Annu. Rev. Pharmacol. Toxicol. 1995. 35:129–44

AN EVALUATION OF THE ROLE OF CALCIUM IN CELL INJURY

Andrew W. Harman and Michael J. Maxwell
Department of Pharmacology, The University of Western Australia, Nedlands, Western Australia 6009

KEY WORDS: oxidative stress, mitochondria, cell death

ABSTRACT

It has been proposed that a number of chemical-induced cell injuries result from disrupton of the ability of the cell to control calcium. Many of the techniques used to develop this theory have relied on indirect measurements of intracellular calcium. The advent of digital imaging fluorescence microscopy has allowed a more direct examination of the relationship between calcium and cell damage. Results indicate that cytosolic calcium does not play a central role in the initiation of oxidative injury in a number of cell types. Changes in calcium homeostasis occur well after the appearance of other indications of cell injury. However, recent studies indicate that a mitochondrial lesion occurs relatively early in the time course of oxidative cell injury. Calcium may play a role in the development of this lesion.

INTRODUCTION

An understanding of the mechanisms by which chemicals damage cells has important implications in the treatment of overdose poisoning and in the design of new drugs and chemicals that are less toxic. These tasks would be simplified if there were some common underlying mechanism for the initiation or progression of chemical-induced cell injury. In recent years much interest has centered on the proposal that calcium ions play a central role in a variety of chemical toxicities.

0362-1642/95/0415-0129$05.00

The calcium hypothesis arose from observations by pathologists that liver damage caused by toxic chemicals was associated with the accumulation of large amounts of calcium. It was proposed that calcium entry into the cell may be involved in tissue damage. Farber et al (1) suggested that there were two steps in the killing process: (*a*) disruption of the integrity of the cell membrane, followed by (*b*) an influx of extracellular calcium across the damaged membrane and down a steep concentration gradient, with a resultant activation of calcium-dependent detrimental processes in the cell. This concept was refined by Orrenius et al (2), who proposed a general scheme for toxic cell injury caused by calcium. Normally, the concentration of intracellular free calcium ions $[Ca^{2+}]_i$ is some four orders of magnitude less than the extracellular level. This concentration gradient is maintained by the transport of calcium out of the cell by ATPases as well as by compartmentalization into intracellular stores, principally within the mitochondria and the endoplasmic reticulum. It was hypothesized that certain chemicals may interfere with these translocation systems and hence precipitate an uncontrolled rise in $[Ca^{2+}]_i$, either from intracellular stores or from the extracellular space. The rise in $[Ca^{2+}]_i$ could then lead to a number of detrimental cellular processes, including the disruption of the cytoskeleton and activation of calcium-dependent catabolic enzymes (proteases, phospholipases, endonucleases). Chemicals believed to be involved in calcium-mediated toxic cell injury include carbon tetrachloride, bromobenzene, chloroform, acetaminophen (paracetamol), cystamine, compounds that are used to model oxidative stress [e.g. *tert*-butylhydroperoxide (tBH), menadione] (3), and compounds that induce chemical hypoxia (4).

However, the calcium hypothesis fails to explain many biological phenomena associated with cell injury. Results from a number of research groups now indicate that calcium plays a more limited role in chemical-mediated toxicities. This review critically examines the basis of the calcium hypothesis and highlights new directions for research in the field.

MEASUREMENT OF INTRACELLULAR FREE CALCIUM

The calcium hypothesis was conceived at a time when the concentration of $[Ca^{2+}]_i$ was usually measured by somewhat indirect methods. Many of these methods have significant limitations when used in toxicological studies. Consequently, the results from many earlier studies may be misleading. The following techniques have been used to assess the role of calcium in toxic cell injury.

Arsenazo III Uptake

Uptake of the dye arsenazo III has been used to study the compartmentalization of calcium into mitochondrial and extramitochondrial pools in hepatocytes

exposed to an oxidative stress (5). However, arsenazo III is a potent inhibitor of the sarcoplasmic reticulum calcium pump and attenuates uptake of calcium into that organelle (6). Because the dye itself can influence calcium distribution, caution should be exercised in interpreting the effects of chemicals on processes involving calcium transport.

Phosphorylase a *Activity*

A number of researchers have used an increase in phosphorylase *a* activity as an indirect measure of an increase in $[Ca^{2+}]_i$ (7–9). This measurement is based on calcium's ability to activate phosphorylase *b* kinase via calmodulin, which in turn, increases phosphorylase *a* activity (10). However, this explanation of the rise in phosphorylase *a* activity is only one among several, the rest of which are independent of changes in $[Ca^{2+}]_i$. For example, cyclic AMP bound to calmodulin can activate phosphorylase *b* kinase with a resultant increase in phosphorylase *a* activity. And respiratory toxins can enhance the rate of glycolysis by interfering with the rate of the phosphorylation-dephosphorylation equilibrium of the cell, resulting in activation of phosphorylase *a* (11). Glycogen phosphorylase activity can also be enhanced by an increase in ADP or AMP or by a fall in ATP (10). These effects are not dependent on changes in $[Ca^{2+}]_i$. In sum, an increase in phosphorylase *a* can result from several biological events, such as a decreased energy supply to the cell, and may not be a calcium-related event.

Quin-2

Quin-2 was one of the first calcium-indicative dyes used to monitor $[Ca^{2+}]_i$ directly in isolated cells. Quin-2's ability to be loaded by a noninvasive technique gives it an advantage over dyes such as aequorin that have to be loaded by microinjection. To load quin-2, the cell suspension is incubated in the presence of a cell-permeant ester of quin-2, which is deesterified by intracellular esterases, thus trapping the impermeant form of the dye in the cell (12). The concentration of $[Ca^{2+}]_i$ is then determined based on a shift in fluorescence properties when calcium binds to the dye (13).

