

Peroxisome Proliferator Activated Receptors α and γ Require Zinc for Their Anti-inflammatory Properties in Porcine Vascular Endothelial Cells¹

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ABSTRACT Zinc is an essential structural component of various proteins and is crucial for the integrity of the vascular endothelium. The present study focused on the effect of zinc deficiency on the anti-inflammatory properties of peroxisome proliferator activated receptor (PPAR) α and γ agonists. Porcine pulmonary-arterial endothelial cells were deprived from zinc by chelator *N,N,N',N'*-tetrakis (2-pyridylmethyl)ethylene diamine. Cells were exposed to TNF- α for 2 h following pretreatment with the PPAR α agonists fenofibrate or ciprofibrate or the PPAR γ agonists thiazolidinedione or troglitazone. The inflammatory response was tested by measuring nuclear factor-kappaB (NF- κ B) and activator protein-1 (AP-1) binding activities as well as by measuring mRNA expression levels of inflammatory genes, such as vascular cell adhesion molecule-1 (VCAM-1) and IL-6. All PPAR agonists tested lost their potency to downregulate the TNF- α -induced inflammatory response in zinc-deficient cells. However, if zinc was added back, all PPAR agonists significantly downregulated the TNF- α -mediated induction of inflammatory transcription factors NF- κ B and AP-1 and significantly reduced the expression of their target genes, VCAM-1 and IL-6. We therefore hypothesize that zinc is required for the PPAR α and - γ DNA binding activity. Indeed, zinc deficiency significantly reduced the agonist-induced binding activity of PPAR α and - γ to the PPAR response element. Our data demonstrate the importance of zinc in PPAR signaling and the requirement of zinc for the anti-inflammatory properties of PPAR α and - γ agonists. J. Nutr. 134: 1711–1715, 2004.

KEY WORDS: • atherosclerosis • vascular endothelial cells • PPAR • zinc • inflammation

Atherosclerotic lesions are thought to be initiated by vascular endothelial cell dysfunction. Factors implicated in the pathogenesis of atherosclerosis include chronic and cumulative metabolic alterations of the endothelium induced by numerous activating molecules, such as lipids, pro-oxidants, and inflammatory cytokines. These risk factors induce certain cell signaling pathways leading to the activation of proinflammatory transcription factors such as nuclear factor- κ B (NF- κ B)³ and activator protein-1 (AP-1) (1). NF- κ B and AP-1 control most adhesion molecules and cytokines like vascular cell adhesion molecule-1 (VCAM-1) and IL-6 in endothelial cells. The expression of cytokines and adhesion molecules further enhances the inflammation by recruiting monocytes and facilitating their binding to and migration through the endothelium. This loss of the endothelial barrier function can

increase the formation of foam cells in the subendothelial space, subsequently leading to the formation of fatty streaks.

Peroxisome proliferator activated receptor (PPAR) α and γ agonists were shown to be protective against these events by downregulating underlying proinflammatory signaling pathways. PPARs were shown to negatively interfere with NF- κ B, signal transducer and activator of transcription, and AP-1 signaling pathways (2,3) and can therefore prevent the expression of inflammatory genes such as adhesion molecules and cytokines. Indeed, clinical and experimental evidence suggests that PPAR activation decreases the incidence of cardiovascular diseases. PPARs appear to act at least in part directly at the level of the vascular wall in addition to correcting metabolic disorders (4).

However, the precise mechanisms by which PPARs can inhibit inflammatory transcription factors are not clear. In addition to ligand-dependent regulation, it was demonstrated that the transcriptional activity of PPARs could be altered by covalent modifications such as phosphorylation (5,6). Furthermore, the activity of nuclear receptors can be influenced by cofactors that modulate signaling and interactions with the basal transcription machinery (7). So far, only a few studies addressed the interactions between PPARs and cofactors, and none of them included trace elements.

