Upregulation of Nitric Oxide Production in Vascular Endothelial Cells by All-trans Retinoic Acid Through the Phosphoinositide 3-Kinase/Akt Pathway

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Background—A natural retinoid all-trans retinoic acid (ATRA) contains various beneficial effects on vasculature, including suppression of neointima formation after balloon injury. However, little is known about the effects of ATRA on vascular endothelial function. We therefore studied its role in nitric oxide (NO) production of vascular endothelial cells (ECs).

Methods and Results—Human dermal microvascular ECs, human umbilical vein ECs, and SV40-transformed rat lung vascular ECs were incubated with or without ATRA (1 μmol/L) for 48 hours. Their NO production was determined with the use of a fluorescent NO indicator, diaminofluorescein-2 diacetate. ATRA significantly increased their basal as well as acetylcholine-induced NO production. Treatment with Nω-nitro-L-arginine methyl ester or carboxy-PTIO suppressed their fluorescence. Increase of NO production was also observed by incubation with retinoic acid receptor (RAR) agonist Am580. ATRA-induced NO increase was abolished by coincubation with RAR antagonist LE540. Moreover, the NO increase was completely inhibited by the phosphoinositide 3-kinase (PI3K) inhibitor wortmannin and LY294002. ATRA as well as Am580 enhanced endothelial NO synthase (eNOS) phosphorylation at Ser-1177 as well as Akt phosphorylation at Ser-473 without changing their protein expression. Overexpression of dominant-negative Akt inhibited the eNOS phosphorylation. Moreover, ATRA increased PI3K activity as well as PI3K catalytic subunit p110β protein expression, which was completely inhibited by LE540 treatment. Real-time polymerase chain reaction analyses demonstrated that ATRA increased PI3K catalytic subunit p110β mRNA expression without affecting its stability. Finally, ATRA-induced NO increase was observed in COS-1 cells transfected with wild-type eNOS and RARα, but not with mutated eNOS whose Ser-1177 was substituted.

Conclusions—ATRA increases NO production by eNOS phosphorylation through RAR-mediated PI3K/Akt pathway activation in vascular ECs and possibly plays beneficial roles in vascular endothelium. Retinoids may therefore be candidates as novel therapeutic agents against vascular disorders with endothelial damage. (Circulation. 2005;112:727-736.)

Key Words: endothelium ■ nitric oxide ■ receptors

All-trans retinoic acid (ATRA), a natural retinoid derived from vitamin A, has recently been reported to induce differentiation and inhibit proliferation, cell migration, inflammation, and extracellular matrix synthesis of vascular smooth muscle cells (VSMCs).1-4 Moreover, ATRA is reported to suppress neointima formation and restenosis after balloon injury in vivo.5,6 In vascular endothelium, ATRA as well as other retinoids is reported to modulate endothelial cell growth and phenotype7,8; to induce antithrombotic genes including urokinase-type plasminogen activator,9 tissue-type plasminogen activator,10 and thrombomodulin11; and to improve acetylcholine (ACh)-induced relaxation in rat aorta.12 ATRA is thus considered to have favorable effects not only in VSMCs but also in vascular endothelium. However, roles of ATRA in endothelium still remain unclear.

Nitric oxide (NO) is a potent vasodilator and signal modulator molecule, and endothelium-derived NO in particular plays important roles in controlling vascular function, including suppression of vascular remodeling.13,14 With regard to the effects of ATRA on NO synthesis in endothelium, although a previous study has demonstrated its increase by ATRA through alteration of the metabolism of endogenous...
NO synthase (NOS) inhibitor asymmetric dimethylarginine (ADMA), little is known about the effects of ATRA on endothelium-derived NO production with regard to endothelial NOS (eNOS) function. We therefore examined the effects of ATRA on NO production as well as eNOS phosphorylation regulation in vascular endothelial cells (ECs).

**Methods**

**Reagents**

ATRA, wortmannin, LY294002, N-nitro-L-arginine methyl ester (L-NAME), and ACh were purchased from Sigma-Aldrich. The synthetic retinoic acid receptor (RAR) agonist Am580, RAR antagonist LE540, and retinoid X receptor (RXR) antagonist HX603 were synthesized as previously described. Diaminofluorescein-2 diacetate (DAF-2DA) was purchased from Daichi Pure Chemicals. Carboxy-PTIO was purchased from Dojindo.

