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Retinoic acid decreases nitric oxide production in endothelial cells: a role of phosphorylation of endothelial nitric oxide synthase at Ser[1179]☆

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Abstract

The effects of retinoic acid (RA) on nitric oxide (NO) production are controversial. Furthermore, it has never been studied whether these effects are mediated by direct modulation of phosphorylation of endothelial nitric oxide synthase (eNOS). Using bovine aortic endothelial cells, we found that all-*trans* RA (atRA) dose- and time-dependently decreased NO production without alteration in eNOS expression. This decrease was accompanied by reduction in eNOS-Ser[1179] phosphorylation. However, atRA did not alter the phosphorylation of eNOS-Ser[116] or eNOS-Thr[497]. Concurrently, atRA also decreased the expressions of vascular endothelial growth factor (VEGF) and its receptor KDR/Flk-1, and Akt phosphorylation. Co-treatment with troglitazone, an activator of VEGF expression, reversed the atRA-induced reductions in eNOS-Ser[1179] phosphorylation and NO production, with concomitant restoration in VEGF expression. Direct treatment with VEGF also reversed these inhibitory effects, suggesting an important role for VEGF. Nonetheless, the RARα antagonist Ro 41-5253 did not block all the inhibitory effects of atRA, indicating that these inhibitory effects are not mediated by the RA response element (RARE). Thus, atRA decreases eNOS-Ser[1179] phosphorylation through a mechanism that depends on VEGF–KDR/Flk-1-mediated Akt phosphorylation but is independent of RARE, leading to reduction in NO production.

Keywords: All-*trans* retinoic acid; Nitric oxide; Endothelial nitric oxide synthase; Vascular endothelial growth factor; Phosphorylation; Endothelial cells

Vitamin A derivatives (retinoids), and in particular all-*trans* retinoic acid (atRA), exert profound effects on the growth, maturation, and differentiation of many cell types in vivo and in vitro [1]. Biological responses to atRA are currently used therapeutically in various human cancers including leukemia, skin cancer, cervical cancer, and neuroblastoma [2,3]. Furthermore, it is also involved in beneficial functions in the cardiovascular system, because it inhibits inflammation, thrombosis, and platelet adhesion and aggregation [3–5].

The actions of atRA are mediated by specific nuclear receptors, namely the RA receptors (RARα, β, and γ) and the retinoid X receptors (RXRα, β, and γ). After atRA activation, RARs form heterodimers with RXRs,

☆ *Abbreviations:* atRA, all-*trans* retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; RARE, RA response element; PPAR, peroxisome proliferator-activated receptor; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; DDAH, dimethylarginine dimethylaminohydrolase; BAEC, bovine aortic endothelial cells; VEGF, vascular endothelial growth factor; VSMC, vascular smooth muscle cells.

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which function as transcriptional regulators. RAR/RXR heterodimers bind to the *cis*-regulatory elements, 5′-AGGTCA-3′, termed RA response element (RARE), located in the regulatory regions of target genes [6]. In addition to heterodimerizing with RARs, RXR also forms either homodimers with RXRs or heterodimers with a number of other nuclear receptors such as the peroxisome proliferator-activated receptor (PPAR), leading to crosstalk between several signaling pathways [7]. atRA exerts three types of action on gene activity. First, atRA modulates (mostly activates) gene transcription by activating RAR/RXR heterodimers. This atRA action is the most well known and characterized by direct interactions between RAR/RXR heterodimers and RARE. Second, atRA also stimulates gene transcription by mediating Sp1 sites, not RARE, located in the regulatory region of target genes [8]. This second action is characterized by interactions between unidentified RAR-mediated protein(s) and Sp1 sites in the promoter of target genes. *Trans*-repression of AP1 is the third mode of action of atRA on gene expression, which is characterized by the antagonism of AP1-dependent gene expression, independently of RARE in target genes [9,10].

Endothelial nitric oxide synthase (eNOS) is an enzyme essential to the maintenance of cardiovascular integrity by producing nitric oxide (NO) in vivo. This is a key signaling molecule with multiple functions, including vasodilation, and many anti-atherogenic properties. Therefore, the dysregulation of eNOS is thought to contribute to the pathogenesis of certain vascular diseases, such as atherosclerosis and hypertension [11]. eNOS is regulated not only at level of expression [12,13] but also nongenomically by phosphorylation [14]. Recently, specific sites of phosphorylation have been identified; among these sites, Ser^{1179} (eNOS-Ser^{1179}; bovine sequence), eNOS-Thr^{497}, and eNOS-Ser^{116} are the most studied. The phosphorylation of eNOS-Ser^{1179} increases NO production, which is mediated by several protein kinases including protein kinase B/Akt [15] and protein kinase A [16,17]. In contrast, the phosphorylations of eNOS-Thr^{497} and eNOS-Ser^{116} decrease eNOS activity, which are mediated by protein phosphatases 1 and 2B [14,18,19].

