Biochemical and Biophysical Research Communications 430 (2013) 476–481

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

B56α subunit of protein phosphatase 2A mediates retinoic acid-induced decreases in phosphorylation of endothelial nitric oxide synthase at serine 1179 and nitric oxide production in bovine aortic endothelial cells

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ARTICLE INFO

Article history:
Received 26 November 2012
Available online 10 December 2012

Keywords:
Endothelial nitric oxide synthase
All-trans retinoic acid
Dephosphorylation
Protein phosphatase 2A
PP2A B56α subunit

ABSTRACT

We previously showed that all-trans retinoic acid (atRA) decreased nitric oxide (NO) production through Akt-mediated decreased phosphorylation of endothelial NO synthase at serine 1179 (eNOS-Ser^{1179}) in bovine aortic endothelial cells (BAEC). Since protein phosphatase 2A (PP2A) was also reported to decrease eNOS-Ser^{1179} phosphorylation, we investigated using BAEC whether PP2A mediates atRA-induced eNOS-Ser^{1179} dephosphorylation and subsequent decreased NO production. Treatment with okadaic acid (5 nM), a selective PP2A inhibitor, or ectopic expression of small interference RNA (siRNA) of PP2A catalytic subunit α (PP2A Cα) significantly increased eNOS-Ser^{1179} phosphorylation and NO production. Each treatment also significantly reversed atRA-induced observed effects, suggesting a role for PP2A. We also found that atRA significantly increased cellular PP2A activity. However, Western blot analysis revealed that atRA did not increase the expression of PP2A Cα, although it significantly increased the level of B56α of PP2A regulatory B subunit (PP2A B56α), but not PP2A B55α and PP2A B56δ. Real-time PCR assay confirmed a significant increase in PP2A B56α mRNA expression in atRA-treated cells. Ectopic expression of siRNA of PP2A B56α significantly reversed atRA-induced inhibitory effects on eNOS-Ser^{1179} phosphorylation and NO production, suggesting a role for PP2A B56α. Our study demonstrates for the first time that atRA decreases eNOS-Ser^{1179} phosphorylation and NO release at least in part by increasing PP2A B56α-mediated PP2A activity in BAEC.

1. Introduction

Endothelial nitric oxide synthase (eNOS) is an enzyme essential to the maintenance of cardiovascular integrity by producing NO in endothelial cells (EC). Since NO serves multiple functions including vasodilation and cell cycle regulation, dysregulation of eNOS is thought to contribute to the pathogenesis of certain diseases such as atherosclerosis, hypertension, and cancer [1–4]. eNOS is mainly regulated at the level of phosphorylation [5]. Several specific sites of phosphorylation have been identified, of which eNOS-Ser^{1179} (bovine sequence) is the most studied. Phosphorylation of eNOS-Ser^{1179} increases NO production, which is mediated by several protein kinases, including Akt [6–8], AMP-activated protein kinase (AMPK) [9,10], calmodulin-dependent kinase II (CaMKII) [11], protein kinase A (PKA) [12,13], and checkpoint kinase 1 [14]. The role for these protein kinases as signaling molecules in eNOS-Ser^{1179} phosphorylation is dependent on several stimuli including vascular endothelial growth factor, bradykinin, shear stress, troglitazone, UV irradiation, and all-trans retinoic acid (atRA) [6,8,10,13–15].

Contrary to protein kinases responsible for eNOS-Ser^{1179} phosphorylation, a few studies have been reported about protein phosphatase-mediated signaling pathways. A series of experiments such as treatment with selective protein phosphatase (PP) inhibitors, transfection with specific phosphorylation site-deficient eNOS mutants, and immunoprecipitation and subsequent PP assay using immunoprecipitates, showed that PP2A was identified as a regulator modifying eNOS-Ser^{1179} phosphorylation [16]. Furthermore, it

