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Inhibition of ATP-dependent Microsomal Ca^{2+} Sequestration during Oxidative Stress and Its Prevention by Glutathione*

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The metallochromic indicator arsenazo III was used to study the effect of oxidative stress on ATP-dependent Ca^{2+} uptake by rat liver microsomes. Addition of ATP caused a rapid increase in ionophore A23187-releasable Ca^{2+} which stabilized in 2-3 min and provided a rapid and very sensitive assay for ATP-dependent Ca^{2+} sequestration. Quantitatively, this fraction was sufficient to account for virtually all of the nonmitochondrial ionophore-releasable Ca^{2+} of rat hepatocytes. Incubation with *t*-butyl hydroperoxide caused a rapid loss in the ability of microsomes to sequester Ca^{2+} in the presence of ATP. Addition of dithiothreitol or a physiological concentration of GSH to these incubations provided effective protection against the oxidative damage. ATP-dependent microsomal Ca^{2+} sequestration is therefore sensitive to oxidative damage and may be a primary site of injury leading to disturbed Ca^{2+} homeostasis during the early stages of drug hepatotoxicity. Intracellular thiols, notably GSH, may prevent these changes by protecting the microsomal Ca^{2+} pump from oxidative damage.

The intracellular free Ca^{2+} concentration is controlled by differential Ca^{2+} transport across mitochondrial, endoplasmic reticular, and plasma membranes. The influx and efflux processes of the mitochondria are known to be able to control the ambient free Ca^{2+} concentration (1, 2), and recent studies by Becker *et al.* (3) have shown that mitochondrial influx-efflux cycling can be adjusted by energy-dependent Ca^{2+} sequestration in microsomes. The Ca^{2+} uptake system of the endoplasmic reticulum has only recently been characterized in a variety of tissues and cell types, including the kidney (4), liver (5), pancreas (6), adipocyte (7), and submandibular gland (8). This system utilizes ATP and is thought to be associated with the microsomal Ca^{2+}-ATPase activity (4, 5). Up to 50% of the total cellular calcium can be located in the endoplasmic reticulum of hepatocytes (9), and therefore, modulation of microsomal Ca^{2+} may have an important regulatory role in maintaining intracellular Ca^{2+} homeostasis.

In recent studies of Ca^{2+} homeostasis in isolated hepatocytes, we found that the Ca^{2+} content of the mitochondrial and extramitochondrial compartments could be quantitated by a relatively simple method. The sequential addition of the protonophore FCCP[1] and the ionophore A23187 in the presence of the metallochromic indicator arsenazo III allowed the spectrophotometric determination of the Ca^{2+} concentrations in these two intracellular compartments (10, 11). Using this method, we have shown that exposure of hepatocytes to oxidative stress, by incubation with *t*-butyl hydroperoxide or the redox-active quinone menadione (2-methyl-1,4-naphthoquinone), causes the mobilization of Ca^{2+} and a decrease in both the mitochondrial and extramitochondrial Ca^{2+} pools (10-12). It is very likely that most of the extramitochondrial pool is Ca^{2+}-sequestered by the endoplasmic reticulum, and since this pool appears to be a primary target for Ca^{2+} mobilization and loss during oxidative stress (11, 12), in this study we have investigated the effects of oxidative stress on microsomal Ca^{2+} sequestration. For these studies, we developed a rapid spectrophotometric method for the measurement of ATP-dependent microsomal Ca^{2+} uptake which measures only the ionophore-releasable pool. The results clearly demonstrate that microsomal ATP-dependent Ca^{2+} sequestration is highly sensitive to oxidative damage caused by *t*-butyl hydroperoxide and that GSH effectively protects against this damage.

