

# Zinc Nutrition and Apoptosis of Vascular Endothelial Cells: Implications in Atherosclerosis

BERNHARD HENNIG, PHD,\* PURUSHOTHAMAN MEERARANI, PHD,\*  
PACHAIKANI RAMADASS, PHD,\* MICHAL TOBOREK, MD, PHD,† ANDRZEJ MALECKI, MD, PHD,†  
RABIH SLIM, PHD,\* AND CRAIG J. McCLAIN, MD‡

From the \*Departments of Nutrition and Food Science, †Surgery, and ‡Medicine, University of Kentucky, Lexington, Kentucky, USA

## ABSTRACT

Little is known about the requirements and function of zinc in maintaining endothelial cell integrity, especially during stressful conditions, such as the inflammatory response in cardiovascular disease. There is evidence that zinc requirements of the vascular endothelium are increased during inflammatory conditions such as atherosclerosis, where apoptotic cell death is also prevalent. Apoptosis is a morphologically distinct mechanism of programmed cell death which involves the activation of a cell-intrinsic suicide program, and there is evidence that factors such as inflammatory cytokines (e.g., tumor necrosis factor [TNF]) and pure or oxidized lipids are necessary to induce the cell death pathway. Because of its constant exposure to blood components, including prooxidants, diet-derived fats, and their derivatives, the endothelium is very susceptible to oxidative stress and to apoptotic injury mediated by blood lipid components, prooxidants, and cytokines. Thus, it is likely that the cellular lipid environment, primarily polyunsaturated fatty acids, can potentiate the overall endothelial cell injury by increasing cellular oxidative stress and cytokine release in proximity to the endothelium, which then could further induce apoptosis and disrupt endothelial barrier function. Our data suggest that zinc deficiency exacerbates the detrimental effects of specific fatty acids (e.g., linoleic acid) and inflammatory cytokines, such as TNF, on vascular endothelial functions. We propose that a major mechanism of zinc protection against disruption of endothelial cell integrity during inflammatory conditions, is by the ability of zinc to inhibit the pathways of signal transduction leading to apoptosis and especially mechanisms that lead to upregulation of caspase genes. *Nutrition* 1999;15:744–748. ©Elsevier Science Inc. 1999

Key words: zinc, apoptosis, endothelial cells, fatty acids, cytokines

## INTRODUCTION

Zinc is an essential component of biomembranes and is necessary for maintenance of membrane structure and function. There is evidence that zinc can provide antiatherogenic properties by preventing metabolic physiologic derangements of the vascular endothelium. Because of its antioxidant and membrane-stabilizing properties, zinc appears to be crucial for the protection against cell-destabilizing agents such as polyunsaturated lipids and inflammatory cytokines. Most importantly, zinc also may be antiatherogenic by interfering with signaling pathways involved in apoptosis (Fig. 1). There is evidence that zinc requirements of the vascular endothelium are increased during inflammatory conditions such as atherosclerosis, where apoptotic cell death is prevalent.

## ENDOTHELIAL CELLS, APOPTOSIS, AND ATHEROSCLEROSIS

Apoptosis is a morphologically distinct mechanism of programmed cell death which involves the activation of a cell-intrinsic suicide program, and is known to play a major role during development, in homeostasis, and in disease processes.<sup>1</sup> All mammalian cells possess the basic machinery to carry out apoptosis, and the decision to undergo apoptosis is made by individual cells in response to intracellular and extracellular stimuli. There is evidence which suggests that apoptosis also is involved in the regulation of aortic intimal thickening during atherosclerosis.<sup>2</sup> In fact, apoptotic cell death is common in atherosclerotic plaques,<sup>3</sup> especially in plaques that show a dense macrophage infiltration.<sup>4</sup> This indicates that the vascular tissue can become susceptible to apoptosis, and there appears to be evidence that additional factors

Correspondence to: Bernhard Hennig, PhD, University of Kentucky, Department of Nutrition and Food Science, 204 Funkhouser Building, Lexington, KY 40506-0054, USA.

