Research article

The effect of hypoxia mimetic cobalt chloride on the expression of EC-SOD in 3T3-L1 adipocytes

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It is well known that adipose tissue is not only a passive reservoir for energy storage but also produces and secretes a variety of bioactive molecules called adipocytokines, including adiponectin and tumor necrosis factor-α (TNF-α). Recently, it has been reported that adipose tissue can suffer a chronic hypoxic condition during hypertrophy of adipocytes, and this condition leads to the dysregulation of adipocytokines. Further, hypoxic adipocytes are in an increased oxidative stress. Extracellular-superoxide dismutase (EC-SOD) is an anti-inflammatory enzyme that protects cells from reactive oxygen species (ROS) by scavenging superoxide anion. Previous reports showed that plasma EC-SOD levels in type 2 diabetes patients were significantly and inversely related to the body mass index, homeostasis model assessment-insulin resistance index; however, the mechanisms of EC-SOD and adiponectin reductions during hypoxia remain poorly understood. Here, we demonstrate that cobalt chloride ($CoCl_2$), a hypoxia mimetic, decreases EC-SOD and adiponectin in 3T3-L1 adipocytes by intracellular ROS-independent, but TNF-α and c-jun N-terminal kinase (JNK)-dependent mechanisms. From these results, it is possible that TNF-α is a key regulator of the reduction of EC-SOD and adiponectin in $CoCl_2$-treated 3T3-L1 adipocytes, and we speculated that the reduction of EC-SOD and adiponectin would lead to and/or promote metabolic disorders.

Keywords: extracellular-superoxide dismutase, adiponectin, tumor necrosis factor-alpha, c-Jun N-terminal kinase, cobalt chloride

Introduction

Obesity is closely linked to a variety of metabolic disorders, including insulin resistance, atherosclerosis and type 2 diabetes.[1] Recent studies have indicated that adipose tissue is not only an energy store but also produces and secretes various bioactive molecules called adipocytokines, such as adiponectin, tumor necrosis factor-α (TNF-α), plasminogen activator inhibitor type 1 and monocyte chemo-attractant protein-1.[2–5] Adiponectin is an anti-inflammatory adipocytokine that plays a pivotal role in the improvement of glucose and lipid metabolism and the prevention of atherosclerosis and inflammation.[6,7] In patients with insulin resistance, obesity or type 2 diabetes, serum adiponectin levels are reduced,[8,9] and previous studies showed that TNF-α and intracellular reactive oxygen species (ROS) decrease adiponectin

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expression.[10–12] On the other hand, increases in adiponectin expression have been reported during adipocyte differentiation and transcriptional factors associated with adipogenesis, including CCAAT/enhancer-binding protein-α (C/EBP-α) and peroxisome proliferator-activated receptor-γ (PPAR-γ), have been shown to up-regulate adiponectin expression.[13]

Recently, adipose tissue has been found to suffer chronic hypoxia during the development of obesity.[14,15] Hypoxic conditions can be induced by the addition of certain chemicals called 'hypoxia mimetics', such as the carcinogenic transition metal cobalt.[16] In these hypoxic conditions, it has been recognized that hypoxia-inducible factor-1α (HIF-1α), a transcriptional factor, is accumulated and increases/decreases a wide variety of genes to ensure adaptation to low-oxygen tension.[17,18] Further, it has been reported that both hypoxia and hypoxia mimetics increase ROS generation and dysregulate adipocytokines, and these conditions lead to and/or promote metabolic disorders.[19,20] To protect cells from oxidative stress, mammals have anti-oxidative enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase.[21,22] SOD is a major anti-oxidative enzyme that protects cells from oxidative stress by scavenging superoxide.[23] There are three SOD isozymes in mammals; copper and zinc containing SOD (Cu,Zn-SOD), manganese-containing SOD (Mn-SOD) and extracellular SOD (EC-SOD).[24] EC-SOD is a secretory enzyme, whereas Cu,Zn-SOD and Mn-SOD are intracellular enzymes found predominantly in the cytoplasm and mitochondria, respectively. The EC-SOD content is very low in the liver, heart, brain and other organs, with the exception of the lung, thyroid gland, and adipose tissue.[25] Moreover, it was found that the plasma EC-SOD levels in type 2 diabetic patients were significantly and inversely related to the body mass index, homeostasis model assessment insulin resistance index.[26] We previously reported that $CoCl_2$ decreases EC-SOD in green monkey kidney COS7 cells via intracellular ROS generation and p38-MAPK, a mitogen-activated protein kinase (MAPK), signaling cascade;[27] however, the mechanisms of EC-SOD and adiponectin reductions during hypoxia remain unclear.