EXPERIMENTAL PROCEDURES

Microsomes were prepared from male Sprague-Dawley rats (190-220 g, fed *ad libitum*) as described by Ernster *et al.* (13). Rats were treated with phenobarbital in drinking water (1 mg/ml) for at least 7 days prior to preparation of microsomes. Microsomes were washed once by resuspending in 0.15 M KCl containing 5 μM EGTA and centrifuging 30 min at 105,000 × *g*. The pellet was resuspended in 0.25 M sucrose containing 10 mM Tris-HCl, pH 7.4, to give a final concentration of 10-15 mg/ml and stored on ice until used. Incubations were performed with approximately 1 mg of protein/ml in 125 mM KCl, 2 mM K_2HPO_4, 4 mM $MgCl_2$, and 25 mM Hepes, pH 7.0. This was the medium used by Becker *et al.* (3) to study Ca^{2+} regulation by both mitochondria and microsomes, except that the components to support mitochondrial Ca^{2+} uptake were omitted. Purified arsenazo III was dissolved in the above medium, and the concentration was determined from the $A_{654-685}$ obtained by adding excess Ca^{2+} to medium which had been titrated with EGTA to remove Ca^{2+} and using the $E_{654-685} = 2.15 \times 10^4\ M^{-1}\ cm^{-1}$ for the Ca^{2+}·arsenazo III complex. This ΔE value was determined from difference spectra of the Ca^{2+}·arsenazo III complex in the above medium. This value corresponded to the ΔE value for these wavelengths calculated from Fig. 1 of Kendrick *et al.* (14), which was obtained using similar conditions. Use of *A* for our studies was advantageous because of the turbidity due to the microsomes, and this approach was used throughout for consistency. The measured K_D was 11.3 μM, in agreement with earlier measurements in similar media (9). The high Mg^{2+} concentra-

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[1] The abbreviations used are: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; EGTA, ethylene glycol bis(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid; arsenazo III, *o*-(1,8-dihydroxy-3,6-disulfonaphthalene-2,7-bisazo)bisbenzenearsonic acid.

tion allowed addition of ATP and measurement of free Ca^{2+} concentration by absorbance changes at the wavelength pair 654 *versus* 685 nm without significant change in the spectral characteristics or sensitivity of the dye due to changes in Mg^{2+} concentration. Use of these wavelengths rather than 675 *versus* 685 nm was therefore preferred because they provided about 10-fold greater sensitivity. Dual wavelength and scanning spectrophotometry was performed with either a Sigma ZWS-11 spectrophotometer or an Aminco DW2a spectrophotometer at room temperature.

Incubations were performed in rotating round bottom flasks at 37 °C. Protein was measured by the method of Lowry *et al.* (15) with bovine serum albumin as a standard. Malonaldehyde formation was measured as the thiobarbituric acid reactive products (16).

Materials—FCCP was purchased from Boehringer Mannheim GmbH, Mannheim, Federal Republic of Germany. Arsenazo III, bovine serum albumin (crystallized), Hepes, EGTA, *t*-butyl hydroperoxide, GSH, and dithiothreitol were purchased from Sigma. The ionophore A23187 was purchased from Calbiochem-Behring. Other chemicals were at least of reagent grade and purchased locally. Deionized water was used for all solutions, except for FCCP and A23187, which were dissolved in dimethyl sulfoxide.

RESULTS AND DISCUSSION

Measurement of exchangeable Ca^{2+} in the endoplasmic reticulum and the activity of the Ca^{2+} translocase has previously been performed using isolated microsomes and either $^{45}Ca^{2+}$ as a tracer (5, 6) or direct determination of Ca^{2+} by atomic absorption spectroscopy (9). A sensitive and rapid approach to measurement of Ca^{2+} in biological systems involves the use of the metallochromic indicator for Ca^{2+}, arsenazo III (14). We have therefore used this compound to develop a new assay for Ca^{2+} sequestration by liver microsomes.