There are two major problems associated with using quin-2 to measure $[Ca^{2+}]_i$ concentration. Firstly, high concentrations of quin-2 are toxic to isolated rat hepatocytes (14). Thus, the interpretation of toxicity studies using this dye are complicated by its own toxic effects. Secondly, quin-2 is a strong chelator of soluble ferric ions (15, 16). Ferric ions play a central role in oxidative cell injury by acting as catalysts for the formation of hydroxyl radicals (17). Hence, quin-2 itself may ameliorate the toxic effects of oxidative stress by decreasing hydroxyl radical formation. The fact that quin-2 has both an inherent toxic effect, as well as a cytoprotective effect against oxidative stress, is a fatal

complication for the interpretation of toxicological studies of other toxins when used together with this dye.

An example of such a complication is illustrated by Nicotera et al (18). Quin-2 was found to delay the onset of bleb formation and loss of viability caused by potassium cyanide (KCN) and iodoacetate in hepatoma 1c1c7 cells. This effect was attributed to the chelation of calcium by quin-2. It was proposed that the chelation by quin-2 prevented the rise in $[Ca^{2+}]_i$ and hence protected the cell from injury. However, it is thought that hydroxyl radical formation plays a role in chemical hypoxic injury (19). Chelation of ferric ions by quin-2 and the resultant decrease in the formation of hydroxyl radicals may account for this protective effect rather than its being the result of calcium chelation.

Use of Suspensions of Isolated Hepatocyte

A number of studies examining the role of calcium in cell injury have been performed in suspensions of isolated hepatocytes preloaded with calcium-indicative dyes such as quin-2 or fura-2. As these cells are used soon after enzymatic disruption of the tissue, it has been argued that the injury suffered during isolation renders them more fragile than cells in culture. In addition, incubation of these isolated cells in an atmosphere of 95% O_2 and 5% CO_2 may further increase susceptibility to oxidative stress because of the high oxygen tension (20, 21).

The fluorescence signal obtained from cell suspensions is the average response from a large number of cells that are in various stages of responding to a toxic insult. Studies of cells in culture now have a major advantage over suspension systems. The advent of fluorescence microscopy techniques allows the concentration of $[Ca^{2+}]_i$ to be followed in single cultured cells (22). The digital imaging technique is superior for measuring the $[Ca^{2+}]_i$ level as it allows greater temporal and spatial resolution than could possibly be achieved using cell suspensions (22, 23). The obvious advantage of this technique is that it clearly defines the events that occur in single cells, rather than giving an overall signal that is the mean result of changes in many millions of cells, as is the case when using fura-2–loaded hepatocytes in suspension. An additional advantage of the digital imaging technique is that changes in $[Ca^{2+}]_i$ can be compared to other morphometric and biochemical parameters in the same cell (22). The use of this technique has resulted in a major reevaluation of the role of calcium in cell injury.

CALCIUM AND OXIDATIVE CELL INJURY

The Source of the Calcium

It has been proposed that oxidative stress causes a decrease in the activity of calcium-translocating enzyme systems in the cell and that this results in an

uncontrolled rise in $[Ca^{2+}]_i$ (2, 3). There are at least three ways in which oxidative damage to the cell might result in a rise in $[Ca^{2+}]_i$.

INTRACELLULAR CALCIUM RELEASE The calcium hypothesis proposes that oxidative injury is associated with the oxidation of sulfhydryl groups on Ca^{2+} ATPases in the cell (3, 24, 25). Inhibition of ATPase activity decreases the ability of organelles such as the endoplasmic reticulum to sequester calcium and of plasma membrane calcium pumps to extrude calcium from the cell. These initial toxic lesions would then result in an increase in $[Ca^{2+}]_i$ because of the inability of the cell to sequester it. This increase in turn activates calcium-dependent degradative pathways that produce, or at least contribute to, cell death (3, 24, 25). The terms *cell death* and *irreversible cell damage* seem to be used interchangeably in the literature and generally describe an irreversible point when the permeability barrier of the cell membrane is lost.

INFLUX OF EXTRACELLULAR CALCIUM The second possibility is that an influx of extracellular calcium, resulting from damage to the plasma membrane caused by the toxin, causes the rise in cytosolic calcium in the cell. This influx is essentially a secondary event preceded by the toxic lesion. Calcium may enter through sodium channels (26) or through pores opened by damage to the cell membrane. If the ability of the cell to pump calcium across the plasma membrane and out of the cell by ATP-dependent processes is compromised, $[Ca^{2+}]_i$ will rise and may then activate degradative processes that contribute to the progression of the toxic lesion.

PATHOLOGICAL INFLUX OF EXTRACELLULAR CALCIUM The third possibility is that the rise in $[Ca^{2+}]_i$ is simply an epiphenomenon that occurs in association with the loss of cell viability. The cell membrane becomes physically damaged and extracellular calcium passes into the cytosol of an already irreversibly damaged cell.

Characteristics of Oxidative Cell Injury

Results from a number of groups studying a variety of cell types indicate that oxidant-induced increases in $[Ca^{2+}]_i$ result from an influx of calcium from the extracellular medium. The following examples of experimental systems illustrate that the source of calcium is the extracellular space. In each case, exposure to a toxic agent caused a rise in $[Ca^{2+}]_i$, yet incubation in a low-calcium buffer prevented this increase:

1. Isolated rat hepatocytes under anoxic conditions (26),
2. Cultured mouse hepatocytes exposed to acetaminophen (27),

3. Rat proximal-tubule epithelial cells incubated in a xanthine-xanthine oxidase system to generate free radicals of oxygen outside the cell (28),
4. Isolated alveolar rat macrophages exposed to hydrogen peroxide and ferric ions (29),
5. Neuronally derived cell line PC12 exposed to cyanide (30).

