GSSG, glutathione disulfide; CYSSG, cysteine glutathione mixed disulfide; PCA, perchloric acid; HPLC, high performance liquid chromatograhy; ISTD, internal standard.

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cellular protective systems. To test this hypothesis we examined, in isolated rat hepatocytes, the effect of the presence or absence of extracellular Ca^{2+} on several indicators of cell injury; namely intracellular $[K^+]$, LDH leakage, lipid peroxidation and glutathione status.

Materials and Methods

Preparation of isolated hepatocytes. Rat parenchymal liver cells were prepared as described previously [4,5]. A known volume of the isolation hepatocyte suspension was added to a 50 ml conical test tube and centrifuged slowly for 2 minutes. The resulting supernatant was discarded and the cells were resuspended in medium containing 0 or 3.5 mM Ca^{2+}. The hepatocyte suspension (3×10^6 cells/ml, 20 ml) was then placed in 125 ml culture flask and slowly rotated at 37 °C under an atmosphere of 95% Air - 5% CO_2 for 5 hours. The Ca^{2+} concentration of medium from 0 mM Ca^{2+} hepatocyte suspensions was less than 0.1 mM as measured by atomic absorption spectrophotometry. The medium was a modified Fischer's formulation [5,6] with the addition of 0.2 mM cystine and 10 mM Hepes immediately before use.

Preparation of cell fractions. In a 1.9 ml microcentrifuge tube, 0.45 ml of dibutyl phthalate was layered over 0.55 ml of 10% PCA. Rapid separation of hepatocytes from medium was accomplished by layering 0.75 ml of hepatocyte suspension over the oil layer and centrifuging for 1 minute at 13,000 g. As a result, intact hepatocytes were forced through the dibutyl phthalate and into 10% PCA thus releasing intracellular contents free from medium contamination [7]. Unlike hepatocytes, medium (extracellular space) remained above the oil layer after centrifugation. The number of cells sampled was determined by analyzing the PCA precipitate for DNA content [8].

Cell viability. Cell viability was monitored hourly by measuring LDH leakage with a Beckman TR Analyzer [9] and intracellular $[K^+]$ with a flame photometer [10]. LDH levels were routinely measured in the medium above the oil while intracellular K^+ was measured in the 10% PCA. Lipid peroxidation was determined by measuring TBA reactants in the cell suspension [11].

Determination of intracellular, extracellular and mitochondrial glutathione. The method of Reed et al.[12] was used with several modifications [5]. Measurement of intracellular GSH and GSSG in this report were obtained by adding 0.5 ml of 10% PCA (lower fraction in microcentrifuge tube) to a tube containing a known amount of γ-glutamyl glutamic acid (ISTD, Vega Biochemicals, Tucson, AZ). Each sample was treated with 0.05 ml of 1,10-phenanthroline (25 mM) and iodoacetic acid (20 mg/ml) followed by 0.45 ml of a KOH (2M) - $KHCO_3$ (2.4M) solution. After incubation at pH 8-9 for 15 minutes, 1 ml of 1% fluorodinitrobenzene in ethanol (v/v) was added to the sample. The reaction mixture was stored in the dark for 24 hours at room temperature and then analyzed (100 µl) for GSH and GSSG by HPLC with an internal standard method. GSSG levels were corrected for 1% GSH oxidation which occurred during the assay procedure.

The efflux of GSH from hepatocytes was determined by taking advantage of the reaction of cystine (0.2 mM), which was added to the medium, with extracellular GSH, resulting in the stoichiometric formation of CYSSG. By measuring CYSSG and GSSG in the medium, the efflux rate of GSH and GSSG respectively were determined [5]. To 0.55 ml of medium (upper fraction in microcentrifuge tube), a known volume of ISTD was added and the mixture was acidified with 0.1 ml of 70% PCA. After freezing, thawing and centrifugation, this solution (0.5 ml) was analyzed for CYSSG, GSSG and GSH as described in the preceeding paragraph.

The mitochondrial glutathione concentration in isolated hepatocytes was determined by the method of Meredith and Reed [13]. In brief, 1 ml of hepatocyte suspension was centrifuged briefly to remove the medium. The cells were resuspended in a digitonin buffer for 2 minutes and then centrifuged

through dibutyl phthalate into 10% PCA. The acid layer was analyzed for glutathione as previously described for intracellular glutathione.

