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Activation of ATM/Akt/CREB/eNOS Signaling Axis by Aphidicolin Increases NO Production and Vessel Relaxation in Endothelial Cells and Rat Aortas

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Abstract

Although DNA damage responses (DDRs) are reported to be involved in nitric oxide (NO) production in response to genotoxic stresses, the precise mechanism of DDR-mediated NO production has not been fully understood. Using a genotoxic agent aphidicolin, we investigated how DDRs regulate NO production in bovine aortic endothelial cells. Prolonged (over 24 h) treatment with aphidicolin increased NO production and endothelial NO synthase (eNOS) protein expression, which was accompanied by increased eNOS dimer/monomer ratio, tetrahydrobiopterin levels, and eNOS mRNA expression. A promoter assay using 5'-serially deleted eNOS promoters revealed that Tax-responsive element site, located at −962 to −873 of the eNOS promoter, was responsible for aphidicolin-stimulated eNOS gene expression. Aphidicolin increased CREB activity and ectopic expression of dominant-negative inhibitor of CREB, A-CREB, repressed the stimulatory effects of aphidicolin on eNOS gene expression and its promoter activity. Co-treatment with LY294002 decreased the aphidicolin-stimulated increase in p-CREB-Ser^{133} level, eNOS expression, and NO production. Furthermore, ectopic expression of dominant-negative Akt construct attenuated aphidicolin-stimulated NO production. Aphidicolin increased p-ATM-Ser^{1981} and the knockdown of ATM using siRNA attenuated all stimulatory effects of aphidicolin on p-Akt-Ser^{473}, p-CREB-Ser^{133}, eNOS expression, and NO production. Additionally, these stimulatory effects of aphidicolin were similarly observed in human umbilical vein endothelial cells. Lastly, aphidicolin increased acetylcholine-induced vessel relaxation in rat aortas, which was accompanied by increased p-ATM-Ser^{1981}, p-Akt-Ser^{473}, p-CREB-Ser^{133}, and eNOS expression. In conclusion, our results demonstrate that in response to aphidicolin, activation of ATM/Akt/CREB/eNOS signaling cascade mediates increase of NO production and vessel relaxation in endothelial cells and rat aortas.

Key Words: Endothelial nitric oxide synthase, Nitric oxide, Vessel relaxation, Aphidicolin, DNA damage response

INTRODUCTION

Nitric oxide (NO) has a variety of biological effects in cells, largely due to the isoforms of NO synthase (NOS) expressed in specific types of cells. For example, NO catalyzed by neuronal NOS (nNOS) controls synaptic transmission in neurons, while NO produced by inducible NOS (iNOS) regulates inflammatory responses in macrophages. NO in endothelial cells (ECs) is produced by the action of endothelial NOS (eNOS), and this NO has a vasodilatory effect (Bruckdorfer, 2005; Forstermann and Sessa, 2012).

Among many regulatory mechanisms underlying eNOS-derived NO production, the two mechanisms are the main determinants; regulation of eNOS phosphorylation and regulation of eNOS gene expression. eNOS contains various phosphorylation sites; serine 1179 (Ser^{1179}) and Tyr^{83} are activatory phosphorylation residues, while Ser^{116} and Thr^{497} are inhibitory (in bovine sequences) (Fleming, 2010; Heiss and Dirsch, 2014; Seo *et al*., 2014). Phosphorylation of these sites is regulated by various stimuli and contributes to acute endothelial NO production and vascular tone (Kou *et al*., 2002; Bae *et al*., 2003; Cho *et al*., 2004; Zhang *et al*., 2006; Park *et al*., 2011; Cho *et al*., 2014). In contrast, the eNOS gene is constitutively expressed at basal levels under normal conditions (Searles, 2006). However, long-term treatment with estrogen or exercise has been reported to be able to induce eNOS gene

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expression (Forstermann *et al*., 1998; Searles, 2006). In this regard, we previously reported that hypoxia induces eNOS expression by activating Tax-responsive element (TRE), a cAMP response element (CRE)-like site in the eNOS promoter (Min *et al*., 2006). Alteration of eNOS expression is likely to affect NO production and vascular tone profoundly. Nonetheless, it is also noted that the levels of eNOS protein are not always directly correlated with NO production (Forstermann *et al*., 2017); under pathological states like atherosclerosis and hypertension which are associated with oxidative stress, eNOS is uncoupled due to deficiency of tetrahydrobiopterin (BH_4), one of the major underlying factors, producing superoxide instead of NO.

DNA damage responses (DDRs) can be induced by various genotoxic stresses such as ionizing irradiation, oxygen radicals, camptothecin, and aphidicolin (Tanaka *et al*., 2007; Ciccia and Elledge, 2010; Poehlmann and Roessner, 2010). These DNA damage agents initiate DDRs by activating upstream DNA damage sensors such as ataxia-telangiectasia mutated kinase (ATM), ATM- and Rad3-related kinase (ATR), and DNA-dependent protein kinase (DNA-PK) (Ciccia and Elledge, 2010; Poehlmann and Roessner, 2010). Cell type, DNA damage agent, and extent of damage ultimately determine the fate and function of cells. Numerous studies of cell dysfunction and death due to DNA damage agents have been conducted in various types of cells, particularly cancer cells (Ciccia and Elledge, 2010; Carrassa and Damia, 2017). Because most DNA damage agents are used as chemotherapeutic agents and ECs are often exposed to them during anticancer chemotherapy, and eNOS-derived NO plays a critical role in maintenance of vascular homeostasis by mediating vascular relaxation (Bruckdorfer, 2005; Forstermann and Sessa, 2012), unraveling the effects of DNA damage agents on NO production and vessel tone, and determining the underlying mechanisms are essential to understand the effect of DNA damage agents used for anticancer chemotherapy *in vivo*.

In this study, we investigated the mechanism by which aphidicolin increases NO production in bovine aortic endothelial cells (BAECs) and rat aortas, and found that activation of ATM/Akt/CRE binding protein (CREB)/eNOS signaling cascade stimulated NO production in aphidicolin-treated ECs and vessel relaxation.

MATERIALS AND METHODS

Materials

Aphidicolin, LY294002, H-89, forskolin, and dimethyl sulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany). Phenylephrine and acetylcholine (ACh) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Antibody against eNOS was purchased from Transduction Laboratories (Lexington, KY, USA). Antibodies against Akt, p-Akt-Ser^{473}, CREB, p-CREB-Ser^{133}, ATM, and p-ATM-Ser^{1981} were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibody against tubulin was purchased from Abcam (Cambridge, MA, USA). Minimum essential medium (MEM), Medium 200 (M200), Dulbecco's phosphate-buffered saline (DPBS), newborn calf serum (NCS), fetal bovine serum (FBS), low serum growth supplement (LSGS), penicillin-streptomycin antibiotics, L-glutamine, trypsin-EDTA solution, and plasticware for cell culture were purchased from Gibco-BRL (Gaithersburg, MD, USA). All other chemicals used were of the purest analytical grade available.

Cell culture and drug treatments

BAECs were isolated and maintained in MEM supplemented with 5% NCS at 37°C under 5% CO_2 in air as described previously (Kim *et al*., 1999). Human umbilical vein endothelial cells (HUVECs) were isolated and cultured in M200 supplemented with LSGS and 10% FBS as reported previously (Hwang *et al*., 2014). Cells were passaged five to nine times for all experiments. BAECs or HUVECs grown to 60% confluence in 60-mm culture dishes were incubated in the absence or presence of various concentrations of aphidicolin for 24 h or 20 μM aphidicolin for the indicated times in fresh MEM containing 5% NCS or M200 supplemented with LSGS and 10% FBS.

Transfection with A-CREB or dominant-negative Akt cDNA

For transfection with A-CREB, a dominant-negative (dn)-CREB, or dn-Akt, 3 μg of the pcDNA3.1 vector containing human A-CREB cDNA (Min *et al*., 2006) or human dn-Akt cDNA (Park *et al*., 2011), respectively, was transfected into BAECs grown to 70% confluence in 60-mm culture dishes using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions with minor modifications. For the control, equivalent amounts of the pcDNA3.1 vector alone were transfected. After incubation for 5 h at 37ºC, the culture medium was removed and cells were further incubated in MEM containing 5% NCS for 24 h before treatment with aphidicolin.

