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Dietary zinc mediates inflammation and protects against wasting and metabolic derangement caused by sustained cigarette smoke exposure in mice

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Abstract In mouse asthma models, inflammation can be modulated by zinc (Zn). Given that appetite loss, muscle wasting and poor nutrition are features of chronic obstructive pulmonary disease (COPD) and that poor dietary Zn intake is in itself accompanied by growth retardation and appetite loss, we hypothesised that dietary Zn limitation would not only worsen airway inflammation but also exaggerate metabolic effects of cigarette smoke (CS) exposure in mice. Conversely, Zn supplementation would lessen inflammation. Mice were exposed to CS [2× 2RF, 3×/day; 15 min/cigarette] and fed diets containing 2, 20 or 140 mg/kg Zn ad libitum. Airway cells were collected by bronchoalveolar lavage (BAL). Plasma Zn was measured by fluorometric assay. Inflammatory, metabolic and Zn transport markers were measured by real-time RT-PCR. Mice fed low Zn diets had less plasma labile zinc (0–0.18 μM) than mice fed moderate (0.61–0.98 μM) or high (0.77–1.1 μM) Zn diets (SDs 0.1–0.4, n = 8–10). Smoke exposure increased plasma and BAL labile Zn (1.5–2.5 fold, $P < 0.001$), bronchoalveolar macrophages (2.0 fold, $P < 0.0001$) and MT-1 (1.5 fold), MIP-2 (2.3 fold) and MMP-12 (3.5 fold) mRNA. Zn supplementation reduced alveolar macrophage numbers by 62 and 52% in sham and smoke-exposed mice, respectively (Zn effect: $P = 0.011$). Gastrocnemius, soleus and tibialis anterior muscle mass were

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affected by both smoke and dietary Zn in the order of 3–7%. The 50–60% reduction in alveolar macrophages in Zn-supplemented mice supports our evolving hypothesis that Zn is an important anti-inflammatory mediator of airway inflammation. Restoring airway Zn levels through dietary supplementation may lessen the severity of lung inflammation when Zn intake is low.

Keywords Zinc · Zinc transporters · Cigarette smoke · Chronic Obstructive Pulmonary Disease (COPD) · Inflammation · Metabolic wasting

Background

Chronic Obstructive Pulmonary Disease (COPD) is a chronic airway inflammatory disease that is predicted to be the leading cause of death by the year 2020 (Haughney and van der Molen 2005). At present, there are few effective treatments and this is partly due to patients presenting with established disease as well as a lack of understanding concerning the basic pathological mechanisms involved.

Reduced nutrition and significant weight loss are phenomena associated with COPD (Schwartz and Weiss 1990). Chen et al. (2004, 2008) have previously used short-term (4 days) and sub-chronic (4 and 12 weeks) smoke exposure to investigate how smoking itself directly affects central feeding regulation and metabolic regulation. Mice exposed to cigarette smoke show reductions in food intake consistent with reduced appetite (Chen et al. 2004, 2008). In addition to appetite suppression, metabolic effects of cigarette smoke included acceleration of proteolysis and lipolysis, increases in the pro-inflammatory cytokine TNF-α in fat and alterations in neurotransmitters known to regulate appetite also contributed to the reduced weight gain observed (Chen et al. 2008). For example, increased expression of muscle specific E3 ligases that help target myofibrillar proteins for breakdown, such as atrogin 1 and muscle ring finger 1 (MuRF1), have been shown in a number of models of muscle wasting (Hansen et al. 2006). Increases in uncoupling protein 3 (UCP3) and adipose triglyceride lipase, proteins involved in lipolysis, also occur in COPD patients within skeletal muscle (Hansen et al. 2006).

Zinc is a dietary trace metal that has been clinically and physiologically linked to airway inflammatory diseases, such as asthma and COPD. Patients with COPD have lower serum, plasma and hair zinc levels than healthy subjects (Karadag et al. 2004). In addition, there is a negative correlation between plasma zinc and wheezing (Karadag et al. 2004). Zinc supplementation had favorable effects on oxidant–antioxidant balance in patients with COPD (Kirkll et al. 2008). Moreover, using a murine model of allergic inflammation, we have observed a marked loss of labile zinc in the inflamed airway epithelium (Truong-Tran et al. 2002). Mice fed moderately low (14 ppm) zinc diets were found to have significantly higher blood and airway inflammatory cells (i.e. eosinophils up by 1.6 fold), more airway mucus cells (1.9 fold) and greater airway irritability (hyperresponsiveness) (up 1.3-fold), than mice fed normal zinc (50 ppm) diets (Truong-Tran et al. 2002). Conversely, zinc supplementation markedly reduced the number of eosinophils (40–80%) and lymphocytes (55–65%) in mice both acutely and chronically exposed to the allergen (Lang et al. 2007). Depletion of airway epithelial cell zinc in vitro increases oxidative stress (Truong-Tran et al. 2003), toxin or cytokine-induced cell death (Bao and Knoell 2006; Truong-Tran et al. 2003) and reduces the beating of respiratory cilia (Truong-Tran et al. 2003).

Zinc levels are tightly controlled by a number of specialised proteins. Metallothioneins (MTs) are cytosolic proteins that regulate zinc homeostasis, while two families of proteins known collectively as zinc transporters, transport zinc across biological membranes. The zinc and iron-related transport protein (ZIP) family (14 members designated ZIP1-14) are most likely involved in control of cellular zinc uptake and the zinc transporter protein (ZnT) family (10 members, designated ZnT1-10) may be involved in zinc efflux and sub-cellular redistribution (Murgia et al. 2006). By mobilizing zinc in intracellular organelles (Wang et al. 2004), ZIP and ZnT proteins may supply the metal to zinc-dependent proteins and interact with cell-signaling pathways (Suzuki et al. 2005). A number of ZIP and ZnT proteins have now been linked to airway inflammation (Lang et al. 2007), including ZIP8, which is upregulated by the cytokine tumor necrosis factor alpha (TNF-α) in airway epithelial cells (Besecker et al. 2008).

The rationale for the current study comes from our previous observations that zinc may play a key role in mediating the severity of inflammation in allergic

airway inflammation and the knowledge that (1) loss of appetite, muscle wasting and poor nutrition are features of smoke exposure and (2) that poor dietary zinc intake is in itself accompanied by growth retardation, loss of appetite and immunodeficiency. Here we aimed to translate our earlier observations using a murine model of allergic airway inflammation to a sub-chronic murine model of cigarette-smoke induced airway inflammation. Given the rationale outlined above, we hypothesised that dietary zinc limitation would significantly worsen airway inflammation in a mouse model of cigarette-smoke exposure and that zinc supplementation would lessen inflammation. Moreover, we hypothesised that mild nutritional zinc deficiency would exaggerate the metabolic and muscle wasting effects of cigarette smoke previously observed in mice exposed to cigarette smoke. The expression of selected zinc transporter proteins and metabolic markers were also studied.

Methods

Reagents

Unless otherwise mentioned, all reagents were purchased from Sigma-Aldrich Chemicals, St Louis, MO, USA and reagents for RNA manipulation and retro-transcription (RT) were purchased from Qiagen Pty Ltd, Australia. Zinc-free buffers were prepared as previously described (Zalewski et al. 2006).

Animals and zinc modified diets

Male Balb/c mice (age 3–5 weeks, pathogen free) were purchased from the University of Adelaide Laboratory Animal Services, Australia. Mice were weight-matched before dividing into experimental groups and housed together in plastic cages containing wire grids (to prevent animals consuming their own faeces). All cages were cleaned weekly and rinsed with ultra-pure water. Mice were given ultra-pure water ad libitum and fed one of three zinc modified diets (developed with Specialty Feeds, Perth, WA, Australia). Food consumption was measured two to three times per week and the body weights of the mice were measured twice weekly throughout the experimental protocol. Mice were acclimatised to the new diets for 5 days prior to beginning the smoking protocol.

