RESEARCH ARTICLE

Suboptimal dietary zinc intake promotes vascular inflammation and atherogenesis in a mouse model of atherosclerosis

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Scope: Cardiovascular health is strongly influenced by diet. Zinc has antioxidant and anti-inflammatory properties but its long-term influence on vascular health at dietary intake levels relevant to the human population in developed countries has not been studied. We investigated the influence of suboptimal zinc intake in a Western-type diet on the development of vascular inflammation and arterial plaque in apoE knock-out (AEKO) mice.

Methods and results: Weanling AEKO and wild-type (WT) controls were given high saturated fat (21% w/w) and high cholesterol (0.15%) semi-synthetic diets containing 3 or 35 mg Zn/kg (AEKO and WT) or 8 mg Zn/kg (AEKO only) for over 6 months. AEKO mice on zinc intakes of 3 and 8 mg Zn/kg (suboptimal zinc) developed significantly ($p < 0.05$) more aortic plaque than AEKO mice consuming 35 mg Zn/kg (adequate zinc). Circulating levels of interleukin-1β, interleukin-6 and soluble vascular adhesion molecule-1 were significantly ($p < 0.05$) raised at the lowest zinc intake in AEKO mice, as compared to zinc-adequate controls. Plasma total cholesterol and total protein were also significantly ($p < 0.05$) increased at the lowest zinc intake.

Conclusion: We propose that suboptimal dietary zinc intake raises circulating pro-atherogenic lipoprotein levels that promote vascular inflammation and enhance arterial plaque formation.

Keywords:
Atherosclerosis / Cholesterol / Inflammation / Western diet / Zinc deficiency

1 Introduction

As a non-redox active divalent metal ion, zinc is utilized in biology to perform structural or catalytic roles in proteins from a diverse range of metabolic and signalling pathways [1]. Its essentiality is reflected by its requirement in about 7% of the proteome and the presence of at least 24 different zinc-specific transporters [2] to help maintain its cellular homeostasis. Reduced homeostatic control of zinc occurs at low dietary zinc intake [3] and decreased zinc status has been detected at dietary zinc intakes within the normal range for developed countries [4]. Since zinc promotes anti-inflammatory and antioxidant effects, low zinc status promotes inflammation and oxidative stress [5]. We and others have therefore proposed that the development of atherosclerosis, which is initiated and driven by inflammatory and oxidative processes, may be accelerated in low zinc status [6, 7].

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Abbreviations: AEKO, apoE knock-out; **CCL**, chemokine (C–C motif) ligand; **HMG-CoA**, 3-hydroxy-3-methylglutaryl-coenzyme A; **ICAM-1**, intercellular adhesion molecule-1; **IFN-γ**, interferon-γ; **MCP-1**, monocyte chemotactic protein-1; **MIP1α**, macrophage inflammatory protein 1α; **OCT**, optimal cutting temperature; **PAI-1**, plasminogen activator inhibitor-1; **PPAR**, peroxisome proliferator activated receptor; **SREBP**, sterol regulatory element binding protein; **TNF-α**, tumour necrosis factor-α; **VCAM-1**, vascular cell adhesion molecule-1; **VSMC**, vascular smooth muscle cell; **WT**, wild-type; **ZA35**, zinc adequate at 35 mg Zn/kg diet; **ZD3, ZD8**, zinc deficient at 3 and 8 mg Zn/kg diet

While zinc status is an obvious candidate to influence atherogenesis, it has received scant attention. This is partly because epidemiological evidence showing a relationship between status and cardiovascular disease is lacking, due to an absence of reliable zinc status biomarkers [8]. In addition, genetically unmodified rats and mice are not good models of atherosclerosis and appropriately large, long-term and well-controlled studies with suitable animal models and zinc intake levels are also lacking. However, in human population studies, a strong negative association was found between dietary Zn intake (as opposed to Zn status) and the incidence of factors related to diabetes and heart disease, particularly in urban localities [9]. Supplementation trials, which are easier to conduct than dietary deficiency interventions, but which do not necessarily indicate changes that would be observed in deficiency, are more frequently published. For example, supplementation of older healthy adult volunteers with 45 mg Zn/day for 6 months decreased markers of vascular inflammation as compared to volunteers receiving a placebo [10].

Observations in human subjects are supported by some animal and cell studies. Hennig et al. have investigated the impact of manipulating the zinc status of endothelial cells in culture on responses to oxidative stress [11]. This group later concluded that the signalling of endothelial peroxisome proliferator activated receptor (PPAR) gamma, which contains zinc finger domains and negatively regulates the expression of adhesion molecules, is impaired in zinc deficiency [12–14]. In a 4-wk study with LDL receptor deficient mice, which develop atherosclerosis, acute Zn deficiency (<1 mg Zn/kg diet) decreased hepatic PPAR gamma binding to DNA and also increased inflammatory markers such as vascular cell adhesion molecule-1 (VCAM-1) in aorta [15]. In this short-term acute deficiency study with young mice, plaque development was not measured and no pair-fed animals were included to control for zinc deficiency induced anorexia. In rabbits, 8 wk of Zn supplementation in combination with a high-fat, high-cholesterol diet reduced total cholesterol levels in aorta and aortic lesion cross-sectional area [16]. Although rats do not develop atherosclerosis, they are good models of zinc deficiency and we have shown that both acute and marginal dietary zinc intake over 6 wk modulates the expression of proteins, such as sm22α, related to smooth muscle cell differentiation in aorta [17]. We proposed that zinc deficiency may modulate the differentiated phenotype of smooth muscle cells.