Knockdown of ATM expression using small interfering RNA (siRNA)

For knockdown of ATM expression, the following siRNA against ATM mRNA was synthesized; 5′-UAUAUCACCUGUUUGUUAGUU-3′ (Dharmacon Research Inc., Lafayette, CO, USA). The non-specific siRNA oligonucleotide, 5′-UAGCGACUAAACACAUCAA-3′, was also designed and synthesized for use in control experiments. BAECs grown to 60% confluence in 60-mm culture dishes were transfected with 100 nM of ATM siRNA or control siRNA using DharmaFECT (Dharmacon Research Inc.). After incubation for 5 h at 37ºC, the DharmaFECT mixtures were washed out and cells were incubated in MEM containing 5% NCS for 24 h before aphidicolin treatment.

eNOS gene promoter luciferase assay

We used eNOS gene promoter constructs designed previously in our laboratory (Min *et al*., 2006). Briefly, the full length 5'-flanking region of the eNOS promoter cDNA and its 5′-serially deleted regions were fused into the luciferase gene reporter plasmid pGL2 to yield the constructs pGL2-eNOS(−1600), pGL2-eNOS(−962), pGL2-eNOS(−873), and pGL2-eNOS(−428) containing eNOS promoter sequences from −1600 to +22, −962 to +22, −873 to +22, and −428 to +22, respectively. For eNOS promoter assays, BAECs grown to 60% confluence in 60-mm culture dishes in the absence or presence of aphidicolin treatment were transfected with each of the above pGL2-eNOS constructs using Lipofectamine 2000 (Invitrogen), and luciferase activity was then determined using the Luciferase Assay System from Promega (Madison, WI, USA).

Western blot analysis

For western blot analysis, BAECs treated with aphidicolin in the absence or presence of various chemicals were lysed in lysis buffer [20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 mM β-glycerophosphate, 1 mM NaF, 1 mM Na_3VO_4, and 1× Protease Inhibitor Cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA)]. In addition to BAECs, aortic tissues were also used; dissected aortas were incubated at 37°C under 5% CO_2 in air in the absence or presence of 20 μM aphidicolin for 24 h, and then aortic proteins were extracted by chopping the aortas with iris scissors in lysis buffer. Protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Rockford, IL, USA). Equal quantities of protein (20 μg) were separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) then transferred onto a nitrocellulose membrane (GE Healthcare Life Sciences, Pittsburgh, PA, USA). Blots were probed with the appropriate primary antibody followed by the corresponding secondary antibody (Invitrogen), and finally developed using enhanced chemiluminescence (ECL) reagents (Amersham Biosciences, Arlington Heights, IL, USA). Dilutions of primary antibodies used in western blot analyses were as follows; eNOS (1:2,000), Akt (1:2,000), p-Akt-Ser^{473} (1:1,000), CREB (1:2,000), p-CREB-Ser^{133} (1:1,000), ATM (1:2,000), p-ATM-Ser^{1981} (1:2,000), and tubulin (1:2,000).

Assessment of eNOS dimerization

Low-temperature SDS-PAGE (LT-PAGE) was performed as previously described (Yang *et al*., 2009) for detection of eNOS dimers and monomers. Briefly, after BAECs were treated with aphidicolin, the cells were lysed in lysis buffer and equal quantities of protein were incubated in 1x Laemmli buffer without reducing agents such as dithiothreitol (DTT) at 37°C for 5 min. The samples were then subjected to SDS-PAGE using a 6% gel. Gels and running buffers were equilibrated at 4°C before electrophoresis, and the buffer tank was placed in an ice bath during electrophoresis to maintain the temperature of the gel <15°C. Subsequent to LT-PAGE, the gels were transblotted and the blots were probed using standard western blotting methods. Samples treated with reducing agent (100 mM DTT) were used to assess levels of total eNOS and tubulin proteins.

Quantitative real-time polymerase chain reaction (qRT-PCR)

After BAECs were treated with aphidicolin at the indicated concentrations for the indicated times, total RNA was isolated using an RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol, followed by a reverse transcription reaction using 200 units of Superscript II reverse transcriptase (Invitrogen), 10 pmol of oligo-dT, and 1 mM of dNTPs in a 20 μL reaction mix containing 1 μg of RNA for 1 h at 42°C. PCR amplification of cDNA encoding eNOS or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed with Power SYBR™ Green Master Mix (Applied Biosystems, Foster City, CA, USA) and a QuantStudio™ 3 Real-Time PCR system (Applied Biosystems). The following PCR primer pairs were used; eNOS-F, 5'-GAGTTACAAGATCCGCTTCA-3' and eNOS-R, 5'-AGTCCGAACACACAGAACCT-3'; GAPDH-F, 5'-ACGTGTCTGTTGTGGATCTG-3' and GAPDH-R, 5'-GTAGCCTAGAATGCCCTTGA-3'. GAPDH was used as a reference gene. The relative expression levels of each mRNA were quantitated using the ΔΔCt method.

NO measurement

Level of NO was measured electrochemically using an NO sensor (ISO-NOP, World Precision Instruments (WPI), Sarasota, FL, USA) connected to an amplifier-recorder (TBR4100 Free Radical Analyzer; WPI). The NO sensor was polarized by placing the sensor tip in a chamber containing 2 mL of 0.1 M $CuCl_2$ overnight at room temperature, and calibrated based on the decomposition of the NO donor S-nitroso-N-acetyl-D, L-penicillamine (SNAP) in 2 mL of 0.1 M $CuCl_2$, according to the manufacturer's instructions. Briefly, after BAECs were treated with aphidicolin or vehicle, 50 μL of the culture medium was loaded into the NO sensor chamber containing 2 mL of 0.1 M $CuCl_2$. The amount of NO was measured via calibration curves constructed with known concentrations of SNAP using the software Lab-Trax (WPI) and normalized with total protein.

BH_4 measurement

The level of BH_4 was measured using a BH_4 enzyme-linked immunosorbent assay (ELISA) kit (MyBioSource, San Diego, CA, USA) according to the manufacturer's protocol. Briefly, ELISA plates coated with monoclonal antibody specific to BH_4 were incubated with 100 μg of cell lysates and 50 μL of BH_4-HRP enzyme conjugate for 1 h at 37°C. After incubation, each well was washed and incubated with 100 μL of 3,3',5,5'-tetramethylbenzidine (TMB) ELISA substrate for 20 min at 37°C. The reaction was terminated by adding 50 μL of stop solution, and the absorbance was measured on a 96-well microplate reader at a wavelength of 450 nm.

Animals

All the animal experiments were conducted in accordance with the approved institutional guidelines for animal care and use in Yeungnam University (Approval No. YUMC-AEC2019-003). Male Sprague-Dawley (SD) rats at 6 weeks of age were maintained for 1 week at the beginning of the experiment in a temperature- and humidity-controlled room (22 ± 1°C and 50 ± 10%, respectively) under a 12-h alternate light/dark cycle. All rats were given water and fed with standard chow (Purina Mills, LLC, St. Louis, MO, USA) *ad libitum* throughout the experiments.

