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Zinc pyrithione induces apoptosis and increases expression of Bim

J. J. Mann and P. J. Fraker

Cell and Molecular Biology Program (J. J. Mann, P. J. Fraker) and Department of Biochemistry & Molecular Biology (P. J. Fraker) Michigan State University, East Lansing, MI 48824, USA

We demonstrate herein that zinc pyrithione can induce apoptosis at nanomolar concentrations. Zinc pyrithione was a potent inducer of cell death causing greater than 40–60% apoptosis among murine thymocytes, murine splenic lymphocytes and human Ramos B and human Jurkat T cells. Conversely, the addition of a zinc chelator protected thymocytes against zinc pyrithione induced apoptosis indicating these responses were specific for zinc. Zinc-induced apoptosis was dependent on transcription and translation which suggested possible regulation by a proapoptotic protein. Indeed, zinc induced a 1.9 and 3.4 fold increase respectively in expression of the BimEL and BimL isoforms and also stimulated production of the most potent isoform, BimS. This increase in Bim isoform expression was dependent on transcription being blocked by treatment with actinomycin D. Overexpression of Bcl-2 or Bcl-xL provided substantial protection of Ramos B and Jurkat T cells against zinc-induced apoptosis. Zinc also activated the caspase cascade demonstrated by cleavage of caspase 9. Addition of specific inhibitors for caspase 9 and caspase 3 also blocked zinc-induced apoptosis. The data herein adds to the growing evidence that free or unbound zinc could be harmful to cells of the immune system.

Keywords: apoptosis; Bcl-2; Bim; caspases; zinc pyrithione.

Introduction

Zinc is an essential biological trace metal vitally important for many cellular activities, therefore, the concentration of zinc is tightly regulated.[1,2] Zinc is critical as a cofactor for both catalytic and structural roles within the cell as well as numerous other processes including gene expression.[1,2] However exposure to excess unbound or extracellular zinc has been shown to have adverse effects at the cellular level through the induction of apoptosis in a number of cell types.[3–6] A variety of disease states are affected by the disruption of zinc homeostasis. During brain damage certain neurons which contain substantial amounts of zinc, up to 300 μM of releasable free zinc within presynaptic boutons, as well as during seizures zinc is released with associated cell death observed in surrounding cells and tissues.[7–10] Addition of zinc chelators results in survival of these surrounding cells demonstrating a proapoptotic role for excess zinc. In the case of Alzheimer's disease, zinc has been shown to be associated with the β-amyloid proteins potentially enhancing aggregation and concomitant formation of plaques.[11,12] Chelation of zinc reduced the onset of Alzheimer's in a murine model making it of interest as a potential intervention in human trials.[13,14] Substantial amounts of zinc are also associated with the insulin stored in pancreatic islet β-cells which when released may act in a paracrine fashion to further accelerate the death of islet cells during the course of diabetes.[15] Intracellular zinc exists as fixed pools of zinc, such as in transcription factors and as structural cofactors, or in labile pools of readily exchangeable zinc such as that bound by metallothionein[16] that are thought to be responsible for the regulation of apoptosis. Another example of this labile pool is the release of zinc from protein kinase C (PKC) during activation in response to diacylglycerol or reactive oxygen species.[17] Furthermore zinc has been demonstrated to be released within cells undergoing early events of apoptosis induced spontaneously or in response to various agents.[18] Thus there are a number of situations where released zinc might have potentially adverse effects sometimes initiating apoptosis.

Zinc-induced apoptosis utilized the mitochondrial apoptotic pathway as will be demonstrated in this present study. The mitochondria are a central focusing point for many diverse signals initiating apoptosis in the intrinsic apoptotic pathway.[19] Upon activation of this pathway cytochrome c is released from the mitochondria resulting in the assembly of the apoptosome which promotes cleavage and activation of caspase 9 and 3.[20,21] Bcl-2 and Bcl-xL are the antiapoptotic proteins that sequester proapoptotic proteins such as Bax. These proteins prevent the multimerization of Bax and its interaction with the mitochondria thereby neutralizing its proapoptotic effects.[22] Conversely proapoptotic BH3 only family

Correspondence to: Dr. P. J. Fraker, Department of Biochemistry & Molecular Biology, Michigan State University, East Lansing, MI 48824, USA. Tel.: 517-353-3513; Fax: 517-353-9334; e-mail: fraker@msu.edu

members such as Bim (BimEL, BimL, BimS) antagonize the antiapoptotic functions of Bcl-2 and Bcl-xL through direct interaction.[23] This interaction neutralizes these antiapoptotic proteins thus allowing Bax to multimerize resulting in the permeabilization of the mitochondrial outer membrane and release of molecules such as cytochrome c resulting in activation of the intrinsic apoptotic caspase cascade.[23] As will be shown herein zinc-induced a substantial increase in Bim.

To further explore the capacity of zinc to induce apoptosis, we demonstrated several years ago that it could readily induce apoptosis in murine thymocytes causing all the classical changes in morphology to include cell shrinkage, chromatin condensation, and DNA fragmentation.[3] Substantial concentrations of zinc sulfate (around 80–200 μM) were required to produce 40% thymic apoptosis probably because of the inefficient uptake of the zinc salts by cells.[3] However addition of zinc pyrithione, an ionophore, induced substantial amounts of apoptosis (40–60%) at 400 nM (normal plasma zinc is $\sim$15–20 μM)[24] in both human and murine immune cells. These compounds caused a cellular increase in zinc of 1.2 and 19.5 ng/1 $\times$ 10^7 cells for zinc pyrithione (400 nM) and zinc sulfate (200 μM) respectively for thymocytes after two hours of treatment. While zinc has been demonstrated to induce apoptosis in a variety of cell types a detailed analysis of the apoptotic death pathway utilized by zinc during apoptosis especially in cells of the immune system has not been fully established. The present study was therefore conducted to investigate the death pathway utilized by zinc in cells of the immune system. Such cells are an ideal model in which to determine the role of free zinc in apoptotic cell death since they are able to undergo zinc-induced apoptosis that is readily quantitated by FACS analysis and have well characterized death pathways.[25] As will be demonstrated herein zinc-induced apoptosis utilized the caspase cascade and was blocked by overexpression of the antiapoptotic proteins Bcl-2 or Bcl-xL. To our knowledge it will also be shown for the first time that zinc induced the transcriptionally dependent BH3 only proapoptotic Bcl-2 family member Bim.

