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Is zinc deficiency a risk factor for atherosclerosis?

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The development of atherosclerosis is influenced by genetic, lifestyle and nutritional risk factors. Zn and metallothionein deficiency can enhance oxidative-stress-related signalling processes in endothelial cells, and since changes in available plasma Zn may affect the Zn status of the endothelium, Zn deficiency could be a risk factor for IHD. Although the association of Zn with many proteins is essential for their function, three key signalling processes are highlighted as being principal targets for the effect of Zn deficiency: the activation of NF-κB, the activation of caspase enzymes and the signalling of NO. The need to develop a reliable indicator of Zn status is critical to any epidemiological approach for studying the relationship between Zn status and disease incidence. Studies using appropriate animal models and investigating how the plasma Zn pool influences endothelial intracellular labile Zn would be helpful in appreciating the importance of Zn deficiency in atherogenesis.

Zinc: Atherosclerosis: Metallothionein

Atherogenesis is a complex process involving mechanical, chemical and biological factors (Bannon et al. 2003). Damage to endothelial cells and activation, attachment and transmigration of monocytes through the endothelium are early events in atherosclerosis. It follows that factors modulating these processes could influence the development of the disease. The formation of atherosclerotic plaque and development of IHD is greatly influenced by lifestyle and nutritional factors, many of which have been clearly identified (Tunstall-Pedoe, 2003; Yancy et al. 2003). The impact of trace metal deficiencies on this process is less well defined and studied, although the influences of Se and Cu on atherogenesis and heart disease have attracted much attention (Strain, 1998; Kohrl et al. 2000; Alissa et al. 2003). Although Zn is regarded as an antioxidant (Powell, 2000), its deficiency has not been firmly associated with disease progression (Strain, 1998), probably due to the lack of a good indicator of Zn status (Hambidge, 2003). Zn binds to metallothionein (MT), which has been ascribed an antioxidant function (Miles et al. 2000), and seems to play an influential role on Zn metabolism during periods of stress (Carey et al. 2000). The possibility that Zn can help protect the vascular endothelium against oxidative stress has therefore been raised (Hennig et al. 1996) and we propose that MT may influence Zn protection. In the present review, we examine whether current evidence supports the proposition that Zn deficiency is a risk factor for atherosclerosis and whether MT may influence atherogenesis.

Zinc requirements and deficiency

It is estimated that Zn deficiency affects one third of the World’s population and is the major factor contributing to 1-4% of all deaths worldwide (WHO, 2002). Although acute deficiency in developed countries is rare, marginal deficiency is thought to be relatively common (Hambidge, 2000). Major impacts of Zn deficiency of importance to health include reduced growth and suppression of immune function (Hambidge, 2000). Zn is required for structural and functional integrity of more than 2000 transcription factors (Coleman, 1992) and 300 enzymes (Vallee & Falchuk, 1993); therefore, almost every signalling and metabolic pathway is in some way dependent on at least one, and often several, Zn-requiring proteins. Many Zn-finger proteins bind Zn relatively tightly and have not been regarded as sensitive to changes in cellular total Zn concentrations, which are in the upper μM to lower mM concentration range. However, recent evidence suggests that ‘free’ Zn, which is most likely to influence activation...
of Zn finger proteins and enzymes, is present at fM concentrations, at least in bacterial cells (Outten & O’Halloran, 2001). Hence, small, localised increases in ‘free’ or labile Zn within cells may have disproportionate influences on Zn-dependent protein activation in relation to the overall cellular Zn concentration. In addition, localised intracellular oxidative stress may enhance the lability of Zn within Zn fingers (Webster et al. 2001). With this in mind, it is quite credible that small changes in Zn homeostasis that result in large changes in the cellular labile Zn may have quite profound effects on intracellular signalling. This raises the possibility that Zn status could influence the progression of chronic diseases such as heart disease, because some transcription factors involved in this process are sensitive to Zn. Due to the lack of a reliable indicator of Zn status, epidemiological study of an association between Zn status and disease incidence is rare (Hambidge, 2003). However, a protective effect of high Zn status on the risk of human CVD is compatible with some published data (Kok et al. 1988). Unfortunately little attempt has been made to study this association in appropriate animal models.

