Zinc protects against apoptosis of endothelial cells induced by linoleic acid and tumor necrosis factor α1–3

P Meerarani, P Ramadass, Michal Toborek, Hans-Christian Bauer, Hannelore Bauer, and Bernhard Hennig

ABSTRACT

Background: Zinc requirements of the vascular endothelium may be increased in inflammatory conditions, ie, atherosclerosis, in which apoptotic cell death is prevalent.

Objective: We hypothesized that zinc deficiency may potentiate disruption of endothelial cell integrity mediated by fatty acids and inflammatory cytokines by enhancing pathways that lead to apoptosis and up-regulation of caspase genes.

Design: Endothelial cells were maintained in low-serum medium or grown in culture media containing selected chelators, ie, diethylene-triaminepentaacetate or N,N',N"-tetrakis(2-pyridylmethyl)-ethylenediamine (TPEN), with or without zinc supplementation. Subsequently, cells were treated with linoleic acid, tumor necrosis factor α (TNF-α), or both. We studied the effect of zinc deficiency and supplementation on the induction of apoptosis by measuring caspase-3 activity, cell binding of annexin V, and DNA fragmentation.

Results: Our results indicated that linoleic acid and TNF-α independently, but more markedly in concert, up-regulated caspase-3 activity and induced annexin V binding and DNA fragmentation. Zinc deficiency, especially when induced by TPEN, dramatically increased apoptotic cell death induced by cytokines and lipids compared with control cultures. Supplementation of low-serum- or chelator-treated endothelial cells with physiologic amounts of zinc caused a marked attenuation of apoptosis induced by linoleic acid and TNF-α. Morphologic changes of cells observed during zinc deficiency were prevented by zinc supplementation. Media supplementation with other divalent cations (eg, calcium and magnesium) did not mimic the protective role of zinc against apoptosis.

Conclusions: Our data indicate that zinc is vital to vascular endothelial cell integrity, possibly by regulating signaling events to inhibit apoptotic cell death. Am J Clin Nutr 2000;71:81–7.

KEY WORDS Zinc, zinc deficiency, zinc supplementation, atherosclerosis, endothelial cells, vascular endothelium, apoptosis, apoptotic cell death, cytokines, cell destabilizing agents, polyunsaturated lipids, linoleic acid, caspase-3, annexin V, tumor necrosis factor α, inflammatory conditions

INTRODUCTION

Apoptosis is a morphologically distinct mechanism of programmed cell death that involves activation of a suicide program intrinsic to cells. Apoptosis is known to play major roles in normal development and homeostasis, and in different disease processes (1). Evidence suggests that apoptosis is also involved in the regulation of aortic intimal thickening during atherosclerosis (2). In fact, apoptotic cell death is common in atherosclerotic plaques (3), especially in plaques that show dense macrophage infiltration (4). This suggests that vascular tissue can become susceptible to apoptosis. Evidence also suggests that additional factors, mainly those derived from macrophages and lipids, are necessary to complete the cell-death pathway (4, 5). Such factors may include inflammatory cytokines such as tumor necrosis factor α (TNF-α) and pure or oxidized lipids.

Vascular endothelial cells provide an antithrombotic and antiinflammatory barrier for normal blood vessel walls. It is well known that dysfunction of the vascular endothelium can promote atherosclerosis, whereas normalization of previously dysfunctional endothelial cells can inhibit its development. Apoptotic endothelial cells have been detected on the luminal surface of atherosclerotic coronary vessels, but not in normal vessels (6), suggesting a link between endothelial cell apoptosis and the pathology of atherosclerosis. It is very likely that increased endothelial cell turnover mediated by accelerated apoptosis may alter the function of the endothelium and thereby promote atherosclerosis.

Reactive oxygen species are known to induce apoptosis in a wide variety of cell culture systems and are believed to play important roles in various pathologic disorders, ie, neurodegenerative diseases that involve apoptosis as an underlying mechanism (7–9). Thus, the cellular redox status and the balance between oxygen free radical generation and detoxification can influence...
apoptosis. Evidence indicates that oxidant-initiated apoptosis requires activation of the transcription factor AP-1 (10, 11) and that apoptosis initiated by hydrogen peroxide was inhibited by down-regulation of c-Jun/AP-1 (12).

