Retinoids Inhibit the Actions of Angiotensin II on Vascular Smooth Muscle Cells

Volker Haxsen, Sylvie Adam-Stitah, Eberhard Ritz, Jürgen Wagner

Abstract—Retinoids are derivatives of vitamin A and powerful inhibitors of cell proliferation and inflammation. Angiotensin II (Ang II) contributes to vascular lesions by promoting cell growth of vascular smooth muscle cells (VSMCs). Therefore, we examined whether retinoids interfere with the proliferative actions of Ang II in VSMCs via AT1 receptor–dependent or activator protein-1 (AP-1)–dependent mechanisms. VSMCs express retinoid receptor proteins, ie, retinoic acid receptor (RAR) α and retinoid X receptor (RXR) α. Long-term exposure to 1 μmol/L all-trans retinoic acid (RA) dose-dependently inhibited Ang II–induced cell proliferation (P < 0.005) as well as DNA and protein synthesis (P < 0.001). All-trans RA blocked Ang II stimulation of transforming growth factor-β1 mRNA (P < 0.005). All-trans RA inhibition of vascular VSMC growth was mediated both via RAR- and RXR-dependent pathways, as shown by receptor-specific synthetic retinoids. Transfection experiments revealed that inhibition of AP-1–dependent gene transcription is one mechanism by which all-trans RA inhibits Ang II action. RARα cotransfection enhanced the anti–AP-1 effects of all-trans RA dose-dependently. AP-1 activity was similarly inhibited by cotransfection with either RARα or RXRα. Ang II–induced gene expression of c-fos was abrogated by all-trans RA treatment (P < 0.005). In VSMCs, all-trans RA downregulated AT1 receptor mRNA (P < 0.01) and reduced Bmax (P < 0.001). All-trans RA repressed Ang II–stimulated AT1 receptor promoter activity. The all-trans RA inhibitory effect was abolished when the AP-1 consensus site on the AT1 receptor promoter was deleted. Our findings demonstrate that retinoids are potent inhibitors of the actions of Ang II on VSMCs. The findings support the notion that retinoids may interfere with proliferative vascular disease. (Circ Res. 2001;88:637-644.)

Key Words: retinoic acid ▪ angiotensin II ▪ vascular smooth muscle cells ▪ activator protein-1 ▪ proliferation

Retinoids, natural and synthetic derivatives of vitamin A, influence cellular differentiation in organ development.11,12 They potently inhibit proliferation of a variety of cell types.13–15 They act, not exclusively but in part, via inhibition of AP-1–induced activation.16 Inhibition of AP-1 activity by retinoids is mediated via different mechanisms, which are cell- and context-dependent. These include direct inhibition of c-jun, c-fos downregulation, interruption of c-fos/c-jun dimerization, or inhibition of binding of AP-1 to DNA.15,16

Retinoid signals are transduced by 2 families of receptors, ie, retinoid acid receptors (RARs) and retinoid X receptors (RXRs). RAR and RXR heterodimers bind to polymorphic cis-acting retinoid acid (RA)–responsive and retinoid X–responsive elements.17 RXRs also form heterodimers with other receptors of the steroid receptor family, such as the peroxisome proliferator–activated receptors and the vitamin D and thyroid hormone receptors, thus enabling crosstalk between different regulatory pathways.18 Although the vascular system is not considered a traditional target for the retinoids, VSMCs are sensitive to their action.11 Expression of RARs by...
VSMCs have been demonstrated on the RNA level. Functional studies were performed after RA transduction using reporter gene assays. The antiangiogenic properties of retinoids have long been known. Retinoids effectively block neointima proliferation induced by balloon arterioplasty injury in the rat. The consideration that Ang II has important nonvasoconstrictive effects on VSMCs and that VSMCs constitute a target for retinoids prompted us to examine whether retinoids interfere with the action of Ang II on VSMCs.

Materials and Methods

Culture of VSMCs

Primary rat aortic SMCs were prepared according to Owens et al and maintained as previously reported in our laboratory. Cells were used between passages 3 and 10 out of 3 different isolates. Cells were rendered quiescent after reaching the appropriate degree of confluence by placing them in DMEM/F12 containing 0.5% FCS for 24 hours unless otherwise stated.