Materials and methods

Materials

Roswell Park Memorial Institute (RPMI-1640) culture media, penicillin-streptomycin, HEPES buffer, sodium pyruvate, L-glutamine, G418 were obtained from Gibco BRL (Rockville, MD). Hanks balanced salt solution (HBSS), ultrapure zinc sulfate heptahydrate, 1-hydroxypyridine-2-thione zinc salt (zinc pyrithione), dexamethasone, bovine serum albumin (BSA) and the antibody for β-Actin were obtained from Sigma Chemical Co. (St. Louis, MO). N,N,N′,N′-tetrakis (2-pyridinylmethyl) ethanediamine (TPEN), actinomycin D, cycloheximide, emetine, puromycin, caspase inhibitors, were obtained from Calbiochem (La Jolla, CA). Certified Australian fetal bovine serum (FBS) was obtained from Hyclone Laboratories, Inc. (Logan, UT). All antibodies used for phenotyping and antibodies to Bax, human Bcl-2, and Bcl-xL were obtained from Pharmingen (San Diego, CA). The antibody able to detect murine Bcl-2 was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The antibodies directed against Bim and caspase 9 as well as the Chaps Cell Extract Buffer were from Cell Signaling Technology (Beverly, MA). Propidium iodide (PI), 4′-6-diaminido-2-phenylindole (DAPI) and merocyanine 540 (MC540) were obtained from Molecular Probes (Eugene, OR). Enhanced Chemiluminescence Plus (ECL^+) and Percoll were obtained from Amersham-Pharmacia (Piscataway, NJ). BCA Protein Concentration Assay was obtained from Pierce Chemical Company (Rockford, IL). The cell lines used Ramos (CRL-1596) and Jurkat (TIB-152) were obtained from American Type Culture Collection (Manassa, VA), murine S49 T cells were obtained from The Cell Culture Facility University of California San Francisco (San Francisco, Ca.). Female A/J or CAF1/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME).

Cell culture

Thymuses or spleens from young mice (8–12 weeks old) were passed through a 100 micron mesh stainless steel screen into harvest buffer (HBSS with 4% heat inactivated FBS). Single cell suspensions of spleens were then layered over a 70% percoll gradient to remove erythrocytes. The resuspended cell suspensions from the thymus or spleen were cultured in RPMI-1640 containing 10% heat inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-mercaptoethanol, 2 mM L-glutamine in 24-well plates at 1–2 $\times$ 10^6 cells per ml for periods up to 8 hours at 37°C in 5% CO_2. Cell lines were cultured up to twelve hours in RPMI-1640 containing 10% heat inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 10 mM HEPES buffer, 1 mM sodium pyruvate, D-glucose at 4.5 g/L. Media for transfected cell lines also contained 500 μg/ml G418. Where applicable, dexamethasone at 1 μM, zinc sulfate (180–200 μM), zinc pyrithione (ZnPy) (200–600 nM) were added at time zero. Inhibitors were added at the concentration indicated one hour prior to the addition of initiators of apoptosis. Viability and recovery were assessed at the beginning and end of culture periods using trypan blue dye exclusion. All use of mice was approved by the University Laboratory Animal Research Committee at MSU.

Immunophenotyping and DNA labeling of thymocytes and splenocytes

Following the designated incubation period, thymocytes and splenocytes were harvested, washed, and resuspended in label buffer (HBSS with 2% heat inactivated FBS and 0.1% sodium azide) at $1–2 \times 10^6$ cells per ml per sample and immunophenotyped for T cell or B cell markers at 4°C. Antibodies used for T cell markers were Phycoerythrin (PE) conjugated anti-CD4 (GK1.5) and Fluorescein Isothiocyanate (FITC) conjugated anti-CD8a (53–6.7) while PE conjugated anti-B220 (RA3-6B2) was used for the B cell marker. Isotype/fluorochrome matched controls to all antibodies were used to determine background. Quantitation of cells in the hypodiploid region of the DNA cell cycle profile was as previously described.[26] Cells were resuspended in one part ice cold 50% FBS in phosphate buffered saline (PBS). Three parts ice cold 70% ethanol were added dropwise with gentle mixing and cells were incubated overnight at 4°C. After washing twice with label buffer the cells were resuspended in 1 ml of PBS with the addition of PI (50 μg/ml), RNaseA (100 units/ml), EDTA (0.1 mM) and 1% Triton X-100. In the case of three color analysis DAPI (1 μg/ml) was used to stain for DNA for 1 hour at room temperature in the dark. Between 10,000–20,000 cells per sample were analyzed.

Detection of apoptosis

Initial gating of thymocytes through PI or DAPI width vs. area allowed for exclusion of debris and cell doublets without affecting the apoptotic region for cell cycle analysis. As previously described, quantitation of apoptosis was carried out by determination of the percentage of cells in the hypodiploid region of DNA cell cycle directly or after gating for phenotype.[27]

Merocyanine 540 (MC540) labeling of cell lines for the detection of apoptosis

Since the hypodiploid peak or apoptotic region obtained with ethanol fixation is generally not well separated from the G_0/G_1 peak in cell lines undergoing apoptosis, it was necessary to stain the cell lines (Jurkat, Ramos, S49) with MC540 for improved identification of apoptotic cells as per past publications.[28] Briefly viable cells were removed from culture washed once in harvest buffer, resuspended in 100 μl MC540 staining solution (30 μM) and incubated 10 minutes in the dark at room temperature. After incubation, 900 μl harvest buffer was added and cells placed on ice in the dark. To differentiate between viable and dead cells, PI was added to a final concentration of 1 μg/ml two minutes prior to analysis. A 660 nm filter was used to detect MC540.