In order to address these issues, we studied the regulation of EC-SOD expression by $CoCl_2$ and examined the role of ROS, inflammatory cytokines and MAPK signaling cascades in these processes. Moreover, we also studied the regulation of adiponectin expression by $CoCl_2$, because we hypothesized that the expression of EC-SOD might be co-regulated with adiponectin in 3T3-L1 cells treated with $CoCl_2$, leading to aggravated metabolic disorders.

Materials and methods

Cell culture

3T3-L1 mouse pre-adipocytes culture and their differentiation into adipocytes were carried out as described in previous reports.[13,28] Briefly, pre-adipocytes were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, and 100 μg/ml streptomycin until 2 days after confluence; then, their differentiation into adipocytes was induced by treating cells for 2 days with 5 μg/ml insulin, 0.5 μM dexamethasone and 0.5 mM isobutylmethyl-xanthine in DMEM, and then for 2 days with insulin (5 μg/ml) in the same medium. The cells were returned to the basal medium, which was replaced every other day. The effects of hypoxia and $CoCl_2$ on gene expression levels were investigated using 8-day differentiated adipocytes. The induction of hypoxia (1% O_2) was carried out in a culture chamber which controls O_2 concentrations by supplying N_2 gas together with 5% CO_2.

RT-PCR analysis

After the adipocytes were treated, the medium was aspirated and the cells were washed twice with ice-cold PBS. The cells were lysed in 1 ml TRIzol® reagent (Invitrogen, CA, USA). cDNA was prepared and RT-PCR performed using the methods described in our previous report.[13] Densitometric analysis of the PCR products was performed with Multi Gauge v3.0 (Fuji Film, Japan).

Measurement of cellular protein

We measured the cellular protein as an index of cell injury. After the adipocytes were treated, the cells were washed twice with ice-cold PBS and then scraped in 1 ml of PBS. The cell suspension was homogenized using an ultrasonic homogenizer. The total protein in the supernatant was assayed using a Bio-Rad protein assay reagent (Bio-Lad Lab., CA, USA).

Measurement of intracellular ROS

After the adipocytes were treated, the medium was aspirated and the cells were washed twice with PBS and incubated with fresh culture medium without serum containing 3 μM 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) for 20 min at 37ºC in 5% CO_2/95% air. The cells were then washed twice with ice-cold PBS

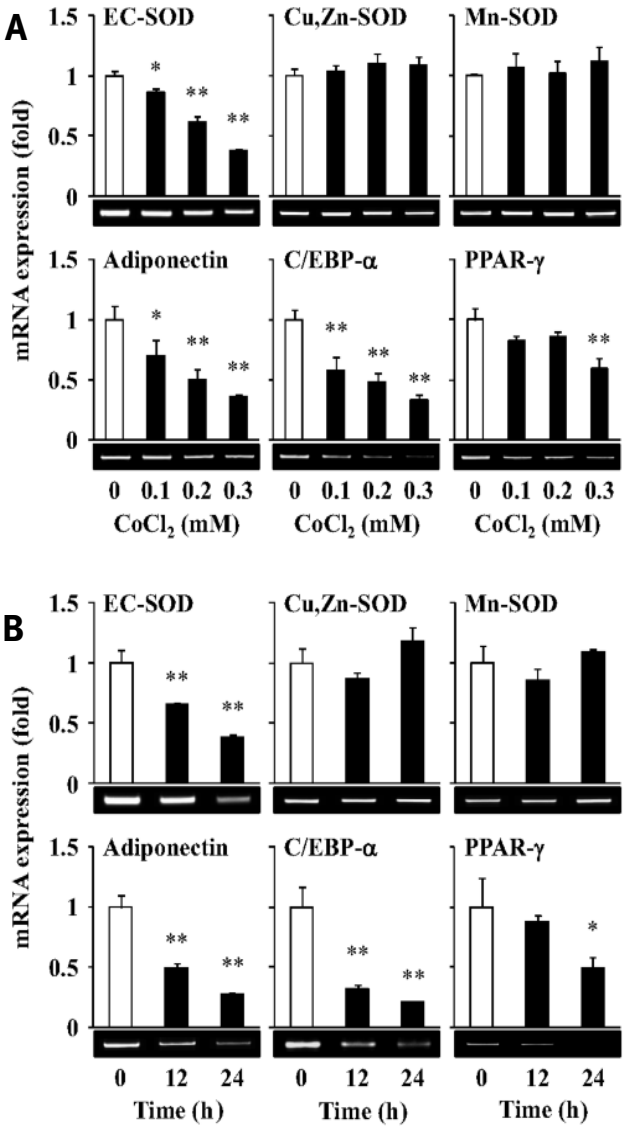

Figure 1 **Dose- and time-dependent effects of $CoCl_2$ on the expression of SODs, adiponectin and transcriptional factors. Fully-differentiated 3T3-L1 adipocytes were treated with the indicated concentrations of $CoCl_2$ for 24 h (A) or with 0.3 mM $CoCl_2$ for the indicated hours (B). After the cells were incubated, RT-PCR was carried out and these data were normalized using β-actin levels. *$P < 0.05$, **$P < 0.01$ compared with untreated adipocytes**

