Marginal dietary zinc deficiency in vivo induces vascular smooth muscle cell apoptosis in large arteries

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Aims Dietary zinc deficiency has been associated with the development of atherosclerosis although the effects on vascular smooth muscle cells (VSMCs), important in maintaining atherosclerotic plaque integrity, are unknown. The main aim of this study was to elucidate the effect of a zinc-deficient environment on VSMCs using an in vivo model.

Methods and results Rats were maintained for 2 weeks on a marginally zinc-deficient diet which resulted in a significant reduction in plasma zinc levels. Large arteries from zinc-deficient rats had significantly increased apoptosis within the VSMC layers compared with arteries from rats on a zinc-adequate diet. This apoptosis occurred in parallel with a known apoptotic pathway, namely dephosphorylation of the pro-apoptotic protein Bcl-2-associated death promoter protein (BAD). Activation of extracellular signal-regulated kinase (ERK)1/2, which maintains BAD phosphorylation as a pro-survival mechanism, was decreased in arteries from zinc-deficient rats. The mechanisms of this in vivo effect were investigated in vitro. Cultured rat VSMCs incubated with plasma from zinc-deficient rats similarly resulted in increased apoptosis in parallel with BAD dephosphorylation and decreased ERK1/2 activation. Further related apoptotic mechanisms induced by plasma from zinc-deficient rats involved a prolonged rise in \([\text{Ca}^{2+}]\), leading to subsequent activation of the phosphatase calcineurin. Calcineurin activation was required to dephosphorylate BAD. In addition, an increase in oxidative stress contributed to the apoptotic effect induced by plasma from zinc-deficient rats.

Conclusion In conclusion, a marginally zinc-deficient diet is pro-apoptotic for VSMCs and this may contribute to cardiovascular disease.

Keywords Vascular smooth muscle cell • Artery • Zinc deficiency • Apoptosis

1. Introduction

The micronutrient zinc is critical to many physiological processes in vivo and has an essential role in health including normal immune system function and anti-oxidant stress responses.¹ Dietary zinc deficiency, which occurs due to inadequate intake, decreased absorption, or increased loss, can therefore be an important factor in many pathological conditions.²,³ This is particularly relevant in the elderly where zinc utilization and absorption are known to be decreased with age.¹,⁴ As zinc is critical to maintaining normal physiological functions, deficiency of this micronutrient is likely to impact on health in the elderly population and may contribute to pathogenesis of age-related diseases including cardiovascular disease.⁴,⁶

Recently, evidence has emerged suggesting that zinc deficiency is associated with the development of atherosclerosis.⁷,⁸ This stems from data demonstrating that the low-zinc status directly correlates with the occurrence of atherosclerosis.⁹ A clinical study has indicated that lower zinc dietary intake is significantly associated with increased levels of low density lipoprotein, a marker for coronary artery disease.⁸ In addition, supplementing zinc decreased the level of C-reactive protein (a predictor of cardiovascular risk) and also lowered levels of inflammatory cytokines and markers of lipid peroxidation.¹⁰

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We have recently demonstrated in ApoE-null mice on a Western diet that zinc deficiency directly contributes to plaque formation and is associated with increased vascular inflammation and increased cholesterol. The cell types and intracellular mechanisms involved in these zinc-dependent atherogenic changes are not known and could be due to effects on inflammatory cells, endothelial cells, and/or vascular smooth muscle cells (VSMCs). To date, in vitro experiments have suggested that zinc deprivation of endothelial cells may be involved. We also have evidence that VSMCs are involved in the atherosclerotic process. Our proteomic study has revealed that the VSMC phenotype in large arteries can be altered in a zinc-deficient animal model which could potentially contribute to vascular disease. As VSMCs have a fundamental role in the pathogenesis of atherosclerosis by stabilizing lipid plaques via formation of a fibrous cap, any changes to the VSMC phenotype could have critical implications for the disease process.

At a cellular level, zinc is integral to the normal function of over 2800 proteins that regulate many aspects of cell behaviour. Zinc is tightly regulated within cells and bound predominantly to zinc-binding proteins. Evidence suggests that the intracellular free-zinc pool which can influence zinc-requiring proteins is very small, and therefore small fluctuations in intracellular zinc could dramatically alter activity of these proteins. Zinc cellular transport is finely controlled by families of specific zinc transporters and is also regulated by metallothioneins which act as zinc homoeostatic buffers. As zinc intracellular transport is highly regulated, it is likely that even modest changes in plasma zinc levels as a result of a marginally zinc-deficient diet could have profound effects on many physiological functions.

