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EXTRACELLULAR CALCIUM ALLEVIATES CELL TOXICITY DUE TO HEPATOTOXINS THAT INDUCE LIPID PEROXIDATION, BUT HAS NO EFFECT ON TOXINS THAT DO NOT CAUSE LIPID PEROXIDATION

A STUDY IN ISOLATED RAT HEPATOCYTES

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Abstract—The effect of extracellular calcium on cell death, induced by hepatotoxins that induce lipid peroxidation [diethyl maleate (DEM), allyl alcohol (AA) and bromoisovalerylurea (BIU)] and hepatotoxins that do not induce lipid peroxidation [disulfiram (DSF), *N*-hydroxy-2-acetyl-aminofluorene (N-OH-AAF) and tetrahydroaminoacridine (THA)] was studied in freshly isolated rat hepatocytes. Extracellular calcium strongly delayed the onset of toxicity of DEM, AA and BIU as detected by lipid peroxidation, depletion of free protein thiol groups and cell death. This protective effect of calcium was decreased at higher concentrations of the toxic compounds. In contrast, no effect of calcium was observed on toxicity induced in the absence of lipid peroxidation by DSF, N-OH-AAF and THA. Addition of calcium was also without effect on the protein thiol depletion. These results indicate that calcium only alleviates cytotoxicity which is induced by thiol depletion resulting from lipid peroxidation. Cytotoxicity as a result of protein thiol depletion through disulfide formation is not affected by extracellular calcium.

The role of intra- and extra-cellular calcium in chemical-induced toxicity in isolated hepatocytes is a highly controversial subject. It has been suggested that many hepatotoxic agents exert their toxic effect via a disruption of the internal calcium homeostasis. Farber and coworkers [1–4] and Chenery *et al.* [5] found that extracellular calcium increased the toxicity of the hepatotoxins carbon tetrachloride, D-galactosamine, phalloidin and 2,4-dinitrophenol in cultured hepatocytes. Elevation of intracellular calcium levels resulting from the influx of extracellular calcium through the injured cell membrane was suggested as a possible mechanism of toxin-induced cell death [1–5]. On the other hand, Smith and others reported that in freshly isolated hepatocytes, toxicity induced by e.g. bromobenzene, carbon tetrachloride, ethyl methanesulfonate and tert-butyl hydroperoxide was less in the presence of extracellular calcium than in its absence [5–11].

The group of Reed has evaluated the interaction between extracellular calcium and vitamin E in relation to chemical-induced toxicity in isolated rat hepatocytes [12–19]. They presented evidence [13] that the presence or absence of vitamin E in the incubation media could explain the above controversial findings on extracellular calcium in the induction of cell death: Reed and coworkers also found a delay in the induction of cell death by extracellular calcium, but only in the absence of vitamin E. Toxicity appeared to depend on cellular vitamin E rather than on the presence of extracellular calcium. Depletion of cellular vitamin E was prevented by extracellular calcium which provided an explanation for the effect of calcium on toxin-induced cell death.

In a previous paper, we have shown that vitamin E protects against toxicity induced by allyl alcohol (AA)†, bromoisovalerylurea (BIU) and diethyl maleate (DEM) [20]. Presumably, vitamin E protected against cell death by preventing lipid peroxidation. Protein thiol depletion, most likely resulting from reaction of lipid peroxidation products with protein thiol groups was thus prevented. Vitamin E was unable, however, to prevent protein thiol depletion and toxicity induced by disulfiram (DSF), a disulfide which does not induce lipid peroxidation. We concluded that cell death can be induced by a lipid peroxidation dependent pathway which is vitamin E-sensitive, and by unrelated routes which do not involve lipid peroxidation and are not vitamin E-sensitive.

In this study we have investigated the effect of extracellular calcium on hepatotoxicity induced by toxins that do or do not cause lipid peroxidation. We demonstrate that calcium only alleviates toxicity when it is induced by toxins that cause lipid peroxidation.