Addition of microsomes to solutions of arsenazo III had no effect on the visible absorption spectrum or extinction coefficient for the Ca^{2+}·arsenazo III complex. Furthermore, the dissociation constant for this complex was unaffected by addition of microsomes, and thus, the free Ca^{2+} concentration could be obtained from the mass action equation,

$$K_D = \frac{[Ca^{2+}\text{ free}]\,[\text{arsenazo III}]}{[Ca^{2+}\cdot\text{arsenazo III}]}$$

where K_D is 11.3 μM, [Ca^{2+}·arsenazo III] was determined by addition of a small excess of EGTA, and total [arsenazo III] was determined by adding excess Ca^{2+}. To measure changes in Ca^{2+} content of microsomes, it was convenient to construct a plot of $\Delta A_{654-685}$ as a function of added Ca^{2+} so that absorbance changes could be directly related to changes in Ca^{2+} content (see below).

Freshly prepared microsomes (10–15 mg of protein/ml) which had been washed with 5 μM EGTA and maintained on ice contained the equivalent of 1–2 nmol of Ca^{2+}/mg of protein, of which 20–40% was detectable only after addition of the ionophore A23187. Dilution of microsomes to 1 mg of protein/ml and incubation at room temperature for 10–15 min or at 37 °C for 5–10 min resulted in the loss of most of the A23187-releasable fraction. Consequently, preincubation was routinely performed to deplete this Ca^{2+} fraction prior to measurement of ATP-dependent Ca^{2+} uptake.

Addition of 3 mM ATP to preincubated microsomes caused a rapid increase in the A23187-releasable Ca^{2+} pool (Fig. 1*A*), which was linear for 90 s and reached a maximal value at 120 s. The amount of Ca^{2+} sequestered (Fig. 1*C*) was calculated from the experimentally derived standard curve (Fig. 1*B*) and was 0.61 ± 0.12 nmol of Ca^{2+}/mg of protein/min for the first minute and 1.03 ± 0.20 nmol of Ca^{2+}/mg of protein for the total uptake (n = 6). In microsomes which were not washed with EGTA, ionophore-releasable Ca^{2+} was typically 2–3 nmol/mg of protein. Assuming 25% of the cellular protein is from the endoplasmic reticulum in cells from phenobarbital-pretreated rat, this would indicate that 0.5–1 nmol of Ca^{2+}/mg of protein should be releasable in hepatocytes. This agrees well with the values of about 1–1.1 nmol of $Ca^{2+}/10^6$ cells (10) for the A23187-releasable pool in hepatocytes which is not released by FCCP treatment when one considers that there are 1.6 mg of protein/10^6 cells (17).

The characteristics of ATP-dependent Ca^{2+} sequestration as measured by this method are similar to those obtained with other approaches (5, 18). The uptake was insensitive to ruthenium red, an inhibitor of mitochondrial Ca^{2+} uptake, and the apparent K_m for ATP (about 0.1–0.2 mM) was somewhat lower than other values (5, 6, 18). At the Ca^{2+} levels present in our system, the sequestration of Ca^{2+} was relatively insensitive to added Ca^{2+} and was therefore consistent with an apparent K_m of about 1 μM total Ca^{2+}. In contrast to these similarities, however, we observed that the microsomal ATP-dependent Ca^{2+} sequestration was insensitive to the protonophore FCCP (Table I). These experiments were performed both by incu-

