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Cobalt chloride decreases EC-SOD expression through intracellular ROS generation and p38-MAPK pathways in COS7 cells

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Abstract

It is known that cells suffer a chronic hypoxic condition during the development of proximal tubulointerstitial disease. However, it is accepted that extracellular-superoxide dismutase (EC-SOD) protects the cells from oxidative stress. The purpose of this study was to elucidate the regulation of EC-SOD expression in cells under hypoxia. The results show that the expressions of EC-SOD mRNA and protein in cobalt chloride ($CoCl_2$)-treated COS7 cells decreased in a dose- and time-dependent manner, whereas the expressions of other SOD isoforms (Cu/Zn-SOD and Mn-SOD) were not changed. The down-regulation of EC-SOD mRNA was suppressed by pre-treatment with the antioxidant trolox and the p38 mitogen-activated protein kinase (p38-MAPK) inhibitor SB203580. It is concluded that the expression of EC-SOD is decreased through ROS and p38-MAPK signalling cascades and that the down-regulation of EC-SOD leads to a decrease in the resistance to oxidative stress of COS7 cells under hypoxia induced by $CoCl_2$.

Keywords: *Extracellular-superoxide dismutase, reactive oxygen species, hypoxia, cobalt chloride, p38 mitogen-activated protein kinase*

Introduction

Reactive oxygen species (ROS) are known to be implicated in a variety of pathological processes including asthma [1], diabetes and atherosclerosis [2]. The superoxide anion, a primary species of ROS, is scavenged by superoxide dismutase (SOD) [3]. In mammalian systems, there are three types of SOD isozymes: copper and zinc containing SOD (Cu/Zn-SOD), manganese containing SOD (Mn-SOD) and extracellular-SOD (EC-SOD) [4]. EC-SOD is a secretary enzyme, whereas Cu/Zn-SOD and Mn-SOD are intracellular enzymes found predominantly in the cytoplasm and mitochondria, respectively. EC-SOD is found in most tissues [5]; but higher amounts of EC-SOD are present in the vascular tissue [6], while EC-SOD is only present in small amounts in the liver, brain and heart compared with Cu/Zn-SOD and Mn-SOD [7]. EC-SOD is known to be produced in renal tubular cells [8] and glomerular mesangial cells [9] and has an affinity for heparin-like substances [10] and the glycosaminoglycans found on vascular endothelial cell surfaces [11]. After secretion, EC-SOD slowly

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diffuses and binds to heparan sulphate proteoglycan ligands, which are found on the surface of most types of cells in the vascular wall. The presence of EC-SOD on the vascular wall might have an important protective effect against the ROS generated in the vascular system [12].

Recently, the number of diabetes patients has been increasing around the world and diabetes induces many kinds of diseases including nephropathy, retinopathy and peripheral neuropathy. It has been reported that proximal tubulointerstitial disease is easily acquired in diabetes patients and it is thought that the tubular cells are under chronic hypoxic conditions during the development of this disease [13]. Hypoxic conditions can be induced by certain chemicals called 'hypoxia mimetics' such as the carcinogenic transition metal cobalt [14]. It has been suggested that cobalt stabilizes hypoxia-inducible factor-1α (HIF-1α) from proteosomal degradation by inhibiting the activity of prolyl hydroxylases (PHDs) through Fe^{2+} substitution [15]. In many cell types, both hypoxia and hypoxia mimetics promote the expression of erythropoietin (EPO), vascular endothelial growth factor (VEGF) and hemeoxygenase-1 (HO-1) through HIF-1α stabilization [16–18] and also increase intracellular ROS generation [17,19]. Further, it has been reported that the mitogen-activated protein kinase (MAPK) pathways are activated in response to ROS [20–23]. In mammalian systems, there are three major sub-families: the extracellular regulated kinases (ERKs), the c-jun N-terminal kinases (JNKs) and p38-MAPK [24,25]. p38-MAPK is activated by $CoCl_2$ through intracellular ROS generation [21–23,26] and is known to regulate cell viability [27,28]. Additionally, we previously reported that p38-MAPK regulates the tumour necrosis factor-α (TNF-α) induced down-regulation of EC-SOD [29]. However, the relationships between EC-SOD-expression, ROS and p38-MAPK signalling cascades in hypoxic conditions 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 and p38-MAPK signalling cascades in these processes.

