all-trans-Retinoic Acid Increases Nitric Oxide Synthesis by Endothelial Cells

A Role for the Induction of Dimethylarginine Dimethylaminohydrolase

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Abstract—all-trans-Retinoic acid (atRA) has important effects on the developing and mature cardiovascular system. Nitric oxide (NO) production has been associated with the atRA-induced differentiation of neuronal cells, and we hypothesized that NO may also mediate certain actions of atRA in the cardiovascular system. We studied the effects of atRA on NO production by endothelial cells and determined whether regulation of enzymes responsible for metabolism of asymmetric dimethylarginine (ADMA) contributed to the effects seen. Murine endothelioma (sEnd.1) cells were incubated with or without atRA. Nitrite production was determined using the Griess reaction. The expression of NO synthase (NOS) and dimethylarginine dimethylaminohydrolase (DDAH) genes was determined by Northern blotting. A reporter gene assay was also used to study the effect of atRA on the DDAH II promoter. atRA significantly increased nitrite production by sEnd.1 cells despite no increase in eNOS expression. atRA also increased DDAH II gene expression and promoter activity and reduced the ratio of ADMA to symmetric dimethylarginine (SDMA) in culture medium. The DDAH inhibitor 4124W significantly reduced the induction of NO synthesis by atRA. The present study demonstrates that atRA increases NO synthesis in endothelial cells without increasing eNOS expression. atRA also increases the expression of DDAH II, the predominant DDAH isoform in endothelial cells. Our data suggests that the induction of NO synthesis by atRA may be facilitated by DDAH II. This pathway may help to explain some of the effects of atRA on the cardiovascular system. (Circ Res. 2002;90:764-769.)

Key Words: asymmetric methylarginine ■ dimethylarginine dimethylaminohydrolase ■ endothelium ■ nitric oxide ■ retinoic acid

Vitamin A derivatives (retinoids), and in particular all-trans-retinoic acid (atRA), inhibit cellular proliferation and promote cellular differentiation. These actions have important effects on the developing as well as the mature cardiovascular system and have potential therapeutic value. atRA regulates angiogenesis,¹ may retard the development of atherosclerosis,² inhibits neointima formation, and alters remodeling after vascular injury or bypass grafting.³⁻⁷ The mechanisms involved are poorly understood and attention has previously focused on vascular smooth muscle cell (VSMC) effects.⁸ However, there are good reasons to suspect that atRA may also affect the endothelium. Endothelial cells are exposed to the highest concentration of circulating atRA, express retinoid receptors, and play a significant role in atRA metabolism compared with other cell types.⁹ atRA also modulates endothelial cell growth, differentiation, and morphology.¹⁰¹¹ Recent data suggests that the effects of retinol (vitamin A alcohol) on VSMC function may depend on an unknown endothelial factor.¹²

NO released by endothelial cells is an important regulator of vascular function. The cellular effects of NO bear some similarity to those of atRA, and NO has been implicated in the atRA-induced differentiation of neuronal cells.¹³ We therefore speculated that atRA might regulate NO production by endothelial cells. With respect to the possible mechanisms involved, we focused on the role of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NO synthase (NOS)¹⁴. ADMA has been described as an inhibitor of angiogenesis¹⁵ and a circulating marker of endothelial dysfunction¹⁶¹⁷ and is metabolized by the enzyme dimethylarginine dimethylaminohydrolase (DDAH). We and others have suggested that regulation of DDAH activity may be a mechanism to regulate NO production.¹⁸¹⁹

Materials and Methods

Cell Culture and Treatment

Five cell types were used. Murine endothelioma cells (sEnd.1)¹⁸ were cultured in Dulbecco’s modified Eagle’s medium (DMEM)
containing 10% fetal calf serum (FCS). sEnd.1 cells stably expressing a DDAH II promoter/reporter plasmid (sEnd.1D2A) were created by cotransfection of the reporter plasmid (pGL3sal) and a plasmid encoding a puromycin resistance gene (pBabe) at a ratio of 1:9 into sEnd.1 cells. sEnd.1D2A cells were cultured in DMEM containing 5% FCS. ECV304 cells (American Type Culture Collection, Manassas, Va) were cultured in M199 medium containing 10% FCS. Primary porcine aortic endothelial cells (PAECs; single donor; rabbit against a DDAH II open reading frame). Sequence was analyzed for the presence of known transcription factor binding sites.

