Relaxant effect of all-trans-retinoic acid via NO-sGC-cGMP pathway and calcium-activated potassium channels in rat mesenteric artery

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Wang Y, Han Y, Yang J, Wang Z, Liu L, Wang W, Zhou L, Wang D, Tan X, Fu C, Jose PA, Zeng C. Relaxant effect of all-trans-retinoic acid via NO-sGC-cGMP pathway and calcium-activated potassium channels in rat mesenteric artery. Am J Physiol Heart Circ Physiol 304: H51–H57, 2013. First published November 2, 2012; doi:10.1152/ajpheart.00240.2012.—Intrapertoneal injection of all-trans-retinoic acid (ATRA) results in a reduction of blood pressure in spontaneously hypertensive rats. However, the mechanisms involved in this effect are not clear. We hypothesized that ATRA may relax resistance arteries. In this study, we found that ATRA relaxed phenylephrine-preconstricted mesenteric arterial rings, which were abrogated by the removal of the endothelium. Pretreatment of endothelium-intact arterial rings with an inhibitor of endothelial nitric oxide synthase, N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME), or soluble guanylyl cyclase, 1H-[1,2,4]-oxadiazole-[4,3-c]-quinazoline-1-one, reduced the vasorelaxant effect of ATRA. Incubation of mesenteric arterial rings with ATRA increased the production of NO and cGMP, which were blocked by N\textsuperscript{G}-nitro-L-arginine methyl ester. The vasorelaxant effect of ATRA was markedly attenuated in the presence of an inhibitor of big conductance calcium-activated potassium channels (charybdotoxin), but not with an inhibitor of voltage-dependent potassium channel (4-aminopyridine) or ATP-sensitive potassium channel (glibenclamide). Activation of retinoic acid receptors (RARs) with CH55 or retinoic X receptors (RXRs) with LGD1069 induced the vasorelaxation of phenylephrine-preconstricted mesenteric arterial rings. The RAR (BMS493) and RXR (UVI3003) antagonists blocked the ATRA-induced vasorelaxation. The vasorelaxant effect ATRA is physiologically relevant because the intravenous infusion of ATRA lowered blood pressure in normotensive rats.

MATERIALS AND METHODS

Preparation of rat mesenteric arterial rings. Male Sprague-Dawley (SD) rats (250–350 g), purchased from Daping Hospital, were anesthetized with pentobarbital sodium (50 mg/kg) and tracheotomized, and blood pressure was determined from the femoral artery. The entire mesenteric bed was carefully removed and placed in ice-cold physiological salt solution (PSS) containing (in mM) 119 NaCl, 4.7 KCl, 2.5 CaCl\textsubscript{2}, 1.17 MgSO\textsubscript{4}, 1.2 H\textsubscript{2}O, 25 NaH\textsubscript{2}CO\textsubscript{3}, 1.18 KH\textsubscript{2}PO\textsubscript{4}, 0.027 EDTA, and 5.5 glucose, adjusted to pH 7.35–7.45. The mesenteric artery was carefully and quickly dissected from the surrounding fat and connective tissues. Third-order branches of the superior mesenteric artery (resting arterial diameter, 250 ± 20 μm) were cut into rings ~2 mm in length and mounted on 40-μm stainless-steel wires in an isometric Mulvany-Halpern small-vessel myograph (model M610, J. P. Trading, Science Park, Aarhus, Denmark) (19). One wire was attached to a force transducer and the other to a micrometer (19, 25). This arrangement enabled the wall tension to be measured at a predetermined internal circumference. The rings were maintained in PSS at 37°C and continuously bubbled with oxygen (95%) and carbon dioxide (5%) (carbogen). All dissecting procedures were done with extreme care to protect the endothelium from inadvertent damage. In some vessels, the endothelium was removed by pulling a hair along the vessel; successful denudation of the endothelium was confirmed by the absence of relaxation with the addition of acetylcholine (ACh, 10\textsuperscript{-6} M) (25). All experiments were approved by the Third Military Medical University Animal Use and Care Committee.

Measurement of isometric vascular tone. Following mounting, the arterial ring was equilibrated in PSS for 1 h at 37°C at a wall tension of 0.1 mN/mm. Based on preliminary data from >100 vessels, we confirmed that a normalized circumference (L\textsubscript{0}) = 0.9 L100 resulted in maximal active force development. The vessels were studied at L\textsubscript{0}, which may participate in the control of blood pressure.