Abbreviations: eNOS, endothelial nitric oxide synthase; EC, endothelial cell(s); NO, nitric oxide; AMPK, AMP-activated protein kinase; CaMKII, calmodulin-dependent kinase II; PKA, protein kinase A; atRA, all-trans retinoic acid; PP, protein phosphatase; PP2A Cα, catalytic subunit α of PP2A; PP2A B55α, regulatory subunit B55α of PP2A; PP2A B56α, regulatory subunit B56α of PP2A; PP2A B56δ, regulatory subunit B56δ of PP2A; PP2A B56γ, regulatory subunit B56γ of PP2A; BAEC, bovine aortic endothelial cells; siRNA, small interference RNA; OA, okadaic acid; DAF-FM, 4-amino-5-methylamino-2′,7′-difluorescein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide.

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http://dx.doi.org/10.1016/j.bbrc.2012.12.011

was also reported that the proteasomal inhibition by MG132 or lactacystin specifically dephosphorylated eNOS-Ser^{1179} through increasing PP2A association to eNOS [17], suggesting a role for PP2A in eNOS-Ser^{1179} dephosphorylation.

PP2A is an ubiquitous and conserved serine/threonine phosphatase with broad substrate specificity and diverse cellular functions. It comprises three distinct functional components: The catalytic subunit C of PP2A (PP2A Cα or Cβ) interacts with the structural A subunit (PP2A Aα or Aβ) making up a dimeric core enzyme, and the association with a variety of regulatory B subunits to the core enzyme results in the formation of heterotrimeric PP2A holoenzyme complexes. We previously reported that atRA decreased NO release by decreasing specifically eNOS-Ser^{1179} phosphorylation [15]. Based on previous studies showing a role for PP2A in eNOS-Ser^{1179} dephosphorylation [16,17], here, we investigated whether PP2A is also involved in decreased eNOS-Ser^{1179} phosphorylation and NO production in atRA-treated cell, and if any, how PP2A regulates this signaling pathway. Our result identifies a specific B subunit, PP2A B56α, which mediates atRA-induced decreases in eNOS-Ser^{1179} phosphorylation and NO release in BAEC.

2. Materials and methods

2.1. Materials

Okadaic acid (OA) and atRA were purchased from Sigma–Aldrich (St. Louis, MO). Antibodies against eNOS, p-eNOS-Ser^{1179}, PP2A Cα, PP2A B56α, were purchased from Transduction Laboratories (Lexington, KY). Antibody against PP2A B55α was purchased from Cell Signaling Technology (Boston, MA). Antibody against PP2A B56δ was prepared as described [18]. β-Actin antibody was purchased from Santa Cruz Biotechnology (La Jolla, CA). Minimal essential medium (MEM), Dulbecco's phosphate-buffered saline (DPBS), newborn calf serum (NCS), penicillin–streptomycin antibiotics, L-glutamine, and trypsin–EDTA solution obtained from Gibco-BRL (Gaithersburg, MD). All other chemicals were of the purest analytical grade.

2.2. Cell culture and drug treatments

Bovine aortic endothelial cells (BAEC) were isolated and maintained in MEM supplemented with 5% NCS at 37 °C under 5% CO_2 as described [19]. Cells between passages 5 and 9 were used. BAEC grown at 80% confluence were incubated without or with atRA (5 μM) for 24 h in fresh MEM containing 1% NCS. In some experiments, cells were co-treated with OA (5 nM).

2.3. Transfections

Small interference RNA (siRNA) oligonucleotides designed against PP2A Cα (Cat. No. L-003598-01) and PP2A B56α (Cat. No. L-009352-00) were purchased from Dharmacon Research Inc. (Lafayette, CO). A non-specific siRNA oligonucleotide (Cat. No. D-001810-01) was also obtained for a control experiment. BAEC grown to 80% confluence in 60 mm culture dishes were transfected with 100 nM of each siRNA oligonucleotide using DharmaFECT (Dharmacon Research Inc.) according to manufacturer's instructions. After incubation for 5 h at 37 °C, DharmaFECT mixtures were washed out and the cells were further incubated in MEM containing 5% NCS for 24 h before atRA treatment.

