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RESEARCH PAPER

TNF-α gene expression is increased following zinc supplementation in type 2 diabetes mellitus

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Abstract Chronic low-grade inflammation in type 2 diabetes mellitus (DM) can elicit changes in whole-body zinc metabolism. The interaction among the expression of inflammatory cytokines, zinc transporter and metallothionein (MT) genes in peripheral blood mononuclear cells in type 2 DM remains unclear. In a 12-week randomized controlled trial, the effects of zinc (40 mg/day) supplementation on the gene expression of cytokines, zinc transporters and MT in women with type 2 DM were examined. In the zinc-supplemented group, gene expression of tumour necrosis factor (*TNF*)-α tended to be upregulated by 27 ± 10 % at week 12 compared to baseline ($P = 0.053$). *TNF*-α fold change in the zinc-treated group was higher than in those without zinc supplementation ($P < 0.05$). No significant changes were observed in the expression or fold change of interleukin (*IL*)-*1β* or *IL-6*. Numerous bivariate relationships were observed between the fold changes of cytokines and zinc transporters, including *ZnT7* with *IL-1β* ($P < 0.01$), *IL-6* ($P < 0.01$) and *TNF*-α ($P < 0.01$). In multiple regression analysis, *IL-1β* expression was predicted by the expression of all zinc transporters and MT measured at baseline ($r^2 = 0.495$, $P < 0.05$) and at week 12 ($r^2 = 0.532$, $P < 0.03$). The current study presents preliminary evidence that zinc supplementation increases cytokine gene expression in type 2 DM. The relationships found among zinc transporters, MT and cytokines suggest close interactions between zinc homeostasis and inflammation.

Keywords Inflammation · Cytokines · Zinc transporters · Metallothionein · Gene expression · Type 2 diabetes mellitus

Introduction

Zinc is involved in the biosynthesis, storage and secretion of insulin within the pancreatic β-cells (Huang 2014). Furthermore, intracellular zinc can act directly on the insulin signalling pathway to improve glucose uptake and insulin sensitivity in peripheral tissues (Haase and Maret 2005; Foster and Samman 2010). Individuals with type 2 diabetes mellitus (DM) are suggested to be of poor zinc status due to the presentation of higher urinary zinc excretion and lower serum zinc concentrations (Jansen et al. 2009). Suboptimal zinc status can affect glycaemic control by compromising the production and secretion of insulin in the pancreas (Huber and Gershoff 1973) and impacting insulin sensitivity in peripheral tissues (Jansen et al. 2009; Kelleher et al. 2011). Hence, the persistent elevation of blood glucose concentration featured in type 2 DM may be attributed partly to perturbed zinc homeostasis.

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Cellular zinc homeostasis is regulated primarily by two families of zinc transporters and metallothionein (MT) (Lichten and Cousins 2009). The Zrt- and Irt-like protein (Zip) (SLC39) family of transporters is responsible for increasing cytoplasmic zinc concentration by transporting zinc from intracellular organelles or the extracellular space. Conversely, the ZnT (SLC30) family of transporters functions to decrease the cytoplasmic zinc concentration by transporting zinc from the cytosol into the extracellular space or internal organelles, such as those involved in secretory pathways. MT acts as a target for zinc ion binding in the cytoplasm and is believed to assist in the trafficking of zinc ions through the cell (Babula et al. 2012). The regulatory control of cellular zinc transporters and MT is complex and has been shown to be influenced by zinc status, systemic glucose and inflammatory cytokines (Lichten and Cousins 2009; Hennigar and Kelleher 2012).

Cellular zinc transporters in different cell types have been shown to be both up- and downregulated to meet the changing demand for zinc in inflammatory conditions (Foster and Samman 2012). The redistribution of zinc from the systemic circulation into cellular compartments is thought to be crucial for immune function. During acute infection, an increase in systemic interleukin (IL)-6 can induce hepatic accumulation of zinc, which contributes to the rapid decrease in the plasma zinc concentration often seen in the acute phase response (Liuzzi et al. 2005). The chronically activated innate immune system in type 2 DM (Pickup 2004) also can elicit changes to whole-body zinc metabolism. Exposure to systemic cytokines, such as IL-1β, have been associated with downregulation of the zinc transporters shown to play a role in insulin production and storage in the pancreatic β-cells (Egefjord et al. 2009).

The expressions of immune markers, such as IL-1β, IL-6 and TNF-α, often are linked to the progression of type 2 DM (Donath and Shoelson 2011). Specifically, increase in IL-1β has been shown to promote pancreatic β-cell destruction (Banerjee and Saxena 2012) which, in combination with TNF-α and IL-6, may synergistically exacerbate the extent of β-cell apoptosis and disease progression (Cnop et al. 2005). Furthermore, TNF-α and IL-6 can promote insulin resistance in peripheral tissues by modulating the expression of key regulators in the insulin signalling pathway (Mlinar et al. 2007). Attenuation of CRP and inflammatory cytokine production may be achieved by dietary supplementation of anti-inflammatory nutrients, such as zinc and n-3 polyunsaturated fatty acids (PUFA) (Calder 2006; Foster and Samman 2012). In a 6-week cross-over intervention study which manipulated the composition of fatty acids, a diet high in α-linolenic acid (ALA) derived from FSO resulted in significant reductions in cytokine production and vascular inflammation in hypercholesterolemic subjects (Zhao et al. 2004, 2007). The effectiveness of a modest level of ALA supplementation on inflammation in type 2 DM is currently unknown.