Measurement of endothelium-dependent vessel relaxation

Endothelium-dependent vessel relaxation was measured in thoracic aortic rings as described previously (Seo *et al*., 2016) with minor modifications. Briefly, male SD rats were euthanized using CO_2 gas and subsequent cervical dislocation. The thoracic aorta was then rapidly and carefully removed, and placed in the Krebs-Henseleit (KH) solution containing 118.1 mM NaCl, 4.7 mM KCl, 2.6 mM $CaCl_2$, 0.6 mM $MgSO_4$, 24.9 mM $NaHCO_3$, 1.2 mM KH_2PO_4, and 5.6 mM glucose. The aorta with intact endothelium was carefully cleaned by removing fat and connective tissues, and then cut into 5-mm ring segments. After the prepared aortic ring segments were incubated in the absence or presence of 20 μM aphidicolin in MEM supplemented with 5% NCS at 37°C under 5% CO_2 in air for 24 h, the aortic rings were then mounted on L-shaped holders in 7 mL organ baths containing warmed (37°C) and oxygenated (95% O2 and 5% CO_2) KH solution. Muscle force was recorded isometrically by means of a force transducer (MP35; BIOPAC system Inc., Goleta, CA, USA) that was connected to a BLS analysis software (BIOPAC system Inc.). The rings

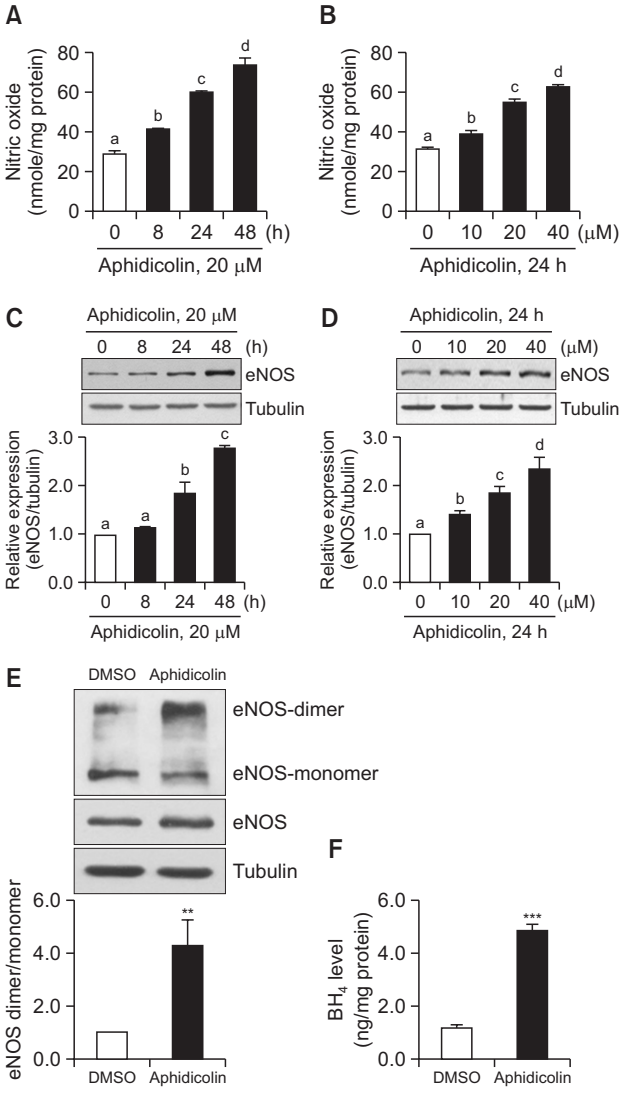

Fig. 1. Aphidicolin increases NO production in BAECs with concomitant increase in expression and dimerization of eNOS protein and BH_4 levels. (A) BAECs were treated with 20 μM aphidicolin or vehicle (DMSO) for the indicated times (0, 8, 24, or 48 h) and the level of NO production was electrochemically measured using an NO sensor (ISO-NOP, WPI), as described in the MATERIALS AND METHODS. (B) BAECs were treated with various doses of aphidicolin (0, 10, 20, or 40 μM) for 24 h and the level of NO production was measured as described in Fig. 1A. (C) After BAECs were treated with 20 μM aphidicolin for the indicated times (0, 8, 24, or 48 h), total protein was obtained and the level of eNOS protein expression was detected using western blot analysis. Nitrocellulose membranes were re-probed using an anti-tubulin antibody to assess equal sample loading. (D) BAECs were treated with various doses of aphidicolin (0, 10, 20, or 40 μM) for 24 h and the level of eNOS protein expression was detected using western blot analysis as described in Fig. 1C. (E) BAECs were treated with 20 μM aphidicolin or vehicle (DMSO) for 24 h, the eNOS dimer and monomer were separated using LT-PAGE, and their ratio was measured using western blot analysis as described in the MATERIALS AND METHODS. (F) In a separate experiment, BAECs were treated as described in Fig. 1E and cellular BH_4 level was measured using a BH_4 ELISA kit (MyBioSource) as described in the MATERIALS AND METHODS. (C, D) Using western blot data, densitometry was done to quantitate eNOS protein expression relative to tubulin expression or (E) level of eNOS dimer relative to monomer. All experiments were performed at least four times independently and blots shown are representative of at least four experiments (n=4). Bar graphs depict mean fold alterations above the controls (± SD). Statistical significance was evaluated using either Student's *t* test or ANOVA. All differences were considered to be statistically significant at a *p* value of <0.05. $^{**}p<0.01$, $^{***}p<0.001$.

were stretched to a resting tension of 10 mN and equilibrated for 30 min in an organ bath filled with KH solution, sequentially exposed to 65 mM KCl and KH solution at least two times, followed by precontraction with 1 μM phenylephrine, and then ACh was cumulatively added to determine endothelium-dependent reactivity.

Statistical analysis

All results are represented as means ± standard deviations (SD) with n indicating the number of experiments. Statistical significance of differences between points was determined using Student's *t* test. Statistical significance among various (more than two) doses or time points was evaluated by one-

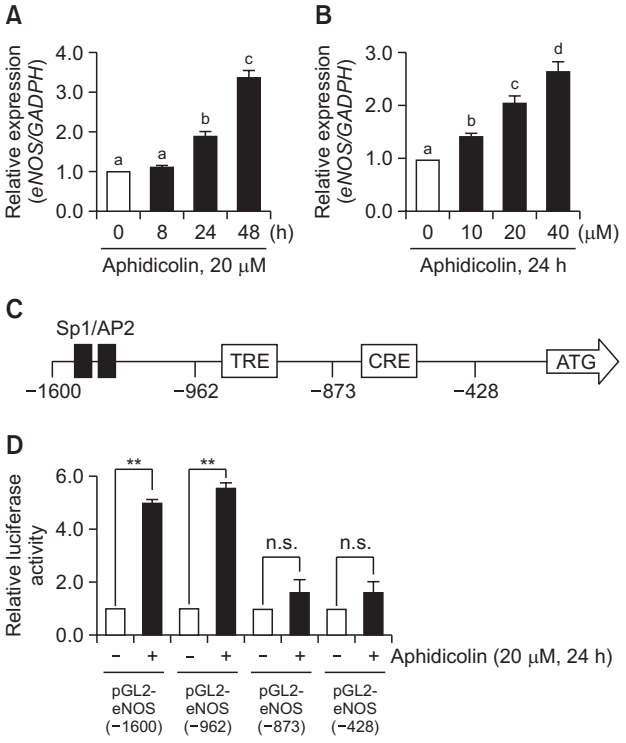

Fig. 2. A Tax-responsive element located at −962 to −873 of the promoter region of eNOS is involved in increasing eNOS mRNA transcription in aphidicolin-treated BAECs. (A) After BAECs were treated with 20 μM aphidicolin for the indicated times (0, 8, 24, or 48 h), total RNA were extracted and cDNA were synthesized via a reverse transcription reaction. Level of eNOS mRNA expression was determined using quantitative real-time PCR (qPCR) as described in the MATERIALS AND METHODS. (B) BAECs were treated with various doses of aphidicolin (0, 10, 20, or 40 μM) for 24 h and the level of eNOS mRNA expression was determined using qPCR as described in Fig. 2A. (C) A schematic illustration of the eNOS gene promoter from −1600 to −428. The eNOS gene promoter contains an Sp1/AP2 site between −1600 and −962, a TRE site between −962 and −873, and a CRE site between −873 and −428. (D) After 5'-serially deleted eNOS promoter cDNAs fused into a luciferase gene reporter plasmid were transfected into BAECs, the cells were incubated for 24 h in the absence or presence of 20 μM aphidicolin. Cells were harvested and luciferase activity was determined as described in the MATERIALS AND METHODS. All experiments were performed at least four times independently (n=4). Bar graphs depict mean fold alterations above the controls (± SD). Statistical significance was evaluated using either Student's *t* test or ANOVA. All differences were considered to be statistically significant at a *p* value of <0.05. ***p*<0.01. n.s., not significant.

way analysis of variance (ANOVA) and DUNCAN analysis as post hoc using SPSS package program (PASW Statistics 18, Hong Kong). All differences were considered significant at a *p* value of <0.05.

