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Probucol *via* inhibition of NHE1 attenuates LPS-accelerated atherosclerosis and promotes plaque stability *in vivo*

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ABSTRACT

Activation of Na^+/H^+ exchanger 1 (NHE1) by lipopolysaccharide (LPS) *via* Ca^{2+}/calpain is responsible in vascular smooth muscle cell (VSMC) apoptosis and to the process of atherosclerosis. Probucol is a lipid-lowering drug which has an anti-atherosclerosis effect. The mechanism remains poorly understood. Here we hypothesized that probucol *via* inhibition of NHE1 in VSMCs attenuates LPS-accelerated atherosclerosis and promotes plaque stability. Our results revealed that treatment of VSMCs with LPS increased the NHE1 activity in a time-dependent manner, associated with the increased $Ca^{2+}{}_i$. Probucol inhibited the LPS-induced increase of NHE1 activity in a dose-dependent manner in VSMCs for 24-hour co-incubation, as well as the change of $Ca^{2+}{}_i$. In addition, LPS enhanced the calpain activity. Both probucol and calcium chelation of Ca^{2+} abolished the LPS-induced increase of calpain activity. Treatment of VSMCs with LPS reduced the expression of Bcl-2 without altering the mRNA level. Probucol inhibited the LPS-reduced expression of Bcl-2 protein in VSMCs. Animal studies indicated administration of probucol suppressed LPS-accelerated apoptosis, atherosclerosis and plaque instability in *Apoe*$^{-/-}$ mice. In conclusion, probucol *via* inhibition of NHE1 attenuates atherosclerosis lesion growth and promotes plaque stability.

Introduction

Atherosclerosis is a common cardiovascular disease characterized by the depositions of fatty substances on and fibrosis of the inner lining of the arteries. Probucol is a unique cholesterol lowering drug with an anti-atherosclerosis effect (Alessio et al., 2011). Probucol trial revealed that probucol reduces coronary restenosis after percutaneous transluminal coronary angioplasty. Probucol observational study illuminating therapeutic impact on vascular events showed that probucol was useful in lowering the risk of cardiovascular events in secondary prevention in spite of causing a decrease in HDL-C levels. Although probucol decreases HDL-C levels, it shows greatly controlled progression of atherosclerosis (Bocan et al., 1992). The molecular mechanism against atherosclerosis still remains unknown.

Lipopolysaccharide (LPS) is a critical glycolipid component of the outer wall of Gram-negative bacteria, and many of the cellular signals activated by Gram-negative bacteria are attributed to LPS (Raetz, 1990). Recent report has shown that LPS can induce VSMC injury by activating calpain (Liu et al., 2008) and accelerate atherosclerosis in mice (C. Wang et al., 2007). The vascular cell is a prime target of the LPS molecule, and vascular complications of septic shock due to Gram-negative bacteria are related to vascular injury (Westhorpe et al., 2012). Indeed, LPS-induced systemic organ failure is triggered initially by vascular injury, characterized by vascular occlusion, perivascular accumulation of leukocytes and cell death. VSMC apoptosis is caused by various mechanisms, such as cytokines, free radicals, death receptors and cell interaction, which is related to atherosclerosis.

The induction of apoptosis by the Na^+/H^+ exchanger (NHE1) activation has been extensively studied. The NHE1 is expressed ubiquitously in the plasma membrane of mammalian cells and exchanges intracellular H^+ for extracellular Na^+ to regulate intracellular pH (pH_i) value and the concentration of $Na^+{}_i$ (Shuang-Xi et al., 2005). The activation of NHE1 increases $Na^+{}_i$ concentration that leads to Ca^{2+} overload through the Na^+/Ca^{2+} exchanger, which is assumed to be the crucial factor in cell injury (Wang et al., 2003). Increase intracellular Ca^{2+} concentration automatically activates calpain, a calcium-dependent protease (Goll et al., 2003). Many proteins in cells are the natural substrates of calpain, including anti-apoptotic family member, Bcl-2 (Gil-Parrado et al., 2002). Previous studies have showed that the inhibition of NHE-1 has anti-apoptotic effects and the role of Bcl-2 in LPS-induced apoptosis (Garciarena et al., 2009; Schelling and Abu Jawdeh, 2008; H.L. Wang et al., 2007).

In this report we hypothesized that LPS stimulates a calpain-mediated death pathway in VSMCs and probucol potentially suppresses

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this pathway to attenuate atherosclerosis progression and formation of vulnerable plaque *via* inhibition of apoptosis. The study provides new insights to understand the effects of probucol on atherosclerosis.

Materials and methods

Materials

Probucol was from Sigma Company. Calcium chelator (BAPTA) was obtained from Invitrogen Corporation. 2-Carboxyethyl-5(6)-carboxyfluorescein (BCECF) were purchased from Calbiochem (USA). Other chemicals were purchased from Sigma Chemical Co, USA. Primary antibodies (Bcl-2, CD68, actin, GAPDH) and secondary antibodies were obtained from Cell signaling company. All chemicals were of reagent grade. All concentrations are the final concentration.

Mice

Male Apo E gene knockout (*Apoe*$^{-/-}$) mice originally purchased from Jackson Laboratories (Bar Harbor, ME), were used at 8 to 10 weeks of age and then fed a high-fat diet (HFD) with or without 0.5% (w/w) probucol and LPS infusion for 4 weeks. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by Harbin Medical University.

