Exendin-4 promotes extracellular-superoxide dismutase expression in A549 cells through DNA demethylation

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Exendin-4 is an agonist of the glucagon-like peptide 1 receptor (GLP-1R) and is used in the treatment of type 2 diabetes. Since human GLP-1R has been identified in various cells besides pancreatic cells, exendin-4 is expected to exert extrapancreatic actions. It has also been suggested to affect gene expression through epigenetic regulation, such as DNA methylation and/or histone modifications. Furthermore, the expression of extracellular-superoxide dismutase (EC-SOD), a major SOD isozyme that is crucially involved in redox homeostasis, is regulated by epigenetic factors. In the present study, we demonstrated that exendin-4 induced the demethylation of DNA in A549 cells, which, in turn, affected the expression of EC-SOD. Our results showed that the treatment with exendin-4 up-regulated the expression of EC-SOD through GLP-1R and demethylated some methyl-CpG sites (methylated cytosine at 5′-CG-3′) in the EC-SOD gene. Moreover, the treatment with exendin-4 inactivated DNA methyltransferases (DNMTs), but did not change their expression levels. In conclusion, the results of the present study demonstrated for the first time that exendin-4 regulated the expression of EC-SOD by reducing the activity of DNMTs and demethylation of DNA within the EC-SOD promoter region in A549 cells.

Key Words: exendin-4, extracellular-superoxide dismutase, epigenetics, DNA demethylation

S uperoxide dismutase (SOD) is an antioxidative enzyme that protects cells against oxidative stress induced by the excessive production of reactive oxygen species (ROS), and a SOD deficiency has been shown to increase the risk of developing various diseases, such as type 2 diabetes, atherosclerosis, and asthma. Extracellular-SOD (EC-SOD), one of the three SOD isozymes in mammals, is secreted into the extracellular space and widely distributed in tissues. EC-SOD is highly expressed in the lung and kidney, and is localized to specific cells in these tissues. However, it currently remains unclear why the expression of EC-SOD is different among cells and/or tissues. EC-SOD was previously reported to be expressed at very low levels in the human lung adenocarcinoma epithelial cell line A549, but at very high levels in normal airway epithelial cells. EC-SOD modulates oxidative stress in the tumor microenvironment and inhibits tumor growth. Therefore, pharmacological therapy that induces the expression of EC-SOD may represent a useful therapeutic approach for cancer chemotherapy.

Epigenetics have been defined as mitotically heritable changes in gene expression that do not change the DNA sequence, and include DNA methylation and histone modifications. DNA methylation, occurring at the 5′ carbon of cytosine within CpG, plays a pivotal role in tissue-specific gene regulation. Regions with a high density of CpG sites are referred to as CpG islands, and are mostly unmethylated. However, gene expression is suppressed by the methylation of some CpG islands, and DNA methylation is involved in the development, differentiation, or canceration of their cells. The methylation of DNA is initiated by the transmethylation of S-adenosylmethionine (SAM), a methyl group donor, which is catalyzed by DNA methyltransferases (DNMTs), including DNMT1, DNMT3A, and DNMT3B in mammals.

Glucagon-like peptide-1 (GLP-1) is an incretin hormone secreted by L-cells located in the distal small intestine. The plasma concentration of GLP-1 rapidly increases within a few minutes of food intake. GLP-1 stimulates the secretion of insulin from pancreatic β-cells, but suppresses that of glucagon from α-cells by binding to the GLP-1 receptors (GLP-1R) on these cells, thereby lowering blood glucose concentrations. However, the biological activity of GLP-1 is tightly regulated because once it is secreted into plasma, it is rapidly cleaved by dipeptidyl peptidase 4 (DPP-4). GLP-1R agonists that are resistant to DPP-4 cleavage have recently been developed and generally enable the strong and steady activation of GLP-1R signaling. Exendin-4 is a GLP-1 receptor agonist, which was initially discovered in the saliva of the Gila monster, Heloderma suspectum, and shares 53% sequence homology with GLP-1. It was approved as a treatment for type 2 diabetes, called “exenatide”, in Japan in 2010. GLP-1 and its receptor agonists have been shown to directly exert extra-pancreatic actions because GLP-1R is also expressed in the heart, intestines, kidney, and brain.