**Cloning and Sequencing of DDAH II**

We identified a human genomic clone that contains the entire DDAH II gene. To study transcriptional regulation, we subcloned and sequenced 1.6 kb of the DNA immediately upstream of the DDAH II open reading frame. Sequence was analyzed for the presence of known transcription factor binding sites.

**Reporter Gene Assay**

A restriction fragment spanning nucleotides −1755 to −216 of the human DDAH II gene was isolated from a human DDAH II genomic clone and cloned into the multiple cloning site of the pGL3basic luciferase vector (Promega) to produce a DDAH II promoter/reporter gene construct (pGL3sal). Sequences containing the putative PPAR/RXR binding sites were desiged to amplify −927 to −216 downstream of PPAR/RXR consensus site. This PCR product was cloned into pGL3 basic to produce pGL3salPPAR and sequenced. Other putative transcription binding sites were sequenced using primer extension experiments. Other statistical analyses were performed using the Student’s t-tailed t test. Values of P<0.05 were regarded as being statistically significant.

**Data Analysis**

Data are expressed as mean±SEM values unless otherwise stated. A 2-way ANOVA test was used to analyze data from time-course experiments. Other statistical analyses were performed using the Student’s 2-tailed t test. Values of P<0.05 were regarded as being statistically significant.

**Results**

**Effect of atRA on NO Synthesis and NOS Expression**

Nitrite production by sEnd.1 cells increased from 9.9±0.6 to 13.7±0.8 mmol/L cells over 24 hours after stimulation by atRA (1 μmol/L) (n=9; P<0.005) (Figure 1A). No significant increase in nitrite generation was seen at 4, 8 (data not shown), or 12 hours. Nitrite was undetectable in media from stimulated and control cells treated with the NOS inhibitor ADMA (n=4), confirming that nitrite arose from NOS activity. The inactive enantiomer of ADMA, SDMA, did not affect nitrite accumulation (data not shown).

To determine whether the time-dependent changes in nitrite production might be associated with changes in NOS expression after atRA treatment, we studied NOS mRNA expression in sEnd.1 cells by Northern blotting. eNOS mRNA levels decreased by 5.8% and 20.3% at 12 and 24 hours, respectively (Figure 1B). Expression of nNOS or iNOS mRNA was not detectable at any time point (data not shown).

**Effect of RA on DDAH Expression**

Low levels of DDAH I mRNA were detectable in sEnd.1 cells and no significant changes in expression were seen between control and atRA-treated cells over the time course.
studied. In contrast, DDAH II mRNA levels in sEnd.1 cells increased in a time-dependent manner after atRA treatment (by 90% and 73% compared with controls at 12 and 24 hours, respectively) (Figure 1B). Western blotting confirmed that DDAH II protein expression was also increased by atRA treatment of sEnd.1 cells (Figure 1B). A similar effect was also seen in the human endothelial cell line SGHEC-7 (data not shown). To test whether atRA also induced DDAH II expression in primary arterial endothelial cells, we used PAECs. In primary PAECs, atRA increased DDAH II mRNA expression in primary arterial endothelial cells, we used the DDAH inhibitor 4124W, which we have previously characterized and shown to alter vascular reactivity in organ bath studies. In this experiment, 4124W reduced nitrite release from control cells by a small but statistically insignificant amount (n=4). In contrast, 4124W reduced the atRA-stimulated increase in nitrite production by 53.5±9.2% (n=4; P<0.02) (Figure 3).

