Effects of Zinc and Cadmium on Apoptotic DNA Fragmentation in Isolated Bovine Liver Nuclei

Ruth D. Lohmann and Detmar Beyersmann

Department of Biology and Chemistry, University of Bremen, Bremen, Germany

Isolated nuclei from mammalian cells contain a calcium-dependent endonuclease. The produced DNA fragmentation is a necessary step in the sequence of events resulting in apoptosis (programed cell death). We report here that zinc and cadmium inhibit the calcium-dependent endonuclease. The essential metal ion zinc may counterbalance the calcium-mediated apoptosis. In contrast to zinc, cadmium alone stimulates the endonuclease by replacing calcium. Thus cadmium exerts a dual effect: micromolar concentrations inhibit the apoptotic endonuclease in the presence but activate the enzyme in the absence of calcium. — Environ Health Perspect 102(Suppl 3):269–271 (1994).

Key words: liver nuclei, calcium, zinc, cadmium, apoptosis, DNA fragmentation, endonuclease

Introduction

Apoptosis, a cellular suicide process, has recently attracted much interest. It shows specific morphologic and biochemical features that are distinct from the pathologic cell death necrosis. The characteristic condensation of the chromatin that occurs during apoptosis has been linked to the activation of an endonuclease that cleaves the DNA at internucleosomal linker regions in fragments of approximately 200 base pairs (*1,2*). The classic example for this type of DNA degradation is found in the apoptotic thymocyte after glucocorticoid treatment (*2,3*). A sustained increase in cytosolic calcium levels has been demonstrated to stimulate both endonuclease activation and cell death (*4*). Pretreatment with intracellular Ca-chelators blocks this process (*5*), but the Ca^{2+} ionophore A23187 stimulates apoptosis in thymocytes (*6*). It is obvious that nuclei contain a constitutive Ca^{2+}-dependent endonuclease, which can be activated by incubation of the nuclei with micromolar free Ca^{2+} concentrations (*1*).

Zinc is the preferential Ca^{2+}-dependent endonuclease blocker. In different cell culture systems (*7–11*) and *in vivo* (*12,13*) zinc inhibits the apoptotic cell death. Spontaneous apoptosis is greatly increased in cells depleted of zinc *in vitro* (*13*). Thus, zinc seems to have an apoptosis suppressing function. Until now, both, the zinc binding site and the mechanism of inhibition have remained obscure. Furthermore, there is no information available about the influence of other heavy metals on Ca^{2+}-dependent DNA fragmentation. In this article we present the results of investigations into Ca^{2+}-dependent DNA fragmentation as influenced by zinc and cadmium. We also studied possible stimulations of DNA fragmentation by divalent cations other than Ca^{2+}.

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Address correspondence to Detmar Beyersmann, University of Bremen, Department of Biology and Chemistry, Leobener, Str., NW2; D-28334 Bremen, Germany. Telephone (421) 218-2377. Fax (421) 218-4042.

Materials and Methods

Preparation of Bovine Liver Nuclei

Nuclei were prepared by the method of Jones et al. (*1*). The highly purified nuclei were resuspended in incubation medium with chelators (25 mM HEPES, 2 mM potassium phosphate, 125 mM KCl, 4 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM HEDTA, 0.5 mM NTA, pH 7.0) and washed once by centrifugation at $200 \times g$ for 5 min. The freshly prepared nuclei were used directly for incubation. The nuclear fraction was virtually free of contamination by plasma membranes, microsomes, and mitochondria, as checked by determination of marker enzyme activities (5′-nucleotidase, alkaline phospodiesterase I, glucose-6-phosphatase, succinate-INT-reductase).

DNA Fragmentation Assay

5×10^6 nuclei were incubated in 1 ml incubation medium containing the calculated free ion concentration at 37°C. After 1 hr the nuclei suspension was added to 1ml ice cold lysis medium (5mM Tris-HCl, 20mM EDTA, 0.5% (w/v) Triton X-100, pH 8.0). Samples were centrifuged for 20 min at $27,000 \times g$ to separate the intact chromatin (pellet) from the fragmented DNA (supernatant). The supernatants were decanted and the pellets were resuspended in 4 ml of 10mM Tris-HCl, 1mM EDTA, 0.2% SDS, pH 8.0. Pellet and supernatant fractions were assayed for DNA content with the diphenylamine reaction (*14*).

