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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1Department of Cardiology, The First Affiliated Hospital of Shantou University Medical College, Chongqing, China; 2Department of Cardiology, Daping Hospital, The Third Military Medical University, Chongqing, China; 3Chongqing Institute of Cardiology, Chongqing, China; 4Division of Nephrology and Hypertension, and Hypertension, Kidney and Vascular Health Center, Georgetown University, Washington, District of Columbia; and 5Division of Nephrology, Department of Medicine, University of Maryland School of Medicine, Baltimore, Maryland

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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.—Intraperitoneal 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 endo-

Recently, the role of ATRA in the development of cardiovascular dysfunctions has gained attention. It has also been shown that ATRA has potent antiproliferative and antioxidant actions (21, 23). A previous study showed that ATRA, given as daily intraperitoneal infusion, lowers blood pressure in spontaneously hypertensive rats (SHRs) (33). However, the mechanisms by which ATRA decreases blood pressure are not clear. To test the hypothesis that ATRA may relax resistance arteries, we studied the vasorelaxant effect of ATRA on phenylephrine (Phe)-preconstricted mesenteric arterial rings from rats. We found that ATRA, via RXR and RAR, induced vasorelaxation via calcium-activated potassium channels that are mediated by endothelium-dependent nitric oxide (NO)-cGMP pathways. The vasorelaxant effect of 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 CaCl2·H2O, 1.17 MgSO4·H2O, 25 NaH2CO3, 1.18 KH2PO4, 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, 10−6 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 circumfernece (L0) = 0.9 L100 resulted in maximal active force development. The vessels were studied at L0
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 1 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 carbenic, 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 1\text{N}o^\text{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% trichloracetic 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, Hurafor). The precipitates were resuspended with 20 mM Tris·HCl buffer (pH 7.4), and the protein concentration was then determined. The 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 \mu M 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, a fluorescent indicator for intracellular NO. (28) 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% trichloracetic 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 8.0% SDS-polyacrylamide gel and then transferred onto polyvinylidene 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).

Materials. ATRA, ACh chloride, glibenclamide, 4-aminopyridine (4-AP), charybdotoxin, Phe HCl, l-NAME, S-methylisothiourea sulfate (SMT), N^{6}-monomethyl-l-arginine, 1H-[1,2,4]-oxidazole-[4,3-α]-quinoxaline-1-one (ODQ), and DAF-2DA were obtained from Sigma-Aldrich (St. Louis, MO). LGD11069, CH55, UVI31003, and BMS493 were from Tocris Bioscience (Ellisville, MO). ATRA, ODQ, 4-AP, glibenclamide, LGD11069, CH55, UVI31003, and BMS493 were dissolved into dimethylsulfoxide.

Statistical analysis. Relaxation responses were expressed as a percent decline from the maximum contractile response to Phe (10^{-5} M). The results are shown as means ± SE. Comparison within groups was made by repeated-measures ANOVA (or paired t-test when only 2 groups were compared), and comparison among groups (or t-test when only 2 groups were compared) was made by one-way ANOVA with Duncan’s test. A value of P < 0.05 was considered significant.

RESULTS

Vasorelaxant effect of ATRA on mesenteric arterial rings preconstricted by Phe. ATRA (1 × 10^{-8}–3 × 10^{-6} M), by itself, had no vasoconstrictor effect but relaxed arterial rings preconstricted with Phe in a concentration-dependent manner (Fig. 1, A and B). The maximal relaxant effect of ATRA was 65.5 ± 10.9%. To determine the role of endothelium on the vasorelaxant effect of ATRA, the endothelium was denuded, which prevented the vasorelaxant effect of ATRA. The graph of the ATRA-induced vasodilation in endothelium-intact arteries is shown in subsequent graphs for comparison with other experiments. Representative tracings of the vasorelaxant effect of ATRA in endothelium-intact and endothelium-denuded arterial rings are shown in Fig. 1A, a and b, respectively.