Similarly, the effect of zinc supplementation on inflammatory biomarkers in type 2 DM remains largely unexplored. While zinc supplementation appears to reduce the level of systemic CRP and IL-6 (Bao and Prasad 2010), conflicting findings are reported for the effect of zinc supplementation on ex vivo cytokine production in stimulated mononuclear cells (Aydemir et al. 2006; Prasad and Beck 2007). In a recent zinc supplementation trial in individuals with type 2 DM (Foster et al. 2013b), we reported that systemic inflammatory markers were associated with the gene expression of zinc transporters and MT, suggesting an interplay between systemic markers of inflammation and cellular zinc transport. Consistent with our observation, a recent study in obese women showed levels of systemic C-reactive protein (CRP) and tumour necrosis factor (TNF)-α to be inversely correlated with a range of zinc transporter gene expressions (Noh et al. 2014).

Peripheral blood mononuclear cells (PBMC) have been used as a candidate target tissue to detect transcriptome changes in response to dietary modification in humans (de Mello et al. 2012). The expression of zinc transporters and MT in PBMC have been described previously in a healthy population (Foster et al. 2011) and those with type 2 DM (Foster et al. 2013b). However, the interactions between the gene expression of inflammatory cytokines, zinc transporters and MT remain unclear. To extend our previous report, specifically the relationships between systemic cytokines (IL-1β, IL-6 and TNF-α) and zinc transporters and MT (Foster et al. 2013b), the gene expressions of *IL-1β*, *IL-6* and *TNF-α* in PBMC were measured to provide further insight into the interactions between zinc and the immune system in type 2 DM. The present study aims to investigate the effect of zinc on the gene expression of inflammatory cytokines in PBMC and explore possible relationships between the gene expression of cytokines, zinc transporters and MT.

Materials and methods

Study design

The study design has been described previously (Foster et al. 2013a, b). In brief, 48 participants were enrolled in a randomized, double-blind, placebo-controlled trial. The primary inclusion criteria were that participants be postmenopausal (>12 months without menses), have normal glomerular filtration rate and microalbumin/creatinine ratio, and were diagnosed with type 2 DM, controlled by either diet and lifestyle or oral hypoglycaemic medication. Postmenopausal women were chosen for this trial as they have been identified as an understudied population group,

and the inclusion of women who suffer from chronic conditions such as type 2 DM contributes biomedical knowledge that advances patient care (Kim et al. 2010). Enrolled participants were randomized into four equal groups to receive a total of 40 mg/day elemental zinc ('Zn Group'), 2,000 mg/day flaxseed oil ('FSO group'), both zinc and flaxseed oil ('Zn + FSO group'), or placebo for 12 weeks. Placebo capsules that were identical in appearance to their active counterparts were given to the placebo groups. Zinc placebo capsules contained cellulose, while olive oil was used as the placebo for FSO. All procedures followed were in accordance with the ethical standards of the Human Research Ethics Committee of the University of Sydney. Informed consent was obtained from all participants for being included in the study. The study protocol was registered at www.clinicaltrials.gov (NCT01505803).

Markers of systemic inflammation, glycaemia and zinc

Venous blood samples from participants were collected at baseline, and at weeks 4, 8 and 12 for the analysis of glucose, haemoglobin A1c (HbA1c), cytokines, CRP and zinc. Serum glucose was measured by glucose hexokinase UV method using the Gluco-quant reagent kit adapted for a Modular PPE auto-analyser (Roche Diganostics, Basel, Switzerland). Serum insulin was determined by chemiluminescent microparticle immunoassay on an Architect i2000SR Analyzer (Abbott Laboratories, Abbott Park, IL, USA). HbA1c was assayed using ion-exchange high performance liquid chromatography (HPLC) on a Variant II analyser equipped with the Variant II NU Program (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's protocol. Plasma zinc was determined using inductively coupled plasma mass spectrometry (Agilent 7500ce ICPMS, Santa Clara, CA, USA). The human cytokine/chemokine Milliplex MAP kit (Millipore, Billerica, MA, USA) was used for the simultaneous quantification of serum IL-1β, IL-6 and TNF-α concentrations, according to the manufacturer's instructions. Samples were analysed on a Luminex 100 Bioanalyser (Luminex Corp., Austin, TX, USA) using Fidis multiplex technology (Biomedical Diagnostics, Marne la Vallée, France). Serum CRP was measured using the Tina-quant CRP (gen.3) immunoturbidimetric method adapted for a Roche Modular PPE analyser (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions.

