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Note

Epigenetic Regulation of Carbonic Anhydrase 9 Expression by Nitric Oxide in Human Small Airway Epithelial Cells

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DNA methylation is a crucial epigenetic modification that regulates gene expression and determines cell fate; however, the triggers that alter DNA methylation levels remain unclear. Recently, we showed that *S*-nitrosylation of DNA methyltransferase (DNMT) induces DNA hypomethylation and alters gene expression. Furthermore, we identified DBIC, a specific inhibitor of *S*-nitrosylation of DNMT3B, to suppress nitric oxide (NO)-induced gene alterations. However, it remains unclear how NO-induced DNA hypomethylation regulates gene expression and whether this mechanism is maintained in normal cells and triggers disease-related changes. To address these issues, we focused on carbonic anhydrase 9 (CA9), which is upregulated under nitrosative stress in cancer cells. We pharmacologically evaluated its regulatory mechanisms using human small airway epithelial cells (SAECs) and DBIC. We demonstrated that nitrosative stress promotes the recruitment of hypoxia-inducible factor 1 alpha to the *CA9* promoter region and epigenetically induces *CA9* expression in SAECs. Our results suggest that nitrosative stress is a key epigenetic regulator that may cause diseases by altering normal cell function.

Key words nitric oxide, human small airway epithelial cell, epigenetics, DNA methylation, carbonic anhydrase 9, hypoxia-inducible factor 1 alpha

INTRODUCTION

DNA methylation is a crucial epigenetic modification that determines the cell fate. Aberrant DNA methylation followed by gene expression has been observed in various diseases such as cancer.[1] DNA methylation levels are affected by diet, smoking, and viral infection[2]; however, the triggering environmental factors remain largely unknown. In this study, we focused on the function of nitric oxide (NO). NO regulates blood pressure and neurotransmission under physiological conditions. However, excessive NO generation during pathological inflammatory reactions can cause various diseases. Protein *S*-nitrosylation, an oxidative modification of the cysteine thiol group by NO, is particularly important. This process alters protein function and localization to regulate physiological functions such as cell proliferation, neurotransmission, and apoptosis.[3–7]

Previously, we showed that excessive nitrosative stress functions as an epigenetic regulator *via* *S*-nitrosylation of DNA methyltransferases (DNMT), which play a central role in epigenetic regulation.[8,9] *S*-Nitrosylation of DNMT attenuates enzyme activity and induces aberrant expression of cancer-related genes. Furthermore, using *in silico* virtual screening, we identified the chemical compound ((*E*)-*N'*-(3,4-dihydroxybenzylidene)-1*H*-benzo[*d*]imidazole-5-carbohydrazide, designated here as DBIC), a specific inhibitor of the *S*-nitrosylation of DNMT3B, without affecting its enzymatic activity. We identified that C651 in the catalytic domain of DNMT3B as the *S*-nitrosylation site. DBIC inhibited *S*-nitrosylation of DNMT3B by binding to small-molecule binding pockets located near C651. DBIC treatment markedly suppressed NO-induced tumorigenesis in an *in vivo* cancer model. However, it is not fully elucidated whether nitrosative stress-induced changes in gene expression also occur in normal cells and whether this mechanism induces cellular changes that are relevant to pathogenesis.

In this study, we focused on respiratory diseases. Respiratory organs such as the airways and lungs are in contact with the external environment. Their functions are affected by smoking, environmental conditions, and viral infections, all of which induce inflammatory stress. The inflammatory response induces inducible NO synthase (iNOS) expression, resulting in excessive NO generation.[10] In our previous study, we found that *S*-nitrosylation of DNMT epigenetically upregulates carbonic anhydrase 9 (CA9) in HeLa cells.[8] CA9 is known as a cancer marker in various organs and a hypoxia marker because it reflects the activation of hypoxia-inducible factor 1 alpha (HIF-1α).[11,12] It is speculated that the hypoxic response is relevant to respiratory organs responsible for oxygen exchange. In this study, we investigated the regulation of CA9 expression by nitrosative stress in normal human small airway epithelial cells (SAECs) through pharmacological evaluation using DBIC. Moreover, we examined the effect of exposure to NO donor on the interaction between HIF-1α and the *CA9* promoter region.

