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Regulation of EC-SOD in Hypoxic Adipocytes

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1. Introduction

Obesity is closely linked to a variety of metabolic disorders, including insulin resistance, atherosclerosis and type 2 diabetes (Eriksson, 2007). 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 (PAI-1) and monocyte chemoattractant protein-1 (MCP-1) (Hotamisligil & Spiegelman, 1994; Shimomura et al., 1996; Berg et al., 2002; Havel, 2004). TNF- α is a major inflammatory adipocytokine that decreases the phosphorylation of insulin receptor substrate-1 (IRS-1), and this event leads to insulin resistance (Hotamisligil et al., 1993). Moreover, because it is well recognized that TNF- α increases the adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and MCP-1, all of which are key mediators involved in atherogenesis, over secretion of TNF- α may induce and accelerate atherosclerosis. On the other hand, adiponectin is a major anti-inflammatory adipocytokine that plays a pivotal role in the improvement of glucose and lipid metabolism and the prevention of atherosclerosis and inflammation (Yamauchi et al., 2002). Further, it has been clarified that adiponectin not only increases insulin sensitivity, but also has anti-atherosclerosis properties which decrease the expression of VCAM-1 and ICAM-1 (Ouchi et al., 1999) and suppresses the proliferation of vascular smooth muscle cells (Arita et al., 2002). In patients with insulin resistance, obesity or type 2 diabetes, serum adiponectin levels are reduced (Arita et al., 1999; Hotta et al., 2000). Further, previous studies showed that TNF- α and intracellular reactive oxygen species (ROS) decrease adiponectin expression (Kim et al., 2005; Soares et al., 2005; Simons et al., 2007). 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 (Adachi et al., 2009).

Adipose tissue has been found to suffer chronic hypoxia during the development of obesity (Brook et al., 1972; Helmlinger et al., 1997), and these conditions may lead to metabolic disorders. Hypoxic conditions can be induced by the addition of certain chemicals called 'hypoxia mimetics', such as the carcinogenic transition metal cobalt (Vincent et al., 1996). It

has been suggested that cobalt stabilizes transcriptional factor, hypoxia-inducible factor-1 α (HIF-1 α), from proteasomal degradation by inhibiting the activity of prolyl hydroxylases (PHDs) through Fe²⁺ substitution (Schofield & Ratcliffe, 2004). In these hypoxic conditions, it has been recognized that accumulated HIF-1 α increases a wide variety of genes including vascular endothelial growth factor (VEGF), heme oxygenase-1 (HO-1), erythropoietin (EPO) and cytochrome p450 (CYP) 3A6 to ensure adaptation to low-oxygen tension (Levy et al., 1995; Lee et al., 1997). On the other hand, the expression of CYP1A2, 2B and 2C are decreased by the activation of HIF-1 α (Olsvik et al., 2006; Fradette et al., 2007). 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 (Schuster et al., 1989; Hosogai et al., 2007).

To protect cells from oxidative stress, mammalian have anti-oxidative enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase (Amstad & Cerutti, 1990; Cerutti et al., 1994). SOD is a major antioxidant enzyme that protects cells from the damaging effects of superoxide by accelerating the dismutation reaction of superoxide by approximately 10,000 times (Faraci, 2003). As shown in Table 1, 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) (McCord & Fridovich, 1969; Keele et al., 1970; Marklund, 1982; Faraci, 2003). Among of three SOD isozymes, EC-SOD is secretory, tetrameric glycoprotein, whereas Cu,Zn-SOD and Mn-SOD are intracellular enzymes found predominantly in the cytoplasm and mitochondria, respectively. EC-SOD is a major SOD isozyme in the extracellular space but is distributed mainly in blood vessel walls (Ookawara et al., 1998). After secretion, EC-SOD slowly diffuses and binds to the heparan sulfate proteoglycans in the glycocalyx on the surface of most cell types in the vascular wall. 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 (Marklund, 1984). 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, but positively related to adiponectin levels (Adachi et al., 2004). Recently, we reported that cobalt chloride (CoCl₂) decreases EC-SOD in green monkey kidney COS7 cells via intracellular ROS generation and p38-MAPK, a mitogen-activated protein kinase

	EC-SOD	Cu,Zn-SOD	Mn-SOD
Molecular mass	135,000	32,000	85,000
Subunit	a4	a2	a4
Metal (atom/subunit)	1Cu, 1Zn	1Cu, 1Zn	1Mn
Rate constant (M ⁻¹ s ⁻¹)	1.25 × 10 ⁹	2 × 10 ⁹	1.25 × 10 ⁹
Location	extracell	cytoplasm	mitochondria
Carbohydrate	+	—	—
Affinity for heparin	+	—	—

Table 1. The properties of SOD isozymes

(MAPK), signaling cascade (Kamiya et al., 2008); however, the mechanisms of EC-SOD and adiponectin reductions during hypoxia remain unclear. Because the EC-SOD content in adipose tissue is comparably high, it is important to elucidate the regulation of EC-SOD may contribute to the control of cytotoxicity induced by intracellular ROS during hypoxia.