Acute zinc deficiency in animals such as rats can induce anorexia [18]. Food intake and therefore growth are reduced and pair feeding of controls is required to eliminate this food intake variable. However, even selection of a pair-feeding methodology is not straightforward due to differences in diurnal feeding patterns of zinc-deficient animals. In addition, acute zinc deficiency in humans is not relevant in affluent countries and we are particularly interested in the accelerated development of atherosclerosis in people consuming high-fat, high-cholesterol diets. For this reason, we have focussed our efforts on understanding how chronic exposure to marginal dietary zinc intake over 25 wk using a Western-type diet can influence the development of atherosclerosis. We have used genetically modified apoE knock-out (AEKO) mice as a model because they develop abundant arterial lesions, and the pathogenesis of atherosclerosis in these animals is very similar to that of the human arterial disease. Our hypothesis was that marginal dietary zinc intakes equivalent to those in up to 20% of adult men and 60% of adult women in the United Kingdom may be pro-atherogenic.

2 Methods

2.1 Animals and diets

Thirty-six weanling AEKO mice (age 5 wk) congenic to a C57BL genetic background were obtained from Charles River (Margate, UK), along with 24 wild-type (WT) controls of the same age and strain. They were individually housed in zinc-free plastic and steel cages and were provided with Perspex tunnels and shredded paper for bedding. The mice were acclimated for 1 week and during this time were given a semi-synthetic diet containing adequate (optimal) zinc levels (35 mg Zn/kg), *ad libitum*. The AEKO animals were then randomly allocated to three groups of 12 mice and were given diets containing 3, 8 or 35 mg Zn/kg for 25 wk. The WT mice were randomly allocated to two groups of 12 mice and were given diets containing 3 or 35 mg Zn/kg for 25 wk. Diets containing 3, 8 and 35 mg Zn/kg are referred to as the ZD3, ZD8, and ZA35 diets, respectively. A pilot study confirmed that neither of the two lowest zinc levels affected food intake or weight gain and so pair-fed groups were not included. Diets and distilled water were given *ad libitum*. All precautions to avoid contaminant sources of zinc were observed. Mice were maintained at an environmental temperature 23°C and a 12-h light–dark cycle.

The diets were custom made by Harlan-Teklad (Indianapolis, IN, USA) and were based on TD.88137 (high-fat, high-cholesterol, Western-type diet). The diet compositions are shown in Table 1 and the original formulation was modified to replace casein with egg albumen and also supplement with additional biotin due to the elevated avidin content of albumen. The diets contained 21% by weight of milk fat, which is high in saturated fat, and energy contribution from fat was 42%. In order to analyse the zinc diet content, samples of the ZD3, ZD8 and ZA35 diets were solubilized using wet ashing with nitric acid and analysed using flame atomic absorption spectrophotometry, and the zinc concentrations recorded were 3.7 ± 0.7, 9.6 ± 4.6 and 30.9 ± 4.1 mg Zn/kg, respectively (mean $\pm$ SE, $n = 3$).

After 25 wk on the diets, mice were killed by exsanguination under terminal anaesthesia. Each mouse was given an intra-peritoneal injection of 0.2 mL of Euthatal (Merial Animal Health, Harlow, UK: dose of 1 g sodium pentobarbital/kg body weight) and anaesthesia was monitored until

Table 1. Composition of the semi-synthetic diets. Values are in g/kg and diets were produced as compressed pellets

Constituent	Dietary Zn (mg/kg)		
	3	8	35
Egg white solids, spray-dried	212	212	212
Sucrose	340.5	338.3	326.5
Corn starch	75	75	75
Maltodextrin	75	75	75
Cellulose	46.2	46.2	46.2
Anhydrous milkfat	210	210	210
Cholesterol	1.5	1.5	1.5
Mineral mix, Zn deficient (81264)[a)]	25.7	25.7	25.7
Calcium Phosphate, dibasic	3.0	3.0	3.0
Chromium Potassium Sulfate, dodecahydrate	0.02	0.02	0.02
1% $ZnSO_4.7H_2O$ in sucrose	1.0	3.2	15.0
Vitamin mix (40060)[b)]	10.0	10.0	10.0
Biotin	0.004	0.004	0.004
Ethoxyquin, antioxidant	0.04	0.04	0.04

a) Number refers to Harlan-Teklad (Indianapolis, IN, USA) catalogue number for mineral mix.
b) Number refers to Harlan-Teklad (Indianapolis, IN, USA) catalogue number for vitamin mix.

breathing ceased and no foot pinch reflex response was observed. All available blood was then removed from the abdominal vena cava using a heparinized syringe and needle. A blood sample was removed for haematology using a Sysmex KX-21N instrument (Sysmex UK Ltd., Milton Keynes, UK), and the remaining blood was immediately centrifuged at 2000×*g* (4°C). Plasma was divided into aliquots and frozen in liquid nitrogen.