Cell Counts

VSMCs (2×10⁶) were plated per well in 24-well dishes, stimulated every 48 hours, and counted in hemocytometers (Neubauer type; Brand).

Measurement of DNA and Protein Synthesis

Cells were plated in 96-well dishes (10⁴ per well) and rendered quiescent for 24 hours. The cells were treated with agonists for another 24 hours and labeled with tritiated thymidine or proline within the final 4 hours of the experiment, as described earlier.

Transient Transfection and Luciferase Assay

Confluent VSMCs in 6-well dishes were transfected with 1 µg/well of the reporter gene construct PatDetect AP-1 cis-reporting system (pAP-1-Luc; Stratagene) with FuGENE6 transfection reagent according to the manufacturer’s protocol (Roche Diagnostics). Transfections were normalized against the reporter gene construct PathDetect AP-1 (29 cycles), and c-fos (31 cycles). For quantification, we used deletion mutants of the respective genes sharing the same primer sequences. The results were expressed as ratio of the optical densities of wild-type versus mutant cDNA signal.

Immunoblotting and Immunoprecipitation

Whole-cell extracts of VSMCs were prepared from transfected cultures and immunoprecipitated with monoclonal anti-mouse RXRα [RXR3A2] or RXRβ [AbβB(F2)] antibodies (IgG1 subclass) covalently linked to protein A beads according to Gaub et al. Proteins with or without previous immunoprecipitation were fractionated by SDS-PAGE, electrotransferred onto nitrocellulose filters (Schleicher & Schuell), and immunoprobed with polyclonal antibodies raised against RARα [F(2)], RARβ [RPβ(F2)], or RXRα [RPXα(A)], as described, and detected via chemoluminescence. The specificity of the reactions was checked by depletion of the specific antibodies from the antisera. COS-1 cell extracts transfected with full-length RARα, RARβ, or RXRα expression vectors served as positive controls, as described.

Statistics

Results are presented as mean±SEM. Data were evaluated using the Mann-Whitney test. The zero hypothesis was rejected at P<0.05.

Results

VSMCs Express RAR and RXR Protein

To examine whether VSMCs contain RAR protein, we analyzed lysates of VSMCs by immunoblotting with polyclonal anti-RAR and anti-RXR antibodies. The presence of RARα protein is documented in Figure 1A. RARα protein was consistently demonstrated by immunoblotting in 10 and 20 µg of lysate. The 51-kDa band migrated at the same position as the RARα protein overexpressed in control COS cell lysates, which had been transiently transfected with a total RARα construct (pCMV-RARα).

RARβ could be demonstrated in VSMCs on the mRNA level only (data not shown). The polyclonal anti-RARβ antibody failed to detect RARβ protein in VSMC lysates. RARβ could also not be detected after immunoprecipitation with specific RARβ antibodies or after stimulation of VSMCs with 1 µmol/L all-trans RA (data not shown). In contrast, RARβ could be demonstrated in transiently transfected RARβ-COS control cell lysates.

RXRα protein concentration in VSMC lysates was below the detection limit of immunoblotting when a polyclonal anti-RXRα antibody was used. Immunoprecipitation yielded a clear signal for RXRα in lysates of VSMCs. Controls were lysates of transiently transfected RXRα COS cells (Figure 1B).

All-trans RA Inhibits Ang II–Induced Proliferation of VSMCs

Figure 2A shows the effect of 10 nmol/L or 1 µmol/L all-trans RA on VSMCs treated with 1 µmol/L Ang II in the presence of 0.5% FCS over 10 days. Dose-dependently lower rates of cell proliferation were observed in the cultures treated with all-trans RA plus Ang II compared with the cultures treated with Ang II alone.

Figure 2B indicates that all-trans RA completely abrogated the Ang II–induced DNA synthesis, as assessed by thymidine incorporation, but no effect of all-trans RA on basal DNA synthesis was noted. Similarly, protein synthesis in response to 1 µmol/L Ang II was abrogated by 1 µmol/L all-trans RA (Figure 2C), but basal protein synthesis was not affected. In an additional 48-hour experiment, the cumulative proline incorporation into the precipitable fraction was determined in the presence of 1 µmol/L all-trans RA, 1 µmol/L Ang II
alone, or both compounds. The results showed significantly less proline incorporation in the presence of all-trans RA (data not shown).