We propose that zinc could be an important factor for the

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³ Abbreviations used: AP-1, activator protein-1; CF, ciprofibrate; DMSO, dimethylsulfoxide; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; FF, fenofibrate; NF- κ B, nuclear factor-kappa B; PPAR, peroxisome proliferator activated receptor; PPRE, peroxisome proliferator response element; TG, troglitazone; TPEN, *N,N,N',N'*-tetrakis (2-pyridylmethyl)ethylene diamine; TZD, thiazolidinedione; VCAM-1, vascular cell adhesion molecule-1.

anti-inflammatory function of PPARs. Our laboratory has substantial data on the protective properties of zinc against inflammatory and pro-oxidative stimuli such as TNF- α or linoleic acid (8–10). Zinc can stabilize the vascular endothelium (11) and protect against TNF-induced disruption of endothelial barrier function (12). However, the protective properties of zinc are not as well understood in inflammatory pathways that are directly induced by a signaling cascade triggered by TNF- α where oxidative stress is not the main inducer of inflammatory transcription factors. Little is known about the requirements and functions of zinc in maintaining the integrity of the vasculature and particularly the vascular endothelium. Zinc might play an important role in endothelial protection against pro-oxidative and pro-inflammatory insults, which are critical events in the early pathogenesis of atherosclerosis.

In this study we aimed to demonstrate that zinc is required for the anti-inflammatory properties of PPAR α and γ and that activation of PPAR α or γ is defective in zinc-deficient cells.

MATERIALS AND METHODS

Cell culture and experimental medium. Endothelial cells were isolated from porcine pulmonary arteries as described previously (13). Arteries obtained during routine slaughter were donated from the College of Agriculture, University of Kentucky. Cells were subcultured in Medium 199 (M-199) containing 10% (v/v) fetal bovine serum (FBS, HyClone Laboratories) using standard techniques.

The experimental media were composed of M-199 enriched with 5% (v/v) FBS. Zinc (20 μ mol/L) was added as zinc acetate from a stock solution in water. *N,N,N',N'*-Tetrakis (2-pyridylmethyl)ethylene diamine (TPEN) (2 μ mol/L) and PPAR agonists (10–25 μ mol/L) were added from stock solutions in ethanol and dimethylsulfoxide (DMSO), respectively. TNF α (0.01 μ g/L) was added directly into the culture medium 2 h prior to termination of treatment. Unless otherwise stated, chemicals were purchased from Sigma. All treatment groups contained an equal amount of DMSO or ethanol. The final DMSO concentration in the medium never exceeded 0.05% (v:v) in all treatment groups. For most experimental settings, cells were treated with zinc and/or TPEN for 24 h, PPAR agonists for 8–18 h, and TNF for 2 h.

Transcription factor (NF- κ B, AP-1, and PPAR γ) activation studies: electrophoretic mobility shift assay (EMSA). Nuclear extracts containing active proteins were prepared from cells according to the method of Dignam et al. (14). Nuclear extracts were incubated for 25 min with ³²P-end-labeled oligonucleotide probes containing enhancer DNA element NF- κ B (5'-AGTTGAGGGGACTTTC-CCAGGC-3'), AP-1 (5'-CGCTTGATGAGTCAGCCGAA-3') (Promega), or PPRE (5'-AGGTCAAAGGTCA-3') (Santa Cruz). Incubation at room temperature was performed in the presence of nonspecific competitor DNA. Following binding, the complexed and uncomplexed DNA in the mixture were resolved by electrophoresis in a 6.5% (w:v) nondenaturing polyacrylamide gel and visualized by autoradiography. Control reactions using a supershift assay were performed to demonstrate the specificity of the shifted DNA-protein complexes for NF- κ B, PPAR γ , and AP-1, respectively. All antibodies used were obtained from Santa Cruz.