Hearts were perfused in situ with DMEM medium (Product 21063, Gibco, Invitrogen, Paisley, UK), excised and frozen in Tissue-Tek optimal cutting temperature (OCT) embedding medium 4583 (Agar Scientific, Stanstead, UK) by immersion in dry ice cooled isopropanol.

Animal procedures were carried out under Institutional, personal and project licences from the UK Home Office and the study was approved by the Rowett Institute small animal ethics committee. The health and welfare of the mice were monitored by trained animal technicians and veterinary surgeons, and the study conformed to the Directive 2010/63/EU of the European Parliament.

2.2 Analysis of blood plasma

Plasma aliquots were defrosted as required and analysed for a range of markers related to cardiovascular disease using Luminex technology (Luminex B.V., Oosterhout NB, the Netherlands) and a Konelab 20 Clinical Chemistry Analyser (Thermo Scientific, Passau, Germany). The advantage of these techniques is that they use very little sample and utilize panels and kits for automated analysis, which reduces analytical error. Analytes assayed using the Konelab instrument included total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, total protein, albumin and creatine kinase. Luminex analyses using mouse multiplex panels of antibody-coated beads (Millipore, Watford, UK) included tumour necrosis factor-α (TNF-α), monocyte chemotactic protein-1 (MCP-1), IL-6, IL-1α, IL-1β, IL-2, interferon-γ (IFN-γ), macrophage inflammatory protein 1α (MIP1α), rantes, resistin, soluble E-selectin, soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule-1 (ICAM-1) and plasminogen activator inhibitor-1 (PAI-1).

2.3 Analysis of plaque area and calcification

Frozen hearts were sectioned using a Leica CM185 cryostat microtome and 10-μm sections were collected onto Polysine-coated slides for staining. Sections at the aortic root were identified for plaque analysis. These sections were fixed with buffered formalin for 5 min, rinsed with water then dipped in 60% isopropanol briefly before being stained for 3 min in 1% Sudan IV (Sigma, Poole, UK). After washing with distilled water, the sections were counterstained in Harris Hematoxilin Solution (Sigma) and washed again before being placed in a 1.35% lithium carbonate solution for 20 s. After a final wash in distilled water, the slides were dried at room temperature. The stained sections were analysed for total aorta area and plaque area using Image Pro Plus image analysis software (Media Cybernetics, Bethesda, MD). A minimum of three sections per animal were analysed. The sequential section immediately before the section used for plaque analysis was used for staining of extracellular Ca and P mineralization.

For Ca staining, tissue sections were fixed with 2% paraformaldehyde and stained with 40 mM Alizarin Red S (pH 4.2). von Kossa staining was used to assess extracellular P accumulation, which co-precipitates Ca ions. Tissue sections were treated with 5% silver nitrate solution incubated under UV light for 1 h at room temperature.

2.4 Statistics

Data were log transformed and analysed using a two-way analysis of variance (genotype and zinc intake). One combination of genotype and zinc was missing (ZD5 for WT mice), and the main effects of zinc were fitted after those of genotype. Differences between group means were assessed by using the Least Significant Difference. Analyses were carried out using Genstat v. 13 (VSN International Ltd., Hemel Hempstead, Hertfordshire, UK).

3 Results

3.1 Growth, haematology and zinc status

AEKO mice given diets ZD3, ZD8 and ZA35 achieved body weights of 31–34 g, but WT mice were very significantly heavier at around 41–42 g (Table 2). There were no statistically

Table 2. Body weight and levels of various plasma analytes related to cardiovascular health in apoE knock-out (AEKO) and wild-type (WT) mice given high saturated fat, high-cholesterol diets with different zinc levels for 25 wk.