Effect of DDAH Regulation on NO Synthesis

To study whether or not the induction of DDAH II might contribute to the increase in nitrite production after atRA treatment, we used the DDAH inhibitor 4124W, which we have previously characterized and shown to alter vascular reactivity in organ bath studies. In this experiment, 4124W reduced nitrite release from control cells by a small but statistically insignificant amount (n=4). In contrast, 4124W reduced the atRA-stimulated increase in nitrite production by 53.5±9.2% (n=4; P<0.02) (Figure 3).

Effect of atRA on DDAH II Promoter Activity

atRA treatment of ECV304 cells increased DDAH II mRNA expression by 45.1±11.9% after 24 hours (n=3; P<0.02). This effect of atRA was due to transcriptional regulation as it was blocked by actinomycin D (Figure 4), atRA treatment of ECV304 cells transiently transfected with a human DDAH II promoter/reporter construct increased promoter activity by 46.6±7.4% (n=4; P<0.05; Figure 5A) compared with controls. Analysis of the promoter region identified consensus binding sites for several transcription factors, including a PPAR/RXR site (Figure 5B). This site is the only candidate site for a direct effect of retinoids. The natural ligand for RXR is 9cisRA and in many cell types atRA can be converted to 9-cisRA. Deletion of the putative PPAR/RXR site had no effect on induction of DDAH II in response to either atRA or 9-cisRA (Figure 5C). Deletion of promoter sequences upstream to this site had no effect on induction of DDAH II by atRA. In contrast, the deletion of promoter sequences downstream to this site abolished basal transcription of DDAH II.

Figure 1. A, Nitrite production by sEnd.1 cells stimulated by atRA (1 μmol/L) for 12, 24, and 48 hours. Results are expressed as the percent change compared with control cells at the same time point and are presented as mean±SEM. "n=5 refers to 5 independent experiments. **P<0.01 by 2-way ANOVA. B, Top Left, Northern analysis of DDAH II mRNA expression by sEnd.1 cells after DMSO (DM) or RA (1 μmol/L) treatment for 24 hours. Top Right, Western analysis of DDAH II protein expression by sEnd.1 cells after stimulation by atRA (1 μmol/L) for 24 hours. Bottom, Expression of DDAH II (solid bars) and eNOS (open bars) mRNA by sEnd.1 cells after stimulation by atRA (1 μmol/L) for 12 and 24 hours. Results are expressed as the percent change in mRNA levels compared with control cells at the same time point and have been corrected for differences in β-actin expression. Values represent means of 2 separate Northern blots using samples from independent experiments.

Figure 2. ADMA/SDMA production by atRA-treated sEnd.1 cells. sEnd.1D2A cells were treated with 10 μmol/L atRA for 48 hours and methylarginine concentrations in the conditioned medium determined by HPLC. Data are presented as the ADMA/SDMA ratio in culture medium. **P<0.005; n=16 from 3 independent experiments.

Figure 3. Nitrite production by resting and atRA-stimulated sEnd.1 cells in the presence or absence of the DDAH inhibitor 4124W at 24 hours. Results are expressed as the percent change compared with control cells treated with neither atRA nor 4124W. **P<0.005 compared with control; ***P<0.02 compared with RA-treated group. Results are presented as mean±SEM (n=4).
making it impossible to exclude an atRA response element within this region. Inhibition of protein synthesis using cycloheximide abolished the atRA-induced increase in DDAH II expression (Figure 5D).

**Discussion**

Retinoic acid is important in the embryonic development of the cardiovascular system and can influence angiogenesis. It has recently been shown that atRA treatment may be beneficial in models of cardiovascular disease although the mechanisms involved are not understood. The present study demonstrates that atRA increases nitrite production by endothelial cells in a time-dependent manner and suggests that the upregulation of the enzyme DDAH II in endothelial cells contributes to this effect. These findings describe a novel vascular effect of atRA and clearly identify this retinoid as the first known transcriptional modulator of DDAH II.