**Cell Culture**

Vascular ECs were cultured on a monolayer plastic dish in a humidified incubator at 37°C with 5% CO2. Human dermal microvascular ECs (HMVECs) were purchased from Kurabo and cultured in HuMedia-EB2 (Kurabo) supplemented with 5% fetal bovine serum (FBS), 10 ng/mL human epidermal growth factor (hEGF), 1 ng/mL hydrocortisone, 10 μg/mL heparin, 50 ng/mL gentamicin, and 50 ng/mL amphotericin-B. HMVECs were used between passages 1 and 4. SV40-transformed rat lung vascular ECs (TRLEC-03) were obtained from microscope (model ECLIPSE E600, Nikon) equipped with ×20 (for HMVECs) or ×60 (for TRLEC-03) objective. Excitation light was passed through a 495-nm filter, and emitted light was passed through a 510-nm filter. Fluorescent images of NO production was determined by measurement of DAF-2T fluorescence per minute after ACh stimulation for 10 minutes (dF/dt).

**RNA Preparation/Reverse Transcription–Polymerase Chain Reaction**

Total RNAs of HUVECs and TRLEC-03 were extracted with the use of RNeasy mini kit (Qiagen) according to the manufacturer’s instructions. The extracted RNAs (0.5 μg) were then subjected to reverse transcription (RT)—polymerase chain reaction (PCR) by PCR Kit (AMV) version 3.0 (Takara Bio) with the use of specific primers for either M3 muscarinic ACh receptor or glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). RT-PCR for human M3 receptor (forward primer: 5′-AAG-TCA-GAT-CAC-TAA-GCG-GA-3′ [from position 1416 to 1435 in human M3 receptor cDNA (GenBank accession number AF498917)]; reverse primer: 5′-GCC-AGT-AGC-CGA-GAT-TCC-AA-3′ [from position 1575 to 1594 in the cDNA]) (PCR product: 179 bp) was performed under the following conditions: 30°C, 30 minutes and 99°C, 5 minutes for RT with the use of oligo(dT)-adaptor primer; 94°C, 2 minutes for 1 cycle; 94°C, 1 minute; 46°C, 1 minute; 72°C, 1 minute for 37 cycles. RT-PCR for human GAPDH (forward primer: 5′-CCA-TGG-AGA-AAG-CTG-GG-3′; reverse primer: 5′-CAA-AGT-TGT-CAT-GGA-TGA-CC-3′) (PCR product: 195 bp) was performed under the following conditions: 37°C, 10 minutes; 42°C, 30 minutes; and 99°C, 5 minutes for RT with the use of random 9-mer; 94°C, 2 minutes for 1 cycle; 94°C, 1 minute; 55°C, 1 minute; 72°C, 1 minute for 35 cycles. RT-PCR for rat M3 receptor (forward primer: 5′-AGG-TTT-GCT-CTC-AAG-ACC-AG-3′ [from position 1393 to 1402 in rat M3 receptor cDNA (GenBank accession number M16408)]; reverse primer: 5′-CAC-AAG-AGG-AGC-GTC-TTG-AA-3′ [from position 1600 to 1679 in the cDNA]) (PCR product: 287 bp) and rat GAPDH (forward primer: 5′-TCC-CTA-AAG-ATT-GTC-AGC-AA-3′; reverse primer: 5′-AGA-TCC-ACA-ACG-GAT-ACA-TT-3′) (PCR product: 308 bp) was performed under the following conditions: 37°C, 10 minutes; 42°C, 30 minutes; and 99°C, 5 minutes for RT with the use of random 9-mer; 94°C, 2 minutes for 1 cycle; 94°C, 1 minute; 56°C, 1 minute; 72°C, 1 minute for 40 cycles. The RT-PCR products were thereafter subjected to electrophoresis on 1.5% agarose gel.

**Real-Time PCR**

TRLEC-03 grown to 80% confluence was incubated either with or without ATRA (1 μmol/L) for 48 hours, and total RNAs were harvested as described above. For determination of mRNA stability, TRLEC-03 incubated with or without ATRA for 48 hours was coinubated with 5 μg/mL actinomycin D (Nacalai Tesque) for an additional 3 or 6 hours before harvesting. Real-time PCR analyses of rat phosphoinositide 3-kinase (PI3K) catalytic subunit p110β and rat GAPDH were then performed with the use of the obtained total RNAs by SYBR RT-PCR Kit (Perfect Real Time) (Takara Bio) according to the manufacturer’s instructions. Briefly, RT reaction (42°C, 15 minutes and 95°C, 2 minutes) was performed with 600 ng total RNAs by random 6-mer. Thereafter, obtained templates (40 ng) were used for real-time PCR reactions (95°C, 10 seconds for 1 cycle; 95°C, 5 seconds; and 60°C, 20 seconds for 45 cycles) with the use of SYBR Green I by Smart Cycler (Cepheid). Standard curves were obtained by real-time PCR with the use of serial dilutions of templates (40 ng, 10 ng, 4 ng, 1 ng, 400 pg, and 100 pg), and threshold cycle values were calculated.

