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S-1-Propenylcysteine augments BACH1 degradation and heme oxygenase 1 expression in a nitric oxide-dependent manner in endothelial cells

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ABSTRACT

Garlic has been demonstrated to exert protective effects against oxidative damage using numerous experimental models. The antioxidant effects of garlic are associated with the activation of Nrf2-dependent gene expression. *S*-1-Propenylcysteine (S1PC) and *S*-allylcysteine (SAC) are two predominant sulfur amino acids present in aged garlic extract; however, the exact roles of these amino acids within the Keap1/Nrf2 system remain unknown. We hypothesized that sulfur-containing amino acids derived from garlic could activate Nrf2 in the presence of nitric oxide (NO). Neither S1PC nor SAC affected gene expression of either heme oxygenase-1 (*HMOX1*) or the glutamate-cysteine ligase modifier subunit (*GCLM*) in human umbilical vein endothelial cells (HUVECs) or human aorta endothelial cells (HAECs). Interestingly, S1PC augmented expression levels induced by nitric oxide donors (NO-donors) such as NOR3 and GSNO. NO-donors were found to induce nuclear accumulation of NRF2 and activation of the eIF2α/ATF4 pathway, whereas S1PC did not further amplify the NO-induced effects on NRF2 or eIF2α/ATF4. Additionally, NO-donors induced the degradation of BTB domain and CNC homolog 1 (BACH1), a transcriptional repressor that can compete with NRF2. In addition, S1PC enhanced BACH1 downregulation within the nucleus. Pretreatment with deferoxamine, an inhibitor of heme synthesis, upregulated BACH1 protein levels and abolished the effect of NO-donors and S1PC on HMOX1 expression. The above results indicate that S1PC could modulate antioxidant gene expression via the NO/heme/BACH1 signaling pathway, thereby suggesting that S1PC-induced degradation of BACH1 may provide a basis for therapeutic applications.

1. Introduction

Garlic supplementation is thought to provide preventive or therapeutic effects against various diseases that are caused by oxidative stress [1]. Several clinical studies reported that the intake of aged garlic extract reduces the risk of cardiovascular disease [2,3]. The sulfur-containing amino acids *S*-1-propenylcysteine (S1PC) and *S*-allylcysteine (SAC) previously isolated from aged garlic extracts have been demonstrated to exhibit high oral bioavailability [4–6]. Additionally, SAC has been shown to increase stress resistance and to reduce the accumulation of reactive oxygen species (ROS) in *Caenorhabditis elegans*-based oxidative stress models. The transcription factor SKN-1, an ortholog of nuclear factor erythroid 2 like 2 (Nfe2l2/Nrf2) in mammals, has been demonstrated to play an integral role in mediating the antioxidant response [7]. The molecular mechanism underlying SKN-1 activation by SAC or other sulfur-containing compounds, however, remain unclear.

Some natural products, such as curcumin, resveratrol, and sulforaphane, are known to activate Nrf2 in both *in vivo* and *in vitro* experimental models where Nrf2-mediated gene expression plays a crucial protective role [8]. Under basal conditions, Nrf2 binds to Kelch-like ECH-associated protein 1 (Keap1) resulting in proteasomal degradation of Nrf2 in the cytoplasm. In the presence of oxidative stress or inducers, Nrf2 is released from the Keap1-dependent complex and accumulates in the nucleus. Within the nucleus, Nrf2 binds to antioxidant response elements (AREs), which are cis-elements essential for the expression of various antioxidant and detoxification genes, including heme oxygenase 1 (*Hmox1*) and glutamate-cysteine ligase modifier subunit (*Gclm*) [8]. Additionally, the BTB domain and CNC homolog 1 (Bach1) heterodimerizes with sMaf proteins and interacts with the AREs of the corresponding genes to prevent Nrf2 binding and to inhibit gene expression in the basal state. In the presence of oxidative stress, arsenite, or heme, Bach1 is released from AREs and facilitates Nrf2 translocation

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Abbreviations: S1PC, *S*-1-propenylcysteine; SAC, *S*-allylcysteine; NO, nitric oxide; HUVECs, human umbilical vein endothelial cells; HAECs, human aorta endothelial cells; NO-donor, nitric oxide donor; ROS, reactive oxygen species; AREs, antioxidant response elements; NOR3, (±)-(*E*)-4-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide; GSNO, *S*-nitrosoglutathione; tBHQ, *tert*-butylhydroquinone; LeptB, leptomycin B

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and subsequent activation of antioxidant gene expression [9,10].

Nitric oxide (NO) is a well-characterized signaling molecule that mediates various physiological effects [11]. In addition to its physiological significance, NO influences the functional states of transcription factors and other signaling molecules and modulates gene expression. Multiple studies have reported that NO upregulates Hmox1 levels via transcriptional upregulation mediated by Nrf2 activation [12]. Although several studies have shown that NO increases nuclear Nrf2 levels by of S-nitrosylation of Keap1 [13] or inhibition of nuclear export of Nrf2 [14], the underlying molecular mechanisms remain unclear. Additionally, further studies are required to determine whether NO is involved in the regulation of Bach1 to suppress the expression of Nrf2-regulated genes.

In the present study, we evaluated the effects of sulfur-containing compounds on NRF2-regulated gene expression. Our results revealed that S1PC enhanced the expression of antioxidant genes in a NO-dependent manner. We identified a novel interaction between S1PC and NO, and we found that NO induces BACH1 degradation in the nucleus and that S1PC enhances BACH1 downregulation. The interaction between NO and S1PC and the role of this interaction in regulating BACH1 protein levels provides a potential novel therapeutic target for improving antioxidant defense status against certain diseases.