Data collection by FACS

All samples were analyzed on a Becton-Dickinson Vantage fluorescence activated cell sorter (FACS) (Becton-Dickinson, San Jose, CA) using CellQuest data acquisition and analysis software. Other software used to analyze data included WinList for Win32v4.0, ModFIT LT, and Microsoft Excel. Analysis of the immunophenotyped samples required use of an argon laser at 488 nm for excitation of FITC, PE, and PI with emission at 530, 575, and 630 nm, respectively. The UV lines of an argon/krypton mixed gas laser were used for simultaneous excitation of DAPI at 350 nm with emission at 470 nm where applicable. For MC540 analysis excitation was at 488 nm with emission at 575 nm and PI, used as a viability stain, was measured at 660 nm.

Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) for the analysis of zinc content

Untreated or zinc pyrithione (400 nM) or zinc sulfate (200 μM) treated thymocytes (2 hours) were washed once with phosphate buffered saline. Samples were then resuspended in Milli-Q water and transferred to acid washed containers and placed in an oven at 95°C to dry. Samples were digested overnight at 95°C with Ultrex II ultrapure nitric acid from JT Baker (Phillipsburg, NJ). The zinc content of each sample was measured at wavelength 213.857 nm using a Varian Vista Axial Inductively Coupled Plasma-Atomic Emission Spectrometer (Palo Alto, CA). Nitric acid digestion of standards containing reagents only (Milli-Q water or PBS) demonstrated no detectible zinc in these reagents while blank samples spiked with zinc pyrithione (400 nM) or zinc sulfate (200 μM) demonstrated the expected concentrations of zinc. The data shown was derived from quadruplicate samples $\pm$ standard error of the mean for each treatment group from one experiment representative of two independent experiments.

Western blot analysis of protein levels

All cells, except for caspase 9 detection, were lysed in NP-40 lysis buffer which contained 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 1% NP-40, phenylmethylsulfonyl fluoride (0.5 mM), sodium bisulfate (1 mM), benzamidine (1 mM), pepstatin A (1 μM), dithiothreitol (1 mM) and placed on ice for 30 minutes. Samples were then centrifuged to remove debris and the protein concentration was determined using the Pierce BCA assay. Cells used for caspase 9 detection were instead lysed in Chaps Cell Extract Buffer with dithiothreitol

(5mM) and phenylmethylsulfonyl fluoride (0.5 mM). 25 μg of protein were loaded per lane for the Western blots performed in extracts from murine thymocytes. For the Bcl-2 blot of human cell lines, 30 μg, 15 μg, 5 μg protein was used while 25 μg, 5 μg, 1 μg protein was used for the Bcl-xL blots. All samples were separated on a 12% resolving SDS-PAGE gel. The proteins were transferred to PVDF and blocked overnight at 4°C in 5% non-fat dry milk in Tris buffered saline with Tween 20 (TBST). The membranes were then incubated with primary antibody diluted in 5% non-fat dry milk in TBST at room temperature on a rocker platform for 1 hour. As per manufacture protocol for the detection of Bim isoforms (BimEL, BimL, BimS), membranes were incubated with primary antibody diluted in 5% BSA in TBST at 4°C overnight. Blots were washed several times in TBST and incubated with secondary antibody horse radish peroxidase (HRP) conjugated in 5% non-fat dry milk in TBST for 1 hour at room temperature on a rocker platform. After multiple washes, the membranes were developed using ECL$^+$ detection agent following the manufacturers instructions and exposed to film for visualization. The relative levels of protein were evaluated by densitometry performed on scanned images using ImageJ by W. Rasband (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/ij/).

Statistical methods

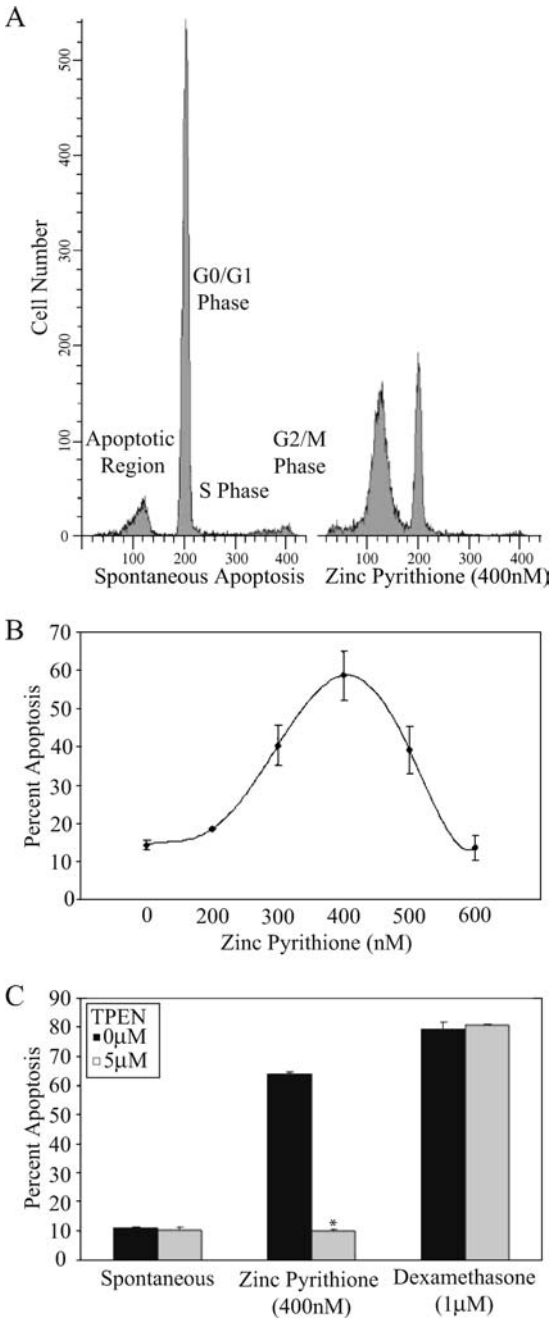
A representative experiment (mean $\pm$ SD) of at least two or more independent experiments is shown throughout. All samples were in triplicate unless otherwise noted. Immunophenotyping data was derived from triplicate samples generated from the assessment of three separate mice. Statistical calculations were made using SigmaStat 3.0 software (SPSS Inc., Chicago, IL). Samples were analyzed by One-way ANOVA with multiple comparison procedures using the Holm-Sidak Method. Data shown is the mean $\pm$ SD. An * indicates significant difference ($p \leq 0.05$) between treated and untreated samples within each group.