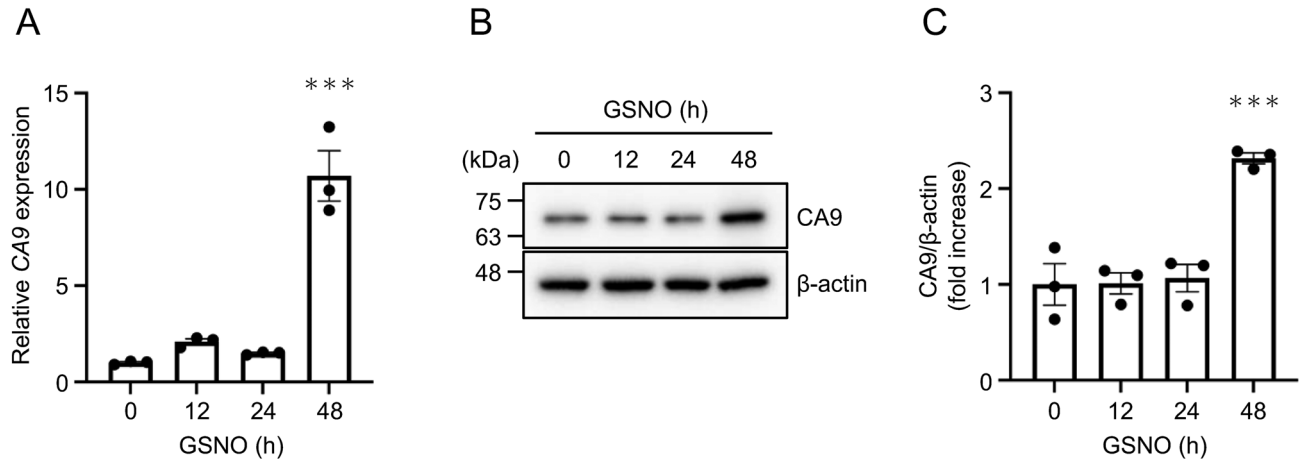

Fig. 1. NO-Induced CA9 Expression in SAECs

(A) NO-induced *CA9* mRNA expression in SAECs. Cells were treated with 100 μM GSNO for the indicated times. RT-qPCR was performed using primers specific for *CA9* mRNA. Values are expressed as the mean ± S.E.M. ($n = 3$; ***$p < 0.001$ by one-way ANOVA with Dunnett's *post hoc* test). (B) NO-induced CA9 protein expression in SAECs. Cells were treated with 100 μM GSNO for the indicated times. The lysates were analyzed by Western blotting with anti-CA9 or anti-β-actin antibodies. (C) The relative intensity of CA9 in (B) was quantified and normalized to that of β-actin. Values are expressed as the mean ± S.E.M. ($n = 3$; ***$p < 0.001$ by one-way ANOVA with Dunnett's *post hoc* test).

MATERIALS AND METHODS

Materials Antibodies against CA9 (11071-1-AP, Proteintech, U.S.A.), β-actin (4970, Cell Signaling Technology, U.S.A.) and HIF-1 alpha (NB100-449, Novus Biologicals, U.S.A.) were purchased from the indicated vendors. Cobalt (II) chloride was purchased from Sigma-Aldrich (232629; U.S.A.). The NO donor, *S*-nitrosoglutathione (GSNO), was freshly prepared before use and kept in the dark. DBIC was synthesized as described previously.[8] SAECs were preincubated with DBIC for 1 h prior to GSNO exposure.