IL-6 and VCAM-1 expression studies. Total RNA was extracted from endothelial cells by the use of TRI reagent (Sigma) according to the manufacturer's protocol. Gene expression was determined through RT-PCR as described earlier (15). The following primers were employed in the PCRs: IL-6 forward: 5'-AAT TCG GTA CAT CCT CGA CG-3', reverse: 5'-GCG CAG AAT GAG ATG AGT TG-3', VCAM-1 forward: 5'-ATGACA TGC TTG AGC CAG G-3', reverse: 5'-GTG TCT CCT TCT TTG ACA CT-3', β -actin forward: 5'-GGG ACC TGA CCG ACT ACC TC-3', reverse: 5'-GGG CGA TGA TCT TGA TCT TC-3'. The amplified PCR products were electrophoresed on a 2% (w:v) Tris-borate EDTA

agarose gel, stained with SYBR Gold (Molecular Probes), and visualized using phosphorimaging technology (FLA-5000; Fuji).

Statistical analysis. The data were quantified and analyzed using Scion Image and Sigma Stat software, respectively. Comparisons between treatments were made by one-way ANOVA with post-hoc comparisons of the means made by Tukey tests. Differences of $P < 0.05$ were considered significant.

RESULTS

PPAR agonists and zinc downregulate the TNF α -induced binding activity of NF- κ B and AP-1. TNF- α increased DNA binding activity of the transcription factor AP-1 and it was further increased by zinc chelation with TPEN (Fig. 1). In the zinc-deficient cells neither the PPAR γ agonist thiazolidinedione (TZD) (25 μ mol/L) nor the PPAR α agonist fenofibrate (FF) (10 μ mol/L) significantly downregulated the TNF- α -mediated AP-1 induction ($P > 0.2$). In cells that received zinc both PPAR agonists significantly decreased the binding activity of AP-1. Similar results were obtained using ciprofibrate (CF) (25 μ mol/L) and troglitazone (TG) (25 μ mol/L) as alternative agonists for PPAR α and γ , respectively (data not shown). Furthermore, TNF- α remarkably increased the DNA binding activity of NF- κ B in zinc-deficient cells (Fig. 2). The addition of either PPAR agonists TZD, CF, TG, or FF (only selected data are shown) or zinc ameliorated NF- κ B activation in zinc-deficient cells ($P > 0.3$). However, significant downregulation was only observed in zinc-sufficient cells treated with PPAR agonists.

PPAR agonists and zinc protect against TNF α -induced IL-6 and VCAM-1 gene expression. IL-6 and VCAM-1 expression was upregulated after a 2-h exposure to TNF- α and was significantly upregulated in zinc-depleted cells exposed to TNF- α (Fig. 3A, B). When administered to zinc-deficient cells, neither zinc nor PPAR γ agonist TZD significantly downregulated VCAM-1 and IL-6 gene expression ($P > 0.4$). TPEN alone did not induce VCAM-1 expression. However, when

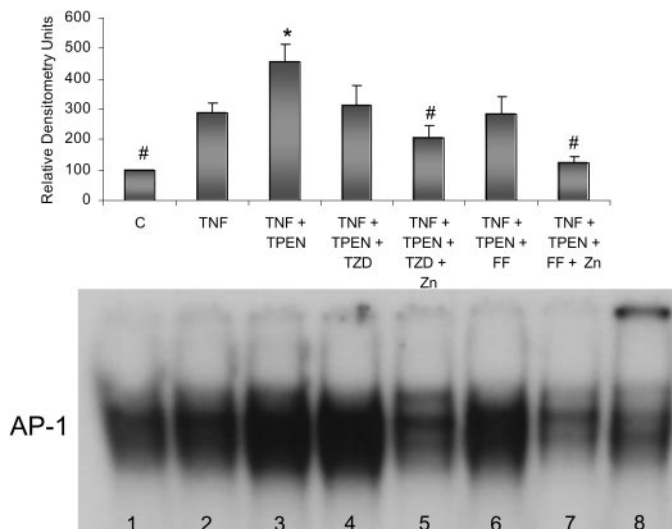


FIGURE 1 TNF- α -mediated induction of AP-1 in primary endothelial cells. Cells were deprived of zinc by chelation with 2 μ mol/L TPEN. Selected treatment groups received 20 μ mol/L zinc and/or PPAR α agonist FF (10 μ mol/L) or PPAR γ agonist TZD (25 μ mol/L) prior to exposure to TNF. Values are means \pm SEM, $n = 3$. Means labeled * differ from those labeled #, $P < 0.05$.

