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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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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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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