Materials and methods

COS7 cell cultures

COS7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), 100 units/mL of penicillin and 100 μg/mL of streptomycin. The cells were kept in an atmosphere of 5% CO_2/95% air at 37°C. The cells were grown to confluence in a 6- or 10-cm culture dish or a 96-well microplate and then the medium was replaced with DMEM supplemented with 0.5% calf serum (CS) and antibiotics 24 h before exposure to the test reagents. The conditioned medium was collected at the time indicated for the assay of EC-SOD concentration and the cells were washed with ice-cold phosphate buffered saline (PBS) and then used for the mRNA assay and measurement of intracellular ROS.

Measurement of cell viability

An MTT assay was used to estimate the cytotoxicity of $CoCl_2$. Following treatment of the COS7 cells in a 96-well microplate with $CoCl_2$ for 24 h, the culture medium was aspirated and the cells were added to 110 μL of 10% FCS-DMEM containing CT01-5 (CHEMICON Int., Inc., CA) and were then incubated for 3 h at 37°C in a humidified atmosphere of 5% CO_2/95% air. After incubation, the cells were added to 100 μL of isopropanol containing 0.04 N HCl and were then mixed thoroughly to dissolve the MTT formazan. Finally, the MTT formazan formed was measured at 595 nm with a reference wave length of 655 nm.

Measurement of intracellular ROS

After the COS7 cells were treated, the medium was aspirated and the cells were washed twice with PBS and incubated with fresh culture medium without serum containing 10 μm dihydroethidium (DHE) for 20 min at 37°C in 5% CO_2/95% air. The cells were then washed twice with ice-cold PBS and then scraped in 1 mL of PBS and centrifuged at 2300 × g for 5 min at 4°C. The pellet was homogenized with 1 mL of ice-cold PBS using an ultrasonic homogenizer and was then centrifuged again at 2300 × g for 10 min at 4°C. The hydroethidium (HE) fluorescence of the supernatant was measured using a fluorometer (excitation at 488 nm and emission at 610 nm). The total protein concentrations were measured using a protein assay reagent (Bio-Lad Lab., CA).

RT-PCR analysis

After the COS7 cells were treated, the medium was aspirated and the cells were washed twice with ice-cold PBS. The cells were lysed in 1 mL of TRIzol® reagent (Invitrogen, CA). The preparation of the cDNA and the RT-PCR were performed by the methods described in our previous report [30]. Densitometric analysis of the PCR products was performed with Multi Gauge V3.0 (Fuji Film, Japan).

ELISA of SODs concentrations

The EC-SOD concentration in the conditioned medium and the cells was determined by enzyme-linked immunosolbent assay (ELISA) described in our previous report [31], with minor modifications. We had confirmed that EC-SOD concentration was

closely correlated with SOD activity [32]. The Cu/Zn-SOD and Mn-SOD concentrations in the cells were determined by using Cu/Zn-SOD ELISA system and Mn-SOD ELISA system (GE Healthcare Biosciences, Tokyo, Japan), respectively.