A central component of apoptotic cell death is a proteolytic system involving a family of proteases called caspases (13). These enzymes participate in a cascade that is triggered in response to proapoptotic signals; the cascade culminates in cleavage of a set of proteins, which results in disassembly of the cell. Caspase-3, also known as CPP32, appears to be the critical factor causing cell death in the Fas-induced pathway and is one of the major activated caspases present in apoptotic cells, suggesting that it plays a prominent role in the cell-death process (14). Apoptosis mediated by caspase-3 may be modulated by cellular oxidative stress. For example, an increase in caspase-3-like protease activity in human endothelial cells was associated with the induction of apoptosis by oxidized LDL (15).

We have shown that zinc is vital to endothelial integrity and that zinc deficiency causes severe impairment of the endothelial barrier function (16, 17). Zinc has been shown to have antioxidand membrane-stabilizing properties (18, 19). We showed previously that disruption of endothelial cell monolayer integrity by TNF-α can be prevented by preenriching cells with zinc (20). Evidence shows that during zinc deficiency, cellular oxidative stress is markedly induced by exposure to linoleic acid, TNF-α, or both and that this oxidative stress can be partially blocked by zinc supplementation (21).

Research indicates that zinc is a potent inhibitor of apoptosis (22, 23) and that zinc deficiency can induce apoptosis (24). The subcellular mechanisms by which zinc affects apoptosis are not well understood and may occur at many levels. Recently, it was reported that the protective effects of zinc may occur in part via inhibition of caspases, such as caspase-3 (25–27). In this article, we provide evidence that suggests that linoleic acid and TNF-α can independently, but more markedly in concert, induce caspase-3 activity in endothelial cells. Furthermore, our data show that zinc can protect against endothelial cell apoptosis induced by linoleic acid, TNF-α, or both.

**MATERIALS AND METHODS**

**Cell culture and experimental media**

Endothelial cells were isolated from porcine pulmonary arteries and were cultured as described previously (28). Cells were subcultured in medium 199 containing 10% fetal bovine serum (FBS; HyClone Laboratories Inc, Logan, UT) by using standard techniques. Purity of the cultures was determined by using morphologic criteria and measuring angiotensin converting enzyme activity, or by measuring their uptake of fluorescent-labeled acetylated LDL (1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate; Molecular Probes Inc, Eugene, OR). For example, uptake of fluorescent-labeled acetylated LDL as measured by flow cytometry analysis revealed that our cultures consisted exclusively of endothelial cells.

The experimental media were composed of medium 199 enriched with 10% FBS and selected chelating agents such as the membrane-impermeable chelator diethylenetriaminepentaacetic acid (DTPA) (29) or the membrane-permeable chelator N,N',N'-tetrais(2-pyridylmethyl)-ethylenediamine (TPEN) (30). Some of these media were also supplemented for 48 h with zinc (10 μmol zinc acetate/L; Sigma, St Louis). In some experiments, zinc deficiency was induced by culture in low-serum media (1% FBS) for ≤ 8 d, after which the designated groups (half of the cultures) were supplemented with zinc for 48 h. After zinc depletion or zinc supplementation, cultures were exposed to linoleic acid (90 μmol/L for 24 h; >99% pure; Nu-Chek Prep, Elysian, MN), TNF-α (500 U/mL for 6 h; Knoll Laboratories, Whippany, NJ), or both. Experimental media containing linoleic acid, TNF-α, or both were prepared as described previously (28, 31).

Zinc concentrations in endothelial cells or surrounding culture media were measured with a Perkin-Elmer 5000 atomic absorption spectrophotometer (Perkin-Elmer, Norwalk, CT) as described previously (17, 32). For example, mean (±SEM) cellular zinc concentrations when cells were cultured for several days in zinc-deficient (low-serum) media and were subsequently supplemented with zinc were 1.2 ± 0.06 and 2.3 ± 0.3 μmol L⁻¹ mg protein⁻¹, respectively (21). Thus, zinc supplementation resulted in a marked increase in cellular zinc content. Zinc concentrations in the medium were significantly lower in medium 199 supplemented with 1% FBS compared with medium 199 supplemented with 10% FBS (0.4 ± 0.07 and 3.4 ± 0.15 μmol/L, respectively).

The rationale for using 10 μmol Zn/L (65 μg zinc/dL) was based on preliminary data from other studies. Compared with normal serum zinc concentrations, which average 12–18 μmol/L, cell culture media are usually low in zinc. We were attempting to perform studies in which physiologic amounts of zinc were added to the culture media. Thus, 10 μmol/L would represent a moderately low serum zinc concentration that might be found in normal volunteers, but would be within a physiologic range and certainly higher than that found in many tissue culture media.