Buffering of Free Ion Concentrations

Free ion concentrations were adjusted in standard incubation medium. The appropriate total concentrations of Ca^{2+}, Zn^{2+}, Cd^{2+}, Ni^{2+}, or Hg^{2+} required to achieve the desired free ion concentrations were calculated by the computer program SPECS of Fabiato (*15*). Absolute stability constants were taken from Smith and Martell (*16*).

Gel Electrophoresis

The fragmented DNA was lysed with proteinase K (100 mg/ml, Boehringer Mannheim, Mannheim, Germany) and 1% SDS for 1 hr at 37°C. Thereafter the DNA fragments were extracted sequentially with equal volumes of phenol, phenol:chloroform (1:1), and chloroform, precipitated in 67% ethanol, 0.17 M NaCl at –20°C for 40 hr, lyophylized for 2 hr, and resuspended in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0, prior to electrophoresis for 10 hr in 1.8% agarose gels. DNA was visualized by fluorescence after staining with ethidium bromide (1 μg/ml).

Results

Incubation of isolated rat liver nuclei with different free Ca^{2+} concentrations induced DNA fragmentation in a concentration-

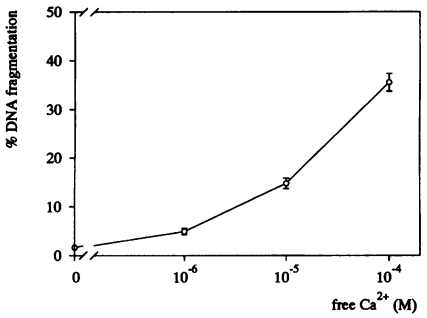

Figure 1. Dependence on free Ca^{2+} of DNA fragmentation in isolated bovine liver nuclei. Mean ± SE are from four different experiments. DNA fragmentation was analyzed by centrifugation and diphenylamine reaction as described in Materials and Methods.

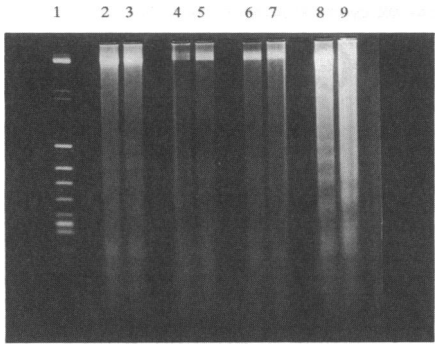

Figure 2. Electrophoresis of DNA fragments from bovine liver nuclei. DNA was visualized by UV fluorescence after staining with ethidium bromide (10 mM/ml). Contents of lanes 1 to 8 were DNA from treated bovine liver nuclei as follows: 1, molecular size markers, digest of Hind III of λ-DNA and Hinc II of ΦX 174 RFA-DNA; 2 and 3, 10 μM free Cd^{2+}; 4 and 5, 100 μM free Ca^{2+} and 10 μM free Cd^{2+}; 6 and 7, control (1.5 mM chelator concentration); 8 and 9, 100 μM free Ca^{2+}. Results of one experiment were typical of three. Reprinted with permission from Lohmann and Beyersmann (*23*).

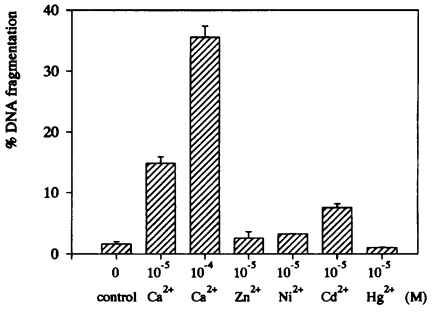

Figure 4. Activating capacity of the DNA fragmentation in bovine liver nuclei by various divalent metals. Mean ± SE are from three separate experiments. Reprinted with permission from Lohmann and Beyersmann (*23*).

dependent manner (*1*). We find this effect also in isolated bovine liver nuclei. The DNA fragmentation after 1 hr incubation of isolated bovine liver nuclei with various concentrations of free Ca^{2+} is shown in Figure 1. Treatment with 1 μM free Ca^{2+} had a fragmentation rate of 5% of the total DNA, increasing to 36% at a concentration of 100 μM free Ca^{2+}. Agarose gel electrophoresis of the DNA fragments showed the typical ladder pattern of internucleosomal cleavage (Figure 2).