In order to address these issues, we studied the regulation of EC-SOD expression by CoCl_2 and examined the role of ROS, inflammatory cytokine such as $\text{TNF-}\alpha$ 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 aggravate metabolic disorders.

2. Methods

2.1 3T3-L1 cell culture

3T3-L1 mouse pre-adipocytes culture and their differentiation into adipocytes were carried out as described in previous report (Adachi et al., 2009). Briefly, pre-adipocytes were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS) until 2 days after confluence; then, their differentiation into adipocytes was induced by treating cells for 2 days with 5 $\mu\text{g/mL}$ insulin, 0.5 μM dexamethasone and 0.5 mM isobutylmethyl-xanthine in DMEM, and then for 2 days with insulin (5 $\mu\text{g/mL}$) in the same medium. The cells were returned to the basal medium, which was replaced every other day. The effects of CoCl_2 and hypoxia on gene expression 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 . After the cells were treated with CoCl_2 or incubated with hypoxia (1% O_2) for 24 h, RT-PCR and measurement of intracellular ROS generation were carried out.

2.2 Oil red O staining

Differentiation of 3T3-L1 mouse pre-adipocytes into mature adipocytes was confirmed by Oil red O staining as described previously (Sakaue et al., 2002). Briefly, the cells were washed twice with ice-cold PBS, and then the cells were fixed with 10% paraformaldehyde for 10 min at room temperature. After fixation, the cells were washed twice with ice-cold PBS and incubated with 60% isopropanol for 1 min followed by incubation with 1.8 mg/mL Oil red O solution for 20 min at room temperature. After the cells were washed twice with ice-cold PBS, Oil red O staining was monitored by microscope.

2.3 Measurement of cellular protein

We measured the cellular protein as an index of cell injury. After 3T3-L1 adipocytes were treated, the medium was aspirated and the cells were washed twice with ice-cold PBS and then scraped in 1 mL 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-Rad Lab., CA, USA).

2.4 Reverse transcriptional polymerase chain reaction (RT-PCR)

After 3T3-L1 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 (Adachi et al., 2009), with minor modifications. Densitometric analysis of the PCR products was performed with Multi Gauge V3.0 (Fuji Film, Japan).

2.5 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 μ M 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H₂DCFDA) for 20 min at 37°C in 5% CO₂/95% air. The cells were then washed twice with ice-cold PBS and then scraped in 1 mL PBS and centrifuged at 2,300 $\times$ g for 5 min at 4°C. The pellet was homogenized with 1 mL PBS using ultrasonic homogenizer and centrifuged at 2,300 $\times$ g for 10 min at 4°C. The DCF fluorescence of the supernatant was measured using a fluorometer (excitation at 493 nm and emission at 527 nm). Total protein concentrations were measured using a protein assay reagent.

2.6 Western blotting

Whole cell extracts were prepared in lysis buffer as described previously (Kamiya et al., 2008). Extracts containing 20 μ g protein were separated by SDS-PAGE on 12% (w/v) polyacrylamide gels followed by transferring electrophoretically onto PVDF membranes. Subsequently, the membranes were incubated with the respective specific primary antibodies (C/EBP homologous protein (CHOP) and Actin). The blots were incubated with biotin-conjugated goat anti-rabbit IgG (for CHOP) or -mouse IgG (for Actin) antibody followed by incubating with ABC reagents (Vector Laboratories, Inc., Burlingame, CA, USA). Finally, the bands were detected using SuperSignal[®] West Pico (Thermo Scientific, Rockford, IL, USA), and imaged using an LAS-3000 UV mini (Fuji Film).

2.7 Statistical analysis

The data shown are the mean $\pm$ 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.

3. Results

3.1 Differentiation of 3T3-L1 mouse pre-adipocytes into mature adipocytes

We first investigated the differentiation of 3T3-L1 pre-adipocytes into mature adipocytes using Oil red O staining. As shown in Fig. 1A, the staining was not observed in 0 day, but observed in 8 days differentiated cells. Further, we measured the expression of adiponectin as an index of differentiation by RT-PCR. The expression of adiponectin was not observed in 0 day, but drastically increased in the 8 day differentiated-adipocytes (Fig. 1B). From these observations, we considered that the cells were differentiated into mature adipocytes, and

we therefore investigated the effect of CoCl_2 on the expression of EC-SOD, other SOD isozymes, adiponectin and transcriptional factors.

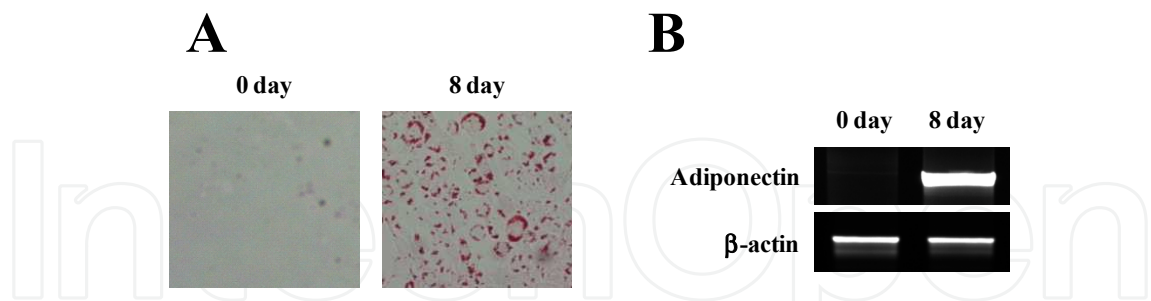


Fig. 1. Differentiation of 3T3-L1 mouse pre-adipocytes into mature adipocytes. 3T3-L1 pre-adipocytes were differentiated by the method described above. After 8 day differentiation, the differentiation degree was evaluated by Oil red O staining (A) and RT-PCR analysis (B).

