Biochemical and Biophysical Research Communications xxx (2015) 1–6

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Berberine improves endothelial function by inhibiting endoplasmic reticulum stress in the carotid arteries of spontaneously hypertensive rats

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ARTICLE INFO

Article history:
Received 30 January 2015
Available online xxx

Keywords:
Carotid artery
Cyclooxygenase
Endoplasmic reticulum stress
Endothelial function
Spontaneously hypertensive rats

ABSTRACT

Activation of endoplasmic reticulum (ER) stress in endothelial cells leads to increased oxidative stress and often results in cell death, which has been implicated in hypertension. The present study investigated the effects of berberine, a botanical alkaloid purified from Coptidis rhizoma, on ER stress in spontaneously hypertensive rats (SHRs) and the underling mechanism. Isolated carotid arteries from normotensive WKYs and SHRs were suspended in myograph for isometric force measurement. Protein phosphorylations and expressions were determined by Western blotting. Reactive oxygen species (ROS) level was measured by DHE staining. SHR carotid arteries exhibited exaggerated acetylcholine-triggered endothelium-dependent contractions (EDCs) and elevated ROS accumulation compared with WKY arteries. Moreover, Western blot analysis revealed the reduced AMPK phosphorylation, increased eIF2α phosphorylation, and elevated levels of ATF3, ATF6, XBP1 and COX-2 in SHR carotid arteries while these pathological alterations were reversed by 12 h-incubation with berberine. Furthermore, AMPK inhibitor compound C or dominant negative AMPK adenovirus inhibited the effects of berberine on above-mentioned marker proteins and EDCs. More importantly, ROS scavengers, tempol and tiron plus DETCA, or ER stress inhibitors, 4-PBA and TUCDA normalized the elevated levels of ROS and COX-2 expression, and attenuated EDCs in SHR arteries. Taken together, the present results suggest that berberine reduces EDCs likely through activating AMPK, thus inhibiting ER stress and subsequently scavenging ROS leading to COX-2 down-regulation in SHR carotid arteries. The present study thus provides additional insights into the vascular beneficial effects of berberine in hypertension.

1. Introduction

Reactive oxygen species (ROS) over-production or increased oxidative stress impairs endothelial function and is one of the primary mediators of the development of hypertension [1–3]. Endothelium-dependent contractions (EDCs) are associated with endothelial dysfunction and correlated with the severity of hypertension [4]. EDCs are induced by endothelium-derived contracting factors including endothelial cyclooxygenase (COX)-derived prostanoids or ROS [5–7]. Reducing oxidative stress can diminish EDCs in renal arteries of spontaneously hypertensive rats (SHR) [6]. Endoplasmic reticulum (ER) stress is among the key players in endothelial dysfunction during hypertension [8] and is caused by the activation of complex cytoplasmic and nuclear signaling pathways, collectively termed the unfolded protein response [9]. Recent studies have shown a close link between ER stress and oxidative stress in cardiovascular pathogenesis [10,11].

Berberine ($[C20H18NO4]^+$), an isoquinoline alkaloid isolated from many medicinal herbs, has long been used in the treatment of gastrointestinal infections and diarrheas, but only until the last few decades its cardiovascular benefits have been reported [12]. Berberine inhibits human immunodeficiency virus protease inhibitor-induced inflammatory response by modulating ER stress

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http://dx.doi.org/10.1016/j.bbrc.2015.02.028
0006-291X/

signaling pathways in murine macrophages [13], ameliorates pro-inflammatory cytokine-induced ER stress in human intestinal epithelial cells [14], and reduces hypoxia/reoxygenation-induced injury by suppressing mitochondria stress and ER stress in human renal proximal tubular cells [15]. Although clinical and experimental studies suggest the anti-hypertensive properties of berberine and its derivative [16–18], the modulatory effects of berberine on ER stress in hypertension remain largely unknown. The present study therefore investigated whether berberine inhibits ER stress to attenuate EDCs in SHR carotid arteries.

2. Materials and methods

2.1. Chemicals

Anti-phospho-AMPKα (Thr172), anti-eIF2α, and anti-AMPKα antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-phospho-eIF2α (Ser51) and anti-GAPDH antibodies were obtained from Bioword Technology (Louis Park, MN, USA). Anti-COX-1 and anti-COX-2 antibodies were purchased from Cayman Chemical (Ann Arbor, MI, USA). Anti-ATF3 and anti-XBP1 antibodies were obtained from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). Anti-ATF6 antibody was from Abcam (Cambridge, MA). HRP-conjugated swine anti-rabbit and anti-mouse IgG were from DakoCytomation (Carpinteria, CA, USA). Immobilon-P polyvinylidenedifluoride (PVDF) membrane was from Millipore (Billerica, MA, USA) and chemiluminescence (ECL reagents) was obtained from Amersham Pharmacia (GE Healthcare Life Sciences, Buckinghamshire, UK). Berberine, compound C, N^G-nitro-L-arginine methyl ester (L-NAME), acetylcholine (ACh), tempol, tiron, diethyldithiocarbamate (DETCA), and 4-phenyl butyric acid (4-PBA) were purchased from Sigma–Aldrich Chemical (St Louis, MO, USA). s18886 was a gift from the Institute RecherchesServier (Suresnes, France). SC560 and NS398 were from Tocris Bioscience (Bristol, UK). Dihydroethidium (DHE) was from Invitrogen (Carlsbad, CA, USA). Tauroursodeoxycholic acid (TUDCA) was from Calbiochem-Novabiochem Corp. (San Diego, CA). L-NAME and ACh were dissolved in distilled water. Other drugs were dissolved in DMSO. DMSO (0.1% v/v) did not modify ACh-induced contraction.