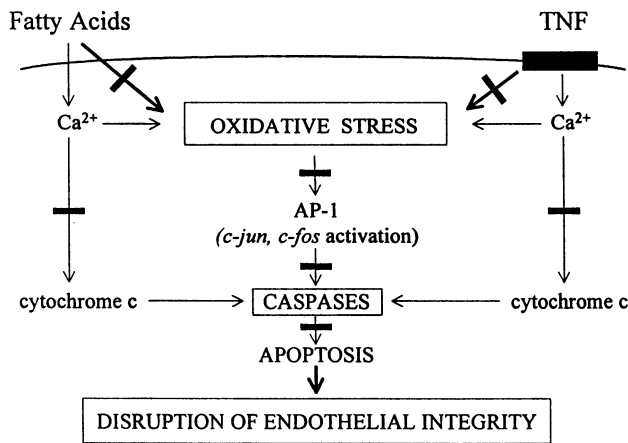


FIG. 1. A schematic diagram illustrating our hypothesis that certain lipids (e.g., fatty acids) can interact with and intensify tumor necrosis factor (TNF)-mediated endothelial cell apoptotic cell death. We postulate that zinc requirements of the vascular endothelium are increased during inflammatory conditions such as atherosclerosis, where apoptosis also is prevalent. We also propose that zinc inhibits the pathways of signal transduction leading to apoptosis and to disruption of endothelial cell integrity. Bold bars represent pathways possibly inhibited by zinc.

(mainly macrophage- and lipid-derived factors) are necessary to complete the cell death pathway.<sup>4</sup> Such factors can include inflammatory cytokines, e.g., tumor necrosis factor (TNF), and pure or oxidized lipids.

Vascular endothelial cells provide an antithrombotic and anti-inflammatory barrier for the normal vessel wall. It is well known that dysfunction of the vascular endothelium can promote atherosclerosis, and normalization of previously dysfunctional endothelial cells can inhibit the pathology of atherosclerosis. Apoptotic endothelial cells have been detected on the luminal surface of atherosclerotic coronary vessels, but not in normal cells,<sup>5</sup> suggesting a link between endothelial cell apoptosis and the pathology of atherosclerosis. It is very likely that increased endothelial cell turnover mediated through accelerated apoptosis may alter the function of the endothelium and, thus, promote atherosclerosis. For example, it has been shown that apoptotic human endothelial cells can become procoagulant by increased expression of phosphatidylserine and the loss of anticoagulant membrane properties.<sup>6</sup> Much evidence suggests that the vascular endothelium is very susceptible to activation and dysfunction during an inflammatory response and that TNF can contribute not only to endothelial necrosis but also to apoptotic cell death.<sup>7</sup> Furthermore, the cellular lipid environment can greatly influence the endothelial inflammatory response.<sup>8,9</sup> In fact, our data suggest that certain unsaturated lipids (e.g., linoleic acid) can potentiate TNF-mediated oxidative stress, disruption of calcium homeostasis, and apoptosis in cultured vascular endothelial cells.<sup>10</sup>

#### IMBALANCE IN OXIDATIVE STRESS/ANTIOXIDANT STATUS AND APOPTOSIS

Reactive oxygen species are known to induce apoptosis in a wide variety of cell culture systems and are believed to play an important role in various pathologic disorders, such as neurodegenerative diseases which involve apoptosis as an underlying mechanism.<sup>11-13</sup> The notion that oxidative stress participates in the regulation of programmed cell death is supported by observations that many agents which induce apoptosis are either oxidants or stimulators of cellular oxidative metabolism. Moreover, many

inhibitors of apoptosis have or enhance antioxidant properties. Such observations are supported by the fact that exposure to low doses of hydrogen peroxide or to xanthine oxidase, which produces superoxide anion, induced apoptosis in a variety of cell types, therefore establishing oxidative stress as a mediator of apoptosis.<sup>14,15</sup> Several studies suggest that TNF-induced apoptosis is associated with the stimulation of TNF receptors, which can provoke an increase in the level of oxygen free radicals especially from the mitochondria.<sup>16</sup> This process can be inhibited by free radical scavengers or through the interference with superoxide generation by respiratory chain inhibitors.<sup>17</sup> N-acetylcysteine, a thiol antioxidant which delivers exogenous cysteine intracellularly and acts as a precursor for the antioxidant glutathione, can diminish programmed cell death in numerous systems.<sup>18</sup> Similarly, many scavengers such as dimethyl sulphoxide, a membrane-permeable hydroxyl radical scavenger, and antioxidants such as vitamins C and E, Trolox, and exogenous catalase, have been shown to prevent apoptosis in experimental systems.<sup>19,20</sup> All of these studies suggest that the cellular redox status and/or the balance between oxygen free radical generation and their detoxification can influence apoptosis. Although the biochemical events in oxidative stress-induced apoptosis are not well understood, it was speculated that this process is partially mediated via gene products such as caspase-3, possibly through oxidative stress-responsive nuclear transcription factors.<sup>12</sup>

