Journal of Pharmacological Sciences xxx (2018) 1–7

Contents lists available at ScienceDirect

Journal of Pharmacological Sciences

journal homepage: www.elsevier.com/locate/jphs

Full paper

Mechanisms underlying the relaxation by A484954, a eukaryotic elongation factor 2 kinase inhibitor, in rat isolated mesenteric artery

Tomoko Kodama, Muneyoshi Okada, Hideyuki Yamawaki*

Laboratory of Veterinary Pharmacology, School of Veterinary Medicine, Kitasato University, Higashi 23 Bancho 35-1, Towada City, Aomori, 034-8628, Japan

ARTICLE INFO

Article history:
Received 27 March 2018
Received in revised form
22 April 2018
Accepted 24 April 2018
Available online xxx

Keywords:
Blood vessel
K^+ channel
Vascular smooth muscle
Vasorelaxation

ABSTRACT

Eukaryotic elongation factor 2 kinase (eEF2K) is a calmodulin-related protein kinase which regulates protein translation. A484954 is an inhibitor of eEF2K. In the present study, we investigated the acute effects of A484954 on contractility of isolated blood vessels. Isometric contraction of rat isolated aorta and main branch of superior mesenteric artery (MA) was measured. Expression of an inward rectifier K^+ (K_{ir}) channel subtype mRNA and protein was examined. A484954 caused relaxation in endothelium-intact [E (+)] and -denuded [E (−)] aorta or MA precontracted with noradrenaline (NA). The relaxation was higher in MA than aorta. The relaxation was partially inhibited by a nitric oxide (NO) synthase inhibitor, N^G-nitro-L-arginine methyl ester (300 μM) in E (+) MA. The relaxation was significantly smaller in MA precontracted with high K^+ than NA. The A484954-induced relaxation was significantly inhibited by a K_{ir} channel blocker, $BaCl_2$ (1 mM) compared with vehicle control in E (−) MA. Expression of $K_{ir}2.2$ mRNA and protein was significantly higher in MA than aorta. We for the first time revealed that A484954 induces relaxation through opening smooth muscle K_{ir} ($K_{ir}2.2$) channel and through endothelium-derived NO in MA.

1. Introduction

Calmodulin (CaM), a Ca^{2+} binding protein, affects various cell functions including muscular contraction, immune response, metabolism and nerve growth through the regulation of CaM-dependent proteins. Eukaryotic elongation factor 2 (eEF2) kinase (eEF2K), also known as CaM-dependent protein kinase III, phosphorylates a specific substrate eEF2. eEF2 mediates protein translation by promoting the movement of translation products from A to P site in the ribosome. Phosphorylation of eEF2 by the activated eEF2K inhibits protein translation by inactivating eEF2.[1–4]

It was previously reported that oxidized low density lipoprotein induced the activation of eEF2K in bone marrow-derived macrophage, leading to the inhibition of protein synthesis and apoptosis during growth factor deprivation.[5] In addition, it was reported that atherosclerotic plaque was decreased in the atherosclerosis model mouse, which was transplanted with bone marrow whose enzyme activity of eEF2K was deactivated, suggesting that eEF2K participated in the development of atherosclerosis.[6] We have also demonstrated that eEF2K mediates the development of hypertension in spontaneously hypertensive rats at least in part through promotion of tumor necrosis factor-α induced vascular inflammation via the activation of c-Jun N-terminal kinase and nuclear factor-κB through the increase of reactive oxygen species (ROS) production.[7] Furthermore, we have demonstrated that eEF2K mediates the platelet-derived growth factor (PDGF)-BB-induced vascular smooth muscle cell proliferation and migration via the activation of extracellular signal-regulated kinase (ERK), Akt, p38/heat shock protein (HSP) 27 signaling.[8] In addition, we have revealed that eEF2K mediates the development of pulmonary arterial hypertension at least in part through the vascular structural remodeling via NADPH oxidase 1/ROS/matrix metalloproteinase 2 pathway.[9] Taken together, these reports indicate that eEF2K might have certain roles in the pathogenesis of vascular diseases.