MATERIALS AND METHODS

Collagenase was obtained from Boehringer (Mannheim, F.R.G.). Bovine serum albumin (BSA)

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† Abbreviations: DEM, diethyl maleate; AA, allyl alcohol; BIU, bromoisovalerylurea; DSF, disulfiram; N-OH-AAF, *N*-hydroxy-2-acetyl-aminofluorene; THA, tetrahydroaminoacridine; BSA, bovine serum albumine; GSH, reduced glutathione.

type V, Ellmann's reagent [5,5′ dithio bis (2-nitrobenzoic acid)] and α tocopherol succinate (vitamin E) were obtained from Sigma Chemical Co. (St Louis, MO). BIU was purchased from Onderlinge Pharmaceutische Groothandel (Utrecht, The Netherlands), AA from Aldrich Chemie (Brussel, Belgium), DEM from Merck (Darmstadt, F.R.G.) and desferal from Ciba Geigy BV (Arnhem, The Netherlands). DSF, (97% purity) and tetrahydroaminoacridine (THA) were obtained from Janssen (Beerse, Belgium). The commercial DSF was recrystallized from ethanol to remove some impurities as described previously [21]. *N*-Hydroxy-2-acetyl-aminofluorene (N-OH-AAF) was synthesized by the method of Westra [22]. All other reagents were of the highest grade of purity available.

Isolation and incubation of hepatocytes. Male Wistar rats of approximately 250 g, who had free access to food (MRH-B, Hope Farms, Woerden, The Netherlands) and tap water were used throughout the study. They were obtained from the Sylvius Laboratories, University of Leiden. Liver parenchymal cells were isolated by collagenase perfusion as described by Seglen [23] with some modifications. After the perfusion with collagenase, one half of the cell suspension was washed three times with cold Hanks–HEPES buffer, pH 7.4, without calcium, and the other half with the same buffer, supplemented with 3.5 mM $CaCl_2$. Finally, the cells were resuspended in washing buffers, supplemented with 1.5% (w/v) BSA. Routinely, more than 95% of the freshly isolated hepatocytes excluded trypan blue. Cells were incubated at a final concentration of 2–4 × 10^6 cells/ml.

DSF, BIU, vitamin E (in ethanol) and DEM (in diethyl ether) were added into the incubation flasks; the solvents were evaporated before the hepatocytes were added. AA, THA, desferal and N-OH-AAF were dissolved in buffer and added directly to the cell suspension. Cells were preincubated with or without vitamin E for 15 min before the hepatotoxins were added. Incubations were carried out in a rotary shaker, 200 cycles/min, at 37° under an atmosphere of 95% O_2/5% CO_2.

Analysis. Cell viability was determined by measuring the leakage of lactate dehydrogenase from the cells into the medium [24]. Intracellular glutathione (GSH) concentrations were measured by the colorimetric thiol method of Saville [25] and protein thiols were determined with Ellmann's reagent by a modification of the method of DiMonte [20, 26]. Lipid peroxidation was measured by the thiobarbituric acid test [27] as the amount of malondialdehyde formed by the hepatocytes, using an absorption coefficient of 1.56 × 10^5/mol/cm [28]. Protein was determined according to Lowry *et al.* [29], using BSA as a standard.

RESULTS

Effect of extracellular calcium on viability, GSH concentration, lipid peroxidation and protein thiols in hepatocytes

Initially, we determined whether incubation of control cells in the absence or presence of calcium led to differences in viability, GSH concentrations, lipid peroxidation and the amount of protein thiols.

During incubation for 6 hr, viability decreased from 95% to about 85%, irrespective of the presence or absence of calcium (Table 1). However, GSH depletion in cells incubated in a calcium-free medium was more outspoken than in cells incubated in a calcium supplemented medium: a loss of 70% (calcium-free) versus a loss of 25% (with 3.5 mM $CaCl_2$) of GSH after 6 hr (Fig. 1). In the absence of calcium, a small amount of lipid peroxidation products was found after 6 hr: about 1.5 nmol MDA/mg protein, while in the calcium supplemented medium no lipid peroxidation was observed (Table 1). The level of free protein thiols in cells, incubated in a calcium supplemented medium was approximately 10% higher (after 6 hr) than in cells incubated in a calcium-free medium (Table 1).

Effect of extracellular calcium on cell death, caused by lipid peroxidation-inducing agents (DEM, AA and BIU)

The induction of cell death by the GSH-depleting agents DEM, AA and BIU is lipid peroxidation-dependent [20]. To investigate the effect of extracellular calcium on cell death induced by these agents, cells were incubated with DEM, AA or BIU in the absence or presence of calcium.