Previous studies reported that EC-SOD activity was decreased in type 2 diabetes. Moreover, EC-SOD expression is known to be suppressed by cytokines such as tumor necrosis factor-α, which plays a role in diabetes. On the other hand, the enhanced expression of EC-SOD has been shown to mitigate streptozotocin-induced diabetic cardiomyopathy by attenuating oxidative stress. As described above, EC-SOD is expressed at very low levels in A549 cells. DNA methylation has been implicated in the low expression of EC-SOD because DNA methylation within the EC-SOD promoter region inhibits binding of the Sp1/3 transcriptional factor to the EC-SOD promoter. Exendin-4 was previously shown to epigenetically regulate (DNA methylation and histone modification) gene expression. This finding prompted us to elucidate the contribution of exendin-4 to the induction of EC-SOD expression in A549 cells because GLP-1R is expressed in this cells, whereas EC-SOD expression is very low. In the present study, we investigated whether exendin-4 regulated the expression of EC-SOD via an epigenetic mechanism.

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Materials and Methods

Cell culture. A549 cells, a human lung adenocarcinoma epithelial cell line, were cultured in Dulbecco’s modified eagle medium (DMEM) containing 10% (v/v) 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. The culture medium was replaced every 2 days.

Real-time reverse transcription-polymerase chain reaction (real-time RT-PCR) analysis. A549 cells (seeded at 4.5 × 10⁵ cells/ml in 60-mm culture dishes) were cultured overnight and treated with exendin-4 (AnaSpec, Fremont, CA), 5-aza-cytidine (Aza, Wako Pure Chem. Ind., Ltd., Osaka, Japan) or exendin-(9-39) (GenScript, Piscataway, NJ). After the treatment for 24 h, the cells were washed with cold phosphate-buffered saline (PBS) and total RNA was extracted from cells with TRIzol reagent (Invitrogen, Carlsbad, CA). The preparation of cDNA was performed by the method described in our previous study.⁵² Real-time RT-PCR was carried out using Thunderbird™ SYBR qPCR Mix (Toyobo, Osaka, Japan) according to the manufacturer’s protocol. The EC-SOD primers were used QuantiTect® Primer Assay HS SOD3 2 S (Qiagen, Chatsworth, CA). The other primer sequences used in real time RT-PCR were as follows: Cu,Zn-SOD, sense 5'-GGC AGC ACG GCC GTT TGC TGG GTG-3'; antisense 5'-TGT GCC GCC AAT GAT GTA ATG-3': Mn-SOD, sense 5'-CGA CCT GCC CTA CGA CTA CGG-3'; antisense 5'-CAG AAC CCC CAC CTG AGC-3': DNMT 3A, sense 5'-GCT GGT AAC TAA GTC ACC-3'; antisense 5'-GTT GCA GTC CTC TGT GAA CAC TGT GG-3': DNMT 3B, sense 5'-AAT GTT ATT CCA CCA AGG AAA GGG-3'; antisense 5'-ACT ACA AAG GAA GTA ATT TTA TAA-3': MSP1-U sense 5'-CCT AAA ACC TAA ATT AAC GCC A-3'; antisense 5'-GAT ATG TAT GTT AAG GTT ATG G-3': MSP2-U sense 5'-ACT AAA ATT CCT AAT CAC AAT-3'; after amplification, aliquots of the PCR mixtures were separated on a 2% (w/v) agarose gel and stained with ethidium bromide. These were subsequently visualized using FLA5100 (Tokyo, Japan).

Bisulfitesequencing of the EC-SOD promoter region. Bisulfite sequencing to analyze the methylation pattern of CpG sites within the EC-SOD promoter was carried out as described previously⁴⁴ with minor modifications. An aliquot of bisulfite-treated genomic DNA (500 ng) was subjected to PCR amplification. The primer sequences used in bisulfite sequencing to amplify the promoter region of EC-SOD were designed using MethPrimer software, and these primers were as follows: sense 5'-TTT TTT GTT GTG TGT TGA TGA TTA T-3', antisense 5'-AAC TCC TCA TAA AAA AAT TTC TCT C-3'. The PCR fragments were subcloned using the pGEM-T Easy Vector System (Promega, Madison, WI) and at least 10 individual plasmid clones were sequenced using CEQ 8000 (Beckman Coulter, Tokyo, Japan).