A selective eEF2K inhibitor, 7-amino-1-cyclopropyl-3-ethyl-2,4-dioxo-1,2,3,4-tetrahydropyrido[2,3-d] pyrimidine-6-carboxamide (A484954), is a novel small molecular weight compound identified from a chemical library which inhibits the activity of eEF2K by an ATP-competitive but CaM-independent manner.[10] We have previously demonstrated that A484954 inhibited PDGF-BB-induced vascular smooth muscle cell proliferation and migration.[8] Furthermore, we have also demonstrated that the long-term

* Corresponding author. Fax: +81 176 24 9456.
E-mail address: yamawaki@vmas.kitasato-u.ac.jp (H. Yamawaki).
Peer review under responsibility of Japanese Pharmacological Society.

https://doi.org/10.1016/j.jphs.2018.04.006

administration of A484954 inhibited the increase of pulmonary arterial (PA) pressure at least in part through the inhibition of PA hypertrophy and fibrosis in monocrotaline-induced PA hypertensive rats.[9] However, it remains to be clarified whether A484954 affects the reactivity of isolated blood vessels. In the present study, we investigated the acute effects of A484954 on contractility of isolated blood vessels and explored underlying mechanisms.

2. Materials and methods

2.1. Materials

Reagent sources were as follows: A484954 (Merck, Darmstadt, Germany), noradrenaline (NA), cimetidine, indomethacin, iberiotoxin (IbTx), glibenclamide, 4-aminopyridine (4-AP) and $BaCl_2$ (Sigma–Aldrich, St. Louis, MO, USA); N^G-nitro-L-arginine methyl ester (L-NAME) (Dojindo, Kumamoto).

Antibody sources were as follows: anti-inward rectifier K^+ (K_{ir}) 2.2 (No. APC-042) (Alomone Labs, Jersulem, Israel); anti-K_{ir}6.2 (No. sc-390104) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (No. GTX00118) (GeneTex, Irvine, CA, USA); anti-rabbit IgG horseradish peroxidase-linked whole antibody (No. NA934) and anti-mouse IgG horseradish peroxidase-linked whole antibody (No. NXA931) (Amersham Biosciences, Buckinghamshire, UK).

2.2. Tissue preparation

Male Wistar rats (6-10-week-old) were anesthetized with urethane (1.5 g/kg, i.p.) and euthanized by exsanguinations. The thoracic aorta and main branch of superior mesenteric artery were isolated. After removal of fat and adventitia, the aorta was cut into strips (approximately 4-mm-wide, 8-mm-long) and mesenteric artery was cut into rings (1–1.5 mm in diameter) for the measurement of isometric contraction as described previously.[11,12] The endothelium was removed by rubbing an intimal surface with a flat face of a pair of tweezers. Removal of the endothelium was confirmed by a lack of relaxation induced by acetylcholine (10 μM). Animal care and treatment were conducted in conformity with the institutional guidelines of The Kitasato University. Animal experiments were approved by the Institutional Animal Care and Use Committee of Kitasato University (Approval no. 16-001 and 17-014).

2.3. Measurement of isometric contraction

The contractility of arterial preparations was measured in normal physiological salt solution, which contained the following compositions (mM): 136.9 NaCl, 5.4 KCl, 1.5 $CaCl_2$, 1.0 $MgCl_2$, 23.8 $NaHCO_3$, 5.6 glucose, and 0.01 ethylene diamine tetraacetic acid. The high-K^+ (72.7 mM) solution was prepared by replacing NaCl with equimolar KCl. These solutions were saturated with a 95% O_2-5% CO_2 mixture at 37 °C and pH 7.4. Smooth muscle contractility was isometrically measured and digitally recorded using a force-displacement transducer (Nihon Kohden, Tokyo) and a PowerLab system (AD instruments, Dunedin, New Zealand) as described previously.[11,12] Each muscle preparation was mounted in a 7 ml (aorta) or 3 ml (mesenteric artery) organ bath under a resting tension of 1.0 g (aorta) or 0.5 g (mesenteric artery). After 30 min equilibration, each preparation was repeatedly exposed to high-K^+ solution until the responses became stable (45–60 min). The change in contractility by A484954 alone-treatment was measured by a cumulative application of A484954 (0.1–10 μM) in the resting state. Concentration-response curves were obtained by a cumulative application of A484954 (0.1–10 μM) to the arteries precontracted with submaximal to maximal concentration of NA (aorta: 100 nM; mesenteric artery: 3 μM) or high-K^+ (72.7 mM). The control experiment was performed by an application of dimethyl sulfoxide (DMSO, 0.1%), which was a solvent. We confirmed that DMSO did not induce relaxation in the arteries. To examine the contribution of endothelium-derived relaxing factor, a nitric oxide (NO) synthase (NOS) inhibitor, L-NAME (300 μM)[13] was pretreated for 15 min before the NA-induced precontraction. To examine the contribution of endothelium-independent relaxing factors, a cyclooxygenase (COX) inhibitor, indomethacin (10 μM)[12–14] or histamine H_2 receptor blocker, cimetidine (30 μM)[12,14] was pretreated for 15 min before the NA-induced precontraction. To examine the contribution of K^+ channel-mediated relaxation, a large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channel blocker, IbTx (100 nM)[13,14] an ATP-sensitive K^+ (K_{ATP}) channel blocker, glibenclamide (1 μM),[12,15] a voltage-gated K^+ (K_V) channel blocker, 4-AP (1 mM)[12,13] or a K_{ir} channel blocker, $BaCl_2$ (1 mM)[12,13] was pretreated for 15 min before the NA-induced precontraction.

