Vol. 176, No. 1, 1991
April 15, 1991
BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 276-279

CALCIUM CHELATION INDUCED GLUTATHIONE EFFLUX FROM TUMOR CELLS AND PREVENTION BY RUTHENIUM RED OR NEOMYCIN

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Received February 19, 1991

Summary: Cultured human lung carcinoma cells (A549) were incubated in a calcium-free medium containing calcium chelators (EGTA, 1-10 mM or BAPTA, 5 mM) for 1 hour at 37 °C. With limited toxicity, the presence of calcium chelators resulted in a decrease of cellular GSH and detachment of the cells from the tissue culture flask. The permeable EGTA tetraacetoxymethyl ester (0.5mM-5 mM) caused a decrease in the cellular GSH content without cell detachment. GSH was not oxidized to GSSG nor formed mixed disulfides with protein thiols. AT-125, a γ-glutamyl transpeptidase inhibitor, prevented detachment, but not the efflux of cellular GSH. Pretreatment with two impermeable compounds (ruthenium red, 100 μM and neomycin, 0.5-10 mM) protected the cells from detachment and prevented the decrease in intracellular GSH. The presence of calcium in the medium during the EGTA and BAPTA treatments also protected the cells. Calcium associated with the cytoplasmic membrane phospholipids or proteins appears important to limit membrane permeability for GSH efflux and to maintain cell attachment.

The role of calcium in cell injury can involve a complex interrelationship between calcium homeostasis and oxidative stress (1). The effect of extracellular calcium on cell injury induced by oxidative damage has been investigated extensively utilizing isolated hepatocytes as a model system (for review see 2). The protective effects of ruthenium red and lanthanum against such injury suggest that the calcium effects may be due in part to calcium movement through the mitochondrial uniport, perhaps involving calcium cycling (3).

With cultured human carcinoma cells (A549) we have used EGTA to decrease the intracellular calcium and to examine whether changes related to calcium homeostasis occur in this cell type as well. We have demonstrated that calcium chelation resulted in decreased intracellular GSH. Ruthenium red and neomycin, cell impermeable compounds which react with negatively charged phospholipids (4) or calcium binding proteins (5), protected the A549 cells from EGTA injury. The mechanism of action in this case, therefore, may involve the cytoplasmic membrane phospholipids or proteins, rather than the mitochondria.

Methods

Human lung carcinoma cells (A549) were obtained from the American Type Culture Collection. The cells were maintained in Nutrient F-12 (Coon's Medium High Zinc, Irvine Scientific) plus 10%

ABBREVIATIONS
Reduced glutathione, GSH; oxidized glutathione, GSSG; lactic dehydrogenase, LDH; t-butylhydroperoxide, tBuOOH; protein-glutathione mixed disulfide, ProSSG.

0006-291X/91 $1.50

fetal bovine serum, 100 U penicillin/ml and 100 μg streptomycin/ml and removed from the culture flask with 0.1% trypsin (Gibco). Cells in logarithmic growth were rinsed with versene (0.14 M NaCl, 5.4 mM KCl, 6.9 mM $NaHCO_3$, 3 mM EDTA and 0.1% dextrose) and incubated 1 hour at 37 °C in formulated Fischer's medium with 10 mM Hepes, and no calcium, sulfur amino acids or glutamate. Additions were made directly to the Fischer's medium, either before the addition of EGTA, or during the incubation, as indicated.

The procedures for preparation of GSH and GSSG samples, separation of mitochondria, high pressure liquid chromatography, cell counting, assay of γ-glutamyl transpeptidase activity, LDH leakage, and trypan blue staining were as previously described (6). For electron microscopy, 4×10^6 cells were fixed in 1.5 ml 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.36) on ice for 2 hours. Post fixation was done with 1% osmium tetroxide for 1 hour. Observations were done on a Zeiss EM\10 A transmission scope. Cell viability was measured by counting cells which attached (indicating ability to grow) after incubation for 4 hours in Primaria tissue culture dishes (Falcon) containing F12 media plus 10% fetal bovine serum, rat tail collagen (3 μg/ml) and antibiotics. In addition, dead cells stained with propidium iodide (15 μM) to stain the DNA, were measured on a Coulter Epics V flow cytometer.

RESULTS

Incubation of A549 cells in culture flasks for 1 hour in formulated Fischer's medium containing EGTA (1-10 mM) resulted in detachment of the cells from the plastic culture flask (Table 1) and decreased cellular GSH (Table 1). The GSH in detached cells (51.8 ± 5.8% of control) was decreased to the same extent as attached cells (56.4 ± 3.7%) after 5 mM EGTA. In a representative experiment with 69% loss of GSH after 5 mM EGTA, 95% of the treated cells were viable as measured by LDH and 91% viable as indicated by trypan blue. In 6 experiments monitoring either propidium iodide staining or cell reattachment, 82 ± 7% of the cells were viable after 5 mM EGTA treatment. When the mitochondrial GSH was analyzed, the percent retained in the mitochondria did not change (control = 8 ± 0%; EGTA treated = 6.25 ± 1.7%). Another calcium chelator, BAPTA (1,2-bis (2-aminophenoxy)ethane N,N,N,N-tetraacetic acid, 5 mM), caused identical effects (data not shown). And, with both EGTA (5 mM) and BAPTA (5 mM), if calcium was included in the medium (5.3 mM), no cell changes occurred.