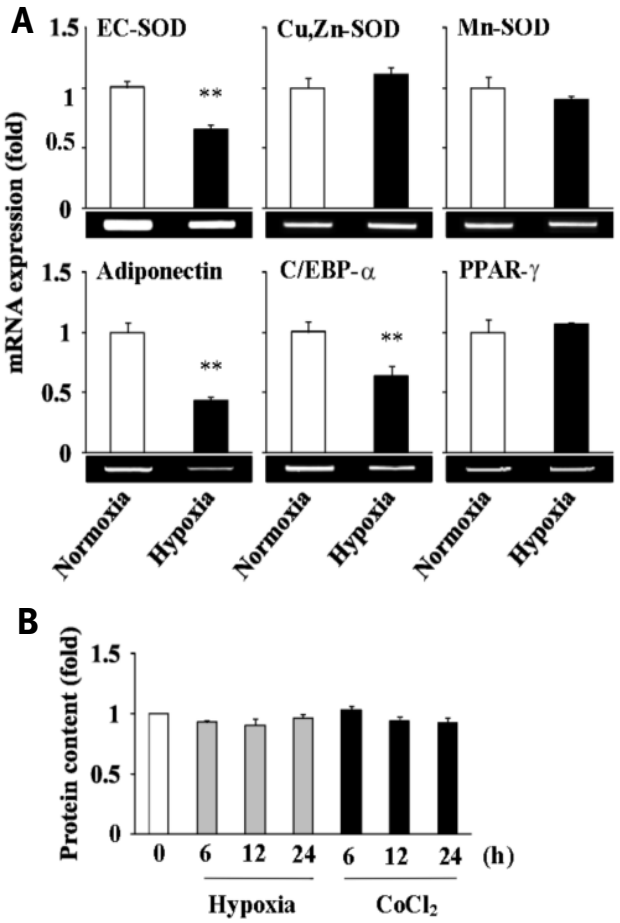

Figure 2 **(A) Expression of SODs, adiponectin and transcriptional factors during hypoxia. Fully differentiated 3T3-L1 adipocytes were incubated under hypoxic condition (1% O_2) for 24 h. After the cells were incubated, RT-PCR was carried out and these data were normalized using β-actin levels. **$P < 0.01$ compared with normoxia. (B) Effect of hypoxia and $CoCl_2$ on the cell viability. Fully-differentiated 3T3-L1 adipocytes were incubated under hypoxic condition (1% O_2) or treated with 0.3 mM $CoCl_2$ for the indicated hours, followed by the assay of cellular protein concentration**

and then scraped in 1 ml ice-cold PBS and centrifuged at 2300 *g* for 5 min at 4ºC. The pellet was homogenized with 1 ml ice-cold PBS using an ultrasonic homogenizer and centrifuged again at 2300 *g* for 10 min at 4ºC. The DCF fluorescence of the supernatant was measured using a fluorimeter (excitation at 493 nm and emission at 527 nm). Total protein concentrations were measured using a protein assay reagent.

Statistical analysis

The data shown are the mean ± SD of three separate experiments. Statistical significance was estimated using ANOVA followed by *post hoc* Bonferroni tests. A *P*-value less than 0.05 was considered significant.

Results

Dose- and time-dependent effects of $CoCl_2$ on the expressions of SODs, adiponectin and transcriptional factors

We first investigated the expression of SODs (EC-SOD, Cu,Zn-SOD and Mn-SOD) in 3T3-L1 adipocytes during $CoCl_2$ treatment. As shown in Figure 1, $CoCl_2$

decreased the EC-SOD mRNA expression in a $CoCl_2$ dose- and time-dependent manner. On the other hand, the other SOD isozymes (Cu,Zn-SOD and Mn-SOD) were not changed when the applied concentration of $CoCl_2$ was 0.3 mM for 24 h. It has been reported that adiponectin is suppressed in mRNA and protein levels during hypoxia;[29] however, the expression of adiponectin during hypoxia induced by $CoCl_2$ was not fully elucidated. Treatment with $CoCl_2$ markedly suppressed the expression of adiponectin in a $CoCl_2$ dose- and time-dependent manner. Additionally, the

