Available online at www.sciencedirect.com
ScienceDirect
Free Radical Biology & Medicine 41 (2006) 1289–1303
www.elsevier.com/locate/freeradbiomed

Original Contribution

Apocynin normalizes hyperreactivity to phenylephrine in mesenteric arteries from cholesterol-fed mice by improving endothelium-derived hyperpolarizing factor response

Takayuki Matsumoto, Kiyoto Miyamori, Tsuneo Kobayashi, Katsuo Kamata *

Department of Physiology and Morphology, Institute of Medicinal Chemistry, Hoshi University, Shinagawa-ku, Tokyo 142-8501, Japan

Received 26 January 2006; revised 5 July 2006; accepted 11 July 2006
Available online 15 July 2006

Abstract

We studied the relationship among endothelial function, oxidative stress, and phenylephrine (PE; α_1-adrenoceptor agonist)-induced contraction in mesenteric arteries from high-cholesterol (HC)-diet-fed mice. In HC mice (vs age-matched normal-diet-fed mice): (1) PE-induced contraction in endothelium-intact rings was enhanced (endothelial denudation increased contraction in "normal-diet" rings, but did not enhance it further in "HC" rings); (2) the enhanced PE-induced contraction was further enhanced in the presence of N^G-nitro-L-arginine (L-NNA; nitric oxide synthase inhibitor) or L-NNA plus indomethacin (cyclooxygenase inhibitor) [to preserve endothelium-derived hyperpolarizing factor (EDHF)], but unchanged in the presence of charybdotoxin plus apamin (to block EDHF); (3) ACh-induced EDHF-type relaxation was reduced; and (4) oxidative stress [indicated by the plasma 8-isoprostane level (reliable systemic marker) and aortic superoxide production] was greater. In HC mice, PE-induced contraction was normalized by apocynin [NAD(P)H oxidase inhibitor] or tempol (superoxide dismutase mimetic), but enhanced by NADH [NAD(P)H oxidase substrate]. Oral dietary supplementation with apocynin (30 mg/kg/day for 4 weeks) corrected the above abnormalities. Hence: (1) PE-induced contraction is modulated by the endothelium, and the enhanced contractility in HC mice results from defective EDHF signaling and elevated oxidative stress, and (2) apocynin normalizes PE-induced contraction in HC mice by improving EDHF signaling.

Keywords: Apocynin; Contraction; EDHF; Hypercholesterolemia; Mesentery; Mice; Oxidative stress; Free radicals

Hypercholesterolemia is associated with an increased risk of vascular complications and atherosclerosis [1]. In contrast to the consistent data indicating a reduced endothelium-dependent relaxation in both hypercholesterolemic humans [2,3] and animals [4–8], the effects of hypercholesterolemia on vascular smooth muscle responsiveness are still controversial. For instance, angiotensin II- and methoxamine-induced contractions of the aorta are reportedly reduced in both hypercholesterolemic rabbits [9,10] and rats [11], but experimental hypercholesterolemia enhanced both the vasoconstriction induced by 5-hydroxytryptamine in porcine coronary arteries [12] and that induced by norepinephrine (NE) in cavernosal smooth muscles [13]. Moreover, in hypercholesterolemic animals the smooth muscle relaxation to nitric oxide (NO) is reduced, whereas the contractility to NE and that to endothelin are increased [14,15].

Abbreviations: 17-ODYA, 17-octadecynoic acid; ACh, acetylcholine; BK_{Ca}, large-conductance Ca^{2+}-activated K^+ channel; ChTX, charybdotoxin; COX, cyclooxygenase; EDCF, endothelium-derived contracting factor; EDHF, endothelium-derived hyperpolarizing factor; EDRF, endothelium-derived relaxing factor; EIA, enzyme immunoassay; eNOS, endothelial nitric oxide synthase; HDL, high-density lipoprotein; HRP, horseradish peroxidase; IbTX, iberiotoxin; K_{Ca}, Ca^{2+}-activated K^+ channel; KHS, Krebs–Henseleit solution; L-NNA, N^G-nitro-L-arginine; NADH, nicotinamide adenine dinucleotide; NBT, nitroblue tetrazolium; NE, norepinephrine; NOS, nitric oxide synthase; PE, phenylephrine; PGE_2, prostaglandin E_2; $PGF_2\alpha$, prostaglandin $F_2\alpha$; PGI_2, prostacyclin; PPOH, 6-(2-propargyloxyphenyl)hexanoic acid; ROS, reactive oxygen species; SNP, sodium nitroprusside; SOD, superoxide dismutase; TXA_2, thromboxane A_2; TXB_2, thromboxane B_2.

* Corresponding author. Fax: +81 3 5498 5856.
E-mail address: kamata@hoshi.ac.jp (K. Kamata).