The zinc-modified mouse diets were designed and formulated based on standard AIN93G rodent diet with raw materials specifically tested and selected to minimize zinc content (i.e. we utilised a relatively zinc free whey protein source rather than a casein protein source which was higher in zinc). Total zinc levels in multiple samples of each diet were estimated by the supplier and confirmed in-house by atomic adsorption mass spectrometry. Zinc levels in the diet were chosen for the following reasons: The low zinc diet, by its formulation, had 2.23 mg zinc per kg. "True" zinc deficiency accompanied by severe growth restriction in mice, is reported in the literature for diets containing <1 mg zinc per kg, and we specifically desired a diet that was zinc limiting, rather than zinc deficient. Mice simply become too sick on "true" zinc deficient diets and mild zinc deficiency from diets low, but not totally deficient, in zinc have more biological relevance, particularly amongst humans in the western world where COPD is prevalent. Standard rodent chows are considered to contain $\sim$40 mg zinc per kg on average, although they do vary greatly from batch to batch. However, final in-house measurements of zinc in the zinc medium diet (aimed at 40 mg zinc per kg) indicated that zinc levels were actually less than this, at 22 mg zinc per kg; but this is still ample zinc to meet daily dietary requirements for zinc in a mouse of 10 mg zinc per kg diet (Subcommittee on Laboratory Animal Nutrition 1995). A high zinc diet formulation of 150 mg zinc per kg was chosen in order to zinc supplement the mice.

Murine model of side-stream cigarette smoke exposure

The model was adapted from that published previously by Chen et al. (2004, 2008). Mice were exposed to smoke produced from two research grade cigarettes (2RF, Kentucky Research and Development Centre, University of Kentucky) three times per day (i.e. at 9 a.m., 12 p.m. and 3 p.m.) for 15 min per cigarette. Each cigarette had a burn time of $\sim$7 min. Mice were exposed to air for 5 min between each cigarette. The smoking protocol was preformed 5 days a week for a period of 8 weeks. Cotinine, a breakdown product of nicotine, was measured in plasma collected from mice at 8 weeks by ELISA

(Bioquant, San Diego, USA) to confirm smoke exposure. All experiments were performed under the University of Adelaide and Central and North Western Adelaide Health Service (CNAHS) Animal Ethics Committees.

Tissue collection

Mice were anaesthetized by an intraperitoneal (i.p.) overdose of pentobarbitone sodium (50 mg/kg). Body weight and naso-anal length was recorded. Whole blood from the tail vein was collected for peripheral blood smears and glucose measurement (Accu-Chek® Advantage, Roche, Basel, Switzerland). Blood was collected in heparin-primed needles and collection tubes (heparin sodium, 30 IU per ~500 µl blood) from the hepatic portal vein. The trachea was cannulated and the lungs lavaged with 2 × 400 µl, followed by 1 × 300 µl ice-cold calcium and magnesium free Dulbecco's Phosphate Buffered Saline (DPBS) to collect the bronchoalveolar lavage fluid (BAL). The average volume of BAL recovered was 994 µl (SD 35, n = 60) and was not significantly affected by smoke exposure or dietary zinc. ~30 mg of lung and liver tissue was dissected and placed directly in RNA later (Qiagen) as per manufacturer's protocol. Remaining liver and lung tissue were snap-frozen in liquid nitrogen. In some mice, one lobe of the lung was collected for formalin fixation. All samples were stored at −80°C prior to analysis. Body fat (interscapular brown adipose tissue (iBAT), right retroperitoneal white adipose tissue (RpWAT) and testicular WAT (TestWAT) were dissected and weighed. Left and right gastrocnemius, tibialis anterior and soleus muscles were dissected and weighed. Tibia length was also measured and recorded.

Measurement of labile zinc in plasma and BAL and total zinc in liver

Plasma was isolated from whole blood containing heparin by centrifugation at 2,000 rpm, 10 min and stored at −80°C until ready for analysis. BAL was centrifuged to remove cells and the cell-free supernatant collected. Labile zinc was measured in plasma and cell-free BAL as previously described (Zalewski et al. 2006). Total zinc in the liver was determined by flame atomic absorption spectroscopy (303 atomic absorption spectrophotometer, Perkin-Elmer), as previously described (Truong-Tran et al. 2002).

Measurement of glutathione in plasma

Total Glutathione was measured, as a marker of oxidative stress, in plasma samples using a Total Glutathione Quantification Kit (Dojindo Laboratories, Kumamoto, Japan).

Measurement of BAL inflammatory cells

BAL was centrifuged to collect the cell pellet and total viable cell counts were conducted using the viable dye, trypan blue. Cells were then cytospun onto slides and fixed in 100% ethanol prior to staining with May Grunwald–Giemsa Stain (IMVS, The Queen Elizabeth Hospital). Differential cell counts revealed that >98% of the inflammatory cells present were alveolar macrophages.

Measurement of TNF-α, IL-6 and MCP-1 in BAL

After removing cells for cytospins, BAL cell-free supernatants were collected and stored at −80°C. Tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and macrophage chemotactic protein-2 (MCP-2) levels were assayed using Ready-Set-Go ELISA kits (Invitrogen, Victoria, Australia). BAL Protein levels were measured using a Biorad DC Protein Assay Kit.

Measurement of gene expression in lung and liver

RNA was isolated using Rneasy minikits (Qiagen, Victoria, Australia), according to the manufacturer's protocol. DnaseI was added to remove any genomic DNA. Concentration and purity of RNA was estimated on a nanodrop spectrophotometer (ND-1000, Biolab, Australia) and by gel electrophoresis. RNA was reverse transcribed using Omniscript RT kits and oligodTs in the presence of Rnase inhibitor (Ambion, Texas, USA), as per the manufacturer's instructions. Both RNA negative and RT-negative RT reactions were included with each RT batch as negative controls. Gene expression of ZIP1, ZIP6, ZIP14, ZnT4, MT-1, MT-2, IL-6, MIP-2 and MMP-12 was determined by real-time RT-PCR using Taqman gene expression assays (Applied Biosystems, Australia).

Relative expression was determined by comparison with the housekeeping gene HPRT. A preliminary screening of housekeeping genes (including GAPDH, β2-microglobulin and 18sRNA) found HPRT to be the most consistent for use with the treatments examined.

ZIP1 western blotting

We used a sheep anti-human ZIP1 polyclonal antibody that cross-reacts with mouse ZIP1 (Gift: Dr Leigh Ackland, Deakin University). Snap-frozen lung tissue was homogenized in ice-cold RIPA buffer (50 mM Tris pH 7.6, 1% v/v NP40, 0.5% w/v sodium deoxycholate, 0.1% w/v SDS, 150 mM sodium chloride) containing EDTA-free protease inhibitor cocktail (Thermo Scientific) and 1 mM PMSF by using a Potter–Elvehjem homogenizer. The 10 μg of tissue lysate was resolved using NuPAGE Bis–Tris 4–12% gels (Invitrogen) and transferred to Hybond-ECL nitrocellulose membrane (Amersham Bioscience). Membranes were probed with ZIP1 antibody and Horseradish peroxidase (HRP) conjugated anti-sheep IgG secondary antibody (R&D Systems). Membranes were reprobed with mouse anti-â-actin clone AC-15 (SIGMA) and HRP conjugated anti-mouse IgG (R&D Systems). Blots were visualised by chemiluminescence detection with ECL Plus (GE Healthcare) using a FujiFilm LAS4000 Imager. MultiGauge software was used to quantitate the chemiluminescent emission from protein bands.

Measurement of metabolic markers in fat and muscle

RNA was isolated from the gastrocnemius skeletal muscle and RpWAT using RNeasy minikits (Qiagen, MD, USA) according to the manufacturer's instructions. Concentration and purity of RNA was estimated on a nanodrop spectrophotometer (ND-1000, Biolab, Australia). RNA (1 μg) was used as a template to generate first-strand cDNA synthesis using SuperScript III (Invitrogen, CA, USA). Gene expression of atrogin-1, MuRF-1, IGF-1, UCP-1, UCP-3, ATGL, MCP-1, IL-6 and TNF-α was determined by real-time RT-PCR using TaqMan gene expression assays (Applied Biosystem, CA, USA) (Bozinovski et al. 2004). Relative expression was determined by comparison with the sham moderate zinc group after standardising to the housekeeping gene 18S rRNA. Relative expression in RpWAT was expressed as a fold difference to iBAT from the sham moderate zinc group.