2. Materials and methods

2.1. Reagents

S1PC, SAC, and *S*-allylmercaptocysteine (SAMC) were synthesized and purified as previously described [15,16]. The NO-donors (±)-(*E*)-4-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide (NOR3) and *S*-nitrosoglutathione (GSNO) were purchased from Dojindo (Kumamoto, Japan). *tert*-Butylhydroquinone (tBHQ), hemin, and deferoxamine mesylate salt were obtained from Sigma-Aldrich (St. Louis MO). Leptomycin B (LeptB) was purchased from LC laboratories (Woburn, MA). Antibodies used included anti-HMOX1 (ab13248, Abcam Cambridge, UK), anti-BACH1 (14018-1-AP, Proteintech, Rosemont, IL), anti-NRF2 (sc-13032, Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-EIF2A (Ser51) (#3398, Cell Signaling Technology, Danvers, MA), anti-EIF2A (#5324, CST), anti-GAPDH (015-25473, Wako pure chemical, Japan), anti-ACTB (20536-1AP, Proteintech), and anti-LMNB1 (66095-1-Ig, Proteintech).

2.2. Cell culture

Commercially available human umbilical vein endothelial cells (HUVECs) (Lonza, Basel, Switzerland) and human aorta endothelial cells (HAECs) (Lonza) were grown in EGM™-2 medium (Lonza) containing supplements and 2% fetal bovine serum (FBS). Confluent cell cultures grown in EBM™-2 medium (Lonza) containing 2% FBS were used for analysis of gene expression or signaling molecules.

2.3. Quantitative real-time RT-PCR

Total RNA was isolated from cells using TRIzol™ reagent (Thermo Fisher Scientific, Waltham, MA) according to manufacturer protocol. First-strand cDNA synthesis was performed using random six-mers, oligo-dT primers, and PrimeScript™ RT reagent (Takara Bio, Otsu, Japan). Real-time PCR was performed using TB Green™ premix (Takara) on a PikoReal™ system (Thermo). The following PCR primer pairs were used for real-time PCR: *HMOX1*, 5′- CTT TCA GAA GGG CCA GGT GAC -3′, 5′- GCG CTC AAT CTC CTC CTC CAG -3′; *GCLM*, 5′- CAG CGA GGA GCT TCA TGA TTG -3′, 5′- TGC ATT CCA AGA CAT CTG GAA A -3′; *BACH1*, 5′- GAG ACG GAC ACC GAA GGA GA -3′, 5′- CGC TGT GCA GCA ATT CTG TT -3′; *NRF2*, 5′- AGT GGA TCT GCC AAC TAC TC -3′, 5′- CAT CTA CAA ACG GGA ATG TCT G -3′; *TXNRD1*, 5′- TTG CAG CTG CGC TCA AAT GT -3′, 5′- ATT TGG GCA CGG AAA CGA GC -3′; *ACTB*, 5′- CGC GAG AAG ATG ACC CAG AT -3′, 5′- GGT GAG GAT CTT CAT GAG GTA GTC -3′. mRNA expression levels were calculated relative to that of the *ACTB* gene according to the comparative $2^{-\Delta\Delta CT}$ method.

2.4. Immunoblot analyses

Whole-cell proteins were extracted using a radio-immunoprecipitation assay (RIPA) buffer (Wako) containing a protease and phosphatase inhibitor cocktail (Thermo). Nuclear and cytosolic fractions were prepared using NE-PER™ reagents according to manufacturer protocol (Thermo). Proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and subjected to immunoblot analysis. Proteins that reacted with primary antibodies were detected using HRP-conjugated secondary antibody and either Amersham™ ECL Prime (GE Healthcare, Menlo Park, CA) or Supersignal™ West Femto (Thermo). Chemiluminescence was visualized and quantified using a ChemiDoc™ MP Imager (Bio-Rad Laboratories, Hercules, CA).

2.5. Small interfering RNA (siRNA) transfection

HUVECs were seeded at 1.4×10^4 cells/well on 48-well plates and subsequently transfected with 2.4 pmol of control (sc-44230, Santa Cruz) or specific siRNA cocktails targeting *BACH1* (sc-37064, Santa Cruz) or *NRF2* (sc-37030, Santa Cruz). Transfections were performed using Lipofectamine™ RNAiMAX (Thermo) in accordance with manufacturer instructions. At 72 h post-transfection, HUVECs were treated with NOR3 and/or S1PC for 6 h, and the expression levels of mRNA were measured by quantitative real-time RT-PCR.

2.6. Statistical analysis

Data are expressed as means ± standard deviation (SD). Group comparisons were performed using a Student's *t*-test or one-way analysis of variance followed by Bonferroni's multiple comparison test. All statistical analyses were performed using WinSTAT (M Sato, Japan). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of S1PC on HMOX1 and GCLM expression in endothelial cells

To evaluate the effect of S1PC on antioxidant gene expression in endothelial cells, *HMOX1* and *GCLM* expression levels were measured in HUVECs treated with S1PC and/or NOR3. Results revealed that NOR3, but not S1PC, induced the expression of *HMOX1* and *GCLM*; however, S1PC more effectively enhanced the NOR3-induced gene expressions compared to enhancement by treatment with NOR3 alone (Fig. 1A). Additionally, results from immunoblot analysis demonstrated the synergistic effects of S1PC and NOR3 in upregulating HMOX1 expression (Fig. 1B). To verify the synergistic effects of S1PC and NO on *HMOX1* induction, similar experiments were performed by exposing HAECs to the NO-releasing compound GSNO. Consistent with previous results, treatment with S1PC alone did not significantly affect *HMOX1* expression, but co-treatment with S1PC and GSNO led to synergistic upregulation of *HMOX1* expression (Fig. 2). Conversely, SAC and SAMC did not exert synergistic effects in combination with GSNO. S1PC, SAC, and SAMC are sulfur-containing amino acids that are abundant in aged garlic extracts [4]. Despite small differences in the *S*-propenyl structures of S1PC and SAC, only S1PC was found to exert synergistic effects on *HMOX1* induction by GSNO (Fig. 2).