Zinc transporter, metallothionein and cytokine gene expressions

PBMC were isolated from blood samples collected at baseline and week 12, processed through to cDNA and stored at −80 °C until quantitative real-time PCR analysis. Unstimulated PBMC from individual samples were extracted, and total RNA was prepared using the RNAqueous Small Scale Phenol-Free Total RNA Isolation Kit (Applied Biosystems-Life Technologies Australia Pty Ltd, Victoria, Australia) according to the manufacturer's instructions. Total RNA was reverse transcribed into cDNA using the Superscript VILO cDNA Synthesis System (Invitrogen-Life Technologies Australia Pty Ltd, Victoria, Australia) following the manufacturer's protocol. Forty complete samples of cDNA from study participants were recovered for cytokine gene expression analysis. Inventoried TaqMan gene expression assays were obtained for *IL-1β, IL-6* and *TNF-α* for relative quantification of cytokine mRNA using TaqMan real-time PCR, as per the manufacturer's instructions (StepOnePlus Real-Time PCR System, Applied Biosystems-Life Technologies Australia Pty Ltd, Victoria, Australia). The selected cytokine transcripts correspond with measures of systemic inflammation that previously showed relationships with expression of zinc transporter genes (Foster et al. 2013b). Relative quantification of zinc transporter mRNA was conducted using TaqMan real-time PCR (ABI 7500 Fast Sequence Detection System; Applied Biosystems-Life Technologies Australia Pty Ltd, Victoria, Australia). Inventoried TaqMan gene expression assays, and one custom-designed assay, were obtained for *ZnT1*, *ZnT5*, *ZnT6*, *ZnT7*, *ZnT8*, *Zip1*, *Zip3*, *Zip7*, *Zip10*, *MT-1A* and *MT-2A* mRNA (Applied Biosystems-Life Technologies Australia Pty Ltd, Victoria, Australia). Messenger RNA expression levels for all genes were normalized to *18S* rRNA expression as an endogenous reference and quantified using the ΔC_P method; fold change relative to baseline was quantified using the $\Delta\Delta C_P$ method.

Statistical analysis

Descriptive statistics were expressed as mean ± SD. Cytokine gene expressions and fold changes were described as mean ± SEM. Differences in group means of baseline characteristics, cytokine expressions and fold changes were assessed by analysis of variance (ANOVA). No significant changes were observed in primary outcomes measured (glycaemia, systemic inflammatory markers or cytokine mRNA expressions) after FSO supplementation, with or without zinc. Hence, post hoc investigations using independent *t*-tests were used to compare groups categorized according to whether participants did or did not receive zinc supplementation. Post hoc analysis according to FSO supplementation was performed and confirmed no effect on cytokine gene expressions when FSO was consumed alone or in combination with zinc.

Bivariate relationships between fold changes of cytokines, zinc transporters and MT were calculated using the Pearson's correlation coefficient. Multiple regression

models were used to explore the relationships among gene expression of *IL-1β, IL-6 and TNF-α* and expression of all zinc transporters and MT measured, at baseline and week 12. The residuals from the regression models were checked to see whether they satisfied the assumptions of normality and homoscedasticity: the initial regressions indicated that analyses of *MT-1A* and *MT-2A* were more appropriately conducted on a log scale. Statistical analyses were carried out using SPSS (PASW) version 18. With analysis of the primary outcomes, a value of $P < 0.05$ was taken to designate statistical significance. In univariate outcomes within multivariate analyses, a more conservative designation of $P < 0.01$ as statistically significant was used due to the large number of statistical tests employed.

Results

Participant characteristics

Characteristics of study participants, biomarker outcomes and zinc transporter expressions have been described previously in detail (Foster et al. 2013a, b). Of the 48 enrolled participants, complete sets of cDNA samples were available from 40 participants for PBMC cytokine gene expression analysis. The age and BMI of participants were (mean ± SD) 65.1 ± 8.0 years and 28.3 ± 5.0 kg/m^2, respectively. The average time since type 2 DM diagnosis was 6.6 ± 5.4 years. The baseline fasting blood glucose concentration, fasting insulin and HbA1c of the participants were 6.7 ± 1.8 mmol/L, 67.2 ± 36.8 pmol/L and 6.7 ± 1.0 %, respectively. The mean plasma zinc concentration was 12.8 ± 2.0 μmol/L, which is within the reference range of 10–18 μmol/L. The baseline serum concentrations of CRP, IL-1β, IL-6 and TNF-α were 1.7 ± 2.2 mg/L, 1.0 ± 1.9 pg/mL, 1.6 ± 2.0 pg/mL and 10.3 ± 3.5 pg/mL, respectively. At baseline, there were no differences in zinc transporter and MT expressions between groups. Participant characteristics and biochemical measures at baseline are shown in Supplemental Table 1.

Markers of systemic inflammation and glycaemia

After 12 weeks of intervention, no significant changes were detected in the measures of systemic inflammation or glycaemia as a result of zinc supplementation. The mean plasma zinc concentration was significantly higher in the zinc-supplemented group when compared to those without zinc treatment after 4 weeks ($P < 0.05$; Supplemental Fig. 1) and remained significantly higher at weeks 8 and 12. There were no significant changes observed in zinc transporter and MT mRNA expressions between treatment groups [reported previously in (Foster et al. 2013b)].