Therefore, from results in a variety of cell types, it appears that the source of calcium is extracellular, and there is little direct evidence to support an increase arising from intracellular stores.

Calcium Does Not Have a Causative Role in Cell Injury

While many studies indicate an association of a rise in $[Ca^{2+}]_i$ with cell toxicity, there is little direct evidence that this rise causes cell injury. It would seem that if a rise in $[Ca^{2+}]_i$ plays a central role in cell injury, then two features of cell injury should always be present: An increase in $[Ca^{2+}]_i$ must occur prior to cell injury, and prevention of such an increase should ameliorate cell injury.

AN INCREASE IN CALCIUM DOES NOT PRECEDE INJURY

Results from a number of cell types indicate that the rise in $[Ca^{2+}]_i$ associated with cell injury does not precede the toxic lesion.

Hepatocytes The formation of cell surface blebs is an early manifestation of injury in hepatocytes (2, 31). It is known that various structural components of the cytoskeleton are influenced to a degree by $[Ca^{2+}]_i$ (32). Orrenius et al have proposed that disruption of calcium homeostasis results in cytoskeletal alterations by causing the dissociation of actin filaments from actin in the plasma membrane and by activating proteases that cleave actin-binding proteins and prevent the anchorage of cytoskeletal elements to the plasma membrane (3). The presence of cell surface blebs was taken as evidence for calcium involvement in this process. The supporting evidence is indirect and includes the facts that calcium ionophore produces a similar cell-blebbing phenomenon (2) and that chelators of calcium largely prevent blebbing (33).

In 1987 Lemasters et al demonstrated that hypoxia, previously thought to be a calcium-mediated injury, was not associated with a rise in $[Ca^{2+}]_i$ concentration (22). Using digital imaging fluorescence microscopy (DIFM), this group subsequently demonstrated that bleb formation precedes changes in $[Ca^{2+}]_i$ in single hepatocytes when exposed to either mercuric chloride or cystamine (34). Thus, bleb formation can be independent of a rise in $[Ca^{2+}]_i$. Axiomatically, an elevation in $[Ca^{2+}]_i$ cannot play a causative role in bleb formation under the conditions of chemical hypoxia in cultured rat hepatocytes. These results were confirmed by Farber et al (35), who, using a similar DIFM

technique, demonstrated that the killing of single cultured rat hepatocytes by tBH was not associated with a sustained increase in $[Ca^{2+}]_i$.

It is difficult to reconcile these results with a calcium-mediated toxic response. Studies that have indicated a causative role for calcium have relied on the protective properties of calcium chelators or the promotional effects of calcium ionophores on toxicity. In this case, what has been attributed to a causative effect of calcium may be related to other properties of such chelators or ionophores. For example, an ionophore may result in some other damage, such as a fall in ATP, and chelating agents may have prevented oxidative stress by chelation of soluble ferric ions and thus prevented hydroxyl radical formation and subsequent oxidative injury.

It has been suggested that elevated $[Ca^{2+}]_i$ plays a key role in acetaminophen toxicity (7–9, 36). However, these studies have relied, in the main, on indirect indicators of calcium, such as measurement of phosphorylase *a* activity. When $[Ca^{2+}]_i$ was measured more directly in single mouse hepatocytes using DIFM, $[Ca^{2+}]_i$ increased only in the late stage of intoxication and at a time well after the initiation of irreversible cell injury (27). Similarly, the toxicity of carbon tetrachloride has been attributed to release of calcium from the endoplasmic reticulum (37). However, it has been shown more recently that when isolated hepatocytes are incubated with 0.172 mM carbon tetrachloride, cell death is not associated with a rise in intracellular calcium. Cell calcium only increased in conjunction with a loss of cell membrane permeability, and neither chelators of calcium nor inhibitors of phospholipases or proteases protected the cell from injury (38).

Results from this laboratory using DIFM have demonstrated that toxicity resulting from exposure to either adriamycin, alloxan, menadione, or tBH was not associated with an increase in $[Ca^{2+}]_i$ (AW Harman & MJ Maxwell, unpublished data). These chemicals have been used as model oxidants in studies of oxidative stress. Figure 1 shows $[Ca^{2+}]_i$ in a single mouse hepatocyte exposed to a toxic concentration of tBH in a calcium-containing buffer from a time before exposure (point A) until the cell membrane ruptures (point D). Morphological changes, thought previously to result from a calcium-dependent disruption of the cell cytoskeleton, occur prior to any change in $[Ca^{2+}]_i$. For example at point B, there are obvious signs of disruption of the cell membrane (blebs), yet $[Ca^{2+}]_i$ is unchanged. Such data demonstrate that the rise in $[Ca^{2+}]_i$ that occurs relatively late in the poisoning process and shortly before cell death results from an influx of extracellular calcium across a damaged cell membrane. Figure 2 shows a typical experiment comparing the effect of exposure to tBH on $[Ca^{2+}]_i$ in a cell incubated in a calcium-containing buffer with that in a cell in a buffer with no added calcium (low-calcium buffer). In the latter case, there was no increase in $[Ca^{2+}]_i$, yet the time course for loss of cell viability was much the same as that for the cell incubated in a calcium-con-

