CoCl₂ Decreases EC-SOD Expression through Histone Deacetylation in COS7 Cells

Shuhei Hattori,⁎ Tetsuro Kamiya,⁎ a Hirokazu Hara,⁎ Masayuki Ninomiya,⁎ b Mamoru Koketsu,⁎ and Tetsuo Adachi⁎

⁎Laboratory of Clinical Pharmaceutics, Gifu Pharmaceutical University; 1–25–4 Daigaku-nishi, Gifu 501–1196, Japan; and ⁎ Department of Chemistry and Biomolecular Science, Faculty of Engineering, Gifu University; 1–1 Yanagido, Gifu 501–1193, Japan.

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Extracellular-superoxide dismutase (EC-SOD), one of the SOD isozymes, is negatively regulated under hypoxic conditions, and decreases in its expression may exacerbate vascular diseases. Moreover, epigenetics, such as DNA methylation and histone modifications, are known to play a critical role in the progression of cancer, type 2 diabetes, and atherosclerosis. We previously investigated the involvement of reactive oxygen species (ROS) and p38 mitogen-activated protein kinase (MAPK) in decreases in EC-SOD expression in hypoxic COS7 cells; however, the role of epigenetics in this process currently remains unknown. In the present study, we demonstrated that the hypoxia mimetic cobalt chloride (CoCl₂) decreased histone acetylation levels, and a pretreatment with 4-phenyl butyric acid (PBA), an inhibitor of histone deacetylation, significantly suppressed CoCl₂-elicited histone deacetylation and decreases in EC-SOD. We found that CoCl₂-elicited decreases in EC-SOD were accompanied by reductions in histone H3 acetylation levels within its promoter region. Furthermore, luteolin, a well-known flavonoid, significantly suppressed the CoCl₂-elicited accumulation of ROS, p38-MAPK activation, and histone deacetylation. Collectively, the results of the present study showed for the first time that CoCl₂ decreases the expression of EC-SOD through its deacetylation and luteolin may be one of the seed compounds that maintain redox homeostasis, even under hypoxic conditions.

Key words extracellular-superoxide dismutase (EC-SOD); epigenetic; hypoxia; luteolin

Reactive oxygen species (ROS) are known to participate in the progression of several kinds of diseases including atherosclerosis, diabetes, and cancer.1–3 Anti-oxidative enzymes such as superoxide dismutase (SOD) and catalase protect cells and tissues from the damaging effects of ROS.4,5 Extracellular-SOD (EC-SOD) is one of the SOD isozymes and plays a pivotal role in the regulation of redox homeostasis, particularly in extracellular spaces.6–8 A previous study reported that the knockdown of EC-SOD exacerbated the progression of atherosclerosis through the accumulation of foam cells in the neointima region;9 on the other hand, the overexpression of EC-SOD has been shown to suppress atherosclerosis.10 Therefore, maintaining the expression of EC-SOD at high levels in vessel walls may prevent the initiation and progression of vascular diseases.

Chronic kidney disease (CKD), defined as kidney injury lasting more than 3 months, is a global public health issue.11,12 During the progression of CKD, kidney cells are exposed to hypoxic stress due to tissue fibrosis, and this stress exacerbates tubulointerstitial injury.13 We previously demonstrated that the expression of EC-SOD is negatively regulated through intracellular ROS signaling in tubular-epithelial COS7 cells.14 Recent studies reported that epigenetics, such as DNA methylation and histone modifications, play pivotal roles in the progression of CKD, and some inhibitors of histone deacetylase (HDAC) exert beneficial effects against CKD.15–17 Moreover, the expression of EC-SOD is known to be regulated by epigenetics in human lung cancer cells,18,19 monocytes/macrophages,20 and retina endothelial cells21; however, limited information is currently available on EC-SOD regulation mechanisms in CKD models and the involvement of epigenetics.

Some flavonoids including luteolin and quercetin exert biological effects, such as anti-oxidative and anti-inflammatory activities.22–24 We previously reported that luteolin significantly suppressed phorbol ester (TPA)-induced human monocytic differentiation into macrophages, and these events were closely associated with the inhibition of intracellular ROS signaling.25 Accordingly, some flavonoids may be seed compounds that maintain redox homeostasis.