3.2 Effect of CoCl_2 and hypoxia on the protein content

In order to elucidate the cytotoxicity of CoCl_2 and hypoxia (1% O_2), we measured the protein content of 3T3-L1 adipocytes treated with 0.3 mM CoCl_2 or incubated under hypoxia for the several hours. As shown in Fig. 2, the protein content was not affected by CoCl_2 and so from this we determined the concentrations of CoCl_2 (0 to 0.3 mM) to use in this study. Moreover, the protein content was not affected under hypoxic condition.

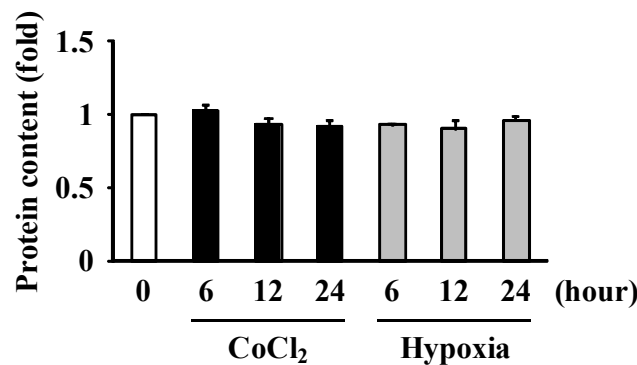


Fig. 2. Effect of CoCl_2 and hypoxia on the protein content. 3T3-L1 adipocytes were treated with 0.3 mM CoCl_2 (closed column) or incubated under hypoxia (1% O_2 ; gray column) for the indicated hours. After the cells were treated, protein contents were measured by using a Bio-Rad protein assay reagent.

3.3 Effect of CoCl_2 on the expression of SODs, adiponectin and transcriptional factors

We next investigated the effect of CoCl_2 on the expression of SODs, adiponectin and transcriptional factors such as C/EBP- α and PPAR- γ by RT-PCR. As shown in Fig. 3, 4, treatment with CoCl_2 decreased the expression of EC-SOD mRNA in a CoCl_2 dose- (Fig. 3) and time-dependent manner (Fig. 4). On the other hand, the other SOD isozymes (Cu,Zn-SOD and Mn-SOD) were not affected 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; 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 CoCl₂ dose- and time-dependent manner. Additionally, the expression of C/EBP- α and PPAR- γ , well recognized as master regulators of adipogenesis and adiponectin, was similar to that of EC-SOD and adiponectin.

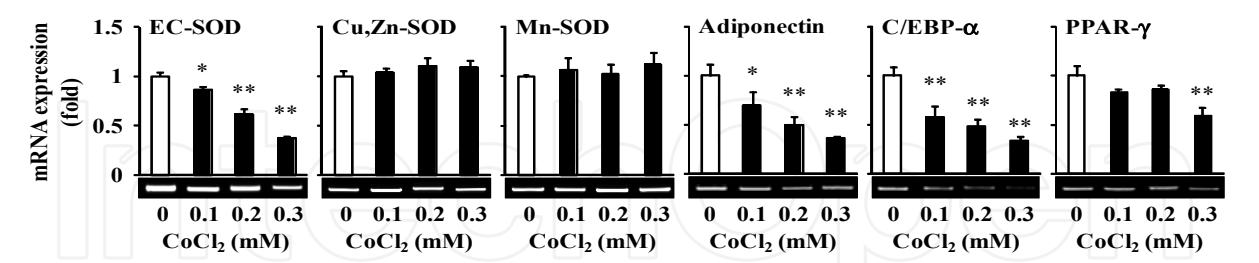


Fig. 3. Dose-dependent effect of CoCl₂ on the expression of SODs, adiponectin and transcriptional factors. 3T3-L1 adipocytes were treated with the indicated concentrations of CoCl₂ for 24 h. After the cells were treated, RT-PCR was carried out and these data were normalized using β -actin levels. (* $p<0.05$, ** $p<0.01$ vs. vehicle).