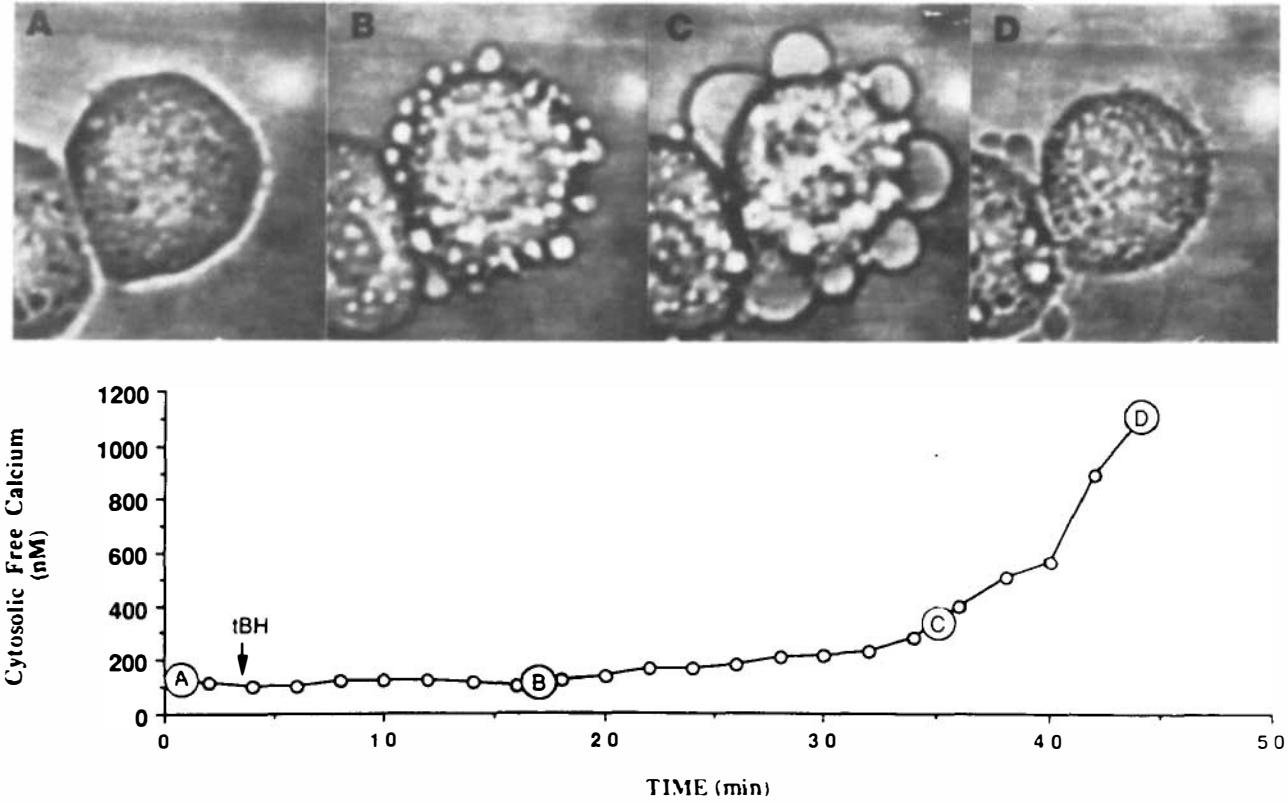

Figure 1 Time course for $[Ca^{2+}]_i$ in a single mouse hepatocyte exposed to *tert*-butylhydroperoxide (tBH) (0.25 mM) in calcium-containing Krebs Ringer Hepes (KRH) buffer (115 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 25 mM Hepes; pH 7.4). Calcium measurements were performed by DIFM using methods described previously (27). Images of cell morphology were recorded: (*A*) prior to exposure to tBH, (*B*) 13 min after addition of tBH, (*C*) 31 min after addition of tBH, and (*D*) 40 min after tBH addition.

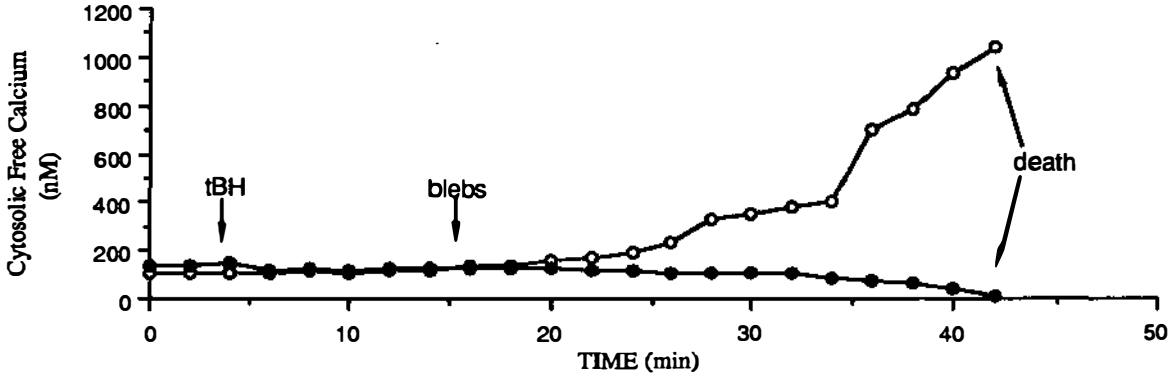

Figure 2 Time course for $[Ca^{2+}]_i$ in single mouse hepatocytes exposed to tBH (0.25 mM) in KRH (*open circles*) and low-calcium KRH (*solid circles*).

taining buffer. This indicates that the toxicity of tBH in cultured mouse hepatocytes is not dependent on a rise in $[Ca^{2+}]_i$.