Analyte	Unit	AEKO			WT	
		ZD3	ZD8	ZD35	ZD3	ZD35
Body weight	g	31.4 ± 1.2^{a}	32.3 ± 0.9^{a}	33.6 ± 0.4^{a}	41.3 ± 1.6^{b}	42.0 ± 1.0^{b}
Albumin	g/L	20.7 ± 0.8^{a}	20.5 ± 0.9^{a}	19.7 ± 0.6^{ab}	18.5 ± 0.7^{ab}	18.4 ± 0.6^{b}
Creatine kinase	U/L	223 ± 21^{a}	223 ± 13^{a}	244 ± 16^{a}	146 ± 12^{b}	131 ± 7^{b}
HDL Chol	mmol/L	9.75 ± 0.21^{a}	9.27 ± 0.18^{a}	9.48 ± 0.22^{a}	3.61 ± 0.36^{b}	3.67 ± 0.28^{b}
LDL Chol	mmol/L	43.0 ± 3.0^{a}	40.3 ± 2.6^{a}	37.7 ± 2.2^{a}	1.1 ± 0.1^{b}	1.0 ± 0.1^{b}
PAI 1 total	pg/mL	1152 ± 177^{a}	944 ± 104^{a}	927 ± 134^{a}	1042 ± 202^{a}	1865 ± 328^{b}
Resistin	pg/mL	2010 ± 148^{a}	2025 ± 106^{a}	2203 ± 95^{ab}	2248 ± 242^{ab}	2518 ± 104^{b}
sE Selectin	ng/mL	35.5 ± 9.5^{a}	24.1 ± 4.8^{a}	25.7 ± 3.5^{a}	84.7 ± 8.7^{b}	73.8 ± 10.2^{b}
sICAM-1	ng/mL	30.1 ± 1.4^{a}	27.2 ± 2.1^{a}	28.6 ± 2.2^{a}	29.8 ± 1.8^{a}	29.5 ± 1.1^{a}
MCP-1 (CCL2)	pg/mL	25.9 ± 3.0^{a}	15.5 ± 2.2^{a}	27.5 ± 3.8^{a}	108.2 ± 13.6^{b}	129.6 ± 21.6^{b}
MIP1α (CCL3)	pg/mL	259 ± 50^{a}	223 ± 54^{a}	265 ± 86^{a}	167 ± 40^{a}	189 ± 34^{a}
Rantes (CCL5)	pg/mL	221 ± 35^{a}	349 ± 158^{a}	151 ± 31^{a}	303 ± 110^{a}	186 ± 44^{a}

ZD3, ZD8 and ZD35 indicate dietary zinc levels of 3, 8 and 35 mg Zn/kg, respectively. Different letter superscripts within the same row indicate statistically significant differences ($p \leq 0.05$)

significant differences relating to zinc intake in either genotype ($p > 0.05$).

Zinc levels in mouse femur were significantly lower ($p < 0.05$) in mice of both genotypes on low dietary zinc intakes compared to the appropriate control mice consuming zinc-adequate diets (data not shown). Zinc deficiency also causes red blood cell fragility [19], and a significant decrease in both red blood cell counts and haematocrit was observed for the ZD3 compared to the ZA35 diet, but only in the AEKO mice (Fig. 1).

3.2 Vascular inflammation and plaque development

By 25 wk, arterial plaque development in AEKO mice was extensive whereas no plaque was visible in WT mice (Fig. 2). The luminal surface of aortas from AEKO mice was covered in lesions from the aortic root to its bifurcation into the two common iliac arteries. The animals showed no adverse signs due to this plaque development and were active and apparently healthy at termination. In contrast, WT mice showed no plaque development. As shown in Fig. 3, plaque area as a proportion of the total aortic root tissue area in stained sections was significantly higher ($p < 0.05$) in AEKO mice consuming the ZD3 and ZD8 diets as compared to the zinc-adequate group (ZA35).

The inflammatory cytokine IL-1β was significantly elevated with the ZD3 diet compared to the ZA35 diet in both genotypes, but IL-6 was only significantly increased at the lowest zinc intake level in the AEKO mice (Fig. 4). Likewise, sVCAM-1 was significantly ($p < 0.05$) raised by low zinc intake only in the AEKO mice (Fig. 4). The other analysed adhesion molecules, namely soluble ICAM-1 and soluble E-selectin, were also raised by low zinc intake, but not significantly (Table 2).

3.3 Plasma lipoprotein

Blood plasma samples from AEKO mice were very turbid in comparison to plasma from WT mice. This is a consequence

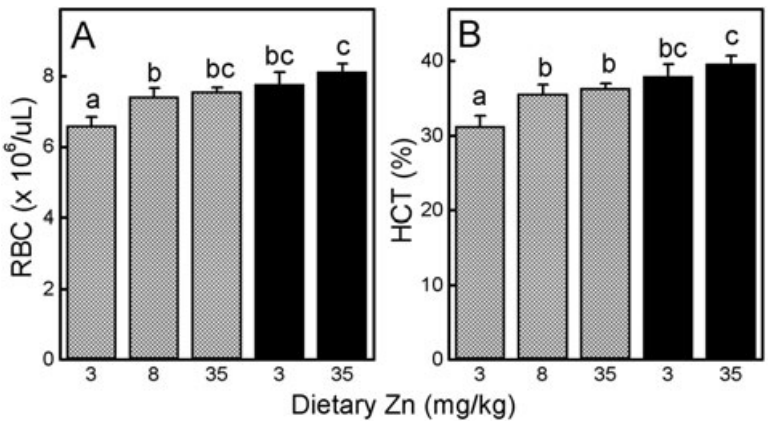

Figure 1. Red blood cell counts (A) and haematocrit (B) in apoE knock-out (AEKO, hatched columns) and wild-type (solid columns) mice given high saturated fat, high-cholesterol diets with different zinc levels for 25 wk. Data are the mean values with vertical lines indicating the standard error. Lower case letters above the columns indicate statistical significance, with dissimilar letters meaning significance at $p \leq 0.05$ and the same letters meaning no significance.