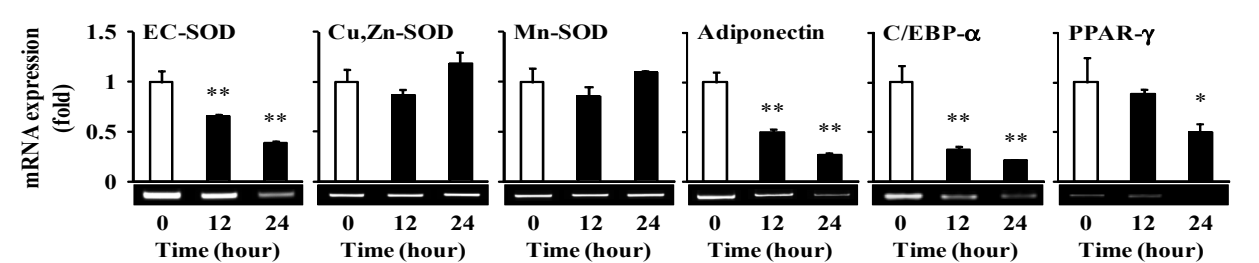


Fig. 4. Time-dependent effect of CoCl₂ on the expression of SODs, adiponectin and transcriptional factors. 3T3-L1 adipocytes were treated with 0.3 mM CoCl₂ for the indicated hours. After the cells were treated, RT-PCR was carried out and these data were normalized using β -actin levels. (* $p<0.05$, ** $p<0.01$ vs. untreated cells).

3.4 Effect of hypoxia on the expression of SODs, adiponectin and transcriptional factors

We further investigated the effect of hypoxia (1% O₂) on the expression of SODs, adiponectin and transcriptional factors. Hypoxia decreased the expression of EC-SOD, adiponectin and C/EBP- α similar to CoCl₂ treatment (Fig. 5); however, Cu,Zn-SOD, Mn-SOD and PPAR- γ were not changed during hypoxia.

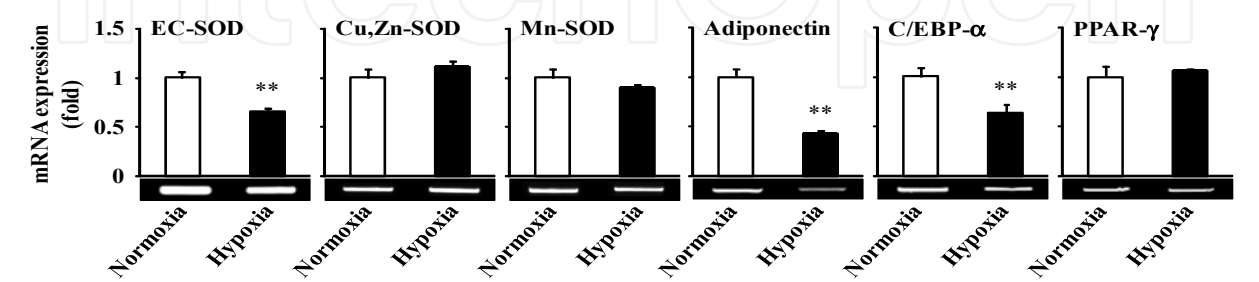


Fig. 5. Effect of hypoxia on the expression of SODs, adiponectin and transcriptional factors. 3T3-L1 adipocytes were incubated under normoxia (20% O₂: open column) or hypoxia (1% O₂: closed column) for 24 h. After the cells were treated, RT-PCR was carried out and these data were normalized using β -actin levels. (** $p<0.01$ vs. normoxia).

3.5 Involvement of ROS in CoCl₂-induced reduction of EC-SOD and adiponectin

We previously reported that intracellular ROS are generated under hypoxic conditions induced by CoCl₂ and lead to decrease the expression of EC-SOD in COS7 cells (Kamiya et al., 2008). Moreover, it has been reported that the expression of adiponectin is also decreased by the accumulation of intracellular ROS in 3T3-L1 adipocytes (Soares et al., 2005). As described in the previous reports, it was speculated that the reduction of EC-SOD and adiponectin was regulated by intracellular ROS-derived signaling. We therefore investigated the involvement of intracellular ROS in these reductions in this study. Treatment with H₂O₂ decreased the expression of EC-SOD and adiponectin, and pretreatment with antioxidant, trolox, partially, but significantly suppressed these reductions (Fig. 6A). We further investigated that generation of intracellular ROS during CoCl₂-treatment using carboxy-H₂DCFDA. As shown in Fig. 6B and C, treatment with CoCl₂ significantly increased intracellular ROS generation in a time-dependent manner and pretreatment with trolox attenuated CoCl₂-triggered ROS generation. However, surprisingly, pretreatment with trolox did not attenuate the reduction of EC-SOD and adiponectin (Fig. 6D), suggesting that the expression of EC-SOD and adiponectin were not regulated by intracellular ROS-derived signaling.