Neuronal cells It has been proposed by some researchers that elevated $[Ca^{2+}]_i$ is associated with hypoxic injury in neuronal cells (39, 40), while others have demonstrated that toxicity precedes increased $[Ca^{2+}]_i$ (41). However, in this instance, as is the case for other cell types, much of the evidence supporting a role for calcium is indirect, as calcium levels are not monitored directly (39). A more recent study measured $[Ca^{2+}]_i$ using DIFM in single spinal neurons (42). Exposure to a cytotoxic concentration of glutamate resulted in an initial transient rise in $[Ca^{2+}]_i$, which was followed by a prolonged period at near basal levels. In lethally injured cells $[Ca^{2+}]_i$ increased to greater than millimolar concentrations shortly before cell death. However, this rise in $[Ca^{2+}]_i$ was not an initiating causative event; rather it was found to be associated with the loss of the ability of the damaged cell to regulate $[Ca^{2+}]_i$.

Clearly an increase in $[Ca^{2+}]_i$ on its own is not sufficient to cause irreversible cell injury. In some cases the opposite appears to be the case. For example, a sustained increase in intracellular calcium in cultured chick embryo ciliary ganglia has been found to promote, rather than decrease, the survival of cultured neuronal cells (43).

Cardiac myocytes Much interest has centered on the role of calcium in myocardial ischemia, particularly in relation to reperfusion injury. It has been proposed that reperfusion injury is caused by the formation of free radicals of oxygen (44) and disruption of calcium homeostasis (45). When cultured neonatal rat cardiac myocytes were exposed to a chemical hypoxia (deoxyglucose plus cyanide), there was an increase in $[Ca^{2+}]_i$ and a decrease in intracellular pH (46). When these cells were washed and subsequently incubated in control medium to simulate the conditions of reperfusion, there was a rapid loss of cell viability associated with a rise in intracellular pH. However, there was no evidence that calcium had a causative role in this type of injury. If the pH rise was blocked with dimethylamiloride, the cell viability was maintained even though $[Ca^{2+}]_i$ increased. Conversely, if myocytes were reperfused in the presence of dichlorobenzamil (a Na^+-Ca^{2+} exchange inhibitor), $[Ca^{2+}]_i$ fell, but there was no protective effect from cell lethality (47). It appears that a change in intracellular pH, rather than calcium overload, was the causative factor in reperfusion injury.

INCUBATION IN A CALCIUM-FREE MEDIUM If a rise in $[Ca^{2+}]_i$ were central to the mechanism of oxidative cell injury, then preventing this rise should protect from toxicity. As discussed above, studies that applied techniques such as chelating calcium failed to take into account the effects that the chelator itself

may have, such as chelating soluble ferric ions and thus decreasing the formation of hydroxyl radicals. On the other hand, studies with isolated cells in low-calcium buffers indicated little or no protective effect from exposure to oxidants despite the absence of a $[Ca^{2+}]_i$ increase (27, 34) (Figure 2). It is clear from these investigations that oxidative cell injury can occur independently of a rise in $[Ca^{2+}]_i$.

NEW DIRECTIONS—ROLE OF MITOCHONDRIAL INJURY

Research into the mechanism of oxidative cell injury is shifting away from the central association of cytosolic calcium with lethal cell injury to focus on adverse effects of oxidants on mitochondria. Two hypotheses attempt to explain lethal cell injury in terms of how $[Ca^{2+}]_i$ may be involved in mitochondrial dysfunction.

Calcium Cycling in the Mitochondria

It has been suggested that excessive cycling of calcium across the inner mitochondrial membrane is responsible for decreased mitochondrial function during oxidative stress. Calcium cycles continuously across the inner membrane; it is taken up via a uniporter in response to the mitochondrial membrane potential and released by an antiport system in exchange for H^+ or Na^+. It has been proposed that if mitochondria were required to take up an excessive amount of calcium, then the mitochondrial membrane potential would collapse. Further, oxidation of intramitochondrial NAD(P)H would result in calcium efflux and accelerate calcium cycling (24, 48).

Experiments that support this hypothesis have used either ethylene glycol-bis(β-aminoethyl ether) (EGTA) or quin-2 to chelate calcium and have found a correlation between the presence of a chelator and protection of the mitochondria (24, 48). As indicated above, both these chelating agents bind ferric ions, and it is also possible that the protection may be associated with reduced formation of hydroxyl radicals rather than an effect of chelating excess calcium.

Calcium cycling would seem to be an unlikely process during oxidative stress. The energy requirement of calcium cycling is modest (49), and very large fluxes of calcium would be required to depolarize the membrane. In such a case, one would expect a substantial rise in $[Ca^{2+}]_i$ to occur prior to mitochondrial depolarization. Research from this laboratory indicates that one of the earlier events in oxidative stress is a mitochondrial depolarization. Figure 3 illustrates that mitochondrial depolarization occurs relatively early in the poisoning process when mouse hepatocytes are exposed to a toxic concentration of alloxan. Rhodamine 123 is distributed electrophoretically into the