A BIOLOGICALLY ACTIVE metabolite of vitamin A, all-trans-retinoic acid (ATRA) has anti-inflammatory, anticancer, and immunomodulatory actions (20, 24, 31). ATRA exerts its biological effects by modulating gene transcription through distinct intracellular proteins, including the retinoic acid receptor (RAR) and retinoic X receptor (RXR) (2, 18), and activating some key transcription factors, such as nuclear factor-κB (20).

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in all subsequent protocols. Relaxation induced by ACh (10^{-6} M) was used to indicate the presence of intact endothelium. After the response to ACh was determined, the vessels were rinsed three times with fresh PSS and allowed to recover to baseline for 15 min. In the first set of experiments, the rings were contracted with Phe (10^{-5} M) and high-potassium PSS (125 mM) to obtain maximal response. After the maximal response to Phe (10^{-5} M) plateaued, the response curves to ATRA were measured by a cumulative concentration-dependent protocol (10^{-8} to 3 \times 10^{-6} M). Response to every single concentration of ATRA was observed for 3 min. The effect of the vehicle for ATRA, dimethylsulfoxide (<1%), was also tested.

Measurement of cGMP levels in mesenteric artery. After equilibration of the mesenteric arterial rings for 30 min in PSS with carbenicillin, endothelium-intact and endothelium-denuded arterial rings were incubated with Phe (10^{-5} M) for 15 min before the addition of ATRA (10^{-6} M). Other groups were treated with N^6-nitro-L-arginine methyl ester (L-NAME; 10^{-4} M) for 30 min before the addition of ATRA (10^{-6} M). The reaction was stopped by freezing the tissues in liquid nitrogen. The tissues were weighed and then homogenized in 6% trichloroacetic acid. The homogenates were centrifuged at 15,000 g for 10 min, and the supernatant was extracted four times with water-saturated diethyl ether and then concentrated in a high-speed refrigerated centrifuge (Neofuge 18R, Harburg). The precipitates were resuspended with 20 mM Tris-HCl buffer (pH 7.4), and the protein concentration was then determined. cGMP content was measured using a cGMP kit (rat cGMP, cGMP ELISA KIT, HuFeng, China). Results were expressed as picomoles of cGMP generated per milligram of protein.

Measurement of NO production in mesenteric artery. NO production in the mesenteric artery was quantified with the use of 4,5-diaminofluorescein-2 (DAF-2) diacetate (DAF-2DA) as a fluorescent indicator for intracellular NO. (28) Third-order branches of the superior mesenteric artery were removed of connective tissue and fat, as described above, and then cut into rectangular pieces and incubated at 37°C with PSS. The pieces of vessels were loaded with 15 μM DAF-2DA for 30 min and washed with PSS three times for 15 min. DAF-2DA permeates the cell membrane and is converted to DAF-2, which reacts with NO and changes to a highly fluorescent triazole form (DAF-2T) that can be quantified (28). The tissue was incubated with ATRA (10^{-6} M) for 30 min before recording DAF-2T fluorescence intensity using a microscope (model ECLIPSE Ti-U, Nikon) and a high-speed video system (MHS-200). The fluorescence intensity was analyzed by a Macintosh computer and the National Institutes of Health Image program. Results were expressed as DAF-2T fluorescence.

Immunoblotting. Mesenteric arterial rings from SD rats were washed three times with cold PSS. Endothelium-intact arterial rings were incubated with ATRA (10^{-6} M) for 20 min in PSS; those treated with vehicle were considered as controls. The reaction was stopped by freezing the tissues in liquid nitrogen. The tissues were weighed and then homogenized in 6% trichloroacetic acid for 1 h. The homogenates were centrifuged at 15,000 g for 10 min. The supernatant was collected and protein concentration was measured using the bicinchoninic acid method (Pierce, Rockford, IL). The proteins in equal amounts of samples were resolved in 8.6% SDS-polyacrylamide gel and then transferred onto polyvinyllidene difluoride membranes. After blocking with 0.5% skim milk, the membranes were incubated with primary antibodies [endothelial NO synthase (eNOS), 1:800 dilution; phospho-eNOS (Ser1177) 1:500 dilution; Akt, 1:800 dilution; and phospho-Akt (Ser473), 1:500 dilution] (Cell Signaling, Beverly, MA) at 4°C overnight. The membrane-bound antibodies were visualized using horseradish peroxidase-conjugated secondary antibodies (1:15,000 dilution, 1 h) and the Odyssey Infrared Imaging System (Li-Cor Bioscience, Bad Homburg). The expression of phosphorylated eNOS and Akt were normalized with total eNOS and Akt, respectively (32).