DNMT activity analysis. Nuclear proteins were isolated from A549 cells using EpiQuik™ Nuclear Extraction kit 1 (Epigentek Group, Farmingdale, NY) according to the manufacturer’s protocol. After nuclear extraction, the protein concentration of the supernatant was assayed using a Bio-Rad protein assay reagent. An aliquot of the nuclear extract (10 μg) was subjected to the measurement of DNA methyltransferase (DNMT) activity. DNMT activity was measured using the EpiQuik™ DNMT Activity/Inhibition Assay Ultra kit (Colorimetric) (Epigentek Group) according to the manufacturer’s protocol.

Data analysis. Data are presented as the mean ± SD from at least three experiments. Data were analyzed by the Mann-Whitney U test. A p value of less than 0.05 was considered significant.

Results

Effects of exendin-4 on EC-SOD expression in A549 cells. The treatment of A549 cells with exendin-4 for 24 h significantly induced the expression of EC-SOD, but not that of Cu,Zn-SOD or Mn-SOD, the other SODs isozymes (Fig. 1A). Furthermore, the treatment with Aza, a DNA methyltransferase inhibitor, also increased the expression of EC-SOD, but not that of Cu,Zn-SOD or Mn-SOD (Fig. 1A). These results suggested that exendin-4 acted as a DNA demethylation agent. Moreover, the up-regulation of EC-SOD by exendin-4 was significantly suppressed by the addition of exendin-(9-39), an antagonist of GLP-1R⁴⁵,⁴⁶ (Fig. 1B), suggesting that exendin-4 induced the expression of EC-SOD by binding to GLP-1R.

DNA methylation within the EC-SOD promoter region by the exendin-4 treatment. Exendin-4 was previously reported to potentially regulate DNA methylation for gene expression.⁴⁶ On the other hand, low expression of EC-SOD in A549 cells is known to be due to DNA methylation. The DNA methylation within EC-SOD promoter region attenuates Sp1/3 transcriptional factor binding to EC-SOD promoter.⁴⁴,⁴⁵ Therefore, we investigated the involvement of DNA methylation in the exendin-4-induced up-regulated expression of EC-SOD. The DNA methylation status was analyzed using methylation-requiring nuclease MsrBC. PCR amplification of EC-SOD promoter regions from −1,208 to −764 (Pair 1) and −278 to +51 (Pair 2) showed that genomic DNA in untreated A549 cells was digested by MsrBC, while genomic DNA in Aza-treated A549 cells was resistant to MsrBC in the Pair 1 and Pair 2 regions (Fig. 2A). Genomic DNA in exendin-4-treated A549 cells was digested by MsrBC in the Pair 1 region, but it in the Pair 2 region resisted MsrBC digestion (Fig. 2A). We then determined whether DNA methylation in the promoter (−173 to −35) and coding regions (+4,014 to +4,241) was changed by exendin-4. The genomic DNA
of A549 cells was treated with bisulfate, and followed by MSP amplification with methylation (M) and non-methylation (U) site primers. As shown in Fig. 2B, the MSP analysis revealed that the treatment with Aza demethylated DNA in the EC-SOD promoter and coding regions. When cells were treated with exendin-4, DNA demethylation was observed in the EC-SOD promoter region, but not in the coding region. We investigated DNA demethylation in the EC-SOD promoter region in exendin-4-treated A549 cells in more detail. We determined that the treatment with exendin-4 or Aza was more likely to demethylate the –149 and –93 CpG sites in the region from –291 to +36 in A549 cells (Fig. 2C). These results suggested that the up-regulated expression of EC-SOD with exendin-4 was induced by DNA demethylation in a narrow range of CpG sites in the EC-SOD promoter region of A549 cells.

**Effects of exendin-4 on DNMTs in A549 cells.** DNA methylation is known to be mediated by DNMTs. The treatment with exendin-4 or Aza for 24 h did not change the expression of DNMTs (Fig. 3A). We next investigated changes in the activities of DNMTs in exendin-4-treated A549 cells. As shown in Fig. 3B, exendin-4 and Aza both significantly reduced the enzymatic activity of DNMT.