Histopathology and ZIP1 localisation in formalin-fixed lung

Lungs fixed in neutral buffered formalin were processed by the histopathology laboratory (IMVS, The Queen Elizabeth Hospital) and lung sections stained with Haematoxylin and Eosin (H&E), martius scarlet blue (MSB) for collagen detection and periodic acid shift (PAS) for detection of mucus. ZIP1 was visualised by immunofluorescence using a sheep anti-human ZIP1 antibody that also cross-reacts with mouse ZIP1 (Gift: Leigh Ackland, Deakin University) and donkey-anti-sheep IgG Alexa Flour 488 (Invitrogen, Australia). At the time of completing this study we did not have a working antibody raised against the other zinc transporters.

Statistical analyses

Data are expressed as mean and standard deviation in the following format: mean (SD). Data was analysed by 2-way ANOVAs and Dunnett's, Bonferroni or Student's *t* tests where appropriate using SPSS^{TM} statistical software, version 15 or GraphPad Prism, version 4.

Results

Confirmation of experimental protocol

To our knowledge, this is the first time that zinc-modified diet formulations utilising a whey protein source, rather than casein or egg protein source, have been described. The whey protein source contained only 1 mg zinc per kg compared to over 40 mg zinc per kg in the other casein protein sources tested, which enabled the production of a base zinc diet containing only 2.23 (SD 0.6) mg zinc per kg to which we added zinc sulphate to achieve the moderate [22.8 (SD 2.3) mg/kg] and high [142.2 (SD 2.3) mg/kg] zinc diet formulations (n = 4–5 samples per diet). Sham mice fed our low zinc diet showed only minor reductions in body weight (<1 g) and nasoanal

length (<0.6 mm) compared to mice fed moderate or high zinc diets confirming that our diets contained enough zinc (defined as >1 mg zinc per kg) to prevent severe zinc deficiency and its associated symptoms (diarrhoea, severe weight loss and growth retardation) whilst still significantly reducing labile zinc in the plasma. Plasma from mice exposed to cigarette smoke tested positive for cotinine (26.2 ng/ml cotinine (SD 7.34, n = 23), while all sham mice tested negative for cotinine, confirming the smoke exposure protocol in the mice. Total glutathione was measured in plasma as a marker of oxidative stress. Cigarette smoke exposure did not significantly affect plasma glutathione levels as measured 24 h after the last smoke exposure, however mice fed both low zinc and high zinc diets had lower total glutathione in plasma than mice fed moderate zinc diets (Table 1). Blood glucose levels were not affected by either diet or smoke exposure (Table 1).

Plasma and BAL labile zinc and total liver zinc

Levels of labile zinc in mouse plasma were sensitive to dietary zinc (Table 1). Mice fed low zinc diets had significantly lower levels of labile zinc (Sham: below detection limits.; Smoke: 0.18 μM zinc (SD 0.3, n = 10)) compared with mice fed both moderate (Sham: 0.61 μM zinc (SD 0.2, n = 9); Smoke: 0.98 μM zinc (SD 0.4, n = 10)) and high zinc (Sham: 0.77 μM zinc (SD 0.3, n = 8); Smoke: 1.1 μM zinc (SD 0.1, n = 10)) diets. Using Dunnett's post-hoc tests, levels of labile zinc in the plasma did not change between mice fed moderate and high zinc diets and this is most likely due to saturation of zinc uptake proteins in the intestine. Labile zinc in mouse plasma increased by ~1.5 fold (2-way ANOVA, $P = 0.001$) with smoke exposure compared with Sham exposure (e.g.: Moderate zinc: 0.6 cf 1.0 μM; High zinc: 0.8 cf 1.1 μM zinc) independent of diet (Table 1). Labile zinc levels in the airway lumen were unaffected by alterations in dietary zinc, but significantly increased by over 2.6 fold with smoke exposure (Table 1). Since labile zinc comprises only 10–20% of total cellular zinc (80–90% of cellular zinc is in a largely fixed pool that turns over very slowly), measurements of total zinc in the liver did not change with dietary zinc intake ($P = 0.424$). However, liver zinc did increase slightly (2 way ANOVA: $P = 0.02$) with smoke exposure (Table 1).

Mouse growth rates and food consumption

Mice fed low zinc diets initially experienced a slower growth rate than mice fed both moderate and high zinc diets (Supplementary Fig. 1). Body weight, tibia length and naso-anal length measured, at the end of the 8 week experimental protocol, were all significantly affected by dietary zinc and exposure to cigarette smoke (Table 1). Average food consumption over the 8 weeks was also lower in mice fed low zinc diets (data not shown).

BAL inflammatory cells

Differential cell counts using May Grunwald–Giemsa stain showed that over 99.5% of the cells collected in BAL were alveolar macrophages in both sham and smoke-exposed mice. BAL cell viability, as determined by exclusion of the vital dye trypan blue, was 91% (SD 4, n = 60) in all cases and was not affected by smoke exposure or by zinc-modified diet. Smoke exposure increased the total number of alveolar macrophages by ~2-fold compared to Sham exposed mice fed the same zinc-modified diet (Fig. 1). Dietary zinc also significantly affected the number of alveolar macrophages collected in BAL fluid. When compared to mice fed zinc moderate diets, mice fed high zinc diets had 62 and 52% lower numbers of alveolar macrophages in both sham and smoke-exposed mice, respectively (Fig. 1).

BAL protein and cytokine content

Total protein recovered in BAL, as measured by Biorad DC protein assay, was ~30% lower in BAL collected from smoke-exposed mice compared to sham exposed mice (Table 2). The protein content of BAL fluid was shown to be predominantly albumin by electrophoresis protein profiling (IMVS Biochemistry Labs, TQEH). When expressed relative to total protein in BAL fluid, levels of TNF-α, IL-6 and MCP-1 were not affected by smoke exposure or dietary zinc.

Gene expression in mouse lung

Cigarette smoke significantly increased the expression of the zinc transporter ZIP1 in mouse lung tissue, but had no effect on ZIP6, ZIP14 or ZnT4 zinc

Table 1 Descriptive data

	Sham			Smoke			Statistics (2-way ANOVA)		
	Low Zn	Mod Zn	High Zn	Low Zn	Mod Zn	High Zn	Diet	Smoke	N
Whole blood glucose (μM)	6.6 (1.2)	7.3 (0.8)	7.1 (0.8)	6.5 (1.7)	6.8 (2.2)	7.5 (2.0)	n.s.	n.s.	18–20
Plasma glutathione (μM)	5.1 (1.3)	6.6 (3.4)	5.0 (2.3)	3.6 (1.0)	8.1 (2.8)	4.4 (2.0)	$P = 0.007$*	n.s.	5–6
BAL Zn (nM)	17.4 (14.9)	17.6 (16.3)	19.9 (11.1)	57.9 (29.2)	45.84 (18.2)	57.32 (35.5)	n.s.	$P < 0.001$*	6–9
Plasma Zn (μM)	N.D.	0.61 (0.2)	0.77 (0.3)	0.18 (0.3)	0.98 (0.4)	1.1 (0.1)	$P < 0.0001$*	$P < 0.001$*	8–10
Liver Zn (μM g dry weight^{-1})	1.40 (0.1)	1.48 (0.2)	1.47 (0.2)	1.52 (0.1)	1.53 (0.1)	1.56 (0.1)	n.s.	$P = 0.02$*	8–10
Growth parameters									
Body weight (g)	22.8 (1.6)	23.9 (1.3)	23.6 (1.2)	20.9 (1.6)	21.9 (1.2)	21.7 (1.2)	$P = 0.003$*	$P < 0.0001$*	18–20
Tibia length (cm)	1.89 (0.05)	1.88 (0.08)	1.94 (0.06)	1.85 (0.06)	1.82 (0.05)	1.86 (0.05)	$P = 0.002$*	$P < 0.0001$*	18–20
Naso-anal length (cm)	9.62 (0.3)	9.77 (0.2)	9.83 (0.2)	9.56 (0.2)	9.59 (0.2)	9.68 (0.2)	$P = 0.025$*	$P = 0.005$*	18–20
Muscles									
Gastrocnemius (mg)	114.0 (7.5)	120.0 (6.6)	119.7 (7.1)	110.2 (10.3)	115.6 (8.2)	115.0 (6.1)	0.0025*	0.0036*	18–20
Gastrocnemius (% body wt)	0.50 (0.02)	0.50 (0.02)	0.51 (0.02)	0.53 (0.02)	0.53 (0.03)	0.53 (0.02)	n.s.	$P < 0.0001$*	18–20
Soleus (mg)	7.0 (0.7)	7.4 (0.6)	7.2 (0.7)	6.55 (0.8)	6.91 (0.58)	6.7 (0.62)	0.0003*	0.0004*	18–20
Soleus (% body wgt)	0.03 (0.002)	0.03 (0.002)	0.03 (0.003)	0.031 (0.003)	0.032 (0.002)	0.031 (0.003)	n.s.	n.s.	18–20
Tibialis anterior (mg)	47.7 (3.91)	50.4 (3.61)	49.0 (3.4)	45.04 (4.4)	47.17 (3.11)	46.85 (2.95)	0.0131*	0.0001*	18–20
Tibialis anterior (% body wgt)	0.21 (0.01)	0.21 (0.01)	0.207 (0.01)	0.216 (0.01)	0.216 (0.011)	0.215 (0.007)	n.s.	0.006*	18–20
Fats									
iBAT (mg)	65.3 (8.8)	72.2 (7.2)	73.4 (10.5)	57.9 (9.03)	60.7 (8.5)	61.4 (8.4)	$P = 0.0103$	$P < 0.0001$*	18–20
iBAT (% body wgt)	0.29 (0.03)	0.30 (0.03)	0.31 (0.04)	0.28 (0.04)	0.28 (0.04)	0.28 (0.03)	n.s.	$P < 0.0001$*	18–20
RpWAT (mg)	55.8 (15.4)	67.4 (14.6)	64.0 (18.0)	31.4 (9.61)	44.0 (13.1)	45.1 (12.0)	$P = 0.0003$*	$P < 0.0001$*	18–20
RpWATp (% body wgt)	0.24 (0.06)	0.28 (0.06)	0.271 (0.07)	0.15 (0.04)	0.2 (0.06)	0.21 (0.05)	$P = 0.0005$*	$P < 0.0001$*	18–20
TestWAT (mg)	430.1 (82.2)	505.2 (107.7)	461.5 (103.2)	283.2 (61.4)	335.0 (75.8)	352.6 (64.1)	$P = 0.003$*	$P < 0.0001$*	18–20
TestWAT (% body wgt)	1.87 (0.28)	2.11 (0.39)	1.95 (0.39)	1.35 (0.25)	1.53 (0.31)	1.61 (0.24)	$P = 0.01$*	$P < 0.0001$*	18–20

