Calcium-dependent and Calcium-independent Mechanisms of Irreversible Cell Injury in Cultured Hepatocytes*

(Received for publication, July 26, 1985)

Pamela E. Starke, Jan B. Hoek, and John L. Farber†

From the Department of Pathology and Laboratory Medicine, Hahnemann University School of Medicine, Philadelphia, Pennsylvania 19102

It has been proposed that alterations in intracellular calcium homeostasis mediate the genesis of lethal cell injury with an acute oxidative stress. It is shown here, however, that such changes can be dissociated by two different means from the cell death occurring with the exposure of cultured hepatocytes to hydrogen peroxide generated either in the medium by glucose oxidase or intracellularly by the mechanism of menadione. The chelation of intracellular ferric iron with deferoxamine inhibits the formation of hydroxyl radicals from hydrogen peroxide and prevents cell killing. Deferoxamine did not prevent, however, an elevation of the cytosolic Ca\(^{2+}\) ion concentration detected as an activation of phosphorylase \(a\). Sulhydryl reagents inhibited the rise in phosphorylase \(a\) activity in deferoxamine-pretreated hepatocytes. Conversely, cultured hepatocytes were depleted of Ca\(^{2+}\) ions by treatment with EGTA in a calcium-free medium. Calcium-depleted cells were not resistant to the toxicity of hydrogen peroxide despite the virtual elimination of the activation of phosphorylase \(a\).

In contrast, it was possible to kill cultured hepatocytes by a mechanism dependent upon a disordered intracellular calcium homeostasis using hepatocytes pretreated in calcium-free medium with the ionophore A23187. These cells were killed in a dose-dependent manner by the addition of calcium ions to the culture medium in concentrations ranging from 0.1 to 2.0 mM. There was a similar dose-dependent activation of phosphorylase \(a\), but phosphorylase \(a\) activities were higher than with \(H_2O_2\) at comparable cell killing. Deferoxamine pretreatment and sulphydryl reagents had no effect on the loss of viability with this calcium-dependent cell killing.

Orrenius and co-workers (1-6) have proposed that a disturbance of intracellular calcium homeostasis is an early event in the genesis of lethal cell injury produced by an acute oxidative stress. This hypothesis is based on experimental data of three sorts. First, exposure of suspensions of rat hepatocytes to \(t\)-butyl hydroperoxide or hydrogen peroxide generated by the redox cycling of menadione impaired the sequestration of calcium by mitochondria and the endoplasmic reticulum. There was an accompanying rise in the cytosolic free Ca\(^{2+}\) concentration as indicated by the activity of glycogen phosphorylase \(a\). This disturbed calcium homeostasis was paralleled by the depletion of glutathione resulting from the reduction of either peroxide by glutathione peroxidase and the oxidation of pyridine nucleotides accompanying the reduction of GSSG by glutathione reductase. Blebbing of the surface membrane followed the alterations in both calcium and glutathione metabolism. Second, certain conditions that protected against cell injury by an oxidative stress, notably the presence of the sulphydryl reagent dithiothreitol, also prevented the increase in phosphorylase \(a\) and the loss of Ca\(^{2+}\) from its intracellular storage pools. The oxidation of GSH and NADPH and the blebbing of the surface membrane of the hepatocytes were similarly prevented by dithiothreitol. Finally, direct mobilization of calcium from intracellular stores by the ionophore A23187 also promoted cell killing, preceded by phosphorylase activation. This process was not sensitive to protection by sulphydryl reagents. On the basis of these observations, a model of oxidative injury was proposed whereby the ultimate loss of viability of the liver cells was a consequence of the sequence of events proceeding from GSH depletion to an elevated cytosolic calcium concentration and leading finally to membrane injury. These same data do not exclude, however, alternative interpretations in which the rise in cytosolic calcium after an oxidative stress is not an essential factor in membrane damage, but rather a consequence thereof or an unrelated epiphenomenon.

In the course of our studies of the mechanisms mediating cellular injury by hydrogen peroxide (7-9), we have developed various methods by which the sensitivity of hepatocytes to this oxidative stress can be manipulated. In particular, we have shown that the toxicity of \(H_2O_2\) is dependent upon a source of intracellular ferric iron (9). Cultured hepatocytes treated with the ferric iron chelator deferoxamine were resistant to the toxicity of \(H_2O_2\). Sensitivity of the cells could be restored by the addition of heavy metal cations to the culture medium. In the present report, we have used this and other manipulations of our experimental system to reassess the role of changes in Ca\(^{2+}\) homeostasis in the genesis of lethal cell injury after an acute oxidative stress.