In the present study, we demonstrated the involvement of epigenetics in cobalt chloride (CoCl₂)-elicited decreases in EC-SOD, and found that a treatment with CoCl₂ reduced the acetylated levels of histone H3 and H4 through intracellular ROS-p38-mitogen-activated protein kinase (MAPK) signaling. Moreover, luteolin significantly ameliorated CoCl₂-elicited decreases in EC-SOD by inhibiting ROS-p38-MAPK signaling and histone deacetylation. Collectively, these results provide a novel insight into the involvement of epigenetics in the regulation of EC-SOD in the kidney, and the moderate uptake of luteolin may contribute to maintaining good redox homeostasis.

MATERIALS AND METHODS

Flavonoids Luteolin (Lut) (>98.0%) and chrysin (Chr) (>98.0%) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and CosmoBio Co., Ltd. (Tokyo, Japan), respectively. Apigenin (Ap), diosmetin (Di), chrysoriel (Chl), 3’,4’-dimethoxy luteolin (Dlu), and tricetin (Tr) were synthesized using previously described methods.26 Their purities were assessed as >95.0% using analytical HPLC. After filtration of flavone solutions (1 mg/mL in MeOH) through 0.20 mm membrane filter (ADVANTEC©, Japan), © 2016 The Pharmaceutical Society of Japan

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samples were analyzed by HPLC (Shimadzu Corp., Japan). Analysis was carried out using a TSKgel ODS-100V analytical column (5 mm, 4.6 × 250 mm). The isocratic mobile phase employed was 1% AcOH aq./MeCN = 1/1. The column eluate was monitored at 330 nm UV absorbance (shown in Supplementary Materials).

**Cell Culture** COS7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 100 units/mL penicillin, and 100 µg/mL streptomycin. Cells were maintained at 37°C in a humidified 5% CO₂ incubator. Culture medium was replaced every 2 d. After 90% confluence, culture medium was replaced with DMEM containing 0.5% calf serum (CS), cultured overnight, and then subjected to experiments.

**Western Blotting** COS7 cells (seeded at 1.2 × 10⁵ cells/35-mm dishes) were treated with CoCl₂ in the presence or absence of 4-phenyl butyric acid (PBA), luteolin, or SB203580 (an inhibitor of p38-MAPK) for the indicated time. After this treatment, histone proteins were collected and purified by the method described in our previous study. In order to assess phospho- or total p38-MAPK levels, COS7 cells (seeded at 3.6 × 10⁵ cells/60-mm dishes) were treated with CoCl₂ in the presence of luteolin for 1 h. Whole cell lysates were then prepared by the method described in our previous study. An equal amount of histone (assessed by Coomassie staining) or a whole cell lysate (20 µg) was separated by polyacrylamide gel electrophoresis (PAGE) or sodium dodecyl sulfate (SDS)-PAGE, respectively. After transferring electrophoretically onto polyvinylidene difluoride (PVDF) membranes, the membranes were incubated with anti-acetyl-histone H3 (06-599, Millipore: 1:2000), anti-acetyl-histone H4 (06-598, Millipore: 1:2000), or anti-phospho-p38 MAPK (#9215, Cell Signaling: 1:1000) overnight. Blots were incubated with a biotin-conjugated goat anti-rabbit or anti-mouse antibody (1:1000) followed by ABC reagents (Vector Laboratories, Inc.: 1:5000). Bands were detected using SuperSignal West Pico (Thermo Scientific, U.S.A.) or ImmunoStar LD (Wako Pure Chemical Industries, Ltd.) and imaged using an LAS-3000 UV mini apparatus (FUJIFILM, Tokyo, Japan).

**Reverse Transcriptional (RT)-PCR Analysis** After cells had been treated, they were lysed in 1 mL TRizol reagent (Invitrogen, Carlsbad, CA, U.S.A.). cDNA was prepared, and RT-PCR was performed by the methods described in our previous study with minor modifications. The primer sequences used were as follows: EC-SOD, sense 5'-AGA AAG CTCT CT TGG AGG AG-3'; antisense 5'-ACC GGG AAG TGT GCG AAG TC-3'; glucose-regulated protein 78 kDa (GRP78), sense 5'-TTT CTGC AT GTT CCT CACT-3'; antisense 5'-CCC AGA TGAG TCT CAC ATT-3'; 18S ribosomal RNA (rRNA), sense 5'-CGG GTA CCA CAT CAA CGG AA-3'; antisense 5'-GCT GGA ATT ACC GCG GCT-3'. After amplification, PCR products were loaded onto 2% (w/v) agarose gels for electrophoresis, and a densitometric analysis of PCR products was performed with Multi Gauge V3.0.