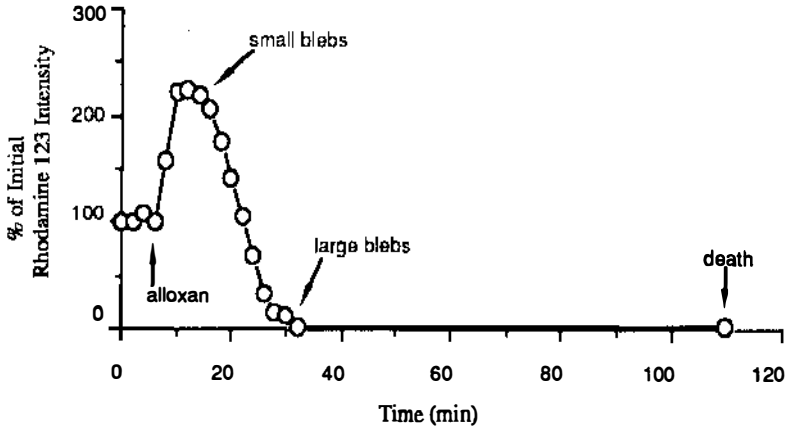

Figure 3 Fluorescence intensity of single mouse hepatocytes preloaded (60 min, 37°C) with 800 nM rhodamine 123 and exposed to alloxan (10 mM). Fluorescence (475 nm excitation; > 520 nm emission) was digitalized and quantitated using *DIFM*. Propridium iodide was used to monitor cell membrane permeability. Loss of membrane viability resulted in fluorescent staining of the cell nucleus (575 nm excitation; 590 nm emission). These data are from five individual hepatocytes, where fluorescence was averaged across each cell every 2 min. In control cells not exposed to alloxan, the fluorescence signal was relatively stable for 120 min (data not shown).

mitochondrial matrix in response to the mitochondrial membrane potential (49a). Following exposure to alloxan there was a lag phase of about 5 min, followed by an increase in rhodamine 123 fluorescence. This increase represented unquenching of the dye as it moved from the mitochondria to the cytosol following loss of the mitochondrial membrane potential (49a). As rhodamine 123 was partially charged, it only slowly diffused out of the cell down its concentration gradient. However, an increase in $[Ca^{2+}]_i$ is a comparatively late event, as illustrated in Figures 1 and 2. We found that with a number of oxidants (alloxan, tBH, adriamycin, menadione), deenergization of mitochondria occurred well before a rise in $[Ca^{2+}]_i$ (AW Harman & MJ Maxwell, unpublished data).

ATP Depletion

It has been known for some time that oxidative stress causes a permeability transition of the inner mitochondrial membrane, which involves increased permeability to ions, mitochondrial swelling, uncoupling of oxidative phosphorylation, and collapse of the mitochondrial membrane potential (50, 51). Evidence suggests that this transition is caused by changes in the deacylation-reacylation cycle of inner mitochondrial phospholipids. Oxidative stress inhibits reacylation of lysophosphatides, and deacylation is stimulated by enhanced

phospholipase A_2 activity in response to increased mitochondrial calcium (52). The accumulation of lysophospholipids and free fatty acids results in a permeability change in the inner mitochondrial membrane. This permeability transition can be stimulated by exogenous lysophospholipids (53) and ameliorated by phospholipase A_2 inhibitors (54) and cyclosporin A (55).

Therefore, it is possible that calcium plays a role in activating phospholipase A_2 activity in the inner mitochondrial membrane. This may lead to increased permeability of the inner mitochondrial membrane and loss of the mitochondrial membrane potential. The calcium homeostasis–thiol oxidation theory does predict that lethal cell injury is in response to activation of degradative enzymes, such as phospholipases (2, 3). However, it predicts an increase in $[Ca^{2+}]_i$ resulting from calcium efflux from mitochondria as a result of oxidation of intramitochondrial NAD(P)H, not an increase in mitochondrial calcium. The source of any such increase in mitochondrial calcium has yet to be determined.

It has recently been proposed that the major determinant of lethal cell injury caused by oxidative stress is ATP depletion. Hepatocytes exposed to tBH can be protected as long as ATP levels are maintained (56). Oxidative stress was found to be associated with inhibition of mitochondrial ATP production. However, hepatocytes were protected by addition of the glycolytic substrate, fructose (to maintain ATP), plus the combination of cyclosporin A and trifluoperazine (a phospholipase A_2 inhibitor), which prevented the mitochondrial permeability transition. Thus, it appears that as long as ATP levels can be maintained and the permeability transition prevented, the cell survives. This would indicate that the permeability transition, as well as the subsequent collapse of the mitochondrial membrane potential and loss of ATP, is central to the mechanism of oxidative stress. Calcium may play a role in activating phospholipase A_2 in this process.

CONCLUSION

The hypothesis that a rise in $[Ca^{2+}]_i$ plays a central role in chemical injuries associated with oxidative stress is not supported by recent data. Studies using DIFM techniques indicate that a rise in $[Ca^{2+}]_i$ is a relatively late event in the poisoning process initiated by oxidants and that the increase in $[Ca^{2+}]_i$ results from influx from the extracellular environment. However, an increase in mitochondrial calcium may play a role in phospholipase A_2 activation and in promotion of a permeability transition in the inner mitochondrial membrane that appears to be associated with oxidant-induced loss of mitochondrial function and ATP depletion.