Calcium had no effect on GSH depletion by these agents (data not shown). Within one hour, all GSH was depleted in the presence of extracellular calcium, identical to our previous observation in the absence of calcium [21]. However, as illustrated for DEM in Fig. 2, the presence of calcium delayed the onset of lipid peroxidation, and less malondialdehyde was formed. This effect of calcium on lipid peroxidation was concomitant with a delay in protein thiol depletion (Fig. 3) and cell death (Fig. 4). Both in the presence and absence of calcium, protein thiol depletion and cell death occurred in parallel. At increasing concentrations of DEM, the effect of calcium on lipid peroxidation, protein thiol depletion and cell death was reduced, indicating that protection by calcium is limited.

Calcium had the same effects on lipid peroxidation, protein thiol depletion and cell death induced by AA and BIU (Table 1): the delaying effects of calcium on the induction of lipid peroxidation again were followed by a delay in the induction of protein thiol depletion and cell death.

Effect of calcium on cell death, caused by agents which do not induce lipid peroxidation

DSF and N-OH-AAF cause cell death in the absence of lipid peroxidation both in the presence and absence of calcium. The induction of cell death by DSF [20] and N-OH-AAF (E. D. Kroese, manuscript in preparation) was not prevented by vitamin E. In marked contrast to its effect on DEM, AA and BIU-induced cell death, the presence of calcium had no effect on DSF (Fig. 5) and N-OH-AAF-induced cell death (Fig. 5). Protein thiol depletion, caused by DSF, which was complete when all cells were dead, was also unaffected by calcium (Fig. 6).

We reported earlier, that THA induces cell death in the absence of calcium [30]. This was accompanied

Table 1. Effects of extracellular calcium (3.5 mM $CaCl_2$) on lipid peroxidation, free protein thiol depletion and cell death induced by AA and BIU. All values were determined after 6 hours of incubation. One experiment typical of three.

Compound	MDA (nmol/mg protein)		Free protein thiols (nmol –SH/mg protein)		Cell death (% LDH leakage)	
	–Ca	+Ca	–Ca	+Ca	–Ca	+Ca
Control	1.5	0.1	85	92	15	15
AA: 0.38 mM	6.0	0.5	40	87	80	20
0.42 mM	11.2	2.2	32	68	100	42
0.46 mM	11.5	10.2	25	30	100	100
BIU: 4.0 mM	12.2	3.5	37	62	100	52

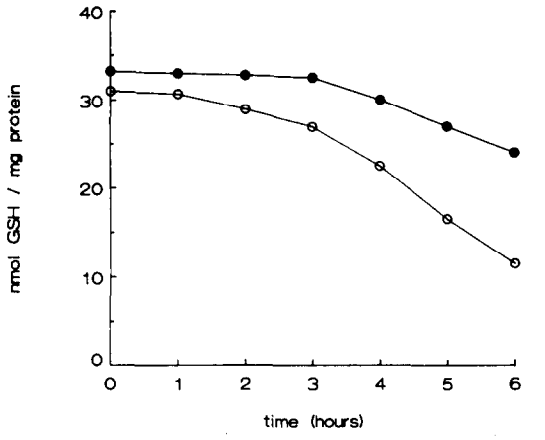

Fig. 1. GSH concentrations in control cells, incubated in the absence or presence of extracellular calcium; (○) without calcium; (●) 3.5 mM $CaCl_2$. One experiment typical of three.

by the formation of a small amount of lipid peroxidation products, but THA-induced cell death does not seem to be caused by lipid peroxidation: vitamin E could not prevent cell death by THA [30], as it could in the case of AA, DEM and BIU. To exclude a role of lipid peroxidation in the cell death caused by THA, we incubated cells with THA in the absence and presence of desferal, an inhibitor of lipid peroxidation [20]. Although desferal prevented the induction of lipid peroxidation (Fig. 7), it had no effect on cell death (Fig. 8), confirming that lipid peroxidation is of no importance in the induction of THA-induced cell death.

Although extracellular calcium delayed the time of onset of THA-induced lipid peroxidation (Fig. 7), this was not accompanied by an effect on the induction of cell death (Fig. 8). In the presence of calcium, vitamin E and desferal had no effects on THA-induced cell death (data not shown).

DISCUSSION

The present data show a different effect of extracellular calcium on cell death caused by hepatotoxins that induce lipid peroxidation and those that are toxic by other pathways.