RESULTS

Aphidicolin increases NO production in BAECs with concomitant increase in expression and dimerization of eNOS protein and BH_4

Because genotoxic agents like aphidicolin have exhibited their clinical effects after a prolonged period of treatment, we examined the long-term effect of aphidicolin on NO production in the present study. Exposure to aphidicolin for up to 48 h increased NO production in a time-dependent manner (Fig. 1A). Furthermore, this effect was also dose-dependent (Fig. 1B). As shown in Fig. 1C and 1D, all these effects were accompanied by time- and/or dose-dependent increases in eNOS protein expression. Because eNOS protein levels do not always assure eNOS-mediated NO production, we examined whether aphidicolin in fact increased functional eNOS, i.e., the dimeric form of eNOS, and BH_4, an essential cofactor for eNOS coupling. Uncoupled eNOS monomer has been reported to produce superoxide instead of NO (Forstermann and Sessa, 2012). As shown in Fig. 1E and 1F, aphidicolin treatment at 20 μM for 24 h significantly increased the levels of eNOS dimer/monomer ratio and of BH_4 by ~4 fold compared to the vehicle control. These results suggest that aphidicolin-increased NO production is stemmed from functional eNOS but not nonspecific NO sources.

A Tax-responsive element located at –962 to –873 of the promoter region of eNOS is involved in increasing eNOS mRNA transcription in aphidicolin-treated BAECs

We next investigated whether these increases in eNOS protein resulted from transcriptional activation of the eNOS gene. As shown in Fig. 2A and 2B, aphidicolin also increased expression of eNOS mRNA in a time-and dose-dependent manner, suggesting that the stimulatory effects of aphidicolin on NO production and eNOS expression occur at the level of eNOS mRNA transcription. To explore which regions of the eNOS gene promoter are responsible for aphidicolin-stimulated eNOS gene transcription, we performed luciferase assays using eNOS gene promoter constructs generated previously in our laboratory (Min *et al*., 2006). Fig. 2C depicts *cis*-elements contained in each promotor construct. Briefly, pGL2-eNOS(−1600) contains Sp1/AP2, TRE, and CRE sites; pGL2-eNOS(−962) contains TRE and CRE sites; pGL2-eNOS(−873) contains the CRE; and pGL2-eNOS(−428) contains no apparent *cis*-element. As described in the MATERIALS AND METHODS, we performed eNOS gene promoter assays using these constructs fused into a luciferase gene reporter plasmid. As shown in Fig. 2D, compared to the vehicle control, aphidicolin significantly increased promoter activity by ~5 fold when cells were transfected with pGL2-eNOS(−1600) or pGL2-eNOS(−962). However, promoter activity returned to almost control levels in BAECs transfected with the constructs pGL2-eNOS(−873) or pGL2-eNOS(−428) (Fig. 2D). These results suggested that the TRE, located at −962 to −873 of the eNOS gene promoter, and CREB, which recognizes this response element, may play a role in aphidicolin-stimulated eNOS gene transcription.

CREB mediates aphidicolin-stimulated eNOS expression

To determine whether CREB plays a critical role in transactivation of the eNOS promoter, we transfected a construct containing a dominant-negative inhibitor of CREB, A-CREB, into

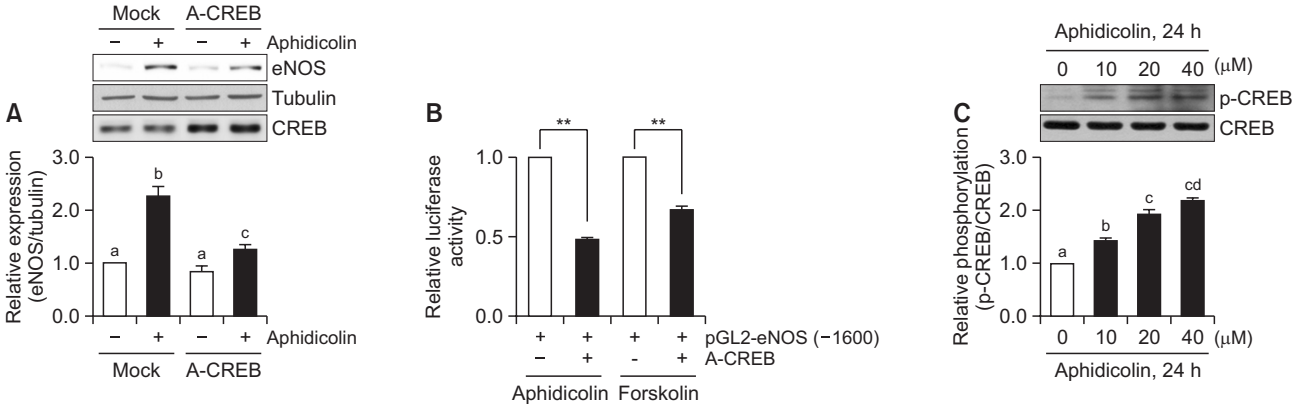

Fig. 3. CREB mediates aphidicolin-stimulated eNOS expression. (A) After the A-CREB construct, a dominant-negative (dn) inhibitor of CREB, or empty vector was transfected into BAECs, cells were incubated for 24 h in the absence or presence of 20 μM aphidicolin. Levels of eNOS and CREB protein expression were measured using western blot analysis as described in Fig. 1. (B) In a separate experiment, after the A-CREB construct or empty vector was transfected into BAECs, the eNOS promoter pGL2-eNOS (−1600) fused into a luciferase reporter plasmid was transfected into the cells. The cells were treated with 20 μM aphidicolin or 20 μM forskolin for 24 h, and an eNOS promoter assay was performed as described in Fig. 2. (C) BAECs were treated with various doses of aphidicolin (0, 10, 20, or 40 μM) for 24 h and the level of p-CREB-Ser^{133} expression was detected using western blot analysis as described in Fig. 1. (A, C) Reprobing and quantitation of eNOS or p-CREB-Ser^{133} relative to tubulin or CREB expression, respectively, were conducted as described in Fig. 1. All experiments were performed at least four times independently and blots shown are representative of at least four experiments (n=4). Bar graphs depict mean fold alterations above/below the controls (± SD). Statistical significance was evaluated using either Student's *t* test or ANOVA. All differences were considered to be statistically significant at a *p* value of <0.05. ***p*<0.01.

BAECs. As shown in Fig. 3A, ectopic expression of the A-CREB construct significantly inhibited aphidicolin-stimulated eNOS expression in BAECs. The higher expression of the CREB protein in cells transfected with A-CREB confirmed successful transfection because the CREB antibody used in the present study can detect both CREB and A-CREB. Furthermore, overexpression of the A-CREB construct significantly inhibited aphidicolin-mediated transactivation of the eNOS promoter pGL2-eNOS(−1600) (Fig. 3B), indicating the involvement of CREB in eNOS gene transcription. We also found that overexpression of A-CREB inhibited forskolin-stimulated eNOS promoter activity (Fig. 3B), further confirming the validity of the use of A-CREB. Lastly, aphidicolin clearly increased p-CREB-Ser^{133} in a dose-dependent manner (Fig. 3C), indicating an increase in CREB activity. Taken together, our data suggest that the stimulatory effect of aphidicolin on eNOS expression in BAECs is mediated at least in part by CREB activation.