In the current study, we have used an in vivo model to assess the effects of a marginally zinc-deficient diet on VSMCs. The VSMCs in large arteries of zinc-deficient rats revealed an increase in apoptosis. VSMC apoptosis was correlated with dephosphorylation of the pro-apoptotic protein Bcl-2-associated death promoter (BAD) which occurred by both an inhibition of phosphorylation and an activation of dephosphorylation. Using an in vitro model, we also established that zinc-deficient plasma alone is sufficient to induce a pro-apoptotic phenotype. These experiments indicate that marginal zinc deficiency, via the induction of VSMC apoptosis, could impact on cardiovascular diseases such as atherosclerosis.

## 2. Methods

Full-methodological details are provided in the Supplementary material online.

### 2.1 Rat zinc diets

All animal procedures were in compliance with UK Home Office animal welfare regulations and approved by the Rowett Institute Research Ethics Committee. This investigation conforms to Directive 2010/63/EU of the European Parliament. Rowett Hooded Lister rats were obtained from a colony in the Institute-specific pathogen-free breeding unit. Rats were acclimatized for 1 week on a semi-synthetic diet containing 35 mg zinc/kg (zinc-adequate diet). The control diet containing 35 mg zinc/kg meets the zinc requirements for rodents as identified by the American Institute of Nutrition. Following acclimatization, rats were divided into two groups (n = 10 for each group) and fed ad libitum with diets containing 3 mg zinc/kg (marginally zinc deficient) or 35 mg zinc/kg (zinc adequate) for 2 weeks as previously described. The dietary zinc levels were confirmed by acid digestion and atomic absorption spectrophotometry.

### 2.2 Tissue and plasma preparation

Animals were fasted for 4 h prior to being killed by isoflurane anaesthesia and exsanguination. Blood was drawn from the vena cava using heparinized syringes. The drawn blood was centrifuged for 15 min at 2000 g. Plasma was then aliquoted and snap-frozen in liquid nitrogen. For each experiment with rat plasma, individual 'n' numbers used plasma from different rats. Rat carotid arteries were dissected and connective tissue was removed in serum-free DMEM. In blood vessels used for assessing ERK1/2 activation, the endothelial layer was removed during dissection.

### 2.3 Measurement of zinc plasma concentrations

Plasma samples were analysed for zinc by atomic absorption spectroscopy as previously described along with a standard reference material.

### 2.4 Cell culture

Rat primary aortic smooth muscle cells were obtained from the rat aorta using enzymatic digestion as previously established.

### 2.5 SDS–PAGE electrophoresis/ immunoblotting

Protein extracts were prepared and subjected to SDS–PAGE followed by immunoblotting as previously described.

### 2.6 Tissue and cell preparation for microscopy

The rat aorta was fixed, flash-frozen, and sectioned as previously described. Immunolabelled sections/cells were imaged using a Zeiss 710-scanning confocal microscope with a 40 x objective. For analysis of immunofluorescent images, four fields of view were taken from each sample and the fluorescence intensity measured using Image J. These intensities were averaged and used as n = 1. This was repeated as indicated for each experiment. During confocal imaging comparing zinc-deficient and zinc-adequate tissue/cells, settings on the microscope were not changed (including contrast, aperture, and laser strength).

### 2.7 Apoptosis assays

Apoptotic cells were labelled using an in situ death detection kit (TUNEL assay). Annexin V/propidium iodide (PI) staining was used in cultured VSMCs. JC-1 staining to determine mitochondrial membrane potential was also used in VSMCs. All the assays were performed according to manufacturers’ instructions. Tissue section/cultured VSMCs were examined by confocal microscopy.

### 2.8 Intracellular calcium imaging

Cultured rat aortic VSMCs were loaded with the fluorescent calcium indicator 5 μM Fura-2 acetylmethoxy as previously described. Cells were mounted at room temperature on a Zeiss Axiovert 200 microscope equipped with a cooled CCD camera and a polychromatic illumination system (T.I.L.L. Photonics) to capture fluorescent images with excitations at 340 and 380 nm. The Metafluor software performed ratiometric imaging expressed as a 340:380 ratio.