Evidence indicates that the cellular lipid environment can contribute to the development of apoptotic cell death. For example, hydroperoxides of unsaturated fatty acids, such as 15-hydroperoxyeicosatetraenoic acid (15-HPETE, a product of arachidonic acid peroxidation) or 13-hydroperoxydodecadienoic acid (13-HPODE, a product of linoleic acid peroxidation) can induce apoptosis, decrease cellular viability, increase intracellular calcium, and induce DNA fragmentation in different T cell lines.<sup>21</sup> It should be noted that HPODE is the major fatty acid oxidation product found in oxidized low density lipoprotein (LDL),<sup>22</sup> and oxidized LDL initiated apoptosis in several vascular cells, including endothelial cells,<sup>23,24</sup> smooth muscle cells, macrophages, and fibroblasts.<sup>25</sup> Aldehyde products of lipid peroxidation, such as 4-hydroxynonenal (4-HNE) also can contribute to cellular apoptosis. We have shown that HNE can induce apoptosis in human umbilical vein endothelial cells (HUVEC).<sup>26</sup> Apoptosis mediated by HNE can be linked to disruption of ion homeostasis, including increased intracellular calcium and dysregulation of Na/K-ATPase.<sup>27</sup> Elevated free and protein-bound forms of HNE are found both in oxidized LDL and in atherosclerotic lesions.<sup>28</sup>

#### TRANSCRIPTION FACTORS AND APOPTOSIS

There is evidence that oxidant-initiated apoptosis requires the activation of the transcription factor activator protein AP-1,<sup>29,30</sup> and hydrogen peroxide-initiated apoptosis was inhibited by down-regulation of c-Jun/AP-1.<sup>31</sup> c-Jun, a signal-transducing transcription factor of the AP-1 family has been recently linked to apoptosis. An increase in c-Jun protein and *c-jun* mRNA has been shown in neurons undergoing apoptosis.<sup>32</sup> Several reports have implicated *c-jun* and *c-fos* in lymphocyte apoptosis, elicited by diverse stimuli.<sup>33</sup> Levels of *c-jun* and *c-fos* mRNA are increased in lymphocyte apoptosis induced by dexamethasone.<sup>34</sup> AP-1 activity can be induced by two distinct signal transduction pathways mediated by different mitogen-activated protein kinase (MAPK). The Ras/Raf/ERK kinase cascade induces the expression of *c-fos* and thereby increases AP-1 activity. The MEKK-SEK/JNK (SAPK) kinase pathway results in phosphorylation and an increase in transactivation activity of c-Jun and ATF-2. Both the ERK and the JNK kinase pathways have been implicated in apoptosis.<sup>35,36</sup>

## CASPASES AND APOPTOSIS

A central component of apoptotic cell death is a proteolytic system involving a family of proteases called caspases.<sup>37</sup> These enzymes participate in a cascade that is triggered in response to proapoptotic signals, which culminates in the cleavage of a set of proteins, resulting in disassembly of the cell. For example, caspase-8 is associated with apoptosis involving death receptors.<sup>1</sup> In contrast, caspase-9 is involved in death induced by cytotoxic agents.<sup>37</sup> Activation of procaspase-8 requires association with its co-factor FADD (Fas-associated protein with death domain) through the DED (death effector domain), while procaspase-9 activation involves a complex with the co-factor APAF-1 through the CARD (caspase recruitment domain). Activation of caspase-9 also requires cytochrome *c* and deoxyadenosine triphosphate, indicating that the caspase activation may require multiple co-factors. Cytochrome *c* is released from mitochondria during apoptosis and can be a co-activator of caspase-3.<sup>38</sup> Furthermore, activated caspase-8 can activate other members of the caspase family, such as caspase-3 and -6, which act downstream in the caspase cascade. Caspase-3 (or CPP32) appears to be the critical death executioner in the Fas-system, as well as one of the major activated caspases present in apoptotic cells, suggesting that it plays a prominent role in the cell death process.<sup>39</sup> In select cell systems, caspase-3 is strictly required for chromatin condensation and DNA degradation, but not for other features of apoptotic cell death.<sup>39</sup> Two proteins, DNA fragmentation factor (DFF) and p21-activated kinase 2 (PAK2) were reported recently to be targets for activated caspase-3.<sup>40,41</sup> These proteins are both activated in response to apoptotic stimuli and have been shown to induce DNA fragmentation.<sup>39</sup> During the execution phase of apoptosis, caspase-3 is responsible either wholly or in part for the proteolysis of a large number of substrates, each of which contain the common Asp-Xaa-Xaa-Asp (DXXD) motif.