TABLE 1
CALCIUM CHELATION INDUCED GSH EFFLUX AND CELL DETACHMENT

Treatment	Cellular GSH (Percent 0 mM EGTA)	Attached Cells (Percent Total)
EGTA:		
1 mM	92.9 ± 6.2 (14)	87.7 ± 5 (11)
3 mM	60 ± 4.9 (8)*	67.1 ± 7 (8)*
5 mM	56.4 ± 3.7 (28)*	51 ± 3.1 (34)*
10 mM	68.7 ± 4.5 (3)*	50.7 ± 14 (4)*
Ruthenium Red:		
100 μM	95.9 ± 7.8 (10)	100
5 μM ± 5 mM EGTA	43.9 ± 7.2 (7)*	70.3 ± 5.2 (11)*
25 μM ± 5 mM EGTA	68.4 ± 4.7 (8)*	86.6 ± 5.3 (11)*
100 μM ± 5 mM EGTA	78 ± 4.7 (7)**	96.7 ± 2.8 (13)

A549 cells were incubated for 1 hour in calcium-free medium with indicated levels of EGTA ± ruthenium red. Cells in the medium were counted to determine the number detached by the treatment. GSH was analyzed by HPLC (6). GSH in the control was 27 ± 2 nmols/10^6 cells. Average ± Standard Error (Number of Experiments).
* Significantly different from 100%, t=95%
** Significantly different from 5 mM EGTA, t=95%

In addition to the presence of calcium, the presence of two polycationic compounds also inhibited the EGTA effect. The results of incubation with ruthenium red (5-100 μM) are shown in Table 1. Cells preincubated for 1 hour in neomycin (0.5-10 mM) before EGTA (5 mM) were also protected. The loss of GSH was significantly inhibited by all doses of neomycin. In these experiments, cellular GSH levels 1 hour after EGTA treatment (5 mM) was 58 $\pm$ 8% control and with neomycin pretreatment, GSH was 85 $\pm$ 6% control. When the cells had been released by trypsin treatment prior to incubation with EGTA, no change of GSH occurred with up to 50 mM EGTA.

Although the GSH concentration decreased in the A549 cells, GSH was not oxidized to GSSG, did not form ProSSG with the cytoplasmic protein thiols, and was not present in the medium. When the cells were incubated with extracellular GSH, degradation of the GSH occurred. A549 cells possess γ-glutamyl transpeptidase activity; incubation with AT-125 for 1 hour inhibited γ-glutamyl transpeptidase activity and GSH was found in the medium after EGTA treatment. At 1 mM AT-125, with only 10% activity of γ-glutamyl transpeptidase remaining, GSH in the medium was still decreased.

Using electron microscopy, no cellular changes could be identified between control and EGTA-treated cells. Ruthenium red-treated cells were coated extracellularly with an electron dense material presumed to be the dye, but no similar material was seen intracellularly.

Two other treatments: the antioxidant, N,N-diphenyl-p-phenylenediamine (0.05-10 mM), and alcian blue (1-2 mM), a stain similar to ruthenium red but with fewer charges (7), were included during the EGTA incubation. Each treatment inhibited cell detachment, but did not greatly affect the loss of GSH, indicating that the GSH decrease is not a result of detachment, nor is it the cause. Lanthanum chloride (50 - 500 μM) did not protect the cells. Treatment of cells with EGTA tetra-acetoxymethyl ester, a permeable form of EGTA, did not result in cell detachment. However, the GSH decreased up to 63% with as low a dose as 0.5 mM of the EGTA ester for 1 hour.

DISCUSSSION

This is the first instance in which a membrane permeability change to GSH has been shown to be caused by treatment with calcium chelation. Similar increased plasma membrane permeability to low molecular weight solutes has been studied in macrophage cells (8). However, their studies differ from the present experiments as extracellular EGTA was not tested without calcium, and the amount of time necessary for the permeability change exceeded the time necessary for the A549 cells to efflux GSH. The macrophage cells required intracellular loading of the chelators for the change to occur. There are several other instances in which other cell functions have been affected by EGTA (9,10,11).

The inorganic dye, ruthenium red, is impermeable to intact cells and is a histochemical stain for acidic glycosaminoglycans and calcium binding proteins (5). As ruthenium red protected the cells from EGTA, which is also impermeable to cells, the two interactions probably occurred with the cytoplasmic membrane. Charuk et al. (5), who developed a protocol for identifying proteins which interact with ruthenium red, concluded that ruthenium red detects acidic calcium-binding proteins. Neomycin, another polycation with six positive charges (12) also protected the cells from EGTA. Spermine, with only two positive charges had no protective effect (data not shown). The strong interaction of ruthenium red and neomycin with the membrane is being used in continuing studies to investigate the mechanism of control of GSH efflux from tumor cells.

In summary, when cultured human lung carcinoma cells (A549) were incubated in calcium chelators, limited toxicity occurred with a decrease in cellular GSH and detachment of the cells from the tissue culture flask. Treatment with the permeable EGTA-ester caused a decrease in cellular GSH without cell detachment. Neither cytoplasmic GSSG nor protein-glutathione mixed disulfide were found. From results obtained using AT-125, a γ-glutamyl transpeptidase inhibitor, it appeared that the GSH was effluxing from the cell. Pretreatment with two cell impermeable compounds (ruthenium red and neomycin) protected the cells from injury. The presence of calcium during the EGTA and BAPTA treatments also protected the cells. The permeability change to GSH appeared to involve cytoplasmic membrane phospholipids or proteins which bind calcium.

ACKNOWLEDGMENTS

The authors would like to thank Kristin Hoffbuhr for technical assistance. An American Cancer Society grant (CH-109) supported this research.

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