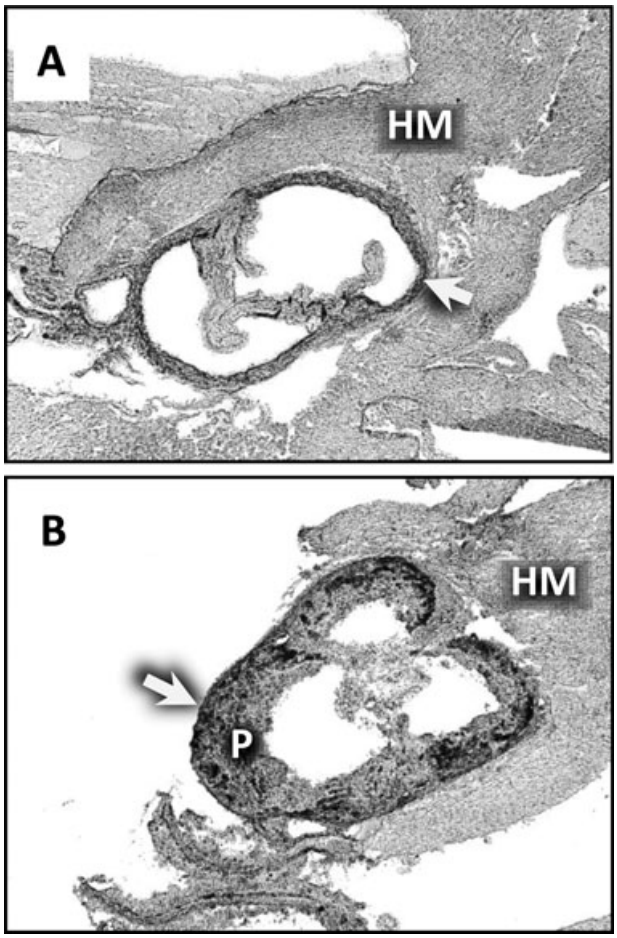

Figure 2. Cross-section of aortic root close to the aortic valve in wild-type (WT) mice (A) and AEKO mice (B) showing the aorta (arrowed), plaque within the aorta (P) and heart muscle (HM). Magnification ×40.

of elevated lipoprotein particle levels since AEKO mice cannot efficiently clear VLDL and chylomicron remnants from circulation [20]. The high levels of total protein, triglycerides, total cholesterol and LDL in AEKO mice compared to the WT controls confirmed the elevated presence of lipoprotein particles (Fig. 5, Table 2). In contrast to total plasma protein, which was 40% higher in AEKO mice compared to WT animals, plasma albumin, which normally constitutes 60% of total plasma protein, was raised by only 10% in AEKO mice ($p > 0.05$ when comparing zinc-adequate animals of each genotype). Consumption of the ZD3 diet significantly increased total protein and cholesterol but not triglyceride levels in AEKO mice (Fig. 5).

3.4 Calcification of plaque

Compared to the ZA35 AEKO mice, animals of the same genotype given the low zinc diets showed a marked increase

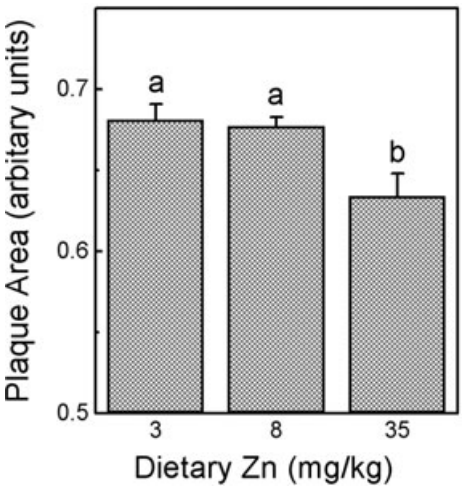

Figure 3. Atherosclerotic plaque areas in AEKO mice given high saturated fat, high-cholesterol diets with different zinc levels for 25 wk. Data are the mean values with vertical lines indicating the standard error. Lower case letters above the columns indicate statistical significance, with dissimilar letters meaning significance at $p \leq 0.05$ and the same letters meaning no significance.

in the presence of intramural calcium and phosphorous deposits in the aortic root close to the aortic valve (Fig. 6). The mineralized areas were shown to underlie the atheromatous plaque.

4 Discussion

Atherogenesis in AEKO mice is accelerated by a combination of elevated saturated fat and cholesterol in the diet [21]. After 15 wk on such a diet, aortic plaque formation is evident at sites of disturbed blood flow, such as at arterial bifurcations and in the aortic arch, but after 25 wk, plaque formation is almost continuous throughout the aorta [22]. We hypothesized that low zinc status might compromise vascular resistance to oxidative stress and inflammation resulting from saturated fat and cholesterol overconsumption. We have demonstrated that AEKO mice of lower zinc status showed significantly higher amounts of plaque in the aortic root than mice consuming adequate amounts of zinc. Although the difference was only around 8%, it is noteworthy that this statistically significant effect was detectable at zinc intakes equivalent to those in 40% of the UK adult human population and approached the UK Reference Nutrient Intake value for adult men of 9.5 mg/day [23]. Not only was there significantly more plaque, but these lesions contained more extensive areas of mineralization, which is known to be associated with decreased plaque stability and increased risk of adverse cardiovascular events [24]. These results confirmed those obtained in a pilot study in our laboratory, which was terminated at 13 wk and showed significantly higher levels of plaque in AEKO mice given the same high-fat,