N.D. below assay limit, i.e. not detected; *n.s.* not significant (i.e. $P > 0.05$); *iBAT* interscapular brown adipose tissue; *TestWAT* testicular white adipose tissue; *RpWAT* retroperitoneal white adipose tissue; *wgt* weight; *Zn* zinc

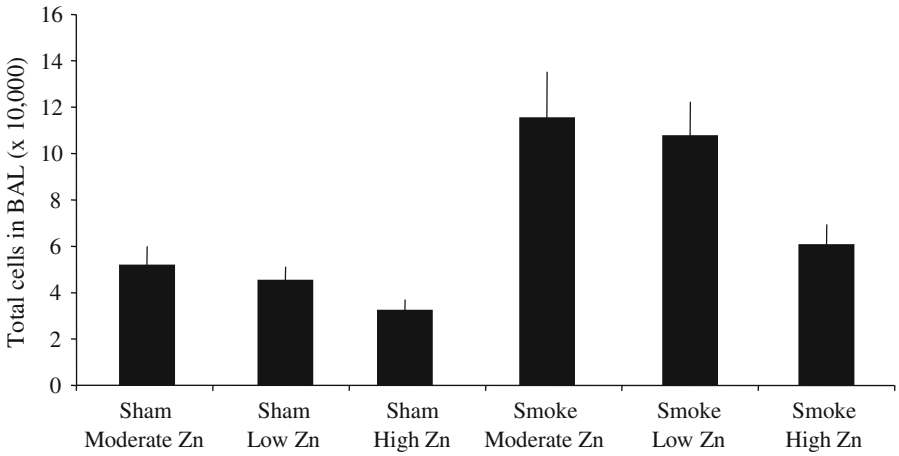


Fig. 1 Total cells in BAL collected from mice lungs. Greater than 99.5% of the total cells detected in BAL from both sham and smoke-exposed mice were alveolar macrophages. Smoke exposure significantly increased by ~2-fold the number of inflammatory cells in BAL (2-way ANOVA, $P < 0.0001$). The number of inflammatory cells in BAL was also significantly affected by dietary zinc (2-way ANOVA, $P = 0.011$). There was no evidence of interaction between smoke and diet effects ($P = 0.287$). There was a significant decrease in BAL inflammatory cells in mice fed high zinc diets compared to mice fed moderate zinc diets (Dunnett's t test, $P = 0.005$). Differences between moderate zinc diets and low zinc diets were not statistically significant (Dunnett's t test, $P = 0.608$)

Table 2 Protein and cytokine content in BAL

	Low Zn	Moderate Zn	High Zn
Total BAL protein (g/ml)			
Sham	199.8 (SD 26.4)	226.1 (51.7)	302.3 (13.9)
Smoke*	139.8 (SD 46)	151.5 (38.8)	153.7 (32.4)
TNF-α (pg/mg protein)			
Sham	175.9 (SD 49.3)	162.0 (SD 59.3)	165.7 (60.6)
Smoke	163.7 (SD 79.3)	150.0 (SD 56.0)	146.3 (SD 99.2)
IL-6 (pg/mg protein)			
Sham	235.9 (SD 65.6)	190.4 (SD 48.3)	224.9 (SD 115.8)
Smoke	219.7 (SD 166.7)	166.4 (SD 41.2)	197.4 (SD 155.7)
MCP-1 (pg/mg protein)			
Sham	1537.4 (SD 970.4)	1657.7 (SD 2100.3)	2172.1 (SD 1471.0)
Smoke	968.8 (624.0)	1237.5 (725.2)	654.2 (314.2)

Data presented as mean (SD), N = 8–10. Total protein measured using Biorad DC protein assay kit. TNF-α, IL-6 and MCP-1 measured by ELISA (Invitrogen)

* Smoke exposure significantly decreased total BAL protein (2-way ANOVA, $P < 0.0001$). Neither TNF-α, IL-6, nor MCP-1 levels were affected by smoke exposure or dietary zinc (2-way ANOVA, $P > 0.05$)

transporter gene expression (Fig. 2). MT-I, MIP-2 and MMP-12 gene expression also increased significantly with smoke exposure (Fig. 3). Dietary zinc affected gene expression of the zinc transporters ZIP6 and ZIP14 in lung tissue (Fig. 2). Regardless of smoke exposure, ZIP6 gene expression increased, albeit slightly, in mice fed low zinc diets and decreased in mice fed high zinc diets, when compared with mice fed moderate zinc. ZIP14 gene expression increased significantly in mice fed both low zinc and high zinc diets compared with mice fed moderate zinc diets (Fig. 2). However, dietary zinc had no effect on ZIP1, MT-I, MIP-2, MMP-12, IL-5 nor MT-II gene expression (Figs. 2, 3).

