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

Jung-Hyun Park¹†, Du-Hyong Cho²†, Yun-Jin Hwang², Jee Young Lee¹, Hyeon-Ju Lee¹ and Inho Jo¹*  
¹Department of Molecular Medicine, Ewha Womans University College of Medicine, Seoul 07804,  
²Department of Pharmacology, Yeungnam University College of Medicine, Daegu 42415, Republic of Korea

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-Ser133 level, eNOS expression, and NO production. Furthermore, ectopic expression of dominant-negative Akt construct attenuated aphidicolin-stimulated NO production. Aphidicolin increased p-ATM-Ser1981 and the knockdown of ATM using siRNA attenuated all stimulatory effects of aphidicolin on p-Akt-Ser473, p-CREB-Ser133, 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-Ser1981, p-Akt-Ser473, p-CREB-Ser133, 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 (Ser1179) and Tyr83 are activatory phosphorylation residues, while Ser116 and Thr497 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 expression.

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-Ser133 level, eNOS expression, and NO production. Furthermore, ectopic expression of dominant-negative Akt construct attenuated aphidicolin-stimulated NO production. Aphidicolin increased p-ATM-Ser1981 and the knockdown of ATM using siRNA attenuated all stimulatory effects of aphidicolin on p-Akt-Ser473, p-CREB-Ser133, 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-Ser1981, p-Akt-Ser473, p-CREB-Ser133, 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 (Ser1179) and Tyr83 are activatory phosphorylation residues, while Ser116 and Thr497 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 expression.

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-Ser133 level, eNOS expression, and NO production. Furthermore, ectopic expression of dominant-negative Akt construct attenuated aphidicolin-stimulated NO production. Aphidicolin increased p-ATM-Ser1981 and the knockdown of ATM using siRNA attenuated all stimulatory effects of aphidicolin on p-Akt-Ser473, p-CREB-Ser133, 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-Ser1981, p-Akt-Ser473, p-CREB-Ser133, 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 (Ser1179) and Tyr83 are activatory phosphorylation residues, while Ser116 and Thr497 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 expression.
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 (BH4), 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; Poelhmann 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; Poelhmann 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), unravelling 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-Ser473, CREB, p-CREB-Ser133, ATM, and p-ATM-Ser1981 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% CO2 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 37°C, 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'-UAUAUCACCU-GUUUGUAGU-U3' (Dharmacon Research Inc., Lafayette, CO, USA). The non-specific siRNA oligonucleotide, 5'-UAGC-GACUAACACAUCA-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 37°C, 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 NaVO₃, 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% CO₂ 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-Ser⁴⁷³ (1:1,000), CREB (1:2,000), p-CREB-Ser¹³⁵ (1:1,000), ATM (1:2,000), p-ATM-Ser¹⁴⁸¹ (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 1× 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 mixture 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 QuantiStudio™ 3 Real-Time PCR system (Applied Biosystems). The following PCR primer pairs were used; eNOS-F, 5’-GAGT-TACAAAGATCCGCTTCA-3’ and eNOS-R, 5’-AGTCCGAACACA-CAGAACCT-3’; GAPDH-F, 5’-ACGTTGCTGTTGATCG-3’ and GAPDH-R, 5’-GTAGCCTAGATGCCTTGA-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 CuCl₂ 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 CuCl₂, 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 CuCl₂. 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.

BH₄ measurement

The level of BH₄ was measured using a BH₄ 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 BH₄ were incubated with 100 µg of cell lysates and 50 µL of BH₄-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 CO₂ gas and subsequent cervical dislocation. The thoracic aorta was then rapidly and carefully removed, and the thoracic 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% CO₂ 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% CO₂) 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
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-
RESULTS

Aphidicolin increases NO production in BAECs with concomitant increase in expression and dimerization of eNOS protein and BH₄.