The following primer sequences were used: rat PI3K catalytic subunit p110β (forward primer: 5′-CAG-TGG-AGA-CAG-TGC-GAA-GTG-A3′ [from position 1557 to 1578 in rat PI3K catalytic subunit p110β cDNA (GenBank accession number NM053481)]; reverse primer: 5′-TCA-GCA-GTA-TTC-CCG-TAG-A3′ [from position 1679 to 1698 in the cDNA]) (PCR product: 142 bp), rat GAPDH (forward primer: 5′-GAC-AAC-TTT-GGC-ATC-GTG-CA-3′ [from position 1339 to 1358 in rat GAPDH cDNA (GenBank accession number NM017608)]; reverse primer: 5′-ATG-CAG-GGA-TGA-TGT-TCT-GG-3′ [from position 1452 to 1471 in the cDNA]) (PCR product: 133 bp).
Protein Preparation/Western Blotting

HMVECs were cultured in a 6-well dish, and TRLEC-03 was cultured in a 100-mm dish. When cells were grown to 80% confluence, they were replaced with media containing 1% stripped FBS. Cells were then incubated with or without ATRA and/or other retinoid for indicated times. Total cellular extracts were prepared as follows. Cells were washed with cold phosphate buffered saline (PBS) 3 times, then 100 µl (for HMVECs) or 300 µl (for TRLEC-03) of cell lysis buffer (in mmol/L: 100 NaCl, 20 Tris-HCl, pH 7.40, 2.5 EDTA, 10 NaF, 1 Na3VO4, 1 Triton X-100, 1% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail [Complete Mini, Roche]) was added. The mixtures were homogenized with Polytron homogenizer for 30 seconds, and cell debris was removed by centrifugation at 14,000 g for 10 minutes. For Western blotting of eNOS and Akt of HMVECs and Akt and PI3K catalytic subunit p110β of TRLEC-03, their crude proteins (30 µg for HMVECs and 100 µg for TRLEC-03) were added to 2x SDS sample buffer (125 mM Tris-HCl, pH 6.80, 4% SDS, 20% glycerol, 100 mM dithiothreitol, 0.02% bromophenol blue) and boiled at 95°C for 5 minutes. For Western blotting of eNOS of TRLEC-03, its crude extracts (500 µg) were added to 75% 2x ADP Sepharose 4B (Amersham Biosciences) and were rotated at 4°C for 3 hours. The beads were washed with cell lysis buffer 3 times, and the bound proteins were eluted in 60 µl of 1x SDS sample buffer (62.5 mM Tris-HCl, pH 6.80, 2% SDS, 10% glycerol, 50 mM dithiothreitol, 0.01% bromophenol blue) at 95°C for 5 minutes. Nuclear extracts of TRLEC-03 for Western blotting of RAR and RXR were prepared as described previously.26

Samples were separated on a 7.5% or a 9.0% SDS-polyacrylamide gel and were transferred onto PVDF membrane (Bio-Rad). The membranes were blocked with 5% nonfat dry milk and thereafter probed with primary antibody against either eNOS (1:1000 dilution) (Sigma); phospho-eNOS (Ser-1177) (1:400 dilution) (Cell Signaling Technology); Akt (1:1000 dilution) (Cell Signaling Technology); phospho-Akt (Ser-473) (1:500 dilution) (Cell Signaling Technology); PI3K catalytic subunit p110β (1:1000 dilution) (Santa Cruz); RARα, β, γ (all 1:400 dilution) (Santa Cruz); or RXRα, β, γ (all 1:200 dilution).26 In an experiment, primary antibody against inducible NOS (iNOS) (1:300 dilution) (Santa Cruz) was also used. Horseradish peroxidase-linked antibodies (Amersham Biosciences) were then used as secondary antibodies. Blotted membranes were visualized with ECL-plus reagent (Amersham Biosciences) and lumino-image analyzer LAS-1000 (Fuji Film).