Results

Induction of apoptosis in murine thymocytes by zinc pyrithione (ZnPy)

By utilizing ZnPy that has ionophore like qualities it was possible to increase the availability of exogenously added zinc to cells. As can be seen in Figure 1A it was possible to induce up to 60% apoptosis in thymocytes using 400 nM zinc pyrithione where FACS DNA analysis was used to quantitate apoptosis. The exogenously added ZnPy

Figure 1. Analysis of induction of apoptosis in thymocytes by zinc pyrithione using FACS to quantitate cells in the hypodiploid or apoptotic region. (A) Analysis of spontaneous levels of apoptosis and zinc pyrithione (400 nM) induced apoptosis after 8 hours in culture. Percent apoptosis is based on the number of cells within the hypodiploid region. (B) Dose response curve for zinc pyrithione induced apoptosis. Thymocytes were incubated with indicated concentrations of zinc pyrithione and apoptosis measured after 8 hours in culture. Zinc pyrithione at 400 nM induced maximal apoptosis. (C) TPEN (N,N,N′,N′-tetrakis (2-pyridinylmethyl) ethanediamine) (5 μM), a cell permeable heavy metal chelator, abolished zinc pyrithione (400 nM) induced apoptosis while having no effect on dexamethasone induced apoptosis. Data shown is the mean $\pm$ SD. An * indicates significant difference ($p \leq 0.05$) between treated and untreated samples within each group.

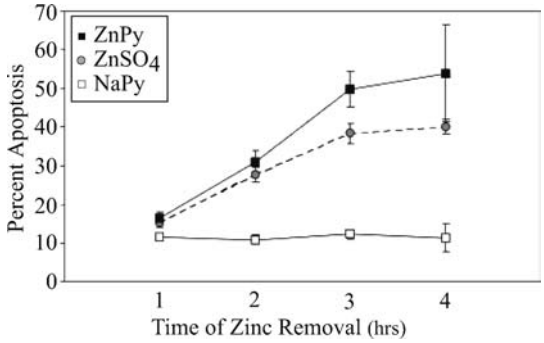


began to induce apoptosis at 250 nM with optimal induction observed at 400 nM for the 8 hr incubation period (Figure 1B). At 600 nM apoptosis declined indicating a moderate range of efficacy for zinc. Thus zinc can induce apoptosis at extremely low concentrations (normal plasma zinc is $\sim$15–20 μM).[24] In previous publications and subsequent figures it will be evident that substantial quantities of zinc salts were required to approach analogous levels of apoptosis.[3] The reduction in apoptosis observed above 400 nM zinc pyrithione was not due to cell loss or necrosis since viability and cell recovery were assessed using trypan blue exclusion. As previously shown, zinc sulfate (0–500 μM) treatment of thymocytes resulted in a similar bell-shaped dose response curve.[29] Prior studies illustrated that higher concentrations of zinc sulfate actually protect against apoptosis (temporarily) which may account for the bell-shape of the dose response curve.[29] This temporary protection from apoptosis by zinc sulfate may also explain the similar dose response curve observed for zinc pyrithione.[29] As also demonstrated in previous publications, zinc caused the standard morphological shrinkage of cells associated with apoptosis as demonstrated by phase contrast microscopy and by forward-side scatter analysis on the FACS (data not shown).[3] Extensive DNA fragmentation also occurred using ZnPy as demonstrated by the appearance of the apoptotic cells in the hypodiploid region of the DNA histogram but at far lower concentrations (Figure 1A). Observe the significant shift of thymocytes, which were predominantly in the G_0/G_1 region, to the low fluorescence or apoptotic region of the histogram subsequent to treatment with ZnPy (Figure 1A). To make certain that the induction of apoptosis was, indeed, caused by zinc a highly specific chelator of zinc N,N′,N′-tetrakis (2-pyridinylmethyl) ethanediamine (TPEN) was added to the cultures just prior to the addition of ZnPy. Additionally, the chelator completely blocked the ability of ZnPy to induce apoptosis while having no effect on dexamethasone induced apoptosis (Figure 1C).

Time of induction of apoptosis by zinc

We next sought to determine the time required for maximal induction of apoptosis. The removal of media containing exogenously added ZnPy (400 nM) or $ZnSO_4$ (180 μM) and replacing it with regular media for the remainder of an 8 hour culture period resulted in no induction of apoptosis above spontaneous levels after removal of zinc by one hour and only 25% apoptosis after a two hour exposure (Figure 2). The zinc content of thymocytes (19.5 $\pm$ 0.2 ng/1 $\times$ 10^7 cells) treated for two hours with zinc pyrithione (400 nM) increased 1.2 ng (20.7 $\pm$ 0.2 ng/1 $\times$ 10^7 cells) while zinc sulfate (200 μM) treated thymocytes increased 19.5 ng (38.9 $\pm$ 3.4 ng/1 $\times$ 10^7 cells) as measured by ICP-AES analysis. The difference

Figure 2. Time of induction of apoptosis by zinc in thymocytes. Thymocytes were incubated in the presence of zinc pyrithione (400 nM), zinc sulfate (180 μM), or sodium pyrithione (400 nM) for indicated times (1, 2, 3, or 4 hours). At each time point the cells were washed to remove exogenously added zinc and replated in fresh media for the remainder of an eight hour total culture period at which time apoptosis was quantitated. Zinc must be present for approximately 3 hours to facilitate maximum apoptosis. Data shown is the mean $\pm$ SD.



observed between the zinc sulfate and zinc pyrithione treated thymocytes may potentially reflect intracellular as well as associated zinc since the zinc sulfate treated cells were exposed to 500 fold more zinc. However, as demonstrated by this data only a small amount of zinc was associated with treated thymocytes. As shown in Figure 2 the sodium salt of pyrithione (400 nM) also failed to induce apoptosis which in addition to the zinc chelator TPEN shown in Figure 1C demonstrated that the response to zinc pyrithione was due to zinc (Figure 2). Overall it was necessary to incubate cells at least 3 to 4 hours with either ZnPy or $ZnSO_4$ to optimize cell death. Now that the time frame for the initiation of apoptosis by zinc was established we next wanted to determine whether other cells of the immune system in addition to murine thymocytes were susceptible to zinc pyrithione induced apoptosis.