Western blotting

After the COS7 cells were treated, the medium was aspirated and the cells were washed twice with ice-cold PBS and then scraped in 200 μL of lysis buffer (20 mm HEPES, pH 7.4, containing 150 mm NaCl, 1 mm EDTA, 1 mm EGTA, 10 mm NaF, 1 mm Na_3VO_4, 20 mm β–glycerophosphate, 1 mm phenylmethylsulphonyl fluoride, 1 mm dithiothreitol (DTT), 2 μg/mL leupeptin and 0.5% Nonidet® P-40). After extraction on ice for 30 min, the samples were centrifuged at 2300 × g for 5 min at 4°C and the total protein concentrations in the supernatant were measured using a protein assay reagent. Fifty micrograms of protein were boiled with sample buffer (62.5 mm Tris-HCl, pH 6.8, containing 2% sodium dodecylsulphate (SDS), 10% glycerol, 50 mm DTT and 0.01% bromophenol blue) for 5 min and separated by SDS-PAGE on 12% (w/v) polyacrylamide gels. After being transferred electrophoretically onto PVDF membranes, their non-specific binding sites were blocked with 1% bovine serum albumin (BSA) in TBS (10 mm Tris-HCl, pH 7.5, containing 100 mm NaCl) for 1 h at room temperature. Subsequently, the membranes were incubated overnight with the appropriate primary antibody (1:1000) at 4°C. After the membranes had been washed twice with TBST (TBS containing 0.1% Tween 20), the blots were incubated with biotin-conjugated goat anti-rabbit IgG antibody (1:1000) for 1 h at room temperature. After the membranes had been washed twice with TBST, the blots were incubated with ABC reagents (Vector Laboratories, Inc., Burlingame, CA) (1:5000) for 30 min at room temperature. After the membranes had been washed with TBST, TBS and water twice, the bands were detected using SuperSignal® West Pico (Thermo Scientific Rockford, IL) and imaged using an LAS-3000 UV mini (Fuji Film, Japan).

Statistical analysis

Data were analysed by the student's *t*-test. A *p*-value less than 0.05 was considered significant.

Results

The effect of $CoCl_2$ on viability and intracellular ROS generation in COS7 cells

In order to elucidate the cytotoxicity of $CoCl_2$, we measured the viability of COS7 cells treated with various concentrations of $CoCl_2$ for 24 h. As shown in Figure 1A, the viability was not affected by $CoCl_2$-treatments below 300 μm and so from this we determined the concentrations of $CoCl_2$ (0–300 μm) to use in this study. As intracellular ROS generation is known to be increased in cells under hypoxic conditions [33–35], we measured the intracellular ROS generation in $CoCl_2$-treated COS7 cells using DHE. $CoCl_2$ increased ROS generation in a dose- (Figure 1B) and time- (Figure 1C) dependent manner. These results indicate that $CoCl_2$ induced intracellular ROS generation at concentrations which did not affect cell viability.

The effect of $CoCl_2$ on HO-1 mRNA expression in COS7 cells

It has been reported that the intracellular antioxidant enzyme HO-1 is induced during hypoxia by the HIF-1α system [16,35]. We confirmed that the expression of HO-1 mRNA was increased in a dose-dependent manner in our experimental conditions (Figure 1D). In the time-course experiment, the expression of HO-1 mRNA was drastically increased at 6 h compared with the untreated cells and the expression was maintained until 24 h (Figure 1E).

The effect of $CoCl_2$ on SODs expression in COS7 cells

We next investigated the effect of $CoCl_2$ on the mRNA expression of SODs (EC-SOD, Cu/Zn-SOD and Mn-SOD) in COS7 cells. When the applied concentration of $CoCl_2$ was 300 μm, the expression of EC-SOD mRNA was significantly decreased after 24 h, whereas the mRNA expression of Cu/Zn-SOD and Mn-SOD were not changed (Figure 2A). Further, we observed a decrease of EC-SOD expression caused by $CoCl_2$ at the protein level (Figure 2B). In the time-course experiment, the expression of EC-SOD was not changed up to 6 h. However, the EC-SOD expression was significantly decreased at 12 h compared with the untreated cells (Figure 2C).

