all-Trans-Retinoic Acid Reduces Neointimal Formation and Promotes Favorable Geometric Remodeling of the Rat Carotid Artery After Balloon Withdrawal Injury

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Background—The multifactorial and unpredictable nature of human restenosis will probably necessitate interventional strategies that target multiple processes involved in acute vascular narrowing. Retinoids (eg, all-trans-retinoic acid, atRA) represent a growing class of pleiotropic biological response modifiers with demonstrable efficacy in managing several pathological conditions. In this report, we have initiated studies to examine the hypothesis that atRA limits neointimal formation after experimental vascular injury.

Methods and Results—Rats were predosed with atRA (30 mg · kg⁻¹ · d⁻¹ PO) or corn oil 4 days before balloon withdrawal injury (BWI) of the left common carotid artery and continued on this drug regimen for an additional 14 days. High-performance liquid chromatographic analysis documented therapeutic levels of atRA in serum and vascular tissue. atRA depressed peak DNA synthesis in the tunica media of BWI vessels (P < 0.05). Histomorphometry revealed atRA-mediated reductions in neointimal area, neointimal cell number, and intimal/medial area ratio as well as significant increases in vessel wall perimeter (P < 0.05). Such changes in vascular architecture contributed to a 35% to 37% increase in the luminal area of BWI vessels exposed to atRA (P < 0.005 compared with controls).

Conclusions—atRA reduces neointimal mass and elicits favorable geometric remodeling of the injured rat carotid artery. (Circulation. 1998;98:1219-1227.)

Key Words: retinoids ■ actins ■ restenosis ■ muscle, smooth ■ cells ■ revascularization

Mechanical injury to the macrovascular wall evokes an ordered series of events, including striking changes in gene expression, that culminate in the formation of a neointima. Medial smooth muscle cell (SMC) dedifferentiation, growth, and migration are salient features of such intimal expansion. Similar activities are thought to underlie the pathogenesis of atherosclerosis and a subset of human restenotic lesions. Despite the intensive study of therapeutic agents aimed at arresting SMC growth and migration, no widely effective treatment exists for the prevention of human restenosis. A likely explanation for this fact is the unpredictable, multifactorial nature of human restenosis. Accordingly, new therapeutic approaches should be aimed at targeting not 1 but several processes implicated in the pathogenesis of acute vascular occlusive disease.

Retinoids are natural and synthetic derivatives of vitamin A that exert their pleiotropic biological effects through receptor-mediated changes in gene expression. Many effects elicited by retinoids are of relevance to the pathogenesis of human restenosis. For example, all-trans-retinoic acid (atRA) promotes differentiation and fibrinolysis and inhibits cell proliferation, migration, thrombosis, angiogenesis, platelet aggregation, and inflammation. Although their clinical efficacy has been documented for some proliferative disorders, virtually nothing is known with respect to retinoids and vascular occlusive disease.

Recently, we documented the presence of retinoid receptor transcripts in cultured rat aortic SMCs and aortic tissue. We also showed atRA-mediated suppression of SMC growth at concentrations that evoked retinoid receptor activation. These and other findings prompted us to begin assessing the effect of atRA on the response of the vessel wall to mechanical injury. Specifically, we hypothesized that atRA would inhibit the development of a neointima after balloon withdrawal injury (BWI) of the rat carotid artery. Here, we present evidence in support of this thesis and discuss potential mechanisms for the observed effects of atRA in the injured vessel wall.