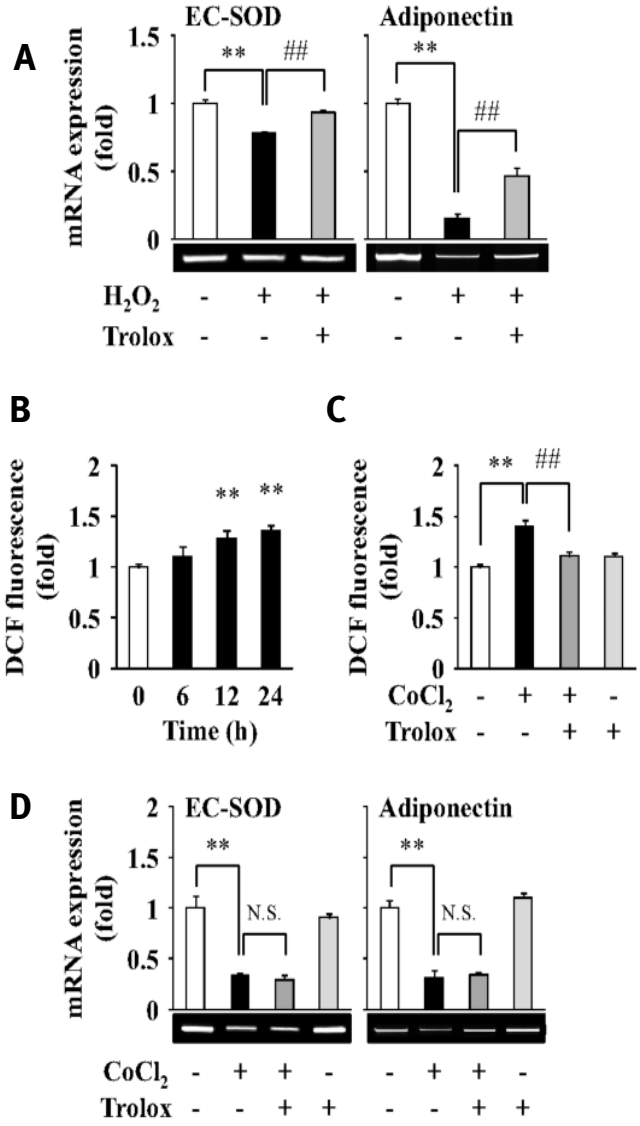

Figure 3 **Involvement of ROS in the $CoCl_2$-induced suppression of EC-SOD and adiponectin. (A) Expression of EC-SOD and adiponectin during H_2O_2 treatment. Fully-differentiated 3T3-L1 adipocytes were pretreated without (–) or with Trolox (+, 0.2 mM) for 2 h prior to further 24 h treatment without (–) or with H_2O_2 (+, 0.5 mM). (B) Fully-differentiated 3T3-L1 adipocytes were treated with 0.3 mM $CoCl_2$ for the indicated time. (C,D) Fully-differentiated 3T3-L1 adipocytes were pretreated without (–) or with Trolox (+, 0.2 mM) for 2 h prior to further 24 h treatment without (–) or with $CoCl_2$ (+, 0.3 mM). After the cells were incubated, RT-PCR was carried out (A,D) and intracellular ROS generation was measured (B,C). All RT-PCR data were normalized using β-actin levels. *$P < 0.05$, **$P < 0.01$ compared with untreated adipocytes, $^{\#\#}P < 0.01$, N.S. not significant compared with $CoCl_2$ alone-treated adipocytes**