**MATERIALS AND METHODS**

Male Sprague-Dawley rats (150-200 g) from Charles River Breeding Laboratories were fasted overnight prior to use. Isolated hepatocytes were prepared by collagenase (Sigma) perfusion according to Seglen (10). Yields of 3-5 x 10^6 cells/liver with 88-96% viability by trypan blue exclusion were routinely obtained. The hepatocytes were plated at a density of 50,000/cm^2 in 25-cm^2 flasks in 3 ml of Williams E medium (Flow Laboratories) containing 10 IU/ml penicillin, 10 \(\mu\)g/ml streptomycin, 0.02 unit/ml insulin, and 10% fetal calf serum (complete Williams E). After incubation for 1/4 h at 37°C in an atmosphere of 5% CO\(_2\)/95% air, the cells were washed with a pre-

* This work was supported by Grants AM-31114, AM-31086, AA-05662, and AA-03442 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1794 solely to indicate this fact.

† To whom to address correspondence.
warmed Hepes \(^1\) (Sigma) buffer, pH 7.4 (0.14 M NaCl, 0.7 mM KCl, 1.2 mM CaCl\(_2\), and 2.4 mM Hepes) to remove unattached dead cells. Complete Williams E medium was replaced (5 ml), and the cells were incubated for 24 h. The cultures were then washed and incubated in Williams E medium minus calcium alone or with either one or a combination of the following additions: 20 mM deferoxamine (Chiba-Geigy, dissolved in 0.9% NaCl) for 60 min, 0.5 μg/ml \[^{3}H\]TPMP\(^+\) (Amer sham Corp.) and 10 mM tetraphenylboron (Sigma, dissolved in distilled water) for 30 min, 1 mM EGTA (Sigma, dissolved in 0.9% NaCl) for 45 min, and 5 μg/ml calcium ionophore A23187 (Sigma, dissolved in dimethyl sulfoxide) for 45 min. The cultures treated with deferoxamine or EGTA were washed two or five times, respectively, with prewarmed Hepes buffer and returned to Williams medium minus calcium. The cultures were then treated with varying reagents for different times as detailed in the text. Viability of cultured cells was assayed by the release of lactic dehydrogenase \(\text{LDH}\) (11). In previous publications (7, 12, 13), we have demonstrated that under the conditions of the present study there is a strict correlation between lactic dehydrogenase release and other indicators of cell viability such as trypan blue exclusion. All experiments were performed on triplicate cultures.

Glucose oxidase, grade I (Sigma), and menadione (Sigma) were dissolved in Hepes buffer and added to the medium at a final concentration of 2.5 units/ml and 250 μM, respectively. Dithiothreitol (Sigma) and N-acetyl-L-cysteine (Calbiochem) were dissolved in 0.9% NaCl and added to the cultures at a final concentration of 200 μM and 4 mM, respectively. Glucose oxidase activity was assayed as the rate of \(\text{H}_2\text{O}_2\) formation, by the scopo llin-horseradish peroxidase assay of Root et al. (14).

Phosphorylase \(\alpha\) activity was assayed by measuring the incorporation of \[^{14}C\text{glucose} 1\text{-phosphate}\) into glycogen under the assay conditions described by Stalmans and Hers (15) with some modifications. The culture medium was suctioned off and replaced with 1 ml of ice-cold stopping media containing 10 mM MES, 50 mM NaF, 25 mM β-glycerophosphate, 10 mM EDTA, and 0.8 M dithiothreitol. The cells were scrapped from the flasks and collected in the stopping medium. Cell extracts (25 μl) were mixed with an equal volume of an extraction medium containing 86 mM glucose 1-phosphate, 85 μM of \[^{14}C\text{glucose} 1\text{-phosphate}\), 2% glycogen, 4.5 mM caffeine, and 50 mM NaF and were incubated at 37 °C. The reaction was stopped after 30 min with the addition of 25 μl of glacial acetic acid. A 75-μl sample was spotted on filter paper and further treated as described by Gilboe et al. (16). Under these conditions, untreated hepatocytes had an activity of 258 ± 25.8 nmol/min/mg of protein. Changes in the mitochondrial membrane potential were assessed by measuring the release of incorporated \[^{3}H\]TPMP\(^+\) in the culture medium. The release of \[^{3}H\]TPMP\(^+\) was measured by counting aliquots (25 μl) of the culture medium. The total calcium content of the cultured hepatocytes was measured by atomic absorption spectroscopy according to the method of Blackmore and Exon (18). The QSH content of the hepatocytes was determined by the method of Sedlak and Lindsey (19).