2.4. Western blot analysis

BAEC before or after atRA treatment were washed with ice-cold DPBS and then homogenized in lysis buffer as described [6]. In some experiments, cells were transfected with siRNA of catalytic C or regulatory B subunit of PP2A before atRA treatment. Proteins (20 μg) in homogenates were separated by SDS–PAGE, and transferred onto nitrocellulose membranes as described [6]. Blots were probed with antibody directed against eNOS, p-eNOS-Ser^{1179}, PP2A Cα, PP2A B55α, PP2A B56α, PP2A B56δ, and β-actin (each 1:1000 dilution), followed by the corresponding secondary antibody, and finally developed using enhanced chemiluminescence (ECL) reagents (Amersham, Buckinghamshire, UK).

2.5. Real-time polymerase chain reaction (PCR)

Total RNA was extracted using RNeasy Plus Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. One microgram of total RNA was converted to cDNA using Superscript II reverse transcriptase and oligo-$(dT)_{12-18}$ primer (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The qRT-PCR was performed in a 20 μl reaction mixture containing 1 μl cDNA, 10 μl SYBR Premix EX Taq (Takara Bio, Otsu, Shiga, Japan), 0.4 μl Rox reference dye (50X, Takara Bio) and 200 nM of primers for each gene using ABI PRISM 7000 sequence detection system (Applied BioSystems, Foster City, CA). The primer sequences were as follows: PP2A Cα-F, 5′-ATA ACT GGT GCC ATG ACC GA-3′ and PP2A Cα-R, 5′-GCT GGG TCA AAC TGC AAG AA-3′; PP2A B55α-F, 5′-GGC CCA TGG ATC TAA TGG TT-3′ and PP2A B55α-R, 5′-TTC CAT ATT GGC AGG CTT GA-3′; PP2A B56α-F, 5′-AGA AAG TGG ACG GCT TCA CC-3′ and PP2A B56α-R, 5′-ATC TTT GAG TTG GGG CAA GG-3′; PP2A B56δ-F, 5′-GAG CGG GAC TTC CTC AAG AC-3′ and PP2A B56δ-R, 5′-AGG AGC TCA GCA ATC CCA TT-3′; β-actin-F, 5′-CTC TTC CAG CCT TCC TTC CT-3′ and β-actin-R, 5′-GGG CAG TGA TCT CTT TCT GC-3′. The reaction was run at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min and a dissociation stage of 1 cycle at 95 °C for 15 s, 60 °C for 20 s and 95 °C for 15 s. All PCRs were performed in triplicates and the specificity of the reaction was detected by the melting curve analysis at the dissociation stage. Comparative quantification of each target gene was performed based on cycle threshold (C_T) normalized to β-actin using the $\Delta\Delta C_T$ method.

2.6. PP2A activity assay

The PP2A activity assay was carried out using the serine/threonine phosphatase Assay System (Promega, Madison, WI) as described [20], with minor modifications. Briefly, BAEC after treatment with 5 μM atRA or vehicle for 24 h were lysed in storage buffer containing 50 mM Tris–HCl (pH 7.5), 1 mM EDTA, 0.1% β-mercaptoethanol, 1% Triton X-100, 0.1 mM PMSF and 1X Protease Inhibitor Cocktail (Roche Molecular Biochemicals), and then centrifuged at 13,000*g* for 1 h. Supernatants were collected and filtered through a Sephadex G25 column to remove free phosphate. Protein concentration was determined using the BCA method (Sigma). Equal quantities of protein (100 μg) were incubated in PP2A-specific reaction buffer (250 mM imidazole pH 7.2, 0.2 mM EGTA, 0.02% β-mercaptoethanol, and 0.1 mg/ml BSA) containing the phosphatase substrate R-R-A-pT-V-A (100 μM) at 37 °C for 3 min. After the end of incubation, the reaction was stopped by adding 50 μl of molybdate dye/additive mixture. The cellular PP2A activity was quantified by measuring the optical density at 630 nm wavelength after color development at room temperature for 30 min.