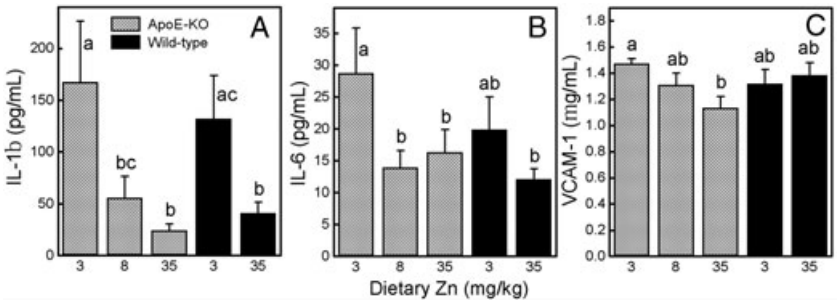

Figure 4. Blood plasma levels of inflammatory cytokines IL-1β (A) and IL-6 (B), in addition to soluble vascular adhesion molecule-1 levels (C) in AEKO and wild-type mice given high saturated fat, high-cholesterol diets with different zinc levels for 25 wk. Data are the mean values with vertical lines indicating the standard error. Lower case letters above the columns indicate statistical significance, with dissimilar letters meaning significance at $p \leq 0.05$ and the same letters meaning no significance.

high-cholesterol diet but containing 5 mg Zn/kg compared to 35 mg Zn/kg in the zinc-adequate group (J. H. Beattie, unpublished observations).

The self-perpetuating process of vascular inflammation leading to increased plaque formation, which in turn causes more inflammation, is characterized by the release of inflammatory cytokines, principally from macrophages. In this study, the ZD3 diet significantly raised plasma IL-1β levels in both AEKO and WT mice, but significantly raised plasma IL-6 levels only in the AEKO mice (twofold increase). Cytokines can originate from several vascular-independent sources, such as adipose tissue, and so increased IL-6 may not necessarily reflect increased vascular inflammation. However, sVCAM-1, the expression of which is regulated by vascular inflammatory cytokine levels, is more vascular-specific in origin and we found that the plasma levels of this adhesion molecule were significantly raised in AEKO mice consuming the ZD3 diet. Resistin is reported to activate endothelial cells and upregulate VCAM-1 expression [25], but in this study, plasma resistin was unaffected by dietary zinc intake (Table 2). Raised aortic VCAM-1 expression was also noted in young LDL receptor deficient mice in acute zinc deficiency [15]. As might be expected, there was no significant effect of zinc intake on sVCAM-1 levels in the WT mice. In AEKO mice, the other analysed soluble adhesion molecules sICAM-1 and sE-selectin showed a zinc intake dependent relationship but group differences were not statistically significant. Chemokines CCL5, CCL2 and CCL3 were also unaffected by zinc intake, but large genotype differences were noted for the latter two chemokines (Table 2). It is reasonable to conclude that vascular inflammation in AEKO mice was raised in response to low dietary zinc intake, with IL-6 being a key cytokine mediator of changes in zinc status.

Since lipoprotein particle size and abundance is a major factor influencing the development of atherosclerosis, we measured total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides and total plasma protein. As expected, AEKO mouse plasma contained much higher levels of all measured constituent components of lipoprotein particles. In these mice, much of the additional cholesterol, triglycerides and protein would be found in VLDL and chylomicron remnants, but given the limited plasma available, it was not practicable to confirm this chromatographically. Clinical assays for LDL and HDL cholesterol used in this study are intended for human sample analysis but can be applied to mouse samples. Unlike human blood, mouse blood does not contain high levels of LDL but due to the presence of VLDL remnants, which have a similar size and density to LDL, a substantial apparent increase in LDL cholesterol levels was found in AEKO mice compared to the WT animals. AEKO mice consuming the ZD3 diet had significantly higher levels of plasma total cholesterol and total protein, indicating that increased plaque formation may have been caused, at least in part, by elevated pro-atherogenic lipoprotein particle levels. Indeed, zinc deficiency induced increase in plasma total cholesterol and protein levels was of a similar magnitude to the increase in aortic sinus plaque. Elevation of plasma total cholesterol has also been noted in acutely zinc-deficient LDL receptor deficient mice compared to non-pair-fed zinc-adequate mice [26]. However, a meta-analysis of randomized controlled supplementation trials with human volunteers revealed that plasma total cholesterol is unchanged in response to zinc supplementation [27]. There is likely a marked