RESULTS

Irreversible Cell Injury by an Oxidative Stress—We have shown recently that the killing of cultured hepatocytes by hydrogen peroxide generated either in the medium by glucose oxidase or intracellularly by the redox cycling of menadione is dependent upon the formation of hydroxyl radicals via a Fenton reaction (9). By chelating intracellular ferric iron with deferoxamine, it was possible to prevent the formation of hydroxyl radicals and, therefore, the cell killing by hydrogen peroxide. We show here that chelation of iron does not prevent the changes in calcium homeostasis that accompany the exposure of hepatocytes to \(\text{H}_2\text{O}_2\).

\(\text{H}_2\text{O}_2\) was determined by the method of Sedlak and Lindsey (19).

The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TPMP\(^+\), triphenylmethylphosphonium cation; EGTA, ethylenediaminetetraacetic acid; MES, 4-morpholineethanesulfonic acid.

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![Fig. 1. Prevention by deferoxamine of the cell killing by glucose oxidase or menadione. Hepatocytes in culture for 24 h were washed and given fresh medium alone or medium containing 20 mM deferoxamine. After 1 h, the cells were washed again, given fresh medium, and exposed to either 2.5 units/ml glucose oxidase or 250 μM menadione. The data illustrate the time course of the subsequent cell killing. The viability of the cells was determined by the release of lactic dehydrogenase into the medium. Results are the means ± S.D. of 3 separate cultures.](https://example.com/fig1.png)
in hydrogen peroxide-intoxicated hepatocytes the alteration in intracellular calcium homeostasis represented by the rise in phosphorylase a does not require the formation of hydroxyl radicals and is not necessarily linked to further events that result in lethal cell injury.

Several possible mechanisms could account for a rise in cytosolic calcium with an acute oxidative stress. Release of sequestered stores of calcium with a consequent rise in phosphorylase activity could result from collapse of the mitochondrial membrane potential secondary to damage to the inner membrane. The intracellular distribution of the nonmetabolizable lipophilic cation triphenylmethylphosphonium has been used to monitor membrane potential gradients in intact hepatocytes (21). The accumulation of TPMP + in the cell reflects the sum of plasma membrane and mitochondrial membrane potentials, in addition to a nonspecific binding contribution. Due to the magnitude of the mitochondrial membrane potential under energized conditions, more than 90% of the accumulated TPMP + is localized in the mitochondria, and the total cellular TPMP + content responds rapidly to changes in the mitochondrial membrane potential (21).

The distribution of [3H]TPMP + between hepatocytes and the culture medium was used, therefore, to determine whether the changes in cytosolic calcium concentration induced by hydrogen peroxide (Fig. 2) could be attributed, at least in part, to a collapse of the mitochondrial membrane potential.

Deferoxamine-pretreated and native hepatocytes were treated with 2.5 units/ml glucose oxidase or 250 μM menadione (Fig. 3). Only 10% of the initial radioactive activity was released within 1 min, and greater than 70% of the [3H]TPMP + initially incorporated by the pretreated cells was retained at the end of 1 h of exposure to hydrogen peroxide.

In order to confirm that release of TPMP + was caused by collapse of the mitochondrial membrane rather than reflecting plasma membrane damage, the release of TPMP + was examined from hepatocytes incubated in a buffer containing 30 mM KCl in the presence of glucose oxidase (Table I). Raising the extracellular potassium concentration will depolarize the plasma membrane. Over the course of 3 h, 30 mM KCl released 33% of the TPMP + with little if any loss of viability. In the presence of 30 mM KCl, glucose oxidase further increased the release of TPMP + with the killing of half of the cells. These data document that glucose oxidase caused the release of TPMP + even when the plasma membrane potential is collapsed, presumably reflecting a decrease in mitochondrial membrane potential.