Evidence indicates that caspase 3-mediated apoptosis can 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.<sup>42</sup> In addition, specific inhibition of caspase-3 activity completely blocked oxidized LDL-induced apoptosis. The antioxidants N-acetylcysteine and the combination of vitamins C and E prevented oxidized LDL-induced apoptosis, abrogated the enhancement of caspase-3 protease activity and inhibited the proteolytic cleavage of caspase-3 into its active subunit p17.<sup>42</sup> Because the caspases are central to most cell death programs, they present an attractive target for therapeutic interventions to inhibit apoptosis.

## ZINC AND ENDOTHELIAL INTEGRITY

We have shown that zinc is vital to endothelial integrity and that zinc deficiency causes a severe impairment of endothelial barrier function.<sup>43,44</sup> Zinc is documented to act as an antioxidant, to have membrane-stabilizing properties, and to block apoptotic cell death. In zinc deficient endothelial cells, barrier function was significantly decreased compared with controls.<sup>43</sup> Media supplemented with physiologic concentrations of zinc completely restored the cell integrity. Supplementation with calcium or magnesium, however, did not restore this function, suggesting a unique role of zinc in maintaining normal endothelial integrity. In our zinc research, we extensively studied methods to determine zinc deficiency in endothelial cells. These methods included cell culture in low-serum medium (where serum was the only source of zinc), as well as in media previously exposed to different types of chelating agents. All these techniques resulted in depletion of intracellular zinc levels and similar metabolic changes.<sup>43-45</sup>

Endothelial barrier function may be more severely compromised during zinc deficiency in the presence of specific lipids. To

test the hypothesis that zinc may protect endothelial cells against fatty acid induced injury, studies were undertaken using media with and without supplemental zinc. Exposure to media enriched with linoleic acid, but not with stearic acid, significantly injured endothelial monolayers. This unsaturated fatty acid induced injury to endothelial cells was prevented when culture media were supplemented with zinc but not with calcium or magnesium.<sup>46</sup> The mechanism of zinc protection is not clear and may be accounted for in part by its property to act as an antioxidant and to block apoptotic cell death. In fact, we have evidence that during zinc deficiency, cellular oxidative stress is markedly induced by exposure to linoleic acid and/or TNF and that this oxidative stress can be partially blocked by zinc supplementation.<sup>47</sup>

The nutritional status of the endothelium is likely to influence its response to TNF,<sup>48,49</sup> fatty acids, and other stresses, and a marginal status of protective nutrients (e.g., zinc) may increase the susceptibility of the endothelial cell towards dysfunction. Enrichment with zinc may attenuate or prevent TNF-induced cell injury. We have evidence that a disruption of endothelial cell monolayer integrity by TNF can be prevented by pre-enriching cells with zinc.<sup>45</sup> The protective mechanism(s) of zinc against cytokine-induced injury requires further clarification. Our data support the hypothesis that zinc may provide several defenses against endothelial cell injury, e.g., antioxidant, membrane stabilizing properties, and antiapoptotic properties, which may or may not be related. We have exciting evidence that TNF-mediated activation of the transcription factor AP-1 can be attenuated by zinc in endothelial cells,<sup>50</sup> suggesting that apoptosis occurs in TNF-mediated disruption of endothelial integrity and that zinc may be a critical nutrient to block TNF-mediated apoptotic cell death.

## ZINC NUTRITION AND APOPTOSIS

There is evidence that zinc is a potent inhibitor of apoptosis<sup>51,52</sup> and that zinc deficiency can induce apoptosis (reviewed in ref. 53). The subcellular mechanisms by which zinc affects apoptosis are not well understood and may occur at multiple levels. Thus, zinc may be antiatherogenic 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.<sup>54</sup> The protective effect of zinc has been attributed to its inhibition of calcium and magnesium-dependent endonucleases, a terminal step and hallmark of apoptosis.<sup>55</sup> Pretreatment of cells with zinc chloride prevented apoptosis in response to various agents.<sup>56</sup> Recently, it has been reported that part of the protective mechanisms of zinc appear to be via inhibition of caspases, such as caspase-3.<sup>57-59</sup> It is known that this enzyme is expressed in cells as an inactive 32 kDa precursor, and proteolytic processing is required to generate the 17 and 12 kDa subunits that form the enzyme that is active during apoptosis.<sup>60</sup> Thus, zinc ions can inhibit the proteases that catalyze the conversion of the precursor of caspase-3 to active apoptosis inducing proteases.