Cell Culture SAECs human small airway epithelial cells (CC-2547, Lonza, Switzerland) were cultured SAGM™ Small Airway Epithelial Cell Basal Medium (CC-3119, Lonza) supplemented with SAGM™ Single Quots™ Supplements and Growth Factors (CC-4124, Lonza) at 37 °C in a humidified atmosphere of CO_2/95% air.

RT-Quantitative PCR (qPCR) Total RNA was extracted from SAECs using TRI reagent (TR118, Molecular Research Center, Inc., U.S.A.), according to the manufacturer's instructions. A ReverTra Ace qPCR RT kit (FSQ-201, TOYOBO, Japan) was used to synthesize cDNAs according to the manufacturer's instructions. qPCR was performed using the KOD SYBR qPCR Mix (QKD-201, TOYOBO) under the following conditions: 98 °C for 2 min, followed by 40 cycles of 98 °C for 10 s, 60 °C for 10 s, and 68 °C for 30 s. All qPCRs amplified single products, as confirmed by the melting curve and electrophoresis using 2% agarose gels with 0.5 ng/mL ethidium bromide. The following primer sets were used: human *CA9* 5′-GAA ATC GCT GAG GAA GGC TC-3′ and 5′-CGG TGT AGT CAG AGA CCC CT-3′; human *ACTB* 5′-TCA CCC ACA CTG TGC CCA TCT ACG A-3′ and 5′-CAG CGG AAC CGC TCA TTG CCA ATG G-3′; human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) 5′-AGG TCG GAG TCA ACG GAT TTG-3′ and 5′-ATG AAG GGG TCA TTG ATG GCA-3′. The $2^{-\Delta\Delta Ct}$ relative quantification method, using ACTB or GAPDH for normalization, was used to estimate the target gene expression. The fold-change was calculated relative to the mRNA expression levels in the control samples.

Western Blotting Cells were washed with phosphate buffered saline (PBS) and lysed in radioimmunoprecipitation assay buffer (50 mM Tris–HCl (pH 7.5), 0.15 M NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), and 1% sodium deoxycholate supplemented with protease inhibitor cocktail (04693116001, Roche, Switzerland)). After quantification of protein concentration by the bicinchoninic acid (BCA) assay (T930A, TaKaRa, Japan) method, protein samples were boiled in 1 × Laemmli SDS sample buffer (62.5 mM Tris–HCl (pH 6.8), 5% (v/v) 2-mercaptoethanol, 2% (w/v) SDS, and 10% (v/v) glycerol) for 5 min and analyzed by Western blotting as described previously.[13]

Chromatin Immunoprecipitation Sequencing (ChIP-seq) Human small airway epithelial cells were fixed by 1% paraformaldehyde (553-87281, FUJIFILM Wako, Japan) at 37 °C for 5 min and then lysed with radioimmunoprecipitation assay buffer (50 mM Tris–HCl (pH 8.0), 0.15 M NaCl, 1% NP-40, 0.1% SDS, and 1% sodium deoxycholate, 2 mM ethylenediaminetetraacetic acid (EDTA) supplemented with EDTA-free protease inhibitor cocktail (04693132001, Roche)). Cells were sonicated 15 times at 50% amplitude for 10 s at 1 min interval using a sonicator (VCX130, Sonics & Materials, Inc., U.S.A.). Samples were incubated with an anti-HIF-1 alpha antibody and Dynabeads Protein G (10003D, Thermo Fisher Scientific, U.S.A.) at 4 °C overnight. Input and immunoprecipitates were incubated at 65 °C for 4 h for reverse cross-linking, and DNA was purified using the QIAquick PCR Purification Kit (28106, Qiagen, the Netherlands). ChIP-seq libraries were generated using a ThruPLEX DNA-seq kit (RB4674, TaKaRa). Next-generation sequencing was performed using the DNBSEQ-G400 platform (BGI) with a 100-bp paired-end read protocol, according to the manufacturer's instructions. Bowtie2 (version 2.5.1;default parameters) was used to map the reads to the reference genome (UCSC/hg38). HOMER (version 4.11) was used for find Peaks.