RAR- and RXR-Specific Agonists Block Ang II–Induced Proliferation of VSMCs

To test whether inhibition of cell proliferation by all-trans RA is mediated via RAR- or RXR-dependent pathways, cells were treated with the RAR-specific agonists AM580 and all-trans RA as well with the RXR-specific agonists BMS649 or 9-cis RA (all reagents 1 μmol/L), respectively. In a 7-day experiment (Figure 3A), 1 μmol/L Ang II stimulated VSMC proliferation 3-fold over controls. In incubations without Ang II, retinoids (all-trans RA, AM580, BMS649, and 9-cis RA) induced a small increase in cell number over control, which was not significant. However, Ang II–induced proliferation was significantly inhibited by all retinoids. There was no difference between RAR- and RXR-specific agonists. To exclude loss of receptor specificity at high concentrations (1

Figure 1. VSMCs express RAR and RXR protein. A, Immuno- blotting indicates expression of RARα protein in 10- and 20-μg cell lysates of VSMCs (lanes 3 and 4). Control cell lysates were of transiently transfected COS cells expressing pCMV-RARα (lane 2). In the presence of nonimmune serum, only background expression could be detected. RARβ: no RARβ protein could be detected by Western blot despite the presence of RARβ mRNA (lanes 3 and 4). RARβ (lanes 1 through 4) was detected in control cell lysates of transiently transfected COS cells expressing pCMV-RARβ only (lane 2) but not in VSMC extracts. B, Immuno-precipitation (IP) indicates a 54-kDa band for RXRα (lane 4, VSMC-IP) but not in VSMC protein extracts (lane 3, VSMC-extr; 20 μg) compared with transiently transfected COS cell lysates (pCMV-RXRα). Control studies in which nonimmune serum was substituted for the primary antibody demonstrated only diffuse and faint background staining (data not shown).

Figure 2. Antimitogenic effect of all-trans RA. A, Cells were seeded at a density of 2×10^4/well (24-well; n=6), stimulated every other day, and counted on days 4 and 10. All-trans RA (tRA) 1 μmol/L abrogated the Ang II–induced cell growth to control levels, whereas the effect of 10 nmol/L was intermediate. **P<0.005 for Ang II plus stimulated cultures vs controls; ##P<0.005 for Ang II plus all-trans RA vs Ang II treatment groups. VSMCs treated for 24 hours with Ang II, all-trans RA, or both (both 1 μmol/L) showed a marked decrease in the incorporation (final 4 hours) of tritiated thymidine (B) and proline (C) when all-trans RA (tRA) was added to Ang II. *P<0.05 for Ang II–stimulated group vs controls (n=12); ###P<0.005 for Ang II plus all-trans RA vs Ang II treatment groups.
we tested AM580 and BMS649 at 3 different concentrations (1 μmol/L, 100 nmol/L, and 1 nmol/L). The inhibitory efficiency was equivalent (Figure 3B).

**All-trans RA Inhibits Ang II–Dependent Activation of TGF-β1 Expression**

After 4 hours of exposure to 1 μmol/L Ang II, the steady-state expression of TGF-β1 mRNA was significantly increased compared with controls. All-trans RA alone (1 μmol/L) reduced basal TGF-β1 mRNA concentration and completely abrogated the Ang II–induced growth in VSMCs. **P<0.005 for Ang II–stimulated group vs controls; ***P<0.005 for Ang II vs retinoid treatment groups. B. Both the RARα-specific agonist AM580 and the RXR-specific agonist BMS649 dose-dependently inhibited Ang II–induced growth in VSMCs. **P<0.005 for Ang II–stimulated group vs controls; ***P<0.005 for Ang II vs retinoid treatment groups.