Results and Discussion

Criteria for determining cellular damage in hepatocytes have focussed on parameters that measure membrane integrity and metabolic performance [14-16]. These parameters include intracellular glutathione, Ca^{2+}, K^+ and ATP content, LDH leakage, lipid peroxidation products, trypan blue staining and morphological appearance. The leakage of intracellular enzymes, such as LDH, and the uptake of trypan blue stain suggest irreversible cell damage [14,15]. Alterations in the other parameters mentioned, however, indicate more subtle changes in metabolic or membrane performance which may be reversible [14-16].

In the present study, the amount of LDH lost from isolated hepatocytes incubated in the presence or absence of extracellular Ca^{2+} was identical; approximately 28% over a 5 hour period (Fig. 1). In contrast, the loss of intracellular K^+ was accelerated in hepatocytes incubated with Ca^{2+} free medium (Fig. 1). The percentage of intracellular K^+ lost after 5 hours was 14% in hepatocytes incubated with medium containing 3.5 mM Ca^{2+} and 32% in hepatocytes incubated with Ca^{2+} free medium. Because the loss of intracellular K^+ is a more sensitive indicator of cell damage than LDH leakage [14,15], these results suggest that incubation in Ca^{2+} free medium injures isolated hepatocytes and that the damage is probably reversible.

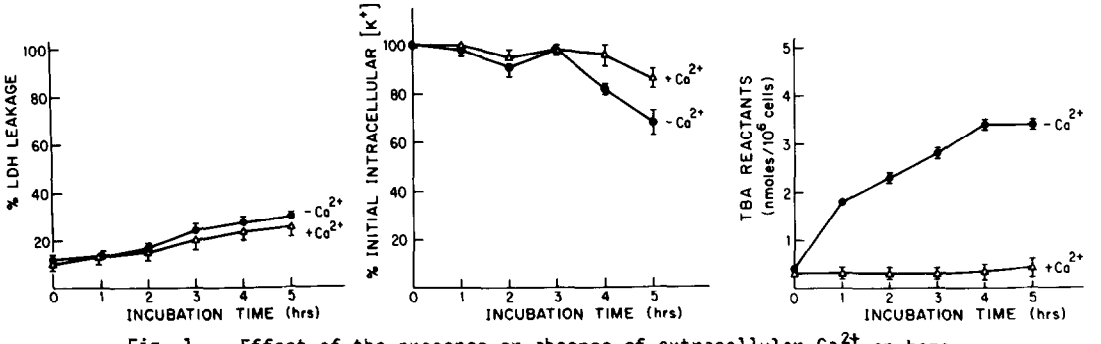

Fig. 1. Effect of the presence or absence of extracellular Ca^{2+} on hepatocyte viability (left and middle) and lipid peroxidation (right). Each point represents the average of 2-6 separate hepatocyte preparations ± SE. Intracellular $[K^+]$ at 0 time was 1.1 ± 0.1 μmoles/10^6 cells in both treatment groups.

Another consequence of eliminating Ca^{2+} from the medium is that lipid peroxidation was markedly increased in isolated hepatocytes. Over a four-hour period, the concentration of malondialdehyde (TBA reactants) in hepatocytes exposed to Ca^{2+} free medium steadily increased from 0.4 to 3.4 nmoles/10^6 cells (Fig. 1). In contrast, malondialdehyde levels in hepatocytes incubated with 3.5 mM Ca^{2+} remained constant throughout the experiment (0.3 nmoles/10^6 cells). Thus, lipid peroxidation was an immediate and continuous consequence of exposing hepatocytes to Ca^{2+} free medium.

The oxidation of membrane lipids has been proposed as the mechanism by which a number of foreign compounds cause cell injury or cell death [17-19]. In the present study, however, the enormous rise in lipid peroxidation in hepatocytes incubated without Ca^{2+} did not cause cell death (LDH leakage, Fig. 1). The concentration of TBA reactants in hepatocytes incubated with Ca^{2+} free medium (3.4 nmoles TBA/10^6 cells, Fig. 1) was greater than or equivalent to levels observed in hepatocytes incubated with extracellular Ca^{2+} and toxic levels of CCl_4 (3.9 nmoles TBA/10^6 cells), bromobenzene (0.3 nmoles TBA/10^6 cells) and ADR/BCNU (1.5 nmoles TBA/10^6 cells) (unpublished data). Consequently, we question the importance of lipid peroxidation in cell death. Because lipid peroxidation is commonly observed in chemically induced cell death, but does not appear to cause it, the cellular events preceeding lipid peroxidation may be the critical events in cell death.