**Measurement of caspase-3 activity**

Caspase-3 activity was measured with a commercially available kit called ApoAlertTM (Clontech Laboratories, Palo Alto, CA; 33). Briefly, 2 × 10⁶ porcine endothelial cells were lysed by incubating them in a lysis buffer in ice for 10 min. The cell lysates were centrifuged at 200 × g for 5 min at 4°C to precipitate cellular debris. The supernates were transferred to new microcentrifuge tubes and 50 μL of 2 times the concentration of the reaction buffer containing DTT, followed by 5 μL of substrate, were added and incubated at 37°C for 1 h in a water bath. The samples were transferred to a 96-well microtitre plate and optical density was measured at 405 nm in an enzyme-linked immunosorbent assay reader. A calibration curve was prepared with different concentrations, ranging from 0 to 20 nmol, of p-nitroanilide (provided in the kit) against optical density at 405 nm.

**Annexin V staining and flow cytometry**

Annexin V binding was measured with a commercially available kit (ApoAlert Annexin V Apoptosis Kit, Clontech Laboratories). The usefulness of annexin V in flow cytometry applications is derived from its selective affinity for negatively charged phospholipids, specifically phosphatidylserine. Because loss of cell membrane phospholipid asymmetry occurs during early phases of apoptosis, ie, cells expose phosphatidylserine to the outer membrane, differential staining of cells with annexin V is a useful tool with which to identify cells undergoing apoptosis. Stained cells were analyzed and sorted with FACStar Plus cell sorter (Becton Dickinson, Franklin Lakes, NJ). Cells were ana-
lyzed by flow cytometry with a single laser-emitting excitation light at 488 nm.

DNA fragmentation studies

The genomic DNA was isolated from cell monolayers by using DNAzol (Genomic DNA Isolation Reagent, Molecular Research Center, Inc, Cincinnati, OH) as described by Chomczynski et al (34). Endothelial cells were grown in culture media enriched with zinc chelators for ≤24 h and were then treated with linoleic acid and TNF-α. Some cultures were also supplemented with zinc. After experimental treatment, the media were removed and 1 mL of DNAzol was added. The cell lysates were passed through Pasteur pipettes several times for complete lysis of cells. Then each lysate was collected in a microcentrifuge tube (Eppendorf, Hamburg, Germany) and centrifuged at 150 × g for 10 min at 4°C. The viscous supernate was removed into another tube and DNA was precipitated by the addition of 0.5 mL 100% ethanol per mL of DNAzol used for isolation. Samples were mixed by inverting the tubes 5–8 times and were left at room temperature for ≈2–3 min. The DNA was pelleted by centrifugation at 200 × g for 15 min at 4°C. The DNA pellets were then washed twice with 0.8–1.0 mL 95% ethanol. Each DNA pellet was dissolved in 50 μL distilled water.

For agarose gel electrophoresis, a 1.6% agarose gel was prepared in 1 × Tris acetate–EDTA buffer. Then 10 μL of sample was loaded into the gel, electrophoresed at 100 volts for about 2–3 h, and viewed in a UV transilluminator.

Statistical analysis

The data were analyzed by using 2-way analysis of variance followed by pairwise comparison with post-hoc Bonferroni analysis (35). A statistical probability of $P < 0.05$ was considered significant.

RESULTS

Caspase-3 activity in endothelial cells during incubation in media with or without added zinc (control condition) and after exposure to linoleic acid, TNF-α, or both is shown in Figure 1. When compared with control cultures, both linoleic acid and TNF-α significantly increased caspase-3 activity, which was partially blocked by zinc supplementation only in linoleic acid-treated cultures. Furthermore, when cells were first exposed to linoleic acid followed by cotreatment with TNF-α, caspase-3 activity was significantly cross-amplified compared with treatment with only linoleic acid or the cytokine.

Cellular zinc deficiency was achieved by either treatment with cholesterol or long-term culture in low-serum medium. Treatment with the membrane-impermeable divalent chelator DTPA before cell exposure to linoleic acid, TNF-α, or both had little additional effect on caspase-3 activity compared with similar experimental protocols without the chelator (data not shown). However, cell exposure to the membrane-permeable chelator TPEN markedly increased caspase-3 activity beyond the treatment effects already induced by linoleic acid, TNF-α, or both (Figure 2). In zinc-deficient endothelial cells that were activated by exposure to fatty acid or TNF-α, apoptosis was completely blocked by zinc supplementation (Figure 2). Supplementation of the media with other divalent cations (eg, calcium or magnesium) did not mimic the protective effect of zinc against apoptosis (data not shown). Caspase-3 activity in control cultures (ie, in the absence of fatty acid and TNF-α) was unaffected by chelation or zinc supplementation (Figure 2).