A maximum increase in nitrite production by sEnd.1 cells (39.7 ± 2.2%) was seen 24 hours after stimulation with atRA (n = 9; P < 0.001). atRA produces many of its effects by regulation of transcription and the time course over which atRA increased nitrite production was consistent with a genomic effect. We therefore studied the effects of atRA on NOS transcription. Neither nNOS nor iNOS mRNA was detectable in sEnd.1 cells and eNOS mRNA expression did not increase after atRA treatment. Indeed, a decrease in NOS expression after atRA treatment has previously been described in other cell types.

An alternative mechanism for the action of atRA might be the induction of another enzyme that indirectly influences the activity of expressed NOS. Our studies focused on those enzymes that regulate ADMA metabolism, namely the DDAH enzymes. ADMA is an endogenous and competitive inhibitor of NOS that is derived from the degradation of intracellular proteins. We and others have suggested that the metabolism of ADMA to citrulline by DDAH may play an important role in the regulation of NO synthesis.

DDAH I was expressed at low levels in endothelial cells and was not altered by atRA. However, we have recently identified a second isoform of DDAH (DDAH II) that is highly expressed in cardiovascular tissues and has a tissue distribution with similarities to that of eNOS. DDAH II expression was induced by atRA with the maximum increase in nitrite production by sEnd.1 cells and have been corrected for differences in β-actin expression. *P < 0.02; n = 3.

![Figure 4](image)

**Figure 4.** Northern analysis of DDAH II mRNA expression by ECV304 after stimulation by atRA (1 μmol/L) for 24 hours in the presence or absence of actinomycin D. Results are expressed as the percent change in mRNA levels compared with control cells and have been corrected for differences in β-actin expression. *P < 0.02; n = 3.

![Figure 5](image)

**Figure 5.** A, DDAH II promoter activity in control and atRA-treated ECV304 cells. Cells transfected with a DDAH II promoter/reporter construct were either treated with atRA or mock treated for 24 hours before determination of luciferase activity. Results are expressed as luciferase light units corrected for β-galactosidase activity to control for transfection efficiency. *P < 0.05 compared with the control group; n = 4. B, Schematic representation of the DDAH II promoter region. The DDAH II translational start site (ATG) lies 1.6 kb downstream of the 3′ end of a nuclear chloride ion channel gene. This 1.6-kb sequence contains three DDAH II exons with the translational start site contained within exon 3. Database searches suggest that at least 3 transcriptional start sites exist within this sequence (filled horizontal arrows). Alternative splicing between exons 1 and 2 and exon 3 (indicated by diagonal lines) generated at least three DDAH II mRNA species that differ in their 5′ UTR sequences. Putative consensus binding sites for STAT (signal transducer and activator of transcription), NF-κB, PPAR/RXR, and IRF1 (interferon regulatory factor-1) are indicated. C, Increase in DDAH II promoter activity induced by atRA (solid bars) or 9-cis-RA (open bars). Cells transfected with a reporter construct containing either the complete DDAH II promoter region (pGL3sΔa) or a truncated promoter sequence lacking the PPAR/RXR consensus binding site (ΔPPAR) were treated with either atRA (10 μmol/L) or 9-cis-RA (10 μmol/L) for 24 hours prior to determination of luciferase activity. Data are expressed as percentage increase in luciferase activity of treated cells over untreated cells corrected for β-galactosidase activity control for transfection efficiency. *P < 0.05 compared with the control group; n = 4. D, Increase in DDAH II promoter activity induced by atRA (solid bars) or 9-cis-RA (open bars). Cells transfected with a reporter construct containing either the complete DDAH II promoter region (pGL3sΔa) or a truncated promoter sequence lacking the PPAR/RXR consensus binding site (ΔPPAR) were treated with either atRA (10 μmol/L) or 9-cis-RA (10 μmol/L) for 24 hours prior to determination of luciferase activity. Data are expressed as percentage increase in luciferase activity of treated cells over untreated cells corrected for β-galactosidase activity control for transfection efficiency. (n = 4). D, atRA induced DDAH II expression in control and cycloheximide-treated ECV304 cells. ECV304 cells were treated with atRA (10 μmol/L) (atRA), atRA and cycloheximide (atRA+CHX), or DMSO for 24 hours prior to Northern blotting. Data are expressed as the percent increase in DDAH II mRNA compared with control and have been corrected for differences in β-actin expression (n = 6 from 2 independent experiments).
seen 12 hours after atRA stimulation. A similar effect was seen in primary PAECs and the human cell lines SGHEC-7 and ECV304. Studies with actinomycin D confirmed that the effect was due to transcriptional regulation rather than the alteration of mRNA stability. This was consistent with the observation that in ECV304 cells transfected with a reporter construct driven by the human DDAH II promoter, atRA treatment increased promoter activity by 46.6±7.4%. Analysis of the promoter region of the DDAH II gene indicated the presence of a PPAR/RXR binding site, which might be a target for the action of atRA. Deletions of the PPAR/RXR site and sequences upstream to it had no effect on the induction of DDAH II by atRA. However, the deletion of sequences downstream to the PPAR/RXR site abolished basal levels of DDAH II transcription, making it impossible to exclude an atRA effect on this region. That atRA may be acting via an intermediate protein was suggested by the observation that cycloheximide treatment blocked the induction of DDAH II gene expression. These findings are consistent with the observation that a mouse cDNA sequence (7u), which we identified as DDAH II, is indirectly upregulated in growth-arrested melanoma cells treated with atRA.