Incubation of hepatocytes in a Ca^{2+} free environment also resulted in the depletion of both mitochondrial and cytoplasmic GSH. The mitochondrial GSH concentration in hepatocytes incubated without extracellular Ca^{2+} fell steadily, to less than 50% of the initial concentration, over a 5 hour period (Fig. 2). In contrast, hepatocytes incubated with Ca^{2+} maintained mitochondrial GSH at approximately 90% of the initial concentration, 2.8 nmoles/10^6 cells. Hepatocytes incubated with Ca^{2+} also maintained the intracellular (cytoplasmic and mitochondrial) GSH concentration at approximately 30 nmoles/10^6 cells for 5 hours (Fig. 2). Incubation of hepatocytes without extra-

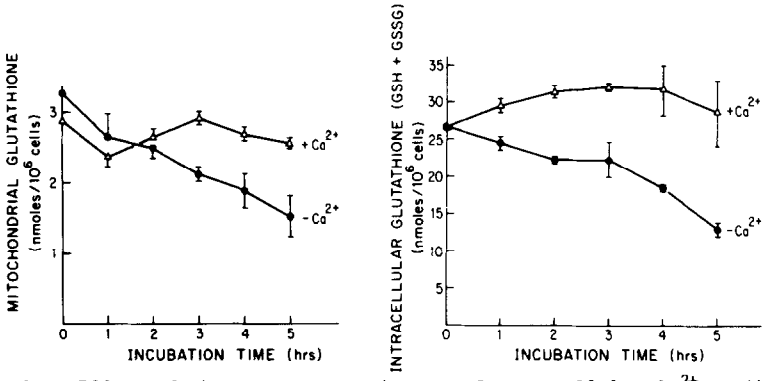

Fig. 2. Effect of the presence or absence of extracellular Ca^{2+} on the concentration of mitochondrial (left) and intracellular (right) glutathione in isolated rat hepatocytes. Each point represents the average of 2-6 separate hepatocyte preparations ± SE. Glutathione levels are the sum of GSH and GSSG concentrations expressed as GSH equivalents. The concentration of intracellular and mitochondrial GSSG did not increase with time and was less than 0.5 nmoles/10^6 cells and 0.2 nmoles/10^6 cells respectively.

cellular Ca^{2+}, however, reduced the intracellular GSH concentration to 13 nmoles/10^6 cells, a 50% reduction over a 5 hour period.

A comparison of the intracellular GSH concentration in hepatocytes incubated in the presence and absence of extracellular Ca^{2+} for 5 hours shows a difference of approximately 15 nmoles/10^6 cells (Fig. 2). Several possible explanations for the accelerated depletion of intracellular GSH in hepatocytes incubated without Ca^{2+} include (1) an enhanced GSH efflux; (2) an enhanced formation and efflux of GSSG or (3) a depressed rate of GSH biosynthesis. When extracellular Ca^{2+} was removed from the medium the efflux of GSH from hepatocytes was indeed accelerated (Fig. 3). Within a 5 hour period, hepatocytes incubated without Ca^{2+} released approximately 7 nmoles/10^6 cells more GSH than cells incubated with Ca^{2+}. Similarly, the formation and release of GSSG from hepatocytes was enhanced. The efflux of GSSG was approximately 4 nmoles/10^6 cells/5 hr greater in hepatocytes incubated without Ca^{2+} than in those incubated with Ca^{2+} (Fig. 3). The rate of GSH biosynthesis, however, was not significantly affected by the elimination of extracellular Ca^{2+}. The glutathione synthesis rate in hepatocytes incubated with or without extracellular Ca^{2+} was identical, approximately 2.8 nMoles/10^6 cells/hr. Thus, the accelerated release of GSH and GSSG from hepatocytes