### 2.9 Intracellular measurement of oxidative stress

VSMCs were incubated with 30 μM 2′,7′-dichlorofluorescein diacetate (DCFDA) for 1 h. After 3 h of stimulation fluorescence was measured at 529 nm.

### 2.10 Statistics

Data are expressed as mean ± SEM. Comparisons were made using Student’s paired t-test or ANOVA as appropriate. P-values of <0.05 were considered statistically significant.
3. Results

3.1 A marginally zinc-deficient diet induces low zinc plasma concentrations in vivo

There was a significant reduction in plasma zinc levels as determined by atomic absorption spectroscopy from zinc-deficient rats fed a 3 mg/kg zinc diet compared with zinc-adequate rats fed a 35 mg/kg zinc diet (Figure 1A). Total body weights were not significantly different between rats on the zinc-adequate diet compared with rats on the marginally zinc-deficient diet (rats on 35 mg/kg zinc diet: Day ‘0’: 294 ± 4.3 g, Day ‘14’: 363 ± 3.3 g; rats on 3 mg/kg zinc diet: Day ‘0’: 290 ± 3.7 g, Day ‘14’: 349 ± 3.7 g).

3.2 Carotid artery smooth muscle cells undergo a switch to a more apoptotic phenotype in vivo under marginally zinc-deficient conditions

To investigate whether a zinc-deficient environment in vivo elicits an effect on VSMCs, fixed rat carotid artery sections were examined for apoptosis using the TUNEL assay. A significant increase in apoptotic VSMCs was observed in the carotid artery of rats on a marginally zinc-deficient diet for 2 weeks (Figure 1B). In VSMCs from rats on a zinc-adequate diet, there was little evidence of apoptosis. A similar apoptotic effect was also observed in the aorta from zinc-deficient rats (results not shown). An apoptotic phenotype was not observed in the

Figure 1 A marginally zinc-deficient diet reduces zinc plasma concentrations and increases apoptosis in VSMCs of rat large arteries. (A) Rats fed on a zinc-adequate diet (35 mg/kg) or marginally zinc-deficient diet (3 mg/kg) for 2 weeks had a significantly reduced plasma zinc concentration. n = 7. (B) Sections of the carotid artery from rats maintained on either a zinc-adequate diet or a zinc-deficient diet were subjected to the TUNEL assay. Arteries from zinc-deficient rats displayed a significant increase in apoptotic VSMCs compared with zinc-adequate rats. Typical images are shown of TUNEL staining with respective nuclear localization (using BOBO-3). Arrows denote examples of apoptotic nuclei. Scale bar = 20 μm. Graph is expressed as mean total TUNEL positive cells, n = 3. All results mean ± SEM, *P < 0.05.
endothelial cell layer. To determine the underlying mechanisms of increased apoptosis in VSMCs from zinc-deficient rats, we examined the pro-apoptotic Bcl-2 family member, BAD. Sections of carotid arteries from rats following a 2-week zinc-deficient diet revealed a significant increase in BAD expression as determined by immunolocalization compared with arteries from zinc-adequate rats (Figure 2A). This had a non-nuclear localization. As BAD dephosphorylation is directly linked to the onset of apoptosis, the phosphorylation state of BAD was also assessed. In carotid artery sections from zinc-deficient rats, BAD dephosphorylation was significantly reduced in VSMCs compared with arteries from zinc-adequate rats (Figure 2B).

The pro-survival ERK1/2 signalling pathway maintains the phosphorylation of BAD under normal conditions, thereby preventing apoptosis. Denuded arteries obtained from 2-week zinc-deficient rats

**Figure 2.** Zinc deficiency results in an increase in BAD expression, BAD dephosphorylation and decreased ERK1/2 activation in VSMCs from large arteries. 