In the absence of calcium the process of lipid peroxidation, caused by the hepatotoxins, begins within the first hour of incubation, followed by free protein thiol depletion and induction of cell death. However, calcium strongly delays the time of onset of lipid peroxidation and the subsequent events. This suggests that extracellular calcium supports protection mechanisms within the cell against lipid peroxidation and subsequent cell death. At increasing concentrations of the hepatotoxins, these protective mechanisms or factors become exhausted and calcium is less effective.

Such a protective factor is cellular vitamin E. It has been reported that cell death was preceded by a decrease of the intracellular vitamin E concentration and a subsequent induction of lipid peroxidation;

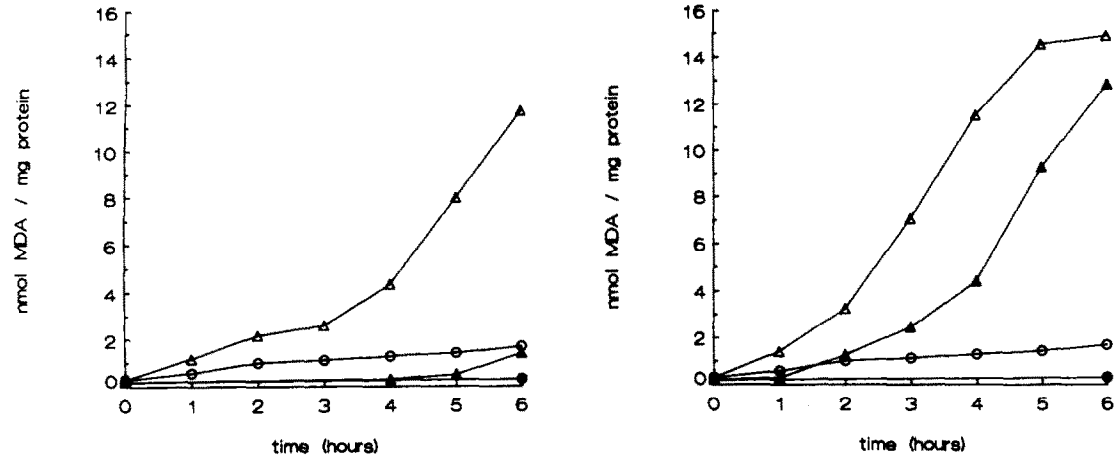

Fig. 2. Effect of extracellular calcium on the induction of lipid peroxidation by 4.2 mM DEM (left) or 6.8 mM DEM (right) in isolated hepatocytes; (○) control, no calcium; (●) control, 3.5 mM $CaCl_2$; (△) DEM, no calcium; (▲) DEM, 3.5 mM $CaCl_2$. One experiment typical of three.

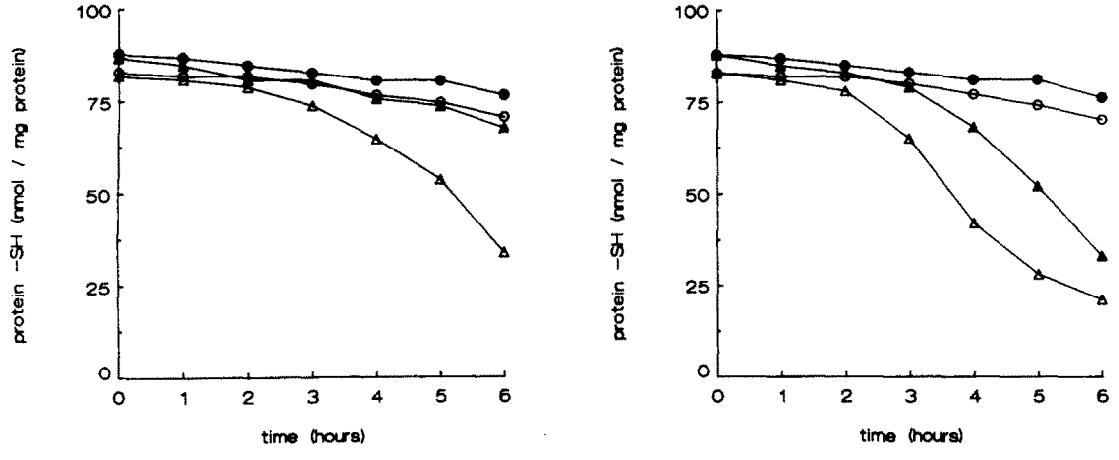

Fig. 3. Effect of extracellular calcium on depletion of free protein thiols by 4.2 mM DEM (left) or 6.8 mM DEM (right) in isolated hepatocytes; (○) control, no calcium; (●) control, 3.5 mM $CaCl_2$; (△) DEM, no calcium; (▲) DEM, 3.5 mM $CaCl_2$. One experiment typical of three.