**Inhibition of Ang II by All-trans RA Is Mediated Via AP-1**

Ang II induced a 24-fold stimulation of luciferase activity of the AP-1 plasmid within 4 hours (Figure 5A). Pretreatment (1 hour) with 1 μmol/L all-trans RA reduced Ang II–dependent AP-1 induction by ~30%. Costimulation of Ang II–treated cells with 1 μmol/L Losartan abrogated luciferase activity to control levels (data not shown). Dose-dependent effects of all-trans RA on AP-1 induction could not be detected, presumably because the overall effect was small. The anti-AP-1 activity of all-trans RA in response to Ang II, however, was markedly increased after cotransfection of plasmids encoding CMV promoter–driven retinoid receptors RARα and RXRα with the AP-1 reporter gene plasmid (Figure 5B). Cotransfection of both retinoid receptors reduced AP-1 luciferase activity by ~50%. When different amounts of RARα were cotransfected, a clear dose-dependency could be demonstrated (Figure 5C).

**All-trans RA Abrogates the Induction of c-fos Expression by Ang II**

Treatment with 1 μmol/L Ang II for both 30 minutes and 1 hour stimulated c-fos RNA expression significantly. Pretreatment with 1 μmol/L all-trans RA completely abrogated the induction of c-fos mRNA by Ang II (data shown after 1-hour Ang II treatment only; Figure 6).

**All-trans RA Reduces the Basal Expression of the AT1 Receptor**

To examine whether all-trans RA changes AT1 receptor gene expression and thus alters Ang II sensitivity, we determined steady-state levels of AT1 receptor mRNA. Reduction in AT1 receptor gene expression reached statistical significance after 24 hours of incubation with 1 μmol/L all-trans RA (P<0.05, Figure 7A).

**All-trans RA Downregulates AT1 Receptor**

After 24 hours of treatment with 1 μmol/L all-trans RA (Figure 7B), B_{max} decreased by 45%, from 995±51 to 564±31 cpm. EC_{50} decreased from 0.31 to 1.1 nmol/L, indicating a negligible rise in affinity of AT1 receptors to DuP753 when treated with all-trans RA.
Inhibitory Action of All-trans RA on Ang II–Induced AT 1 Receptor Promoter Activity Is Dependent on AP-1

The effects of 1 μmol/L all-trans RA on the Ang II–induced AT 1 receptor promoter activity were assessed in transiently transfected VSMCs. All-trans RA had no significant effect on the low basal promoter activity of the wild-type AT 1 receptor promoter under quiescent conditions. VSMCs were transfected with an AT 1 receptor promoter luciferase reporter gene construct either with (AT 1 -luc) or without (AT 1 -luc ΔAP-1) a functionally active AP-1 consensus site. All-trans RA abrogated the Ang II–induced (1 μmol/L) increase of luciferase activity in the wild-type (AT 1 -luc) AT 1 promoter construct (Figure 7C). In transfected VSMCs carrying the construct without a functional AP-1 site (AT 1 -luc ΔAP-1), all-trans RA was no longer able to inhibit the Ang II–dependent stimulation of AT 1 promoter activity (Figure 7D).

Discussion

The data document that all-trans RA inhibits the effects of Ang II in VSMCs. This inhibition may involve AP-1 repression and downregulation of the AT 1 receptor. VSMCs express both RAR and RXR subtypes on the RNA level and, as shown here, on the protein level. These observations suggest that VSMCs may respond not only to RAR ligands, such as all-trans RA, but also to RXR ligands, such as 9-cis RA or BMS649. Expression of RXRs allows for crosstalk with other receptors of the steroid superfamily, such as thyroid hormone, vitamin D, and peroxisome proliferator activator receptors. These receptors also act on cardiovascular tissues and cell growth.

The induction of growth in VSMCs by Ang II has been studied intensively. Ang II may evoke hypertrophic or hyperplastic responses, depending on the balance of antiproliferative and proliferative autocrine factors, ie, TGF-β, PDGF, FGF, and others. Our data confirm that Ang II stimulates c-fos expression time-dependently and AP-1
activity by a factor of about 20 to 30 using a luciferase vector containing AP-1 consensus sites.\textsuperscript{8}

Repression of c-fos gene expression has been identified as a mechanism of retinoid inhibition of AP-1 activity.\textsuperscript{15,33} All-trans RA has been shown to inhibit the serum-induced stimulation of c-fos and c-jun by serum in mesangial cells\textsuperscript{15,33,34} and in our VSMCs.