Measurement of PI3K Activity

PI3K activity was measured by enzyme-linked immunosorbent assay (ELISA). When TRLEC-03 cultured in 100-mm dish was grown to 80% confluence, it was replaced with media containing 1% stripped FBS. Cells were thereafter incubated with or without ATRA for 48 hours. Their total cellular extracts were then prepared and subjected to Western blotting as described above.

Statistical Analyses

All data are presented as means±SEM. Statistical analyses were performed with ANOVA followed by post hoc Tukey test.

Results

Effects of NO Scavenger and NOS Inhibitor on NO Increase

To confirm whether the DAF-2T fluorescence increase was specifically derived from NO increase, effects of specific NO scavenger, carboxy-PTIO65 were examined. In the presence of carboxy-PTIO, both basal [ATRA(−)] (Figure 3A and 3B, lane 2) and ATRA-induced [ATRA(+)] (Figure 3A and 3B, lane 5) DAF-2 fluorescence was completely suppressed. We next examined the effects of the NOS inhibitor L-NAME to determine whether the NO increase was obtained from NOS-derived de novo synthesis. Treatment with L-NAME diminished basal [ATRA(−)] NO level (Figure 3A and 3B, lane 3, reverse: 5'-GGAAATCCAGGGGCTGTTGCTGCTGAAGAGCCTG-3'; forward: 5'-CCCAAGCTTACCATGGGCAACTTGAAGAGCCTG-3'. The isolated cDNA was confirmed to be identical to the previously reported clone (NM_000603) by sequencing and was subcloned into pcDNA3.1 expression vector (Invitrogen) (pcDNA3/ wt-eNOS). The wild-type eNOS cDNA was then subjected to PCR-based site-directed mutagenesis to substitute Ser-1177 to Ala (pcDNA3/mut-eNOS). COS-1 cells grown on a microcoverglass (Matsumura Glass) in a 100-mm culture dish were transfected with 4 µg of human RARα cDNA expression vector (pSG5[RARα]) and 16 µg of pcDNA3/wt-eNOS or pcDNA3/mut-eNOS with the use of Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After transfection, cells were incubated with or without ATRA (1 µmol/L) for 48 hours. Cells were then subjected to measurement of NO production with the use of DAF-2DA as described above in vascular ECs.

Statistical Analyses

All data are presented as means±SEM. Statistical analyses were performed with ANOVA followed by post hoc Tukey test.

Effects of ATRA on NO Production in Vascular ECs

Effects of ATRA on NO production were first examined. ATRA treatment (1 µmol/L, 48 hours) significantly increased NO production in HMVECs [Figure 1A and 1B; 173±4.0% of ATRA(−)], HMVECs [Figure 1A and 1B; 259±14% of ATRA(−)], and TRLEC-03 [Figure 1A and 1B; 173±19% of ATRA(−)]. RARα agonist Am580 also increased NO production in HMVECs [Figure 1C; 166±5.7% of Am580(−)] and HMVECs [Figure 1C; 237±18% of Am580(−)]. ACh-induced NO release was also significantly enhanced by ATRA in both HMVECs [Figure 1D and 1E; 187±31% of ATRA(−)] and TRLEC-03 [Figure 1D and 1E; 159±21% of ATRA(−)]. Expression of M3 muscarinic ACh receptor, functioning predominantly in vasculature,27 was confirmed both in HMVECs (Figure 1F, lane 2) and TRLEC-03 (Figure 1F, lane 7).

ATRA increased NO production in a concentration-dependent manner (Figure 2A; 157±22% of 0 mol/L at 10−7 mol/L and 183±10% of 0 mol/L at 10−6 mol/L). Time course analyses (Figure 2B) demonstrated that ATRA increased NO production later than 48 (171±18% of 0 hour) and 96 (162±9.1% of 0 hour), but not by 24 hours (104±11% of 0 hour).

Effects of NO Scavenger and NOS Inhibitor on NO Increase

To confirm whether the DAF-2T fluorescence increase was specifically derived from NO increase, effects of specific NO scavenger carboxy-PTIO were examined. In the presence of carboxy-PTIO, both basal [ATRA(−)] (Figure 3A and 3B, lane 2) and ATRA-induced [ATRA(+)] (Figure 3A and 3B, lane 5) DAF-2 fluorescence was completely suppressed. We next examined the effects of the NOS inhibitor L-NAME to determine whether the NO increase was obtained from NOS-derived NO production with the use of DAF-2DA as described above in vascular ECs.
25±3.8% of lane 1) as well as ATRA-induced [ATRA(+) NO increase (Figure 3A and 3B, lane 6, 20±2.4% of lane 4).