The effects of hypoxia (1% O_2) and desferioxamine on the ROS generation and expression of HO-1 and EC-SOD

In order to elucidate the participation of HIF-1α on the down-regulation of EC-SOD, we investigated the ROS generation, HO-1 and EC-SOD expressions by hypoxia (1% O_2) or desferioxamine (DFO), a hypoxia mimetic. It is well known that DFO stabilizes HIF-1α from degradation by inhibiting the activity of the PHDs through Fe^{2+} substitution and not generate intracellular ROS [36]. In this study, hypoxia (1% O_2) increased ROS generation and HO-1 mRNA expression and decreased EC-SOD mRNA expression (Figure 3A and B). On the other hand, DFO did not generate intracellular ROS (Figure 3C), but increased HO-1 expression (Figure 3D). Interestingly, DFO did not affect the expression of EC-SOD (Figure 3D). From these results, we

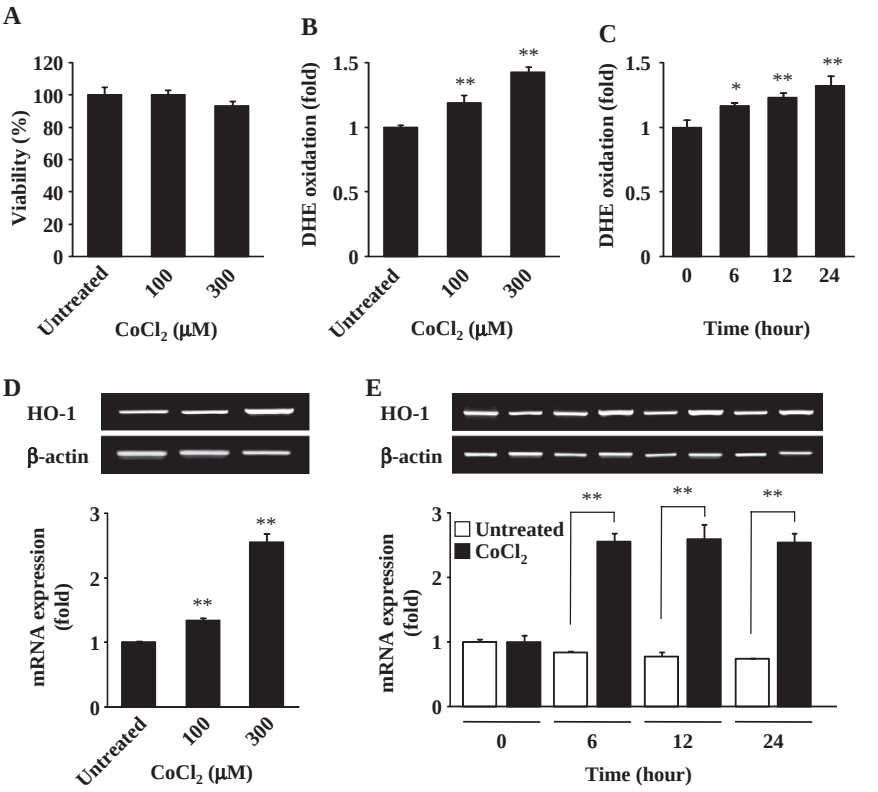

Figure 1. The effect of $CoCl_2$ on cell viability and the induction of HO-1 in COS7 cells. COS7 cells were treated with the indicated concentrations of $CoCl_2$ for 24 h (A, B and D) or with 300 μm $CoCl_2$ for the indicated time (C and E). After treatment, the cell viabilities (A) and ROS generation (B and C) were measured, and RT-PCR (D and E) was carried out and these data were normalized using β–actin levels (* $p<0.05$, ** $p<0.01$ vs untreated cells).