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 BH₄, 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 BH₄ 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 promoter construct. Briefly, pGL2-eNOS(−1600) contains Sp1/AP2, TRE, and CRE sites; pGL2-eNOS(−962) contains TRE and CRE sites; pGL2-eNOS(−873) contains 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 transcription of the eNOS promoter, we transfected a construct containing a dominant-negative inhibitor of CREB, A-CREB, into
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-activated 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-Ser133 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-Ser133

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-Ser133 (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-Ser133 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-Ser133 (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-Ser473, p-CREB-Ser133, and eNOS. Furthermore, it also significantly inhibited aphidicolin-stimulated NO production (Fig. 4E). To confirm the 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-Ser133 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. Expected, aphidicolin activated ATM, evidenced by the increase in level of p-ATM-Ser1981 (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-Ser1981, p-Akt-Ser473, p-CREB-Ser133, and eNOS (Fig. 5B-5C4). 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
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-Ser1981, p-Akt-Ser473, and p-CREB-Ser133 (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 EC50 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-Ser1981, p-Akt-Ser473, and CREB-Ser133 (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, p-CREB, CREB, and Tubulin 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/below the controls (± SD). Statistical significance was evaluated using 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.

https://doi.org/10.4062/biomolther.2020.007
were treated as described above, aortic proteins were extracted as described in the MATERIALS AND METHODS. Levels of eNOS, p-ATM-Ser 133, p-Akt-Akt, and p-CREB 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 eNOS activation. We 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 (Forsterrmann 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 (Romano et al., 1991; Yn and Gaynor, 1999).

In addition to eNOS expression, we also found that aphidicolin increased the levels of BH4, an essential cofactor for 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 (Romano et al., 1991; Yn and Gaynor, 1999).
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 BH₄ 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 BH₄ 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-Ser1981 (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 Ser473 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 technology (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
acutely acting procedures 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 vessel 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 used in the current study is reasonably concentration and incubation times with which aphidicolin 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/m² to 2250 mg/m² (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

This work was supported by National Research Foundation grants (2016R1A2B2002062, 2018R1D1A1B07050732, and 2017R1D1A1B03034131) from the Korean government and an RP-Grant in 2016 from Ewha Womans University.

REFERENCES

Bae, S. W., Kim, H. S., Cha, Y. N., Park, Y. S., Jo, S. A. and Jo, I. (2003) Rapid increase in endothelial nitric oxide production by bradykinin is mediated by protein kinase A signaling pathway. Biochem. Biophys. Res. Commun. 306, 981-987.

Braunweiler, A., Garli, P., Franklin, A. A., Giebler, H. A. and Nyborg, J. K. (1995) A molecular mechanism for human T-cell leukemia virus latency and Tax transactivation. J. Biol. Chem. 270, 12814-12822.

Bruckdorfer, R. (2005) The basics about nitric oxide. Mol. Aspects Med. 26, 3-31.

Carrassa, L. and Damia, G. (2017) DNA damage response inhibitors: mechanisms and potential applications in cancer therapy. Cancer Treat. Rev. 60, 139-151.

Cho, D. H., Choi, Y. J., Jo, S. A. and Jo, I. (2004) Nitric oxide production and regulation of endothelial nitric-oxide synthase phosphorylation by prolonged treatment with troglitazone: evidence for involvement of peroxisome proliferator-activated receptor (PPAR) gamma-dependent and PPARgamma-independent signaling pathways. J. Biol. Chem. 279, 2490-2506.

Cho, D. H., Park, J. H., Lee, E. J., Won, K. J., Lee, S. H., Kim, Y. H., Hwang, S., Kwon, J. K., Shin, C. Y., Song, K. H., Jo, I. and Han, S. H. (2014) Valproic acid increases NO production via the SH-PPTP1-CDK5-eNOS-Ser(116) signaling cascade in endothelial cells and mice. Free Radic. Biol. Med. 76, 96-106.

Ciccia, A. and Elledge, S. J. (2010) The DNA damage response: making it safe to play with knives. Mol. Cell 40, 179-204.

Du, K. and Montminy, M. (1998) CREB is a regulatory target for the protein kinase Akt/PKB. J. Biol. Chem. 273, 32377-32379.

Fleming, I. (2010) Molecular mechanisms underlying the activation of eNOS. Pflugers Arch. 459, 793-806.

Forstmann, U., Boissel, J. P. and Kleinert, H. (1998) Expression control of the ‘constitutive’ isoforms of nitric oxide synthase (NOS I and NOS III). FASEB J. 12, 773-790.

Forstmann, U. and Sessa, W. C. (2012) Nitric oxide synthases: regulation and function. Eur. Heart J. 33, 829-837, 837a-837d.