Involvement of Retinoid Receptors in NO Increase
We examined involvement of retinoid receptors in the ATRA-induced NO increase. Western blotting revealed that TRLEC-03 expressed RARα, RARβ (Figure 4A), and RXRα (Figure 4B). Effects of selective RAR and/or RXR antagonists were next examined (Figure 4C). ATRA-induced NO production (lane 2, 180±14% of lane 1) was suppressed by cotreatment with RAR antagonist LE540 (lane 3, 124±12% of lane 1), whereas RXR antagonist HX603 showed little effect (lane 4, 176±20% of lane 1). Cotreatment with both LE540 and HX603 did not show further suppression compared with LE540 alone (lane 5, 109±14% of lane 1). These data suggest that RAR, rather than RXR, is involved in the NO increase.

Effects of PI3K Inhibitor on NO Increase
Because several reports demonstrated involvement of PI3K in NO increase after various stimuli, including vascular endo-

Figure 1. Effects of ATRA on NO production in ECs. A, Basal NO production visualized with DAF-2 DA. HMVECs, HUVECs, or TRLEC-03 was incubated with [ATRA(±)] or without [ATRA(±)] ATRA (1 μmol/L) for 48 hours, and NO production was determined by DAF-2T fluorescence (B). C, Effects of Am580 on NO production in ECs. HMVECs or HUVECs were incubated with [Am580(±)] or without [Am580(±)] Am580 (1 μmol/L) for 48 hours, and NO production was determined by DAF-2T fluorescence. D, ACh-stimulated NO production visualized with DAF-2 DA. HUVECs or TRLEC-03 was incubated with [ATRA(±)] or without [ATRA(±)] ATRA (1 μmol/L) for 48 hours, and cells were thereafter stimulated with ACh (10 μmol/L). ACh responsiveness was determined by increment of DAF-2T fluorescence per minute after 10-minute ACh stimulation (dFl/dt) (E). F, Expression of M3 muscarinic ACh receptor in HUVECs and TRLEC-03. Lanes 1 and 6, 100-bp DNA ladder; lane 2, human M3 receptor; lane 3, human GAPDH; lane 4, human M3 receptor without RT; lane 5, human GAPDH without RT; lane 7, rat M3 receptor; lane 8, rat GAPDH; lane 9, rat M3 receptor without RT; lane 10, rat GAPDH without RT. Results in B, C, and E represent mean±SEM of 3 independent experiments (n=18). In B, C, and E, *P<0.01 vs ATRA(−) or Am580(−).
thelial growth factor\textsuperscript{29} and estradiol,\textsuperscript{30} we next examined the effects of the PI3K inhibitor wortmannin and LY294002 (Figure 4D). ATRA-induced NO increase (lane 2, 159±6.9% of lane 1) was completely abrogated by treatment with wortmannin (30 nmol/L) (lane 4, 75±4.8% of lane 1) or LY294002 (lane 5, 99±16% of lane 1), suggesting involvement of PI3K in the NO increase.

Effects of ATRA on eNOS Phosphorylation at Ser-1177

To examine involvement of eNOS in the NO increase, Western blotting with the use of antibody against phosphorylated eNOS (Ser-1177) or eNOS was performed. ATRA significantly increased eNOS phosphorylation at Ser-1177 in HMVECs (Figure 5A, lane 2, 272±16% of lane 1) and TRLEC-03 (Figure 5B, lane 2, 300±39% of lane 1). In contrast, ATRA did not affect eNOS protein expression (Figure 5A and 5B, lower blots) as well as eNOS mRNA expression determined by RT-PCR (data not shown). RAR agonist Am580 also increased eNOS phosphorylation at Ser-1177 in HMVECs without changing its protein expression (Figure 5A and 5B, lower blots) as well as eNOS mRNA expression determined by RT-PCR (data not shown). RAR antagonist LE540 (Figure 5A, lane 3, 151±5.2% of lane 1) and wortmannin (100 nmol/L) (Figure 5A, lane 4, 98±7.0% of lane 1). Time course analyses (Figure 5C) revealed that ATRA increased eNOS phosphorylation after 48 hours (lane 3). ATRA did not increase iNOS protein expression (data not shown). These data suggest that ATRA enhances eNOS phosphorylation at Ser-1177, mediated through RAR and PI3K, later than 48 hours.