2.4. RT-PCR analysis

RT-PCR was performed as described previously.[16] Aorta and mesenteric artery were isolated from rats as described above. The endothelium-denuded aorta and mesenteric artery were immediately frozen in liquid nitrogen and crushed by the Cell destroyer (PS1000; Bio Medical Science, Tokyo). Total RNA was extracted with a TRI Reagent (Molecular Research Center, Cincinnati, OH, USA). DNase treatment was conducted as follows; total RNA was mixed with 1 μl 10 x DNase I buffer (10 mM Tris-Cl, pH 7.6, 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$), 1 μl RNase-free DNase I (5 U/μl) and nuclease-free water (total 10 μl), which was left on ice for 30 min. Alternatively, DNase-treated total RNA was obtained after a total RNA was heat-treated at 65 °C for 5 min and cooled on ice, mixed with 2 μl 4 x DN Master Mix containing gDNA Remover (TOYOBO, Osaka) and nuclease-free water (total 10 μl), and incubated at 37 °C for 5 min. The first-strand cDNA was synthesized using a ReverTra Ace qPCR RT Master Mix kit or ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO) at 37 °C for 15 min and 98 °C for 5 min. PCR amplification was performed using a Quick Taq HS DyeMix (TOYOBO). The sequences of the primers which we used were listed in supplemental table. After an initial activation at 94 °C for 2 min, 25–35 cycles of amplifications at 94 °C for 30 s, 58 °C for 30 s and 68 °C for 1 min were done with a thermal cycler (PC707; ASTEC, Fukuoka). The PCR products were separated by electrophoresis on a 0.1% ethidium bromide containing 2% agarose gel. We visualized the bands with a UV trans-illuminator and analyzed using a CS analyzer 3.0 software (ATTO, Tokyo).

2.5. Quantitative RT-PCR (qPCR) analysis

After cDNA was synthesized as described above, qPCR amplification was performed using a THUNDERBIRD SYBR qPCR Mix (TOYOBO) with the pair of gene-specific primers (described in supplemental table) as described previously.[17] qPCR analysis was done using a PikoReal 96 Real-time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). After an initial activation at 95 °C for 1 min, 40 cycles of amplifications at 95 °C for 15 s and 60 °C for 30 s were done. Melting curve was analyzed from 65 °C to 95 °C. The relative mRNA level to GAPDH was calculated using $2^{-\Delta\Delta Cq}$ values and shown as fold increase relative to aorta.

2.6. Western blotting

Western blotting was performed as described previously.[9] Aorta and mesenteric artery were isolated from rats as described above. The endothelium-denuded aorta and mesenteric artery were immediately frozen in liquid nitrogen and mashed with a lysis

Please cite this article in press as: Kodama T, et al., Mechanisms underlying the relaxation by A484954, a eukaryotic elongation factor 2 kinase inhibitor, in rat isolated mesenteric artery, Journal of Pharmacological Sciences (2018), https://doi.org/10.1016/j.jphs.2018.04.006