2.7. Measurement of NO in culture media

NO production was measured as nitrite (a stable metabolite of NO) concentration in cell culture supernatants as described [6] with minor modifications. BAEC were grown on 60 mm dish in

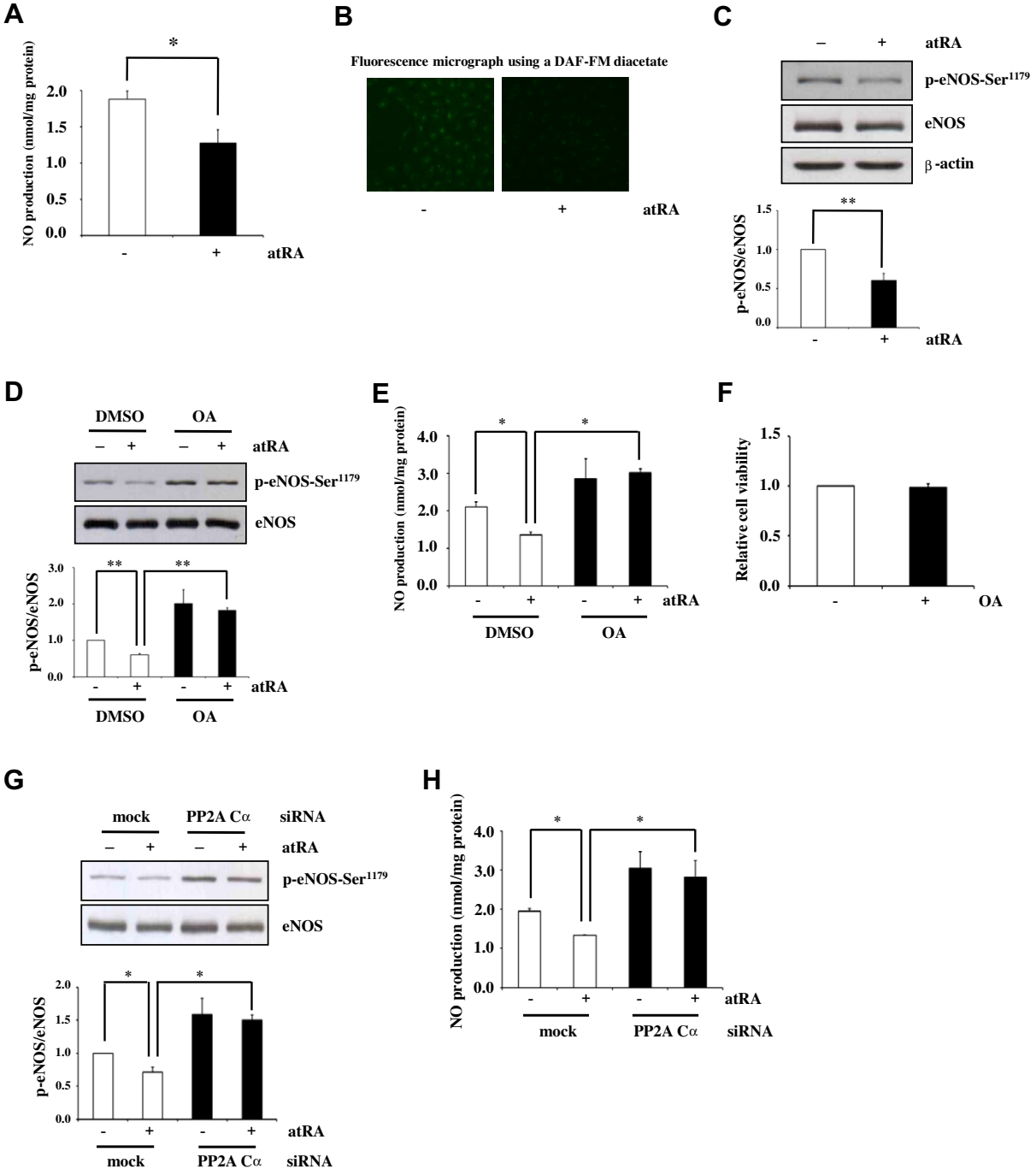

Fig. 1. OA and ectopic expression of siRNA of PP2A Cα reversed atRA-induced decreases in eNOS-Ser^{1179} phosphorylation and NO production. BAEC grown at 80% confluence were treated with 5 μM of atRA for 24 h (A, B, C). In separate experiments, cells were also co-treated with 5 nM OA (D, E, F) or transfected with siRNA (100 nM) of PP2A Cα before atRA treatment (G, H). For the control experiments, cells were treated vehicle (DMSO) only or transfected with non-specific siRNA (mock). NO levels in culture media were measured by the Griess method ($n = 4$) (A, E, H). Differences are statistically significant at $^{*}P < 0.05$. Using a NO-specific fluorescence probe DAF-FM diacetate, intracellular NO levels were also measured in cells treated without (left) and with atRA (right) (B). Cell viability after OA treatment was measured by MTT assay, and the ratio of the relative cell viability in OA-treated cells over that in control cells is shown (F). In some experiments, cells were lysed, and cellular proteins were separated on a SDS–polyacrylamide gel and electrophoretically transferred onto nitrocellulose membranes. p-eNOS was measured by Western blot analysis with antibody specific for eNOS phosphorylated at Ser^{1179} (C, D, G). Nitrocellulose membranes were reprobed with antibody detecting total eNOS to monitor equal loading of samples. The ratios of the relative p-eNOS-Ser^{1179} in treated cells over that in control cells are shown. The data represent means ± SD of 4 independent experiments. Differences are statistically significant at $^{*}P < 0.05$ and $^{**}P < 0.01$.