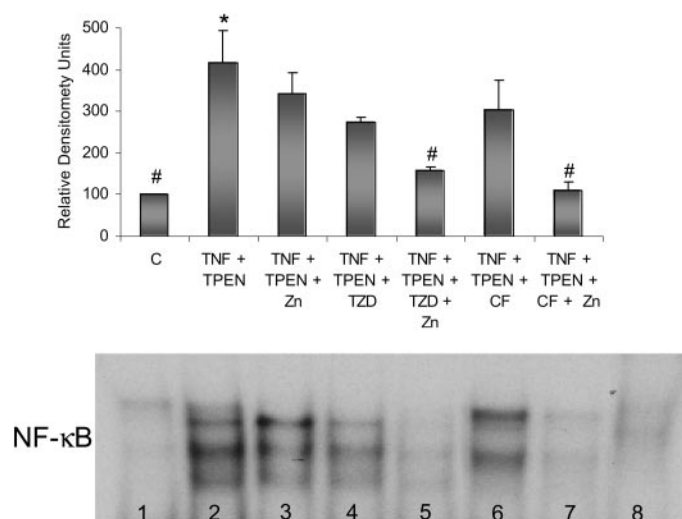


FIGURE 2 TNF- α -mediated induction of NF- κ B in porcine endothelial cells. Cells were deprived of zinc by chelation with 2 μ mol/L TPEN. Selected treatment groups received 20 μ mol/L zinc and/or were treated with PPAR α agonist CF (25 μ mol/L) or γ agonist TZD (25 μ mol/L) prior to exposure to TNF- α . Values are means \pm SEM, $n = 3$. Means labeled * differ from those labeled #, $P < 0.05$.

zinc and TZD were added in concert to zinc-deficient cells, IL-6 and VCAM-1 mRNA levels decreased significantly.

PPAR agonists induced PPAR binding activity only in zinc-sufficient cells. PPAR γ agonist TZD and PPAR α agonist CF both induced binding to the peroxisome proliferator response element (PPRE) as determined by EMSA (Fig. 4A, B). Zinc depletion by TPEN alone did not affect binding to the PPRE. However, cotreatment of cells with TPEN and PPAR agonists resulted in a significant decrease in PPAR DNA binding activity.

DISCUSSION

Zinc was shown to be essential to the structure and function of a large number of macromolecules and for over 300 enzymatic reactions (16). It has both catalytic and structural roles in enzymes, while in zinc finger motifs it provides the scaffold that organizes protein subdomains for the interaction with either DNA or other proteins (16).

Zinc is a critical component of biomembranes and is essential for proper membrane structure and function (17). In addition, zinc is required for cellular repair processes and could therefore play a critical role in protecting the vasculature from cell injury and loss of barrier function—both critical initial steps in the pathogenesis of atherosclerosis (1). In the current study we show that adequate amounts of zinc are required for the anti-inflammatory properties of PPAR α and - γ agonists. In zinc-deficient cells, PPAR agonists failed to downregulate the TNF- α -induced NF- κ B and AP-1 transcription factor binding activity as well as the expression of the pro-inflammatory genes VCAM-1 and IL-6. TPEN alone did not induce any of the inflammatory markers, indicating that zinc deficiency does not induce inflammation by itself; however, it increases the susceptibility to inflammatory inducers, such as TNF- α . The involvement of zinc in PPAR is a novel concept and the mechanism of the interaction is not known.

PPAR α and - γ are nuclear receptors expressed in a wide variety of tissues, including vascular endothelial cells (18).

PPARs regulate lipid and lipoprotein metabolism and glucose homeostasis as well as cell proliferation and differentiation. In addition, PPARs also modulate the inflammatory response. In addition to ligand binding, cofactor recruitment appears to play a key role in the transcriptional regulation by PPARs. In fact, recent reports demonstrated that different subsets of cofactors are recruited to the PPAR transcription factor machinery dependent on the nature of the PPAR ligand (4). The difference in cofactors appears to contribute to a ligand-specific response in gene transcription. Because zinc is known to be essential for protein structure, zinc might be important in the assembly and stability of the PPAR-RXR-cofactor complexes.