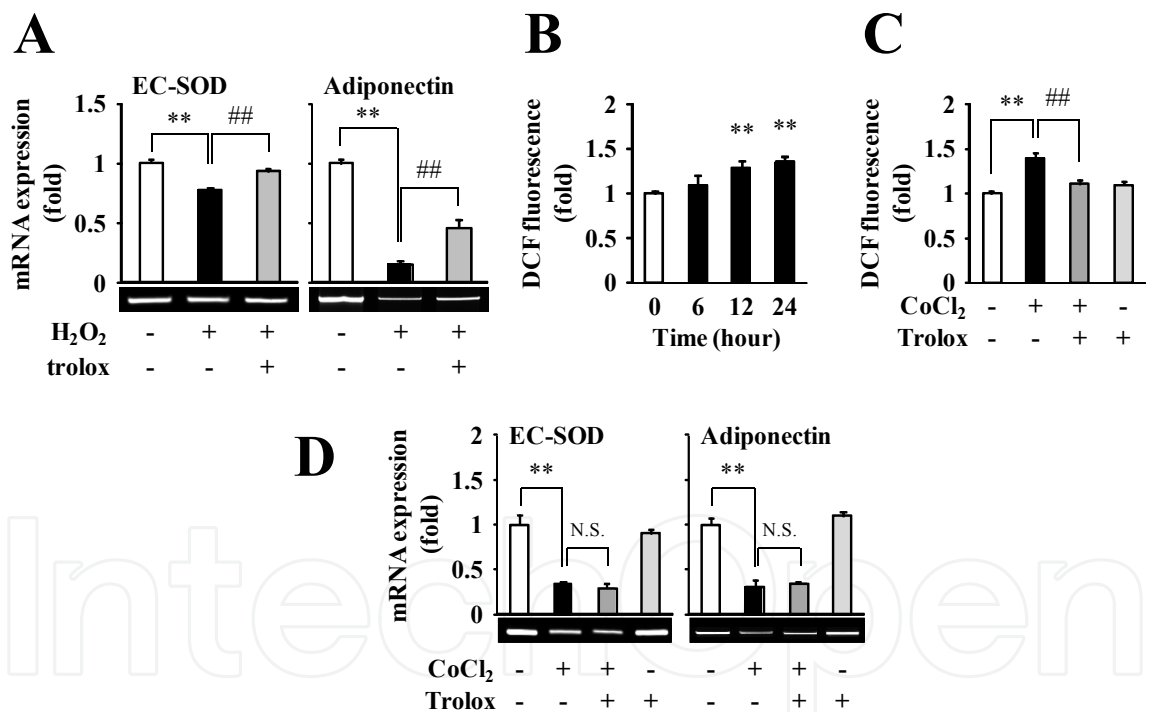


Fig. 6. Involvement of ROS in CoCl₂-induced reduction of EC-SOD and adiponectin. (A) 3T3-L1 adipocytes were pretreated without (–) or with (+) 0.2 mM trolox for 2 h, and then the cells were treated without (–) or with (+) 0.5 mM H₂O₂ for 24 h. (B) The cells were treated with 0.3 mM CoCl₂ for the indicated hours. (C, D) The cells were pretreated without (–) or with (+) 0.2 mM trolox for 2 h, and then the cells were treated without (–) or with (+) 0.3 mM CoCl₂ for 24 h. After the cells were treated, RT-PCR (A, D) and assay of intracellular ROS generation (B, C) were carried out. All RT-PCR data were normalized using β-actin levels. (** *p*<0.01 vs. vehicle (A, C and D) or untreated cells (B), ## *p*<0.01, N.S.: not significant vs. H₂O₂-treated cells (A) or CoCl₂-treated cells (C, D)).

3.6 Involvement of TNF-α in CoCl₂-induced reduction of EC-SOD and adiponectin

It is well recognized that the inflammatory adipocytokines including TNF-α are increased during adipocytes hypertrophy and hypoxic conditions (Ye et al., 2007). Moreover, we previously reported that exposure to TNF-α decreased EC-SOD in several kinds of cells (Adachi et al., 2006, 2009) and adiponectin in 3T3-L1 adipocytes (Adachi et al., 2009). Accordingly, we next investigated the involvement of TNF-α in the reduction of EC-SOD and adiponectin by CoCl₂. Treatment with CoCl₂ increased the expression of TNF-α at the mRNA (Fig. 7A), and pretreatment with actinomycin D, an inhibitor of mRNA synthesis, blocked the CoCl₂-triggered reduction of EC-SOD and adiponectin (Fig. 7B). Further, pretreatment with infliximab, a chimeric monoclonal antibody against TNF-α, partially, but significantly, suppressed the CoCl₂-triggered reduction of these genes (Fig. 7C), indicating that TNF-α plays an important role in the CoCl₂-induced suppression of EC-SOD and adiponectin.