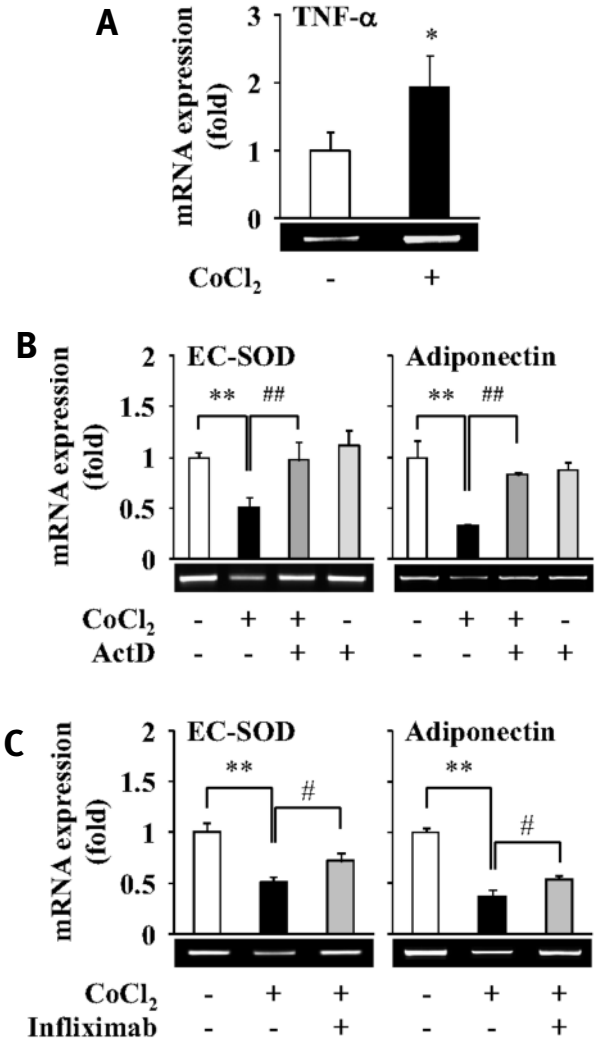

Figure 4 **Involvement of TNF-α in the $CoCl_2$-induced suppression of EC-SOD and adiponectin. (A) Fully-differentiated 3T3-L1 adipocytes were treated with 0.3 mM $CoCl_2$ for 24 h. Cells were pretreated without (–) or with actinomycin D (ActD; +, 1 μg/ml) for 1 h (B), infliximab (+, 10 μg/ml) for 1 h (C) prior to further 24 h treatment without (–) or with $CoCl_2$ (+, 0.3 mM). After the cells were treated, RT-PCR was carried out and these data were normalized using β-actin levels. *$P < 0.05$, **$P < 0.01$ compared with untreated adipocytes, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with $CoCl_2$ alone-treated adipocytes**

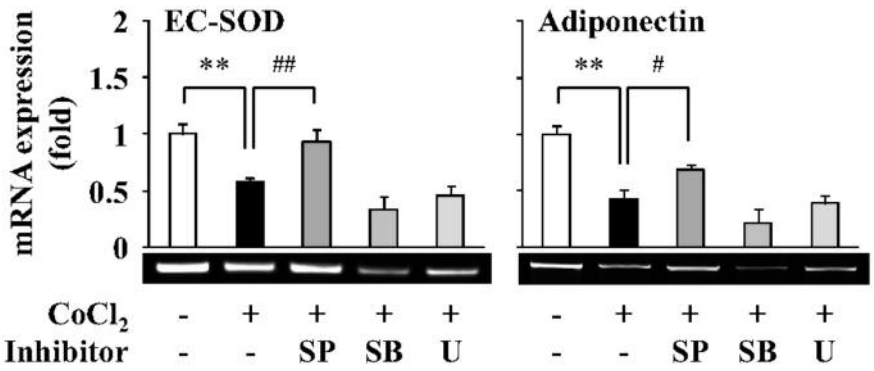

Figure 5 Involvement of MAPK in the $CoCl_2$-induced suppression of EC-SOD and adiponectin. Fully-differentiated 3T3-L1 adipocytes were pretreated without (–) or with SP600125 (SP, 50 μM), SB203580 (SB, 50 μM) or U0126 (U, 50 μM) for 30 min prior to further 24 h treatment without (–) or with $CoCl_2$ (+, 0.3 mM). After the cells were treated, RT-PCR was carried out and these data were normalized using β-actin levels. $^{**}P < 0.01$ compared with untreated adipocytes, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with $CoCl_2$ alone-treated adipocytes

expression of C/EBP-α and PPAR-γ, well recognized as master regulators of adiponectin, was similar to EC-SOD and adiponectin.

Expression of SODs, adiponectin and transcriptional factors during hypoxia

We further investigated the effect of hypoxia (1% O_2) on the expression of SODs, adiponectin and transcriptional factors in 3T3-L1 adipocytes. Hypoxia decreased the expression of EC-SOD, adiponectin and C/EBP-α similar to $CoCl_2$ treatment (Fig. 2A); however, Cu/Zn-SOD, Mn-SOD and PPAR-γ were not changed during hypoxia.

Effect of hypoxia and $CoCl_2$ on cell viability

In order to investigate cell injury, we next measured cellular protein of 3T3-L1 adipocytes incubated under hypoxic condition (1% O_2) or treated with 0.3 mM $CoCl_2$ for up to 24 h. Both hypoxia and $CoCl_2$ did not affect cellular protein content, indicating that the conditions used in this study did not induce cell death (Fig. 2B).