**Chromatin Immunoprecipitation Assay (ChIP)** ChIP assays were performed as described in our previous study. The abundance of EC-SOD promoter regions in ChIP precipitates was quantified using a PCR analysis. The primer sequences for EC-SOD were sense 5'-GGT GAG GCC ACA TCT TA-3'; antisense 5'-CTT GTA GCC GCA GTG CAG GA-3'. After amplification, these PCR products were loaded onto a 1.2% (w/v) agarose gel for electrophoresis and visualized using FLA5100, and a densitometric analysis of the PCR products was then performed with Multi Gauge V3.0.

**Measurement of ROS Accumulation** COS7 cells (seeded at 1.2 × 10⁵ cells/35-mm dishes) were treated with CoCl₂ for 1 h in the presence of luteolin, washed with PBS twice, and incubated with PBS containing 10 µM 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H₂DCFDA) for 30 min in a CO₂ incubator. After cells had been washed with PBS three times, they were visualized under an HS All-In-One fluorescence microscope BZ-9000 (KEYENCE, Osaka, Japan).

**Statistical Analysis** Data are expressed as the mean ± standard deviation (S.D.) of three independent experiments. A statistical analysis of data was performed using ANOVA followed by post hoc Bonferroni tests. A p value less than 0.05 was considered significant.

**RESULTS**

**Involvement of Histone Deacetylation in CoCl₂-Elicited Decreases in EC-SOD** We previously reported that hypoxia (1% O₂) and the hypoxia mimetic, CoCl₂ decreased the expression of EC-SOD in COS7 cells. Moreover, EC-SOD expression is known to be regulated by epigenetics such as DNA methylation and histone modifications, particularly by histone deacetylation in human lung cancer, human endothelial cells, and monocytes/macrophages. Accordingly, decreases in EC-SOD elicited by an exposure to CoCl₂ may be regulated by epigenetics. In order to elucidate the involvement of epigenetics in CoCl₂-elicited decreases in EC-SOD, histone acetylation levels were measured after the exposure to CoCl₂. As shown in Fig. 1A, treatment with CoCl₂ decreased the levels of acetylated histone H3 and H4 in a dose-dependent manner. These data are consistent with our previous finding, in which CoCl₂ did not decrease EC-SOD expression at 100 µM CoCl₂. HDAC has been shown to play a pivotal role in the regulation of gene expression through chromatin remodeling. As expected, the pretreatment with PBA, an inhibitor of HDAC, significantly suppressed CoCl₂-elicited decreases in EC-SOD and was accompanied by the inhibition of histone deacetylation (Figs. 1B, C). On the other hand, PBA has the potential to inhibit endoplasmic reticulum (ER) stress, and ER stress has been shown to regulate the expression of EC-SOD in COS7 cells and 3T3-L1 adipocytes. Our results demonstrated that CoCl₂ induced GRP78, an ER stress marker, however, PBA did not affect its induction (Fig. 1C). These results suggest that CoCl₂-elicited decreases in EC-SOD were regulated in an ER stress-derived signal-independent manner. We also found decreases in acetylated histone enrichment within the proximal promoter region of EC-SOD following the exposure to CoCl₂ (Fig. 1D), suggesting that CoCl₂ decreases EC-SOD through histone deacetylation within its promoter region.

**Involvement of p38-MAPK in CoCl₂-Elicited Histone Deacetylation** MAPK including p38-MAPK, extracellular-regulated kinase (ERK), and c-jun N-terminal kinase (JNK) play pivotal roles in a large number of physiological processes such as apoptosis, proliferation, and differentiation. Moreover, we previously reported that p38-MAPK is involved in the regulation of EC-SOD in COS7 cells under hypoxic condi-
tions.\textsuperscript{14}) Therefore, we investigated the involvement of p38-MAPK in CoCl\textsubscript{2}-elicited histone deacetylation. The pretreatment with SB203580, an inhibitor of p38-MAPK, suppressed CoCl\textsubscript{2}-elicited histone H3 and H4 deacetylation (Fig. 2), suggesting that histone deacetylation is mediated by p38-MAPK-related signaling in COS7 cells.