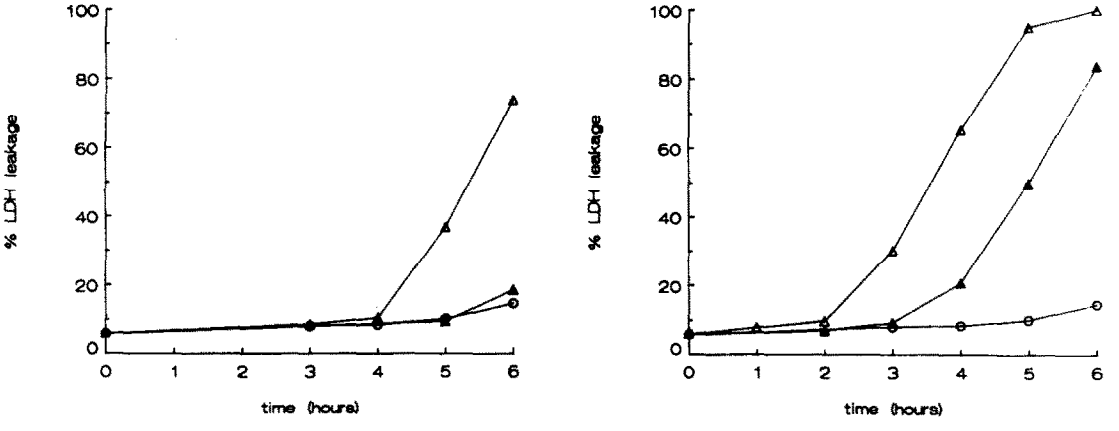

Fig. 4. Effect of extracellular calcium on the induction of cell death by 4.2 mM DEM (left) or 6.8 mM DEM (right) in isolated hepatocytes; (○) control without or plus 3.5 mM $CaCl_2$; (△) DEM, no calcium; (▲) DEM, 3.5 mM $CaCl_2$. One experiment typical of three.

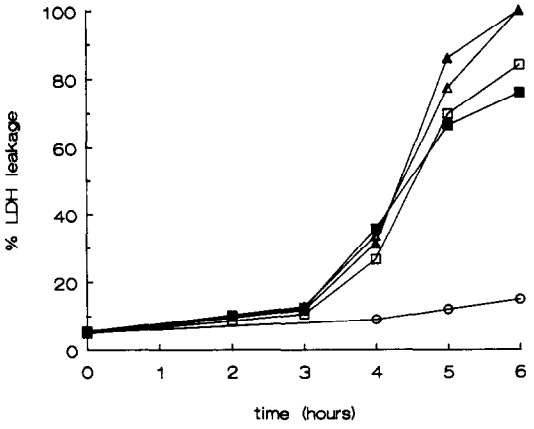

Fig. 5. Effect of extracellular calcium on the induction of cell death by 10 mM DSF or 1 mM N-OH-AAF in isolated hepatocytes; (○) control without or plus 3.5 mM $CaCl_2$; (△) DSF, no calcium; (▲) DSF, 3.5 mM $CaCl_2$; (□) N-OH-AAF, no calcium; (■) N-OH-AAF, 3.5 mM $CaCl_2$. One experiment typical of three.

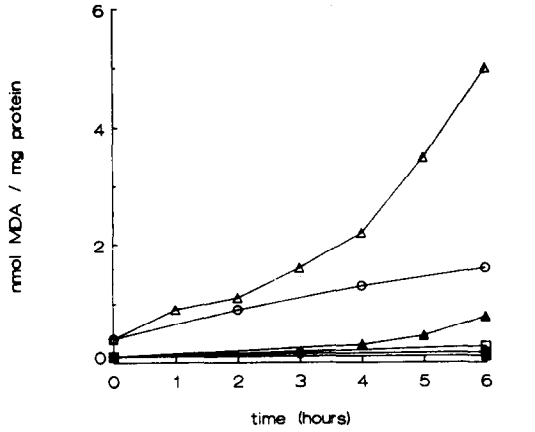

Fig. 7. Effect of extracellular calcium, vitamin E and desferal on the induction of lipid peroxidation by 3 mM THA in isolated hepatocytes. Note difference in y-axis between Figs. 2 and 8. (○) Control, no calcium; (●) control, 3.5 mM $CaCl_2$; (△) THA, no calcium; (▲) THA, 3.5 mM $CaCl_2$; (■) THA plus 75 μM vitamin E, no calcium; (□) THA plus 5 mM desferal, no calcium. One experiment typical of three.