**Cellular zinc homeostasis**

As regards initiation of atherogenesis, a crucial question to be addressed is whether under physiological conditions, endothelial or mononuclear cells would ever become deficient in the pool of labile Zn that regulates transcription factor and enzyme activation. Although plasma contains <0.1% whole-body Zn, it is the only medium for Zn transport, and so plasma Zn is turned over very rapidly (about 150 times per d in human subjects; King et al. 2000). The most labile Zn in human plasma is that bound to albumin (about 70% plasma total Zn), with the remainder being more firmly associated with higher molecular mass proteins, predominantly α-2-macroglobulin (Chesters, 1997). Tissues such as liver also contain mobile reserves of Zn, but the kinetics of Zn turnover are slower than those in plasma and this tissue Zn is regarded as a separate pool in compartmental models of Zn metabolism (Lowe et al. 1993). Zn shows a rapid uptake into endothelial cells and may involve endocytosis of albumin-bound Zn (Rowe & Bobilya, 2000). The lability of albumin-bound Zn and the case with which it is transported into endothelial cells suggests that the vascular endothelium may be particularly influenced by changes in plasma Zn levels, which are affected both by whole-body Zn homeostasis (Hambidge & Krebs, 2001) and by changes in the partitioning of Zn into different compartments (Philcox et al. 1995). Normal physiological levels of plasma Zn in developed countries are approximately 13–15 μM (Lentner, 1984) and Zn homeostasis is normally tightly regulated over a wide range of Zn intake (Hambidge & Krebs, 2001). However, increasing age >25 years (Hotz et al. 2003), for example, is associated with lower plasma Zn levels. Tissue MT1, induced by a wide variety of factors including bacterial endotoxin and ethanol, draws and binds Zn from the plasma and has a profound, albeit transient effect to suppress plasma Zn (Philcox et al. 1995; Carey et al. 2000). How endothelial cell Zn homeostasis is affected by plasma Zn levels will depend on issues relating to the regulation of Zn influx and efflux from the endothelial cells and the intracellular binding and compartmentalisation of absorbed Zn.

**Monocyte activation**

Once activated, peripheral blood monocytes form macrophages, which then secrete cytokines and form part of the immune response against bacterial, viral, fungal and neoplastic pathogens. During activation, they release inflammatory cytokines such as IL-1 and -6, and TNF-α. These cytokines stimulate vascular endothelial cell production of adhesion factors, which encourage monocyte adhesion and diapedesis through the endothelium. Recent studies using the monocyte–macrophage cell line HL-60 show that Zn deficiency increases phagocytosis-stimulated IL-1 and TNF-α gene expression, which if translated into secretion of these proteins, may enhance localised inflammatory effects of monocyte activation (Bao et al. 2003). However, the same group have previously reported that plasma IL-1 levels in elderly subjects of marginal Zn status are significantly lower than in younger subjects, and can be corrected by supplementation with Zn (Prasad et al. 1993). The major contribution to plasma IL-1 may not come from lymphocytes, the predominant mononuclear cell type in blood, and so changes in plasma IL-1 may not reflect expression of this cytokine by monocytes–macrophages. Attachment of monocytes to damaged endothelium is a normal part of the repair process, but macrophage ingestion of oxidised LDL can result in the intimal formation of foam cells. Over time, this results in the formation of fatty streaks in the vasculature, and ultimately in the formation of atherosclerotic plaque. It has been suggested that physiological levels of Zn can prevent oxidation of LDL and its uptake into macrophages (Wilkins & Leake, 1994), although this is controversial and has yet to be confirmed.