Table 1 Relative mRNA expression and fold changes of *IL-1β*, *IL-6* and *TNF-α* in PBMC (mean ± SEM)

	Placebo ($n = 10$)	FSO ($n = 10$)	Zn ($n = 11$)	Zn + FSO ($n = 9$)
IL-1β				
Week 0	0.96 ± 0.11	1.72 ± 0.39	1.04 ± 0.15	0.91 ± 0.14
Week 12	0.97 ± 0.02	1.33 ± 0.06	1.46 ± 0.01	0.98 ± 0.06
Fold change	1.08 ± 0.24	0.88 ± 0.43	1.51 ± 0.30	1.21 ± 0.35
IL-6				
Week 0	0.10 ± 0.12	0.13 ± 0.25	0.05 ± 0.36	0.17 ± 0.13
Week 12	0.08 ± 0.02	0.14 ± 0.08	0.08 ± 0.02	0.26 ± 0.10
Fold change	0.97 ± 0.30	1.24 ± 0.29	1.46 ± 0.48	1.38 ± 023
TNF-α				
Week 0	2.86 ± 0.15	3.85 ± 0.16	2.77 ± 0.26	2.89 ± 0.18
Week 12	3.01 ± 0.12	3.16 ± 0.27	3.64 ± 0.25	3.09 ± 0.23
Fold change	1.10 ± 0.12	0.88 ± 0.12	1.38 ± 0.16	1.14 ± 0.10

FSO flaxseed oil, *IL-1β* interleukin-1β, *IL-6*, interleukin-6, *TNF-α* tumour necrosis factor-α, *PBMC* peripheral blood mononuclear cells

Relative mRNA expression expressed as copies of mRNA per 10^6 *18S* rRNA

Fold change data are calculated using the $\Delta\Delta C_p$ method and expressed on a log scale

Cytokine mRNA expression and fold change

At baseline, no differences were observed in the mRNA expression of *IL-1β*, *IL-6* or *TNF-α* among the intervention groups. *TNF-α* was the most highly expressed mRNA transcript of the cytokines measured, expressing at almost threefold of *IL-1β*. Separated into the four treatment groups, no significant changes were found in cytokine gene expressions after 12 weeks of intervention (Table 1).

Secondary analyses were conducted with the four treatment groups differentiated according to whether participants received zinc treatment (Zn group and Zn + FSO group) or no zinc treatment (FSO group and placebo). In the zinc-treated group, mRNA expression levels at week 12 tended to be higher than baseline for *TNF-α* (per cent increase 27 ± 10 %; mean ± SEM; $P = 0.053$), *IL-6* (42 ± 17 %; $P = 0.066$), but not *IL-1β* (37 ± 16 %; $P = 0.19$). In those without zinc treatment, no significant differences were found between baseline and week 12 cytokine mRNA expressions. When the data were expressed as fold change, *TNF-α* was significantly higher ($P = 0.037$) as a result of zinc supplementation (Fig. 1). *IL-1β* fold change was marginally higher ($P = 0.054$) in the zinc-treated group, while no differences were observed in the fold change of *IL-6* ($P = 0.17$).

When separated into those groups who did receive FSO (FSO and FSO + Zn group) or did not receive FSO (placebo and Zn group), *IL-1β*, *IL-6* and *TNF-α* gene expressions were not significantly different between week 0 and 12. Fold change of *IL-1β*, *IL-6* and *TNF-α* were not

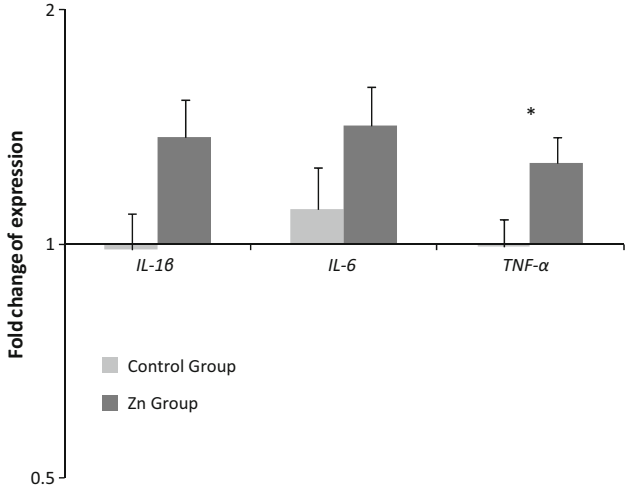

Fig. 1 Fold change of *IL-1β*, *IL-6* and *TNF-α* in PBMC separated by whether participants received zinc treatment ($n = 20$) or no zinc treatment ($n = 20$). Fold change data (mean ± SEM) are calculated using the $\Delta\Delta C_p$ method and expressed on a log scale. *$P < 0.05$ by independent *t*-test

significantly different in the FSO group when compared to those who did not receive FSO.