(A) Sections of the carotid artery from rats fed a 2-week zinc-deficient diet or zinc-adequate diet were immunolabelled with anti-BAD antibody and visualized by fluorescence confocal microscopy. Typical micrographs are shown together with respective nuclear localization, scale bar = 20 μm. BAD expression was significantly increased in arteries from zinc-deficient rats, n = 3 (8). Phospho-BAD was decreased in arteries from zinc-deficient rats compared with zinc-adequate rats, n = 3, scale bar = 20 μm. (C) Activation of ERK1/2 by either 1 μM S1P or 50 ng/mL rat PDGF for 15 min was determined by immunoblot using anti-phospho-ERK1/2 antibodies. Typical immunoblots are shown, n = 3. Both S1P- and PDGF-induced ERK1/2 activation was significantly decreased in carotid arteries from marginally zinc-deficient rats compared with zinc-adequate rats. All the results in (A), (B), and (C) are expressed as fold change normalized to control values. * P < 0.05.
were stimulated ex vivo immediately following dissection with either 1 μM sphingosine 1-phosphate (S1P) or 50 ng/mL platelet-derived growth factor-BB (PDGF) for 15 min at 37°C and ERK1/2 activation was examined. Both S1P and PDGF are known agonists for ERK1/2 activation in VSMCs. In arteries from zinc-deficient rats, there was a significant decrease in ERK1/2 phosphorylation following stimulation with either S1P or PDGF (Figure 2C). Immunoblotting of whole cell homogenates indicates that overall ERK1/2 expression was not significantly altered based on equal protein loading (assessed by GAPDH, not shown) in arteries from 2-week zinc-deficient rats compared with control rats (Figure 2C).

3.3 Plasma from marginally zinc-deficient rats increases apoptosis in VSMCs in vitro
Cultured rat primary aortic VSMCs were incubated for 24 h with 10% plasma extracted from rats on either the marginally zinc-deficient (3 mg/kg) or zinc-adequate (35 mg/kg) diet. TUNEL assays indicated that significant apoptosis occurred in VSMCs incubated for 24 h with plasma from zinc-deficient rats compared with cells incubated with zinc adequate plasma (Figure 3A). To confirm this apoptotic effect, simultaneous annexin V/PI staining was used. VSMCs incubated as above with plasma from zinc-deficient rats had a significantly increased annexin V staining but minimal PI staining (Figure 3B). In addition, changes in the mitochondrial membrane potential were determined by JC-1 staining. VSMCs incubated with plasma from zinc-deficient rats had significantly more depolarized mitochondrial membranes as evidenced by the shift from red to green fluorescence compared with cells incubated with plasma from zinc-adequate rats (Figure 3C). Caspase-3 activation, as assessed by immunoblotting, was also increased in VSMCs following incubation with plasma from zinc-deficient rats (Figure 3D).

3.4 VSMC apoptosis induced by plasma from zinc-deficient rats occurs via mechanisms involving BAD phosphorylation
Expression of the protein BAD was increased in cells incubated for 24 h in 2-week zinc-deficient plasma as assessed by immunolocalization (Figure 4A). Using identical conditions, the phosphorylation of BAD was examined. VSMCs incubated with plasma from zinc-deficient rats revealed significant dephosphorylation compared with VSMC incubated with zinc-adequate plasma (Figure 4B). Similarly, the activation of ERK1/2 was also decreased following stimulation with either 1 μM S1P or 50 ng/mL PDGF for 15 min in VSMCs pre-incubated for 24 h in plasma from zinc-deficient rats (Figure 4C).

To further delineate the mechanisms involved in the observed decrease in BAD phosphorylation induced by plasma from zinc-deficient rats, we examined the potential role of the protein phosphatase 2B calcineurin, which acts as a BAD phosphatase.24 VSMCs were incubated as above in zinc adequate or zinc-deficient plasma for 24 h with 10 μM cyclosporin A, a calcineurin inhibitor. In VSMCs incubated in plasma from zinc-deficient rats, cyclosporin A prevented the increase in apoptosis (Figure 4D). Cyclosporin A also significantly decreased BAD dephosphorylation (Figure 4E).

3.5 A prolonged increase in [Ca$^{2+}$]$^{i}$ is involved in VSMC apoptosis induced by zinc deficiency
As calcineurin is activated by an increase in calcium, the effects of plasma from zinc-deficient rats on [Ca$^{2+}$]$^{i}$ were examined. Cultured VSMCs loaded with the fura-2 were stimulated with 10% plasma from zinc-deficient and zinc-adequate rats. Plasma from zinc-adequate rats induced a typical transient rise in [Ca$^{2+}$]$^{i}$ (Figure 5A), however plasma from zinc-deficient rats produced a Ca$^{2+}$ rise which had a significantly increased peak and prolonged Ca$^{2+}$ elevation (Figure 5A). Treatment with 10 μM BAPTA-AM for 30 min to chelate intracellular Ca$^{2+}$ significantly decreased the apoptosis in VSMC induced by plasma from zinc-deficient rats but did not alter apoptotic levels in cells incubated with zinc-adequate plasma (Figure 5B).