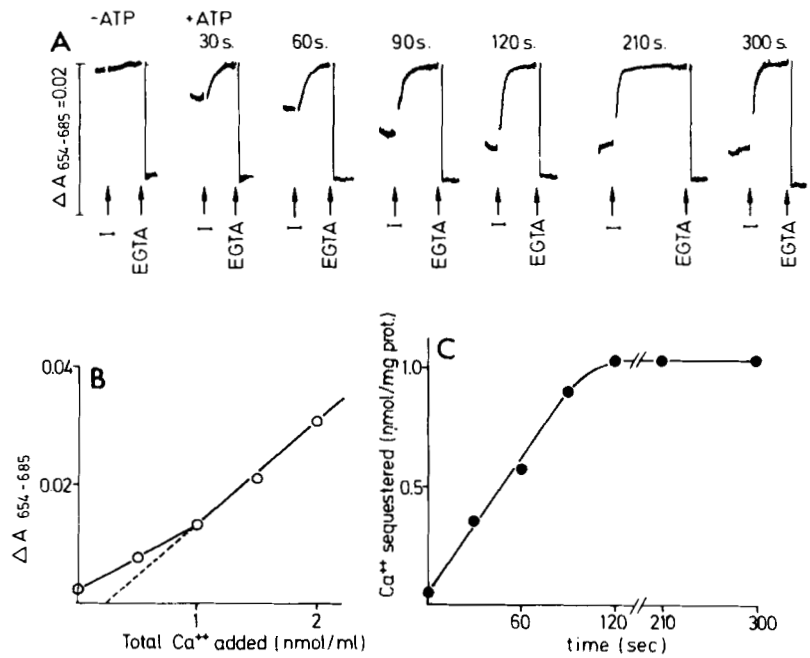

FIG. 1. **Measurement of the ATP-dependent microsomal Ca^{2+} sequestration by the A23187 release method with arsenazo III.** *A*, microsomes (0.8 mg of protein/ml) were preincubated for 20 min at room temperature to release residual sequestered Ca^{2+}. ATP (3 mM) was added prior to the ionophore A23187 (*I*) at the intervals indicated, and arsenazo III was added 30 s prior to addition of A23187. Excess EGTA was added as indicated to give zero Ca^{2+} and thus a measure of total Ca^{2+}·arsenazo III in the system. *B*, $\Delta A_{654-685}$ was calibrated relative to additions of known amounts of Ca^{2+} to microsomes, which had been depleted of Ca^{2+} by addition of EGTA. This curve allowed simple calculation of the amount and rate of Ca^{2+} sequestration. *C*, Ca^{2+} sequestration as a function of time. Data are from a single experiment representative of data from six microsomal preparations.

bating with FCCP before adding ATP and by adding FCCP after ATP-dependent Ca^{2+} uptake had occurred and provided no evidence for a sensitivity of this system to the respiratory uncoupler.

Recent studies in our laboratory have shown that the metabolism of *t*-butyl hydroperoxide by isolated hepatocytes can lead to altered Ca^{2+} homeostasis (10–12). In these studies, two intracellular Ca^{2+} pools were distinguished by treatment first with FCCP and then ionophore A23187 in the presence of arsenazo III. The FCCP-releasable pool has been identified as being mitochondrial in origin. The remaining extramitochondrial Ca^{2+}, released by subsequent addition of A23187, was thought to be located predominantly in the endoplasmic reticulum, largely on the basis of subfractionation studies (9). The results using the present approach show that the Ca^{2+} sequestering capacity of the microsomal fraction is quantitatively sufficient to account for virtually all of the "extramitochondrial" Ca^{2+} of liver cells.

Effects of t-Butyl Hydroperoxide on Microsomal Ca^{2+} Sequestration—*t*-Butyl hydroperoxide is rapidly metabolized by glutathione peroxidase in liver cells, resulting in the oxidation of GSH and pyridine nucleotides, as well as a loss in ionophore-releasable Ca^{2+} and, at high concentrations (4 mM), cell death (10). Incubation of microsomes with *t*-butyl hydroperoxide resulted in rapid loss of their ability to sequester Ca^{2+} in the presence of ATP (Fig. 2*A*). This loss in activity was reflected both in the rate and the maximal amount of Ca^{2+} sequestered (Fig. 2*B*). Both the net rate of uptake and the amount sequestered are a function of the balance of the uptake and release processes. To test whether *t*-butyl hydroperoxide altered the microsomal ability to retain Ca^{2+}, microsomes, which had not been preincubated to deplete Ca^{2+}, were incubated with and without the hydroperoxide, and the rate of loss of the A23187-releasable pool was measured. These results indicate that the hydroperoxide had no effect on the net rate of Ca^{2+} loss from microsomes in short term incubations in the absence of ATP, but that at longer time points an increased release was observed, especially in microsomes preincubated with ATP (data not shown). Therefore, we presently cannot distinguish between a possible inhibition of uptake as opposed to a stimulation of release. Indeed, both processes may occur simultaneously. *t*-Butyl hydroperoxide is largely metabolized by glutathione peroxidase but is also a substrate for cytochrome P-450 (19). Investigation of this activity of cytochrome P-450 indicates that a free radical is generated (19) which could account for the observed effect of the hydroperoxide on Ca^{2+} sequestration. Addition of metyrapone, a well known inhibitor of cytochrome P-450, prevents the inactivation of Ca^{2+} sequestration by *t*-butyl hydroperoxide (Table I). To distinguish between this process and radical-mediated lipid peroxidation, experiments were performed with added Mn^{2+}, an inhibitor of free radical-mediated lipid peroxidation.[2] The results (Fig. 3) show that Mn^{2+} inhibits malonaldehyde production following *t*-butyl hydroperoxide treatment but has little effect on the hydroperoxide-induced loss of Ca^{2+} sequestration. These results suggest that the inactivation of Ca^{2+} sequestration may be a direct effect of a free radical product on the Ca^{2+} pump and not mediated through a generalized peroxidation of membrane lipids.