3.2. Effects of S1PC and NOR3 on signaling molecules involved in HMOX1 expression

A number of signaling molecules and transcription factors have

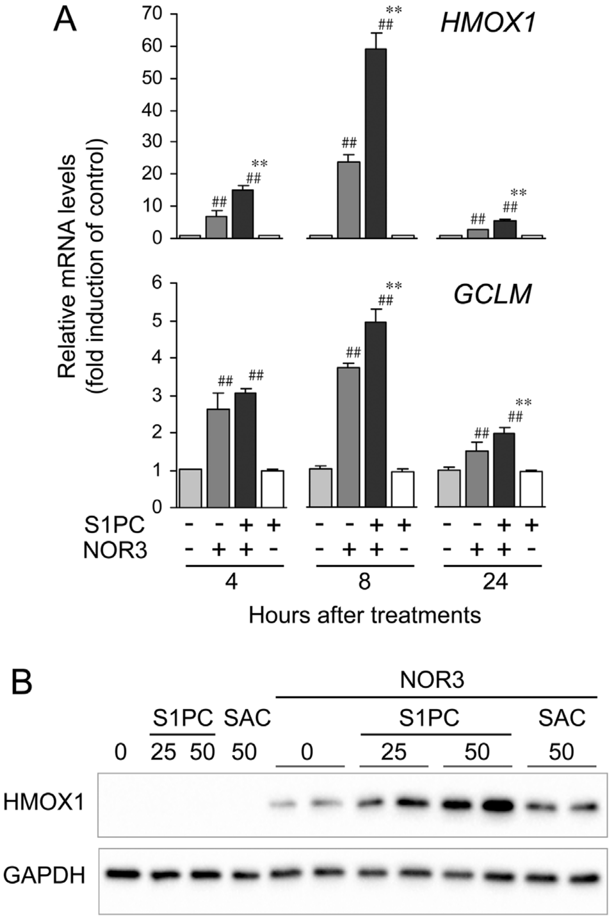

Fig. 1. Effects of S1PC on NOR3-induced *HMOX1* and *GCLM* expression in HUVECs. (A) HUVECs were treated with NOR3 (25 μM) and/or S1PC (50 μM) for 4, 8, or 24 h. Relative mRNA levels of *HMOX1* and *GCLM*, normalized to *ACTB* levels, were determined by real-time PCR. Data are expressed as mean ± SD (n = 3). Significant differences relative to the control cells (##p < 0.01) or NOR3-treated cells were determined (**p < 0.01) using one-way ANOVA followed by Bonferroni's multiple comparison test. (B) HUVECs were treated with NOR3 (50 μM) and/or S1PC (25 or 50 μM) and SAC (50 μM) for 16 h. Whole-cell lysates were subjected to immunoblot analysis. Results of HMOX1 and GAPDH are shown.

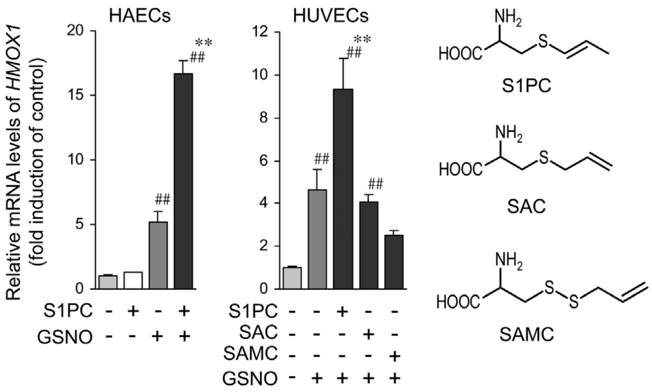

Fig. 2. Effects of sulfur-containing compounds on GSNO-induced *HMOX1* expression in endothelial cells. Endothelial cells were treated with GSNO (100 μM) and/or S1PC, SAC, SAMC (100 μM) for 6 h. Relative mRNA levels of *HMOX1*, normalized to *ACTB* levels, were determined by real-time PCR. Data are expressed as mean ± SD (n = 3). Significant differences relative to the control (##p < 0.01) or GSNO-treated cells were determined (**p < 0.01) using one-way ANOVA followed by Bonferroni's multiple comparison test.