**Effects of Flavonoids on CoCl\textsubscript{2}-Elicited Decreases in EC-SOD**

Previous studies reported that some flavonoids including luteolin exhibit potent anti-oxidative activities, and may be seed compounds against cancer and atherosclerosis.\textsuperscript{22–24} In this study, we attempted to clarify whether some flavonoids have the ability to suppress CoCl\textsubscript{2}-elicited decreases in EC-SOD. Among the 7 flavonoids examined in the present study (Fig. 3A), chrysin and luteolin significantly suppressed CoCl\textsubscript{2}-elicited histone H3 and H4 deacetylation (Fig. 2), suggesting that histone deacetylation is mediated by p38-MAPK-related signaling in COS7 cells.

DISCUSSION

Epigenetics, such as DNA methylation and histone modifications, play important roles in cell differentiation and cellular development.\textsuperscript{29,37,38} DNA methylation, which is mediated by DNA methyltransferase (DNMT) with S-adenosyl-methionine as a methyl donor, has been shown to regulate gene expression and genomic imprinting.\textsuperscript{39–41} Recent studies revealed that global DNA hypomethylation and hypermethylation are both involved in several physiological processes, including tumorigenesis and CKD.\textsuperscript{42–44} Based on a vast amount of findings, genes, which may be regulated by DNA methylation,
are known to possess CpG islands within proximal promoter regions. We previously reported that the expression of EC-SOD in basal human monocyte THP-1 cells was silenced, and attributed this to CpG hypermethylation within its promoter region. As noted above, DNA methylation processes may be associated with CoCl₂-elicted decreases in EC-SOD in COS7 cells; however, a treatment with 5-azacytidine, an inhibitor of DNMT, did not suppress these decreases (data not shown). These results indicate that CoCl₂-elicted decreases in EC-SOD are regulated in a DNA methylation-independent manner.

Histone modifications in the N-terminal tail, including acetylation, methylation, and phosphorylation, play critical roles in gene regulation as well as DNA methylation. Histone acetylation at lysine residues is closely related to gene induction, and these phenomena are regulated by histone acetyltransferase (HAT) and HDAC, two opposing groups of enzymes involved in chromatin remodeling. Histone acetylation neutralizes the positive charge and facilitates the binding of transcription factors to nucleosomal DNA, thereby inducing its transcription. Acetylation-dependent gene induction has been shown to play a pivotal role in the progression of cancer invasion, metastasis, and kidney fibrosis. We previously reported that the excessive production of ROS induced epithelial-mesenchymal transition (EMT), which is considered to be critically involved in cancer metastasis and fibrosis and is mediated by histone acetylation in the human breast cancer cell line MCF-7. Therefore, obtaining a deeper understanding of the role of ROS and regulation of redox homeostasis may lead to novel CKD therapies. In the present study, we examined the involvement of histone H3 deacetylation in CoCl₂-elicted decreased in EC-SOD, which is closely associated with ROS-p38-MAPK signaling. It has been reported that p38-MAPK induces serine phosphorylation of p300. In the present study, we tested whether some flavonoids suppress CoCl₂-elicted decreases in EC-SOD, and found that luteolin exerted a potent inhibitory effect against this phenomenon. These results are consistent with our previous findings, in which luteolin exhibited potent radical scavenger activity and significantly suppressed monocytic differentiation into macrophages. These results indicate that luteolin maintains good redox homeostasis, particularly in extracellular spaces.

In the present study, we identified a significant role for histone de/acetylation in the regulation of EC-SOD in COS7 cells. However, unfortunately, we could not determine a critical role of histone de/acetylation in the regulation of EC-SOD under hypoxic condition (1% O₂, data not shown). Accordingly, we consider that additional studies will be necessary to determine the exact molecular mechanisms governing the regulation of EC-SOD under hypoxia. On the other hand, we demonstrated that the knockdown of EC-SOD increased sensitivity against ROS and induced cell death in human fibroblasts (data not shown); therefore, our results provide evidence for luteolin contributing to the maintenance of redox homeostasis.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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