2.2. Animal protocols

Male SHR (32–40 weeks old) and WKY rats (32–40 weeks old) were used in compliance with the Institutional Authority for Laboratory Animal Care, Peking University Health Science Center, China. This study conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.3. Artery preparation and functional assay

WKY and SHRs were sacrificed by CO_2 suffocation. Carotid arteries from rats were removed and placed in ice-cold Krebs solution (mmol/L): 119 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 1.2 KH_2PO_4, and 11 D-glucose. Arteries were cleaned of adhering tissue and cut into ring segments of 2 mm in length and suspended in myograph (Danish Myo Technology, Aarhus, Denmark) for recording of changes in isometric tension. Briefly, to visualize the endothelium-dependent contractions, rings with endothelium were exposed for 30 min to 100 μmol/L L-NAME to eliminate the relaxant effect of endothelium-derived nitric oxide before the application of ACh (0.01–100 μmol/L). The effects of COX-1 inhibitor (SC560, 10 nmol/L), COX-2 inhibitor (NS398, 1 μmol/L) or TP receptor antagonist (s18886, 0.1 μmol/L) were tested on ACh-induced contractions following 30-min incubation with L-NAME. In some experiments, SHR carotid arteries were incubated with ROS scavengers [tempol (100 μmol/L) or tiron (1 mmol/L) plus DETCA (100 μmol/L)], ER stress inhibitors [4-PBA (10 μmol/L) or TUDCA (20 μmol/L)] and compound C (10 μmol/L, AMPKα inhibitor) along with berbeine (1 μmol/L) for 12 h in Dulbecco's Modified Eagle's Media (DMEM) with 10% fetal bovine serum, 100 IU penicillin, and 100 μg/mL streptomycin.

2.4. Western blot analysis

Carotid arteries from WKY and SHRs were cultured and then homogenized in RIPA lysis buffer containing 1 μg/mL leupeptin, 5 μg/mL aprotinin, 100 μg/mL PMSF, 1 mmol/L sodium orthovanadate, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L sodium fluoride, and 2 μg/mL β-glycerolphosphate. The homogenated were centrifuged at 20,000 × g for 20 min at 4 °C. Protein lysates (10 μg) were separated by electrophoresis and transferred onto PVDF membrane. Blots were blocked with 1% bovine serum albumin or 5% non-fat milk for 1 h and incubated overnight at 4 °C with primary antibodies. After washing, blots were incubated with HRP-conjugated swine anti-rabbit or anti-mouse IgG. Immunoreactive bands were visualized by chemiluminescence and exposed to Kodak Image Station 440 for densitometric analysis.

2.5. Dihydroethidium (DHE) staining

Frozen sections of rat carotid arteries on glass coverslips were loaded with 5 μmol/L DHE at 37 °C for 10 min. ROS fluorescence was measured by a confocal scanning unit (Olympus) at excitation 515 nm and emission 585 nm. Data were analyzed by the Fluoview software (Olympus).

2.6. Adenoviral infection

Recombinant virus was produced in human embryonic kidney (HEK) 293A cells. SHR carotid arteries were infected with a dominant negative AMPK (DN-AMPK) adenovirus, using a protocol of 4-h exposure to 1.5 μl of adenovirus to 24-well plate (1×10^8 plaque forming units/ml). 4 h after infection, arteries were cultured for 20 h in full DMEM medium and then treated with or without berberine (1 μmol/L) for additional 12 h. Thereafter, arteries were collected for functional study, Western blotting, and DHE staining.

2.7. Statistical analysis

The contraction was expressed as percentage of 60 mmol/L KCl-induced tension. Results are means ± SEM and n represents carotid arteries from different rats. Statistical significance was determined by two-tailed Student's t-test and nonparametric test. $P < 0.05$ was considered significantly different.

3. Results

3.1. Berberine attenuates endothelium-dependent contractions in SHR carotid arteries

Endothelium-dependent contractions (EDCs) are associated with endothelial dysfunction in hypertension [4,6]. ACh-triggered EDCs in carotid arteries from SHR were exaggerated compared with WKY arteries, and 12-h incubation with berbeine (1 μmol/L) markedly attenuated EDCs from SHR arteries without affecting those from WKY (Fig. 1A). The EDCs were likely mediated through COX-2-dependent mechanism since they were inhibited or abolished by selective COX-2 inhibitor NS398 (1 μmol/L) or selective TP

Please cite this article in press as: L. Liu, et al., Berberine improves endothelial function by inhibiting endoplasmic reticulum stress in the carotid arteries of spontaneously hypertensive rats, Biochemical and Biophysical Research Communications (2015), http://dx.doi.org/10.1016/j.bbrc.2015.02.028