culture media and incubated with atRA or vehicle. After 24 h incubation, 200 μl of culture medium was carefully transferred into a 96-well plate, with the subsequent addition of 100 μl of Griess reagent (50 μl of 1% sulfanilamide containing 5% phosphoric acid and 50 μl of 0.1% *N*-[1-naphthyl] ethylenediamine). After color development at room temperature for 10 min, absorbance was measured on a microplate reader at a 520 nm wavelength. Levels of NO in culture media were expressed as nmol/mg total cellular protein. A calibration curve was plotted using known amounts of sodium nitrate solution.

2.8. Measurement of intracellular NO production

Intracellular NO was detected by using 4-amino-5-methylamino-2′,7′-difluorescein (DAF-FM) diacetate (Invitrogen) according to manufacturer's protocol. Briefly, BAEC grown on coverslips were treated with atRA or vehicle for 24 h. Cells were then incubated with 5 μM DAF-FM diacetate for 20 min at room temperature and fixed with 4% (wt/vol) paraformaldehyde in DPBS for 10 min. Images of intracellular NO were photographed using a confocal microscope (LSM5 Pascall, Carl ZEISS).

2.9. Cell viability assay

Cell viability assay was carried out as described [14] using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma). BAEC were grown in 96-well culture plates at 37 °C. After treatment with 5 nM OA or vehicle for 24 h, cells were incubated with 5 mg/ml MTT and further incubated for 2 h at 37 °C. Dimethyl sulfoxide (DMSO; 200 μl) was added to the cells and incubated for 10 min more and absorbance read at 570 nm using a 96-well microtiter plate reader.

2.10. Statistical analysis

All results are expressed as means ± standard deviation (SD) with n indicating the number of experiments. Statistical significance of difference was determined using Student's t test for paired data. A value of $P < 0.05$ was considered significant.