The anti–AP-1 activity of retinoids has been intensively studied and may be cell-type specific.\textsuperscript{16,35,36} All-trans RA reduced Ang II–induced activation of AP-1 modestly but significantly in reporter gene assays. This suggested low natural abundance of the receptors. Cotransfection with an RAR\textsuperscript{a} expression vector dose-dependently inhibited Ang II–dependent AP-1 activation by all-trans RA.

All-trans RA dose-dependently blocked Ang II–stimulated cell proliferation, as indicated by proliferation markers, such as cell counts or thymidine and proline incorporation. All-trans RA alone generally had no effect on cell proliferation, although, depending on cell preparation and culture conditions, occasionally a minor increase was observed, which was also described by Chen and Gardner.\textsuperscript{37} In all doses of all-trans RA used in experiments, no sign of toxicity was present; signs of toxicity were only present at \(\geq 10\) \(\mu\text{mol/L}\).

To clarify whether RAR\textsuperscript{a} inhibits Ang II–dependent AP-1 activation alone or with RAR\textsuperscript{a} pathways, we assessed retinoid receptor–specific ligands and cotransfection of either RAR\textsuperscript{a} or RXR\textsuperscript{a} expression plasmids. Receptor-specific ligands, such as AM580 (which is specific for RAR) or BMS649 and 9-cis RA (which are specific for RXRs [Figures 3A and 3B]), showed that both receptor ligands were able to block Ang II–induced cell proliferation. RAR-dependent inhibition of VSMC proliferation is well known.\textsuperscript{13,14} Our data support these findings. In our cells, RXR agonists, 9-cis RA (Figure 3A), and BMS649 (Figures 3A and 3B) were as efficient at inhibiting Ang II–induced cell proliferation as RAR agonists.

We used a highly specific RXR agonist, BMS649, which cannot isomerize into other retinoids as 9-cis RA. Its \(K_d\) for RXR\textsuperscript{a} is 4.9 mmol/L, for RXR\textsuperscript{b} is 2.9 mmol/L, and for RXR\textsuperscript{g} is 98 mmol/L. In contrast, the \(K_d\) of BMS649 for RAR\textsuperscript{a} is 42 500 mmol/L, for RAR\textsuperscript{b} is 17 500 mmol/L, and for RXR\textsuperscript{g} is 14 000 mmol/L, which is about 10 000-fold higher than for RXRs. Furthermore, in COS-1 cell test systems comparable to Brand et al,\textsuperscript{38} transient transfections of

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\caption{All-trans RA alters AT\textsubscript{1} receptor. A, Treatment with 1 \(\mu\text{mol/L}\) all-trans RA (tRA) significantly lowered AT\textsubscript{1} receptor steady-state mRNA levels by \(\sim 50\%\) after 24 hours. AT\textsubscript{1} receptor mRNA was determined by quantitative RT-PCR using an AT\textsubscript{1} receptor deletion mutant with the same primer sequences as internal standard. Results are expressed as ratio of the optical densities (OD) of the wild-type and the mutant amplicon. \(\star\textsuperscript{P}<0.005\) for all-trans RA–stimulated group vs controls. B, Confluent VSMCs treated with 1 mmol/L all-trans RA (tRA) for 24 hours were incubated with 50 mmol/L tritiated DuP753 and various concentrations of unlabeled DuP753 to assess a competitive binding assay. \(B_{\text{max}}\) decreased by \(\sim 45\%\), corresponding to a marked decrease in AT\textsubscript{1} receptor numbers. C and D, VSMCs were transfected with AT\textsubscript{1} promoter luciferase reporter gene constructs with wild-type (AT\textsubscript{1}-luc) or point-mutated AP-1 (AT\textsubscript{1}-luc\textsubscript{DAP-1}) consensus sites. After 1 hour of pretreatment with 1 mmol/L all-trans RA, cells were stimulated with 1 mmol/L Ang II for another 24 hours. C, Ang II–induced wild-type AT\textsubscript{1}-luc luciferase activity is completely abrogated by all-trans RA. D, Ang II–induced AT\textsubscript{1}-luc DAP-1 activity was not inhibited by all-trans RA. C and D, Representative experiments from 5 experiments using 3 cell isolates. Values are the mean of triplicates and are from a representative experiment.}
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specific retinoid receptor subtype CAT constructs had shown no stimulation of CAT activity of RAR by BMS649 (C. Zusi, unpublished data, 1991). These data are different from those provided by Neuville et al., who found no RXR-dependent growth inhibition in SMCs. These differences may be explained by the use of 10% serum instead of Ang II and specific SMC populations derived from injured vasculature.