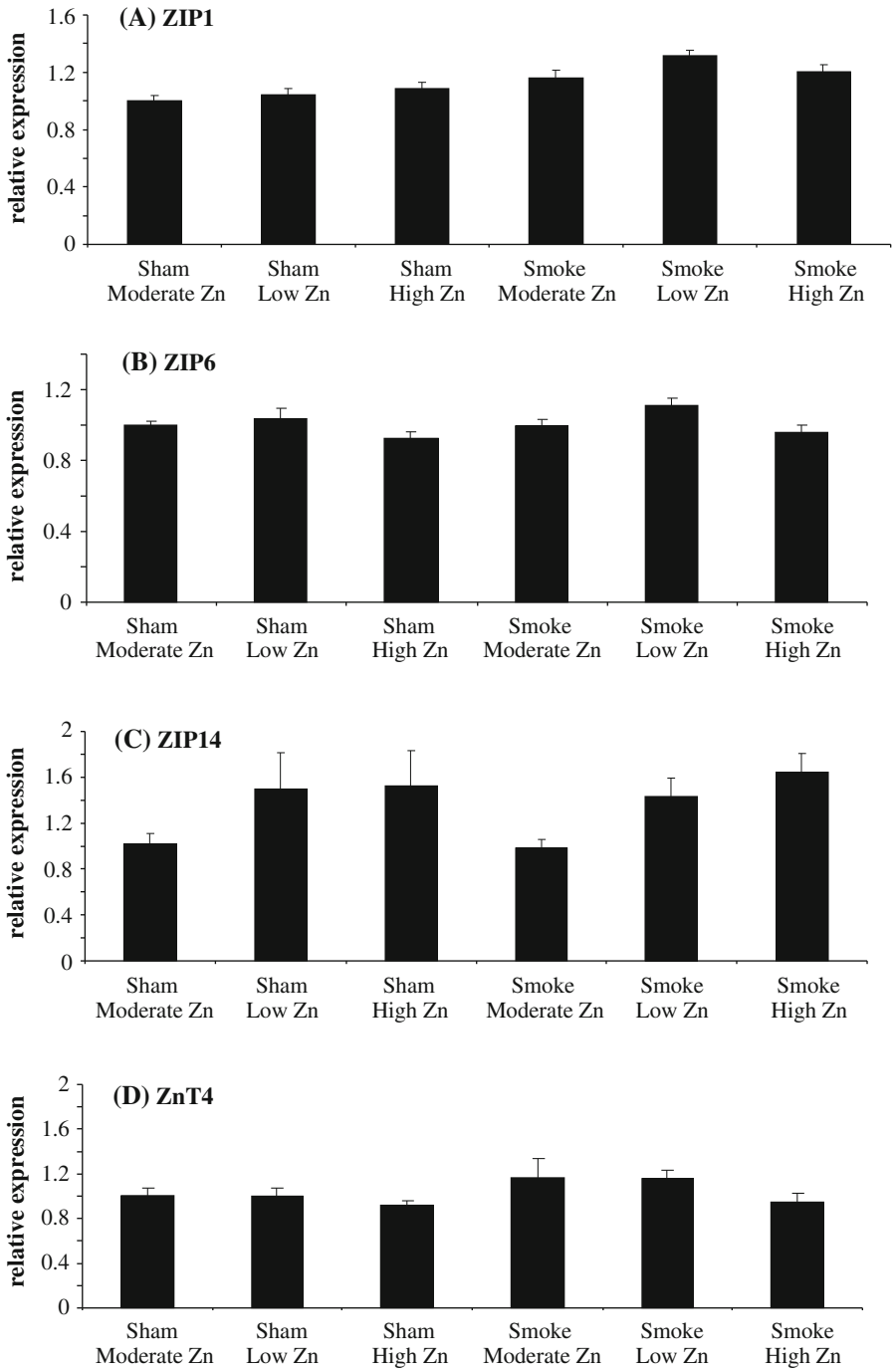


Fig. 2 The effect of smoke exposure and dietary zinc on the gene expression of selected zinc transporters in mouse lung. Gene expression of **a** ZIP1, **b** ZIP6, **c** ZIP14 and **d** ZnT4 in mouse lung. Data presented as the expression relative to Sham Moderate zinc (after first standardising to the housekeeping gene, HPRT). N = 5–6. Data was analysed using 2-way ANOVAs and Dunnett's 2-sided *t* test where appropriate. In mouse lung tissue, smoke exposure increased ZIP1 ($P < 0.0001$) gene expression but not ZIP14, ZIP6 or ZnT4 gene expression. Dietary zinc affected both ZIP6 ($P = 0.012$) and ZIP14 ($P = 0.014$) gene expression, but not ZIP1 or ZnT4 gene expression. For ZIP14, there was a significant increase in ZIP14 gene expression in mice fed both low ($P = 0.050$) and high ($P = 0.011$) zinc diets compared to moderate zinc diets

Gene expression in mouse liver

Mouse livers expressed the zinc transporters ZIP1, ZIP6, ZIP14 and ZnT4. Relative to the housekeeping gene HPRT, ZIP14 had the lowest transcript abundance (−0.65 (0.89), n = 35) and ZIP6 had the highest transcript abundance (6.41 (0.22), n = 23). Transcript abundance of ZIP1 and ZnT4 was 5.94 (0.22) (n = 36)

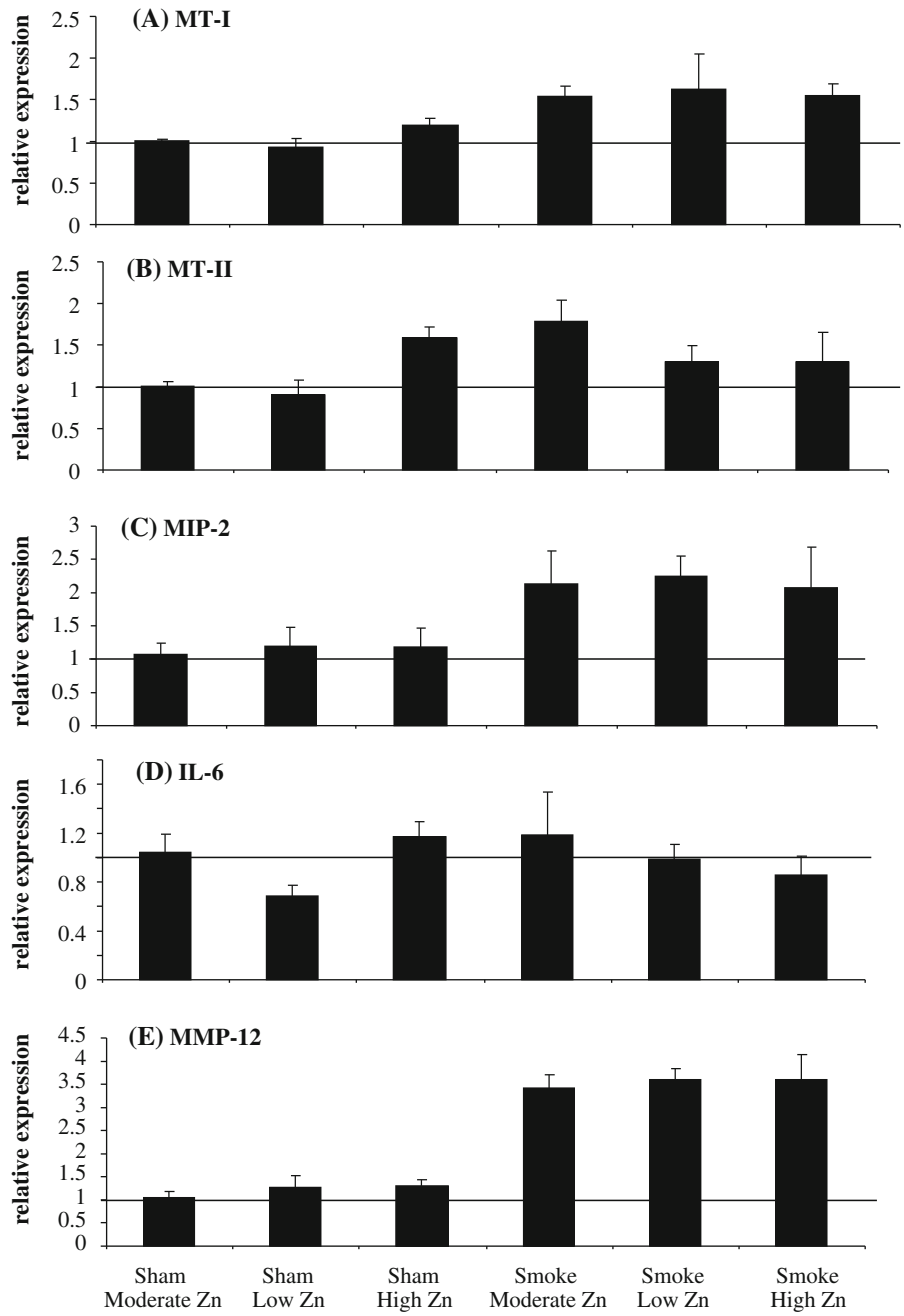


Fig. 3 The effect of smoke and zinc on the gene expression of MT-I, MT-II, MIP-2, IL-6 and MMP-12 in mouse lung. Data are presented as the expression relative to Sham Moderate zinc (after first standardising to the housekeeping gene, HPRT). N = 5–6. Data was analysed using 2-way ANOVA and Dunnett's 2-sided *t* tests where appropriate. Smoke exposure significantly increased MT-I ($P = 0.003$), MIP-2 ($P = 0.005$) and MMP-12 ($P \leq 0.0001$)) gene expression, but not MT-II nor IL-6 expression. Dietary zinc had no effect on MT-I, MT-II, MIP-2, IL-6 and MMP-12 gene expression in lung tissue

and 4.86 (0.27), n = 24, respectively. The gene expression of zinc transporters, ZIP1, ZIP6, ZIP14 and ZnT4 in mouse livers were not affected by cigarette smoke or dietary zinc levels (data not shown). MT-I and MT-II gene expression were affected by both cigarette smoke and dietary zinc (Fig. 4).