3.6 Plasma from zinc-deficient rats induces oxidative stress which contributes to VSMC apoptosis
We tested the possible role of reactive oxygen species in the observed apoptotic effect. Oxidative stress, as measured by the fluorescent indicator DCFDA, was significantly increased in VSMCs incubated with 10% plasma from zinc-deficient rats over a 3 h timecourse (Figure 6A). Co-incubation with 15 μM of the anti-oxidant N-acetyl cysteine (NAC) prevented this plasma-induced rise in oxidative stress (Figure 6A). To determine whether the increased oxidative stress contributes to VSMC apoptosis induced by plasma from zinc-deficient rats, we incubated cells with plasma from either zinc-deficient or zinc-adequate rats in the presence of NAC. Inclusion of this anti-oxidant prevented the increase in apoptosis with plasma from zinc-deficient rats (Figure 6B).

4. Discussion
Determining the in vivo effects of zinc deficiency in the cardiovascular system is essential to fully understand its potential role in the pathogenesis of cardiovascular disease.7,8 In the current study, we have used an established animal model of zinc deficiency and examined the effects on large arteries. Our results demonstrate that marginal dietary zinc deficiency induces apoptosis in VSMCs in vivo. This occurs in parallel with a previously characterized pro-apoptotic signalling mechanism, BAD dephosphorylation. In addition activation of ERK1/2, which maintains BAD phosphorylation as part of a cellular pro-survival mechanism, is decreased in VSMCs from large arteries of marginally zinc-deficient animals. Plasma from zinc-deficient rats induces similar pro-apoptotic changes to VSMCs in vitro. Using this in vitro model, two further apoptotic mechanisms were uncovered. First, the plasma-derived apoptotic effect involves activation of the phosphatase calcineurin which results in dephosphorylation of BAD. This activation of calcineurin probably occurs via a sustained Ca$^{2+}$ rise induced by the plasma from zinc-deficient animals. Secondly, plasma from zinc-deficient animals induces an increased oxidative stress which also has a primary role in the apoptotic effect. Zinc deficiency in vivo therefore results in the activation of multiple pathways leading to VSMC apoptosis.