Relationships among various cytokines and zinc transporters

Bivariate correlations between fold changes of individual zinc transporters and *IL-1β*, *IL-6* and *TNF-α* are shown in Table 2. Positive correlations between *TNF-α* fold change and fold changes of *ZnT5*, *ZnT6, ZnT7*, *Zip1*, *Zip3*, *Zip7*, *Zip10*, *MT-1A* and *MT-2A* were observed when all participants were considered ($P < 0.05$). Similarly, *IL-1β* fold change was positively correlated to the fold change of *ZnT7* ($r = 0.44$, $P < 0.01$; Fig. 2a) and *Zip1* ($r = 0.42$, $P < 0.01$; Fig. 2b). The treatment of zinc abolished a number of correlations between fold changes of cytokines and zinc transporters; in particular, fold changes of *IL-6* and *ZnT5* (control group, $r = 0.61$, $P < 0.01$; zinc group, $r = -0.13$, $P > 0.05$; Fig. 3), *ZnT6* (control group, $r = 0.49$, $P < 0.05$; zinc group, $r = 0.09$, $P > 0.05$) and *ZnT7* (control group, $r = 0.54$, $P < 0.05$; zinc group, $r = 0.32$, $P > 0.05$).

Using multiple regression analysis for all participants at baseline, *IL-1β* expression was predicted by expressions of all zinc transporters and MT measured ($r^2 = 0.495$, $P = 0.04$, Table 3), with marginally significant univariate correlations observed with *Zip1* ($P = 0.02$) and *Zip7* ($P = 0.01$). At week 12, overall significant multivariate relationship was maintained between expressions of *IL-1β* and zinc transporters and MT ($r^2 = 0.532$, $P = 0.02$) with a single significant univariate relationship found between *IL-1β* and *ZnT7* ($P = 0.002$); *ZnT7* expression explained 36 % of the variability in *IL-1β* expression. Multiple regression analyses using expression of zinc transporters and MT as predictors of *IL-6* or *TNF-α* expression did not show any significant relationships (data not shown).

Discussion

The key observation in the present study is an upregulation of cytokine gene expression in PBMC after zinc

Table 2 Bivariate Pearson's correlations between fold changes of cytokines, zinc transporters and metallothionein when analysed in all participants ($n = 38$[a]) and according to whether participants received (zinc, $n = 20$) or did not receive (control, $n = 18$) zinc supplements for 12 weeks

	ZnT1	*ZnT5*	*ZnT6*	*ZnT7*	*ZnT8*[b]	*Zip1*	*Zip3*	*Zip7*	*Zip10*	*MT-1A*	*MT-2A*
IL-1β											
All	0.28	0.28	0.33*	0.44**	−0.03	0.42**	0.38*	0.24	0.14	0.19	0.36
Zinc	0.33	0.49*	0.27	0.57**	−0.23	0.51*	0.31	0.27	0.24	0.10	0.37
Control	0.31	0.24	0.42	0.29	0.04	0.35	0.70**	0.33	−0.06	0.38	0.32
IL-6											
All	0.07	0.21	0.29	0.43**	0.05	0.06	0.38*	0.22	0.42**	0.51**	0.14
Zinc	−0.08	−0.13	0.09	0.32	−0.13	0.10	0.46*	0.31	0.43	0.59**	0.24
Control	0.29	0.61**	0.49*	0.54*	0.32	0.04	0.41	0.18	0.39	0.40	−0.01
TNF-α											
All	0.31	0.39*	0.45**	0.56**	0.10	0.52**	0.34*	0.44**	0.33*	0.33*	0.60**
Zinc	0.31	0.44	0.41	0.60**	0.11	0.64**	0.49*	0.48*	0.40	0.28	0.65**
Control	0.42	0.55*	0.49*	0.57*	−0.12	0.46	0.36	0.54*	0.22	0.43	0.55*

* $P < 0.05$; ** $P < 0.01$

[a] Missing data for expression of zinc transporters and metallothionein for two participants

[b] *ZnT8* mRNA expression was detected in seven participants supplemented with zinc and nine participants not supplemented with zinc

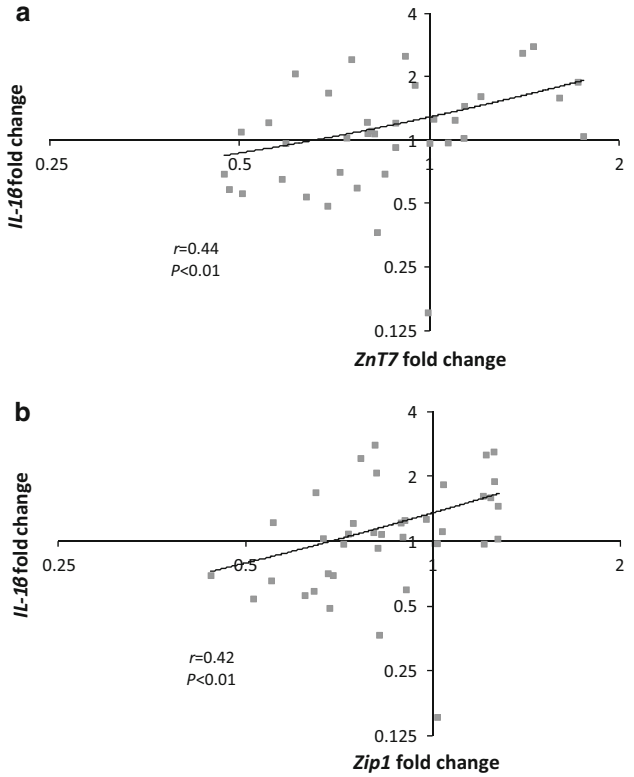

Fig. 2 Bivariate correlations between fold changes of *IL-1β* and *ZnT7* (Fig. 2a), and *IL-1β* and *Zip1* (Fig. 2b) in all participants ($n = 38$). Fold change data are calculated using the $\Delta\Delta C_p$ method and expressed on a log scale