The data in Fig. 3 and Table I indicate that an early loss of mitochondrial membrane potential precedes the cell killing by hydrogen peroxide. Since the deferoxamine pretreatment reduced the extent of this collapse of the mitochondrial mem-
brane potential, the increase in phosphorylase a activity (Fig. 2) could not be explained by the rise in cytosolic calcium ion concentration that would accompany a loss of the mitochondrial membrane potential.

A rise in cytosolic calcium occurring under the present circumstances may be the consequence of the acute metabolism of toxic concentrations of either hydrogen peroxide or t-butyl hydroperoxide. The metabolism of these agents leads to depletion of both GSH and NADPH. Continued sequestration of calcium ions in the mitochondria and endoplasmic reticulum may depend upon an adequate supply of both GSH and NADPH. In that case, the pretreatment with deferoxamine used in the experiments illustrated in Figs. 1 and 2 should have no effect upon such depletion of either GSH or NADP. Table II shows that pretreatment with 20 mM deferoxamine for 1 h did not prevent the depletion of GSH occurring in cultured hepatocytes exposed to hydrogen peroxide generated by the addition to the culture medium of 5 units/ml glucose oxidase. Deferoxamine alone had no effect on the GSH content of the hepatocytes.

The data in Fig. 4 support a conclusion that the rise in phosphorylase a activity is related to this depletion of GSH. The thiol reagent dithiothreitol was shown by Orrenius and co-workers to prevent the changes in calcium homeostasis occurring in freshly isolated hepatocytes treated with t-butyl hydroperoxide (1) or menadione (5, 6). Fig. 4 details the time course in the presence or absence of two different sulfhydryl reagents of the increase in phosphorylase a activity in deferoxamine-pretreated hepatocytes treated with glucose oxidase. Both N-acetyl cysteine and dithiothreitol prevented the increase in phosphorylase a. By adjusting the concentration of either sulfhydryl reagent, therefore, it was possible to manipulate up or down the changes in phosphorylase a activity induced by hydrogen peroxide in deferoxamine-pretreated hepatocytes in the absence of any changes in the viability of the cells.

In further experiments, it was possible to show that the cells could be injured by an oxidative stress without an increase in cytosolic calcium concentration. Cultured hepatocytes can be depleted of much of their cell-associated calcium by treating them with 1 mM EGTA (Table III). Such calcium-depleted cells showed no loss of viability as measured by lactic dehydrogenase release over a 3-h incubation period in culture medium containing no added calf serum and less than 20 μM total calcium ions. Calcium-depleted hepatocytes were not resistant to the toxicity of hydrogen peroxide generated by either glucose oxidase or by the metabolism of menadione. The same number of cells were killed in cultures with or without the pretreatment with EGTA. There were, however, differences in calcium metabolism between the EGTA-treated and control cells (Fig. 5). The basal level of phosphorylase a activity in the calcium-depleted hepatocytes was half that of the controls (Fig. 5). In addition, the rise in phosphorylase a activity in response to an acute oxidative stress was much reduced in the EGTA-pretreated hepatocytes (Fig. 5). After 15 min, the phosphorylase activity in the EGTA-pretreated cells had risen only to the basal level of the native hepatocytes. After 1 h, in the EGTA-pretreated cells the phosphorylase activity had risen to only 35 and 40% of the levels in control cells given glucose oxidase and menadione, respectively. These data indicate that cells can be killed by an acute oxidative stress even though a rise in cytosolic calcium ion concentration is largely prevented.

Calcium-dependent Irreversible Cell Injury—In the final series of experiments, we sought to further define the role of calcium metabolism in toxic cell death by examining a situation where alterations in calcium homeostasis could be related to cell killing as measured by lactic dehydrogenase release. This was achieved by rendering hepatocytes sensitive to the calcium ion concentration in the medium by incubating them for 45 min in calcium-free medium containing 5 μg/ml calcium ionophore A23187. This concentration of A23187 increased the permeability to calcium but was not sufficient to equilibrate intracellular and extracellular calcium concentrations. The cells were then exposed to varying concentrations of calcium for 15 and 60 min. The left panel of Fig. 6 shows the cell killing measured at 15 and 60 min after the addition of Concentrations of calcium ions from 0.1 to 2.0 mM. There was no loss of viability in the cultures treated with the ionophore.