3. Results

3.1. PP2A mediates atRA-induced decreases in eNOS-Ser^{1179} phosphorylation and NO production

Like our previous study [15], we found that atRA (5 μM for 24 h) significantly decreased NO production in culture media (Fig. 1A), intracellular NO levels (Fig. 1B) and eNOS-Ser^{1179} phosphorylation (Fig. 1C). Under this condition, no alterations in eNOS expression (Fig. 1C) and the phosphorylations at other two sites, Thr^{497} and Ser^{116}, (data not shown) were found. Since PP2A was reported to be involved in dephosphorylation of eNOS-Ser^{1179} in response to endostatin [21] and proteasomal inhibition [17], we tested whether PP2A is also involved in atRA-induced decreases in

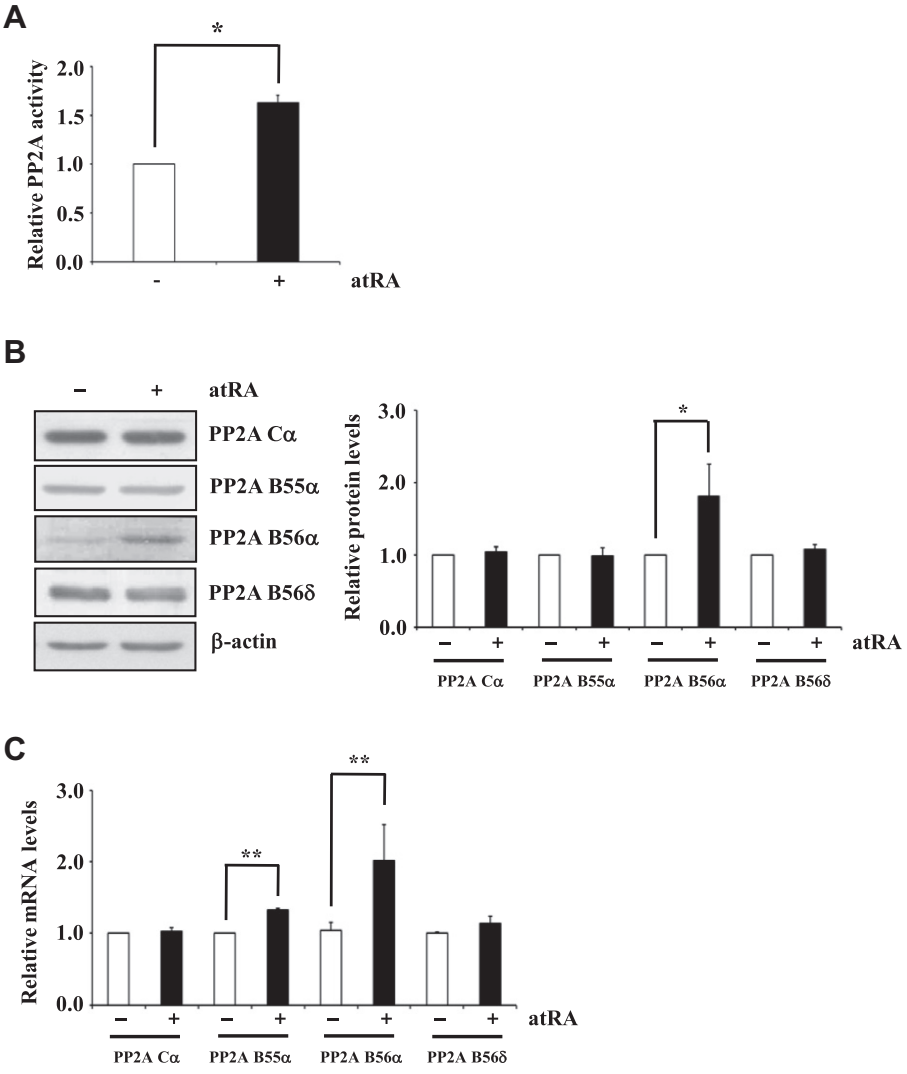

Fig. 2. atRA increases PP2A activity and PP2A B56α expression, but not PP2A Cα in BAEC. After treatment with 5 μM atRA for 24 h, cells were lysed and collected in PP2A storage buffer. Total cellular PP2A activity was measured by serine/threonine phosphatase assay kit (A), as described in Section 2. Cellular proteins after lysis were also subjected to assess the levels of PP2A B subunits using Western blot analysis with antibodies specific for PP2A Cα, PP2A B55α, PP2A B56α and PP2A B56δ (B). Total RNA was used for the detection of each B subunit mRNA transcript using real-time PCR assay (C), as described in Section 2. Bar graphs depict the ratios of the relative levels of PP2A activity (A), protein (B), and mRNA (C) in atRA-treated cells over that in control cells. The data represent means ± SD of 4 independent experiments. Differences are statistically significant at $^{*}P < 0.05$ and $^{**}P < 0.01$.