Role of NO-cGMP on the vasorelaxant effect of ATRA on mesenteric arterial rings preconstricted by Phe. Because ATRA has been reported to increase NO production in endothelial cells (1, 28), we next studied the role of NO on the vasorelaxant effect of ATRA (10^{-8}–3 × 10^{-6} M). In the presence of a NO synthase (NOS) inhibitor N^{6}-monomethyl-l-arginine (10^{-5} M), the vasorelaxant effect of ATRA was significantly reduced (Fig. 2). To determine the specific NOS isoform involved, we used specific NOS isoenzyme inhibitors, i.e., l-NAME, an NOS inhibitor, and SMT, an inducible NOS (iNOS) inhibitor. We found that the vasorelaxant effect of ATRA was blocked by l-NAME (10^{-4} M) but not by SMT (10^{-5} M) (12), indicating that eNOS, but not iNOS, was involved in the ATRA-mediated vasorelaxation (Fig. 2). DAF-2T fluorescence studies showed that ATRA induced NO production in a time-dependent manner, which was maximal at 15 min and lasted for at least 1 h (Fig. 3A).

A previous study showed that ATRA increased NO production in vascular endothelial cells by phosphorylation of eNOS through the phosphatidylinositol 3-kinase/Akt pathway (28). In our current study, using mesenteric arterial rings, we also found that ATRA (10^{-6} M/20 min) increased the phosphorylation of eNOS and Akt, indicating that Akt-eNOS was involved in the ATRA-mediated increase in NO production (Fig. 3, B and C).

As a downstream signaling molecule of NO (7), we studied the role of cGMP on the vasorelaxant effect of ATRA. We found that ODQ (10^{-5} M), a selective inhibitor of soluble guanylyl cyclase (sGC), markedly decreased the vasorelaxant effect of ATRA (10^{-6} M) (Fig. 4). Incubation of mesenteric arterial rings with ATRA (10^{-6} M) increased cGMP production that was blocked by l-NAME (10^{-4} M). Moreover, the stimulatory effect of ATRA on
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,
Fig. 2. Role of nitric oxide (NO) synthase (NOS) in ATRA-induced vasorelaxation. Phe-preconstricted mesenteric arterial rings were treated with ATRA (10^{-8}–3 \times 10^{-6} M) in the presence of N\textsuperscript{\textgamma}-monomethyl-l-arginine (l-NMMA; 10^{-5} M, n = 4), a NOS inhibitor; N\textsuperscript{\textdelta}-nitro-l-arginine methyl ester (l-NAME; 10^{-4} M, n = 5), an endothelial NOS (eNOS) inhibitor; or S-methylisothiourea sulfate (SMT; 10^{-5} M, n = 5), an inducible NOS inhibitor. Each value represents the mean ± SE. *P < 0.01 vs. l-NMMA + ATRA or l-NAME + ATRA.

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}–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 LGI069 (10^{-8}–3 \times 10^{-6} M) or CH55 (10^{-8}–3 \times 10^{-6} M) (Fig. 9), indicating that the presence of eNOS (p-eNOS; Fig. 3) and Akt (p-Akt; Fig. 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) 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.

Fig. 4. Effect of 1H-[1,2,4]-oxadiazole-[4,3-\textalpha]-quinazoline-1-one (ODQ), an inhibitor of soluble guanylyl cyclase, on ATRA-induced relaxation of Phe-preconstricted mesenteric arterial rings. Phe-preconstricted mesenteric arterial rings were incubated with ODQ (10^{-7} M, n = 5) for 30 min and then treated with ATRA (1 \times 10^{-8}–3 \times 10^{-6} M) (n = 8 for the ATRA alone). Each value represents the mean ± SE. *P < 0.01 vs. ATRA.

NO was involved in both RAR- and RXR-mediated vasodilation.

Effect of ATRA on blood pressure in SD rats. To determine the physiological significance of our findings, we infused ATRA intravenously (5 µg·kg\(^{-1}\)·min\(^{-1}\)) in SD rats. We found that systolic, diastolic, and mean arterial blood pressures tended to decrease after 10 min of infusion of ATRA and significantly decreased after 20 min. The decrease in blood pressure was accompanied by a slight decrease in the heart rate (Table 1).