Akt mediates eNOS promoter transactivation by inducing p-CREB-Ser^{133}

Next, we made an effort to identify upstream signaling molecules responsible for aphidicolin-induced CREB activation and subsequent eNOS promoter activation. Because protein kinase A (PKA) is known to activate CREB by phosphorylating CREB-Ser^{133} (Gonzalez and Montminy, 1989; Shaywitz and Greenberg, 1999), we first performed an inhibitor study using H-89, a PKA inhibitor, and found that H-89 had no effect on the level of p-CREB-Ser^{133} or eNOS expression in aphidicolin-treated cells (Fig. 4A-4B2), suggesting that PKA is not involved in aphidicolin-mediated eNOS expression. Akt has also been reported to activate CREB by inducing p-CREB-Ser^{133} (Du and Montminy, 1998), and therefore we investigated the involvement of Akt in aphidicolin-mediated CREB activation and eNOS expression. As shown in Fig. 4C-4D3, co-treatment with LY294002, a phosphoinositide 3-kinase inhibitor, significantly attenuated aphidicolin-stimulated levels of p-Akt-Ser^{473}, p-CREB-Ser^{133}, and eNOS. Furthermore, it also significantly inhibited aphidicolin-stimulated NO production (Fig. 4E). To confirm a role for Akt in aphidicolin-stimulated NO production, we transfected a dominant-negative Akt construct into BAECs. As shown in Fig. 4F, ectopic expression of the dominant-negative form of Akt significantly attenuated aphidicolin-stimulated NO production. These results suggest that Akt-stimulated p-CREB-Ser^{133} mediates the aphidicolin-induced increases in eNOS expression and NO production.

ATM is upstream of the aphidicolin-stimulated Akt/CREB/eNOS/NO signaling pathway

Because aphidicolin, a well-known DNA polymerase inhibitor, activates ATM, which is considered to be the most upstream regulator of DDR signals, we examined the involvement of ATM in the stimulatory effects of aphidicolin. As expected, aphidicolin activated ATM, evidenced by the increase in level of p-ATM-Ser^{1981} (Fig. 5A). Furthermore, ectopic expression of siRNA against the ATM gene significantly blocked ATM expression and attenuated the aphidicolin-induced increase in levels of p-ATM-Ser^{1981}, p-Akt-Ser^{473}, p-CREB-Ser^{133}, and eNOS (Fig. 5B-5C4). Furthermore, we found that aphidicolin-stimulated NO production was significantly reversed by overexpression of ATM siRNA (Fig. 5D). Collectively, all these data suggest that aphidicolin increases NO production at least in part by increasing ATM/Akt/CREB/eNOS signaling.

Aphidicolin also increases NO production and eNOS expression through activation of ATM/Akt/CREB/eNOS signaling pathway in HUVECs

So far, we revealed that aphidicolin increased eNOS expression via activation of ATM/Akt/CREB signaling pathway and consequently promoted NO production in BAECs. Therefore, we examined whether these our findings obtained from

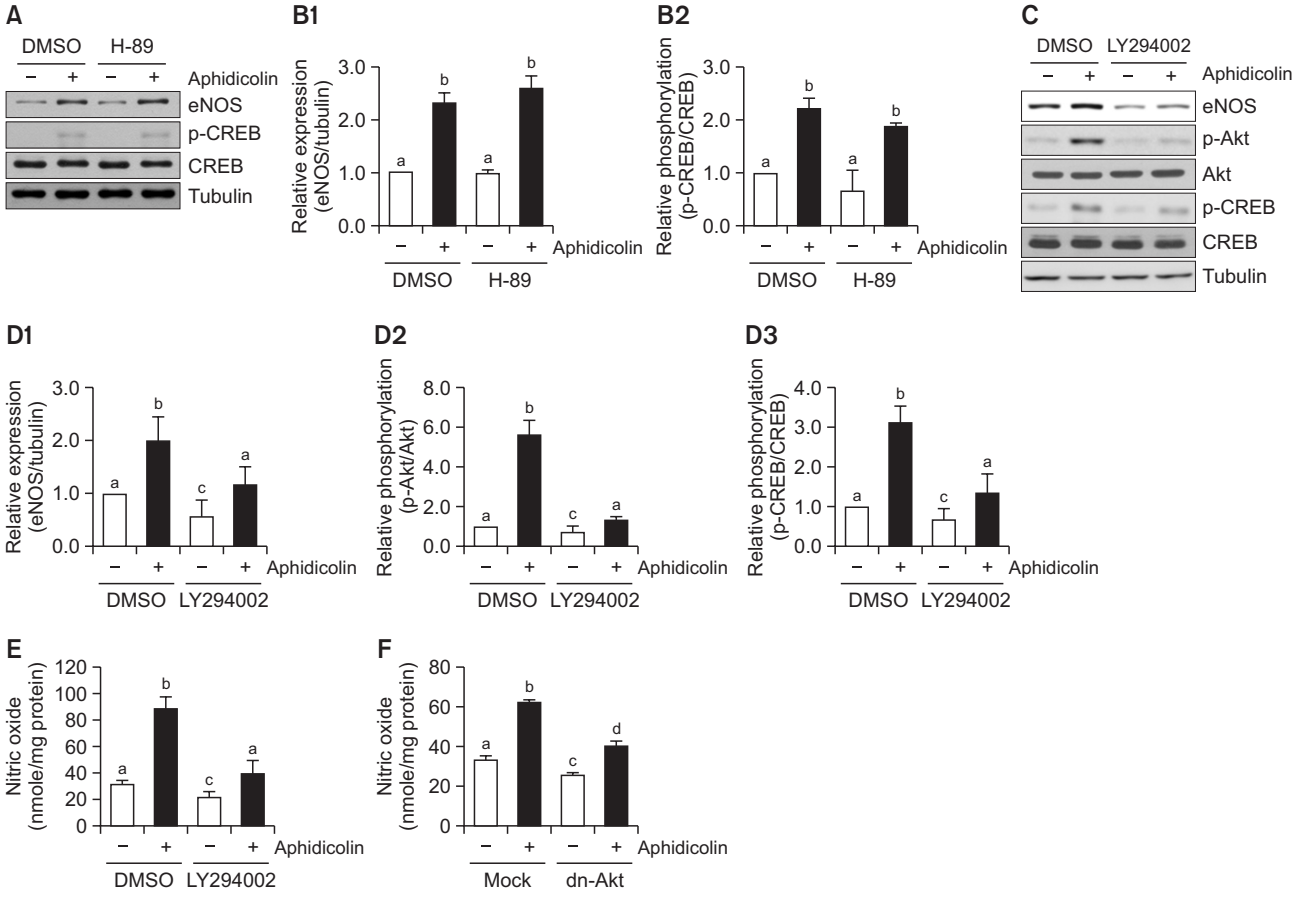

Fig. 4. Akt mediates eNOS promoter transactivation by inducing p-CREB-Ser^{133}. (A-B2) BAECs were treated with 20 μM aphidicolin or vehicle (DMSO) for 24 h in the absence or presence of 10 μM H-89, a PKA inhibitor, and then, levels of eNOS protein and p-CREB-Ser^{133} were assessed using western blot analysis as described in Fig. 1. (C-D3) BAECs were treated with 20 μM aphidicolin or vehicle (DMSO) for 24 h in the absence or presence of 5 μM LY294002, a phosphoinositide 3-kinase inhibitor, and then, levels of eNOS protein, p-Akt-Ser^{473}, and p-CREB-Ser^{133} were detected using western blot analysis as described in Fig. 1. (A-D3) Reprobing and quantitation of eNOS, p-Akt-Ser^{473}, or p-CREB-Ser^{133} were done as described in Fig. 1. (E) After BAECs were treated with 20 μM aphidicolin or vehicle (DMSO) for 24 h in the absence or presence of 5 μM LY294002, NO release was measured as described in Fig. 1. (F) In a separate experiment, after cDNA encoding dn-Akt or an empty vector was transfected into BAECs, the cells were treated with 20 μM aphidicolin or vehicle (DMSO) for 24 h and then, NO production was measured as described in Fig. 1. All experiments were performed at least four times independently and blots shown are representative of at least four experiments (n=4). Bar graphs depict mean fold alterations above/below the controls (± SD). Statistical significance was evaluated using ANOVA. All differences were considered to be statistically significant at a *p* value of <0.05.

BAECs hold true for a different kind of ECs originated from other species. To achieve this, we performed experiments using HUVECs which are derived from human umbilical cord. In line with results obtained from BAECs, treatment with 20 μM aphidicolin for 24 h in HUVECs significantly increased NO production, eNOS expression, and levels of p-ATM-Ser^{1981}, p-Akt-Ser^{473}, and p-CREB-Ser^{133} (Fig. 6). These results undoubtedly showed that aphidicolin also increased eNOS expression and NO production by activating ATM/Akt/CREB signaling pathway in HUVECs.