Besides retinoid receptor–specific ligands, cotransfection with either RARα or RXRα caused inhibition of AP-1 activation by Ang II. This is in good agreement with studies where both RAR and RXR expression vectors were able to confer anti–AP-1 activity. The data do not allow conclusions on differences in potency and efficacy between RAR- and RXR-specific compounds, because the effects varied slightly between different cell preparations, and differences were subtle at best.

Ang II induction of cell growth is modulated by autocrine factors, particularly TGF-β1, which significantly contributes to the hypertrophic response of VSMCs to Ang II. Our data of a modest but consistent stimulation of TGF-β1 gene expression by Ang II are in accordance with the literature, where an increase of 1.5- to 8-fold, depending on the cellular context, was reported. All-trans RA completely abrogated this induction and even reduced basal TGF-β1 mRNA levels in these cells. The effects of retinoids on TGF-β are cell-specific: all-trans RA stimulated TGF-β1 mRNA in chondrocytes, where basal TGF-β is low, and inhibited TGF-β in myocytes, where basal expression is high. The TGF-β promoter contains 3 AP-1 sites, which are able to confer AP-1–mediated stimulation of gene transcription. Salbert et al demonstrated that both RARs and RXRs inhibit the TGF-β gene promoter via inhibition of AP-1. Morishita et al demonstrated that Ang II–stimulated TGF-β induction in VSMCs is mediated via AP-1 using an anti–AP-1 oligodeoxynucleotide decoy approach. These findings strongly suggest, but do not provide final proof, that abrogation of Ang II–dependent TGF-β induction by retinoids may be mediated via AP-1.

The proliferative response to Ang II is mediated via the AT1 receptor involving protein kinase C and induction of proto-oncogenes. Our data of a 50% reduction of AT1 receptor binding confirm the findings of Takeda et al, who also found a reduction in Bmax but not Kd values. Holzmeister et al previously demonstrated that the AT1 receptor promoter contains a functional AP-1 binding site. It is thus conceivable that downregulation of this receptor by retinoids might be mediated via AP-1. To test this hypothesis, we point-mutated the AP-1 binding site on the human AT1 receptor promoter. All-trans RA had no significant effect on the low basal AT1 receptor promoter activity of the AT1 receptor under quiescent conditions. This is in contrast to findings of Takeda et al, where at 10% FCS, all-trans RA reduced AT1 receptor promoter activity, but inhibition was not AP-1 dependent. In our study, all-trans RA did not inhibit serum-induced AP-1 activity in the presence of 5% or 10% FCS (data not shown). Additionally, under serum conditions of >2%, no Ang II effects could be observed. This suggests that the AP-1 dependency of retinoid effects may be overrun under high-serum stimulation and prompted us to examine the effects of retinoids under low-serum conditions.

In contrast to the wild-type AT1 receptor construct, the inhibition of Ang II–dependent promoter activity by all-trans RA was now abolished. These findings support the notion that the AP-1 binding site of the AT1 receptor promoter mediates Ang II stimulation, as has previously been shown for tissue plasminogen activator stimulation by Holzmeister et al.

We have shown that retinoids inhibit the proproliferative action of Ang II on different levels, first by inhibiting AP-1–dependent gene transcription and second by reducing responsiveness to Ang II via reduced expression of the AT1 receptor. Although retinoids belong to the steroid superfamily, they clearly differ in this respect from glucocorticoids. The latter sensitize VSMCs to vasopressors and upregulate the AT1 receptor.

The effects of retinoids on Ang II are an additional example of their influence on the expression action and of vasoactive factors. Inhibition of endothelin-1, PDGF, inducible nitric oxide synthase, and others by retinoids has been reported in myocytes or mesangial cells. Retinoids also inhibit neointima formation in response to vascular balloon injury in the rat. Retinoids, therefore, are potentially interesting candidate drugs to modulate agonist-induced transcription in the cardiovascular system.

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