Effects of ATRA on Akt Phosphorylation

Akt, a serine/threonine protein kinase that lies downstream of PI3K and is a major target of PI3K, is known to directly phosphorylate eNOS at Ser-1177, which results in enhancement of eNOS activity and increase of NO production from vascular ECs.\textsuperscript{31,32} We therefore examined involvement of Akt in the ATRA-induced eNOS phosphorylation (Figure 5D). When TRLEC-03 was transfected with wild-type Akt, ATRA enhanced eNOS phosphorylation (lane 2, 398±47% of lane 1). However, when cells were transfected with dominant-negative Akt, ATRA could not enhance eNOS phosphorylation (lane 3, 175±39% of lane 1), indicating involvement of Akt in the ATRA-induced eNOS phosphorylation. Effects of ATRA on Akt phosphorylation were next examined. Western blotting with the use of antibody against phosphorylated Akt (Ser-473) or Akt revealed that ATRA significantly increased Akt phosphorylation at Ser-473 in HMVECs (Figure 6A, lane 2, 308±48% of lane 1) and TRLEC-03 (Figure 6A, lane 8, 313±43% of lane 7) without changing its protein expression (Figure 6A, lower blots). ATRA-induced Akt phosphorylation in both cells was diminished...
Figure 4. Involvement of RAR and PI3K in ATRA-induced NO increase in TRLEC-03. Expression of retinoid receptors in TRLEC-03. Ten micrograms of TRLEC-03 nuclear extracts was electrophoresed, and Western blotting with the use of antibody against RARα, β, γ (A) or RXRα, β, γ (B) was performed. C, Effects of RAR/RXR antagonists on NO increase in TRLEC-03. Cells were incubated with [ATRA(−)] or without [ATRA(−)] ATRA (1 μmol/L) for 48 hours. Cells were incubated with [ATRA(+) or without [ATRA(−)] ATRA (1 μmol/L) for 48 hours. Dimethyl sulfoxide (vehicle), wortmannin (30 nmol/L), or LY294002 (20 μmol/L) was added 30 minutes before DAF-2 DA loading. Results represent mean ± SEM of DAF-2T fluorescence of 3 independent experiments (n=18). *P<0.01 vs lane 1; †P<0.01 vs lane 2; ‡P<0.01 vs lane 2. D, Effects of PI3K inhibitor wortmannin or LY294002 on NO increase in TRLEC-03. Cells were incubated with ATRA (1 μmol/L) for indicated times. E, Effects of Akt on ATRA-induced eNOS phosphorylation. TRLEC-03 transfected either with wild-type eNOS and wild-type Akt (WT-Akt) (lanes 1 and 2) or wild-type eNOS and dominant-negative Akt (DN-Akt) (lane 3) was incubated with (lanes 2 and 3) or without (lane 1) ATRA (1 μmol/L) for 48 hours. *P<0.01 vs lane 1; †P<0.01 vs lane 2. In A to D, Western blotting was performed with the use of antibody against phospho-eNOS (Ser-1177) (upper blots, p-eNOS) or eNOS (lower blots, eNOS). In A, B, and D, phosphorylation of eNOS at Ser-1177 was determined by densitometry of phospho-eNOS or of eNOS (mean ± SEM; n=6).

Effects of ATRA on PI3K Activity/Catalytic Subunit p110β Protein Expression

Effects of ATRA on PI3K activity in TRLEC-03 were next examined. PI3K activity assay revealed that ATRA markedly increased PI3K activity after 48 hours (Figure 7A, 460±28% of 0 hour) but not by 24 hours. Because ATRA is known to induce expression of PI3K catalytic subunit p110β protein and increase PI3K activity in a neuroblastoma cell line,33 we next examined effects of ATRA on p110β protein expression in TRLEC-03. ATRA significantly increased p110β protein expression.
Effects of ATRA on PI3K Catalytic Subunit p110β mRNA Expression

Effects of ATRA on PI3K catalytic subunit p110β mRNA expression in TRLEC-03 were next examined by real-time PCR with the use of SYBR Green I. As shown in standard curves of Figure 7E, the threshold cycle values for the mRNA of p110β and GAPDH showed good correlation with the amount of templates in the real-time PCR analyses, indicating the reliability of the obtained data. As shown in Figure 7F, ATRA treatment (1 μmol/L, 48 hours) significantly increased p110β mRNA expression (205 ± 21% of ATRA(-)). To examine ATRA effects on p110β mRNA stability, we next treated the cells with actinomycin D for an additional 3 or 6 hours. As shown in Figure 7G, p110β mRNA equally decayed either in the absence or presence of ATRA. These data indicate that ATRA induces increase of PI3K catalytic subunit p110β mRNA expression without affecting its stability.