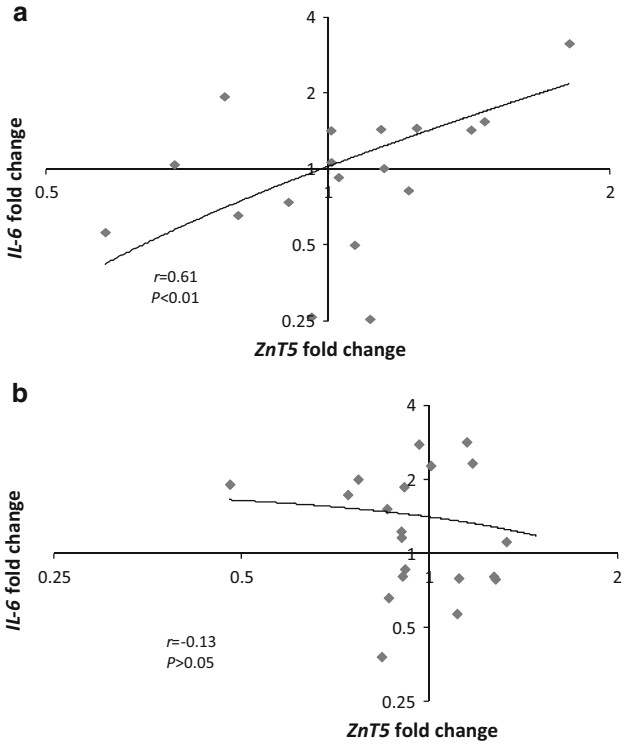

Fig. 3 Bivariate correlations between fold changes of *IL-6* and *ZnT5* in the control group ($n = 18$, Fig. 3a) and in the zinc group ($n = 20$, Fig. 3b). Fold change data are calculated using the $\Delta\Delta C_p$ method and expressed on a log scale

supplementation in postmenopausal women with type 2 DM. Specifically, higher fold change of *TNF-α* was observed, with *IL-1β* fold change, and *IL-6* expression tending to increase after 12 weeks of zinc supplementation. A range of bivariate relationships were found among fold changes of various cytokines and zinc transporters. In addition, zinc transporter and MT gene expressions were shown to predict the expression of *IL-1β*. Collectively, the observations support a relationship between zinc and immune response in type 2 DM.

The significant upregulation observed in *TNF-α* expression after zinc supplementation in the present study is in agreement with several in vitro studies (Scuderi 1990; Wellinghausen et al. 1996; Chang et al. 2006). In cultured cells, changes in gene expression and secretion of cytokines as a result of zinc treatment have been reported. Increases in TNF-α, IL-1β and IL-6 secretion have been shown in otherwise unstimulated PBMC incubated with high levels of zinc (≥100 μM) in the media (Wellinghausen et al. 1996; Chang et al. 2006). Similar effects were observed with zinc added at physiologically relevant levels; *TNF-α* and *IL-1β* mRNA expressions were shown to be upregulated with 30 μM of zinc added to the incubating media (Chang et al. 2006). Limited data are available from human trials on the effects of zinc supplementation on cytokine production in non-activated PBMC. The gene expressions of *TNF-α* and *IL-1β* remain unchanged in unstimulated leucocytes extracted from healthy subjects given 15 mg/day of zinc for 4 days (Aydemir et al. 2006). The present results are novel, and to our knowledge, there are no previous reports of the effect of zinc supplementation on unstimulated cytokine expressions in postmenopausal women with type 2 DM.

Perturbation in zinc metabolism is often reported in type 2 DM (Jansen et al. 2009). The increase in the plasma zinc concentration seen in zinc-supplemented participants in the present study suggests that zinc is available for intracellular zinc replenishment in previously zinc-deficient tissues. We hypothesize that an increase in cellular zinc content in PBMC may explain these observations. The classic transcription factor, nuclear factor-kappa B (NF-κB), has been proposed to be influenced by increased zinc availability. NF-κB represents the converging signalling molecule in immune cells; the propagation of Toll-like, IL-1 and TNF receptor signalling pathways is reliant on the activation of NF-κB. Zinc has been proposed to enhance or inhibit NF-κB activity, dependent on the different health and/or zinc status of the studied population (Foster and Samman 2012).