eNOS-Ser^{1179} and NO production in BAEC. Treatment with a PP2A inhibitor OA (5 nM) at a concentration specific for PP2A reversed all the inhibitory effects induced by atRA treatment (Fig. 1D and E). Under this condition, OA did not affect endothelial cell viability (Fig. 1F). The same effects were also manifested when siRNA of PP2A Cα was ectopically expressed in cells (Fig. 1G and H). These results indicate a role for PP2A in atRA-induced decreases in eNOS-Ser^{1179} and NO production.

3.2. All-trans retinoic acid increases PP2A activity and PP2A B56α expression

We next explored how PP2A mediates atRA-induced decreases in eNOS-Ser^{1179} phosphorylation and NO production. As shown in Fig. 2A, atRA significantly increased total cellular PP2A activity. However, no alteration in PP2A Cα level was found in atRA-treated cells (Fig. 2B), which was not in agreement with increase in PP2A activity. Interestingly, we found that atRA significantly increased the expression of PP2A B56α among three B subunits of PP2A tested in this study. The levels of other two subunits, B55α and B56δ, were not altered (Fig. 2B). Real-time PCR assay also confirmed that atRA increased PP2A B56α mRNA (Fig. 2C), although PP2A B55α also slightly increased.

3.3. PP2A B56α mediates atRA-induced decreases in eNOS-Ser^{1179} phosphorylation and NO production in BAEC

Finally, we examined whether PP2A B56α plays a role in decreasing eNOS-Ser^{1179} phosphorylation and NO production in atRA-treated BAEC. As shown in Fig. 3, ectopic expression of siRNA of PP2A B56α reversed completely all the inhibitory effects by atRA treatment, indicating a physiological role for PP2A B56α.

4. Discussion

Unlike kinases, the identification of phosphatases regulating eNOS activity has been limitedly studied. Only a few data showed that PP2A is involved in decreased NO production in part through eNOS-Ser^{1179} dephosphorylation [17,21], but the underlying mechanism is not fully understood. In this study, we report that PP2A plays an important role in atRA-induced decreases in eNOS-Ser^{1179} phosphorylation and NO production. Furthermore, PP2A B56α is involved in this inhibitory signaling pathway, which may provide the molecular mechanism underlying atRA-mediated cell migration and angiogenesis that may be NO-dependent in EC.

The most important finding in this study is that the specific B subunit of PP2A, PP2A B56α, mediates atRA-induced decreased eNOS-Ser^{1179} phosphorylation. It is well known that PP2A activity is largely due to PP2A Cα although PP2A Cβ also contributes to the activity but to a much lesser extent [22]. Apparently, the expression of catalytic subunit of PP2A is tightly regulated in cells to ensure to maintain relatively constant levels, and therefore PP2A activity is likely to be controlled by other subunits. As expected, in this study, we were also unable to find the alteration in the levels of PP2A Cα, a major catalytic form of PP2A, in atRA-treated cells although atRA significantly increased PP2A activity. Hence, it has been now recognized that B subunits of PP2A appear to mainly regulate PP2A activity. For example, we previously found that the down-regulation of PP2A B56δ expression by RNAi decreases PP2A activity, which in turn increases the phosphorylation of tyrosine hydroxylase at serine 40 and subsequent dopamine synthesis in rat mesencephalic neuronal N27 cells [23]. Similarly, overexpression of PP2A B56γ in NIH3T3 cells was also reported to significantly increase PP2A activity, decreasing p27 phosphorylation at threonine 187 and consequent cell proliferation through a delay of G1/S transition [24]. In this regard, our present data show that atRA significantly increases the level of PP2A B56α, which would provide the mechanism underlying atRA-induced dephosphorylation of eNOS-Ser^{1179}.