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cGMP production also disappeared in the endothelium-denuded mesenteric arterial rings (Fig. 5).

To determine whether or not prostanoids were involved in the vasorelaxant effect of ATRA, we studied the vasorelaxant effect of ATRA in the presence of the cyclooxygenase inhibitor indomethacin (10⁻⁵ M). It resulted that the vasorelaxant effect of ATRA was not influenced by indomethacin (data not shown).

Role of potassium channels in the vasorelaxant effect of ATRA. Because the vasorelaxant effect of cGMP could be via potassium channels (27, 29), we studied the effect of different potassium channel blockers: glibenclamide (10⁻⁵ M) (14), an ATP-sensitive potassium channel blocker; 4-AP (10⁻⁴ M) (14), a voltage-dependent potassium channel blocker; and charybdotoxin (10⁻⁵ M), a calcium-activated potassium channel blocker. In the presence of charybdotoxin (10⁻⁵ M), the vasorelaxant effect of ATRA in arterial rings preconstricted with Phe was blocked (Fig. 6), indicating the importance of calcium-activated potassium channels in the vasorelaxant effect of ATRA. ATRA could not relax arterial rings preconstricted with high potassium chloride (125 mM) (data not shown), further indicating the importance of potassium channels in the vasorelaxant effect of ATRA. Moreover, neither glibenclamide nor 4-AP blocked the vasorelaxant effect of ATRA in Phe-preconstricted mesenteric arterial rings (data not shown), indicating that ATP-sensitive potassium channel and voltage-dependent potassium channel were not involved in the vasorelaxant effect of ATRA.

Involvement of ATRA receptor subtypes in ATRA-mediated vasorelaxation of mesenteric arterial rings preconstricted by Phe. Retinoid signals are transduced by the RAR and RXR. We found that CH55 (10⁻⁸ M–3 × 10⁻⁶ M), an RAR agonist,
relaxed mesenteric arterial rings preconstricted by Phe, but to a lesser extent than ATRA. Similar results were obtained with an RXR agonist LGD1069 (10^{-8} M – 3 \times 10^{-6} M). However, simultaneous stimulation of RXR and RAR with CH55 and LGD1069 produced a vasorelaxant effect similar to ATRA (Fig. 7, A and B), indicating that both RAR and RXR can contribute to the relaxation produced by ATRA. This interpretation was confirmed by our study in which the combination of RAR antagonist (BMS493) and RXR antagonist (UV13003) completely blocked, whereas either antagonist only partially blocked, the ATRA-induced vasorelaxation in mesenteric arterial rings preconstricted by Phe (Fig. 8). The role of NO on the RAR- and RXR-mediated vasodilation was studied in the presence of the eNOS inhibitor l-NAME (10^{-4} M), which blocked the vasodilatory effect mediated by LG1069 (10^{-8} – 3 \times 10^{-6} M) or CH55 (10^{-8} – 3 \times 10^{-6} M) (Fig. 9), indicating that p-eNOS and p-Akt were quantified by immunoblotting. The mean ± SE of the ratio of phosphorylated and total eNOS or Akt is shown. *P < 0.01 vs. control; n = 5.

Fig. 3. Effect of ATRA on NO production in mesenteric arterial tissue. A: effect of ATRA on NO production in mesenteric arterial tissue. Mesenteric arterial tissues were treated with ATRA (10^{-6} M, n = 3) at the indicated duration. NO production was quantified by measuring 4,5-diaminofluorescein-2-triazole (DAF-2T) high fluorescence. Each value represents the mean ± SE. *P < 0.01 vs. control (0 min) or vehicle. B and C: effect of ATRA on the phosphorylation of eNOS (p-eNOS; B) and Akt (p-Akt; C) in mesenteric arterial tissue. Mesenteric arterial tissues were treated with ATRA (10^{-6} M) for 20 min; total and p-eNOS and p-Akt were quantified by immunoblotting. The mean ± SE of the ratio of phosphorylated and total eNOS or Akt is shown. *P < 0.01 vs. control; n = 5.
plaque formation (30). A previous study (33) demonstrated that daily intraperitoneal injection of retinoic acid for 1 mo reduced the elevated blood pressure and attenuated the myocardial damage in the SHR. We hypothesized that the reduction of blood pressure in the SHR with retinoic acid may be mediated, in part, by a direct relaxant effect on systemic arterial resistance vessels. The results of this study suggest a series of signaling pathways from the ATRA receptor to Akt-eNOS, resulting in the generation of NO. NO, generated by the endothelial cells, diffuses into vascular smooth muscle cells and activates sGC, which increases intracellular cGMP concentrations (15, 17). In the presence of sGC inhibitors, NO-dependent processes may be blocked, and the ATRA-induced relaxation and ATRA-induced increase in cGMP levels would be abolished.