Methods

Materials
atRA was generously supplied by Dr Louise Foley (Hoffmann-La Roche, Nutley, NJ) and was prepared under reduced lighting conditions.
3-amino-9-ethylcarbazole or 3,3'-IHC was carried out with immunoperoxidase staining using either tissue were extracted with chloroform/methanol (2:1), and the minor modifications. Briefly, serum and homogenized vascular tissue was handled in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication 8523, revised 1985) and the Laboratory Animals Program V1.27 (developed at the University of Texas Health Science Center at San Antonio and available from the Internet by anonymous FTP from maxrad6UTHSCSA.edu). Intimal and medial cells of the middle carotid artery segment were counted manually and are expressed as the number of cells per cross section. The perimeter of the endoluminal border, the internal elastic lamina (IEL), and the external elastic lamina (EEL) were carefully traced and the following areas computed: luminal area (area bound by endoluminal perimeter), intimal area (area bound by IEL perimeter – luminal area), medial area (area bound by EEL perimeter – area bound by IEL perimeter), and vessel wall area (area bound by EEL perimeter). An image of a 1-mm slide micrometer was used to calibrate all measurements. A minimum of three 100-μm spaced sections per segment per animal were analyzed for each of the above parameters. An average of the 3 measurements for each parameter was then calculated for each animal, and a final average was calculated based on day 7 was significantly lower than 2-hour measurement (*P < 0.0004). *Two-hour measurement on day 7 was statistically significantly different from 6-hour measurements as determined by HPLC. All three 2-hour measurements were statistically significantly different from each other (P = 0.0004). 

**Figure 2.** Serum concentration of atRA over a 14-day time course. Blood was withdrawn from animals (n = 4) either 2 or 6 hours after oral dose of atRA on indicated days after BWI as described in Methods. Molar concentration of atRA was then determined by HPLC. All three 2-hour measurements were statistically significantly different from 6-hour measurements as determined by 2-way ANOVA (P = 0.0004). *Two-hour measurement on day 7 was significantly lower than 2-hour measurement at 14 days after BWI (P < 0.05).
on either 12 (corn oil) or 13 (atRA) animals. A second independent observer (T.A.N.) randomly analyzed 20 vessel segments for each parameter. The interobserver variability was ~10%.

**Immunohistochemistry**

Studies were carried out to assess the effect of BWI on peak DNA synthesis in the media and perivascular compartment (defined as the region of the carotid artery extending from the EEL to the nearest neighboring nerve, muscle, or adipose tissue). Animals were injected with 30 mg/kg BrdU at 18, 12, and 6 hours before perfusion-fixation on the days indicated in Figure 1. The middle third of the injured and sham-operated contralateral carotid arteries was cut in half and embedded for IHC. Sections of intestinal crypt epithelium were included as a positive control for every animal. After dewaxing, cross sections of tissues were incubated with 3% H₂O₂ in methanol for 20 minutes at room temperature and rinsed in PBS 3 times. Antigen retrieval was achieved with a 30-minute incubation in trypsin (0.1 mg/mL). After rinsing with distilled water, sections were incubated in 2 mol/L HCl at 37°C for 20 minutes, rinsed 3 times with PBS, briefly dried, and then circumscribed with a PAP pen. Sections were then blocked for 5 minutes in 3% horse serum containing 0.05% Triton X-100. A 1:100 dilution of anti-BrdU antibody was then applied to the sections for 6 hours in a humidified chamber, and the immunoreactive product was visualized with the Vectorstain ABC System. Adjacent sections were stained with either hematoxylin and eosin or a monoclonal antibody to SM α-actin (1:1250).

The BrdU index was calculated in the medial (2 and 4 days), perivascular (2 days), and neointimal (14 days) compartments of the vessel wall by 2 of the authors (J.M. and L.K.). Total BrdU-positive cells were manually counted in the media and neointima and expressed as a percentage of the total number of nuclear profiles. An average perivascular (see definition above) BrdU index was determined by counting the percent total perivascular nuclear profiles staining positive for BrdU in 4 independent high-magnification video images from each of 2 vessel segments per animal. All photomicrographs were shot on Kodak Gold Max self-adjusting film with an Olympus IX-50 microscope equipped with an Olympus SC-35 camera.

**Statistical Analyses**

Data were analyzed with GraphPad Prism Software (version 2.01, GraphPad Software Inc). All data are presented as the mean±SEM. A Kolmogorov-Smirnov test for normality was performed for all data sets. A 2-factor ANOVA (using Duncan’s multiple range post hoc test for differences between means) was performed for the pharmacokinetic study (Figure 2). All other comparisons were made with an unpaired, 1-tailed or 2-tailed t test. The difference between means was considered statistically significant if P<0.05.