Forstmann, U., Xia, N. and Li, H. (2017) Roles of vascular oxidative stress and nitric oxide in the pathogenesis of atherosclerosis. Circ. Res. 120, 713-735.

Gonzalez, G. A. and Montminy, M. R. (1989) Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. Cell 59, 675-680.

Heiss, E. H. and Dirsch, V. M. (2014) Regulation of eNOS enzyme activity by posttranslational modification. Curr. Pharm. Des. 20, 3503-3513.

Hwang, S., Lee, D. H., Lee, I. K., Park, Y. M. and Jo, I. (2014) Far-infrared radiation inhibits proliferation, migration, and angiogenesis of human umbilical vein endothelial cells by suppressing secretory clusterin levels. Cancer Lett. 346, 74-83.

Inoue, N., Venema, R. C., Sayegh, H. S., Obara, Y., Murphy, T. J. and Harrison, D. G. (1995) Molecular regulation of the bovine endothelial cell nitric oxide synthase by transforming growth factor-beta 1. Arterioscler. Thromb. Vasc. Biol. 15, 1255-1261.

Kim, H. P., Lee, J. Y., Jeong, J. K., Bae, S. W., Lee, H. K. and Jo, I. (1999) Nongenomic stimulation of nitric oxide release by estrogen is mediated by estrogen receptor alpha localized in caveolae. Biochem. Biophys. Res. Commun. 263, 257-262.

Kleinert, H., Wallerath, T., Eichhofer, C., Ihrig-Biedert, I., Li, H. and Forstmann, U. (1998) Estrogens increase transcription of the human endothelial NO synthase gene: analysis of the transcription factors involved. Hypertension 31, 582-588.

Kou, R., Greif, D. and Michel, T. (2002) Dephosphorylation of endothelial nitric-oxide synthase by vascular endothelial growth factor. Implications for the vascular responses to cyclopasin A. J. Biol. Chem. 277, 29669-29673.

Mazouzi, A., Stukalov, A., Muller, A. C., Chen, D., Wiedner, M., Prokhorova, J., Chiang, S. C., Schuster, M., Breitwieser, F. P., Pi- chlmair, A., El-Khamsy, S. F., Bock, C., Kralovics, R., Colinge, J., Bennett, K. L. and Loizou, J. I. (2016) A comprehensive analysis of the dynamic response to aphidicolin-mediated replication stress uncovers targets for ATM and ATMIN. Cell Rep. 15, 893-908.

Min, J., Jin, Y. M., Moon, J. S., Sung, M. S., Jo, S. A. and Jo, I. (2006) Hypoxia-induced endothelial NO synthase gene transcriptional activation is mediated through the tax-responsive element in endothelial cells. Hypertension 47, 1189-1196.

Nagane, M., Kuppasamy, M. L., An, J., Mast, J. M., Gogna, R., Yasui, H., Yamamori, T., Inanami, O. and Kuppasamy, P. (2018) Ataxia-
telangiectasia mutated (ATM) kinase regulates eNOS expression and modulates radiosensitivity in endothelial cells exposed to ionizing radiation. *Radiat. Res.* **189**, 519-528.

Nguyen, G. H., Dexheimer, T. S., Rosenthal, A. S., Chu, W. K., Singh, D. K., Mosedale, G., Bachrati, C. Z., Schultz, L., Sakurai, M., Savitsky, P., Abu, M., McHugh, P. J., Bohr, V. A., Harris, C. C., Jadhav, A., Gileadi, O., Maloney, D. J., Simeonov, A. and Hickson, I. D. (2013) A small molecule inhibitor of the BLM helicase modulates chromosome stability in human cells. *Chem. Biol.* **20**, 55-62.

Park, J. H., Kim, W. S., Kim, J. Y., Park, M. H., Nam, J. H., Yun, C. W., Kwon, Y. G. and Jo, I. (2011) Chk1 and Hsp90 cooperatively regulate phosphorylation of endothelial nitric oxide synthase at serine 1179. *Free Radic. Biol. Med.* **51**, 2217-2226.

Poehlmann, A. and Roessner, A. (2010) Importance of DNA damage checkpoints in the pathogenesis of human cancers. *Pathol. Res. Pract.* **206**, 591-601.

Searles, C. D. (2006) Transcriptional and posttranscriptional regulation of endothelial nitric oxide synthase expression. *Am. J. Physiol. Cell Physiol.* **291**, C803-C816.