Compared with culture in control media (5% FBS), prolonged culture in low-serum media (1% FBS) caused a slight activation of caspase-3 that was blocked by zinc supplementation (Figure 3). Similar to treatment with the chelator TPEN (Figure 2), linoleic acid and TNF-α activated caspase-3 activity when cultured in low-serum media compared with control media. This activation was markedly enhanced in cells cultured in low-serum medium and was significantly blocked by zinc supplementation.

Annexin V binding to the outer leaflet of the plasma membrane in response to apoptotic stimuli also was determined in cultures treated with linoleic acid and TNF-α; annexin V binding was markedly up-regulated under these treatment conditions (Figure 4). Annexin V binding was further enhanced after exposure to TPEN (ie, during intracellular zinc deficiency), whereas the apoptosis induced by the lipid and cytokine was reduced during zinc supplementation. Similar results were observed during DNA fragmentation studies (Figure 5). Zinc deficiency induced the characteristic DNA ladders that appear to be caused by internucleosomal cleavages observed during late stages of apoptotic cell death, whereas zinc supplementation prevented these cleavages.

The effects of zinc deficiency and supplementation on cell morphology, as observed under a phase-contrast microscope, are shown in Figure 6. In low-serum media, endothelial cells lacked their characteristic cobblestone morphology, and more frequent cellular detachments were observed (Figure 6A). Such morphologic changes were completely blocked by supplementation of the low-serum media with zinc (Figure 6B).
substances and protective nutrients, can interact with enzymes, receptors, and adhesion and transport molecules located on the luminal surface of endothelial cells, resulting in further communication between blood-borne cells and ablumenal tissues. It is clear that maintaining an adequate and constant supply of protective nutrients such as zinc to the tissues vulnerable to injury (eg, the vascular endothelium) may make important contributions to the protective mechanisms that prevent or reduce atherosclerosis.

Evidence indicates that both excessive endothelial cell necrosis and apoptosis are critical factors in the etiology of atherosclerosis. Necrosis refers to a range of morphologic changes resulting from enzymatic digestion of cells, disruption of cellular membranes, and the denaturing of proteins that accompanies cell death (5). This kind of cell death is common during an inflammatory response. Apoptosis, in contrast, is a programmed, active, and highly selective mechanism of cell death that allows for the removal of cells that are redundant or excessively damaged. Numerous stimuli, including lipids and cytokines, can activate both necrotic and apoptotic types of cell death. Activation of the cellular suicide pathway leading to apoptosis of endothelial cells may be an initial step in the pathology of atherosclerotic lesion formation (43). In addition, evidence indicates that macrophage- and lipid-derived factors such as inflammatory cytokines and pure and oxidized lipids are necessary for the initiation and completion of cell-death pathways. In fact, our data suggest that certain unsaturated lipids (eg, linoleic acid) can potentiate TNF-α-mediated oxidative stress, disruption of calcium homeostasis, and apoptosis in cultured vascular endothelial cells (44).

**DISCUSSION**

Zinc is a critical component of biomembranes because of its roles in proper membrane structure and function; zinc is also essential for the activity of numerous enzymes (19). Factors implicated in the pathogenesis of atherosclerosis include chronic and cumulative metabolic alterations of the endothelium caused by certain lipids and inflammatory cytokines (36–38). Little is known about the requirements for and functions of zinc in maintaining the integrity of the vasculature, particularly the vascular endothelium. Because zinc is required for normal cellular repair processes, and because atherosclerosis is believed to begin with endothelial cell injury or dysfunction, low zinc concentrations in the plasma or vascular tissues may be involved in initiation of cell injury, potentiation of oxidative stress and inflammatory response, or inadequate protection against apoptosis. These events may have important implications in the pathogenesis of atherosclerosis (39) and also during infections and other stressors, when plasma zinc concentrations may be lowered resulting from redistribution of body zinc pools.

The etiology of atherosclerosis involves damage to or dysfunction of the vascular endothelium (40–42). It is now widely recognized that the endothelium is not merely a passive, blood compatible surface but that it plays an active role in the physiologic processes of blood vessel tone regulation and vascular permeability. Endothelial cells are constantly exposed to blood, which contains various kinds of cells, soluble components, vasoactive substances, toxic wastes, and factors involved in hemostasis, thrombosis, and immune reactions. Various circulating components, including injurious

**FIGURE 2.** Effect of linoleic acid (LA), tumor necrosis factor α (TNF-α), or both on caspase-3 activity in zinc-deficient or zinc-adequate endothelial cells. Cultures were incubated for 48 h in either control media (10% fetal bovine serum), control media exposed to N,N,N',N'-tetrakis(2-pyridylmethyl)-ethylenediamine (TPEN), or control media exposed to TPEN with added zinc (10 μmol/L) before treatment for 24 h with LA (90 μmol/L), TNF-α (500 U/mL), or LA for 18 h plus TNF-α for 6 h. Values are mean ± SEM (n = 6). † Significantly different from respective control cultures. †† Significantly different from cultures treated with either LA or TNF-α alone. Significantly different from activated cultures exposed to TPEN without added zinc.