**Results**

**atRA Accumulates Rapidly in Serum and Vascular Tissue**

The results depicted in Figure 2 show micromolar increases in serum atRA 2 hours after dosing on the day of (day 0) and 7 and 14 days after BWI. The rapid rise in serum atRA was transient, because nearly undetectable levels were noted 6

![Figure 3.](http://circ.ahajournals.org/)

**Figure 3.** atRA-mediated changes in vascular caliber after BWI. Shown are low-power photomicrographs of injured common carotid arteries (middle segment) from corn oil–treated (top) and atRA-treated (bottom) animals. Each pair of vessels from independent study. Note pronounced vasoconstriction in A. Magnification ×40.
hours after dosing ($P=0.0004$). Samples of serum obtained from rats administered corn oil contained essentially no measurable atRA. Samples of vascular tissue from corn oil–treated rats contained only low levels of atRA on day 0 (0.007 nmol/g) and day 14 (0.01 nmol/g). In contrast, injured vascular tissue from atRA-treated rats exhibited much higher levels of atRA on the day of (0.508 nmol/g) and 14 days after (0.370 nmol/g) BWI. Interestingly, elevated atRA levels were also observed in both the uninjured contralateral carotid artery (day 0, 0.697 nmol/g and day 14, 0.451 nmol/g) and the aorta (day 0, 0.606 nmol/g and day 14, 0.451 nmol/g). The majority of retinoic acid in serum and vascular tissue was in the all-trans configuration (>$80\%$), with low quantities of the 13-cis and 9-cis stereoisomers (data not shown).

Table 1 summarizes the results of several terminal blood chemistry measurements in animals receiving atRA or corn oil. atRA treatment evoked significant elevations in serum triglycerides and alkaline phosphatase activity. All other measurements were statistically equivalent between groups. Mild cheilitis was noted in many of the atRA-treated rats, although other dermatological signs of retinoid toxicity (eg, alopecia) were rarely observed. No significant change in body weight gain was noted between treatment groups (20.0±11.5 g for atRA rats versus 29.3±6.9 g for corn oil rats, $P=0.501$). Moreover, atRA had no effect on systemic blood pressure or heart rate, and histological studies of the kidney and liver revealed no pathological changes in tissue architecture (data not shown).

**atRA Effects Favorable Changes in Vascular Histomorphometry After BWI**

Figure 3 reveals several qualitative changes in injured vessels exposed to atRA, including reduced neointimal mass, greater luminal area, and a higher-caliber vessel compared with control vessels. Contralateral vessels exposed to atRA exhibited no such alterations in vessel geometry (data not shown). A high-power micrograph shows a decrease in neointimal cellularity and extracellular matrix with atRA treatment (Figure 4). Histomorphometry of vessels from 3 independent studies is presented in Table 2. Medial area and cell number were essentially identical between treatment groups. The intimal area and intimal/medial area ratio of the middle
TABLE 2. Histomorphometry of Injured Carotid Artery Segments at 14 Days