Various cells of the immune system are susceptible to zinc-induced apoptosis

Various cells of the immune system undergo apoptosis in response to zinc pyrithione treatment (400 nM) shown in Table 1. These cells include primary murine splenic T and B cells and the murine S49 T cell line as well as both human Jurkat T cell and human Ramos B cell lines all of which readily undergo apoptosis in response to zinc pyrithione. The experiments shown in Figures 1 and 2 utilized thymocytes that, in fact, consist of four distinct cell subsets that include the earliest of T cells or the pro-T cells, pre-T cells that are involved in the gene arrangement of the TCR, mature helper T cells, and mature cytolytic T cells.[3] It is known that pre-T cells are very vulnerable to apoptosis, possible due to the lower expression of Bcl-2,

Table 1. Susceptibility of various cells of the immune system to zinc pyrithione (400 nM) induced apoptosis

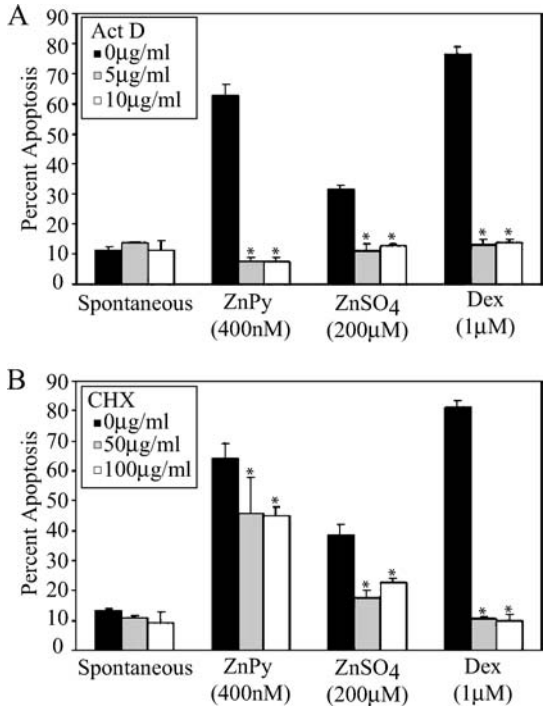
	Origin	Untreated (%)	Zinc pyrithione (%)	Time of incubation (hrs)
Primary cells				
Thymocytes				
Pro T-cells	murine	17	24	8
Pre T-cells	murine	13	60	8
Helper T-cells	murine	4	39	8
Cytolytic T-cells	murine	7	39	8
Spleen				
Helper T-cells	murine	7	48	8
Cytolytic T-cells	murine	3	66	8
B-cells	murine	15	33	8
Cell lines				
S49 T-cell	murine	11	51	12
Jurkat T-cell	human	6	43	12
Ramos B-cell	human	5	44	8

and succumb more readily to treatment with glucocorticoids, irradiation, etc., when compared to the pro-T, mature Helper T or Cytolytic T cells.[3,25,30] Thus, the pattern of sensitivity of thymocytes to ZnPy treatment, of which the pre-T cells undergo 60% apoptosis shown in Table 1, partially paralleled the previously reported expression of the antiapoptotic protein Bcl-2 raising the possibility that zinc-induced apoptosis might utilize the Bcl-2 regulated pathway.[3,25,31]

Dependence of ZnPy induced apoptosis on transcription and translation

Many of the classical apoptotic pathways are dependent on transcription and translation.[25] Using dexamethasone induced apoptosis as a standard, it was evident in Figure 3A that both ZnPy and zinc sulfate induced apoptosis require transcription. Actinomycin D at 5 μg/ml completely inhibited both zinc and dexamethasone induced apoptosis. Conversely in Figure 3B, 50 to 100 μg cycloheximide which completely blocked dexamethasone induced cell death, indicating the inhibitor was functioning, only partially blocked zinc-induced death for either ZnPy or the zinc salt. Higher concentrations of cycloheximide were toxic to thymocytes but other inhibitors of translation such as emitine and puromycin also failed to completely block zinc-induced apoptosis for either form of zinc (data not shown). Since the inhibitor of transcription completely blocked zinc pyrithione induced apoptosis and the translation inhibitor at least partially blocked apoptosis this indicated that the apoptotic signaling pathway required for zinc-induced apoptosis were dependent upon an increase in the production of both mRNA and protein.

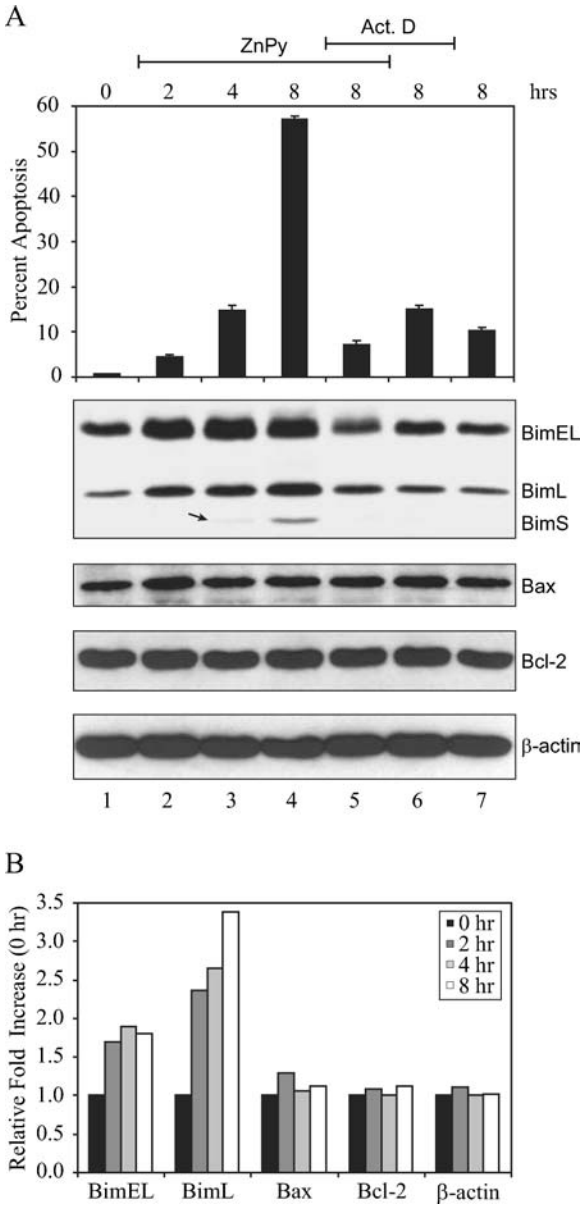
Figure 3. Zinc-induced apoptosis is dependent upon transcription and translation in thymocytes. (A) The addition of actinomycin D, an inhibitor of transcription, completely abolished zinc-induced apoptosis. (B) Cycloheximide, an inhibitor of translation, partially blocked zinc-induced apoptosis. Data shown is the mean $\pm$ SD. An * indicates significant difference ($p \leq 0.05$) between treated and untreated samples within each group.



