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Capsaicin protects endothelial cells and macrophage against oxidized low-density lipoprotein-induced injury by direct antioxidant action

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ABSTRACT

Atherosclerosis is a chronic inflammatory vascular disease. It is characterized by endothelial dysfunction, lipid accumulation, leukocyte activation, and the production of inflammatory mediators and reactive oxygen species (ROS). Capsaicin, a biologically active compound of the red pepper and chili pepper, has several anti-oxidant, anti-inflammatory, anti-cancer, and hypolipidemic biological effects. However, its protective effects on foam cell formation and endothelial injury induced by oxidized low-density lipoprotein (oxLDL) remain unclear. In this study, we evaluated the anti-oxidative activity of capsaicin, and determined the mechanism by which capsaicin rescues human umbilical vein endothelial cells (HUVECs) from oxLDL-mediated dysfunction. The anti-oxidative activity of capsaicin was defined by Apo B fragmentation and conjugated diene production of the copper-mediated oxidation of LDL. Capsaicin repressed ROS generation, as well as subsequent mitochondrial membrane potential collapse, cytochrome c expression, chromosome condensation, and caspase-3 activation induced by oxLDL in HUVECs. Capsaicin also protected foam cell formation in macrophage RAW 264.7 cells. Our results suggest that capsaicin may prevent oxLDL-induced cellular dysfunction and protect RAW 264.7 cells from LDL oxidation.

1. Introduction

Atherosclerosis is a complex inflammatory process characterized by the accumulation of lipids on the inner surfaces of blood vessels, activation of macrophages, and production of reactive oxygen species (ROS) [1–3]. The oxidative modification of low-density lipoprotein (LDL) plays a major risk factor in the pathogenesis of atherogenesis [4]. ROS modify LDL by producing oxidized LDL (oxLDL). OxLDL results in the formation of atherosclerotic plaque in arteries, and can ultimately lead to infarction. Endothelial dysfunction is an early event in atherosclerotic disease, preceding clinical manifestations and complications. OxLDL activates the cellular suicide pathway in endothelial cells, including increased ROS [5] and reduction of the mitochondrial transmembrane potential, induction expression of cytochrome c and subsequent activation of caspase-3 and cleavage of poly ADP-ribose polymerase (PARP), leading to apoptosis. Monocyte-derived macrophages play a key role in the inflammatory events that occur in the initiation and progression of atherosclerosis [6,7]. Macrophages are recruited to the deposit site and start accumulating large amounts of lipids through the uptake of oxidized lipoproteins; they then become foam cells [8]. The accumulation of foam cells in the arterial intima represents the initiation of atherosclerosis.

Capsaicin, a spicy component found in red pepper and chili pepper, has anti-inflammatory activities by inhibiting IκB-alpha degradation and inactivated NF-κB in lipopolysaccharide (LPS)-stimulated murine macrophages [9]. Capsaicin also protects against the development of adjuvant-induced arthritis and ethanol-induced oxidative damage in rats [10,11]. Dietary capsaicin protects against iron-induced LDL oxidation and hepatotoxicity and carrageenan-induced inflammation in rats [12]. Capsaicin demonstrates potential benefits for treating obesity and insulin

Abbreviations: LDL, low-density lipoprotein; HUVECs, human umbilical vein endothelial cells; OxLDL, oxidized low-density lipoprotein; DAPI, 4′-6-diamidino-2-phenylindole; TBARS, thiobarbituric acid-reacting substances; REM, relative electrophoretic mobility; ROS, reactive oxygen species.

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resistance, both in animal models and clinical studies [13–15]. Capsaicin represses the transcriptional activity of beta-catenin in human colorectal cancer cells, and induces cell death through the mitochondrial pathway and activation of caspase-7 in human breast cancer [16,17]. Although the *in vivo* and *in vitro* anti-inflammatory and anti-oxidant effects of capsaicin are well known, the molecular mechanism of capsaicin function in human umbilical vein endothelial cells (HUVECs) stimulated by oxLDL remains unclear. In this study, we examined whether capsaicin has a protective effect on endothelial cells, particularly with regard to oxLDL-induced endothelial dysfunction, and whether capsaicin modulates macrophage foam cell formation in macrophages. We also investigated the molecular mechanisms underlying the effects of capsaicin in HUVECs.