Table 3 Multiple regression models using expression of zinc transporters and metallothionein to predict *IL-1β* expression at baseline (model 1) and week 12 (model 2) for all participants

Factors	Model 1 (week 0) Regression coefficient	$n = 38$ Standard error	P value	Model 2 (week 12) Regression coefficient	$n = 37$ Standard error	P value
ZnT1	−0.02	0.04	0.58	0.02	0.07	0.75
ZnT5	−0.01	0.34	0.97	−0.64	0.39	0.12
ZnT6	−0.19	0.23	0.41	−0.09	0.30	0.78
ZnT7	0.06	0.07	0.38	0.30	0.09	0.002
ZnT8	−1.38	2.31	0.55	−4.81	4.55	0.30
Zip1	0.18	0.07	0.02	−0.01	0.09	0.89
Zip3	0.11	0.11	0.35	0.12	0.14	0.41
Zip7	−0.34	0.13	0.01	−0.17	0.26	0.53
Zip10	−0.01	0.09	0.91	−0.11	0.12	0.34
MT-1A	−0.11	0.10	0.28	−0.14	0.16	0.40
MT-2A	0.05	0.22	0.82	0.32	0.44	0.48
Overall	$r^2 = 0.495$		0.04	$r^2 = 0.532$		0.02

In zinc-deficient subjects, NF-κB activation was suppressed with the consequent reduction in the cytokine production of immune cells (Prasad et al. 2006), and both of these phenomena were reversed by zinc supplementation.

Increased plasma zinc concentration, per se, may be contributing to the observed upregulation of cytokines. Higher systemic zinc concentrations may initiate intracellular signalling through an extracellular zinc receptor, GPR39. This novel zinc sensor is implicated in the regulation of cell proliferation and growth (Cohen et al. 2014), through modulation of the extracellular regulating kinase (ERK) 1/2 and Akt pathways. Although the direct effect of GPR39 on intracellular signalling in the immune cells is unclear, it is conceivable that the activation of GPR39 by increased extracellular zinc may trigger downstream signalling eventuating in upregulation of cytokine expression. Given that a state of zinc deficiency appears to co-exist with type 2 DM (Jansen et al. 2009), the current findings after zinc supplementation may be evidence of improved immune response through increased efficiency in signalling pathways and thereby upregulation of cytokine gene expressions.

The multiple bivariate correlations found in the present study between fold changes of cytokine, zinc transporter and MT mRNA suggest coordination between fluxes of cellular zinc ions and cytokine production in PBMC, an important function of immune cells. These findings support previous observations of a relationship between zinc transporter gene expression and systemic inflammatory biomarkers in humans (Foster et al. 2013b; Noh et al. 2014). Systemic inflammatory biomarkers, such as CRP, IL-6 and TNF-α, were able to predict the gene expressions of zinc transporter and MT in PBMC; in particular, increases in systemic IL-6 concentration were related to increases in the expression of a cluster of zinc transporters, *ZnT5, ZnT7, Zip1, Zip7* and *Zip10*, which are responsible largely for the uptake of extracellular zinc and transport of zinc into intracellular organelles and secretory pathways in PBMC. Considering that inflammatory biomarkers in the systemic circulation are the cumulative secretion of many peripheral tissues, the present study is unique in elucidating the relationship between zinc transporter, MT and cytokine gene expression within PBMC.

At both baseline and week 12, *IL-1β* gene expression was predicted by zinc transporter and MT gene expressions. Furthermore, *IL-1β* expression was strongly predicted by the gene expressions of *Zip1* and *Zip7* at baseline and *ZnT7* at week 12 in univariate analyses. Considering their proposed localization within the cell (Huang and Tepaamorndech 2013; Jeong and Eide 2013), these zinc transporters are likely to be involved in either increasing cytoplasmic zinc concentration or the transport of zinc into the golgi apparatus. Taken together with the previously observed relationships between systemic IL-6 with gene expression of *ZnT7*, *Zip1* and *Zip7* (Foster et al. 2013b), we hypothesize that systemic cytokines have a regulatory control of zinc transporter transcription which influences the flux of zinc into the secretory pathway, thereby assisting in immune cell function such as *IL-1β* transcription. This hypothesis is supported by recent findings that suggest external stimuli such as systemic cytokines may regulate zinc transporter expression through the JAK-STAT pathways (Miyai et al. 2014). In particular, cytokines were found to induce the expression of Zip10, thereby providing an influx of intracellular zinc. The uptake of zinc via this particular zinc transporter appears to be critical for the survival, function and signalling of B-lymphocytes (Hojyo et al. 2014). In a similar vein, we have previously reported an association between *Zip10* gene expression and glycaemic control (Foster et al. 2014), whereby the change in fasting glucose was correlated with the change in *Zip10* expression. These observations