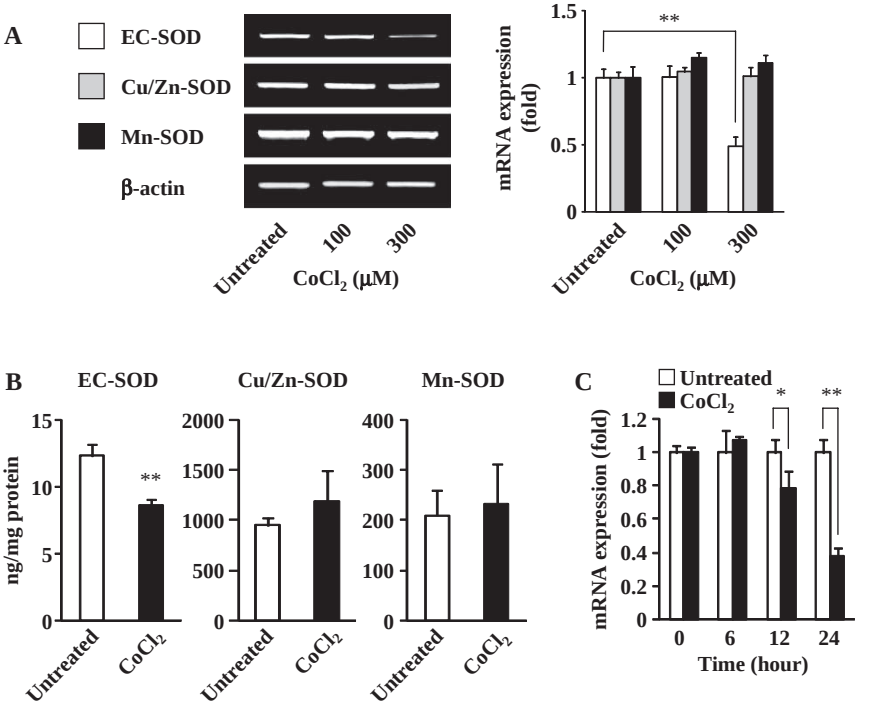

Figure 2. The effect of $CoCl_2$ on the expression of SODs in COS7 cells. COS7 cells were treated with the indicated concentrations of $CoCl_2$ (A), 300 μm $CoCl_2$ (B) for 24 h or they were treated with or without 300 μm $CoCl_2$ for the indicated time (C). After treatment, RT-PCR (A and C) and ELISA (B) were carried out. All RT-PCR data were normalized using β–actin levels (* $p<0.05$, ** $p<0.01$ vs untreated cells).