Statistical Analysis Quantitative data are presented as mean ± standard error of the mean (S.E.M.). Statistical analyses were performed using the GraphPad Prism software version 10.2.2 (GraphPad Software, San Diego, CA, U.S.A.). Multiple comparisons were performed using one-way ANOVA with Dunnett's *post hoc* test or Tukey's *post hoc* test.

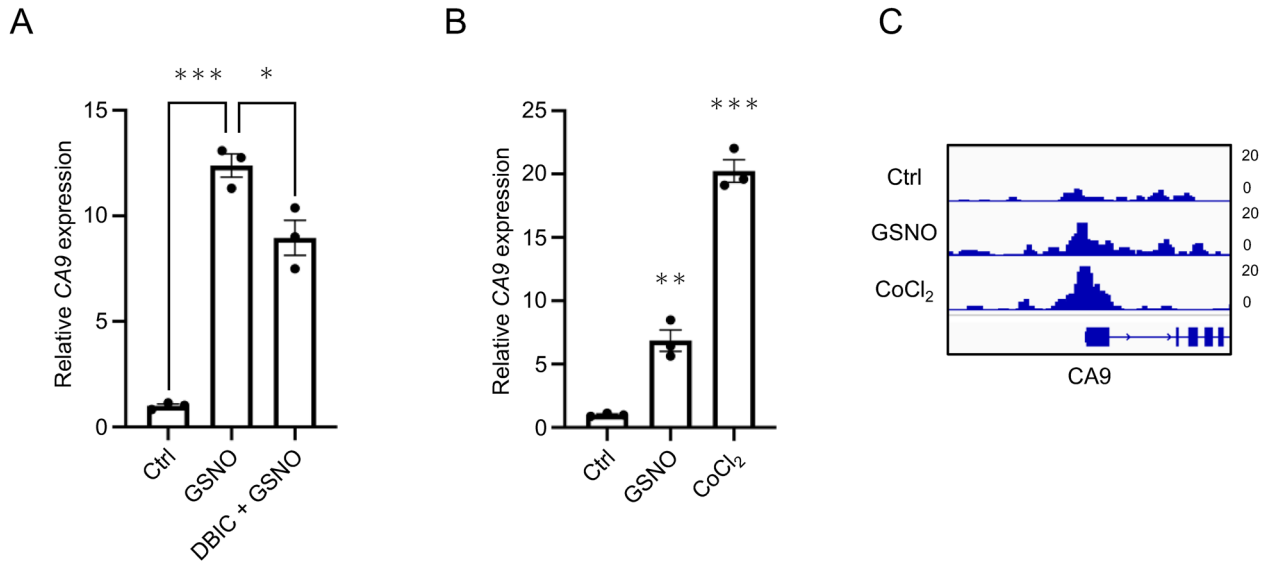

Fig. 2. Epigenetic Regulation of *CA9* Expression in SAECs

(A) Pharmacological effects of DBIC on NO-induced *CA9* expression. Cells were treated with 100 μM GSNO for 48 h. Cells were preincubated with 10 μM DBIC for 1 h prior to GSNO exposure. Values are expressed as the mean ± S.E.M. ($n = 3$; $^{*}p < 0.05$, $^{***}p < 0.001$ by one-way ANOVA with Tukey's *post hoc* test). (B) $CoCl_2$-induced *CA9* mRNA expression in SAECs. Cells were treated with 300 μM $CoCl_2$ for 24 h. Values are expressed as the mean ± S.E.M. ($n = 3$; $^{**}p < 0.01$, $^{***}p < 0.001$ by one-way ANOVA with Tukey's *post hoc* test). (C) HIF-1α binding peaks in the *CA9* promoter region of SAECs. Cells were treated with 100 μM GSNO for 48 h and 300 μM $CoCl_2$ for 24 h. Peak graphs were acquired using the Integrative Genomic Viewer.

Statistical significance was set at $p < 0.05$.