<table>
<thead>
<tr>
<th>Segments</th>
<th>Corn Oil (n=12)</th>
<th>atRA (n=13)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medial area, mm²</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prox</td>
<td>0.1680 ± 0.0051</td>
<td>0.1689 ± 0.0124</td>
<td>0.978</td>
</tr>
<tr>
<td>Mid</td>
<td>0.1462 ± 0.0049</td>
<td>0.1557 ± 0.0057</td>
<td>0.223</td>
</tr>
<tr>
<td>Distal</td>
<td>0.1515 ± 0.0053</td>
<td>0.1597 ± 0.0112</td>
<td>0.526</td>
</tr>
<tr>
<td>Intimal area, mm²</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prox</td>
<td>0.1562 ± 0.0194</td>
<td>0.1155 ± 0.0157</td>
<td>0.114</td>
</tr>
<tr>
<td>Mid</td>
<td>0.1909 ± 0.0153 †</td>
<td>0.1115 ± 0.0096</td>
<td>0.002</td>
</tr>
<tr>
<td>Distal</td>
<td>0.1505 ± 0.0155</td>
<td>0.1007 ± 0.0156</td>
<td>0.033</td>
</tr>
<tr>
<td>Intima/medial</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prox</td>
<td>0.9191 ± 0.097</td>
<td>0.693 ± 0.082</td>
<td>0.087</td>
</tr>
<tr>
<td>Mid</td>
<td>1.3121 ± 0.105†</td>
<td>0.722 ± 0.060</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Distal</td>
<td>1.0111 ± 0.112</td>
<td>0.636 ± 0.078</td>
<td>0.012</td>
</tr>
<tr>
<td>Luminal area, mm²</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prox</td>
<td>0.3392 ± 0.0226</td>
<td>0.4643 ± 0.0323</td>
<td>0.0047</td>
</tr>
<tr>
<td>Mid</td>
<td>0.2847 ± 0.0241</td>
<td>0.3919 ± 0.0176</td>
<td>0.0014</td>
</tr>
<tr>
<td>Distal</td>
<td>0.3760 ± 0.0245</td>
<td>0.5062 ± 0.0311</td>
<td>0.0035</td>
</tr>
<tr>
<td>EEL perimeter, mm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prox</td>
<td>3.015 ± 0.061</td>
<td>3.347 ± 0.120</td>
<td>0.024</td>
</tr>
<tr>
<td>Mid</td>
<td>3.029 ± 0.065</td>
<td>3.217 ± 0.060</td>
<td>0.046</td>
</tr>
<tr>
<td>Distal</td>
<td>3.052 ± 0.100</td>
<td>3.517 ± 0.081</td>
<td>0.0014</td>
</tr>
<tr>
<td>Total area, mm²</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prox</td>
<td>0.5973 ± 0.0316</td>
<td>0.7031 ± 0.0456</td>
<td>0.073</td>
</tr>
<tr>
<td>Med</td>
<td>0.6219 ± 0.0223</td>
<td>0.6673 ± 0.0215</td>
<td>0.157</td>
</tr>
<tr>
<td>Distal</td>
<td>0.6202 ± 0.0480</td>
<td>0.7966 ± 0.0362</td>
<td>0.007</td>
</tr>
<tr>
<td>Medial cell no.</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Mid</td>
<td>441 ± 107</td>
<td>425 ± 99</td>
<td>0.460</td>
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<tr>
<td>Intimal cell no.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mid</td>
<td>961 ± 236</td>
<td>520 ± 75</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*The indicated P values were obtained from an unpaired 2-tailed test. A 2-factor ANOVA showed treatment differences that were consistent with those reported in the table.
†These parameters were significantly higher than the corresponding proximal and distal segments as assessed by 2-factor ANOVA and post hoc testing.

Vascular DNA Synthesis and SM α-Actin Immunoreactivity

Systemic delivery of BrdU was confirmed in all animals by positive BrdU immunostaining of intestinal crypt epithelium (data not shown). The majority of carotid artery cells staining positive for BrdU resided in the perivascular compartment of the vessel wall 2 days after BWI (Figure 5A). Some of these cells (outer perivascular compartment) stained positive for SM α-actin (Figure 5B). Virtually no SM α-actin immunoreactivity was noted in the inner perivascular compartment (closely juxtaposed to the EEL, Figure 5B and data not shown). The composition of cells in the perivascular compartment at 2 days included various inflammatory and fibroblastic cells, many of which incorporated BrdU (data not shown). The number of BrdU-positive cells in the perivascular compartment was greatly diminished 14 days after BWI, and only scattered neointimal cells stained positive for BrdU at this time (Figure 5C). We noted a dramatic remodeling of the inner perivascular compartment 14 days after BWI, as evidenced by an increase in the number of SM α-actin-positive cells (below arrow in Figure 5D). IHC and Western blotting failed to reveal any change in SM α-actin expression between the atRA and corn oil groups (data not shown).

Quantitative BrdU immunostaining of the media revealed a significant decrease in peak DNA synthesis with atRA treatment (Figure 6A). No such decrease was noted in the perivascular compartment (Figure 6B). The BrdU indices were essentially the same between treatment groups at 4 and 14 days after BWI (data not shown).