The cellular effects of NO bear some similarity to those of atRA, and NO has been implicated in the atRA-induced endothelial cell migration. Thus, it is likely that atRA may regulate endothelial NO production. Previously, atRA was reported to either decrease NO production in oocytes by down-regulating expressions of eNOS mRNA and protein [12] or increase in endothelioma cells by up-regulating dimethylarginine dimethylaminohydrolase (DDAH) II expression regardless of eNOS expression [20]. However, to date, there is no report demonstrating that atRA modulates NO production by directly regulating eNOS phosphorylation, although eNOS is known to be a multiple phosphorylated protein and its activity is regulated by phosphorylation states. In this study, we investigate the molecular mechanism for the atRA-induced regulation of eNOS phosphorylation and NO production in bovine aortic endothelial cells (BAEC). Our data demonstrate for the first time that atRA decreases eNOS-Ser^{1179} phosphorylation through a mechanism that depends on vascular endothelial growth factor (VEGF)–KDR/Flk-1-mediated Akt phosphorylation but is independent of RARE, leading to reduction in NO production.

Materials and methods

Materials. atRA was purchased from Sigma Chemical (St Louis, MO). RARα antagonist Ro 41-5253 was kindly provided by Dr. E-M Kutknecht at F. Hoffmann-La Roche (Basel, Switzerland). Troglitazone was obtained as a gift from Sankyo (Tokyo, Japan). Recombinant human VEGF was purchased from R&D Systems (Minneapolis, MN). Antibodies against eNOS and Akt were obtained from Transduction Laboratories (Lexington, KY) and New England Biolabs (Beverly, MA), respectively. Antibodies against VEGF and its receptor KDR/Flk-1 were purchased from Sigma and Santa Cruz Biotechnologies (La Jolla, CA), respectively. Antibodies against phosphorylated Akt at Ser^{473} (p-Akt-Ser^{473}) and p-eNOS-Ser^{1179} were obtained from Cell Signaling Technology (Beverly, MA), and those against p-eNOS-Thr^{497} and p-eNOS-Ser^{116} were from Upstate Biotechnology (Lake Placid, NY). Collagenase (type 2) was purchased from Worthington Biochemical Corporation (Freehold, NJ). Minimal essential medium (MEM), Dulbecco's phosphate-buffered saline (DPBS), newborn calf serum (NCS), penicillin–streptomycin antibiotics, L-glutamine, trypsin–EDTA solution, and plastic ware for cell cultures were purchased from Gibco-BRL (Gaithersburg, MD). All other chemicals were of the purest analytical grade.

Cell culture, drug treatments, and measurement of NO release. BAEC were isolated and maintained in MEM supplemented with 5% NCS at 37 °C under 5% CO_2 in air, as previously described [21]. When BAEC had grown to confluence, they were maintained for the indicated times in MEM supplemented with 0.5% NCS that contained various doses of atRA in the absence or presence of the indicated drugs. NO production by BAEC was measured as nitrite (a stable metabolite of NO) concentration in cell culture supernatants as previously described [21].

Transfection and luciferase assay. When BAEC had grown to 60% confluence in 60 mm culture dish, they were transfected with 2 μg $(\beta_2RARE)_2$-luc construct (a kind gift from Dr. Pierre Chambon, CNRS/INSERM/ULP, CU de Strasbourg, France) using lipofectin reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. After transfection for 5 h at 37 °C, cells were further incubated in fresh MEM for 24 h. Luciferase activity was determined using the Luciferase Assay System (Promega, Madison, WI) in the absence or presence of atRA (5 μM for 24 h). In some experiments, cells were pre-treated for 1 h with RARα antagonist Ro 41-5253 (5 μM) before atRA treatment.

Western blot analysis. Protein samples, treated with atRA in the absence or presence of various chemicals, were subjected to SDS–PAGE and transferred onto nitrocellulose membranes as described [21]. The blots were then probed with the appropriate antibody directed against VEGF (1:500), KDR/Flk-1 (1:1000), Akt (1:4000), eNOS (1:4000), p-Akt-Ser^{473} (1:2000), p-eNOS-Ser^{1179} (1:1000), p-eNOS-Thr^{497} (1:2000), or p-eNOS-Ser^{116} (1:4000), followed by the corresponding secondary antibody, and finally developed using enhanced chemiluminescence (ECL) reagents (Amersham, DE).