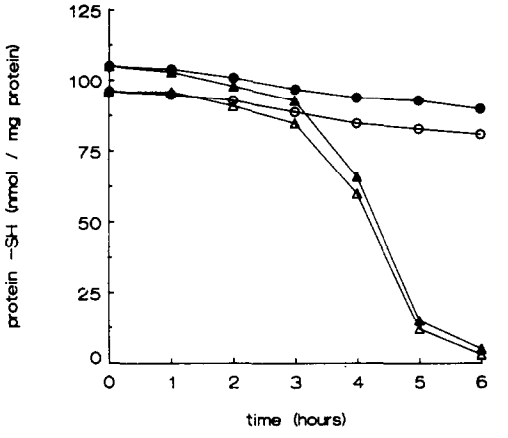

Fig. 6. Effect of extracellular calcium on depletion of free protein thiols by 10 mM DSF; (○) control, no calcium; (●) control, 3.5 mM $CaCl_2$; (△) DSF, no calcium; (▲) DSF, 3.5 mM $CaCl_2$. One experiment typical of three.

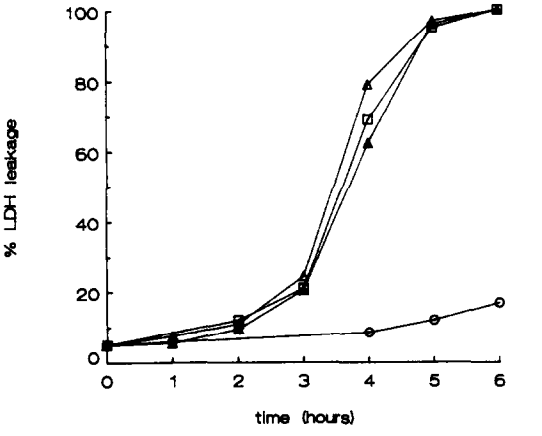

Fig. 8. Effect of extracellular calcium and desferal on the induction of cell death by 3 mM THA in isolated hepatocytes; (○) control without or plus 3.5 mM $CaCl_2$; (△) THA, no calcium; (▲) THA, 3.5 mM $CaCl_2$; (□) THA plus 5 mM desferal, no calcium. One experiment typical of three.

extracellular calcium protected against a rapid decrease of the cellular vitamin E concentration [14, 16] which resulted in increased protection against lipid peroxidation and cell death. This could explain our results because vitamin E completely protects against cell death whereas calcium only (strongly) delays the induction of cell death caused by hepatotoxins which also induce lipid peroxidation. Calcium has no effect on toxicity induced by hepatotoxins that do not induce lipid peroxidation and are insensitive to the action of vitamin E.

THA induces cell death and a low extent of lipid peroxidation [30], which most likely is a consequence of cell death because neither vitamin E nor desferal can prevent THA-induced cell death. As with DSF and N-OH-AAF, calcium has no effect on THA-induced cell death. The fact that calcium decreased the low level of lipid peroxidation but did not affect cell death, is a further indication that lipid peroxidation is of no importance in the THA-induced cell death.

Recently, Thomas and Reed reported [17] that the omission of calcium from the incubation medium caused a high extent of lipid peroxidation and was highly toxic for their cells. Cell death and lipid peroxidation could be prevented by extracellular calcium or vitamin E. In our hands, however, there were no differences in viability of cells, incubated in a calcium free or calcium supplemented medium. The difference may be due to experimental

conditions; Thomas continually oxygenated cells which may result in a state of oxidative stress [17].

In a recent paper [20] we suggested that protein thiol groups, critical for maintaining viability of cells, could be modified by two types of reactions. One is formation of mixed disulfides because in this case the depletion of thiols was fully reversible with dithiothreitol. The other type was not reversible with dithiothreitol and we proposed that this was due to formation of adducts between thiol groups and reactive aldehydes, resulting from peroxidation of endogenous lipids. The results in this paper are in agreement with this suggestion because extracellular calcium only affects protein thiol depletion caused by lipid peroxidation derived products whereas it has no effect on lipid peroxidation independent thiol depletion and cell death.

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