Incubation of nuclei with varying concentrations of zinc resulted in an inhibition of the Ca^{2+}-induced DNA fragmentation. Cadmium, an element that reacts in a manner closely related to zinc (e.g., the ability to substitute for zinc in some zinc enzymes), also displayed an inhibitory effect on the Ca^{2+}-dependent DNA fragmentation. Figure 3 shows that the inhibition by cadmium was much stronger than by zinc. A concentration of 10 μM free Cd^{2+} almost completely blocked the DNA fragmentation even in the presence of high Ca^{2+} concentrations (100 μM). The influence of calcium on the inhibitory effect of zinc is demonstrated in Figure 3*A*. In the presence of 100 μM free Ca^{2+} there was less inhibition than in the presence of 10 μM free Ca^{2+}. In contrast to zinc, cadmium acted almost independent of the concentration of Ca^{2+}. It showed nearly the same degree of inhibition in the presence of 100 μM and 10 μM free Ca^{2+} (Figure 3*B*).

We further investigated whether divalent ions can substitute for Ca^{2+}, and we observed a significant increase in DNA fragmentation in the presence of 10 μM Cd^{2+} in a Ca^{2+}-free system. The effect was comparable to the fragmentation seen with 10 μM Ca^{2+} (Figure 4). Further exploration of the stimulating effect of Cd^{2+} revealed that it occurred only at concentrations around 10 μM, which represents an optimum. Increasing concentrations of Cd^{2+} did not result in any appreciable fragmentation. Possibly there are two different mechanisms, a stimulating and an inhibiting one, which result in this dual effect of cadmium. Ni^{2+}, Zn^{2+}, and Hg^{2+} did not exhibit any DNA fragmentation in isolated bovine liver nuclei at a concentration of 10 μM free ions (Figure 4). Also, at concentrations up to 500 μM, zinc did not stimulate DNA degradation in this system.

Presuming that the DNA fragments found after incubation with Cd^{2+} depend on the activation of the endonuclease, we separated the fragments by agarose gel electrophoresis. Figure 2 shows the isolated DNA fragments in a 1.8% agarose gel displaying the expected ladder pattern in the case of activation by 100 μM Ca^{2+} or 10 μM Cd^{2+}. In contrast, a nearly complete inhibition of the DNA fragmentation by 10 μM free Cd^{2+} is observed in the presence of 100 μM free Ca^{2+} although 10 μM Cd^{2+} without Ca^{2+} had an activating effect.

Aurintricarboxylic acid, a potent inhibitor of nucleases, protected nuclei from Ca^{2+}-induced DNA fragmentation even by 100 μM free Ca^{2+}. This action has been described for whole cells (*6*). The Cd^{2+}-induced DNA fragmentation was also inhibited by 200 μM aurintricarboxylic acid. This result is consistent with the interpretation that Cd^{2+}, like Ca^{2+}, is able to stimulate the apoptotic endonuclease.