Activation of ATM/Akt/CREB/eNOS signaling cascade by aphidicolin increases ACh-induced vessel relaxation in rat aortas

Finally, in an attempt to determine whether the data obtained from our *in vitro* findings agree with *in vivo* results, we performed ACh-induced vessel relaxation assay and western blot analyses in an isolated rat aorta *ex vivo* model. As shown in Fig. 7A, 7B, aphidicolin treatment at 20 μM for 24 h significantly increased ACh-induced aortic vessel relaxation compared to vehicle control; the EC_{50} value of ACh in the aphidicolin treatment group was significantly lower than that in the vehicle control group (0.11 μM and 1.25 μM, respectively). Similar to *in vitro* results, aphidicolin also increased eNOS expression in rat aortas, which was accompanied by increased levels of p-ATM-Ser^{1981}, p-Akt-Ser^{473}, and p-CREB-Ser^{133} (Fig. 7C-7D4). All these results suggest that the aphidicolin-induced NO production caused by activation of ATM/Akt/CREB/eNOS signaling axis, which is mediated possibly by similar mechanisms *in vitro*, *ex vivo*, and perhaps *in vivo*, has relevance to physiological function.

DISCUSSION

One of the most important findings in the present study is that

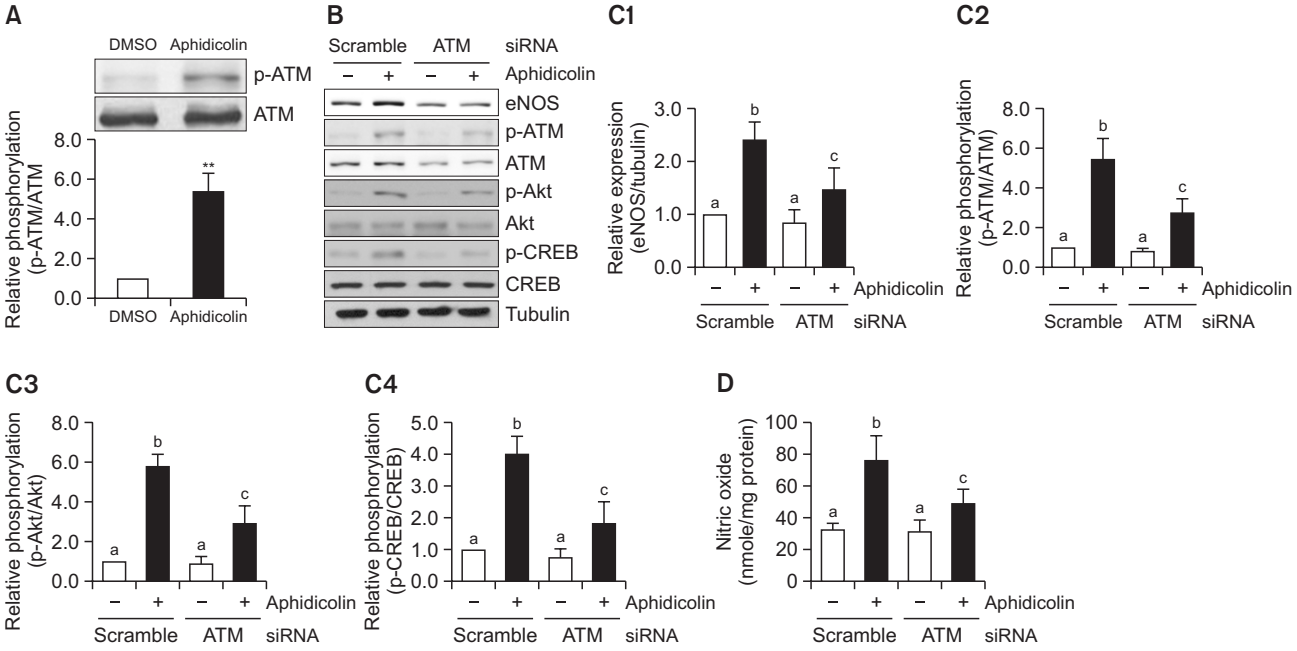

Fig. 5. ATM is upstream of the aphidicolin-stimulated Akt/CREB/eNOS/NO signaling pathway. (A) BAECs were treated with 20 μM aphidicolin or vehicle (DMSO) for 24 h, and the level of p-ATM-Ser^{1981} was assessed using western blot analysis as described in Fig. 1. (B-C4) After 100 nM siRNA specific for the ATM gene or scrambled siRNA was transfected into BAECs, the cells were treated with 20 μM aphidicolin or vehicle (DMSO) for 24 h, and then levels of eNOS, p-ATM-Ser^{1981}, p-Akt-Ser^{473}, or p-CREB-Ser^{133} were measured using western blot analysis as described in Fig. 1. (A-C4) Reprobing and quantitation of eNOS, p-ATM-Ser^{1981}, p-Akt-Ser^{473}, and p-CREB-Ser^{133} was done as described in Fig. 1. (D) After BAECs were prepared as described in Fig. 5B, level of NO production was measured as described in Fig. 1. All experiments were performed at least four times independently and blots shown are representative of at least four experiments (n=4). Bar graphs depict mean fold alterations above/below the controls (± SD). Statistical significance was evaluated using either Student's *t* test or ANOVA. All differences were considered to be statistically significant at a *p* value of <0.05. ***p*<0.01.

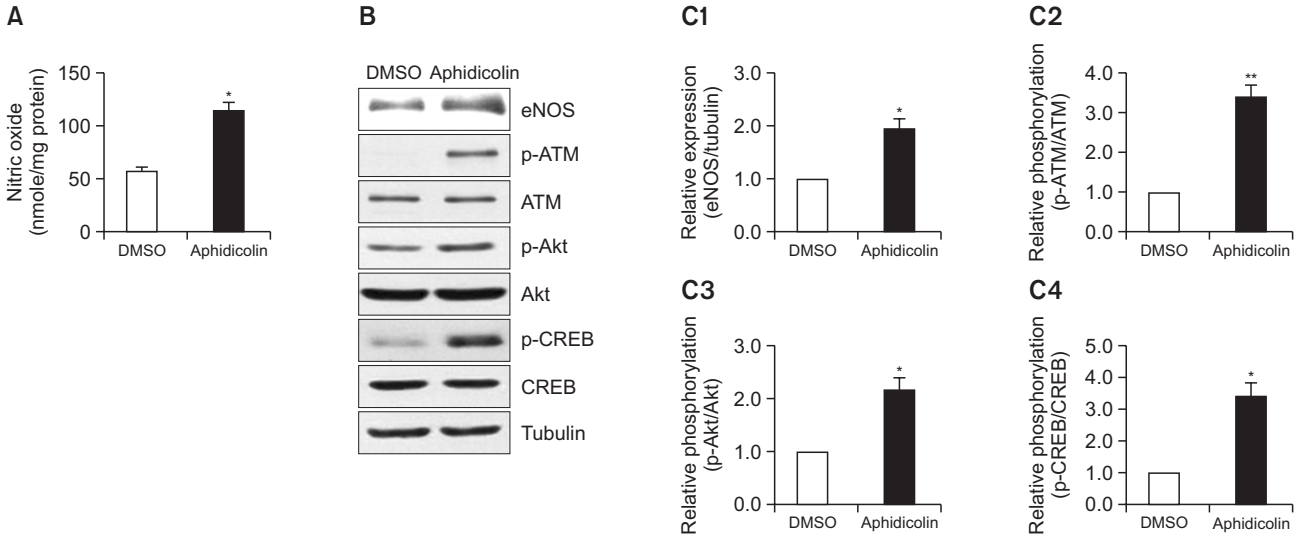

Fig. 6. Aphidicolin also increases NO production and eNOS expression through activation of ATM/Akt/CREB/eNOS signaling pathway in HUVECs. (A) HUVECs were treated with 20 μM aphidicolin or vehicle (DMSO) for 24 h and level of NO production was measured as described in Fig. 1. (B) HUVECs were treated with 20 μM aphidicolin or vehicle (DMSO) for 24 h, and then levels of eNOS, p-ATM-Ser^{1981}, p-Akt-Ser^{473}, or p-CREB-Ser^{133} were measured using western blot analysis as described in Fig. 1. (B-C4) Reprobing and quantitation of eNOS, p-ATM-Ser^{1981}, p-Akt-Ser^{473}, and p-CREB-Ser^{133} was done as described in Fig. 1. All experiments were performed at least four times independently (n=4). Bar graphs depict mean fold alterations above the controls (± SD). Statistical significance was evaluated using Student's *t* test. All differences were considered to be statistically significant at a *p* value of <0.05. **p*<0.05 and ***p*<0.01.