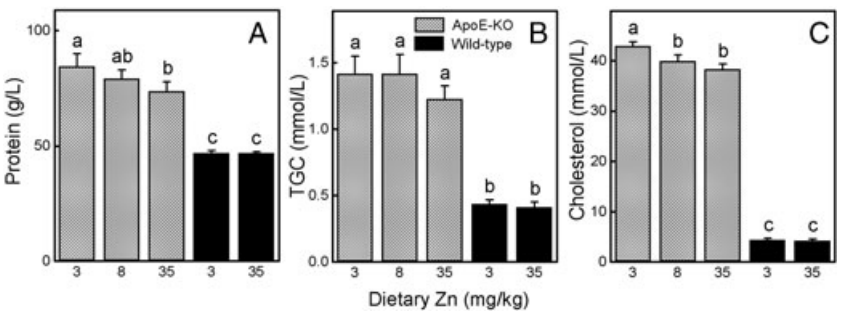

Figure 5. Blood plasma levels of total protein (A) triglycerides (B) and total cholesterol (C) in AEKO and wild-type mice given high saturated fat, high-cholesterol diets with different zinc levels for 25 wk. Data are the mean values with vertical lines indicating the standard error. Lower case letters above the columns indicate statistical significance, with dissimilar letters meaning significance at $p \leq 0.05$ and the same letters meaning no significance.

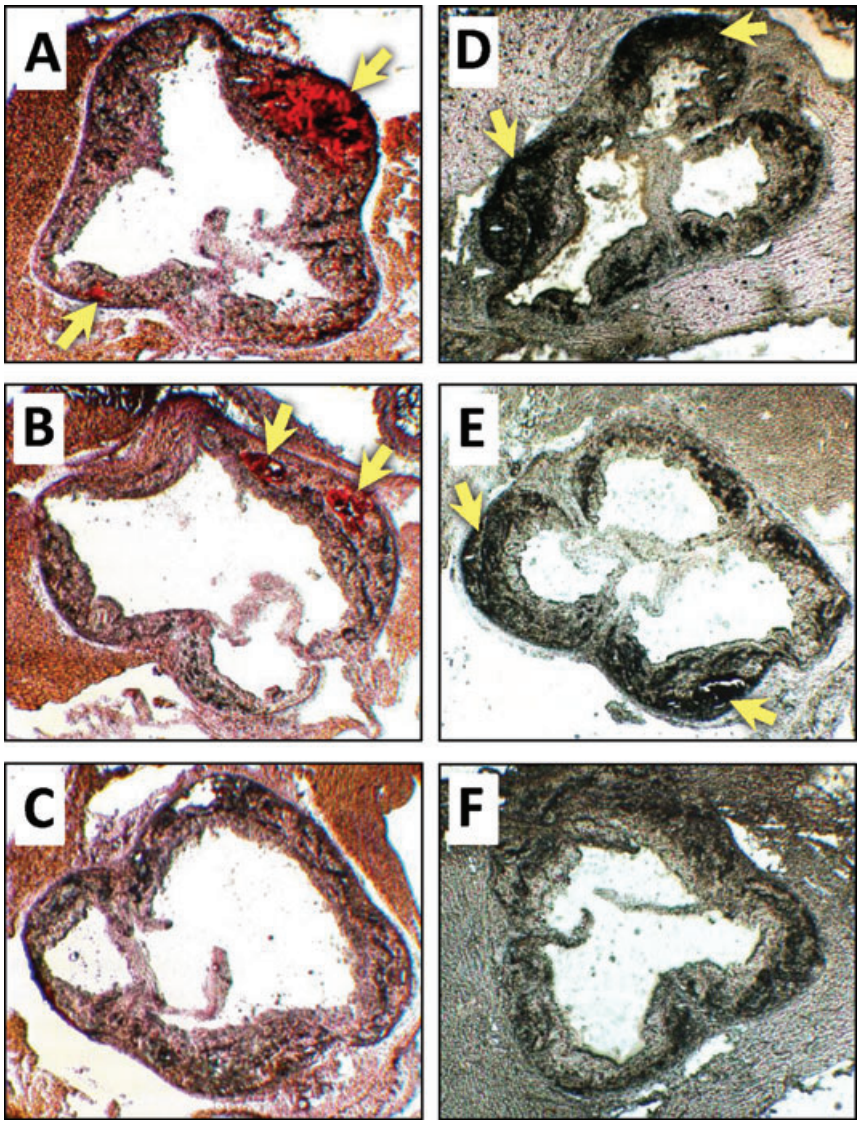

Figure 6. Calcification of plaque in the aortic root of AEKO mice given a high-fat, high-cholesterol diet for 25 wk. Tissue sections (cross-section) stained for calcium using Alizarin Red (A, B and C) and von Kossa stain for phosphorous (D, E and F) were from mice given diets containing 3 (A and D), 8 (B and E) and 35 mg Zn/kg (C and F). Magnification ×40.

difference in the influence of zinc supplementation and zinc deficiency on cholesterol metabolism, with supplementation levels in intervention studies often being high enough to be unachievable through dietary means. It should not be assumed that supplementation above normal dietary levels of zinc intake, and deficiency below those intake levels, have opposite effects on the same metabolic systems. In the present study, LDL cholesterol was unaffected by zinc intake, but LDL is relatively insignificant in the mouse lipoprotein particle profile and also in its importance in promoting atherosclerosis in AEKO mice relative to VLDL remnants. In rats fed a 4% fat, 1% cholesterol diet, plasma HDL was previously reported to be significantly decreased in response to low dietary zinc (2.8 mg Zn/kg diet) [28], but no significant differences were observed in the present mouse study.