Treatment with ZnPy up-regulates the BH3 only proapoptotic protein Bim

Since transcription and translation are required for zinc-induced apoptosis in murine thymocytes the identity of possible candidate proteins responsible for apoptosis was investigated. We were especially interested in possible roles for Bcl-2 family members, as shown in Figure 4 the BH3 only proapoptotic protein Bim was up-regulated in response to zinc treatment. To our knowledge this is the first time that the transcriptionally dependent up-regulation of Bim has been shown in response to zinc treatment. Thymocytes were dosed with 400 nM ZnPy and placed into culture which at the times indicated 0, 2, 4, or 8 hours samples were removed and processed for FACS analysis or Western blot analysis (Figure 4A). Densitometry was performed on the scanned Western blot images and the protein levels at time 0 hour (lane 1) for each protein (BimEL, BimL, Bax, Bcl-2, β-actin) were set to one for comparison purposes across the time course as

Figure 4. The BH3 only proapoptotic protein Bim is up-regulated in response to zinc pyrithione treatment in contrast to Bax or Bcl-2. (A) Murine thymocytes were untreated (lanes 1 and 7) or treated with 400 nM zinc pyrithione (lanes 2–5) and/or 5 μg/ml actinomycin D (lanes 5 and 6) for indicated times (0–8 hours) at which point samples were processed for FACS or Western blot analysis. FACS analysis demonstrated the percentage of cells undergoing apoptosis for each sample at the indicated times. Western blot analysis was performed to determine the protein levels of the Bcl-2 family members: Bim (three isoforms: BimEL, BimL, BimS), Bax and Bcl-2. β-actin was used as a loading control. The arrow shown in lane 3 denotes the most potent proapoptotic Bim isoform BimS detected as a faint band within 4 hours of zinc pyrithione treatment. (B) Densitometry was preformed on lanes 1–4 from part A and the relative fold increase in protein levels compared to the 0 hr sample (lane 1) is shown for each protein. Data shown for FACS analysis is the mean $\pm$ SD. Overall data shown is one biological replicate representative of two independent experiments.

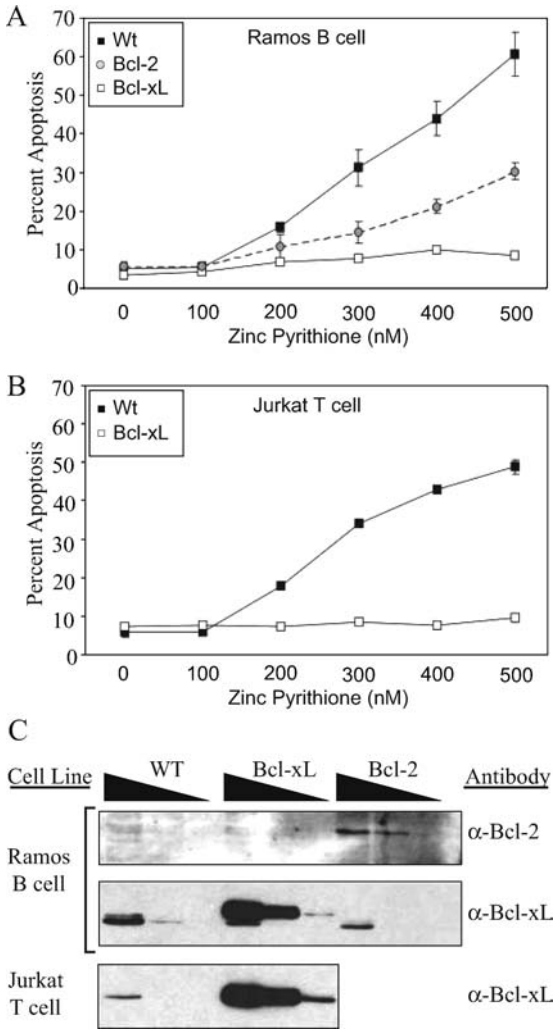


shown in Figure 4B. Three isoforms of Bim exist of which BimEL and BimL are present in murine thymocytes starting at time 0 hour (Figure 4A lane 1). Within 2 hours (lane 2) of exposure to ZnPy BimEL and BimL were strongly up-regulated 1.7 and 2.4 fold respectively. By 4 hours (lane 3) BimEL increased 1.9 fold while BimL increased 2.6 fold. Similar results were observed for zinc sulfate treated thymocytes (data not shown). Additionally detection of the strongest proapoptotic isoform BimS, which wasn't observed at 0 hour, appeared by 4 hours while the strongest levels occurred by 8 hours (lane 3 and 4). At the 8 hour time point BimEL was increased 1.8 fold and BimL was increased 3.4 fold. The overall up-regulation of Bim in response to zinc treatment corresponded to the induction of apoptosis as measured by FACS analysis. Addition of actinomycin D (5 μg/ml) which was shown to block zinc-induced apoptosis (Figure 3A) blocked the strong up-regulation of BimEL, BimL and BimS as well as apoptosis induced by zinc (lane 5). However, no increase in Bim expression was observed with actinomycin D treatment alone (lane 6) or in the untreated thymocytes (lane 7) analyzed for spontaneous apoptosis after 8 hours in culture. β-actin was used as a control to demonstrate equal loading of protein for each sample. As demonstrated in Figure 4B Bim levels increased in response to zinc treatment while Bax and Bcl-2 levels did not change. Since Bim functions by neutralizing antiapoptotic proteins such as Bcl-2 or Bcl-xL the increase in Bim levels would be expected to shift the cells fate in favor of promoting apoptosis, which was observed in lanes 1-4 of Figure 4A. It was also anticipated that overexpression of Bcl-2 or Bcl-xL would result in the protection of cells from apoptosis induced by ZnPy treatment.