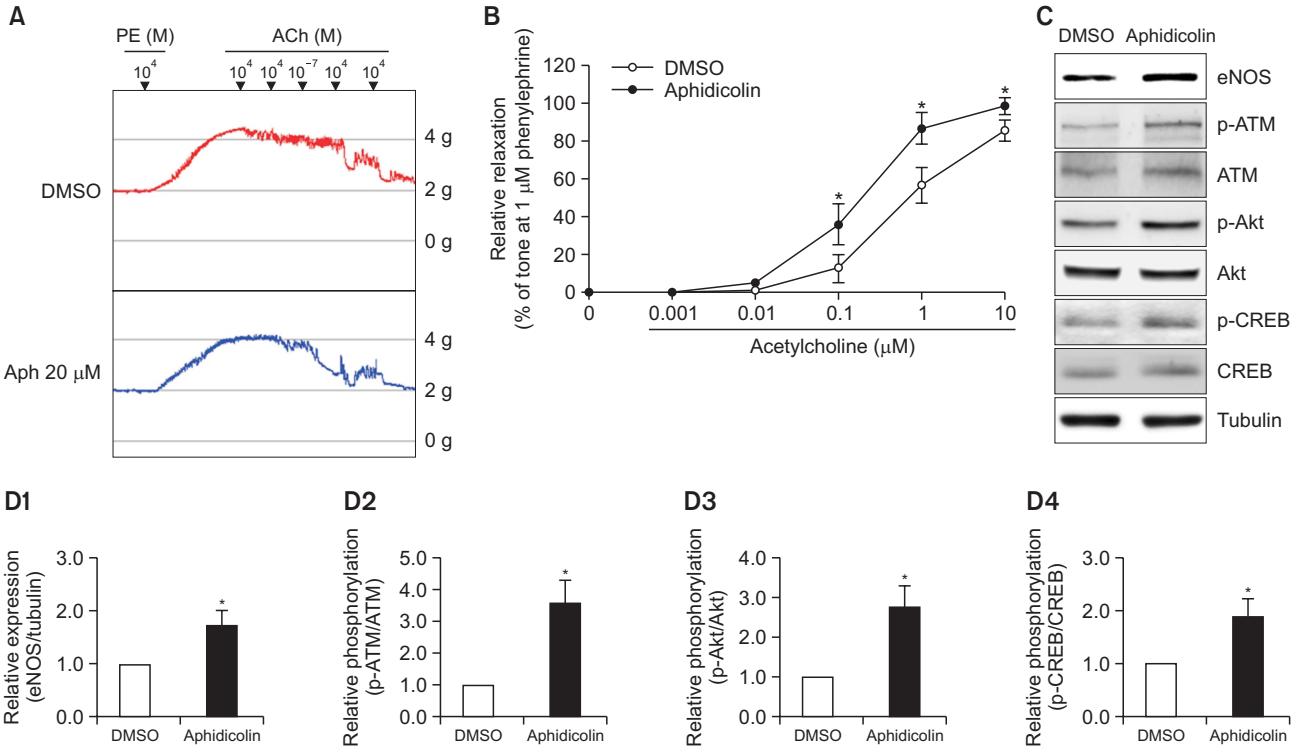

Fig. 7. Activation of ATM/Akt/CREB/eNOS signaling cascade by aphidicolin increases ACh-induced vessel relaxation in rat aortas. (A, B) Rat thoracic aortas were prepared and vessel relaxation assay was performed as described in the MATERIALS AND METHODS. Endothelium-intact aortic rings were treated with 20 μM aphidicolin or vehicle (DMSO) for 24 h and were precontracted with 1 μM phenylephrine, and then treated cumulatively with ACh (0.001-10 μM). The contractile level with phenylephrine (1 μM) immediately before treatment with ACh was considered as 100%. The tension curves indicate ACh-induced aortic relaxation in response to 20 μM aphidicolin or vehicle (DMSO) (A). The line graph represents the mean ± SD at each point (n=6) (B). (C-D4) In a separate experiment, after endothelium-intact aortic tissues were treated as described above, aortic proteins were extracted as described in the MATERIALS AND METHODS. Levels of eNOS, p-ATM-Ser^{1981}, p-Akt-Ser^{473}, or p-CREB-Ser^{133} were measured using western blot analysis as described in Fig. 1. All experiments were performed at least four times independently and blots shown are representative of at least four experiments (n=4). The bar graph depicts mean fold alterations above the controls (± SD). Statistical significance was evaluated using Student's *t* test. All differences were considered to be statistically significant at a *p* value of <0.05. **p*<0.05.

activation of eNOS gene transcription is a major mechanism by which prolonged exposure of ECs to aphidicolin increases NO production. Most recently, another genotoxic stress ionizing irradiation was also reported to increase eNOS transcription and NO generation by ATM-mediated signaling pathway in BAECs, resulting in EC senescence (Nagane *et al*., 2018), but the detailed mechanism underlying genotoxic stress-activated eNOS transcription has not been fully explored. Here, we identified the TRE (located between −962 and −873) in the eNOS promoter as an important site for aphidicolin-stimulated NO production (Fig. 2). Furthermore, our data also showed that ATM/Akt-stimulated p-CREB-Ser^{133} was characterized as the upstream signaling component of TRE, which contributed to increase of endothelial eNOS expression in response to prolonged exposure to aphidicolin (Fig. 5). In addition to TRE, previous studies showed that several other putative *cis*-elements including the Sp1 site, the GATA motif, the estrogen-responsive element, the nuclear factor (NF)-1 element, the CRE, the NF-κB binding site, and the activator protein-1 (AP-1) and AP-2 binding sites regulate eNOS expression (Forstermann *et al*., 1998; Searles, 2006). For example, estrogen increases eNOS expression in human endothelial EA.hy926 cells by enhancing the binding activity of Sp1 (Kleinert *et al*., 1998), and transforming growth factor-β1 upregulates eNOS expression in BAECs via NF-1 activation (Inoue *et al*., 1995). Our finding that TRE was involved in eNOS transcription is largely consistent with the results of a previous study reporting that hypoxia induces eNOS expression by augmenting CREB-TRE binding in the eNOS promoter (Min *et al*., 2006). Together with this previous finding, our present data indicate that the TRE site may be important for the regulation of eNOS expression under various circumstances other than in viral infection, because Tax, a unique viral transactivator originally known to interact with HTLV-1 21-bp repeats in long terminal repeats, recruits cellular CREB onto viral TREs (Brauweiler *et al*., 1995; Yin and Gaynor, 1996).

In addition to eNOS expression, we also found that aphidicolin increased the levels of BH_4, an essential cofactor for eNOS dimerization, and as expected, levels of dimeric eNOS (Fig. 1E, 1F). It is well known that increased eNOS protein alone with no concomitant increase in BH_4 ultimately results in eNOS uncoupling and produces superoxide instead of NO (Forstermann and Sessa, 2012; Forstermann *et al*., 2017). Once eNOS dimers are disrupted, the cellular redox state is known to be shifted from the normally balanced redox state to a harmful oxidative stress state due to excessive super-

oxide produced from monomeric eNOS, which extensively damages intracellular components including DNA, proteins, and lipids, and ultimately leads to endothelial dysfunction, EC damage, and various vascular diseases (Forstermann and Sessa, 2012; Forstermann *et al*., 2017). Based on previous reports and our data showing that aphidicolin stimulates the levels of BH_4 and functional eNOS dimer, it is very likely that aphidicolin-induced NO contributes to maintenance of vascular homeostasis including vessel relaxation and EC viability at least in part by balancing the cellular redox state as well as the intrinsic vascular protective effects of NO. The mechanism by which aphidicolin increases BH_4 levels in ECs is an interesting question, and further studies are needed to solve this issue.