The loss of the A23187-releasable Ca^{2+} pool in hepatocytes following *t*-butyl hydroperoxide treatment occurs only after GSH depletion and can be prevented by dithiothreitol (10). We therefore examined the effect of GSH and dithiothreitol on ATP-dependent Ca^{2+} sequestration by microsomes. Incubation of microsomes with GSH or dithiothreitol did not change the ability of the microsomes to sequester Ca^{2+} in the presence of ATP (data not shown). However, addition of either of these thiols to microsomes incubated with 200 μM *t*-butyl hydroperoxide provided a complete protection against loss of ATP-dependent Ca^{2+} sequestration for up to 30 min (Fig. 4). This protection is probably not due to glutathione

TABLE I

Effects of inhibitors on microsomal Ca^{2+} sequestration

Liver microsomes were incubated for 10 min as described under "Experimental Procedures" with additions as indicated.

Additions	Ca^{2+} sequestered
	nmol/mg protein
None	0.98 ± 0.05
FCCP (10 μM)	0.85 ± 0.07
Ruthenium red (20 μM)	0.82 ± 0.04
Metyrapone (100 μM)	0.84 ± 0.03
$MnCl_2$ (250 μM)	0.81 ± 0.08
t-Butyl hydroperoxide (200 μM)	0.13 ± 0.09
t-Butyl hydroperoxide + Metyrapone (100 μM)	0.61 ± 0.07
t-Butyl hydroperoxide + $MnCl_2$ (250 μM)	0.17 ± 0.05

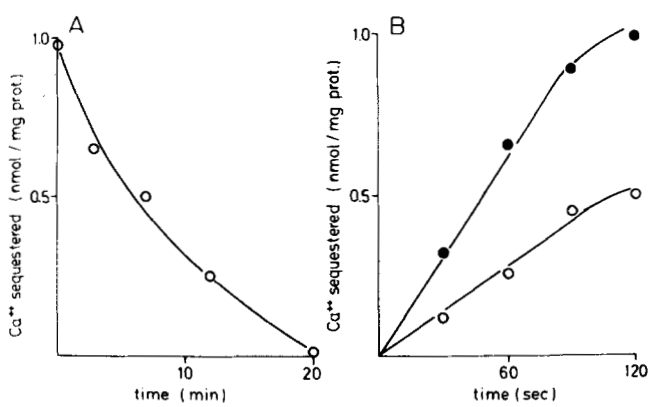

FIG. 2. **Inhibition of microsomal Ca^{2+} sequestration by *t*-butyl hydroperoxide.** *A*, microsomes were incubated with 200 μM *t*-butyl hydroperoxide for 1, 5, 10, and 18 min prior to addition of ATP. 90 s after addition of ATP, arsenazo III was added, and after 30 s, the $\Delta A_{654-685}$ due to addition of A23187 was recorded. Time points are, therefore, expressed as the total incubation time after addition of the hydroperoxide. *B*, time course of Ca^{2+} sequestration following addition of ATP to microsomes incubated 5 min either without (●) or with (○) 200 μM *t*-butyl hydroperoxide.