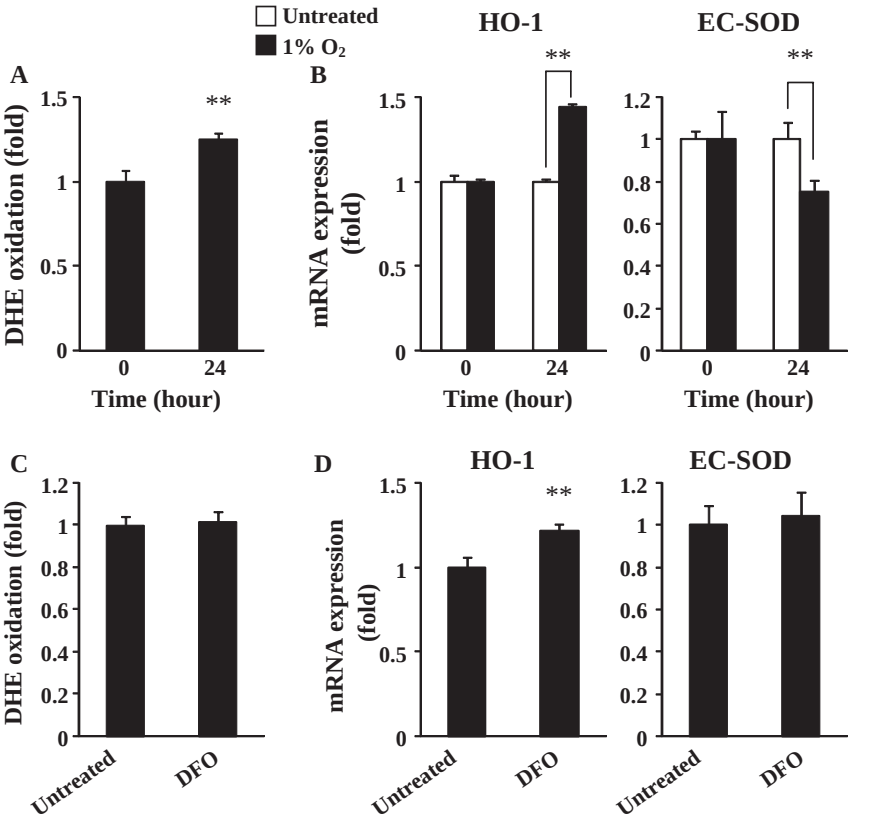

Figure 3. The effects of hypoxia and DFO on the intracellular ROS generation and the expression of HO-1 and EC-SOD in COS7 cells. COS7 cells were treated with 1% O_2 for the indicated time (A, B) or with 300 μm DFO for 24 h (C, D). After treatment, ROS generation (A, C) was measured and RT-PCR (B, D) was carried out. All RT-PCR data were normalized using β–actin levels (** $p < 0.01$ vs untreated cells).

speculated that the expression of EC-SOD is not regulated by HIF-1α system, but regulated by another pathway such as ROS signalling cascade.

The effects of ROS and p38-MAPK signalling cascades on EC-SOD expression in COS7 cells

To clarify the contribution of intracellular ROS on EC-SOD expression, we investigated the effect of the antioxidant reagent trolox, a water-soluble analogue of vitamin E. Trolox mitigated $CoCl_2$-induced intracellular ROS generation (Figure 4A) and suppressed the down-regulation of EC-SOD (Figure 4B). We observed that the expression of EC-SOD mRNA was decreased by 70% in the presence of 300 μm H_2O_2 compared to the untreated cells (Figure 4C). From these results, we confirmed that the expression of EC-SOD is regulated through intracellular ROS generation.

It has been reported that p38-MAPK is activated in hypoxic conditions and regulated by intracellular ROS [21]. We next investigated the effect of p38-MAPK on EC-SOD expression. Results show that $CoCl_2$ induced the phosphorylation of p38-MAPK at 1 h (Figure 5A). We further investigated the effect of the p38-MAPK inhibitor SB203580 on EC-SOD expression. SB203580 did not affect the expression of HO-1 (Figure 5B), but mitigated $CoCl_2$-induced EC-SOD down-regulation (Figure 5C). From these results, the expression of EC-SOD is also regulated through p38-MAPK signalling cascades.

Discussion

The present study demonstrates for the first time that EC-SOD in COS7 cells was suppressed by the addition of $CoCl_2$, a hypoxia mimetic reagent, and is inversely regulated by the activation of p38-MAPK.

It is well known that $CoCl_2$ induces hypoxic conditions in a variety of cells and increases the expressions of EPO, VEGF, HO-1 and cytochrome P-450 (CYP) 3A6 through HIF-1α stabilization [37–39]. On the other hand, it has been reported that the expressions of CYP1A2, 2B and 2C are suppressed by the HIF-1α system [40,41] and changes of expression are observed in these genes a short time after hypoxia and/or the appearance of $CoCl_2$ [39,42]. In this study, $CoCl_2$ (Figure 1D and E), hypoxia (Figure 3B) and DFO (Figure 3D) increased HO-1 expression. On the other hand, $CoCl_2$ and hypoxia increased ROS generation and decreased EC-SOD expression, but DFO did not