Modulation of ZnPy induced apoptosis by Bcl-2

In order to test whether zinc-induced apoptosis could be suppressed by overexpression of Bcl-2 or Bcl-xL two cell lines, Ramos B cells and Jurkat T cell lines, were acquired that overexpressed these antiapoptotic proteins. As demonstrated earlier (Table 1) both wild type Ramos B cells and Jurkat T cells were susceptible to zinc pyrithione induced apoptosis. As observed in Figure 5A and B it was evident that the wild type Ramos B cell and Jurkat T cells were very sensitive to ZnPy which resulted in 50–60% apoptosis as measured by changes in membrane order using MC540 fluorescence.[28] MC540 analysis for the identification of apoptosis was undertaken since the analysis of the hypodiploid peak used for thymocytes can sometimes be difficult to cleanly separate from the G_0/G_1 peak in human cell lines. Our lab has previously shown that MC540 detects equivalent amounts of apoptosis as compared to hypodiploid analysis and Annexin V staining in murine thymocytes.[28] Therefore results obtained

Figure 5. The antiapoptotic Bcl-2 family of proto-oncogenes regulate zinc-induced apoptosis. (A) The human Ramos B cell line overexpressing Bcl-2 or Bcl-xL, and (B) the human Jurkat T cell line overexpressing Bcl-xL were analyzed for their sensitivity to zinc-induced apoptosis. Apoptosis was measured using MC540 fluorescence at eight and twelve hours, respectively. Error bars are shown for every sample being not apparent in some cases where variation was very small. (C) A Western blot was performed to verify expression levels of the antiapoptotic proteins in the transfected cell lines. Data shown is the mean ± SD.

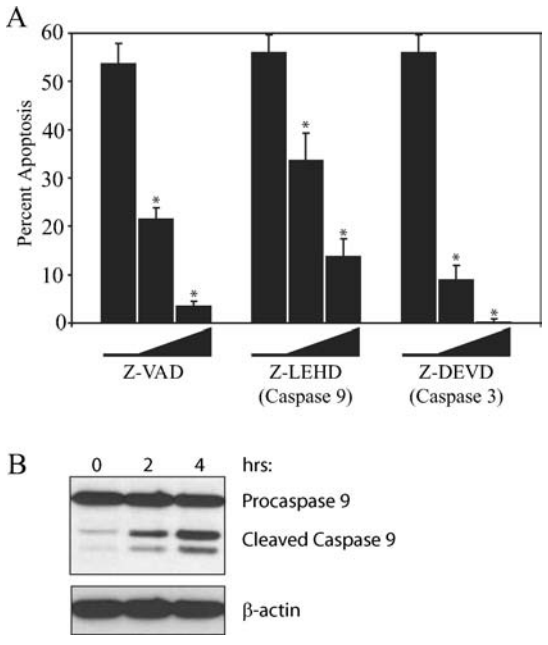


utilizing MC540 analysis in these cell lines was comparable. In the case of both Ramos B cells and Jurkat T cells, Bcl-xL provided complete protection against zinc-induced death (Figure 5A and B). Partial protection was provided in Ramos B cells since Bcl-2 was more moderately expressed (Figure 5A and C). A Western blot was performed to verify the degree of expression of these antiapoptotic proteins (Figure 5C). Overexpression of either Bcl-2 or Bcl-xL was able to suppress apoptosis induced by zinc further indicating that a Bcl-2 proapoptotic family member such as Bim was involved in activation of the intrinsic mitochondrial apoptotic pathway.

Caspase activation by ZnPy

In this paper we have thus far demonstrated that the BH3 only proapoptotic protein Bim (Figure 4) was upregulated in response to zinc which was blocked by the transcriptional inhibitor actinomycin D (Figure 4). Overexpression of Bcl-2 or Bcl-xL that can block the effects of Bim blocked zinc-induced apoptosis (Figure 5). This data clearly indicated that the mitochondrial apoptotic pathway was utilized during zinc-induced apoptosis. Proapoptotic activation of the mitochondria results in the release of various molecules including cytochrome c which activates the apoptosome with further activation of caspase 9 and 3. To further investigate this pathway the caspase family of cysteine proteases which play key roles as executioners during mitochondrial induced apoptosis[25] were examined in response to zinc treatment. As shown in Figure 6A murine thymocytes were treated with the broad spectrum caspase inhibitor Z-VAD-FMK (2 μM and 10 μM) which was able to completely block ZnPy induced apoptosis in thymocytes. The more specific caspase 9 inhibitor Z-LEHD-FMK (10 and 80 μM) was tested and

Figure 6. The dependency of zinc-induced apoptosis on the caspase cascade. (A) The broad spectrum caspase inhibitor (Z-VAD-FMK), the caspase 9 inhibitor (Z-LEHD-FMK), and the caspase 3 inhibitor (Z-DEVD-FMK) were evaluated for their impact on zinc pyrithione induced apoptosis. Zinc pyrithione (400 nM) induced apoptosis in thymocytes was reduced in the presence of increasing concentrations of these caspase inhibitors: Z-VAD (2 μM, 10 μM), Z-LEHD (10 μM, 80 μM), Z-DEVD (10 μM, 80 μM). (B) Procaspase 9 cleavage resulting in active caspase 9 occurs in response to zinc pyrithione treatment in thymocytes. β-actin was used as a loading control. Data shown is the mean ± SD. An * indicates significant difference ($p \leq 0.05$) between treated and untreated samples within each group.



reduced the degree of zinc-induced cell death to 35 and 12%, respectively (Figure 6A). Z-DEVD-FMK (10 and 80 μM), a specific inhibitor of caspase 3 the executioner caspase downstream of initiator caspases, was able to block zinc-induced cell death. Together this data indicated that the mitochondrial caspase cascade was involved in zinc-induced apoptosis. Additionally further analysis demonstrated that cleavage of procaspase 9 to active caspase was observed within two hours in response to zinc pyrithione (400 nM) treatment in thymocytes with stronger cleavage occurring by four hours (Figure 6B). These results provide additional evidence that the intrinsic mitochondrial apoptotic pathway was active and utilized in response to zinc treatment.