**Table II**

<table>
<thead>
<tr>
<th>GSH*</th>
<th>μg/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20.5 ± 0.4</td>
</tr>
<tr>
<td>Glucose oxidase (5 units/ml)</td>
<td>9.4 ± 2.0</td>
</tr>
<tr>
<td>Glucose oxidase (5 units/ml) + deferoxamine pretreatment</td>
<td>11.0 ± 2.2</td>
</tr>
</tbody>
</table>

* Results represent the mean ± S.D. of the determinations from 3 separate cultures.

![Graph](image)

**Fig. 4. Effect of sulfhydryl reagents on the activation of phosphorylase a in deferoxamine-pretreated hepatocytes exposed to 2.5 units/ml glucose oxidase.** N-Acetyl cysteine or dithiothreitol (DTT) was present at 4 mM and 200 μM, respectively. Results are the means ± S.D. of 3 separate cultures.

**Table III**

<table>
<thead>
<tr>
<th>Calcium content</th>
<th>mmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>9.3 ± 0.5</td>
</tr>
<tr>
<td>EGTA (1 mM)*</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>A23187 (5 μg/ml)*</td>
<td>1.9 ± 1.1</td>
</tr>
</tbody>
</table>

* Cultured hepatocytes were washed and placed in calcium- and serum-free medium containing 1 mM EGTA for 45 min. The cultures were then incubated with calcium for 30 min. Results represent the mean ± S.D. of 3 separate experiments.

* Cultured hepatocytes were washed and placed in calcium- and serum-free medium containing 5 μg/ml A23187 for 45 min. The cultures were then washed twice and then scraped into NaCl (150 mM) for calcium determinations.
FIG. 5. Phosphorylase a activation in calcium-depleted hepatocytes. Cultured hepatocytes were treated with 1 mM EGTA in calcium- and serum-free medium for 45 min, then washed and placed in fresh medium. Control cells were kept in fresh complete medium for 45 min. Both the EGTA-treated and control cultures were then exposed to either 2.5 units/ml glucose oxidase or 250 μM menadione. There was no difference in the extent of cell killing as measured by lactic dehydrogenase release at 3 h (55% of the cells dead) with either toxin and with or without the EGTA pretreatment. Illustrated are the time courses of changes in phosphorylase a. Results are the means ± S.D. of 3 separate cultures.

Fig. 6. Dependence on the extracellular calcium ion concentration of the cell killing as measured by the release of lactic dehydrogenase (left panel) and phosphorylase a activation (right panel) in cultured hepatocytes pretreated in serum- and calcium-free medium with 5 μg/ml A23187. Results are the means ± S.D. of 3 separate cultures.

DISCUSSION

Previous studies (1–6) had suggested that the lethal liver cell injury that may accompany exposure to an acute oxidative stress is mediated, at least in part, by an alteration in intracellular calcium homeostasis with an elevated cytosolic calcium ion concentration following the release of these ions from their intracellular sequestered stores. The data presented above, however, have dissociated the loss of liver cell viability with an acute oxidative stress from such changes in intracellular calcium homeostasis. It is important to emphasize that this was achieved by two independent means. On the one hand, changes in calcium homeostasis were shown to occur without there being any accompanying irreversible liver cell injury. On the other, irreversible cell injury was achieved...
by an iron-catalyed Haber-Weiss reaction (9). The formation of hydroxyl radicals from the reaction of hydrogen peroxide with ferrous iron was prevented, the changes in calcium homeostasis were not associated with any loss of viability of the hepatocytes. In contrast to the hydroxyl radical-dependent cell killing that occurs with hydrogen peroxide, cultured liver cells could be lethally injured by another mechanism that is related to the flooding of the cytosol with calcium ions. When the hepatocytes were rendered permeable to extracellular calcium ions by preincubating them with the calcium ionophore A23187 in calcium-free medium, the total cell-associated calcium was reduced by as much as 90%, and the basal phosphorylase a activity was reduced by 50%. These changes occurred without any loss of viability. When calcium ions (0.1–2.0 mM) were then added to the culture medium, there was a dose-dependent killing of the hepatocytes that was accompanied by a parallel dose-dependent rise in phosphorylase a activity. It is obvious that the liver cells were killed as a result of the alteration in calcium homeostasis associated with the flooding of their cytosol with calcium ions.

