Communication

Zinc Is a Potent Inhibitor of the Apoptotic Protease, Caspase-3

A NOVEL TARGET FOR ZINC IN THE INHIBITION OF APOPTOSIS*

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The prevention of apoptosis by Zn2+ has generally been attributed to its inhibition of an endonuclease acting in the late phase of apoptosis. In this study we investigated the effect of Zn2+ on an earlier event in the apoptotic process, the proteolysis of the “death substrate” poly(ADP-ribose) polymerase (PARP). Pretreatment of intact Molt4 leukemia cells with micromolar concentrations of Zn2+ caused an inhibition of PARP proteolysis induced by the chemotherapeutic agent etoposide. Using a cell-free system consisting of purified bovine PARP as a substrate and an apoptotic extract or recombinant caspase-3 as the PARP protease, Zn2+ inhibited PARP proteolysis in the low micromolar range. To rule out an effect of Zn2+ on PARP, a protein with two zinc finger domains, we used recombinant caspase-3 and a chromogenic tetrapeptide substrate containing the caspase-3 cleavage site. In this system, Zn2+ inhibited caspase-3 with an IC50 of 0.1 μM. These results identify caspase-3 as a novel target of Zn2+ inhibition in apoptosis and suggest a regulatory role for Zn2+ in modulating the upstream apoptotic machinery.

Apoptosis is a genetically programmed process of cell death characterized by a series of distinct morphological changes (1). Normal development and tissue remodeling of multicellular organisms are dependent on apoptosis, whereas defects in this process have been implicated in a number of pathological conditions. The agents which induce cells to undergo apoptosis are diverse and include extracellular agents such as TNF-α,1 the Fas ligand, and chemotherapeutic agents. Intracellular agents which regulate apoptosis include proteases, phosphatases, and kinases, products of lipid metabolism, and the cations Ca2+ and Zn2+.

The influence of Zn2+ on apoptosis is a well known phenomenon (2). In both in vitro and in vivo models, Zn2+ supplementation prevents apoptosis induced by a variety of agents (3, 4). Moreover, cells grown under conditions of Zn2+-deficiency will undergo spontaneous apoptosis (5–7).

The protective effect of Zn2+ has been attributed to its inhibition of a Ca2+- and Mg2+-dependent endonuclease (8), thereby causing inhibition of DNA fragmentation, a terminal step and hallmark of apoptosis. However, a number of observations suggest the existence of other and perhaps more relevant targets for Zn2+. First, the concentrations of Zn2+ used to demonstrate anti-apoptotic effects in either intact cells or isolated nuclei have ranged from micromolar to millimolar levels. However, the concentrations of Zn2+ used for inhibition of the purified Ca2+-dependent endonuclease were in the millimolar range (9). Second, it has recently been reported that in L929 cells, Zn2+ inhibited both TNF-α- and etoposide-induced cytotoxicity, as assessed by methylene blue staining, prior to effects on DNA fragmentation (10). Third, it is becoming more evident that the endonuclease functions in the “execution” rather than “regulation” phase of apoptosis, and therefore its inhibition may not prevent cell death. Fourth, Zn2+ has been demonstrated to inhibit the protease responsible for cleavage of laminas in cell-free extracts (11).

Recent studies have pointed to a role for a family of caspase proteases (formerly the ICE/Ced-3 proteases) (12) in apoptosis that act upstream of the endonuclease. Proteases in apoptosis came to the forefront with studies on the proteolysis of poly(ADP-ribose) polymerase (PARP), initially described in cells induced to undergo apoptosis by various chemotherapeutic agents, including etoposide (13–15). This event was later determined to be catalyzed by the protease resembling ICE (16). The human homolog of this protease has been cloned and is now known as caspase-3 (CPP32/yama/apopain).

To determine if Zn2+ inhibits an event upstream of endonuclease activation, we investigated the effect of Zn2+ on etoposide-induced apoptosis using PARP proteolysis as an indicator of this process. We show that Zn2+ is a potent inhibitor of PARP proteolysis in intact cells. We also demonstrate in a cell-free system that Zn2+ potently inhibits PARP proteolysis induced both by a caspase-3-containing apoptotic extract and by purified recombinant caspase-3. These results identify caspase-3 as a novel and proximal site of Zn2+ inhibition in the apoptotic pathway. Moreover, in light of results demonstrating a labile pool of Zn2+ in cells which is in rapid equilibrium with the extracellular medium (17), these findings raise the possibility that the cell may utilize Zn2+ as a regulator of the upstream apoptotic machinery.

EXPERIMENTAL PROCEDURES

Cell Culture—Molt4 cells from ATCC (Rockville, MD) were maintained under subconfluent conditions in RPMI medium with 10% fetal calf serum. For experiments, cells were diluted to 5 × 104/ml in RPMI 18530 This paper is available on line at http://www.jbc.org

1 The abbreviations used are: TNF, tumor necrosis factor; ICE, interleukin-1β-converting enzyme; PARP, poly(ADP-ribose) polymerase; ACDEVD-pNA, N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide.
with 2% fetal calf serum and treated with either etoposide delivered in a solution of 50% ethanol, 50% Me2SO or vehicle control.  