RESULTS

NO-Induced CA9 Expression in SAECs In a previous study, we conducted a transcriptomic analysis to identify NO-dependent differentially expressed genes in HeLa cells and found that CA9, which catalyzes the hydration of carbon dioxide to carbonic acid, was the most significantly upregulated gene. Furthermore, exposure to NO donor induced hypomethylation of the *CA9* promoter region, suggesting that *CA9* expression is epigenetically regulated.[8] We initially tested whether NO donor induced *CA9* expression in normal human cells. Exposure to the physiological NO donor GSNO markedly enhanced the level of *CA9* mRNA in SAECs (Fig. 1A). We then examined the effects of GSNO exposure on CA9 protein expression in SAECs using Western blotting. The results showed similar trends to those of the changes in mRNA expression (Figs. 1B, C). These results suggest that NO-induced CA9 expression occurs not only in cancer cells, but also in SAECs. We previously showed that nitrosative stress promoted hypomethylation of the *CA9* promoter region 48 h after treatment. This suggests that NO-induced CA9 expression regulates *via* epigenetic regulation in SAECs.

Epigenetic Regulation of *CA9* Expression in SAECs Next, we evaluated whether NO-induced *CA9* expression in SAECs occurred through epigenetic regulation resulting from *S*-nitrosylation of DNMT using DBIC.[8] We treated DBIC for one hour before GSNO exposure, which significantly suppressed GSNO-induced *CA9* expression (Fig. 2A). Our results suggest that upregulation of *CA9* expression by GSNO exposure is partly dependent on *S*-nitrosylation of DNMT3B. Under hypoxic conditions, HIF-1α regulates *CA9* transcription.[12] We showed that cobalt (II) chloride, which is an HIF-1α inducer,[14] markedly increased *CA9* mRNA levels, suggesting that HIF-1α regulates *CA9* transcription in SAECs (Fig. 2B). We hypothesized that NO-induced DNA hypomethylation affects the interaction of HIF-1α with the *CA9* promoter region. To confirm this hypothesis, we performed ChIP-seq with an HIF-1α antibody. Interestingly, exposure to GSNO enhanced the intensity of the HIF-1α peaks in the *CA9* promoter region (Fig. 2C). These results suggest that NO-induced DNA hypomethylation may promote the recruitment of HIF-1α to the *CA9* promoter region and induce transcription of its target genes.

DISCUSSION

In this study, we investigated whether nitrosative stress induces *CA9* expression *via* epigenetic regulation using DBIC in SAECs. We demonstrated that DBIC treatment significantly suppressed the NO-induced *CA9* expression. Moreover, ChIP-seq analysis showed that exposure to GSNO promotes the recruitment of HIF-1α to the *CA9* promoter region. Interestingly, previous studies have reported that the level of DNA methylation regulates HIF-1α binding.[15] Additionally, because DNA methylation levels affect the interactions of various transcription factors,[16] nitrosative stress may affect their activity. Previous studies have reported elevated iNOS expression in respiratory diseases associated with inflammation.[17,18] Moreover, the chronic inflammatory response has been implicated as a factor in DNA methylation alterations and HIF-1α activation.[19,20] Considering previous studies and our results, epigenetic regulation by nitrosative stress may contribute to pathogenesis.

Our results showed that DBIC treatment partially suppressed NO-induced *CA9* expression. This suggests that other pathways driven by GSNO exposure contribute to the upregulation of *CA9* expression in SAECs. Therefore, it is necessary to investigate NO-induced DNA methylation changes in the *CA9* promoter region and their contribution to transcriptional activation. To address this issue, we also need to perform

ChIP-seq analysis using DBIC to investigate the direct effect of *S*-nitrosylation of DNMT3B on HIF-1α binding in the future. Altogether, our study provides insights into epigenetic regulation in normal cells by nitrosative stress, resulting in the *S*-nitrosylation of DNMT.

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Conflict of Interest The authors declare no conflict of interest.

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