**Discussion**

Various cancer genes and cancer suppressor genes induce genetic alterations during carcinogenesis. DNA methylation in a gene promoter region represents an inactivating mechanism for cancer suppressor genes as well as gene mutations and chromosomal deletions. EC-SOD was previously shown to inhibit tumor growth in pancreatic cancer. However, EC-SOD is known to be expressed at very low levels in the human lung adenocarcinoma epithelial cell line A549 due to the hypermethylation of CpG islands in the EC-SOD promoter region. The main results of the present study is that exendin-4 up-regulated the expression of EC-SOD by its binding to GLP-1R in A549 cells. Pinney et al. recently demonstrated that exendin-4 regulated gene expression through an epigenetic mechanism. They showed that the administration of exendin-4 induced pancreatic and duodenal homeobox-1 (Pdx1) transcriptional factor in intrauterine growth retardation (IUGR) pancreatic islets in an in vivo experiment. Exendin-4 induced the histone acetylation and demethylation of promoter DNA in the Pdx1 gene. The GLP-1 receptor is widely expressed in various tissues/organs besides the pancreas, suggesting that GLP-1 analogues regulate various genes through an epigenetic mechanism. We herein found that exendin-4 induced the expression of EC-SOD in the A549 lung cancer cell line through the demethylation of DNA in its promoter region.

To elucidate the epigenetic mechanism associated with DNA methylation within the EC-SOD promoter region in A549 cells, we investigated whether the expression and activities of DNMTs were changed by exendin-4. DNA methylation has been classified into maintenance methylation and de novo methylation. DNMT1 (maintenance DNMT) has been shown to copy methylation to the daughter strand by selectively methylating hemi-methylated DNA following semiconservative DNA replication. DNMT1 (maintenance DNMT) has been shown to copy methylation to the daughter strand by selectively methylating hemi-methylated DNA following semiconservative DNA replication. DNMT1 is characterized by higher enzyme activity against hemi-methylated DNA than unmodified DNA. A previous study reported that Aza irreversibly bound DNMT1, and DNMT1 then became a DNA adduct and was degraded. In the present study, the treatment with Aza and also exendin-4 significantly demethylated
DNA within the EC-SOD promoter and induced the expression of EC-SOD (Fig. 1A and 2) with a significant decrease enzymatic activity of DNMT (Fig. 3B). The basal and inducible transcription of human EC-SOD is regulated by Sp1/Sp3 transcriptional factors, which bind to the proximal promoter.\(^{(2,3)}\) However, a CpG site only exists downstream of the Sp1/Sp3 binding region. As shown
in Fig. 2C, the treatment with exendin-4 was more likely to demethylate the –149 and –93 CpG sites in this region, indicating that the expression of EC-SOD may be susceptible to the methylation of CpG sites downstream of the Sp1/Sp3 binding region. The principle structure of eukaryotic chromatin is the core nucleosome, which consists of an octamer of histones. Chromatin exists in two different states, an open and closed configuration, and these are closely associated with the acetylation/deacetylation of histones by histone acetyltransferase (HAT) and histone deacetylase (HDAC). The acetylation of histone neutralizes the positive charge and facilitates the binding of transcription factors to nucleosomal DNA, thereby enhancing its transcription. We previously reported that the 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced expression of EC-SOD in monocytic THP-1 cells was positively related with the acetylation status of histone H3 and H4. Moreover, a treatment with the HDAC inhibitor trichostatin A (TSA) significantly induced the expression of EC-SOD. However, the expression of EC-SOD in A549 cells was not induced by the TSA treatment (data not shown), suggesting that histone modifications did not contribute to the regulation of EC-SOD expression in A549 cells.

In conclusion, the results of the present study suggest that exendin-4-inducible EC-SOD expression in A549 cells was regulated by DNA demethylation through the reduction in the activity of DNMTs. We were unable to clarify the mechanisms by which DNMTs were reduced in the activity by exendin-4. Therefore, additional studies are needed to determine the precise molecular mechanism underlying DNA demethylation within the EC-SOD promoter region. Exendin-4 has been widely approved as a treatment for type 2 diabetes and exerts its pancreatic and extrapancreatic effects by binding to GLP-1R, which is distributed in many tissues. GLP-1 and its analogues may function to maintain physiological homeostasis by epigenetically regulating various genes.

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Abbreviations

Aza 5-azacytidine  
DNMT DNA methyltransferase  
DPP-4 dipeptidyl peptidase 4  
EC-SOD extracellular-superoxide dismutase  
GLP-1 glucagon-like peptide 1  
GLP-1R glucagon-like peptide 1 receptor  
HAT histone acetyltransferase  
HDAC histone deacetylase  
IUGR intrauterine growth retardation  
MSP methylation-specific polymerase chain reaction  
Pdx1 pancreatic and duodenal homeobox-1  
ROS reactive oxygen species  
SAM S-adenosylmethionine  
TPA 12-O-tetradecanoylphorbol-13-acetate  
TSA trichostatin A

Conflict of Interest

No potential conflicts of interest were disclosed.
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