been identified to regulate HMOX1 expression [17,18]. To explore the synergistic effects of S1PC and NO in the context of antioxidant gene expression, we analyzed protein expression and activation of signaling molecules including NRF2, the alpha subunit of the eukaryotic initiation factor 2 (eIF2α/EIF2A), mitogen-activated protein kinases (MAPKs), and nuclear factor-kappa B (NF-κB). Activation of the KEAP1/NRF2 system was examined by immunoblotting for NRF2. We observed no significant NRF2 accumulation in HUVECs treated with S1PC alone. NOR3 was found to induce NRF2 accumulation, but cells co-treated with NOR3 and S1PC showed no significant differences in NRF2 protein levels compared to those in cells treated only with NOR3 (Fig. 3). Activation of the endoplasmic reticulum (ER) stress response or the integrated stress response (ISR) was assessed by measuring increases in EIF2A phosphorylation and subsequent increases in the protein levels of activating transcription factor 4 (ATF4) [19]. NOR3 treatment increased EIF2A phosphorylation, but cells co-treated with NOR3 and S1PC showed no significant differences in E1F2A phosphorylation compared to that of cells treated with NOR3 alone (Fig. 3). ATF4 protein levels were induced in response to NOR3 in the nuclear fraction. Our results confirmed that S1PC exerted no synergistic effects in combination with NOR3 (Fig. S1). Additionally, we analyzed NRF2 phosphorylation at serine 40 as a marker of ER stress response. In this context, treatment with NOR3 and/or S1PC did not affect NRF2 phosphorylation levels (Fig. S2). Immunoblot analysis revealed that cells treated with NOR3 alone and cells co-treated with NOR3 and S1PC exhibited no significant differences in P-ERK5, P-JNK, and P-AKT levels (data not shown) and nuclear accumulation of NF-κB p65 (Fig. S1).

BACH1 and FRA1 play suppressive roles in NRF2-dependent ARE-driven transcriptional responses [20,21]. NOR3 caused a reduction in BACH1 protein levels in HUVECs, whereas S1PC did not affect BACH1 levels. S1PC did, however, enhance BACH1 downregulation upon co-treatment with NOR3 (Fig. 3). Additionally, BACH1 downregulation was observed in the presence of cycloheximide, a translation inhibitor, and BACH1 was degraded post-translationally (Fig. S3). FRA1 protein levels were analyzed in the nuclear fraction, and results indicated that NOR3 and S1PC did not affect FRA1 protein expression (Fig. S4).

3.3. Subcellular localization and downregulation of BACH1 following treatment with S1PC and NOR3

Nuclear localization of BACH1 is essential for this protein to exert suppressive effects on ARE-driven transcription. To investigate BACH1 downregulation following treatment with NO-donor or S1PC, we examined the changes in the nuclear and cytosolic distribution of BACH1. BACH1 was present in both the nuclear and the cytosolic fractions in control cells, and BACH1 levels in the cytosol were higher than those in the nucleus (Fig. 4A). BACH1 levels were significantly downregulated by NOR3 treatment in both the nuclear and cytosolic fractions, and they were further downregulated following co-treatment with S1PC and NOR3 (Fig. 4B). BACH1 has been reported to be exported from the nucleus and ubiquitinated and degraded in response to oxidative stress or heme exposure [22]. To evaluate whether the NOR3-induced or S1PC-enhanced degradation of BACH1 is dependent on its nuclear export, cells were pretreated with leptomycin B (LeptB), an inhibitor of chromosomal region maintenance 1 (CRM1/exportin-1), and were analyzed for BACH1 protein levels in the nuclear and cytosolic fractions. BACH1 nuclear levels were found to be upregulated in control cells treated with LeptB when compared to that in untreated cells. Despite LeptB treatment, nuclear BACH1 levels were significantly downregulated by treatment with NOR3 alone or in combination with S1PC (Fig. 4A and B). Conversely, cytosolic BACH1 levels were downregulated in LeptB-treated control cells relative to untreated cells. Co-treatment with NOR3 and S1PC did not significantly influence BACH1 levels in the cytosol compared to treatment with LeptB alone (Fig. 4A and B). The above results indicate that NOR3 and S1PC induce BACH1 degradation in the nucleus.