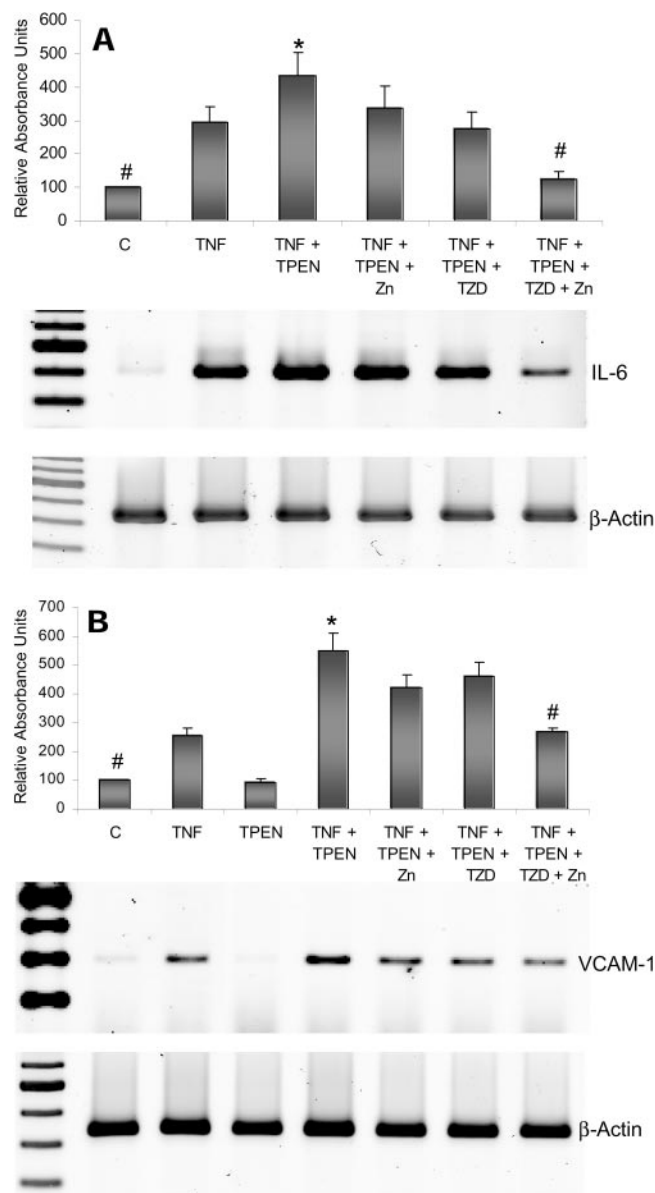


FIGURE 3 Effects of TNF- α , zinc depletion/supplementation, and PPAR agonists on VCAM-1 (A) and IL-6 (B) mRNA levels in endothelial cells. Endothelial cells were pretreated with PPAR agonists and zinc/zinc chelator TPEN (18 h) and exposed to TNF- α for 2 h. β -Actin was used as a housekeeping gene. Values are means \pm SEM, $n = 3$. Means labeled * differ from those labeled #, $P < 0.05$.