A relationship between zinc intake and circulating cholesterol levels has been noted in several studies over many years, particularly in relation to copper [29, 30]. A long-standing controversy over the efficacy of zinc supplementation in influencing plasma cholesterol levels has recently been evaluated in a meta-analysis of 20 human studies, with the conclusion that total cholesterol levels are not affected with supplements up to 100 mg/day, but that HDL cholesterol in healthy subjects is decreased [27]. The influence of zinc deficiency on lipoprotein particle cholesterol has been less well investigated, with most studies reporting results from acute zinc deficiency, which inhibits food intake and requires the inclusion of appropriate pair-fed controls. Body cholesterol levels are maintained by a complex balance between synthesis, secretion, clearance, excretion and absorption/re-absorption. Being a major centre of cholesterol synthesis and excretion, the liver is an obvious focal point for evaluating factors affecting plasma cholesterol levels. Gut mucosa is also an important site of cholesterol synthesis and mucosal 3-hydroxy-3-methyl-glutaryl-CoA

(HMG-CoA) reductase, which regulates cholesterol synthesis and is the direct target for statins, was also shown to be a direct or indirect target for zinc deficiency, and its enzyme activity was significantly increased by acute zinc deficiency compared to pair-fed controls [31]. In an 8-week rat study using similar dietary zinc intakes to those used in the present mouse study, free and esterified plasma cholesterol levels increased significantly but the gene expression of HMG-CoA reductase in lung tissue was decreased in response to limiting dietary zinc intake [32]. The latter makes biological sense, since elevated cholesterol downregulates HMG-CoA reductase expression through regulation of sterol regulatory element binding protein (SREBP) activation [33]. Nevertheless, zinc deficiency may stimulate the enzyme activity, or tissue differences may account for the gene expression and enzyme activity discrepancies.

Increased synthesis of cholesterol is just one way in which plasma levels may become elevated. More efficient absorption of dietary cholesterol might be expected to raise plasma levels, but rats given a marginally zinc-deficient diet (3.6 mg Zn/kg diet) actually showed significantly lower absorption of radiolabelled cholesterol into the lymphatic system [34]. The authors concluded that impaired absorption may be associated with defective formation of chylomicrons in the intestinal mucosa. Secretion of synthesized cholesterol into circulation is dependent on lipoprotein particle assembly, and removal from circulation is likewise dependent on the efficiency of particle clearance, which in the case of AEKO mice is impaired for VLDL and chylomicron remnants. In order to resolve the mechanism for raised cholesterol in low zinc status, future studies may focus, for example, on the clearance of labelled cholesterol in suitable animal models and the regulation of cholesterol synthesis.

In addition to the pro-atherogenic driving force of raised circulating cholesterol levels in the present study, our previous proteomic analysis of vascular protein responses to acute and marginal dietary zinc intake in rats shows that zinc deficiency may have direct or indirect effects on arterial cell function [17]. Specifically, we identified zinc-status related changes in markers of smooth muscle cell differentiation, such as sm22α, indicating a change in the vascular smooth muscle cell (VSMC) phenotype. In the present study, the detection of increased circulating IL-1β in AEKO and WT mice on a ZD3 diet might indicate a mechanism for changes in sm22α expression, since this inflammatory cytokine promotes VSMC proliferation.

Proliferation of VSMCs is thought to stabilize plaque [35], and so zinc deficiency induced VSMC proliferation might not in itself indicate a poor prognosis, because critical events more normally relate to plaque instability [36]. However, our observations in this study suggest that zinc deficiency promotes aortic calcification, which is associated with adverse consequences due to plaque destabilization [24]. In cell culture studies, we have shown previously that calcification of bone matrix is adversely affected by zinc depletion [37,38] but that the opposite effect may be observed in VSMC-mediated calcification of atherosclerotic plaque due to increased apoptosis in zinc deficiency (I. S. Kwun, unpublished data). We therefore propose that the observed effects of reduced zinc status on the pathogenesis of atherosclerosis in AEKO mice in this study are not beneficial. Evaluating the longevity of the animals would test this tentative conclusion.

For the first time, the present study makes a tentative link between reduced zinc intake, increased plasma lipoprotein levels, increased vascular inflammation and increased plaque formation in mice consuming dietary zinc levels relevant to human populations. Mouse models of atherosclerosis are useful for demonstrating nutritional influences on atherogenesis but where these influences may be mediated through changes in lipoprotein particle profiles, some caution is required in the interpretation, because the null mutation for apoE and indeed LDL receptor have their own substantial impact on these profiles. However, there is evidence that reduced zinc intake is associated with raised plasma cholesterol levels and raised markers associated with increased risk of cardiovascular disease as well as increased mortality in human subjects [9]. We are therefore currently investigating the influence of zinc status, as distinct from zinc intake, on lipoprotein particle profiles and markers of vascular inflammation in human subjects. Concluding from the present study, it seems likely that low zinc status may significantly promote atherosclerosis and plaque calcification, which are characteristic consequences of consuming Western-type diets high in saturated fat and cholesterol over extended periods of time.

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