It has been known for some time that apoptosis of VSMCs occurs in cardiovascular disease, both during restenosis and atherosclerosis, although the factors which initiate this apoptosis are not clear.25 In an indiscutable in vivo model of VSMC apoptosis, it has been demonstrated that even marked apoptosis in otherwise healthy mice does not alter vascular physiological properties (at least in the shorter term).16 A significant effect of the VSMC apoptosis was only apparent when these mice were crossed with ApoE$^{-/-}$ mice. In this atherosclerotic model, the experimentally induced vascular apoptosis had a significant effect on markers of plaque stability (including thinning of the cap region and loss of extracellular matrix).17 Our findings now clearly indicate a
Plasma from rats on a marginally zinc-deficient diet induced apoptosis in cultured VSMCs. Primary cultured rat aortic VSMCs were incubated for 24 h with plasma from either marginally zinc-deficient rats (3 mg/kg) or zinc-adequate rats (35 mg/kg). (A) Using the TUNEL assay, apoptotic VSMCs increased significantly in cells incubated with plasma from zinc-deficient rats, n = 3. (B) VSMCs incubated with plasma from zinc-deficient rats had a significantly increased staining of annexin V (green) compared with cells incubated with plasma from zinc-adequate rats. Simultaneous staining with PI (red) did not label nuclei of cells incubated with plasma from either zinc-deficient or zinc-adequate rats. A positive control using cells incubated for 24 h with 500 μM H₂O₂ demonstrated staining for annexin V and nuclear labelling with PI (red), n = 3, scale bar = 10 μm. (C) The mitochondrial membrane potential was assessed using JC-1. VSMCs incubated with plasma from zinc-deficient rats demonstrated an increase in mitochondrial membrane depolarization (shift from red to green fluorescence) compared with cells incubated with plasma from zinc-adequate rats. VSMCs incubated for 24 h with 500 μM H₂O₂ were used as a positive control, n = 3, scale bar = 5 μm. (D) VSMCs incubated with plasma from zinc-deficient rats had increased caspase-3 activation compared with cells incubated with plasma from zinc-adequate rats as assessed by immunoblotting, typical blot shown (densitometry expressed as fold change from control: 3 mg/kg zinc plasma—1.1 ± 0.3; 35 mg/kg zinc plasma—6.7 ± 0.8, n = 3). * P < 0.05.
Figure 4 Decreased BAD dephosphorylation induced by plasma from zinc-deficient rats in VSMCs may involve both ERK1/2 and calcineurin. (A) Expression of BAD was significantly increased in cells incubated with plasma from zinc-deficient rats as assessed by immunofluorescence, \( n = 3 \), scale bar = 5 μm. (B) BAD phosphorylation was decreased in VSMCs incubated with plasma from zinc-deficient rats compared with zinc-adequate rats, \( n = 3 \), scale bar = 5 μm. (C) VSMCs were incubated with plasma from zinc-deficient or zinc-adequate rats for 24 h followed by stimulation with 1 μM S1P or 50 ng/mL PDGF for 15 min. ERK1/2 activation by either S1P or PDGF, determined by immunoblotting, was significantly decreased in VSMCs incubated with plasma from zinc-deficient rats. Typical blots are shown, \( n = 3 \). (D) Inhibition of calcineurin by pre-treating with cyclosporin A for 30 min significantly decreased the apoptosis induced by incubation with 10% plasma from zinc-deficient rats, \( n = 3 \). (E) Cyclosporin significantly inhibited the dephosphorylation of BAD by plasma from zinc-deficient rats (\( n = 3 \)). Results in (A)–(C) and (E) are expressed as fold change normalized to control values. * \( p < 0.05 \).
novel initiator of apoptosis in VSMCs in vivo. This has specific relevance to zinc deficiency in the presence of cardiovascular disease. For example, this would directly relate to zinc deficiency in the elderly, where at least some development of atherosclerosis would be expected. We have previously observed significantly increased development of the aortic plaque, increased plasma cholesterol levels and vascular inflammation in ApoE−/− mice given mildly zinc-deficient diets for 6 months compared with zinc-adequate mice. Although vascular cell apoptosis was not analysed, increased inflammation and atherosclerosis could indicate increased apoptosis. The current study suggests that where zinc deficiency occurs in parallel with atherosclerosis, there would be an increased VSMC apoptosis leading to a weakened cap.
region in atherosclerotic plaques. This would lead to an increased risk of a plaque rupture. These results indicate a potential need to evaluate zinc deficiency in atherosclerotic patients.

The process of apoptosis is associated with an up-regulation of several death-associated pathways typically leading to the activation of caspases. We now demonstrate via several different mechanisms that apoptosis occurs in VSMCs incubated with plasma from zinc-deficient rats. This includes activation of at least one caspase (caspase-3). In addition, this effect does not appear to involve necrosis, as PI staining was not evident. There are several mechanisms which can produce programmed cell death and this includes an inhibition of pro-survival pathways. One such survival pathway which has been previously described involves regulation of the pro-apoptotic Bcl-2 family member, BAD which resides in the outer mitochondrial membrane. BAD is now known to be a key switch between survival and programmed cell death. Inactive BAD is in a phosphorylated state and this phosphorylation is maintained by the pro-survival kinases, ERK1/2. When ERK1/2 activity is decreased, this leads to decreased phosphorylation of BAD and the resultant active (dephosphorylated) BAD protein allows aggregation of other pro-apoptotic proteins. This aggregation induces activation of caspases. To date this mechanism has not been studied in detail with respect to VSMCs. In a zinc-deficient environment in vivo, we now demonstrate for the first time that the resultant apoptosis observed in VSMCs could be due to two novel zinc-dependent mechanisms which regulate BAD phosphorylation; (i) a decrease in activation of ERK1/2, and (ii) activation of the phosphatase calcineurin via prolonged rise in \([\text{Ca}^{2+}]_i\). Decreased ERK1/2 activation would decrease BAD phosphorylation leading to activation of caspases and apoptosis. Although the mechanisms which lead to the decrease in ERK1/2 activation are unknown at present, this is unlikely to be related to activation via a single agonist. Both a 7-transmembrane G-protein-coupled receptor agonist (S1P) and a receptor tyrosine kinase agonist (PDGF) showed similar effects on ERK1/2 activation suggesting that this may be a general mechanism which could affect multiple ERK1/2-activating mediators. Our study also reveals for the first time that plasma from zinc-deficient rats induces a prolonged rise in \([\text{Ca}^{2+}]_i\). Increases in intracellular \([\text{Ca}^{2+}]_i\) are known to have several effects which contribute to the apoptotic process, including activation of calcineurin. Calcineurin can dephosphorylate BAD switching BAD signalling towards apoptosis. As inhibition of calcineurin activation blocks BAD dephosphorylation and VSMC apoptosis induced by zinc deficiency, it is likely that calcineurin has a key role in this process. In addition to altering the phosphorylation status of BAD, VSMCs from rats on a zinc-deficient diet also have significantly increased expression of BAD. Although the mechanisms of these altered protein levels are not yet known, increased BAD expression has previously been observed to correlate with apoptosis in VSMCs.