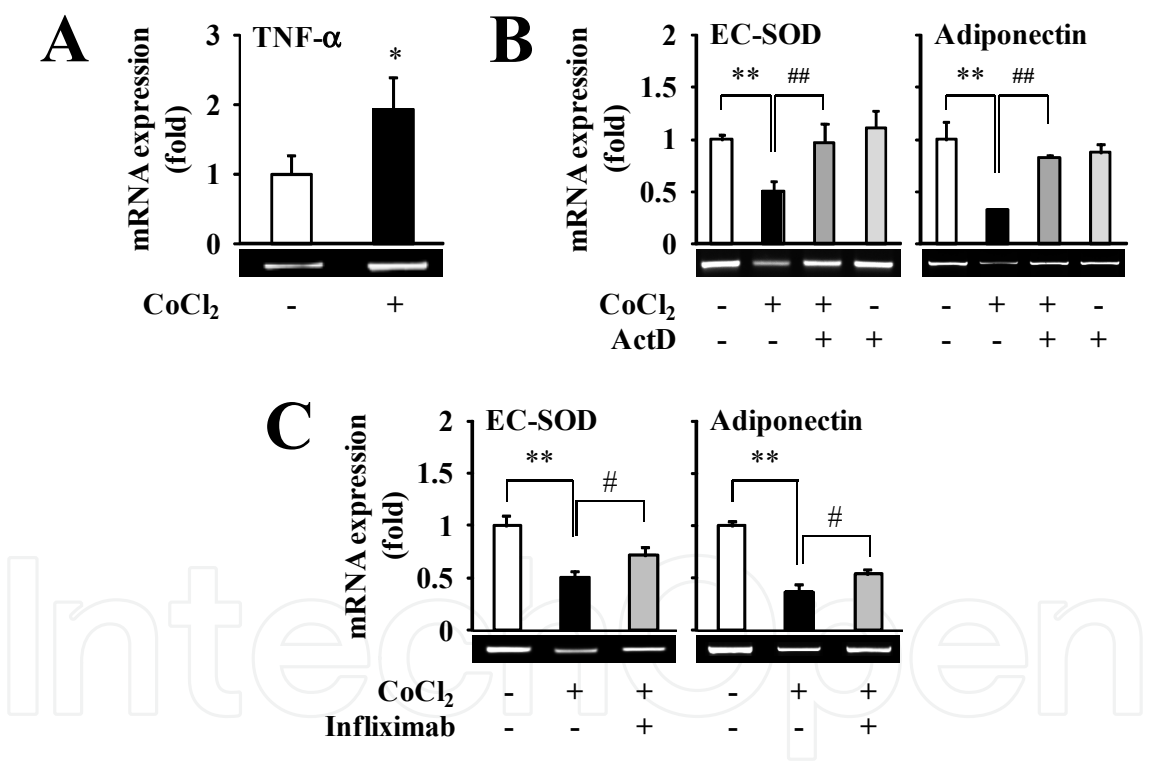


Fig. 7. Involvement of TNF-α in CoCl₂-induced reduction of EC-SOD and adiponectin. (A) 3T3-L1 adipocytes were treated without (–) or with (+) 0.3 mM CoCl₂ for 24 h. (B, C) The cells were pretreated without (–) or with (+) 1 μg/mL actinomycin D (ActD) for 1 h (B) or 10 μg/mL infliximab for 1 h (C), and then the cells were treated without (–) or with (+) 0.3 mM CoCl₂ for 24 h. After the cells were treated, RT-PCR was carried out. All RT-PCR data were normalized using β-actin levels. (* *p*<0.05, ** *p*<0.01 vs. vehicle, # *p*<0.05, ## *p*<0.01 vs. CoCl₂-treated cells).

3.7 Involvement of MAPK in CoCl₂-induced reduction of EC-SOD and adiponectin

In mammalian, there are three types of MAPK, such as c-jun N-terminal kinase (JNK), p38-MAPK and extracellular regulated kinase (ERK) (Kyriakis & Avruch, 2001, Pearson et al., 2001), and play several physiological roles including cell proliferation, differentiation and death. Additionally, we and others reported that activation of MAPK plays important roles in the TNF- α -induced reduction of EC-SOD in vascular smooth muscle cells (VSMCs) and adiponectin in 3T3-L1 adipocytes (Adachi et al., 2006, Kim et al., 2005). We, therefore, investigated the effect of MAPK inhibitors, such as SP600125 (for JNK), SB203580 (for p38-MAPK) and U0126 (for ERK) on the reduction of EC-SOD and adiponectin. Pretreatment with SP600125, SB203580 or U0126 did not affect the basal expression of EC-SOD and adiponectin (data not shown), and SP600125 significantly suppressed the CoCl₂-triggered reduction of EC-SOD and adiponectin, but others had no effects (Fig. 8), indicating that the expression of EC-SOD and adiponectin are regulated by JNK signaling cascades.

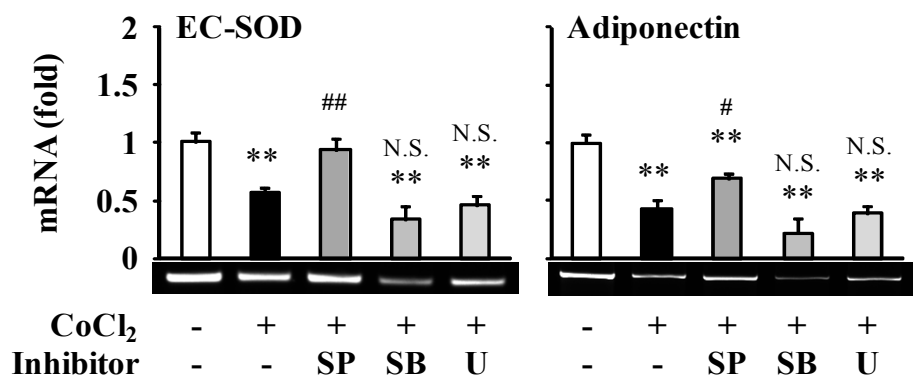


Fig. 8. Involvement of MAPK in CoCl₂-induced reduction of EC-SOD and adiponectin. 3T3-L1 adipocytes were pretreated without (–) or with 50 μ M SP600125 (SP), SB203580 (SB) or U0126 (U) for 30 min, and then the cells were treated without (–) or with (+) 0.3 mM CoCl₂ for 24 h. After the cells were treated, RT-PCR was carried out. All RT-PCR data were normalized using β -actin levels. (** $p < 0.01$ vs. vehicle, # $p < 0.05$, ## $p < 0.01$, N.S.: not significant vs. CoCl₂-treated cells).