Literature Cited

1. Schanne FAX, Kane AB, Young EE, Farber JL. 1979. Calcium dependence on toxic cell death: a final common pathway. *Science* 206:700–2
2. Jewell SA, Bellomo G, Thor H, Orrenius S. 1982. Bleb formation in hepatocytes during drug metabolism is caused by disturbances in thiol and calcium ion homeostasis. *Science* 217:1257–59
3. Orrenius S, McConkey DJ, Bellomo G, Nicotera P. 1989. Role of Ca^{2+} in toxic cell killing. *Trends Pharmacol. Sci.* 10: 281–85
4. Trump BF, Berezesky IK. 1985. The role of calcium in cell injury and repair: a hypothesis. *Surv. Synth. Pathol. Res.* 4:248–56
5. Bellomo G, Jewell SA, Thor H, Orrenius S. 1982. Regulation of intracellular calcium compartmentation: studies with isolated hepatocytes and t-butyl hydroperoxide. *Proc. Natl. Acad. Sci. USA* 79:6842–46
6. Riollet S, Champeil P. 1987. The calcium-sensitive dye arsenazo III inhibits calcium transport and ATP hydrolysis by sarcoplasmic reticulum calcium pump. *Anal. Chem.* 162:160–62
7. Long RM, Moore L. 1988. Evaluation of the calcium mobilizing action of acetaminophen and bromobenzene in rat hepatocyte cultures. *J. Biochem. Toxicol.* 3:353–62
8. Tsokos-Kuhn JO. 1989. Evidence in vivo for elevation of intracellular free Ca^{2+} in the liver after diquat, acetaminophen and CCl_4. *Biochem. Pharmacol.* 38:3061–65
9. Moore MH, Thor G, Moore G, Nelson SD, Moldeus P, Orrenius S. 1985. The toxicity of acetaminophen and N-acetyl-p-benzoquinoneimine in isolated hepatocytes is associated with thiol depletion and increased cytosolic Ca^{2+}. *J. Biol. Chem.* 260:13035–40
10. Hems DA, Whitton PD. 1980. Control of hepatic glycogenolysis. *Physiol. Rev.* 60:1–50
11. Jakob A, Diem S. 1974. Activation of glycogenolysis in perfused rat livers by glucagon and metabolic inhibitors. *Biochim. Biophys. Acta* 362:469–79
12. Tsien RY. 1981. A non-disruptive technique for loading calcium buffers and indicators into cells. *Nature* 290: 527–28
13. Grynkiewicz G, Poenie M, Tsien RY. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260: 3440–50
14. Carpenter-Deyo L, Duimstra JR, Hedstrom O, Reed DJ. 1991. I. Toxicity to isolated hepatocytes caused by the intracellular calcium indicator, quin-2. *J. Pharmacol. Exp. Ther.* 258:739–46
15. Carpenter-Deyo L, Reed DJ. 1991. II. Involvement of calcium and iron in quin-2 toxicity to isolated hepatocytes. *J. Pharmacol. Exp. Ther.* 258:747–52
16. Schnellmann RG. 1991. Intracellular calcium chelators and oxidant-induced renal proximal tubule cell death. *J. Biochem. Toxicol.* 6:299–303
17. Halliwell B, Gutteridge JMC. 1984. Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem. J.* 219:1–14
18. Nicotera P, Thor H, Orrenius S. 1989. Cytosolic-free Ca^{2+} and cell killing in hepatoma 1c1c7 cells exposed to chemical hypoxia. *FASEB J.* 3:59–64
19. Gores GJ, Flarsheim CE, Dawson TL, Nieminen A-L, Herman B, Lemasters JJ. 1989. Swelling, reductive stress, and cell death during chemical hypoxia in hepatocytes. *Am. J. Physiol.* 26:C347–54
20. Farber JL. 1990. The role of calcium in lethal cell injury. *Chem. Res. Toxicol.* 3:503–16
21. Smith MW, Phelps, PC, Trump, BF. 1991. Injury-induced changes in cytosolic Ca^{2+} in individual rabbit proximal tubule cells. *Am. J. Physiol.* 262:F647–655
22. Lemasters JJ, DiGuiseppi J, Nieminen A-L, Herman B. 1987. Blebbing, free calcium and mitochondrial membrane potential preceding cell death in hepatocytes. *Nature* 325:78–81
23. DiGuiseppi J, Inman R, Ishihara A, Jacobson K, Herman B. 1985. Applications of digitized fluorescence microscopy to problems in cell biology. *BioTechniques* 3:394–403
24. Nicotera P, Bellomo G, Orrenius S. 1990. The role of Ca^{2+} in cell killing. *Chem. Res. Toxicol.* 3:484–94
25. Nicotera P, Bellomo G, Orrenius S. 1992. Calcium-mediated mechanisms in chemically induced cell death. *Annu. Rev. Pharmacol. Toxicol.* 32:449–70
26. Gasbarrini A, Borle AB, Van Thiel DH. 1993. Ca^{2+} antagonists do not protect isolated perfused rat hepatocytes from anoxic injury. *Biochim. Biophys. Acta* 1177:1–7