Seo, J., Cho, D. H., Lee, H. J., Sung, M. S., Lee, J. Y., Won, K. J., Park, J. H. and Jo, I. (2016) Citron Rho-interactive kinase mediates arsenite-induced decrease in endothelial nitric oxide synthase activity by increasing phosphorylation at threonine 497: mechanism underlying arsenite-induced vascular dysfunction. *Free Radic. Biol. Med.* **90**, 133-144.

Seo, J., Lee, J. Y., Sung, M. S., Byun, C. J., Cho, D. H., Lee, H. J., Park, J. H., Cho, H. S., Cho, S. J. and Jo, I. (2014) Arsenite acutely decreases nitric oxide production via the ROS-protein phosphatase 1-endothelial nitric oxide synthase-Thr(497) signaling cascade. *Biomol. Ther. (Seoul)* **22**, 510-518.

Sessa, C., Zucchetti, M., Davoli, E., Califano, R., Cavalli, F., Frustaci, S., Gumbrill, L., Sulkos, A., Winograd, B. and D’Incalci, M. (1991) Phase I and clinical pharmacological evaluation of aphidicolin glycinate. *J. Natl. Cancer Inst.* **83**, 1160-1164.

Sessa, W. C., Pritchard, K., Seyedi, N., Wang, J. and Hintze, T. H. (1994) Chronic exercise in dogs increases coronary vascular nitric oxide production and endothelial cell nitric oxide synthase gene expression. *Circ. Res.* **74**, 349-353.

Shaywitz, A. J. and Greenberg, M. E. (1999) CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. *Annu. Rev. Biochem.* **68**, 821-861.

Shen, W., Zhang, X., Zhao, G., Wolin, M. S., Sessa, W. and Hintze, T. H. (1995) Nitric oxide production and NO synthase gene expression contribute to vascular regulation during exercise. *Med. Sci. Sports Exerc.* **27**, 1125-1134.

Sun, D., Huang, A., Koller, A. and Kaley, G. (2002) Decreased arterial sensitivity to shear stress in adult rats is reversed by chronic exercise activity. *Microcirculation* **9**, 91-97.

Tanaka, T., Huang, X., Halicka, H. D., Zhao, H., Traganos, F., Albino, A. P., Dai, W. and Darzynkiewicz, Z. (2007) Cytometry of ATM activation and histone H2AX phosphorylation to estimate extent of DNA damage induced by exogenous agents. *Cytometry A* **71**, 648-661.

Vesela, E., Chroma, K., Turz, Z. and Mistrik, M. (2017) Common chemical inducers of replication stress: focus on cell-based studies. *Biomolecules* **7**, E19.

Viniegra, J. G., Martinez, N., Modirassani, F., Hernandez Losa, J., Parada Cobo, C., Sanchez-Arevalo Lobo, V. J., Aceves Luquero, C., Alvarez-Vallina, L., Ramon y Cajal, S., Rojas, J. M. and Sanchez-Prieto, R. (2005) Full activation of PKB/Akt in response to insulin or ionizing radiation is mediated through ATM. *J. Biol. Chem.* **280**, 4029-4036.

Wright, G. E., Hubscher, U., Khan, N. N., Focher, F. and Verri, A. (1994) Inhibitor analysis of calf thymus DNA polymerases alpha, delta and epsilon. *FEBS Lett.* **341**, 129-130.

Yang, Y. M., Huang, A., Kaley, G. and Sun, D. (2009) eNOS uncoupling and endothelial dysfunction in aged vessels. *Am. J. Physiol. Heart Circ. Physiol.* **297**, H1820-H1836.

Yin, M. J. and Gaynor, R. B. (1996) Complex formation between CREB and Tax enhances the binding affinity of CREB for the human T-cell leukemia virus type 1 21-base-pair repeats. *Mol. Cell. Biol.* **16**, 3156-3169.

Zhang, Y., Lee, T. S., Kolb, E. M., Sun, K., Lu, X., Sladek, F. M., Kassab, G. S., Garland, T., Jr. and Shyy, J. Y. (2006) AMP-activated protein kinase is involved in endothelial NO synthase activation in response to shear stress. *Arterioscler. Thromb. Vasc. Biol.* **26**, 1281-1287.

https://doi.org/10.4062/biomolther.2020.007