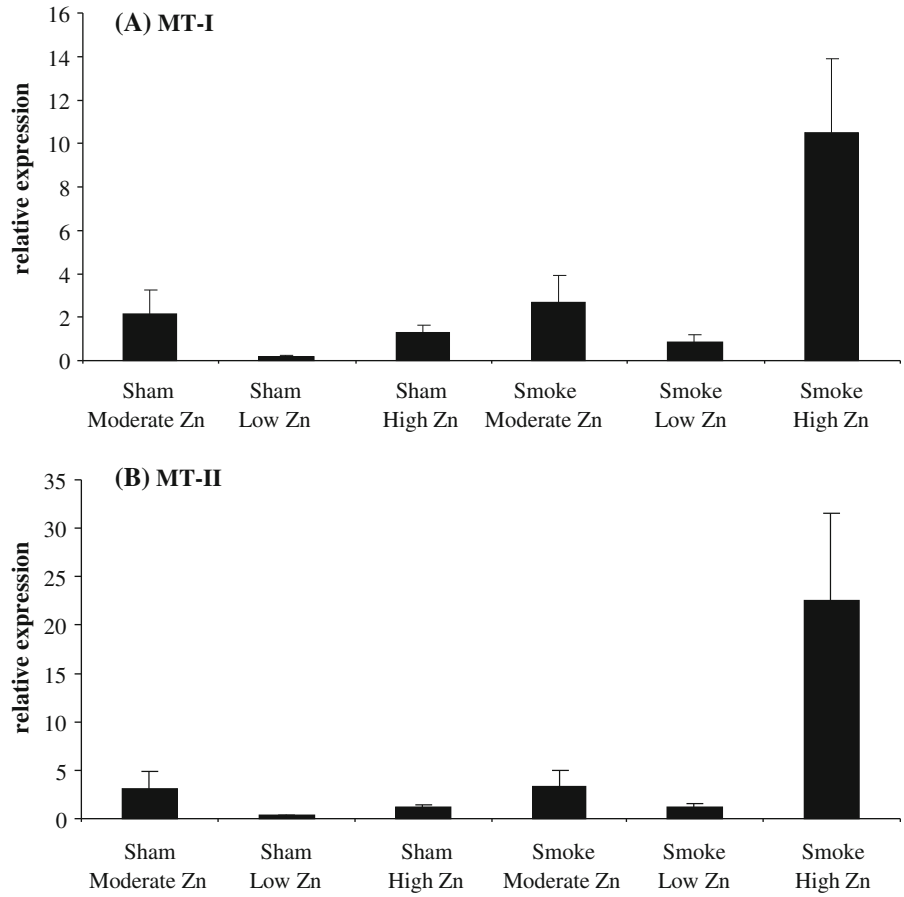


Fig. 4 The effect of smoke and zinc on metallothionein I (MT-I) and metallothionein-II (MT-II) gene expression in mouse livers. Data are expressed as gene expression relative to Sham Moderate Zn (after standardising to the housekeeping gene, HPRT). N = 6. Smoke exposure significantly increased MT-1 ($P = 0.011$) and MT-II ($P = 0.034$) gene expression. Dietary Zn also had a significant effect on MT-1 ($P = 0.031$) and MT-II ($P = 0.027$) gene expression

Localisation and western blotting of ZIP1 protein in mouse lung

ZIP1 is localised to the apical surface of airway epithelial cells in mouse airways (Fig. 5). The protein expression of ZIP1, as detected by western blotting, was not affected by smoke or dietary zinc (Fig. 6).

Skeletal muscle mass

Gastrocnemius, soleus and tibialis anterior (Table 2) skeletal muscle mass were significantly decreased by smoke. Mice fed low zinc diets had significantly less muscle mass when compared with mice fed moderate zinc diets. There was no difference between muscle weights of mice fed moderate and high zinc diets. Mice exposed to smoke had significantly lower skeletal muscle mass than sham exposed mice, regardless of zinc diet.

Skeletal muscle gene expression

Cigarette smoke and dietary zinc had no effect on the gene expression of atrogin-1, MuRF-1, IGF-1, ATGL or UCP-3 in gastrocnemius skeletal muscle (data not shown). Smoke exposure significantly increased UCP-3 gene expression ($P < 0.0005$) and post hoc significance was achieved for the high zinc group ($P < 0.001$), however a significant interaction was observed ($P < 0.0295$).

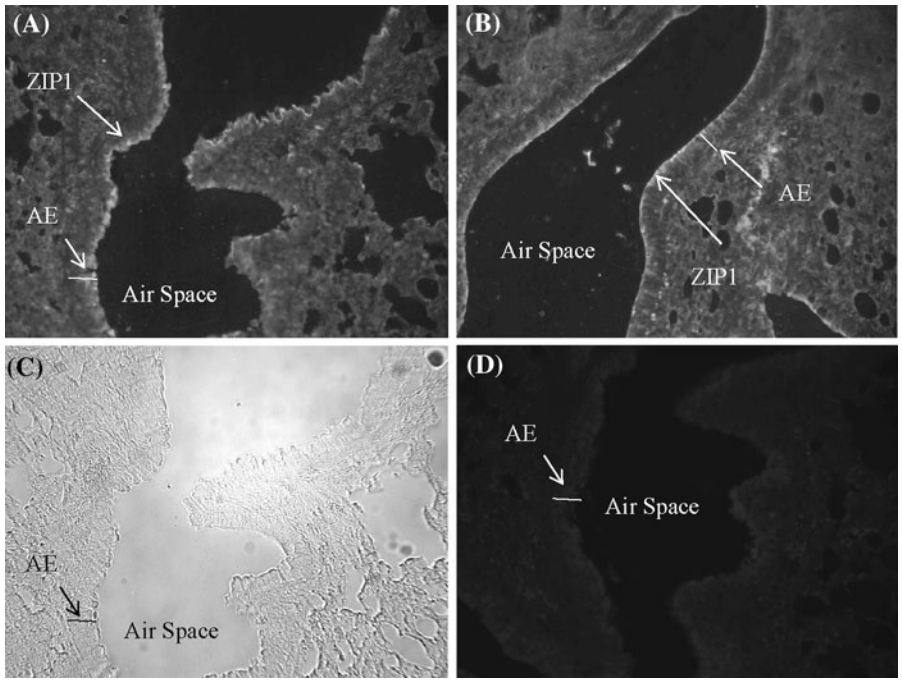


Fig. 5 Localisation of ZIP1 in mouse lung tissue. **a** and **b** Image showing the localisation of ZIP1 to the apical surface of the airway epithelium in the same region as we have previously reported for labile Zn (Truong-Tran et al. 2002). **c** Light image of tissue section shown in A. **d** Fluorescent image showing staining with secondary antibody alone. All fluorescence images were taken with a ×20 fluorescence lens (FITC wavelength) with same camera settings of intensity, gamma and gain. *AE* airway epithelium. The width of the airway epithelium is shown by the *white line* and *arrow* AE

Body fat

The amount of interscapular brown adipose tissue (iBAT), retroperitoneal white adipose tissue (RpWAT) and testicular white adipose tissue from around both testes (TestWAT) was significantly affected by both smoke and dietary zinc (Table 1). Mice fed low zinc diets had significantly less iBAT, RpWAT and TestWAT compared to mice fed moderate zinc diets. There was no difference in fat mass between mice fed moderate zinc and high zinc diets. Smoke exposure significantly decreased RpWAT, iBAT and TestWAT masses, regardless of zinc diet. When fat mass is expressed as a percentage of total body weight, the effect of dietary zinc remains significant in RpWAT and TestWAT, but not iBAT. However, the effect of smoke exposure remains significant in all three fats examined (iBAT, RpWAT and TestWAT).