Antioxidant Trolox did not suppress $CoCl_2$-induced suppression of EC-SOD and adiponectin

We previously reported that $CoCl_2$ suppressed the expression of EC-SOD through intracellular ROS generation in COS7 cells.[27] Accordingly, we next investigated the effect of ROS on the expression of EC-SOD and adiponectin. Treatment with H_2O_2 decreased the expression of EC-SOD and adiponectin, and pretreatment with Trolox partially, but significantly, suppressed these reductions (Fig. 3A). We further investigated that generation of ROS during $CoCl_2$ treatment using carboxy-H_2DCFDA. As shown in Figure 3B,C, treatment with $CoCl_2$ increased intracellular ROS generation and Trolox attenuated $CoCl_2$-induced ROS generation; however, pretreatment with Trolox did not attenuate $CoCl_2$-induced suppression of EC-SOD and adiponectin (Fig. 3D).

Involvement of TNF-α and MAPK in $CoCl_2$-induced suppression of EC-SOD and adiponectin

Our previous reports showed that exposure to TNF-α decreased EC-SOD in smooth muscle cells and adiponectin in 3T3-L1 adipocytes.[13,30] We, therefore, investigated the involvement of TNF-α in the suppression of EC-SOD and adiponectin. Treatment with $CoCl_2$ increased the expression of TNF-α at the mRNA level (Fig. 4A), and actinomycin D, an inhibitor of mRNA synthesis, blocked the $CoCl_2$-induced suppression of EC-SOD and adiponectin (Fig. 4B). Further, infliximab, a chimeric monoclonal antibody against TNF-α, partially, but significantly, attenuated the $CoCl_2$-induced suppression of EC-SOD and adiponectin (Fig. 4C). These results indicate that TNF-α plays an important role in the $CoCl_2$-induced suppression of EC-SOD and adiponectin. It has been reported that activation of MAPK plays an important role in the TNF-α-induced suppression of adiponectin in 3T3-L1 adipocytes.[10] To investigate the involvement of MAPK in the $CoCl_2$-induced suppression of EC-SOD and adiponectin further, we next investigated the effect of SP600125 (JNK inhibitor), SB203580 (p38 inhibitor) and U0126 (extracellular regulated kinases (ERK) inhibitor) on this reaction. Suppression of these genes by $CoCl_2$ was significantly attenuated by pretreatment with SP600125, but not with the other inhibitors (Fig. 5).

Discussion

Obesity is closely related to a variety of metabolic disorders, including insulin resistance, atherosclerosis and type 2 diabetes.[1,31] Further, it has been reported that adipose tissue suffers chronic hypoxia during the development of obesity.[19] It has been shown that inflammatory adipocytokines, such as TNF-α, interleukin (IL)-1 and IL-6, are increased, while anti-inflammatory adipocytokines, such as adiponectin, are decreased in hypoxic adipocytes.[29] In this study, $CoCl_2$ decreased the expression of adiponectin and adipogenic master regulators, including C/EBP-α and PPAR-γ, in a $CoCl_2$ dose- and time-dependent manner (Fig. 1A,B), and these observations were similar to hypoxia (1% O_2 treatment), except for PPAR-γ expression. From these observations and previous reports,[19] we speculated that hypoxia mimetic decreases adiponectin via C/EBP-α and/or PPAR-γ signaling cascades, similar to hypoxia.

On the other hand, it is known that both hypoxia and hypoxia mimetics increase ROS generation via mitochondria-dependent or -independent mechanisms, respectively.[18,32] EC-SOD is the major SOD isozyme in extracellular fluids and protects cells from oxidative stress.[23] Our previous report showed that the expression levels of EC-SOD through the differentiation of 3T3-L1 cells changes in a similar manner to C/EBP-α and PPAR-γ, and down-regulation of these proteins might induce and/or promote the pathogenesis of metabolic syndrome and atherosclerosis.[13] Recently, we reported that $CoCl_2$ decreases EC-SOD in COS7 cells via intracellular ROS generation and p38-MAPK signaling cascades.[27] In this study, $CoCl_2$ (Fig. 1) and H_2O_2 (Fig. 3A) decreased the expression of EC-SOD and adiponectin, whereas the expressions of Cu/Zn-SOD and Mn-SOD were not changed (Fig. 1); however, an antioxidant, Trolox, did not affect the reduction of EC-SOD and adiponectin by $CoCl_2$. From these results, it is possible that $CoCl_2$ decreases EC-SOD and adiponectin by intracellular ROS-independent mechanisms.