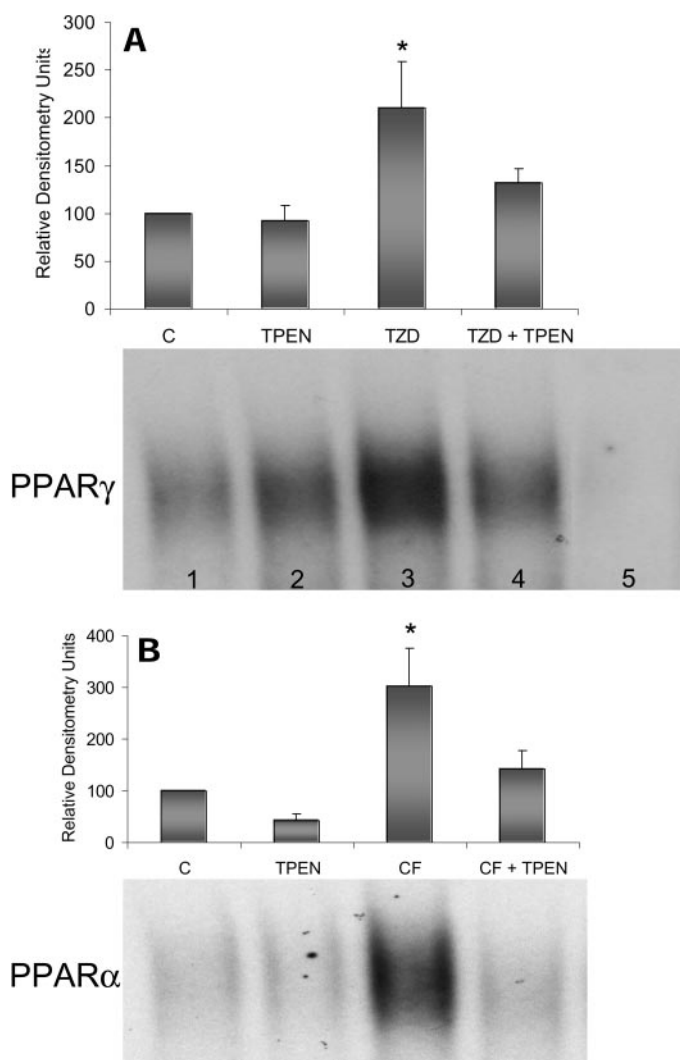


FIGURE 4 Effects of zinc depletion on PPAR γ (A) and PPAR α (B) binding activity in endothelial cells. Primary endothelial cells were zinc deprived by chelation and treated with PPAR α agonist TZD (A) or PPAR α agonist CF (B). Values are means \pm SEM, $n = 3$. *Different from all other means, $P < 0.05$.

In fact, the DNA binding domain of PPARs consists of 2 sets of zinc fingers (19). The specificity and polarity of PPAR-DNA binding seem to be at least in part due to features in the zinc finger domains of PPAR (20). The binding partner of PPAR, RXR, also has a DNA binding domain with 2 zinc fingers involved (21). Because zinc is an essential constituent of the DNA binding domains of both PPAR and RXR, zinc deficiency could impair the function of these transcription factor complexes. In addition, as a critical component of the cells' anti-oxidative defense system, zinc appears to be important for maintaining an environment that facilitates normal protein-protein interaction. It has been suggested that zinc deficiency induces alterations in the intracellular redox state that can lead to the oxidation of thiol groups, thus impairing protein function (22). In fact, zinc finger structures require 2 zinc-coordinated cysteine sulfhydryl groups, and oxidation of these can eliminate DNA binding and transcriptional functions (23). This might be relevant for optimal functioning of the PPAR-RXR protein complex.

Although the effect of oxidative stress on PPAR is controversial, oxidative stress was reported to downregulate PPAR α mRNA in skeletal muscle cells (24). Zinc could change PPAR mRNA levels by preventing the generation of reactive oxidative species. Indeed, we reported recently that zinc deficiency decreased PPAR γ mRNA and protein levels (25).

Overall, our data suggest that zinc is a potent anti-inflammatory substance, which can help protect the endothelium against inflammatory events mediated by TNF- α . The data further demonstrate the importance of zinc for the anti-inflammatory properties of PPAR α and - γ agonists. In zinc-deficient cells, several PPAR agonists tested failed to downregulate the activation of inflammatory transcription factors and the expression of inflammatory genes. Furthermore, the DNA binding activity of both PPAR α and - γ to the PPRE was greatly impaired in cells deprived of zinc. Because dietary zinc intake of certain population groups is still below dietary recommendations (26), these data underline the importance of adequate zinc intake during the administration of PPAR-targeting drugs to treat chronic diseases such as hyperlipidemia or diabetes.

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