Fat gene expression

In RpWAT, cigarette smoke significantly increased gene expression of ATGL ($P < 0.0029$), but significantly reduced MCP-1 gene expression ($P < 0.0013$) (Fig. 7). There was no effect of cigarette smoke on IL-6, TNF-α, UCP-1 and UCP-3 gene expression in RpWAT. Cigarette smoke significantly increased the gene expression of IL-6 in iBAT ($P < 0.0049$) (Fig. 7), but had no effect on the other genes examined. Dietary zinc had no effect on the gene expression of MCP-1, IL-6, TNF-α, UCP-3 and ATGL in RpWAT and iBAT. Dietary zinc significantly increased the gene expression of UCP-1 ($P < 0.048$) in iBAT, but had no effect on UCP-1 expression in RpWAT.

Histopathology of the lung

As previously observed in a mouse model of allergic airway inflammation, zinc supplementation had no effect on any of the histopathological features (data not shown). Airway thickness and collagen deposition was also measured but were not affected by smoke exposure (data not shown). Mucous hyperplasia, as determined by increased PAS-staining of mucopolysaccharide-containing goblet cells in airway epithelium, was not a

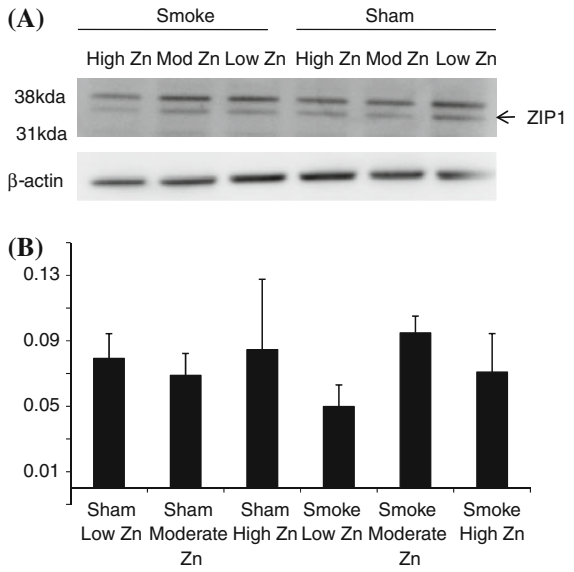


Fig. 6 Western blotting of ZIP1 in mouse lung tissue (**a**). Lung tissue lysates were separated by SDS-PAGE, and ZIP1 was detected by Western blot analysis using a sheep anti-human ZIP1 antibody. *Top*: representative Western blot of ZIP1 protein in mouse lung tissue for each of the treatments tested. *Bottom*: β-actin loading control. **b**. ZIP1 protein normalised to β-actin loading control. ZIP1 expression was not affected by dietary zinc or smoke exposure (2-way ANOVA, $P \gg 0.05$; N = 3)

feature of the airways in smoke-exposed mice (data not shown).

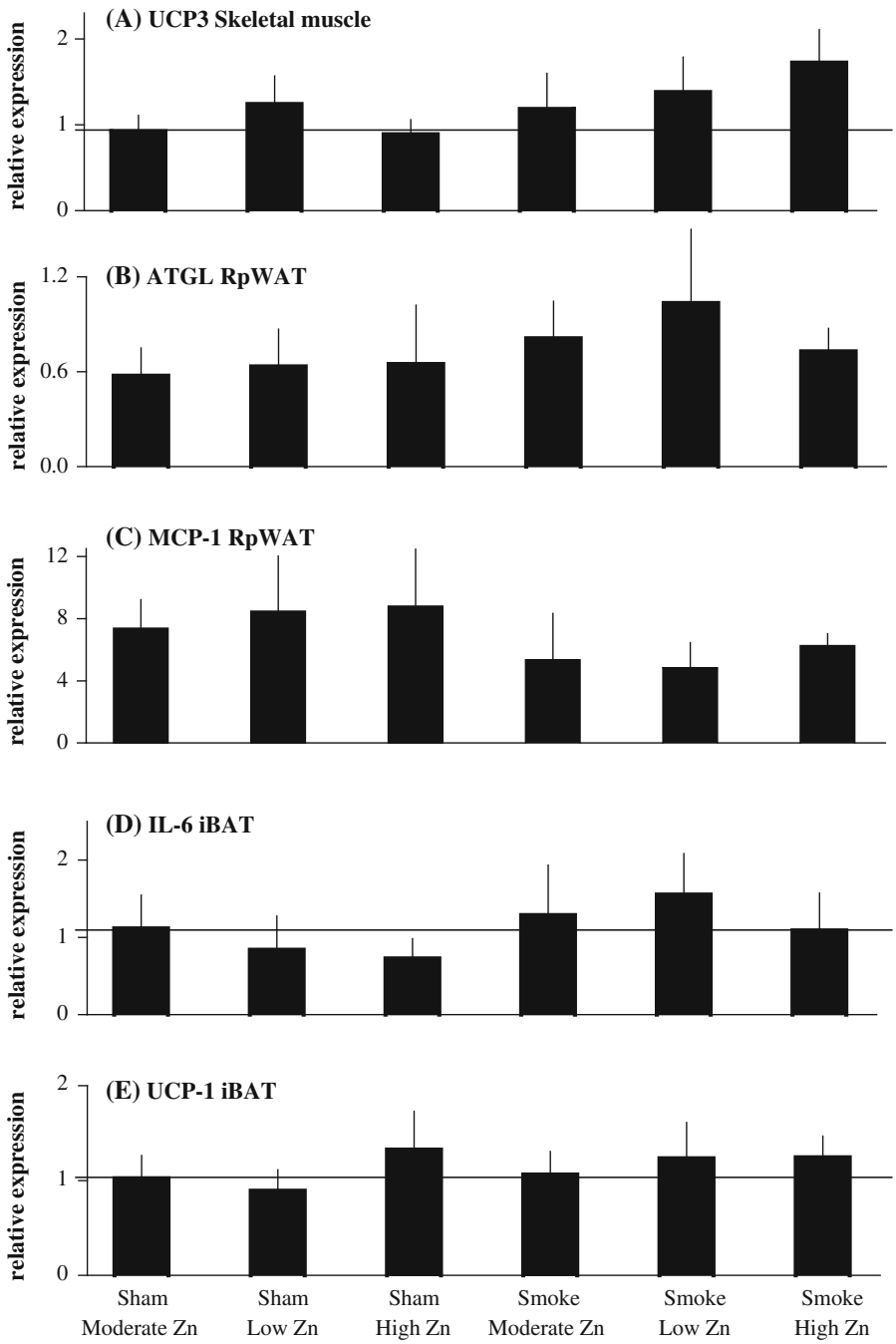
Discussion

Our most notable finding is that alveolar macrophage numbers in the airways of mice were significantly affected by modifications in dietary zinc. Regardless of whether mice were exposed to cigarette smoke or not, we observed a 50–60% reduction in the number of BAL alveolar macrophages in mice fed the high zinc (142 mg/kg) diet compared to the moderate zinc (22 mg/kg) diet. Since plasma labile zinc levels increased with increasing dietary zinc such that when plasma zinc is highest, macrophage numbers are reduced, this finding supports our evolving hypothesis that zinc is an important mediator of airway function.

Relationships between dietary, plasma, liver, lung and airway luminal zinc levels in the body are complex and may depend on a variety of factors, including the presence of transport proteins such as albumin and metallothionein as well as the cell and tissue specific distributions, abundance and activity of up to 14 ZIP and 9 ZnT zinc transporter proteins. Transient fluxes between plasma and liver zinc stores can also occur during inflammation or infection (Liuzzi et al. 2005). Here, labile zinc levels in the airway lumen were unaffected by alterations in dietary zinc and this was not surprising given that labile zinc exerts many of its anti-inflammatory and zinc signalling effects within cells and not in extracellular fluids. Our cell-free BAL samples contain only minimal amounts of labile zinc (i.e. nM concentrations) compared to that in plasma (μM concentrations). In the liver, total zinc levels were also not affected by alterations in dietary zinc. However, since it is the labile zinc pool that is most susceptible to dietary zinc deficiency (Tapiero and Tew 2003) and this labile pool comprises only 10% of the total zinc pool detected by atomic adsorption mass spectrometry, we are less likely to be able to detect changes in total liver zinc levels, particularly given that we did not induce severe or prolonged zinc deficiency in our mice for ethical reasons.