It has been well recognized that TNF-α is one of the most important adipocytokines, inducing insulin resistance, atherosclerosis and type 2 diabetes. Our previous reports and others showed that TNF-α decreases EC-SOD and adiponectin via p38-MAPK and JNK signaling cascades, respectively.[10,30] In this study, $CoCl_2$ increased TNF-α expression (Fig. 4A), and infliximab partially, but significantly, attenuated the reduction of EC-SOD and adiponectin by $CoCl_2$ (Fig. 4C). Further, actinomycin D blocked the reduction of these expressions (Fig. 4B). These results suggested that $CoCl_2$ decreases EC-SOD and adiponectin via *de novo* protein synthesis; however, because the inhibition rate of EC-SOD and adiponectin reductions by infliximab is partial, we speculated that the mechanisms of these reductions by $CoCl_2$ involve other factors. We next investigated the role of MAPK in the reduction of EC-SOD and adiponectin. In mammalian systems, there are three major subfamilies: ERK, JNK and p38-MAPK.[33,34] In this study, pretreatment with a JNK inhibitor, SP600 125, suppressed the reduction of EC-SOD and adiponectin but SB203580 and U0126 did not affect these reductions (Fig. 5). From these results, we concluded that $CoCl_2$ decreases EC-SOD and adiponectin via JNK signaling cascades.

Conclusions

We investigated the expression of EC-SOD and adiponectin in 3T3-L1 adipocytes during hypoxia. The present study demonstrates for the first time that EC-SOD in 3T3-L1 cells was suppressed by $CoCl_2$ and co-regulated with adiponectin through intracellular ROS-independent, but TNF-α and JNK-dependent mechanisms. From our observations, it is speculated that the reduction of EC-SOD by $CoCl_2$ is similar to adiponectin reduction and leads to a decrease in the resistance to oxidative stress. These findings contribute to the control of metabolic disorder exacerbation and knowledge about cytotoxicity induced by intracellular ROS during hypoxia.

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References

1. Eriksson JW. Metabolic stress in insulin's target cells leads to ROS accumulation – a hypothetical common pathway causing insulin resistance. *FEBS Lett* 2007; **581**: 3734–3742.
2. Hotamisligil GS, Spiegelman BM. Tumor necrosis factor α: a key component of the obesity-diabetes link. *Diabetes* 1994; **43**: 1271–1278.
3. Shimomura I, Funahashi T, Takahashi M *et al*. Enhanced expression of PAI-1 in visceral fat: possible contributor to vascular disease in obesity. *Nat Med* 1996; **2**: 800–803.
4. Berg AH, Combs TP, Scherer PE. ACRP30/adiponectin: an adipokine regulating glucose and lipid metabolism. *Trends Endocrinol Metab* 2002; **13**: 84–89.
5. Havel PJ. Update on adipocyte hormones: regulation of energy balance and carbohydrate/lipid metabolism. *Diabetes* 2004; **53**