3.8 CoCl₂ did not induce ER stress in 3T3-L1 adipocytes

It is well recognized that hypoxia is associated with endoplasmic reticulum (ER) stress, which induces metabolic disorders (Ozcan et al., 2004). Because the inhibitory effect of infliximab and actinomycin D on the reduction of EC-SOD and adiponectin by CoCl₂ was partial, it was speculated that the involvement of other mechanisms, such as ER stress. Therefore, we finally investigated the involvement of ER stress in the reduction of EC-SOD and adiponectin. Treatment with ER stress inducer, thapsigargin, significantly increased the expression of ER stress markers, glucose regulated-protein 78 kDa (GRP78) and CHOP; however, treatment with CoCl₂ did not affect the expression of these genes (Fig. 9A, B), suggesting that CoCl₂ decreased the expression of EC-SOD and adiponectin through ER stress-independent mechanisms.

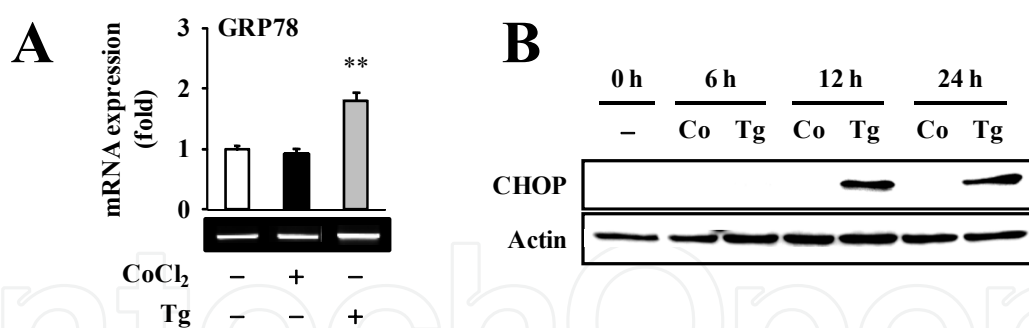


Fig. 9. Effect of CoCl₂ and thapsigargin on the expression of GRP78 and CHOP. (A) 3T3-L1 adipocytes were treated with 0.3 mM CoCl₂ or 1 μ M thapsigargin (Tg) for 24 h. (B) The cells were treated with 0.3 mM CoCl₂ (Co) or 1 μ M Tg for the indicated hours. After treatment, RT-PCR (A) and western blotting (B) were carried out. RT-PCR data were normalized using β -actin levels. (** $p < 0.01$ vs. vehicle).

4. Discussion

It is well known that obesity, especially visceral fat accumulation, is closely related to a variety of metabolic disorders, including insulin resistance, atherosclerosis and type 2 diabetes (Fox et al., 2007). Further, 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 during adipocyte hypertrophy (Ye et al., 2007), all of which might lead metabolic disorder exacerbation. Additionally, it has been reported that adipose tissue suffers chronic hypoxia during above process (Brook et al., 1972; Helmlinger et al., 1997), and lead to disturb adipose homeostasis by excessive production of ROS and TNF- α , or decrease of adiponectin. In this study, chemical hypoxia mimetic, CoCl₂, decreased the expression of adiponectin and adipogenic master regulators, including C/EBP- α and PPAR- γ , in a CoCl₂ dose- and time-dependent manner (Fig. 3, 4), and these observations were similar to hypoxia (1% O₂ incubation), except for PPAR- γ (Fig. 5). From these observations and previous reports, 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 has been well known that both hypoxia and hypoxia mimetics increase intracellular ROS generation through mitochondria-dependent and -independent mechanisms, respectively (Agani et al., 2000; Chandel et al., 1998). Similar to previous reports, we confirmed the intracellular ROS accumulation during CoCl₂ treatment (Fig. 6B), suggesting that hypoxic adipocytes suffered from oxidative stress. To protect cells from the damaging effect of ROS, mammalian have several kinds of anti-oxidative enzymes, such as SOD, catalase and glutathione peroxidase. EC-SOD is the major SOD isozyme in extracellular fluids and protects cells from superoxide. We previously reported that the expression level of EC-SOD through the differentiation of 3T3-L1 adipocytes changes in a similar manner to adiponectin, C/EBP- α and PPAR- γ , and down-regulation of these proteins might induce and/or promote the pathogenesis of metabolic syndrome and atherosclerosis. In fact, it was found that the plasma EC-SOD levels in type 2 diabetic patients were significantly and positively related to adiponectin levels (Adachi et al., 2004). Because, the EC-SOD content of adipose tissue was relative highly expressed compared to other organs, such as liver, heart and brain, it might be important to elucidate the regulation

mechanism of that gene during hypoxia for the control of metabolic disorders. Recently, we reported that CoCl_2 decreases the expression of EC-SOD in COS7 cells via intracellular ROS generation. In this study, treatment with CoCl_2 (Fig. 3, 4), hypoxia (Fig. 5) and H_2O_2 (Fig. 6A) significantly decreased the expression of EC-SOD and adiponectin, but not of Cu,Zn-SOD and Mn-SOD. However, interestingly, an antioxidant, trolox, did not affect the reduction of EC-SOD and adiponectin by CoCl_2 in spite of suppressing the effect of H_2O_2 on these genes (Fig. 6A). From these observations, it is speculated that CoCl_2 decreases EC-SOD and adiponectin by intracellular ROS-independent, but another mechanisms.