Discussion

The in vivo relationship between retinoids and vascular occlusive disease has been largely unexplored. One possible explanation for this may be the contradictory data that exist with respect to coronary heart disease and the intake of β-carotene, a parent molecule of natural retinoids. Early observational studies suggested that β-carotene offered some protection against coronary heart disease.33,34 Moreover, a recent preclinical study in rabbits failed to show an ameliorating effect of β-carotene supplementation on atherosclerosis.35 Subsequently, however, double-blind, placebo-controlled clinical trials showed either no effect or increased coronary heart disease mortality in patients taking supplemental β-carotene.36–39 Moreover, a recent preclinical study in rabbits failed to show an ameliorating effect of β-carotene supplementation on restenosis.40 Because retinoids were not measured in the above-described experimental and clinical studies, the question as to whether they exhibit any beneficial effect on the response of the vessel wall to injury remains open. In this report, we assessed the ability of a potent retinoid, atRA, to reduce neointimal formation after BWI of the rat carotid artery. The results reveal both a reduction in neointimal mass and an increase in vessel wall...
caliber with circulating levels of atRA that permeate the vessel wall with only mild, expected toxicity. These findings provide a platform for further scientific inquiry relating to retinoids and vascular occlusive disease.

**atRA Pharmacokinetics and Side Effects in the Rat BWI Model**

The pharmacokinetics of natural retinoids have been analyzed both experimentally and clinically. Our observation of a rapid and transient micromolar elevation in serum atRA is consistent with data in rats as well as humans undergoing atRA induction therapy for cancer. Interestingly, we did not observe “retinoid resistance” in the rat, which is a major obstacle for long-term atRA therapy in humans. Retinoid resistance is thought to be a consequence of the autoinductive catabolism of atRA via the cytochrome P450 isozyme family. One possible explanation for sustained elevations of atRA in both serum and the intravascular wall may be the high dose of atRA we used in this study (30 mg \( \cdot \) kg\(^{-1} \cdot \) d\(^{-1} \)). The micromolar serum concentration of atRA observed here is within the range we and others have previously used to document atRA-mediated SMC growth inhibition in vitro. Moreover, our dose of atRA evoked few signs of retinoid toxicity (mild cheilitis, hypertriglyceridemia, and elevated alkaline phosphatase activity). These side effects have been observed in humans undergoing atRA therapy for cancer. We conclude, therefore, that transient elevations of atRA are sustained over a 2-week period, with only mild and entirely expected side effects.

**atRA and Neointimal Formation**

On the basis of our previous study, we hypothesized that atRA would curtail neointimal formation after BWI of the rat carotid artery. Our results support this thesis and suggest that attenuated peak medial SMC DNA synthesis may, in part, account for the observed decrease in neointimal mass. We emphasize, however, that atRA probably suppresses additional pathways contributing to neointimal formation. For example, a wealth of data supports an important role for SMC migration in the evolution of a neointima, and atRA has been shown to suppress SMC migration in vitro, apparently through an inhibition of the AP-1–dependent proteases collagenase and stromelysin. Because there is mounting evidence supporting a critical role for retinoid receptors in modulating such gene expression, an examination of retinoid receptors within the normal and injured vessel wall should be a future goal.

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**Figure 5.** DNA synthesis and SM \( \alpha \)-actin immunoreactivity in injured carotid artery. Typical photomicrographs of neighboring carotid artery sections taken at 2 days (A and B) or 14 days (C and D) after BWI and stained with either anti-BrdU (A and C) or anti–SM \( \alpha \)-actin (B and D) antibodies. B and D were lightly counterstained with hematoxylin. Arrow in each panel denotes EEL or medial–inner perivascular boundary. Note absence of BrdU staining in perivascular coat at 14 days (C) and elevated SM \( \alpha \)-actin within its inner compartment (D). Inner perivascular compartment in D also appears to be more cellular than 2-day section (B). Magnification \( \times 80 \).
Another possible mechanism for atRA-mediated reduced intimal mass may relate to accelerated cell death. It is firmly established that injury to rat blood vessels evokes SMC apoptosis.\(^{39-55}\) Retinoids are potent inducers of apoptosis and appear to regulate several apoptotic genes, including bcl-2 and tissue transglutaminase.\(^{55}\) Thus, we must entertain the possibility that atRA-induced decreases in intimal cell number may be a consequence of accelerated apoptosis.