Cell Fractionation—The apoptotic extract was obtained by harvesting the cells after treatment with 20 μM etoposide for 6 h, resuspending once in phosphate-buffered saline, and then resuspending in cell-free buffer (10 mM HEPES, pH 7.4, 100 mM KCl, 3 mM NaCl, 4 mM MgCl2) at a concentration of 106 cells/ml. Cells were then lysed by N2 cavitation for 10 min at 450 p.s.i. The lysate was centrifuged at 100,000 × g for 30 min, and the supernatant was retained. PARP protease activity was retained in this fraction for at least 6 months when kept frozen at −80 °C.

Cell-free Incubations—Reactions in the cell-free system were initiated by the addition of 25 ng of purified bovine PARP to either 10 μM of the apoptotic extract or to 40 ng of caspase-3 in a total volume of 15 μl of cell-free buffer. After 20 min at 37 °C, the reactions were stopped by the addition of Laemmli buffer. For kinetic studies, the apoptotic extract or recombinant caspase-3 was added to buffer containing the respective cations immediately prior to the addition of the PARP substrate.

Production of Recombinant Caspase-3—A full-length cDNA encoding caspase-3 was cloned into pET23b from Invitrogen (San Diego, CA) and expressed in Escherichia coli strain BL21(DE3)pLysS as described previously (18). The caspase contained a C-terminal His6-tag to facilitate purification by affinity chromatography on Ni2+-NTA-agarose from Qiagen (Chatsworth, CA) according to the manufacturer’s instructions.

Western Blotting—Samples from intact cells and the cell-free system or the apoptotic extract from etoposide-treated cells were boiled in Laemmli buffer and loaded onto 6 or 15% polyacrylamide gels for determination of PARP or pro-caspase-3 proteolysis, respectively. After transfer to nitrocellulose membrane, the membranes were incubated with a rabbit polyclonal antibody (1:2000) to an epitope in the auto-modification domain of PARP or with a mouse polyclonal antibody from Transduction Laboratories (Lexington, KY) (1:1000) to a peptide in the p17 domain of caspase-3. Detection was accomplished using a horseradish peroxidase conjugate of a goat anti-rabbit (1:5000) or goat anti-mouse antibody (1:5000) from Bio-Rad for PARP and pro-caspase-3 proteolysis, respectively, and the ECL detection system from Amersham.

Cleavage of a Caspase-3 Peptide Substrate—N-Acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA) from Biomol (Plymouth Meeting, PA) at a concentration of 200 μM was incubated with either 20 μg of the apoptotic extract from cells treated with 20 μM etoposide for 6 h or with 280 ng of recombinant caspase-3 for 10 min at 37 °C in the presence or absence of varying concentrations of ZnCl2 in the cell-free buffer. Substrate cleavage was quantitated by measuring the absorbance at 405 nm in a Shimadzu spectrophotometer.

RESULTS AND DISCUSSION

To determine the effect of Zn2+ on etoposide-induced PARP proteolysis in intact cells, Molt4 culture medium was supplemented with varying doses of ZnCl2 prior to treatment of the cells with etoposide. The data in Fig. 1 indicate that Zn2+ caused a dose-dependent inhibition of PARP proteolysis with complete inhibition occurring at 100 μM ZnCl2. Zn2+ treatment alone had no effect on PARP proteolysis (data not shown).

To ascertain if the inhibitory action of Zn2+ was directly on PARP proteolysis, we used a cell-free system consisting of a soluble extract from etoposide-treated cells and bovine PARP as a substrate. We have previously used this system to study PARP proteolysis (19). The soluble extract contains processed caspase-3 as shown by the data in Fig. 2. Caspase-3 is synthesized as an inactive 32-kDa zymogen (20). During activation, the zymogen is cleaved to yield two subunits of 17 and 12 kDa which dimerize to form an active enzyme (21). Using an antibody which recognizes both the zymogen and the 17-kDa subunit, the immunoblot in Fig. 2 demonstrates that processing of the zymogen was initiated between 2 and 3 h of etoposide treatment as assessed by the appearance of the 17-kDa subunit. This closely correlates with the induction of PARP proteolysis by etoposide in this cell line (19).

Using an apoptotic extract from cells that had been treated for 6 h with etoposide, the results of Fig. 3 show that Zn2+ and Cu2+ caused complete inhibition of PARP proteolysis at a concentration of 10 μM. The effect was independent of the cation as both ZnCl2 and ZnSO4, as well as CuCl2 and CuSO4, gave identical results. Furthermore, other cations including Ba2+, Ca2+, Mn2+, and Fe3+ had no effect on PARP proteolysis. These results strongly suggest that the means by which Zn2+ inhibits PARP proteolysis is by acting directly on the PARP proteolysis step, either by inhibiting the protease(s) or by rendering PARP insensitive to proteolysis.