0891-5849/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.freeradbiomed.2006.07.012

The endothelium plays an important role in maintaining vascular homeostasis by synthesizing and releasing several vasodilating factors [endothelium-derived relaxing factors (EDRFs), which include vasodilator prostaglandins, NO, and the still-unidentified endothelium-derived hyperpolarizing factor (EDHF)] [16–22]. Agonist-mediated contractile responses are modified by removal of or damage to the endothelium in many blood vessels [23–26]. In most cases, hyperreactivity in disease states is related to decreased NO bioavailability; indeed, in control vessels inhibition of NO synthase (NOS) increases the vascular reactivity to α-adrenoceptor agonists. The possibility that an endothelial factor other than NO is responsible for the generation of vasomotion and/or for modulating phenylephrine (PE)-induced contraction has been examined recently [25,27], and the results indicated that EDHF is also involved in vasomotion. Moreover, Mauban and Wier [28] demonstrated that EDHF is essential for the development of adrenergically induced vasomotion. However, no study has yet investigated the relationship between EDHF and PE-induced contraction in mesenteric arteries from hypercholesterolemic mice.

A considerable body of evidence implicates oxidative stress as an important pathogenic element in hypercholesterolemic vascular abnormalities [29,30]. Further, emerging evidence indicates that reactive oxygen species (ROS) are important molecules in the control of vascular reactivity [31–33]. Indeed, superoxide increases vascular tone via an inactivation of endothelium-derived NO. Several investigators have reported that NAD(P)H oxidase is the primary generator of ROS within the vasculature [34–37]. NAD(P)H oxidase is a membrane-associated enzyme that generates superoxide by transferring electrons to molecular oxygen via a flavin-containing "Nox" catalytic subunit. Within the systemic circulation, NAD(P)H oxidase has undergone extensive study, and it is now believed that excess superoxide generation by this enzyme contributes to the endothelial dysfunction known to be associated with numerous cardiovascular diseases [37–41]. Although the importance of oxidative stress in endothelial dysfunction has thus been recognized, the relationship between oxidative stress and PE-induced contraction in mesenteric arteries in hypercholesterolemic mice has not previously been investigated.

Apocynin (4-hydroxy-3-methoxyacetophenone) is a catechol that inhibits neutrophil oxidative burst activity and reduces neutrophil-mediated oxidative damage [42,43]. The anti-inflammatory activity of apocynin has been demonstrated in a variety of cell and animal models of inflammation [44]. Apocynin, after metabolic conversion, inhibits the assembly of NAD(P)H oxidase [37]. It is therefore extensively used to reveal the role of this enzyme in cell and experimental models (whether or not they are characterized by an inflammatory component) [45–47].

For the present study, we designed experiments to characterize any diet-induced alterations in the PE-induced contraction of mesenteric arteries isolated from high-cholesterol-diet-fed mice (versus a normal-diet-fed group). We were especially interested in the relationship between EDHF and/or oxidative stress and PE-induced contraction in these mice. We also asked whether the hyperreactivity to PE in mesenteric arteries from established hypercholesterolemic mice might be normalized by chronic administration of apocynin.

Materials and methods

Reagents

N^G-Nitro-L-arginine(L-NNA),PE,sodiumnitroprusside(SNP), apamin,iberiotoxin(IbTX),carboxy-PTIO[(2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide)], TRAM-34 [1-[(2-chlorophenyl)diphenylmethyl]-1*H*-pyrazole], 17-octadecynoic acid (17-ODYA), 6-(2-propargyloxyphenyl)hexanoic acid (PPOH), charybdotoxin (ChTX), indomethacin, β-nicotinamide adenine dinucleotide (NADH), nitroblue tetrazolium (NBT), protease-inhibitor cocktail, and monoclonal β-actin antibody were all purchased from Sigma Chemical Co. (St. Louis, MO, USA), whereas acetylcholine chloride (ACh) was from Daiichi Pharmaceuticals (Tokyo, Japan). The NAD(P)H oxidase inhibitor apocynin and the superoxide dismutase (SOD) mimetic 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (tempol) were from Calbiochem-Novabiochem Corp. (La Jolla, CA, USA). All drugs were dissolved in saline, except where otherwise noted. All concentrations are expressed as the final molar concentration of the base in the organ bath. Horseradish peroxidase (HRP)-linked secondary anti-mouse antibody was purchased from Promega (Madison, WI, USA), whereas an antibody against endothelial NOS (eNOS) was obtained from BD Biosciences (San Jose, CA, USA).

Animals and experimental design

Male ICR mice age 4 weeks were housed under constant climatic conditions (room temperature 21–22°C, room humidity 50 ± 5%), and food and water were given ad libitum to all animals. This study was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* adopted by the Committee on the Care and Use of Laboratory Animals of Hoshi University (which is accredited by the Ministry of Education, Culture, Sports, Science, and Technology, Japan). The study consisted of two protocols.

Protocol 1

Mice were randomly allocated to one of two groups as follows. After 1 week of familiarization, mice were fed for 10 weeks either a normal diet (normal) or a diet supplemented with high cholesterol (HC; 2% cholesterol and 0.5% cholic acid), as in our previous studies [5,6].

Protocol 2

Mice were randomly allocated to one of three groups as follows. Starting 10 weeks after the dietary intervention described above (Protocol 1), HC mice were fed for 4 weeks an HC diet either containing (HC-apocynin) or not containing (HC-cont) apocynin (30 mg/kg/day). Age-matched control mice (control) were fed a normal diet throughout.