Involvement of eNOS Ser-1177 in NO Increase

To examine direct involvement of eNOS Ser-1177 in the ATRA-induced NO increase, we next transfected either wild-type eNOS or mutated eNOS with RARα into COS-1 cells and incubated the cells with or without ATRA (1 μmol/L) for 48 hours. As shown in Figure 8, ATRA induced NO increase (lane 2, 205 ± 14% of lane 1) in the presence of wild-type eNOS. In contrast, ATRA could not induce NO production in the presence of mutated eNOS whose Ser-1177 was substituted to Ala (lanes 3 and 4). These data indicate direct involvement of eNOS Ser-1177, most likely by its phosphorylation, in the ATRA-induced NO increase.

Discussion

In the present study we demonstrated increase of NO production by ATRA mediated by eNOS phosphorylation through activation of the PI3K/Akt pathway in vascular ECs. We first demonstrated ATRA-induced increase of NO production. The fluorescent NO indicator DAF-2 DA was used to determine NO production, and its specificity was confirmed by inhibition of its fluorescence by treatment with either the NOS inhibitor L-NAME or the specific NO scavenger carboxy-PTIO. ATRA increased NO production in a concentration-dependent manner from 10⁻⁰ to 10⁻⁸ mol/L. The physiological concentration of basal plasma ATRA is ≈10⁻⁸ mol/L, and the pharmacological concentration of plasma ATRA after oral administration in patients with leukemia or solid tumor reaches <10⁻⁶ mol/L. Therefore, ATRA can induce NO increase not only at pharmacological concentrations but also at physiological concentrations. In addition to the increase of basal NO production, ATRA was also demonstrated to enhance ACh-induced NO release. Because expression of M3 muscarinic ACh receptor, which is known to mediate ACh action predominantly in the vasculature, has been demonstrated in both HUVECs and TRLEC-03, the observed ACh-induced NO release may be mediated through the M3 receptor. Our data are consistent with the previous report describing improvement of ACh-induced relaxation response of blood vessel by ATRA treatment. Because attenuation of ACh-induced relaxation response is usually observed under

expression (Figure 7B, lane 2, 206 ± 20% of lane 1), which was completely inhibited by cotreatment with the RAR antagonist LE540 (Figure 7B, lane 3, 57 ± 0.0% of lane 1). Time course analyses (Figure 7C) revealed that ATRA increased p110β protein expression after 48 hours. Moreover, ATRA increased p110β protein expression around 10⁻⁰ to 10⁻⁶ mol/L (Figure 7D), consistent with the ATRA-induced NO increase. These data suggest that ATRA increases PI3K catalytic subunit p110β protein expression and PI3K activity, mediated through RAR, later than 48 hours.
endothelial dysfunction, the ATRA effect may be beneficial for recovery of responsiveness to ACh in cases of endothelial damage.

We also demonstrated that the ATRA-induced NO increase was mediated by eNOS phosphorylation at Ser-1177 through the PI3K/Akt pathway, as previously observed in vascular endothelial growth factor29 and estradiol.30 Because a previous study has demonstrated NO increase by ATRA through alteration of the metabolism of the endogenous NOS inhibitor ADMA,15 ATRA may affect NO production through a dual mechanism. The PI3K/Akt pathway is reported to play important roles in vascular ECs, including cell survival35 and migration.36 ATRA may therefore be able to affect endothelial function other than eNOS activation through the PI3K/Akt pathway. Time course analyses demonstrated that NO increase as well as eNOS phosphorylation was observed later than 48 hours after ATRA treatment. Consistent with their time course, Akt phosphorylation, PI3K activity increase, and PI3K catalytic subunit p110β protein expression were also observed later than 48 hours after ATRA treatment.