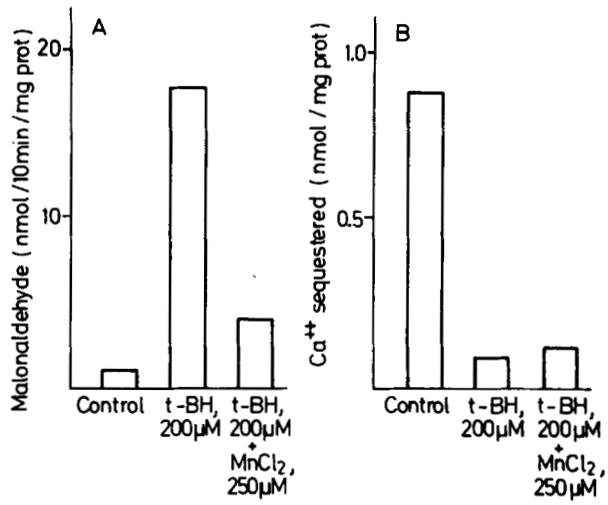

FIG. 3. **Effect of Mn^{2+} on the *t*-butyl hydroperoxide (*t-BH*)-dependent initiation of lipid peroxidation (*A*) and Ca^{2+} sequestration (*B*) in rat liver microsomes.** Lipid peroxidation was measured in terms of malonaldehyde formation (16), and Ca^{2+} sequestration was measured as described in the legend to Fig. 1.

[2] S. Orrenius and L. Ernster, unpublished results.

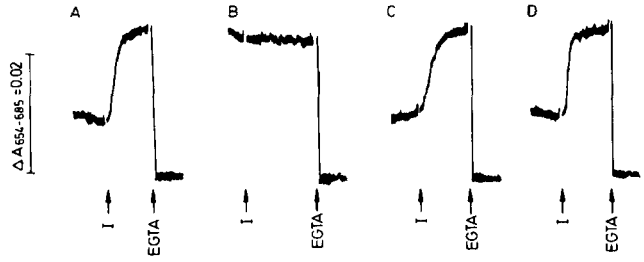

FIG. 4. **Prevention of the *t*-butyl hydroperoxide inhibition of microsomal Ca^{2+} sequestration by GSH and dithiothreitol.** Microsomes (1 mg of protein/ml) were incubated at 37 °C for 15 min with the treatments indicated prior to addition of ATP and measurement of the A23187-releasable Ca^{2+}. *A*, control; *B*, 200 μM *t*-butyl hydroperoxide; *C*, 200 μM *t*-butyl hydroperoxide and 2 mM dithiothreitol; *D*, 200 μM *t*-butyl hydroperoxide and 1 mM GSH. Ionophore A23187 (*I*) and EGTA were added as indicated by the *arrows*.

peroxidase activity associated with the microsomes because dithiothreitol is not a substrate for glutathione peroxidase (20). Previous studies have shown that the microsomal Ca^{2+} pump is sensitive to sulfhydryl reagents (5), and we also found that 1 mM diamide inhibited its activity (data not shown). It has further been shown by Moore and co-workers (5, 21-23) that the microsomal Ca^{2+} pump is inhibited during carbon tetrachloride and carbon disulfide metabolism in liver microsomes. It therefore appears that thiols, notably GSH in liver cells, may protect the Ca^{2+} sequestering system of the endoplasmic reticulum by preventing the oxidation of thiol group(s) critical for Ca^{2+}-ATPase activity.

These results are consistent with our recent finding that the extramitochondrial Ca^{2+} pool is regulated in hepatocytes by the glutathione redox state and is susceptible to alteration by oxidative stress (10-12). The Ca^{2+} sequestering system of the liver endoplasmic reticulum may be a primary target in both hepatotoxic injury and in the physiological response of liver cells to drugs and hormones. Its further characterization therefore seems an area worthy of future investigation.

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