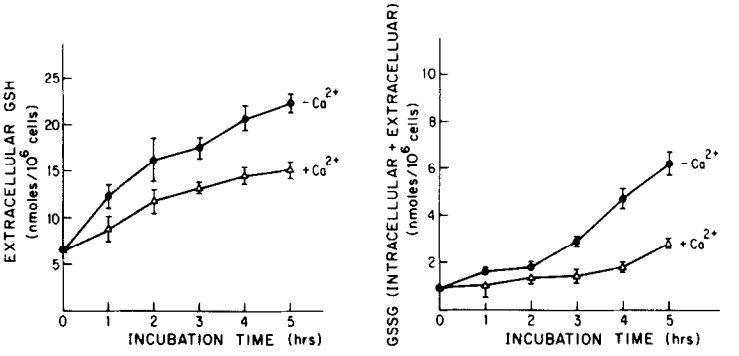

Fig. 3. Effect of the presence or absence of extracellular Ca^{2+} on the efflux of GSH (left) and the formation of GSSG (right) in isolated rat hepatocytes. Each point represents the average of 2-6 separate hepatocyte preparations ± SE. GSSG formation is the sum of intracellular and extracellular concentrations of GSSG. The concentration of intracellular GSSG did not increase with time and was less than 0.5 nmoles/10^6 cells.

incubated without Ca^{2+} appears to account for the depletion of intracellular GSH observed in these cells.

The release of GSSG from the liver is observed under conditions of "oxidative stress" where GSSG formation and release are proportional to the rate of H_2O_2 reduction by glutathione peroxidase [20,21]. Consequently, the increase in GSSG formation (Fig. 3) suggests that a Ca^{2+} free environment causes an "oxidative stress" in isolated rat hepatocytes. This environment also resulted in an accelerated efflux of GSH but its significance is unknown because the purpose and regulation of GSH efflux is poorly understood. It has been suggested, however, that the rate of GSH efflux is simply a reflection of the intracellular GSH concentration [22]. This hypothesis is not supported by the present study in which hepatocytes with the lowest intracellular GSH concentrations showed the highest GSH efflux rates (Fig. 2 and 3). These results suggest that the export of GSH is a dynamic process which possibly functions to maintain the extracellular redox status and to protect the integrity of the plasma membrane [23].

In isolated rat hepatocytes, the presence of extracellular Ca^{2+} appears to be required for maintenance of normal metabolic functions and protective systems. This requirement for extracellular Ca^{2+}, however, may be only a reflection of the total intracellular concentration of Ca^{2+}, since the addi-

tion of extracellular Ca^{2+} (3.5 mM) to Ca^{2+} free medium increased the total intracellular [Ca^{2+}] from 2.5 to 12 nMoles/10^6 cells. Consequently, the increase in total intracellular Ca^{2+} ions might explain the protection afforded isolated hepatocytes incubated with extracellular Ca^{2+}.

Previous studies have shown that chemically induced cell death is accelerated in hepatocytes incubated without extracellular Ca^{2+} [1-3]. One possible explanation for this enhanced toxicity is that the absence of extracellular Ca^{2+} results in the depletion of intracellular GSH (Fig. 2). It is well known that intracellular GSH is an important cellular protective system against reactive oxygen intermediates and highly electrophilic compounds [24]. Indeed, one major function of intracellular GSH is to maintain membrane protein sulfhydryl groups. These protein thiols are important in many metabolic and membrane processes such as calmodulin function, cell shape and Ca^{2+} sequestration [16,25]. In fact, recent studies suggest that the impairment of Ca^{2+} sequestration in the endoplasmic reticulum is the crucial event leading to hepatocellular injury [26]. Consequently, hepatocytes incubated without extracellular Ca^{2+} and thus depleted of intracellular GSH (Fig. 2), might be more susceptible to the toxic effects of compounds which form oxygen free radicals or electrophilic metabolites.

Meredith and Reed [13,27] demonstrated that chemically induced cell death correlates better with the depletion of mitochondrial GSH than with that of cytosolic GSH. These investigators suggest that chemically induced cell death occurs in hepatocytes only after the GSH pool in mitochondria has been eliminated. In view of this hypothesis, the depletion of mitochondrial GSH observed in hepatocytes incubated without Ca^{2+} (Fig. 2) may also explain the increased susceptibility of these cells to chemically induced cell death.

We have shown conclusively that the absence of extracellular Ca^{2+} adversely affects the metabolic and membrane integrity of isolated rat hepatocytes. Consequently, the presence of extracellular Ca^{2+} or possibly total intracellular Ca^{2+} appears to serve a protective role in isolated rat hepatocytes. One can only speculate as to how the intracellular injury in

hepatocytes incubated without Ca^{2+} might increase the susceptibility of these cells to chemically induced cell death. Because the absence of extracellular Ca^{2+} results in an increase in lipid peroxidation, an oxidative stress and mitochondrial and cytoplasmic GSH depletion, we suggest that these events either individually or collectively are responsible for the acceleration of chemically induced cell death in isolated rat hepatocytes.

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