**FIGURE 3.** Caspase-3 activity in endothelial cells during zinc deficiency and supplementation and after exposure to linoleic acid (LA) and tumor necrosis factor α (TNF-α). Cells were cultured for 8 d in media supplemented with 1% fetal bovine serum, and some cultures were then enriched with zinc (10 μmol/L) for 48 h. Cells were treated with LA (90 μmol/L) for 18 h plus TNF-α (500 U/mL) for 6 h, for a total of 24 h. Values are mean ± SEM (n = 6). † Significantly different from respective control cultures. †† Significantly different from LA + TNF-α–treated 5% serum cultures. † Significantly different from activated cells cultured in 1% serum without added zinc.
measuring caspase-3 activity, we were able to show that both linoleic acid and TNF-α independently, but much more dramatically in combination, can induce apoptosis of endothelial cells. Caspases are believed to be critical in the regulation of the signaling pathways of apoptosis. These apoptotic events, induced by lipids and cytokines, were further potentiated during zinc deficiency and were blocked by zinc supplementation. Thus, zinc may have antiatherogenic effects by interfering with signaling pathways involved in apoptosis. For example, the intracellular pathways leading to apoptotic cell death can be modulated by selective manipulation of intracellular zinc in intact cells (30). The protective effect of zinc has been attributed to its inhibition of calcium- and magnesium-dependent endonucleases, a terminal step and hallmark of apoptosis (45). Pretreatment of cells with zinc chloride prevented apoptosis in response to various agents (46). Recently it has been reported that some of the protective effects of zinc appear to involve inhibition of caspases, such as caspase-3 (25–27). It is known that this enzyme is expressed in cells as an inactive 32-kDa precursor, and that proteolytic processing is required to generate the 17- and 12-kDa subunits that form the enzyme that is active during apoptosis (47). Thus, zinc ions can inhibit the proteases that catalyze conversion of the precursor of caspase-3 to active apoptosis-inducing proteases. Our data clearly show that zinc provides protection from multiple pathways of apoptosis by blocking caspase-3 activity and DNA fragmentation.

In addition, zinc-induced attenuation of apoptotic cell death could be related to the antioxidant properties of this nutrient. Our data suggest that both linoleic acid and TNF-α can independently, but more markedly in concert, induce oxidative stress in endothelial cells (31). Evidence indicates that oxidant-initiated apoptosis requires activation of the transcription factor AP-1 (10, 11) and that hydrogen peroxide–initiated apoptosis was inhibited by down-regulation of c-Jun/AP-1 (12). c-Jun, a signal-transducing transcription factor of the AP-1 family, plays an important role in the induction of apoptosis through the c-Jun N-terminal kinase/stress-activated protein kinase pathway. Increases in c-Jun protein and c-Jun mRNA have been shown in neurons undergoing apoptosis (48). We have...
previously shown that zinc supplementation can significantly block TNF-α-mediated activation of AP-1 (32) and that up-regulation of AP-1 mediated by zinc deficiency and linoleic acid treatment was markedly reduced by zinc supplementation. Thus, our data suggest that zinc may protect against linoleic acid– or TNF-α-mediated apoptosis by down-regulating the c-Jun N-terminal kinase/stress-activated protein kinase pathway.

In summary, our findings provide further evidence that zinc is a potent inhibitor of apoptosis and that some of the protective mechanisms of zinc appear to occur via inhibition of caspases such as caspase-3. A link between endothelial cell apoptosis and the pathology of atherosclerosis has also been proposed. We have shown that zinc is vital to endothelial cell integrity and that zinc deficiency causes severe impairment of endothelial barrier function (16, 20).

Our data provide additional evidence that compromised control of cytokine activity and accelerated endothelial apoptosis may be critical signs of zinc deficiency and impending loss of endothelial integrity. We have also provided evidence that the presence of lipids, specifically unsaturated fatty acids, is a critical regulatory component of endothelial apoptosis. These data further support an antiatherogenic role of zinc in which this nutrient prevents linoleic acid– and TNF-α-mediated activation of the vascular endothelium, thereby stabilizing endothelial cell integrity.

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