**Endothelial cell integrity**

The vascular endothelium has a barrier function and must resist a range of chemical and mechanical stresses from the flow of blood containing potentially pro-oxidant and toxic compounds. Atherogenesis is associated with endothelial cell apoptosis, which may be initiated directly by oxidant stress from oxidised LDL or PUFA such as linoleic acid. It may also be initiated by inflammatory cytokines from activated monocytes–macrophages. Recent evidence suggests that Zn may have a protective effect on the vascular endothelium at a number of different levels. Apart from its direct proposed role as an antioxidant and stabiliser of cell membranes, Zn participates in the activity of numerous endothelial signalling processes, several of which are important to the maintenance of cell integrity.

**NF-κB**

NF-κB is a key transcription factor that regulates the expression of many genes and signalling pathways related to apoptosis and the innate and inflammatory responses.
(May & Ghosh, 1998). Its role in vascular cell function has been well characterised and it is activated by several factors including IL-1, TNF-α, lipopolysaccharide, homocysteine and direct oxidative stress (De Martin et al. 2000). NF-κB activity is regulated by Zn, but while the effect of Zn is inhibitory in endothelial cells (Hennig et al. 1999a), it is stimulatory in a Th(0) cell line (Prasad et al. 2002). The reason for this cell-specific influence of Zn is unclear, but the marked inhibition of NF-κB by Zn pyrithione, a Zn ionophore, in endothelial cells (Kim et al. 1999) is striking evidence for the efficacy of this interaction. The binding of NF-κB to DNA is Zn-dependent (Kudrin, 2000), but the precise nature of the protein–Zn interaction is not known. A similarity between the DNA-binding domain of the p53 transcription factor, which binds Zn and is Zn dependent, and that of NF-κB has been noted; unlike p53, however, NF-κB does not contain structural Zn (Hainaut & Mann, 2001). Endothelial cells are activated to express the adhesion molecules intercellular adhesion molecule-1 and vascular adhesion molecule-1 and E-selectin by macrophage secreted TNF-α, encouraging further recruitment to the serosal endothelial surface and transmigration of monocytes through the endothelium. NF-κB is a key signalling process in adhesion molecule gene upregulation (De Martin et al. 2000), but the influence of endothelial Zn status on adhesion molecule expression and other NF-κB-dependent gene expression has not been studied.

Caspase-mediated apoptosis

Many, but not all, cells in culture show characteristic signs of apoptotic cell death when deprived of Zn (Truong-Tran et al. 2001). This was initially attributed to release of the inhibitory action of Zn on endonuclease activity (Duvall & Wyllie, 1986). However, DNA fragmentation and condensation of intact nuclei by cytospins from cells primed to undergo apoptosis was inhibited by Zn at lower levels than required to inhibit endonuclease activity, suggesting that Zn-mediated suppression of apoptosis may occur by independent mechanisms upstream of endonucleases. In addition, Zn inhibition of apoptosis can occur even in the absence of DNA fragmentation (Truong-Tran et al. 2000). Using purified substrate and recombinant enzyme, caspase-3, a pivotal enzyme for several apoptotic pathways, was found to be sensitively inhibited by Zn (Perry et al. 1997). Inhibition may occur by binding of Zn to a sulphydryl essential for enzyme activity (Maret et al. 1999). Zn is also a potent inhibitor of other caspsases such as caspase-6 and -9 (Truong-Tran et al. 2001).

Atherosclerosis involves apoptosis of endothelial cells; activation of caspase enzymes, particularly in response to oxidative stress, plays a major role in that process (Geng, 2001). Porcine endothelial cells made Zn-deficient by treatment with the Zn-chelator N′,N′,N′,N′-tetraakis (2-pyridylmethyl)ethylenediamine show a considerably higher level of apoptotic cell death and caspase-3 activity when challenged with oxidative stress-inducing conditions such as treatment with linoleic acid and TNF-α (Meerarani et al. 2000). Likewise, caspase-3 activity in human aortic endothelial cells showed an inverse relationship with media Zn concentrations (Fanzo et al. 2002). There is therefore merit in the idea that Zn deficiency may enhance atherogenesis-related apoptosis of endothelial cells (Hennig et al. 1999b), but evidence supporting this in vivo is currently lacking.