Many DNA damage agents initiate DDRs by activating upstream DNA damage sensors such as ATM, ATR, and DNA-PK. Aphidicolin has been reported to repress DNA replication and certain forms of DNA repair by inhibiting DNA polymerases α, δ, and ε (Wright *et al*., 1994), which leads to DDRs. Consistent with this notion, we found that aphidicolin initiated DDRs as evidenced by increased levels of p-ATM-Ser^{1981} (Fig. 5A), which consequently activated eNOS gene expression by increasing ATM/Akt/CREB signaling axis (Fig. 5). In support of our results, it was reported that ATM is able to fully activate Akt by phosphorylating at Ser^{473} in response to insulin or ionizing radiation (Viniegra *et al*., 2005) and that CREB is a regulatory target of Akt (Du and Montminy, 1998). Based on these reports and results of the present study, therefore, it is reasonably accepted that aphidicolin-initiated DDRs are able to increase eNOS expression and NO production by activating ATM/Akt/CREB signaling axis.

Recently, the patterns of aphidicolin-induced gene expression and protein phosphorylation were comprehensively analyzed in a time-resolved manner using transcriptomics and quantitative mass spectrometry-based phosphoproteomics technologie (Mazouzi *et al*., 2016). Consistent with our results, the above report showed that eNOS gene expression is elevated by ~2.3 fold at 24 h after aphidicolin treatment in $ATM^{+/+}$ mouse embryonic fibroblasts (MEFs) but not at 4 h, or in $ATM^{-/-}$ MEFs, indicating that ATM is essentially required to induce aphidicolin-stimulated eNOS gene expression. Furthermore, the authors of the previous study also reported a role for ATM interactor (ATMIN) in regulating eNOS gene expression; aphidicolin-induced eNOS gene expression at 24 h post aphidicolin treatment is completely impaired in $ATMIN^{-/-}$ MEFs compared with a ~1.6 fold increase in $ATMIN^{+/+}$ MEFs. ATMIN was reported to affect Akt phosphorylation in aphidicolin-treated MEFs (Mazouzi *et al*., 2016). Based on this previous report, together with our present data, we hypothesize that aphidicolin activates ATM via an ATMIN-dependent pathway, which stimulates the Akt/CREB signaling cascade, resulting in increased eNOS gene expression. However, further experiments are needed to identify the detailed mechanism underlying the role of ATMIN in inducing eNOS gene expression, which are beyond the scope of the present study.

Data obtained in the present study revealed that increased eNOS expression by prolonged exposure to aphidicolin is a major mechanism contributing to increase of NO production and vessel relaxation. So far, two mechanisms regulating endothelial NO production have been well-established; regulation of eNOS phosphorylation, and regulation of eNOS expression. It is now usually accepted that phosphorylation-associated NO regulation achieves acute vascular homeostasis; once the levels of eNOS phosphorylation at specific sites are altered in response to a variety of stimuli, eNOS activity and NO production are acutely and accordingly altered. In contrast to eNOS phosphorylation, a few studies of eNOS expression-associated NO production have been reported, because eNOS is largely known to be constitutively expressed in ECs and eNOS mRNA has a long half-life (10−35 h) (Searles, 2006). Nonetheless, stimuli such as exercise, hypoxia, high glucose, and estrogen treatment have been reported to increase eNOS expression (Forstermann *et al*., 1998; Searles, 2006). It is important to note that prolonged rather than

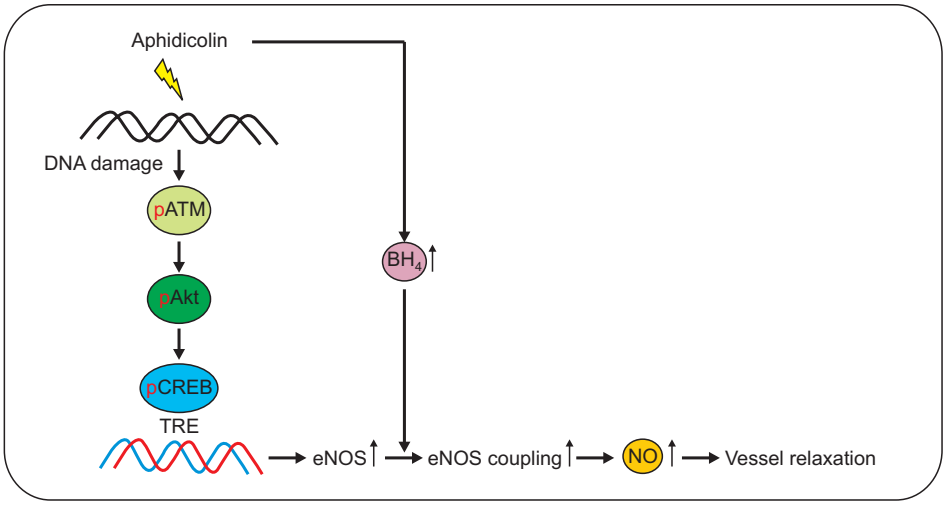

Fig. 8. A schematic illustration of aphidicolin-stimulated NO production and vessel relaxation. Aphidicolin activates ATM, the most upstream DNA damage sensor, followed by Akt activation. Akt in turn activates CREB that transactivates the eNOS promoter through the TRE site, resulting in increased eNOS expression. Aphidicolin simultaneously increases cellular levels of BH_4, and eNOS becomes coupled. Finally, this leads to NO production that induces vessel relaxation.

acute exposure to the aforementioned conditions is required to increase eNOS expression. For example, several animal studies have shown that continuous and repeated exercise gives rise to an increase in eNOS expression in coronary arteries, leading to vascular relaxation (Sessa *et al*., 1994; Shen *et al*., 1995; Sun *et al*., 2002). Based on this notion, it is reasonable to hypothesize that once eNOS expression is altered, its effects on NO production and consequently on vasculature are continuous and profound. In accordance with this concept, increased NO derived from eNOS gene expression by prolonged treatment with aphidicolin showed beneficial effects on regulation of vascular tone; aphidicolin actually increased ACh-induced vassel relaxation (Fig. 7A, 7B). In addition, treatment with aphidicolin for a relatively long period of time more closely mimics the clinical situation and aphidicolin should be considered as a new stimulant capable of inducing eNOS expression.

Previously, it has been reported that aphidicolin is used for *in vitro* studies in concentration range from 0.01 μM to 200 μM (Vesela *et al*., 2017). Various cellular effects including stalled replication forks, S phase arrest, inhibition of DNA synthesis and DNA repair, and irreversible senescence induction are observed in a variety of cancer cells, dependently to the concentrations and incubation times with which aphidicolin are treated (Vesela *et al*., 2017). For instance, treatment with 5–25 μM of aphidicolin for 24 h inhibited replicative polymerases in Werner syndrome cells and Bloom syndrome cells (Nguyen *et al*., 2013). Furthermore, clinical trial phase I study using aphidicolin-glycinate reported that peak serum levels of aphidicolin in patients are observed in range from 2.6 μg/mL to 63.7 μg/mL (equivalent to 7 μM and 172 μM, respectively), dependently to the initial infused doses in range from 290 mg/m^2 to 2250 mg/m^2 (Sessa *et al*., 1991). Therefore, the concentration of aphidicolin used in the current study is reasonably considered to be compatible with the concentrations used in many other *in vitro* and *in vivo* investigations including clinical study.

Taken together, our data demonstrated that prolonged treatment with aphidicolin increased eNOS expression at least in part via activation of ATM/Akt/CREB signaling cascade, which increased NO production and vessel relaxation in ECs and rat aortas (Fig. 8).

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

ACKNOWLEDGMENTS

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