(Suppl. 1): S143–S151.
6. Yamauchi T, Kamon J, Minokoshi Y *et al*. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat Med* 2002; **8**: 1288–1295.
7. Ouchi N, Kihara S, Arita Y *et al*. Novel modulator for endothelial adhesion molecules: adipocyte-derived plasma protein adiponectin. *Circulation* 1999; **100**: 2473–2476.
8. Arita Y, Kihara S, Ouchi N *et al*. Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem Biophys Res Commun* 1999; **257**: 79–83.
9. Hotta K, Funahashi T, Arita Y *et al*. Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. *Arterioscler Thromb Vasc Biol* 2000; **20**: 1595–1599.
10. Kim KY, Kim JK, Jeon JH, Yoon SR, Choi I, Yang Y. c-Jun N-terminal kinase is involved in the suppression of adiponectin expression by TNF-α in 3T3-L1 adipocytes. *Biochem Biophys Res Commun* 2005; **327**: 460–467.
11. Soares AF, Guichardant M, Cozzone D *et al*. Effects of oxidative stress on adiponectin secretion and lactate production in 3T3-L1 adipocytes. *Free Radic Biol Med* 2005; **38**: 882–889.
12. Simons PJ, van den Pangaart PS, Aerts JM, Boon L. Pro-inflammatory delipidizing cytokines reduce adiponectin secretion from human adipocytes without affecting adiponectin oligomerization. *J Endocrinol* 2007; **192**: 289–299.
13. Adachi T, Toishi T, Wu H, Kamiya T, Hara H. Expression of extracellular superoxide dismutase during adipose differentiation in 3T3-L1 cells. *Redox Report* 2009; **14**: 34–40.
14. Brook CG, Lloyd JK, Wolf OH. Relation between age of onset of obesity and size and number of adipose cells. *BMJ* 1972; **2**: 25–27.
15. Helmlinger G, Yuan F, Dellian M, Jain RK. Interstitial pH and pO_2 gradients in solid tumors *in vivo*: high-resolution measurements reveal a lack of correlation. *Nat Med* 1997; **3**: 177–182.
16. Vincent F, Hagiwara K, Ke Y, Stoner GD, Demetrick DJ, Bennett WP. Mutation analysis of the transforming growth factor β type II receptor in sporadic human cancers of the pancreas, liver, and breast. *Biochem Biophys Res Commun* 1996; **223**: 561–564.
17. Lee PJ, Jiang BH, Chin BY *et al*. Hypoxia-inducible factor-1 mediates transcriptional activation of the heme oxygenase-1 gene in response to hypoxia. *J Biol Chem* 1997; **272**: 5375-5381.
18. Levy AP, Levy NS, Wegner S, Goldberg MA. Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia. *J Biol Chem* 1995; **270**: 13333–13340.
19. Hosogai N, Fukuhara A, Oshima K *et al*. Adipose tissue hypoxia in obesity and its impact on adipocytokine dysregulation. *Diabetes* 2007; **56**: 901–911.
20. Schuster SJ, Badiavas EV, Costa-Giomi P, Weinmann R, Erslev AJ, Caro J. Stimulation of erythropoietin gene transcription during hypoxia and cobalt exposure. *Blood* 1989; **73**: 13–16.
21. Furukawa S, Fujita T, Shimabukuro M *et al*. Increased oxidative stress in obesity and its impact on metabolic syndrome. *J Clin Invest* 2004; **114**: 1752–1761.
22. Roberts CK, Barnard RJ, Sindhu RK, Jurczak M, Ehdaie A, Vaziri ND. Oxidative stress and dysregulation of NAD(P)H oxidase and antioxidant enzymes in diet-induced metabolic syndrome. *Metabolism* 2006; **55**: 928–934.
23. Faraci FM, Didion SP. Vascular protection: superoxide dismutase isoforms in the vessel wall. *Arterioscler Thromb Vasc Biol* 2004; **24**: 1367–1373.
24. Faraci FM. Vascular protection. *Stroke* 2003; **34**: 327–329.
25. Marklund SL. Extracellular superoxide dismutase in human tissues and human cell lines. *J Clin Invest* 1984; **74**: 1398–1403.
26. Adachi T, Inoue M, Hara H, Maehata E, Suzuki S. Relationship of plasma extracellular-superoxide dismutase level with insulin resistance in type 2 diabetic patients. *J Endocrinol* 2004; **181**: 413–417.
27. Kamiya T, Hara H, Yamada H, Imai H, Inagaki N, Adachi T. Cobalt chloride decreases EC-SOD expression through intracellular ROS generation and p38-MAPK pathways in COS7 cells. *Free Radic Res* 2008; **42**: 949–956.
28. Mori T, Sakaue H, Iguchi H *et al*. Role of Kruppel-like factor 15 (KLF15) in transcriptional regulation of adipogenesis. *J Biol Chem* 2005; **280**: 12867–12875.
29. Ye J, Gao Z, Yin J, He Q. Hypoxia is a potential risk factor for chronic inflammation and adiponectin reduction in adipose tissue of ob/ob and dietary obese mice. *Am J Physiol* 2007; **293**: E1118–E1128.
30. Adachi T, Toishi T, Takashima E, Hara H. Infliximab neutralizes the suppressive effect of TNF-α on expression of extracellular-superoxide dismutase *in vitro*. *Biol Pharm Bull* 2006; **29**: 2095–2098.
31. Evans JL, Goldfine ID, Maddux BA, Grodsky GM. Are oxidative stress-activated signaling pathways mediators of insulin resistance and β-cell dysfunction? *Diabetes* 2003; **52**: 1–8.
32. Richard DE, Berra E, Gothie E, Roux D, Pouyssegur J. p42/p44 mitogen-activated protein kinases phosphorylate hypoxia-inducible factor 1α (HIF-1α) and enhance the transcriptional activity of HIF-1. *J Biol Chem* 1999; **274**: 32631–32637.
33. Kyriakis JM, Avruch J. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol Rev* 2001; **81**: 807–869.
34. Pearson G, Robinson F, Beers Gibson T *et al*. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev* 2001; **22**: 153–183.