To further investigate the mechanism of Zn2+ inhibition and its target of action, we examined the effects of Zn2+ and other cations on PARP proteolysis catalyzed by purified recombinant caspase-3. The results in Fig. 4 indicate that the extent of inhibition of recombinant caspase-3 by Zn2+ is similar to the extent of inhibition observed on PARP protease activity using the apoptotic extract and that the cation profile is identical to that obtained in the apoptotic extract (Fig. 3). Inhibition was also obtained with Cd2+ and Hg2+ (data not shown). These results demonstrate that Zn2+ directly inhibits PARP cleavage induced by caspase-3.

PARP is a nuclear enzyme that contains two zinc finger domains near its amino terminus. It has been demonstrated that Zn2+ is required for PARP binding to damaged DNA (22); however, the influence of Zn2+ on the ability of PARP to serve as a substrate for caspase-3 has not been investigated. To eliminate the possibility that Zn2+ binding to the zinc finger domains of PARP may render it insensitive to proteolysis, we utilized a chromogenic tetrapeptide substrate, Ac-DEVD-pNA, containing the caspase-3 cleavage site in PARP and lacking the zinc finger domains. The results from Fig. 5 demonstrate that Zn2+ inhibited the cleavage of this substrate by the apoptotic extract with an IC50 value of nearly 1 μM (filled circles). A more potent inhibition by Zn2+ (IC50 ~0.1 μM) was observed when recombinant caspase-3 was used as the protease (open circles). These results provide strong evidence that the Zn2+ inhibition of PARP proteolysis is due to its inhibition of caspase-3 and not to a modification of the PARP substrate.

The mechanism by which Zn2+ inhibits caspase-3 awaits further studies. However, investigations on the crystal structure of ICE have suggested that His-237 and Cys-285 are involved in catalysis (23). These residues are conserved in all the caspase family members identified to date (24). Given the affinity of these amino acids for Zn2+, it is possible that Zn2+ inhibits caspase-3 by coordinating with one or both of these conserved sites. Moreover, Zn2+ inhibition of caspase-3 in the mid-nanomolar to low micromolar range is indicative of Zn2+...
binding to a single (or perhaps two) amino acid residue, whereas the tetrahedral coordination of Zn$^{2+}$ found in many Zn$^{2+}$-finger domains is characterized by a much greater affinity ($K_d \approx 10^{-9} - 10^{-12}$ M).

It has previously been reported that Zn$^{2+}$ does not inhibit caspase-3 activity (11). The reasons for the discrepancy with our results is unclear but a possible explanation is that we have used purified recombinant caspase-3 in our study, whereas in the referred study an E. coli lysate containing the recombinant enzyme was used. Therefore, it is possible that the lysate contained factors which were responsible for mitigating the inhibitory effect of Zn$^{2+}$ on caspase-3.

In the family of caspase proteases, caspase-3 has the highest homology to the Ced-3 protease which is required for developmental cell death in Caenorhabditis elegans (20). It is also activated by a variety of agents that induce apoptosis including TNF-α (25), Fas (26), and chemotherapy agents such as etoposide (27). Moreover, in a cell-free system comprised of apoptotic extracts and healthy nuclei, the presence of caspase-3 in the apoptotic extract was necessary to induce apoptotic changes in the nuclei (21). Also, it has recently been demonstrated that knockout mice which are lacking caspase-3 incur severe abnormalities in the development of the nervous system (28). Thus, the relevance of these data in demonstrating a novel inhibitory site for Zn$^{2+}$ in apoptosis is underscored by the fact that caspase-3 is both an important and common effector of the apoptotic machinery.

The question arises as to whether the concentrations of Zn$^{2+}$ required to inhibit caspase-3 in this study are attainable within the cell. It has been determined that serum Zn$^{2+}$ concentrations are 10–15 μM (29) and that the free Zn$^{2+}$ concentration in serum is approximately 1 μM (30). Furthermore, the intracellular Zn$^{2+}$ concentration has been estimated at 100 μM (17). Nearly 10% of this (~10 μM) exists in a labile pool which is in equilibrium with various Zn$^{2+}$-binding proteins. Therefore, the intracellular levels of free Zn$^{2+}$ may be in the range necessary to inhibit caspase-3. Currently, little is known about changes in intracellular Zn$^{2+}$ or its movement between compartments, but new classes of Zn$^{2+}$-responsive fluorophores appear to be promising in gaining new insight into this area (31). The results reported herein, in conjunction with a recent report demonstrating that treatment of T cells with a Zn$^{2+}$ chelator is able to induce caspase-3 activation (32), suggest that physiologic modulation of intracellular Zn$^{2+}$ may have important consequences for the regulation of caspases and the induction of apoptosis.

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