Nitric oxide signalling

Changes in NO production have been linked to the development of atherosclerosis (Anderson, 2003). Recent studies have shown that Zn plays a crucial role in endothelial NO synthase function and in NO signalling. NO synthases are catalytically active only as a dimer of two subunits, the association of which is stabilised by the tetrahedral binding co-ordination of Zn with thiol ligands at the dimer interface (Zou et al. 2002). The activity of NO synthase is strongly inhibited by the formation of peroxynitrite, a product of superoxide radical reaction with NO, and is directly related to peroxynitrite-induced release of NO synthase-bound Zn. Since NO synthase expression is dependent on NF-κB activation, it is also possible that Zn deficiency could influence NO synthase by this mechanism.

NO also stimulates release of Zn from proteins by thiol nitrosylation. Treatment of endothelial cells with NO increases intracellular labile Zn, which can be detected using the Zn-binding probe Zinquin (Berendji et al. 1997). In particular, thiol nitrosylation of MT was found to release MT-bound Zn (Kroncke et al. 1994). A critical observation is that mesenteric arteries of mice with a knock-out mutation of MT-1 and -2 genes showed no myogenic reflex to increased luminal pressure. Treatment with the NO synthase inhibitor nitro-L-arginine methyl ester abolished the difference in myogenic response between arteries from MT-deficient and MT-normal mice, indicating that NO interaction with MT was an important factor in this reflex. It is hypothesised that an enhanced relaxing effect of NO during the myogenic reflex in MT-deficient vessels may be due to a lack of releasable metals from MT that normally would produce contraction (Pearce et al. 2000a).

Perspective

With so many Zn-binding ligands in endothelial cells, it is not an easy task to predict which proteins from which signalling or metabolic pathways might be most sensitive to changes in intracellular labile Zn under physiological conditions. Modulation of processes highlighted in the present review, namely NF-κB, caspase and NO activity, by physiological changes in Zn levels has been observed in isolated cell systems, but the relative effect of Zn at any particular level on all three processes simultaneously is unknown. However, Zn deficiency may influence localised pools of Zn differentially, which is clearly critical to effects on spatially localised or compartmentalised Zn-sensitive proteins. In some types of cell, labile intracellular Zn is rapidly compartmentalised within Zn containing vesicles (Haase & Beyersmann, 2002), and the presence of these vesicles has been demonstrated in endothelial cells (Pearce et al. 2000b).

Regulation of Zn homeostasis, both at a systemic and cellular level, is very efficient and so large changes in Zn
balance tend to be transitory. For example, an endotoxin-stimulated decrease in plasma Zn may persist for only a matter of hours. However, this may be sufficient to deplete vascular endothelial cells of labile Zn and stress factors such as endotoxin may then cause a more profound response when Zn sensitive processes involved in atherogenesis are more active. As discussed in the present review, MT may regulate labile Zn pools either through oxidative mechanisms, such as S-nitrosylation by NO (St Croix et al. 2002; Gow & Ischiropoulos, 2002), or by sequestering excess Zn released from other cellular ligands or derived from cellular import. In this regard, metal transcription factor-1, which is a cellular sensor of labile Zn, directly regulates MT transcription (Lichten & Schaffner, 2001).

As yet, there is no direct evidence linking Zn deficiency and HHD. However, there is now considerable evidence that Zn deficiency renders endothelial cells more susceptible to the effects of oxidative stress. The age-old problem of developing a reliable diagnostic indicator of Zn status needs to be re-addressed and resolved in order to critically evaluate the relationship between Zn status and human heart disease. In addition, considerably more effort should be devoted to animal studies of Zn deficiency, using appropriate models of heart disease, such as ApoE-null or LDL-receptor-null mice. Study of the dynamic relationship between available plasma Zn and endothelial labile Zn, vesicular or otherwise, would clarify whether Zn-sensitive signalling was likely to be influenced by systemic changes in Zn balance and status. Stress-induced endothelial dysfunction mediated through the signalling processes identified in the present review may predispose individuals to CHD, which we propose could be modulated by dietary Zn.

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