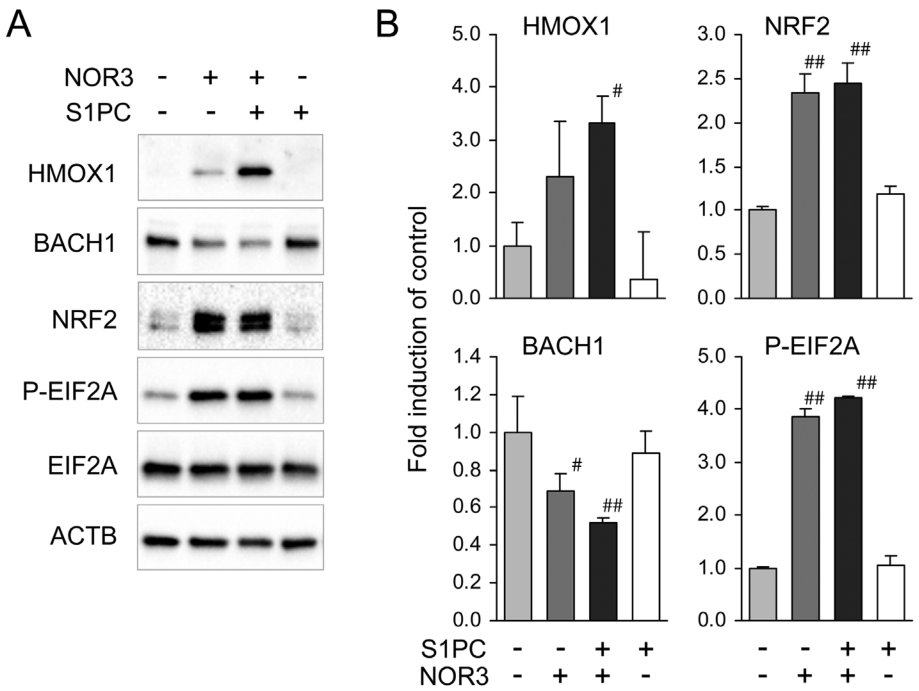

Fig. 3. Effects of S1PC and NOR3 on signaling molecules related to HMOX1 expression. HUVECs were treated with NOR3 (25 μM) and/or S1PC (50 μM) for 6 h. Whole-cell lysates were extracted using RIPA buffer and subjected to immunoblot analysis. (A) Representative immunoblots of HMOX1, BACH1, NRF2, phospho-EIF2A, EIF2A, and ACTB are shown. (B) Immunoblot signals of HMOX1, BACH1, and NRF2 were quantified and normalized to those of ACTB. Phospho-EIF2A signals were normalized to those of EIF2A. Data are expressed as mean ± SD (n = 3). Significant differences relative to the control cells were determined (#p < 0.05, ##p < 0.01) using one-way ANOVA followed by Bonferroni's multiple comparison test.

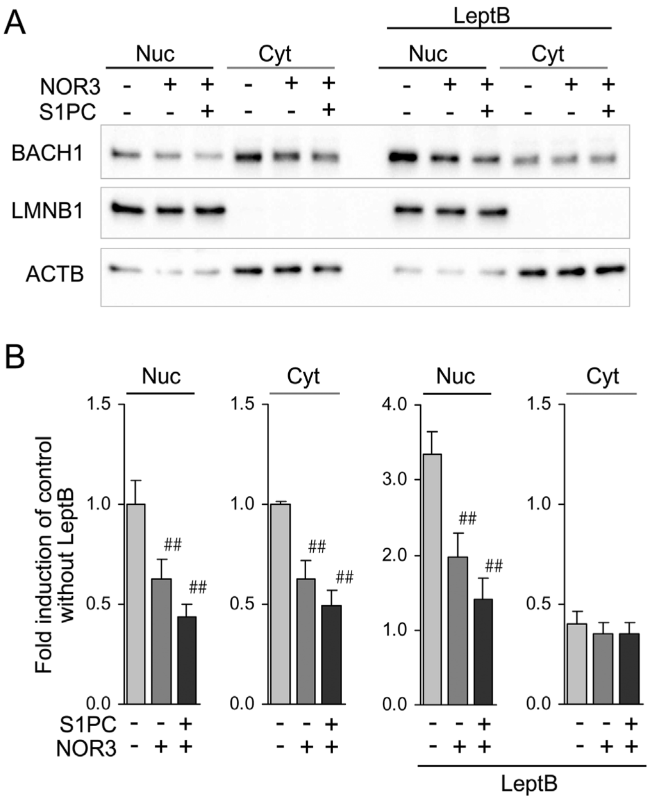

Fig. 4. Effects of S1PC and NOR3 on subcellular localization of BACH1 in HUVECs. Cells were treated with NOR3 (25 μM) and/or S1PC (50 μM) with or without leptomycin B (40 nM) for 4 h. Nuclear and cytosolic extracts were subjected to immunoblot analysis. The lysates were prepared from an equal amount of cells. (A) Representative immunoblots of BACH1, LMNB1, and ACTB are shown. (B) Immunoblot signals of BACH1 were quantified and normalized to those of LMNB1 for nuclear extracts or ACTB for cytosolic extracts. Data are expressed as mean ± SD (n = 3). Significant differences relative to the control cells were determined (##p < 0.01) using one-way ANOVA followed by Bonferroni's multiple comparison test.

3.4. Effects of S1PC on HMOX1 and TXNRD1 expression in HUVECs treated with NOR3, hemin, or tBHQ

Both NRF2 and BACH1 are known to interact with ARE motifs; however, several lines of evidence indicate that the mechanisms underlying these interactions with DNA are not identical for all genes regulated by AREs. The *HMOX1* gene has been demonstrated to be suppressed upon BACH1 binding to the two ARE motifs located in the upstream region of the gene; however, a single ARE motif found in the upstream region of the *TXNRD1* gene showed no interaction with BACH1 [23]. To further demonstrate that BACH1 is a key molecule regulated by S1PC, we examined changes in *TXNRD1* gene expression. *TXNRD1* gene expression was significantly induced in NOR3-treated cells relative to that in untreated cells, whereas S1PC showed no synergistic effect in combination with NOR3 (Fig. 5). As mentioned above, co-treatment with S1PC and NOR3 synergistically upregulated *HMOX1* expression. Hemin and tBHQ act as inducers of BACH1 degradation and KEAP1/NRF2 activation [23,24]. Consistent with previous findings, we confirmed that hemin induced *HMOX1* expression but not *TXNRD1* expression. Additionally, tBHQ treatment induced the expression of both *HMOX1* and *TXNRD1*; however, hemin- or tBHQ-induced *HMOX1* expression was not affected by S1PC (Fig. 5). These results indicate that S1PC activity is specific to BACH1-regulated genes and dependent upon the effects of NO.