So far, it has been reported that the expression of EC-SOD was regulated by several kinds of cytokines, such as $\text{TNF-}\alpha$, transforming growth factor- β (TGF- β), IL-4 and interferon- γ (IFN- γ) (Marklund, 1992, Str  lin & Marklund, 2000). Among of them, it has been well recognized that $\text{TNF-}\alpha$ is one of the most important adipocytokines involved in metabolic disorders, including insulin resistance, atherosclerosis and type 2 diabetes by decreasing the phosphorylation of IRS-1 and inducing adhesion molecules, such as VCAM-1, ICAM-1 and MCP-1. Moreover, we and others showed that $\text{TNF-}\alpha$ decreases EC-SOD and adiponectin in VSMCs and 3T3-L1 cells, respectively (Adachi et al., 2006, Kim et al., 2005). Accordingly, we investigated the involvement of $\text{TNF-}\alpha$ in the reduction of EC-SOD and adiponectin by the addition of CoCl_2 . Treatment with CoCl_2 significantly increased $\text{TNF-}\alpha$ expression (Fig. 7A), and infliximab partially, but significantly, attenuated the reduction of EC-SOD and adiponectin by CoCl_2 (Fig. 7C). Additionally, an inhibitor of mRNA synthesis, actinomycin D, blocked the reduction of these expressions (Fig. 7B). These results suggested that CoCl_2 decreases EC-SOD and adiponectin via $\text{TNF-}\alpha$; 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. Recently, it has been well known that hypoxia induces ER stress, which induces the apoptotic process (Kuznetsov et al., 1996). To assess the induction of ER stress by CoCl_2 , we measured the expression of ER stress marker, GRP78 and CHOP. Treatment with ER stress inducer, such as thapsigargin, drastically increased the expression of GRP78 and CHOP at mRNA and protein levels, but treatment with CoCl_2 did not induce the ER stress (Fig. 9), suggesting that CoCl_2 decreased the expression of EC-SOD and adiponectin through ER stress-independent mechanisms.

We finally investigated the role of MAPK in the reduction of EC-SOD and adiponectin. There are three major subfamilies: JNK, p38-MAPK and ERK. Moreover, we previously reported that the expression of EC-SOD was regulated by p38-MAPK signaling cascades in CoCl_2 -treated COS7 cells (Kamiya et al., 2008). However, in this study, pretreatment with a JNK inhibitor, SP600125, suppressed the reduction of EC-SOD and adiponectin but others, SB203580 and U0126, did not affect these reductions, indicating that CoCl_2 decreases EC-SOD and adiponectin through JNK signaling cascades. In our previous report, we showed the involvement of ROS and p38-MAPK in the regulation of EC-SOD in COS7 cells (Kamiya et al., 2008); however, in this study, we did not observe the involvement of intracellular ROS and p38-MAPK in the reduction of EC-SOD by CoCl_2 . From these observations, we speculated that the difference of signal molecules leads the activation of different MAPK involved in the regulation of EC-SOD. It has been shown that the expression of mouse EC-SOD is regulated by transcriptional factors such as Sp1, Sp3, Ets, Kruppel-like and myeloid zinc finger-1 those bind to the corresponding cis-element in the promoter region (Zelko et

al., 2003, 2004, 2008). According to previous reports, we hypothesized that the regulation of EC-SOD might be also mediated by these transcriptional factors.

5. Conclusion

The EC-SOD content is very low compared to other SODs in parenchymal cells. However, because it is known that adipose tissue has a moderately high content of EC-SOD, EC-SOD might have an important protective role as an anti-inflammatory factor. In this study, we demonstrated that the expression of EC-SOD was co-regulated with adiponectin through TNF- α and JNK signaling cascades. From these observations, it is speculated that the reduction of EC-SOD by hypoxic conditions is similar to adiponectin and leads to a decrease in the resistance to oxidative stress. Overall, it is speculated that the reduction of EC-SOD leads to decreased resistance to oxidative stress and accelerates ROS-derived diseases, and prevents the reduction of EC-SOD, contributing to the control of redox homeostasis under hypoxic conditions.

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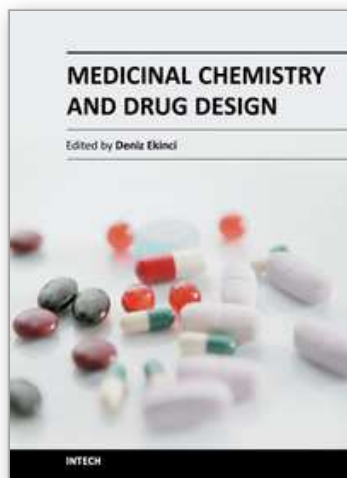
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