Zinc levels in the plasma (labile) and liver (total) were up approximately 1.5 fold in smoke exposed mice compared to sham mice which correlates with increases in MT-I and MT-II mRNA levels in both the lung and liver. Increases in MT-I and MT-II gene expression were further amplified by 10 or 25 fold, respectively, in the liver in smoke exposed mice fed the high zinc diet suggesting that a diet high in zinc may assist the liver in being able to respond rapidly to inflammatory signals or influxes of harmful agents into the blood stream that occur upon inhalation of cigarette smoke. Metallothionein is known to be important in mediating metal ion detoxification as well as zinc homeostasis (Wesselkamper et al. 2006).

In the airway lumen labile zinc levels increased by well over 2.6 fold from ~17 to 45 nM with smoke exposure. This increase could be due, in part to the presence of the heavy metal cadmium in cigarette smoke. Cadmium can be detected by the fluorophore, zinquin, which we used to measure labile zinc (Zalewski et al. 2006) i.e. when zinquin is added in excess in an isolated system, cadmium accounts for 15% of the fluorescence detected. Given that zinc levels are much lower in BAL fluid (in the nM range) than they are in plasma, where zinc is in the μM range and likely to swamp any effect of cadmium, it



is possible that the presence of cadmium may contribute to some of the increases in labile zinc reported in the BAL fluid here. However, even when a potential effect of cadmium [to 15% as based on (Zalewski et al. 2006)] is taken into account, labile zinc levels were still significantly higher (up 245%) in the BAL of smoke exposed mice. Thus, we suggest that at least some of the increase observed in BAL labile zinc levels may be due to the release of zinc by the inflamed or irritated airway epithelium after smoke exposure. We have previously reported that airway epithelial cells are rich in labile zinc (Truong-Tran et al. 2000) and the zinc efflux transporter, ZnT4 is highly expressed in the apical cytoplasm of airway

◀ **Fig. 7** Effect of smoke exposure and dietary zinc on the gene expression of selected metabolic markers in skeletal muscle and fat. Gene expression of **a** UCP-3 in gastrocnemius skeletal muscle, **b** ATGL in RpWAT, **c** MCP-1 in RpWAT, **d** IL-6 in iBAT and **e** UCP-1 in iBAT. Data are presented as the expression relative to Sham Moderate zinc (after first standardising to the housekeeping gene, 18S rRNA). Results are expressed as mean ± SD. N = 8 per group. Data was analysed using 2-way ANOVA and Bonferroni post hoc tests where appropriate. Smoke exposure significantly increased the gene expression of UCP-3 in gastrocnemius ($P < 0.0005$), ATGL in RpWAT ($P < 0.0029$) and IL-6 in iBAT ($P < 0.0049$), whereas MCP-1 gene expression in RpWAT ($P < 0.0013$) was significantly reduced. Dietary zinc significantly altered UCP-1 gene expression in iBAT ($P < 0.0481$). For UCP-3 gene expression in the gastrocnemius post hoc analysis revealed a significant difference between the high zinc groups, but a significant interaction ($P < 0.0295$) was also observed. Post hoc analysis also revealed a significant effect of smoke exposure between the low zinc groups for the gene expression of ATGL in RpWAT and IL-6 in iBAT

epithlial cells, just beneath the cilia (unpublished observations). Increases in zinc levels in the airway lumen, either via the release of zinc from airway epithelial cells or transfer from the blood supply, upon an inflammatory stimulus such as cigarette smoke would allow zinc to be taken up by macrophages or other immune cells and contribute to anti-inflammatory and anti-oxidant functions of these cells. Numerous genes needed for host defence have been identified as zinc-responsive in human macrophages including cytokine receptors and genes associated with the Th1 immune response (Cousins et al. 2003).

Proteins regulating zinc uptake, storage, transport and function within mammalian cells include members of the ZIP and ZnT protein families. Here, we report for the first time, that the Zn transporter protein ZIP1 is localised to the apical surface of the airway epithelium in mouse airways. ZIP1 has been shown to have specific zinc influx activity (Kambe et al. 2004) and may function to bring zinc into the airway epithelium, where it is likely stored in the ER/Golgi or in vesicles at the apical surface (Hogstrand et al. 2009; Truong-Tran et al. 2000). Previously we have reported the up-regulation of zinc influx transporters (e.g. ZIP1 up 9.5-fold; ZIP14 up 5.3-fold) and down-regulation of the Zn efflux transporter, ZnT4 in a murine model of allergic airway inflammation (Lang et al. 2007). Moreover, ZIP8 and 14 have been found to bring environmental cadmium (a contaminant of cigarette smoke) into the organism via alveolar and intestinal cells and may function endogenously to combat inflammation (He et al. 2009). However, in the current experimental model only very small, unconvincing changes were observed in the zinc transporters examined. We believe that this is due to the length of time that had passed between the last smoke exposure and tissue collection (16–24 h) and the fact that the inflammatory response observed with our model was relatively mild (due we believe to the use of research grade cigarettes (2RF, Kentucky Research Cigarettes) which are milder (0.85 mg nicotine per cigarette) than commercially available Winfield Reds (approx 1.2 mg nicotine per cigarette) used by the earlier studies of Vlahos and Chen et al. (Chen et al. 2008; Vlahos et al. 2006) on which our protocol was adapted).

Weight loss, muscle wasting and metabolic changes have been reported in human smokers and COPD patients (Schwartz and Weiss 1990) as well as in murine models of smoke exposure (Chen et al. 2004, 2008). Indeed, in the current study, all growth parameters including growth rates, body weight, nasal–anal length and tibia length were reduced by both cigarette smoke exposure and low zinc diets in an additive manner, such that mice fed both low zinc diets and exposed to cigarette smoke had proportionately lower outcomes in terms of growth parameters. The average food consumption over the 8 weeks was also lower in mice fed low zinc diets and exposed to cigarette smoke and this is consistent with previous smoke exposure studies in mice (Chen et al. 2008; Vlahos et al. 2006).

In addition to reduced food intake, smoke exposure is thought to alter proteolysis and lipolysis in muscle and fat, respectively. Increased expression of the muscle specific ligases atrogin 1 and MuRF1 that break down muscle proteins into amino acids for use by the body, have been shown in a number of models of muscle wasting (Hansen et al. 2006). Moreover, after a similar smoke exposure period Hansen et al. (unpublished observations) have previously found a reduction in IGF-1 gene and protein expression. Since IGF-1 increases skeletal muscle proliferation and differentiation and inhibits atrogin-1 and MuRF1 expression, down-regulation of IGF-1 may partially explain the muscle wasting observed. However, in the current study we did not see such any alterations in the gene expression of atrogin-1, MuRF-1, IGF-1, ATGL or UCP-3 in gastrocnemius skeletal muscle

and this may be because 8 weeks exposure is normally at the threshold for muscle wasting to occur. In terms of lipolysis, adipose triglyceride lipase, which participates in the release of free fatty acids, mRNA levels did increase slightly in RpWAT in mice exposed to cigarette smoke. This small but significant increase in ATGL may increase lipolysis and contribute to WAT loss in these animals. Moreover, the low zinc group had the greatest percent reduction in RpWAT mass compared to sham animals in the same dietary group (44% vs. 35 and 30% in the moderate and high zinc groups, respectively) and the greatest increase in ATGL mRNA expression.

Conclusions

Increases in dietary and therefore, plasma labile zinc, markedly reduced macrophage numbers recovered from the airway lumen from both sham and smoke exposed mice. This observation supports our evolving hypothesis that the trace element zinc is an important mediator of airway function in both healthy and inflamed airways. Here, we demonstrated for the first time that ZIP1 is located in the airway epithelium of lung tissue, in a similar location to that previously reported for labile zinc. Although muscle wasting itself was minimal in this experimental model, the impact of both cigarette smoke exposure and low zinc diets on the growth parameters of the mice was additive such that mice fed both low zinc diet and exposed to cigarette smoke had proportionately lower outcomes in terms of growth parameters. From a clinical perspective, intervention with zinc supplements may be both preventative and therapeutic for respiratory conditions such as asthma and COPD because firstly, maintaining airway zinc levels might lower the risk of developing inflammation and secondly, restoring airway zinc levels in those who already have airway damage may lessen the severity of inflammation and aid tissue repair particularly when zinc intake is low due to loss of appetite and poor nutrition as seen in COPD patients.

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