3.5. Effects of BACH1 depletion on S1PC-induced HMOX1 expression in HUVECs

To further evaluate the role of BACH1 and NRF2 on S1PC-induced *HMOX1* expression, we used HUVEC cells transfected with siRNA targeting BACH1 or NRF2. The siRNAs-transfected cells showed partial depletion of *BACH1* mRNA or *NRF2* mRNA, with levels reaching approximately 50% or 20% of those of the control siRNA-transfected cells, respectively (Fig. S5). BACH1 depletion resulted in a 5-fold increase in *HMOX1* expression in basal or NOR3-treated cells compared that observed in control siRNA-transfected cells. Co-treatment with S1PC and NOR3, however, resulted in no significant enhancement of *HMOX1* expression compared to that in NOR3-treated cells (Fig. 6). Conversely, HMOX1 expression was severely reduced in the NRF2-depleted cells;

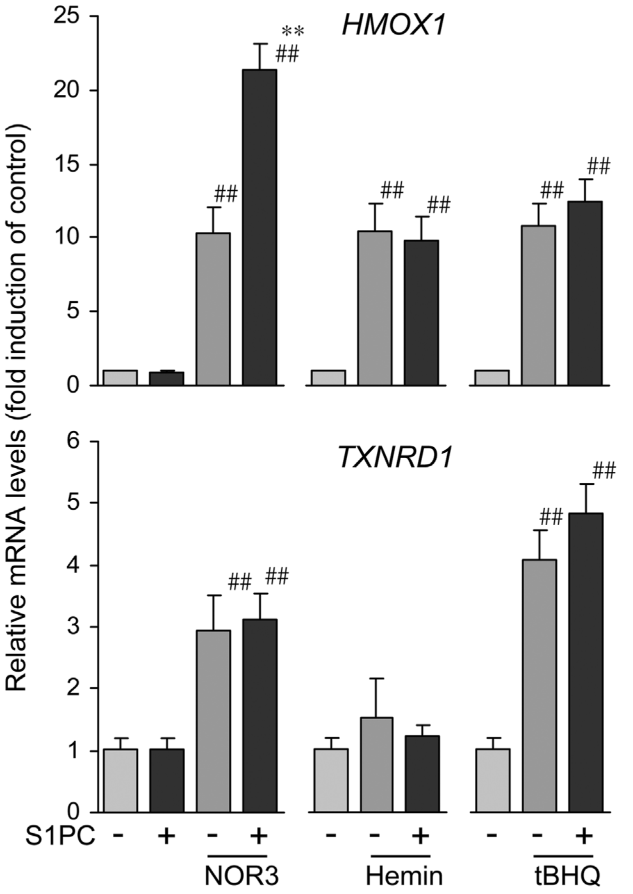

Fig. 5. Effects of S1PC on *HMOX1* and *TXNRD1* expression in HUVECs treated with hemin, tBHQ, or NOR3. Cells were treated with NOR3 (25 μM), hemin (0.5 μM), or tBHQ (5 μM) with or without S1PC (100 μM) for 6 h. Relative mRNA levels of *HMOX1* and *TXNRD1*, normalized to *ACTB* levels, were determined by real-time PCR. Data are expressed as mean ± SD (n = 3). Significant differences relative to control (##p < 0.01) or NOR3-treated cells (**p < 0.01) were determined using one-way ANOVA followed by Bonferroni's multiple comparison test.

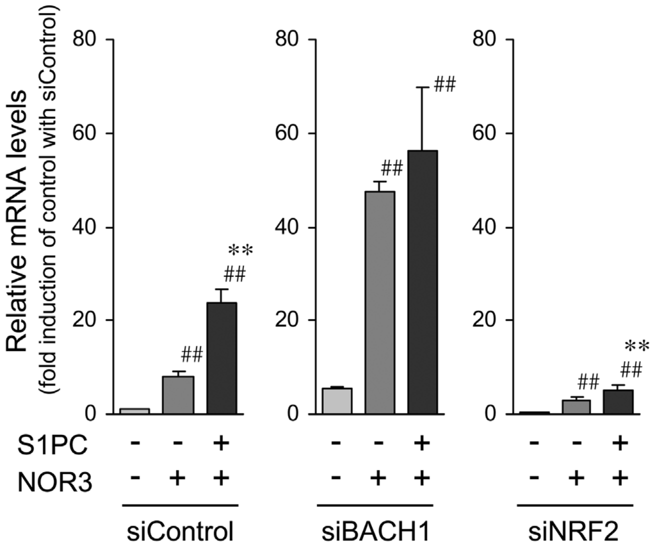

Fig. 6. Effect of *BACH1 depletion* on S1PC-induced *HMOX1* expression in HUVECs. HUVEC cells were transfected with siRNA targeting *BACH1* or *NRF2*. At 72 h post transfection, cells were treated with NOR3 (25 μM) with or without S1PC (50 μM) for 6 h. Relative mRNA levels of *HMOX1* normalized to *ACTB* levels were determined by real-time PCR. Data are expressed as mean ± SD (n = 4). Significant differences relative to control (##p < 0.01) or NOR3-treated cells (**p < 0.01) were determined using one-way ANOVA followed by Bonferroni's multiple comparison test.

however, co-treatment of S1PC with NOR3 still significantly induced HMOX1 expression (Fig. 6). These results indicated that BACH plays a pivotal role in the synergistic effect of S1PC and NOR3 on *HMOX1* induction.