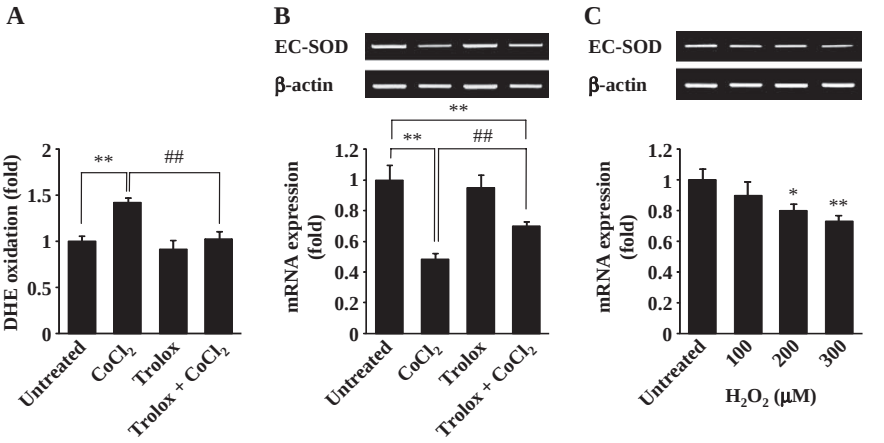

Figure 4. The effects of ROS on the expression of EC-SOD in COS7 cells. COS7 cells were pretreated with or without 200 μm trolox for 2 h and then treated with or without 300 μm $CoCl_2$ for 24 h. ROS generation (A) was measured and RT-PCR was carried out (B). COS7 cells were treated with the indicated concentration of H_2O_2 for 24 h. After treatment, RT-PCR was carried out (C). All RT-PCR data were normalized using β–actin levels (* $p<0.05$, ** $p<0.01$ vs untreated cells, ## $p<0.01$ vs $CoCl_2$-treated cells).

affect ROS generation and EC-SOD expression (Figure 1–3). From these results, we speculated that the expression of EC-SOD was not regulated by HIF-1α system, but regulated by another pathway such as ROS signalling cascades.

It has been reported that ROS are generated in many kinds of diseases and are released into the extracellular fluid; where the ability to resist oxidative stress is poorer than that of the intracellular fraction. There are several extracellular antioxidants such as EC-SOD that play an important role [6], but many researchers have focused on the role of intracellular antioxidants such as Cu/Zn-SOD and Mn-SOD that eliminate ROS from