Establishing the precise molecular mechanism of this indirect atRA effect would be of interest because manipulation of ADMA levels is seen as a potential therapeutic approach in certain disease states.

ADMA is a substrate for DDAH, whereas SDMA, also produced during protein turnover, is not. The ADMA/SDMA ratio therefore reflects DDAH activity and was decreased in cell culture medium after atRA treatment, consistent with an increase in DDAH activity. In order to establish whether the increased NO synthesis might be related to the induction of DDAH II and metabolism of ADMA, we used the DDAH II transfection and sequences upstream to it had no effect on the induction of DDAH II by atRA. However, the deletion of sequences downstream to the PPAR/RXR site abolished basal levels of DDAH II transcription, making it impossible to exclude an atRA effect on this region. That atRA may be acting via an intermediate protein was suggested by the observation that cycloheximide treatment blocked the induction of DDAH II gene expression. These findings are consistent with the observation that a mouse cDNA sequence (7u), which we identified as DDAH II, is indirectly upregulated in growth-arrested melanoma cells treated with atRA.

Establishing the precise molecular mechanism of this indirect atRA effect would be of interest because manipulation of ADMA levels is seen as a potential therapeutic approach in certain disease states.

The actions of atRA within the cell are regulated by cytoplasmic RA binding proteins and RA receptors, all of which are expressed and subject to transcriptional regulation in human endothelial cells. Wang et al have demonstrated that retinol can influence vascular smooth muscle function through an endothelium-dependent mechanism. The results of this study demonstrate that (1) atRA increases NO synthesis by endothelial cells despite no increase in eNOS mRNA expression, (2) atRA induces the expression of DDAH II by endothelial cells and reduces the ADMA/SDMA ratio, and (3) the inhibition of DDAH significantly reduces NO synthesis in atRA stimulated cells only. We infer from this that the regulation of DDAH II expression by atRA may in turn regulate NO synthesis by endothelial cells. Raised ADMA levels are associated with endothelial dysfunction and atherosclerosis, and intracellular concentrations of ADMA are increased within endothelial cells that repopulate denuded areas after experimental balloon injury. Induction of DDAH II may lower ADMA levels and restore NO production in these and other conditions.

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References


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