3.6. Effect of iron deficiency on HMOX1 expression induced by NOR3 and S1PC

The binding of heme to the BACH1 C-terminal domain has been demonstrated to inhibit its DNA binding activity, trigger its export from the nucleus, and induce its ubiquitination and subsequent degradation [25]. Therefore, iron deficiency induced by deferoxamine, a ferric iron chelator, inhibits heme synthesis in cells and results in decreased heme levels and induced nuclear accumulation of BACH1 [26]. Here, we used an iron deficiency model to verify the relationship between BACH1 and HMOX1 expression levels induced by NO and S1PC. HUVECs were pretreated with deferoxamine to induce excessive BACH1 accumulation. Cells exhibiting upregulated BACH1 expression were used for further analysis. Deferoxamine treatment to induce iron deficiency caused upregulation of BACH1 levels by more than twofold relative to those of the controls (Fig. 7). BACH1 protein levels were downregulated in cells with NOR3 and S1PC but showed no significant differences in control cells without deferoxamine treatment. Despite the observed BACH1 downregulation induced by NOR3 and S1PC, the BACH1 protein still suppressed HMOX1 expression under deferoxamine treatment (Fig. 7). Analysis of mRNA expression levels also indicated that iron deficiency inhibited the upregulation of *HMOX1* expression induced by co-treatment with NOR3 and S1PC (Fig. 8). In contrast, NOR3-induced *TXNRD1* expression was unaffected in deferoxamine-treated cells (Fig. 8).

4. Discussion

Garlic supplementation is well known to exert a wide range of beneficial effects, particularly in the prevention of cardiovascular disease [27]. Several studies indicated that the beneficial effects of garlic supplementation can be attributed to the antioxidant activities of compounds present in the garlic preparations. Nrf2-mediated antioxidant gene expression is one of the primary molecular mechanisms underlying the beneficial effects of garlic supplementation [1,28]. In the present study, we demonstrated that S1PC, a sulfur-containing amino acid isolated from aged garlic extract, enhanced ARE-mediated transcription of the genes such as *HMOX1* and *GCLM* in a NO-dependent manner. Additionally, S1PC did not enhance NRF2 accumulation, but instead promoted the degradation of BACH1, a repressor of ARE-mediated transcription, in the presence of NO. These results shed light on the potential role of BACH1 as a target protein of sulfur-containing amino acids present during antioxidant gene response and suggest that S1PC acts in coordination with NO.

NO is an intracellular messenger gas that plays a key role in numerous physiological and pathological processes. In addition to its physiological roles, NO is known to modulate the expression of various genes [29]. One mechanism underlying NO-mediated regulation of gene expression is the activation of the KEAP1-NRF2 pathway, which can be triggered by NO-mediated S-nitrosylation of KEAP1 [13]. Another mechanism is the activation of multiple serine-threonine kinases, such as those controlling the unfolded protein response that leads to eIF2α/EIF2A phosphorylation and stress-dependent translation of ATF4 [30]. At higher ATF4 levels, ATF4 can dimerize with NRF2 and bind to AREs present in the *HMOX1* gene [31]. In our current study, NO-donors were found to induce NRF2 accumulation and EIF2A phosphorylation, indicating that NO could activate these signaling pathways in HUVECs.

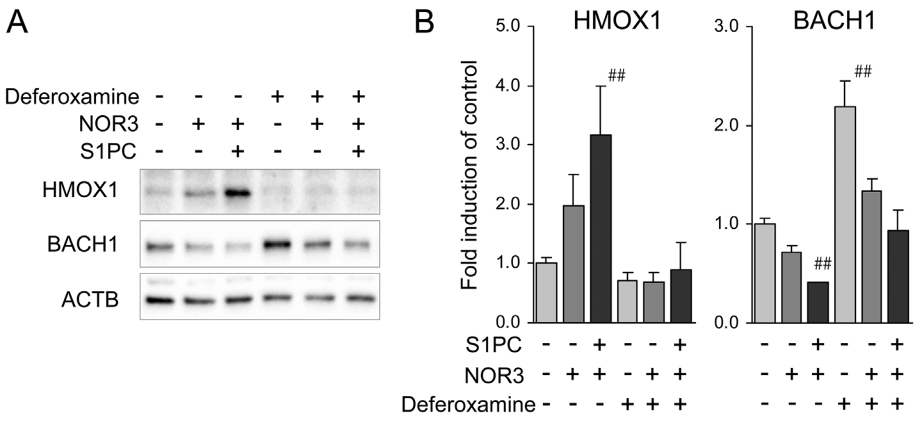

Fig. 7. Effects of NOR3 and S1PC on BACH1 and HMOX1 under iron-deficient conditions. HUVECs were pretreated with deferoxamine (100 µM) for 15 h and then treated with NOR3 (25 µM) and/or S1PC (50 µM) for 6 h. Whole-cell lysates were extracted using RIPA buffer and subjected to immunoblot analysis. (A) Representative immunoblots of HMOX1, BACH1, and ACTB are shown. (B) Immunoblot signals of HMOX1 and BACH1 were quantified and normalized to those of ACTB. Data are expressed as mean ± SD (n = 3). Significant differences relative to the control cells were determined (##p < 0.01) using one-way ANOVA followed by Bonferroni's multiple comparison test.

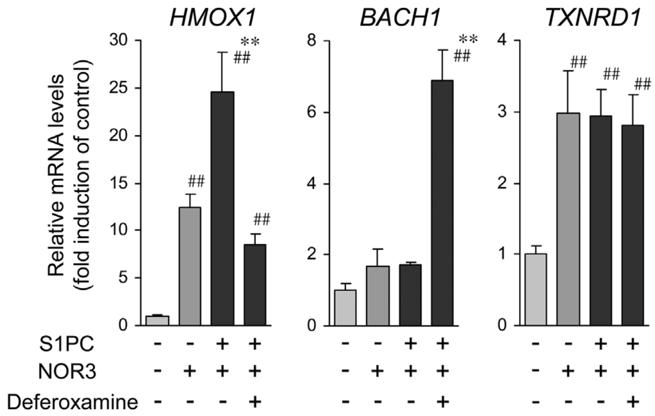

Fig. 8. Effects of iron deficiency on *HMOX1* expression induced by NOR3 and S1PC. HUVECs were pretreated with deferoxamine (100 µM) for 15 h and subsequently treated with NOR3 (25 µM) and/or S1PC (50 µM) for 6 h. Relative mRNA levels of *HMOX1*, *BACH1*, and *TXNRD1*, normalized to *ACTB* levels, were determined by real-time PCR. Data are expressed as mean ± SD (n = 4). Significant differences relative to the control (##p < 0.01) or NOR3-treated cells were determined (**p < 0.01) using one-way ANOVA followed by Bonferroni's multiple comparison test.