In addition to the regulation of B subunit expression, the strikingly features of B subunits stemming from distinct localization and intracellular trafficking in cells also play roles in regulating PP2A activity. It is reported that PP2A B56α modifies PP2A activity towards ceramide-stimulated Bcl2 dephosphorylation at least by increasing the translocation of PP2A B56α to mitochondria where Bcl2 is mainly expressed [25], providing a mechanism underlying ceramide-induced cell death. Another study also shows that PP2A B56δ regulates PP2A activity responsible for Cdc25 dephosphorylation, resulting in 14-3-3 protein release from Cdc25 and subsequent mitosis control [26]. Like these previous studies, our study demonstrates for the first time that PP2A B56α is identified as a new regulator mediating eNOS-Ser^{1179} dephosphorylation and NO decrease in response to atRA treatment. Whether PP2A B56α is co-localized and translocated into eNOS in response to atRA treatment needs further experiments. *In silico* analysis (available at http://www.signaling-gateway.org) reveals that PP2A B56α

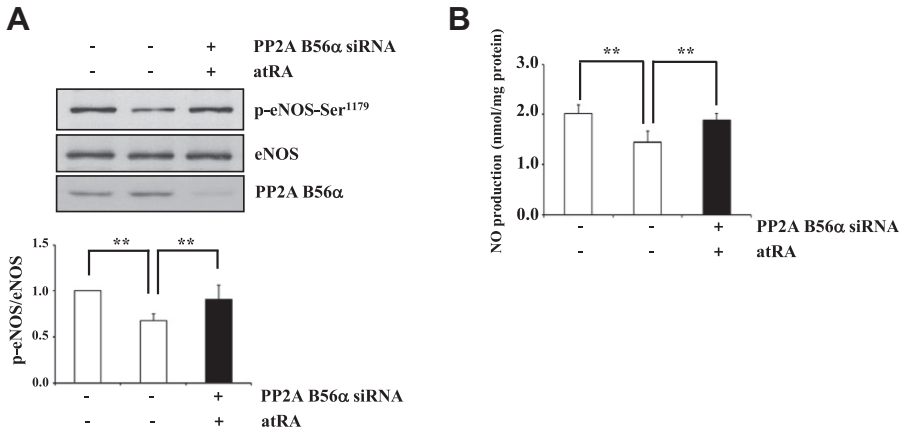

Fig. 3. Ectopic expression of siRNA of PP2A B56α reversed decreases in eNOS-Ser^{1179} phosphorylation and NO production in atRA-treated BAEC. BAEC were transfected for 5 h with 100 nM siRNA specific for PP2A B56α gene or non-specific siRNA (mock). After transfection, cells were further incubated in fresh MEM containing 5% NCS for 24 h before atRA treatment. The levels of p-eNOS-Ser^{1179}, eNOS, and PP2A B56α were measured using corresponding antibodies as described in the legends of Fig. 1 and 2. The blot shown is representative of at least 4 experiments (A). NO release was measured by the Griess method and analyzed (B), as described in the legend of Fig. 1. Bar graphs show the mean ± SD of at least 4 experiments, as described in the legend of Fig. 1. Differences were statistically significant at $^{**}P < 0.01$.

interacts with eNOS, but further study such as co-immunoprecipitation assay is needed to clarify this issue.

In summary, this study is the first to show that PP2A B56α mediates atRA-induced decreases in eNOS-Ser^{1179} phosphorylation and NO production. Because both atRA and NO play a role in cell cycle regulation, proliferation, migration and angiogenesis in several cells including EC, the present study may extend our understanding of the molecular mechanism involved in atRA-mediated and NO-related chronic diseases such as cancer and atherosclerosis.

Acknowledgments

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2011-0027347) and RP-Grant 2011 of Ewha Womans University.

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