An important feature distinguishes the disordered calcium homeostasis related to cell killing from that accompanying an oxidative stress. With the ionophore A23187 and extracellular calcium, the phosphorylase a activities were significantly higher than after exposure to hydrogen peroxide. The phosphorylase response (measured at 60 min) actually saturated at doses of extracellular calcium close to 0.5 mM, while the cell killing continued to increase with 1.0 and 2.0 mM calcium. With 0.5 mM extracellular calcium, the phosphorylase a activity after 1 h was more than twice that with either glucose oxidase or menadione. At the same time, the number of dead cells at 1 or 3 h with 0.5 mM calcium was only half that with hydrogen peroxide. This would further support the conclusion that the cell killing with hydrogen peroxide is unrelated to the changes in calcium homeostasis. To achieve comparable cell killing to that occurring with either glucose oxidase and menadione required 2.0 mM extracellular calcium. With this calcium concentration, the phosphorylase a was more than twice that with hydrogen peroxide. Furthermore, the actual level of cytosolic calcium with 2.0 mM calcium was most likely even higher than the level of phosphorylase would imply since phosphorylase a activation saturated at about 0.5 mM extracellular calcium. It can be concluded, therefore, that the cytosolic calcium ion concentration needed to kill 50% of the hepatocytes with the ionophore A23187 and extracellular calcium is much higher than that induced with doses of either glucose oxidase or menadione that killed the same number of cells. An elevated cytosolic calcium ion concentration may be associated with irreversible liver cell injury, but at much higher calcium concentrations than can be detected with the phosphorylase system.

Both the cell killings by an A23187-induced altered calcium homeostasis and by an acute oxidative stress were associated with very early evidence of mitochondrial dysfunction as shown by a collapse of the mitochondrial membrane potential. Despite this correlation of a common manifestation of cellular injury with the subsequent death of the hepatocytes, one has to be cautious in interpreting such a manifestation of cellular injury as having any necessary relationship to the death of the hepatocytes. In the case of the hydroxyl radical-dependent cell killing with hydrogen peroxide, one might expect other membranes, whose integrity was not similarly assessed, to be damaged either simultaneously with or shortly after the inner
mitochondrial membrane. Collapse of the mitochondrial membrane potential may simply reflect more generalized membrane damage and may not contribute directly to the sequence of events producing loss of cellular viability.

In the situation where the cytosol of the hepatocyte was flooded with calcium ions from the extracellular medium, there are several potential mechanisms that can account for a collapse of the mitochondrial membrane potential. When liver mitochondria are incubated with an energy source and relatively large concentrations of calcium, a period of active accumulation of calcium ions is followed by a collapse of the mitochondrial membrane potential with discharge of the sequestered calcium ions back into the medium. Activation of a phospholipase activity with degradation of the phospholipids of the inner mitochondrial membrane has been proposed as a mechanism to alter the permeability properties of this membrane such that an electrochemical gradient can no longer be maintained (22–24). Alternatively, the collapse of the mitochondrial membrane potential in the face of very high cytosolic calcium ion concentrations could be a consequence of the dissipation of the energy gradient across the inner membrane in a futile uptake of calcium on the electrogenic uniporter and re-release of calcium due to the presence of the ionophore A23187 (25).

Whatever the mechanism, there is no evidence that links the loss of mitochondrial energization with the subsequent manifestations of irreversible cell injury. Clearly, however, the early manifestation of mitochondrial dysfunction in both the situations studied here whereby hepatocytes could be lethally injured would demand further consideration of the role that it may potentially play in the killing of the cells.

In conclusion, in this paper two methods of lethally injuring cultured hepatocytes have been contrasted and compared. In the first case, an alteration in intracellular calcium homeostasis could be dissociated from the hydroxyl radical-dependent mechanisms of cell injury occurring with an acute oxidative stress. In the second, an altered calcium homeostasis was directly imposed on the hepatocytes, and the accompanying cell killing could be attributed to the flooding of the cytosol of the hepatocytes with calcium ions. Whether the finding in both models of early loss of the mitochondrial membrane potential is an important event in the genesis of lethal cell injury or mere coincidence remains to be considered.

REFERENCES