## FATTY ACIDS, TNF, AND ENDOTHELIAL CELL APOPTOSIS—PROTECTIVE ROLE OF ZINC

Even though the involvement of endothelial cell apoptosis in the development or the progression of atherosclerosis is not well understood, large numbers of apoptotic cells are usually present in neointima of atherosclerotic vessels.<sup>61</sup> This suggests that apoptosis might participate in the remodeling of the vessel wall during apoptosis.<sup>61</sup> Moreover, endothelial cells are a major site of inflammatory reactions, and the intermediates of such reactions, such as reactive oxygen species,<sup>62</sup> TNF,<sup>63</sup> or elastase<sup>64</sup> trigger endothelial cell apoptotic processes. We have evidence that the lipid environment may markedly influence a TNF-mediated increase in cellular oxidative stress and intracellular calcium and, thus, potentiate

endothelial cell apoptosis mediated by this cytokine.<sup>10</sup> In fact, TNF-mediated endothelial cell apoptosis was markedly accelerated when cells were pre-enriched first with linoleic acid. It is not clear how changes in the cellular lipid environment can potentiate TNF-mediated apoptosis, but part of the mechanism may be via a fatty acid mediated increase in cellular oxidative stress and disruption of ion homeostasis. It was shown that apoptosis is preceded by a decrease in cellular glutathione<sup>65</sup> or can be induced by oxidation of thiol groups.<sup>66</sup> To support the role of thiols in fatty acid/cytokine-mediated cellular apoptosis, we have shown that endothelial cell exposure to linoleic acid or TNF and, in particular, to linoleic acid plus TNF markedly decreased intracellular glutathione content.<sup>67</sup> An increase in intracellular calcium can induce cellular apoptosis,<sup>68</sup> and the role of elevated intracellular calcium levels in fatty acid plus TNF-induced endothelial cell apoptosis is supported by the observation that BAPTA-AM, an intracellular calcium chelator, partially protected against apoptosis in these experimental settings.<sup>10</sup>

We now have preliminary data which suggest that linoleic acid and TNF can independently, but more markedly in concert, induce caspase-3 activity in endothelial cells. The endothelial cells were cultured in medium enriched with linoleic acid and/or TNF and then assayed for caspase-3 activity. In cells treated with both linoleic acid and TNF, caspase-3 activity was markedly increased when compared to cells treated only with either linoleic acid or TNF.

Most important, our data also demonstrate that zinc can protect against linoleic acid and/or TNF-induced endothelial cell apoptosis.<sup>69</sup> Endothelial cells were first cultured in medium enriched with

the metal ion chelator, diethylenetri-nitropentaacetate (DTPA), to deplete the cells of zinc. Then some of the cultures were supplemented with physiologic concentrations of zinc and then treated with linoleic acid and/or TNF. Subsequently, caspase activity was measured in each apoptotic extract. Our preliminary data clearly show that zinc supplementation can inhibit caspase activity and thus prevent lipid/cytokine induced apoptosis.

#### CONCLUSION

There is evidence that zinc is a potent inhibitor of apoptosis and that part of the protective mechanism of zinc appears to be via inhibition of caspases, such as caspase-3. A link between endothelial cell apoptosis and the pathology of atherosclerosis also has been proposed. Little is known about the requirements and functions of zinc in maintaining the integrity of the vasculature and particularly the vascular endothelium. We have shown that zinc is vital to endothelial integrity and that zinc deficiency causes a severe impairment of endothelial barrier function. Zinc-deficient media supplemented with physiologic concentrations of zinc completely restored endothelial cell integrity. Our preliminary data provide evidence that a critical sign of zinc "deficiency" and subsequent loss of endothelial integrity may be a compromised control of activation of transcription factors, cytokine activity, endothelial cell inflammatory response, and accelerated apoptotic death. Thus, the continued discoveries of the unique protective properties of zinc, possibly as a membrane stabilizer, and antioxidant, and inhibitor of signaling pathways involved in inflammatory response and apoptosis, warrant further research in the role of zinc in endothelial cell metabolism and atherosclerosis.

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