Figure 7. Effects of ATRA on PI3K in TRLEC-03. A, PI3K activity in TRLEC-03. Cells were incubated with ATRA (1 μmol/L) for indicated times, and their PI3K activity was measured by ELISA. *P<0.01 vs 0 hour. B, Effects of ATRA on PI3K catalytic subunit p110β protein expression in TRLEC-03. Cells were incubated for 48 hours either with ATRA (1 μmol/L) (lane 2), without ATRA (lane 1), or with ATRA and LE540 (1 μmol/L) (lane 3). Results represent mean±SEM (n=6). *P<0.01 vs lane 1; †P<0.01 vs lane 2. C, Time course analyses of PI3K catalytic subunit p110β protein expression in TRLEC-03. Cells were incubated with ATRA (1 μmol/L) for indicated times. D, Dose-response analyses of PI3K catalytic subunit p110β protein expression in TRLEC-03. Cells were incubated with ATRA for 48 hours at indicated concentrations. In B to D, Western blotting with the use of antibody against PI3K catalytic subunit p110β protein (p110β) was performed. E, Standard curves for real-time PCR analyses of PI3K catalytic subunit p110β (PI3K p110β) and GAPDH mRNAs. Serial dilutions of templates (40 ng, 10 ng, 4 ng, 1 ng, 400 pg, and 100 pg) were used for real-time PCR, and obtained threshold cycle (Ct) values of each mRNA were plotted against amounts of templates. The equations of the lines for each mRNA were calculated by linear regression analyses. PI3K p110β: y = -1.2214x + 50.43; GAPDH: y = -0.9817x + 31.829. F, Effects of ATRA on PI3K catalytic subunit p110β mRNA expression in TRLEC-03 examined by real-time PCR. Cells were incubated for 48 hours either with ATRA (1 μmol/L) (lane 2), without ATRA (lane 1), or with ATRA and LE540 (1 μmol/L) (lane 3). Results represent mean±SEM (n=6) of p110β mRNA levels normalized by GAPDH mRNA levels. *P<0.05 vs ATRA(-). G, Effect of ATRA on PI3K catalytic subunit p110β mRNA stability examined by real-time PCR. TRLEC-03 incubated either with or without 1 μmol/L ATRA for 48 hours was further incubated with actinomycin D (5 μg/mL) for an additional 3 or 6 hours. Lines represent mean±SEM (n=4) of p110β mRNA levels normalized by GAPDH mRNA levels.
We showed the expression of both retinoid receptors, RAR and RXR, in vascular ECs. ATRA binds to and activates RAR. However, ATRA easily converts to 9-cis retinoic acid, which binds to and activates both RAR and RXR, by intracellular reciprocal isomerization. Therefore, administration of ATRA would activate both retinoid receptors. We thus used receptor-selective antagonists to define the retinoid receptors involved in the ATRA-induced NO increase. The RAR agonist LE540, but not the RXR agonist HX603, suppressed ATRA-induced NO increase. Because HX603 concentration (1 μmol/L) is enough for inhibiting RXR agonist-mediated RXR transactivation, we speculate that RXR may not be involved in the NO increase. Furthermore, ATRA-induced eNOS phosphorylation, Akt phosphorylation, and PI3K catalytic subunit p110β protein increase were all inhibited by LE540. Moreover, the RAR agonist Am580 was shown to induce NO increase, eNOS phosphorylation, and Akt phosphorylation. This therefore indicates that ATRA-activated RAR induces PI3K catalytic subunit p110β protein expression to increase PI3K activity, resulting in sequential phosphorylation of Akt and eNOS. Real-time PCR analyses demonstrated a significant increase of PI3K catalytic subunit p110β mRNA expression by ATRA, indicating that the ATRA-induced p110β protein increase was most likely due to its mRNA increase. The ATRA-induced increase of p110β mRNA expression may possibly be due to RAR-mediated increase of gene transcription, because p110β mRNA stability was not affected by ATRA. Further studies are needed to examine ATRA/RAR effects on PI3K catalytic subunit p110β gene transcription by analyzing its promoter activity.

Antiproliferative effects of retinoids, including ATRA, have recently been reported in VSMCs. ATRA inhibits VSMC proliferation by modulation of cell cycle regulators, downregulation of angiotensin II type 1 receptor expression, and induction of apoptosis. Moreover, ATRA suppresses neointima formation and restenosis after balloon injury in vivo. In contrast to VSMCs, effects of retinoids on vascular ECs have been less investigated. Our study has therefore provided a novel action of retinoids on vascular ECs in terms of improvement of endothelial function through NO production. Retinoids are thus beneficial for both VSMCs and vascular ECs against cardiovascular disorders. Recently, new synthetic retinoids with fewer side effects such as Am80 were shown to induce NO increase, eNOS phosphorylation, and Akt phosphorylation, and all-trans retinoic acid-mediated growth inhibition in vascular smooth muscle cells. Circulation. 1996;93:1886–1895.


Upregulation of Nitric Oxide Production in Vascular Endothelial Cells by All-trans Retinoic Acid Through the Phosphoinositide 3-Kinase/Akt Pathway
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