Discussion

Retinoids are a group of potent natural or synthetic molecules that exert important roles in angiogenesis and the embryonic development of the cardiovascular system (6, 16). Retinoids also inhibit cellular proliferation, increase elastin synthesis, and stimulate metalloproteinase inhibitor production by fibroblasts that may maintain the stability of atherosclerotic plaques. In the present study, we demonstrate that ATRA increases NO production and relaxes preconstricted arterial rings in vitro. To our knowledge, this is the first report of ATRA-mediated vasorelaxation in vivo. It has been shown that ATRA inhibits the proliferation and migration of vascular smooth muscle cells (VSCs) in culture (17), and this effect is associated with the inhibition of the extracellular signal-regulated kinase (ERK) pathway (18). In addition, ATRA inhibits the expression of matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP) in VSCs (19), which would decrease the activity of the metalloproteinase system and increase the stability of atherosclerotic plaques. It is possible that ATRA may act as a negative regulator of atherosclerotic plaque stability through NO-mediated vasorelaxation and inhibition of VSC proliferation and migration. Further studies are needed to determine the role of ATRA in atherosclerotic plaque stability in vivo.
Fig. 5. Effect of L-NAME on ATRA-induced increase in cGMP levels in mesenteric arterial rings with intact or denuded endothelium. Mesenteric arterial rings were incubated with ATRA (10^{-8} M) in the presence or absence of L-NAME (10^{-5} M), an NOS inhibitor. Each value represents the mean ± SE. *P < 0.01 vs. control (Endo−, ATRA−, and L-NAME−). **P < 0.01 vs. ATRA group (Endo+, ATRA+, and L-NAME+); n = 6.

Fig. 6. Effect of L-NAME and/or charybdotoxin (ChTX), a calcium-activated potassium channel blocker, on ATRA-induced relaxation in Phe-preconstricted mesenteric arterial rings. Phe-preconstricted mesenteric arterial rings were incubated with L-NAME (10^{-4} M) with or without ChTX (10^{-5} M) for 30 min and then treated with ATRA (1 × 10^{-8}–3 × 10^{-6} M). Each value represents the mean ± SE. *P < 0.01, ATRA vs. other groups; n = 5.

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 were incubated with CH55 (10^{-8}–3 × 10^{-6} M, n = 6), an RAR agonist, or LGD1069 (10^{-8}–3 × 10^{-6} 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.

plagues (30). A previous study (33) demonstrated that the 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, increases intracellular cGMP concentrations (15, 17). In our study, pretreatment with the sGC inhibitor ODQ reduces the ATRA-induced relaxation and ATRA-induced increase in cGMP (8–3 and then treated with ATRA (1 × 10^{-8}–3 × 10^{-6} M). Each value represents the mean ± SE. *P < 0.01 vs. ATRA group (Endo+, ATRA+, and L-NAME+); n = 6. of big conductance calcium-activated potassium channels, resulting in vasorelaxation.

Smooth muscle cells undergo vasorelaxation that is mediated by the endothelium through a variety of direct and indirect pathways, including hyperpolarization and opening of potassium-sensitive channels in smooth muscle cells or endothelial cells (8, 26). Our current study shows that removal of the endothelium abolishes the vasorelaxation caused by ATRA, indicating the necessity of an intact endothelium in this effect. The endothelium responds to various neurohumoral and physical stimuli by releasing endothelium-dependent vasconstrictor and vasorelaxant factors, such as endothelium-derived relaxing factor (11) and prostacyclin (9). To determine the mechanism by which the endothelium mediates the vasorelaxant effect of ATRA, we studied the effects of NOS inhibitors on the ATRA-induced vasorelaxation. Pretreatment of mesenteric arterial rings with the eNOS inhibitor L-NAME, but not the iNOS inhibitor SMT, abolishes the vascular relaxant effect. Previous studies have demonstrated that sGC, an NO-dependent and constitutively active enzyme, is also responsible for the conversion of GTP to the second messenger, cGMP (15, 17). In our study, pretreatment with the sGC inhibitor ODQ reduces the ATRA-induced relaxation and ATRA-induced increase in
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 charybdoxin 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 charybdoxin 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 apopto-

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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