Cell culture

VSMCs were purchased from ATCC and cultured according to the method described previously (Wang et al., 2008a). VSMCs were grown in M200 (Clonetics Inc. Walkersville, MD) supplemented with 10% FBS, 12.5 mg/ml ECGF, 1 mg/ml hydrocortisone, 100 μ/ml penicillin and 100 mg/ml streptomycin. The cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Culture medium was replaced twice a week, and cells were sub-cultured when confluent. Cultures were expanded by brief trypsinization using 0.25% trypsin in phosphate-buffered saline (PBS) containing 0.02% EDTA. Cells at passage 3–8 were used for all experiments.

Western blot

After treatment, VSMCs were lysated in cell-lysis buffer (Cell Signaling Company). The protein content was assayed by BCA protein assay reagent (Pierce, USA). 20 μg of protein was loaded to SDS-PAGE and then transferred to membrane. Membrane was incubated with a 1:1000 dilution of primary antibody, followed by a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody. Protein bands were visualized by ECL (GE Healthcare). The intensity (area × density) of the individual bands on western blots was measured by densitometry (model GS-700, Imaging Densitometer; Bio-Rad). The background was subtracted from the calculated area.

RT-PCR analysis for Bcl-2 mRNA

Total RNA was extracted from cells after treatment by using a TRIZOL (Life Technologies, USA) reagent according to the manufacturer's protocol (Kyronlahti et al., 2008). The primer sequences were: Bcl-2 sense, 5′-GTGGATGACTGAGTACCTGAACC-3′; Bcl-2 antisense, 5′-AGCC AGGAGAAA TCAAACAGAG-3′; and GAPDH sense, 5′-TCATTTCCTGGTAT GACAACG-3′; GAPDH antisense, 5′-TTACTCCTTGGAGGCCATGT-3′. The PCR protocol for Bcl-2consisted of denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 2 min, and final extension at 72 °C for 15 min. The protocol for GAPDH was the same except for annealing at 65 °C for 1 min and amplification of 25 cycles.

Measurement of NHE1 activity

NHE1 activity in VSMCs *in situ* was determined by measuring the initial rate of pH_i recovery following intracellular acidification by using NH_4Cl pulse method (Wang et al., 2006). Briefly, VSMCs were washed with HCO_3^--free HBS buffer (mM: NaCl, 140; KCl, 5; $CaCl_2$, 1; $MgCl_2$, 1; glucose, 5; HEPES, 6; pH 7.4). After the incubation of HUVECs with HBS containing 10 μM BCECF at 37 °C for 30 min and the removal of free-BCECF by washing, 40 mM NH_4Cl was added into HBS, incubated for 5 min, and washed out with Na^+-free HBS buffer. Cells were acid-loaded and pH_i decreased. When 100 mM NaCl was added, intracellular H^+ was pumped out *via* Na^+/H^+ exchange and pH_i increased linearly during the initial 40 s. This initial rate of pH_i recovery (dpH_i/dt) was considered to reflect the Na^+/H^+ exchange activity.

Calpain activity assay

The calpain activity was measured by using the fluorogenic peptide Suc-Leu-Leu-Val-Tyr-AMC as a substrate following the procedure described previously with slight modification (Dong et al., 2006). Shortly, cells were cultured in 24-well plates in medium with different treatments. After being washed twice with PBS, fluorogenic peptide was added to a final concentration of 80 μM in PBS. Immediately after the addition of fluorogenic peptide, fluorescence was recorded at 2 min intervals for 20 min at an excitation of 360 nm and an emission of 460 nm using a Synergy HT Multi-Detection Microplate Reader (BIO-TEK Instruments Inc.). The initial rate of peptidyl-AMC hydrolysis was used as the velocity of enzyme activity.

Measurement of $Ca^{2+}{}_i$ concentration

The $Ca^{2+}{}_i$ concentration was measured by using a Fluo-4 NW kit from Invitrogen following kit protocol (Wang et al., 2008b). Briefly, VSMCs were treated as indicated, the cell culture medium was aspirated, washed with HEPES buffer (pH 7.4) once, and 1 ml of HEPES buffer with fluorescent dye was added to cultured cells. After 30 min incubation, fluorescence strength was measured in wavelength of excitation/emission of 485/520 nm.

Atherosclerotic lesion analysis

The ascending aorta tissue from to the ideal bifurcation was removed and fixed in 4% paraformaldehyde for 16 h. The adventitia was then thoroughly cleaned under a dissecting microscope. For each mouse, four consecutive sections were stained with HE. Images of plaques were captured using an Olympus microscope connected to a QImaging Retiga CCD camera. The aortic lesion size in each animal was obtained by averaging the lesion areas in the four sections. The lesion area, from the aortic arch to 5 mm distal to the left subclavian artery, was quantified using Alpha Ease FC software (Version 4.0, Alpha Innotech).

Determination of plaque vulnerable index

The plaque vulnerable index was determined by using the ration of CD68-positive (%) plus Oil Red (%) to α-actin (%) plus collagen (%) as described previously (Dong et al., 2013). Two different lesion areas were selected to account in each segments and the mean was used in statistical analysis.

Immunohistochemistry

The aortic section was dissected, fixed in 4% paraformaldehyde for 16 h, and embedded in paraffin. Four micron-thick sections were deparaffinized, rehydrated, and microwaved in citrate buffer for antigen retrieval. Sections were successively incubated in endogenous