27. Harman AW, Mahar SO, Burcham PC, Madsen BW. 1992. Level of cytosolic free calcium during acetaminophen toxicity. *Mol. Pharmacol.* 41:665–70
28. Swann JD, Smith MW, Phelps PC, Maki A, Berezesky IK, Trump BF. 1991. Oxidative injury induces influx-dependent changes in intracellular calcium homeostasis. *Toxicol. Pathol.* 19:128–37
29. Rojanasakul Y, Wang L, Hoffman AH, Shi X, Dalal NS, et al. 1993. Mechanisms of hydroxyl free radical–induced cellular injury and calcium overloading in alveolar macrophages. *Am. J. Respir. Cell Mol. Biol.* 8:377–83
30. Johnson JD, Conroy WG, Isom GE. 1987. Alteration of cytosolic calcium levels in PC12 cells by potassium cyanide. *Toxicol. Appl. Pharmacol.* 88:217–24
31. Lemasters JJ, Thurman RG. 1981. Centrilobular injury following hypoxia in isolated perfused rat liver. *Science* 213: 661–63
32. Weeds A. 1982. Actin-binding proteins—regulators of cell architecture and motility. *Nature* 296:811–16
33. Mirabelli F, Salis A, Vairetti M, Bellomo G, Thor H, Orrenius S. 1989. Cytoskeletal alterations in human platelets exposed to oxidative stress are modulated by oxidative and Ca^{2+}-dependent mechanisms. *Arch. Biochem. Biophys.* 270:478–88
34. Lemasters JJ, Nieminen A-L, Gores GJ, Wray BE, Herman B. 1989. Cytosolic free calcium and cell injury in hepatocytes. In *Cell Calcium Metabolism*, ed. G Fiskum, pp. 46–70. New York: Plenum
35. Sakaida I, Thomas AP, Farber JL. 1991. Increases in cytosolic calcium ion concentration can be dissociated from the killing of cultured hepatocytes by *tert*-butyl hydroperoxide. *J. Biol. Chem.* 266: 717–22
36. Tsokos-Kuhn JO, Hughes H, Smith CV, Mitchell JR. 1988. Alkylation of the liver plasma membrane and inhibition of the (Ca^{2+}, Mg^{2+})-ATPase by acetaminophen. *Biochem. Pharmacol.* 37: 2125–31
37. Long RL, Moore L. 1986. Elevated cytosolic calcium in rat hepatocytes exposed to carbon tetrachloride. *J. Pharmacol. Exp. Ther.* 238:186–91
38. Albano E, Carini R, Parola M, Bellomo G, Goria-Gatti L, et al. 1989. Effects of carbon tetrachloride on calcium homeostasis. A critical reconsideration. *Biochem. Pharmacol.* 38:2719–25
39. Choi DW. 1988. Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage. *Trends Neurosci. Sci.* 11:465–69
40. Farooqui AA, Horrocks LA. 1991. Excitatory amino acid receptors, neural membrane phospholipid metabolism and neurological disorders. *Brain Res. Rev.* 16:171–91
41. Price MT, Olney JW, Samson L, Labruyere J. 1985. Calcium influx accompanies but does not cause excitotoxin-induced neuronal necrosis in retina. *Brain Res. Bull.* 14:369–76
42. Tymianski M, Charlton MP, Carlen PL, Tator CH. 1993. Secondary Ca^{2+} overload indicates early neuronal injury which precedes staining with viability indicators. *Brain Res.* 607:319–23
43. Collins F, Schmidt MF, Guthrie PB, Kater SB. 1991. Sustained increase in intracellular calcium promotes neuronal survival. *J. Neurosci.* 11:2582–87
44. McCord JM. 1985. Oxygen-derived free radicals in post-ischemic tissue injury. *N. Engl. J. Med.* 312:159–63
45. Nayler WG, Poole-Wilson PA, Williams A. 1979. Hypoxia and calcium. *J. Mol. Cell Cardiol.* 11:683–706
46. Bond JM, Chacon E, Herman B, Lemasters JJ. 1993. Intracellular pH and Ca^{2+} homeostasis in the pH paradox of reperfusion injury to neonatal rat cardiac myocytes. *Am. J. Physiol.* 34:C129–37
47. Bond JM, Harper IS, Chacon E, Reece JM, Herman B, Lemasters JJ. 1994. The pH paradox in the pathophysiology of reperfusion injury to rat neonatal cardiac myocytes. *Ann. NY Acad. Sci.* 723:25–37
48. Moore GA, Jewell SA, Bellomo G, Orrenius S. 1983. On the relationship between Ca^{2+} efflux and membrane damage during tert-butyl hydroperoxide metabolism by liver mitochondria. *FEBS Lett.* 153:289–92
49. Stucki JW, Ineichen A. 1974. Energy dissipation by calcium recycling and the efficiency of calcium transport in rat liver mitochondria. *Eur. J. Biochem.* 48: 365–75

49a. Emaus RK, Grunwald R, Lemasters JJ. 1986. Rhodamine 123 as a probe of transmembrane potential in isolated rat-liver mitochondria: spectral and metabolic properties. *Biochim. Biophys. Acta* 850:436–48

50. Hunter DR, Haworth RA, Southard JH. 1976. Relationship between configuration, function, and permeability in calcium-treated mitochondria. *J. Biol. Chem.* 251:5069–77
51. Gunter TE, Pfeiffer DR. 1990. Mechanisms by which mitochondria transport calcium. *Am. J. Physiol.* 258:C755–86

52. Beatrice MC, Steirs DL, Pfeiffer DR. 1984. The role of glutathione in retention of Ca^{2+} by mitochondria. *J. Biol. Chem.* 259:1279–87
53. Dalton S, Hughes BP, Barritt GJ. 1984. Effects of lysophospholipids on Ca^{2+} transport in rat liver mitochondria incubated at physiological Ca^{2+} concentrations in the presence of Mg^{2+}, phosphate and ATP at 37°C. *Biochem. J.* 224:423–30
54. Broekemeier KM, Schmid PC, Schmid HHO, Pfeiffer DR. 1985. Effects of phospholipase A_2 inhibitors on ruthenium red–induced Ca^{2+} release from mitochondria. *J. Biol. Chem.* 260:105–13
55. Fournier N, Ducet G, Crevat A. 1987. Action of cyclosporine on mitochondrial calcium fluxes. *J. Bioenerg. Biomembr.* 19:297–303
56. Imberti R, Nieminen A-L, Herman B, Lemasters JJ. 1993. Mitochondrial and glycolytic dysfunction in lethal injury to hepatocytes by t-butylhydroperoxide: protection by fructose, cyclosporin A and trifluoperazine. *J. Pharmacol. Exp. Ther.* 265:392–400