Fig. 7. Concentration-response curves of retinoic acid receptor (RAR) and retinoic X receptor (RXR) agonists on Phe-preconstricted endothelium-intact mesenteric arterial rings. Phe-preconstricted mesenteric arterial rings incubated with CH55 (10^{-8} M, n = 6), an RAR agonist, or LGD1069 (10^{-8} M, n = 7), an RXR agonist (n = 8 for ATRA alone) (A) or the combination of CH55 and LGD1069 (n = 4) (B) for 1 min. Each value represents the mean ± SE. *P < 0.05, DMSO (vehicle) vs. other group.
Fig. 8. Concentration-response curves of RAR and RXR antagonists on the ATRA-induced vasorelaxation in Phe-preconstricted mesenteric arterial rings. Phe-preconstricted mesenteric arterial rings were incubated with BMS493 (10⁻⁵ M), an RAR antagonist; UV13003 (10⁻⁵ M), an RXR receptor antagonist; or the combination of BMS493 and UV13003 for 30 min and then incubated with ATRA. Each value represents the mean ± SE. *P < 0.01 vs. other groups; n = 8.

cGMP production, indicating the importance of the NO-cGMP pathway in the vasorelaxation of the mesenteric artery caused by ATRA. In our study, the vasorelaxation induced by ATRA is abolished by high concentration of potassium chloride or charybdotoxin indicating that activation of potassium channels, specifically, big conductance calcium-activated potassium channels in vascular smooth cells, which are presumably downstream of NO-cGMP, is important in ATRA-mediated vasorelaxation. Due to the nonselective property of charybdotoxin on intermediate and big conductance calcium-activated potassium channels, whether or not the intermediate channel is involved in the signal pathway is not clear, which needs to be confirmed in the future.

Retinoid signals are transduced by the RAR and RXR. These receptors, belonging to the nuclear hormone receptor superfamily (3, 10, 22), may mediate the effects of retinoic acid mentioned above, i.e., cell growth, differentiation, and apoptosis. Haxsen et al. (13) reported that retinoic acid could inhibit the proliferative effect of angiotensin II on vascular smooth muscle cells via RAR- and RXR-dependent pathways. RAR and RXR are also involved in preventing angiotensin II- and stretch-induced reactive oxygen species generation and stretch-induced apoptosis in cardiomyocytes. However, in endothelial cells, the retinoic acid-induced prostaglandin 1 synthase is mediated by RAR and not RXR (4). In our study, we used retinoid receptor-selective agonists and antagonists to determine the retinoic receptor involved in the ATRA-mediated vascular relaxation. An RAR- or an RXR-specific agonist partially reproduces the concentration-dependent vasorelaxant effect of ATRA in rat mesenteric arterial rings preconstricted by Phe. The vasorelaxant effect of ATRA is only partially blocked by either the RAR or RXR antagonist. In contrast, the concurrent use of both RAR and RXR agonists fully reproduces the vasorelaxant effect of ATRA, whereas the concurrent use of both RAR and RXR antagonists completely blocks the vasorelaxant effect of ATRA. Thus both RXR and RAR are involved in the vasorelaxant effect of ATRA.

Table 1. Effect of ATRA on blood pressure and HR in SD rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Basal</th>
<th>10 min</th>
<th>20 min</th>
</tr>
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<tbody>
<tr>
<td>SBP, mmHg</td>
<td>124.3 ± 6.6</td>
<td>113.0 ± 11.2</td>
<td>104.3 ± 10.3*</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>97.3 ± 8.5</td>
<td>87.3 ± 9.6</td>
<td>81.3 ± 6.1*</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>108.8 ± 7.6</td>
<td>99.0 ± 9.3</td>
<td>90.3 ± 8.7*</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>362.3 ± 5.3</td>
<td>357.0 ± 3.7</td>
<td>346.0 ± 3.1*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4 Sprague-Dawley (SD) rats. ATRA, all-trans-retinoic acid; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial blood pressure; HR, heart rate. *P < 0.01 vs. basal.

In conclusion, ATRA relaxes resistance vessels through an endothelium-dependent NO-cGMP pathway that is dependent on both RAR and RXR. The vasorelaxant effect of ATRA is physiologically relevant because the intravenous infusion of ATRA could lower blood pressure.

GRANTS

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