As zinc is an important anti-oxidant in cells, it is not surprising that zinc deficiency results in an increase in oxidative stress in VSMCs. We now reveal that this oxidative stress is required for apoptosis induced by plasma from zinc-deficient rats. Zinc deficiency has previously been observed to induce oxidative stress in a number of different cell types. In VSMCs, different sources of oxidative stress can have opposing effects; hydrogen peroxide can induce apoptosis, whereas superoxide formation results in proliferation. It therefore seems likely that hydrogen peroxide production may be involved in the oxidative stress in the current study although this remains to be determined.

**Figure 6** Plasma from zinc-deficient rats induces oxidative stress in VSMCs. (A) VSMCs loaded with DCFDA were incubated with plasma from zinc-deficient rats and fluorescence measured. After 3 h fluorescence was significantly increased compared with VSMCs incubated with plasma from zinc-adequate rats. Pre-incubation with 15 \(\mu\)M NAC significantly decreased the oxidative stress. Typical images are shown, scale bar = 5 \(\mu\)m, \(n = 3\). (B) Apoptosis in VSMCs induced by plasma from zinc-deficient rats was inhibited by pre-incubation with 15 \(\mu\)M NAC, \(n = 3\). * \(P < 0.05\).
The addition of the cysteine pro-drug N-acetyl cysteine, which increases cellular glutathione levels, inhibited the oxidative stress and reduced VSMC apoptosis in the presence of plasma from zinc-deficient rats. 32 Though these results do not directly reveal an association with specific apoptotic pathways, such as dephosphorylation of BAD, a recent study has demonstrated that hydrogen peroxide-induced apoptosis does increase BAD expression in human VSMCs. Further research will be required to uncover this link to apoptosis induced by zinc deficiency.

In the current study, we demonstrate that plasma from marginally zinc-deficient animals is sufficient to induce apoptosis in VSMCs in vitro and produces similar effects to those observed in vivo. The mediators of this apoptotic effect are therefore present in plasma from marginally zinc-deficient rats. The apoptosis induced by this plasma is unlikely to be directly due to decreased zinc in the plasma as exogenous zinc added to zinc-deficient plasma (to equate with levels observed in rats on a zinc adequate diet) did not inhibit the apoptotic effect (results not shown). This reveals that the apoptosis observed in vivo is potentially due to mediators released into the blood as a consequence of marginal zinc deficiency. The source of such mediators could be from several different cell types within the vascular system and are likely to be a complex milieu. The identity of these mediators is still unknown and further intensive research will be required.

In summary, this study has revealed that a zinc-deficient diet induces VSMC apoptosis in large arteries. The mechanism of this effect occurs via BAD. Both an increase in activity of the phosphatase calcineurin (an identified BAD phosphatase) and a decrease in activity of ERK1/2 (a recognized BAD kinase) contribute to this dephosphorylation. BAD expression is also increased. In addition oxidative stress from zinc deficiency contributes to apoptosis in VSMCs. The likely relevance of these findings will be in assessing the changes to cardiovascular pathology during zinc deficiency.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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