### atRA and Vascular Remodeling

Remodeling of the vessel wall appears to be a major determinant of vascular stenosis. For example, vasoconstriction is a dominant feature of early (14 days) luminal narrowing of the injured rat carotid artery\(^{33}\) (Figures 3A and 4A). Such functional remodeling subsequently wanes, and later luminal narrowing occurs through neointimal extracellular matrix accretion.\(^{33}\) We observed an increase in the EEL perimeter of injured carotid arteries exposed to atRA. The EEL perimeter of atRA-treated BWI vessels varied little from those measured in the contralateral carotid artery (data not shown). Thus, atRA may prevent early vasoconstrictive remodeling. Alternatively, atRA may restore vessel caliber toward preinjury dimensions only after prolonged exposure. Support for the latter hypothesis is offered by studies in which the duration of atRA administration was shortened from 14 to 7 days after BWI. Although this regimen significantly reduced neointimal formation, it did not elicit significant increases in vascular caliber (data not shown). These findings suggest that the mechanism of atRA-mediated vessel enlargement is not simply an early inhibition of injury-induced vasoconstriction.

The tunica adventitia has recently received attention as an important mediator of remodeling and neointimal formation.\(^{54}\) In the present report, we observed BrdU immunostaining in perivascular cells 48 hours after BWI despite the absence of medial tearing. This perivascular response included a number of inflammatory cells (primarily polymorphonuclear cells). Fourteen days after BWI, the perivascular inflammatory infiltrate and BrdU index were supplanted by inner perivascular remodeling. The latter observation was based on a distinct change in nuclear morphology and increased SM \(\alpha\)-actin immunoreactivity (see Figure 5D), both of which are suggestive of a myofibroblastic phenotype.\(^{54}\) The perivascular responses noted in this study, which are consistent with those reported by others in a pig model of restenosis,\(^{55,56}\) were qualitatively the same between treatment groups. Thus, it is difficult at this time to pinpoint the underlying mechanism(s) for the increase in vessel caliber after atRA treatment. It is noteworthy, however, that similar atRA-mediated changes in vessel caliber have been reported in a rabbit model of vasculostenosis.\(^{57}\)

### Significance and Limitations of the Study

The majority of pharmacological agents tested in animal models of intimal disease target a limited number of processes involved in luminal narrowing (eg, antiproliferatives). Given the multifactorial and unpredictable nature of human vasculostenoses, it is reasonable to surmise that successful therapeutic modalities will probably be those that interrupt numerous pathways implicated in vessel narrowing. Retinoids are attractive candidates for the potential treatment of human restenosis because they represent a large class of pan-acting biological response modifiers that target numerous processes long recognized to play critical roles in the development of vascular occlusive disease.\(^{16-23,26-29}\) The findings in this report permit us to add yet another important activity associated with atRA, namely, favorable geometric remodeling of the injured vessel wall. Collectively, these attributes of atRA beg for its further testing in more complex settings of vascular disease, particularly those associated with multiple, nonoverlapping pathways leading to vasculostenosis.

Enthusiasm for more advanced testing of retinoids, however, should be tempered with the following caveats. First, BWI of the rat carotid artery is not a model of restenosis, and any effects of atRA observed here may not predict outcome in more complex settings of vascular narrowing. Second, although atRA-treated animals exhibited only mild toxicity, the dose of atRA used in this study was higher than that administered to humans undergoing induction therapy for neoplastic disease. Additional studies may therefore be necessary to define a minimally effective dose of atRA that favorably remodels the arterial wall after mechanical injury. In addition, local delivery protocols should be contemplated, including those associated with coated stents. Finally, the results reported here offer little mechanistic insight into the mode of action of atRA. It is hoped that future studies will begin to unveil some of the mechanisms discussed above for the effects of atRA in the vessel wall as a prelude to more refined studies using synthetic retinoids that exhibit higher specificity and lower toxicity.

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