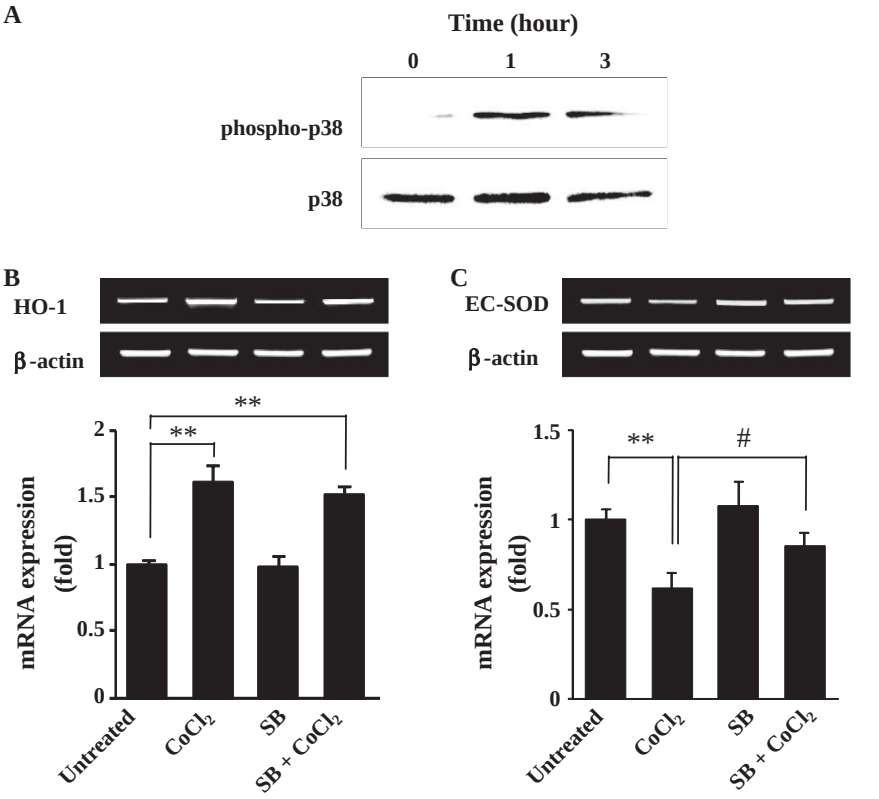

Figure 5. The effects of p38-MAPK on the expression of HO-1 and EC-SOD in COS7 cells. COS7 cells were treated with 300 μm $CoCl_2$ for the indicated time. The cell lysates were analysed by Western blot using the indicated antibodies (A). COS7 cells were pre-treated with or without 1 μm of SB203580 (SB) for 30 min and then treated with or without 300 μm $CoCl_2$ for 24 h. After treatment, RT-PCR was carried out. All RT-PCR data were normalized using β–actin levels (B and C) (** $p<0.01$ vs untreated cells, # $p<0.05$ vs $CoCl_2$-treated cells).

the intracellular environment. In this study, the expression of EC-SOD, but not Cu/Zn-SOD and Mn-SOD, was decreased in COS7 cells treated with $CoCl_2$ (Figure 2A and B). Further, it has been reported that EC-SOD has a protective role against systemic inflammation induced by lipopolysaccharides (LPS) and survival rates in the transgenic mice that constitutively express EC-SOD were enhanced compared to wild-type mice after LPS injection [43]. From these results, we speculated that ROS accumulation and the decline of antioxidative ability of EC-SOD induce a vicious cycle that aggravates cytotoxicity in COS7 cells during hypoxia.

To elucidate the mechanism that down-regulates EC-SOD expression, we investigated the effect of ROS on the expression of EC-SOD. It has been reported that the expression of EC-SOD was decreased by several cytokines, such as TNF-α [29,44] and increased by interferon-γ [45], whereas the effects of ROS on the expression of EC-SOD were not apparent. It has been reported that the expression of the antioxidant enzymes HO-1 and Cu/Zn-SOD were decreased by the excess generation of intracellular ROS [46,47]. From our observations that the down-regulation of the EC-SOD mRNA level was partially suppressed by trolox pretreatment (Figure 4B) and that the expression of EC-SOD was decreased by the addition of H_2O_2 (Figure 4C), it is possible that the expression of EC-SOD could be regulated by intracellular ROS generation. We further investigated the mechanisms that regulate EC-SOD expression. It has been reported that p38-MAPK, one of the MAPK family, is activated in hypoxic conditions by intracellular ROS [21]. Moreover, we have previously reported that the expression of EC-SOD is regulated by p38-MAPK in human smooth muscle cells stimulated by TNF-α [29]. In this study, the phosphorylation of p38-MAPK was increased by $CoCl_2$ (Figure 5A) and the p38-MAPK inhibitor SB203580 mitigated $CoCl_2$-induced EC-SOD down-regulation (Figure 5C). From these results, we concluded that the expression of EC-SOD is also regulated through p38-MAPK signalling cascades.

In this study, we investigated the expression of EC-SOD in COS7 cells during hypoxia. Our results suggest that the expression of EC-SOD is decreased by the addition of $CoCl_2$ and that the down-regulation of EC-SOD is regulated through intracellular ROS generation and p38-MAPK signalling cascades. In proximal tubulointerstitial disease, it is known that intracellular ROS induces cytotoxicity in the kidney and peripheral tissues [13,34]. From our results, it is speculated that the reduction of EC-SOD leads to a decrease in the resistance to oxidative stress and these findings contribute to the control of kidney disease aggravation and knowledge about cytotoxicity induced by intracellular ROS during hypoxia.

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