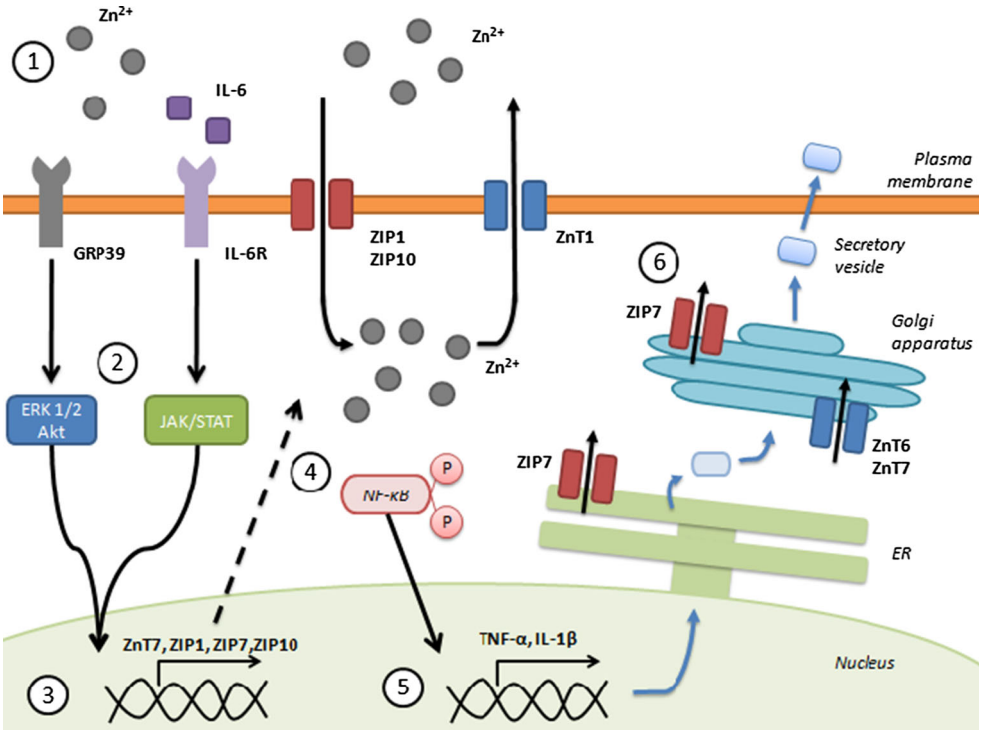

Fig. 4 Potential zinc-mediated pathways by which external stimuli (such as systemic glucose, cytokines and zinc) may act on cellular function. External stimuli such as systemic IL-6, glucose and extracellular zinc bind to respective receptors on the plasma membrane (*1*). This triggers intracellular signalling events (ERK 1/2, Akt and JAK/STAT pathways; *2*), resulting in modulation of zinc transporters and MT gene expressions (*3*). Changes in intracellular zinc concentration can influence NF-κB activation (*4*) and subsequent expression of cytokines, such as TNF-α and IL-1β (*5*). Eventual cytokine secretions may be related to the expression of zinc transporters implicated in the secretory pathway (*6*)

highlight the range of external stimuli that can influence zinc flux and function in the cell. Figure 4 shows the hypothesized interaction between external stimuli, zinc transport and cellular function.

The PBMC transcriptome has been shown to be reflective of gene expressions in metabolically active tissues, such as liver (Powell and Kroon 1994) and muscle (Rudkowska et al. 2011), both of which play a role in glucose and zinc homeostasis. Furthermore, modifications in dietary macronutrients and antioxidants seem to effect changes in the PBMC transcriptome (de Mello et al. 2012), hence presenting it as a promising target tissue in nutrigenomic studies. From the current study, it is evident that zinc supplementation impacts on the gene expression of cytokines and affects the relationships between cytokine and zinc transporter transcription in PBMC. In particular, the relationships between fold change of *IL-6* with *ZnT5*, *ZnT6* and *ZnT7* fold changes in the control group were not observed in the zinc-treated group. Whether zinc supplementation will elicit similar effects in cell types other than PBMC, such as those of the pancreas and other metabolically active tissues in type 2 DM, requires further investigation.

The efficacy of zinc supplementation in the management of type 2 DM has been investigated recently. Meta-analyses have shown that zinc supplementation in type 2 DM results in lower fasting glucose (Capdor et al. 2013) and increased high density lipoprotein concentrations (Foster et al. 2010). In the current study, zinc supplementation upregulates gene expression of cytokines in PBMC despite no changes being observed in systemic levels of the corresponding inflammatory markers (Foster et al. 2013b). Differences in the derivation of samples may provide an explanation in the discrepancy of results. In PBMC, the measure of cytokine gene expressions is upstream of cytokine secretion. Regulatory processes at post-transcriptional, translational and post-translational levels are required prior to eventual secretion of cytokines into the systemic circulation. In addition, the systemic cytokine concentration is reflective not only of PBMC cytokine secretion but from a variety of sources, such as liver, muscle and adipose tissue. Hence, the effect of increased cytokine gene expressions in PBMC remains uncertain in the context of the whole-body inflammatory status in type 2 DM.

The current study presents preliminary data on the effects of zinc supplementation on the expression of cytokines and their relationships with zinc transporter gene expressions in type 2 DM. The present observations are novel in that they provide evidence of interactions between zinc transport and cytokine transcription in PBMC, which add to our previous reports (Foster et al. 2013a, b). The study is limited by the lack of proteomic and intracellular zinc analyses. The ultimate function and activity of transcribed genes are influenced by a number of post-transcriptional regulatory processes, and further research is needed to explore the multiple possible sub-cellular localization sites of zinc transporter proteins which are influenced by the host's zinc status (Lichten and Cousins 2009).

In summary, the current study presents preliminary evidence that zinc supplementation increases *TNF-α* gene expression in postmenopausal women with type 2 DM. The multiple relationships found between gene expression and fold change of zinc transporters, MT and cytokines suggest close interactions between zinc homeostasis and inflammation.

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Conflict of interest Anna Chu, Meika Foster, Dale Hancock, Kim Bell-Anderson, Peter Petocz, and Samir Samman declare that they have no conflict of interest.

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