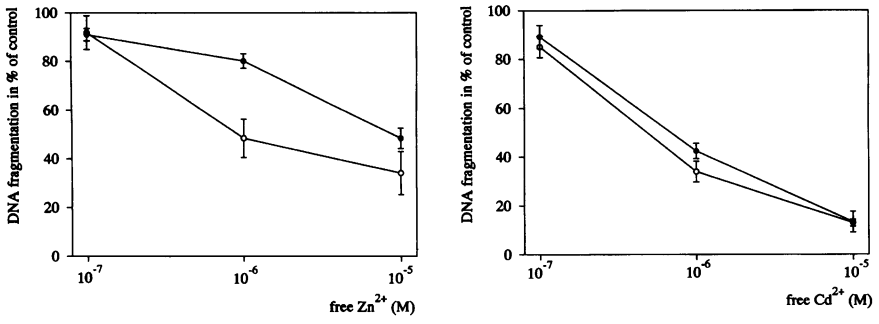

Figure 3. Comparison of the inhibitory effect of zinc and cadmium on DNA fragmentation in bovine liver nuclei. Values are given as a percentage of the fragmentation obtained in the presence of free Ca^{2+} without metal ions. Mean ± SE are from three separate experiments. (*A*) Inhibition of the DNA fragmentation by various zinc concentrations in the presence of 100 μM free Ca^{2+} (●) and 10 μM free Ca^{2+} (○). (*B*) Inhibition of the DNA fragmentation by various cadmium concentrations in the presence of 100 μM free Ca^{2+} (●) and 10 μM free Ca^{2+} (○). Reprinted with permission from Lohmann and Beyersmann (*23*).

Discussion

The Ca^{2+}-mediated DNA fragmentation in mammalian cells is due to a Ca^{2+}/Mg^{2+}-dependent endonuclease, which is constitutive in nuclei and not lysosomal (*17*). To investigate the mechanism of this type of DNA fragmentation, the use of bovine liver nuclei is an appropriate system, because there is almost no fragmentation by incubation

without Ca^{2+} (control) and there is a clear dependence on rising Ca^{2+} concentrations. To explain the inhibitory influence of zinc on the DNA fragmentation, two different mechanisms are discussed. Inhibition by occupying a putative zinc binding site at the endonuclease is one point (*17,18*). Gaido and Cidlowski (*17*) reported the inhibition by zinc of the pure isolated endonuclease. Alternatively, the interactions between zinc and phosphate groups of the DNA stabilize the macromolecular structure, as described by Koizumi and Waalkes (*19*). The modification of the chromatin structure by polyamines can prevent DNA fragmentation and apoptosis in thymocytes (*20*). This suggests that the endonuclease-mediated internucleosomal cleavage occurs only when the linker regions are accessible.

Our experiments indicate that the inhibition by zinc depends on the concentration of free calcium. Higher Ca^{2+} concentrations cause a decreased inhibition by zinc. This fact suggests a competing reaction of calcium and zinc at the same intracellular site. Zinc occurs in smaller amounts than calcium in cells and the exchange of zinc in biological molecules is slower than by Ca^{2+}. To displace zinc, higher Ca^{2+} concentrations are required. We propose that the balance between Ca^{2+}/Zn^{2+} is responsible for the regulation of the endonuclease.

As known for other enzymes, Cd^{2+} could replace zinc (*21*). The inhibitory effect of cadmium on the DNA fragmentation led to the suggestion that Cd^{2+} could substitute for zinc. The stronger inhibitory effect of cadmium may be explained in terms of a higher affinity of cadmium to the putative intracellular site. In contrast to the inhibition by zinc, cadmium inhibits the endonuclease in a Ca^{2+}-independent manner. This may suggest another method of inhibition. In spite of the strong inhibitory effect of Cd^{2+} in the presence of Ca^{2+}, Cd^{2+} can also stimulate the endonuclease in a Ca^{2+}-free system. A Ca^{2+}/Cd^{2+} substitution is possible which leads to an active endonuclease with a slower fragmentation of the double-stranded DNA. This substitution is not unusual; it is known that calmodulin, where Cd^{2+} replaces Ca^{2+}, is capable of its biological function (*22*). The appearance of a maximum, i.e., a decline of the endonuclease activity at higher Cd^{2+} concentrations, emphasizes that Cd^{2+} inhibits the Cd^{2+} activation itself.

The two effects of Cd^{2+}, inhibiting and stimulating the enzyme, indicate the possibility of two "Cd^{2+} binding sites". As shown by the plots of relative inhibition by zinc and cadmium (Figure 3*A,B*), the kinetics differ. This is a further indication for an inhibitory Cd^{2+} binding site, distinct from the Ca^{2+}site for and the probable zinc binding site on the endonuclease.

Investigations of toxic effects of heavy metals should take into consideration cell death and interference with the regulation of cell survival.

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