Although S1PC treatment upregulated HMOX1 expression, it did not affect NRF2 accumulation and EIF2A phosphorylation. Conversely, S1PC augmented BACH1 degradation. S1PC treatment also elevated the expression of *HMOX1* and *GCLM*, but this treatment did not affect *TXNRD1*. This is in agreement with a previous study that demonstrated that the ARE motif of *TXNRD1* is not regulated by BACH1 [23]. Additionally, siRNA-mediated depletion of BACH1 abolished the S1PC-dependent enhancement on the *HMOX1* expression. Together, these findings suggest that BACH1 is the primary target of S1PC during the upregulation of ARE-mediated genes.

In the present study, BACH1 downregulation did not correlate with nuclear accumulation of NRF2, suggesting that these changes were triggered via an independent mechanism. BACH1 is a transcriptional repressor that is regulated by heme. Heme binding to the BACH1 C-terminal domain inhibits BACH1 DNA binding activity, triggers its export from the nucleus, and induces its ubiquitination and subsequent degradation [25]. Heme has been proposed to bind to the CP motif of BACH1, an area that contains dipeptide sequences of cysteine and proline [32]. Multiple studies reported that protein-bound heme further binds gas molecules, such as NO, oxygen, and carbon monoxide. Therefore, the interaction of heme with the cysteine residue of the CP motif could serve as a potential target for NO. The assumption that heme binds more strongly to BACH1 in the presence of higher NO concentrations, however, could not be explained by current models and requires further investigation.

tBHQ has been reported to induce the phosphorylation of tyrosine 486 of BACH1, thereby enhancing its nuclear export in a CRM1-dependent manner [33]. Additionally, diamide, a sulfhydryl oxidizing agent, was reported to induce the dissociation of BACH1 from AREs. During this process, it is thought that the cysteine residue of BACH1 (C574) acts as a sensor of redox status [34]. In the case of NO, the cysteine residue of BACH1 (C578) can serve as a target of nitrosylation to induce nuclear export of BACH1. In the present study, however, BACH1 degradation induced by the combination of NO and S1PC was observed in the nucleus. The observed nuclear degradation of BACH1 is inconsistent with previously reported findings [22,33,35], and our findings suggested a novel mechanism for BACH1 downregulation. Some cysteine residues of BACH1 are likely to be targets of nitrosylation, and this modification can induce conformational changes and subsequent degradation of BACH1. Similarly, S1PC could interact with the *S*-nitrosothiols of BACH1 to influence protein stability. The mechanisms underlying these interactions require further investigation.

Various sulfur compounds derived from several sulfur-containing amino acids originally found in raw garlic are responsible for the characteristic properties of garlic supplements [36]. S1PC and SAC isolated from aged garlic extracts have been demonstrated to exhibit remarkable oral bioavailability [4,5]. In the present study, S1PC was observed to uniquely interact with NO to modulate the expression of ARE-regulated genes. In contrast, SAC, a stereoisomer of S1PC, did not affect the expression of ARE-regulated genes. Additionally, a recent study indicated that S1PC, but not SAC, increases immunoglobulin A production *in vivo* and *in vitro* [37]. The above results indicate that the structure of *S*-1-propenyl is critical for the unique physiological effects of S1PC. The above findings also provide evidence that the unique feature of the sulfur atom in the *S*-1-propenyl moiety is responsible for the stereospecific activity of S1PC in the context of BACH1 degradation.

Although Bach1 is expressed in diverse cell types, no clear defects are observed in *Bach1*-deficient mice under normal conditions [10]. *Bach1*-deficient mice also exhibit elevated disease tolerance in various models, including atherosclerosis in *ApoE* double-knockout mice [38], lung damage after high oxygen exposure [39], osteoarthritis [40], and chemically induced colitis [41]. Multiple studies have demonstrated the involvement of Hmox1 in this resistance. This protein is highly expressed in diverse tissues in *Bach1*-deficient mice. Additionally, recent studies have reported that Bach1 is a critical regulator of the immune system, specifically in the context of macrophage polarization. Genetic ablation of *Bach1* was found to promote the activation of M2 macrophages and ameliorate lupus nephritis in mice [42]. The above results indicate that downregulation of BACH1 levels and upregulation of HMOX1 levels may provide promising therapeutic approaches for the treatment of inflammatory diseases.

In conclusion, our findings demonstrated that S1PC augments HMOX1 expression in a NO-dependent manner, and its effects are associated with the enhancement of BACH1 degradation. In addition, S1PC and NO potentially degrade BACH1 based on the observation that nuclear degradation of BACH1 differs from what was observed in previous studies [22,33,35]. Although the mechanism by which S1PC interacts with NO remains unclear, the inhibition of BACH1 expression by S1PC provides novel insight into potential therapeutic strategies for the treatment of various inflammatory diseases.

Conflicts of interest

The authors have declared that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.niox.2019.01.003.

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