Discussion

Modulation of cellular zinc, either resulting in deficiency or excess, has been shown to be involved in the regulation of cell death in various types of cells.[32] During deficiency cells become more susceptible to numerous inducers of apoptosis, while the addition of exogenous zinc may reverse these apoptotic effects.[32] Supra-physiological concentrations of zinc (mM) have been shown to suppress apoptosis in response to various inducers of apoptosis;[32] however as demonstrated in murine thymocytes the protection afforded by zinc was only temporary as cell death still resulted several hours later.[29] Furthermore lower concentrations of zinc were shown to induce apoptosis in murine thymocytes.[3] Other labs have established that zinc can induce cell death in the case of cells of the lung, liver, prostate, etc.[4–6] Thus, a wide array of cell types can enter an apoptotic death phase upon exposure to exogenous or free zinc. Moreover among trace elements, this phenomenon appears to be somewhat peculiar to zinc since nickel, copper, cadmium and gold, did not initiate apoptosis in thymocytes in our hands.[29]

Using zinc pyrithione it was possible to show that 400 nM exogenously added zinc could induce apoptosis in various cells of the immune system from primary thymocytes and splenic B and T cells to the human Ramos B and Jurkat T cell lines. As shown in Table 1, a variety of cell types can undergo apoptosis in response to zinc pyrithione (400 nM) treatment. ICP-AES analysis of thymocytes treated with zinc pyrithione (400 nM) or zinc sulfate (200 μM) demonstrated that a relatively small amount of zinc (1.2 and 19.5 ng/1 $\times$ 10^7 cells) was associated with the treated cells. Zinc pyrithione induced apoptosis was demonstrated herein to be a zinc specific event through the inhibition of apoptosis by the zinc chelator TPEN and the lack of induction of apoptosis by sodium pyrithione. We further determined that zinc must be present for approximately three hours to maximize activation of the death pathway in thymocytes. Activation of this death pathway required transcription and translation. The inhibitor of transcription, actinomycin D, provided extensive blockage of apoptosis. However, cycloheximide and other translation inhibitors gave only partial inhibition in contrast to earlier studies in our lab with zinc salts where substantial inhibition was noted.[3] It is not uncommon for these inhibitors to not only give moderate inhibition, but actually become inducers dependent on the cell lines and culture conditions.[30] Overall though, the inhibition of zinc-induced apoptosis by the transcription and translation inhibitors suggested regulation by induction or increased expression of potentially proapoptotic genes.

In thymocytes zinc pyrithione had a preferential effect on pre-T cells that have been shown to contain little Bcl-2.[31] which potentially indicated regulation by the mitochondrial based Bcl-2 pathway. We therefore investigated possible Bcl-2 family members in the regulation of zinc-induced apoptosis. Indeed, all three isoforms of the BH3 only proapoptotic family member Bim were increased. Zinc-induced a 1.9 and 3.4 fold increase in expression of the BimEL and BimL isoforms respectively and also stimulated production of the most potent isoform, BimS. In contrast the levels of the proapoptotic protein Bax and the antiapoptotic protein Bcl-2 were unaffected. As others have previously shown Bim functions through neutralization of Bcl-2 or Bcl-xL thereby allowing Bax or other proapoptotic proteins already present to initiate mitochondrial apoptosis.[23] Since Bim functions by neutralizing antiapoptotic proteins an increase in Bim levels with no increase in Bcl-2 levels would be expected to shift the cells fate in favor of promoting apoptosis which was observed herein. Moreover, as would be expected overexpression of the antiapoptotic Bcl-2 or Bcl-xL proteins, which have been shown to block Bim dependent apoptosis,[23] prevented zinc initiated apoptosis. The difference in the ectopic expression of these antiapoptotic proteins may also explain the variation observed between the complete suppression of apoptosis in the Bcl-xL overexpressing cells and the protection provided by the Bcl-2 overexpressing cells. It has been shown that in response to cytokine withdrawal or treatment with taxol $bim^{-/-}$ pre-T cells survived 10–30 times better than wild type cells while heterozygous cells responded in between wild type and $bim^{-/-}$ cells.[33] The amount of the proapoptotic protein Bim or the level of overexpression of the antiapoptotic proteins such as Bcl-2 or Bcl-xL might therefore have profound effects on the induction of apoptosis. Overall, suppression of zinc-induced apoptosis by overexpression of Bcl-2 or Bcl-xL provides further evidence of the mitochondrial apoptotic pathway in apoptosis.

Initiation of the mitochondrial pathway results in the release of cytochrome c and the assembly of the apoptosome which promotes cleavage and activation of caspase 9 and 3.[20,21] This caspase cascade was shown herein to be required for zinc-induced apoptosis where inhibition

of caspase 9, the initiator caspase of the mitochondrial pathway,[34] and the executioner caspase 3 provided effective blockage of zinc-induced apoptosis. Cleavage of procaspase 9 to active caspase 9 also occurred in response to zinc treatment by two hours further demonstrating activation of the Bcl-2 family regulated mitochondrial apoptotic death pathway.

Conclusion

Excess free zinc has been implicated in various disease states having roles from neuronal damage[7–9] to Alzheimer's disease[11,12] and diabetes.[15] While zinc has been shown to induce apoptosis in a variety of cell types[4–6] a detailed analysis of the apoptotic death pathway utilized by zinc during apoptosis especially in cells of the immune system was not fully established. This is the first study to demonstrate the transcriptionally dependent upregulation of Bim leading to the caspase dependent mitochondrial pathway in the induction of apoptosis by a very low concentration of zinc.

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