2. Materials and methods

2.1. Materials and chemicals

Capsaicin, ethylenediaminetetraacetic acid (EDTA), cupric sulfate, sodium dodecyl sulfate (SDS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 4′-6-diamidino-2-phenylindole (DAPI), endothelial cell growth supplement (ECGS), and polyacrylamide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Medium 199, RPMI 1640, fetal bovine serum (FBS), and trypsin–EDTA were obtained from Gibco Invitrogen Corporation (Barcelona, Spain). A monoclonal antibody against β-actin was purchased from Sigma (St. Louis, MO, USA). A goat polyclonal antibody against caspase-3, were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Mouse monoclonal antibodies against Bcl-2, and Bax, cytochrome c were purchased from Cell signaling Technology (Danvers, MA, USA). A rabbit polyclonal antibody against PARP was purchased from BD Transduction Laboratories (San Diego, CA, USA). An Immobilon Western Chemiluminescent HRP Substrate kit was obtained from Millipore Corporation (Billerica, MA, USA).

2.2. Cell cultures

HUVECs were obtained from the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan), and cultured on gelatin-coated culture dishes in medium 199 with 10% FBS, 25 U/mL heparin, 30 μg/mL ECGS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Subcultures were performed with trypsin–EDTA. Cells from passages 5–10 were used. Media were refreshed every other day. RAW 264.7 macrophage cells were obtained from the BCRC, and cultured in RPMI 1640 with 10% FBS containing 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. All cell cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

2.3. Treatment of HUVECs with oxLDL

LDL was diluted with 10 mM PBS to a final concentration of 1 mg protein/mL, and incubated at 37 °C in the presence of $CuSO_4$ (10 μM) for 16 h. Cu^{2+} was removed by gel filtration (PD-10 gel) with 10 mM PBS. After extraction, oxLDL was added to the culture medium of HUVECs to obtain a final concentration of 200 μg/mL, with or without the addition of capsaicin.

2.4. Lipoprotein separation and oxidation

Human plasma was obtained from the Taichung Blood Bank, and LDL was isolated using sequential ultracentrifugation [20]. LDL (q = 1.019–1.210 g/mL) in KBr solution containing 30 mM EDTA was stored at 4 °C in a sterile, dark environment and used within 3 d. Immediately before oxidation tests, LDL was separated from EDTA and diffusible low molecular mass compounds by gel filtration on PD-10 gel (Pharmacia, St-Quentin, France) in 10 mM PBS (pH 7.4). Protein quantification was measured by Bradford protein assay (Bio-Rad Lab., Richmond, CA, USA) [18].

2.5. Lipid peroxidation assay

Malondialdehyde (MDA) production was assessed as an indicator of lipid peroxidation according to the procedures of Camejo et al. [21]. To each tube containing 0.55 mL of the incubated LDL, 0.5 mL of 25% (w/v) trichloroacetic acid was added to denature protein. The samples were then centrifuged (10,000 rpm) at 10 °C for 30 min to remove pellets. Thiobarbituric acid (TBA; 1%, 0.5 mL) in 0.3% NaOH was added to the supernatant, and the mixed reagents were allowed to react at 90–95 °C for 40 min in the dark. After completing the reaction, samples were analyzed using a Hitachi F2000 spectrophotofluorometer (excitation wavelength at 532 nm and emission wavelength at 600 nm). The concentration of MDA or TBA-reacting substance was expressed as equivalents of 1,1,3,3-tetraethoxypropane, which served as a standard [6].

2.6. Conjugated dienes determination

Conjugated dienes formation was monitored by second derivative spectroscopy (220–300 nm) based on the method described by Bourne and Rice-Evans. The second derivative spectrum was subtracted from the second derivative spectrum of the matching control sample without Cu^{2+}. The increase in conjugated dienes expressed in relative unit was obtained from the amplitude of the peak at 254 nm.

2.7. Relative electrophoretic mobility (REM) shift assays

LDL (200 μg/mL) was pretreated with the indicated concentrations of capsaicin for 2 h, followed by incubation with 10 μM $CuSO_4$ at 37 °C for 16 h. LDL modifications were assessed by agarose electrophoresis to detect the increase in electrophoretic mobility of the modified LDL relative to native LDL. In brief, native or modified LDL (8–10 μg) were loaded into 0.6% agarose gels and electrophoresed for 40 min at 100 V. The gel was fixed in 75% ethanol and 5% acetic acid for 15 min, stained with 1% oil red O (in 60% isopropanol) for 30 min, and rinsed with 30% isopropanol to visualize LDL bands. The distance migrated by each LDL band was measured and expressed as an arbitrary REM value compared with native LDL [18].

2.8. Electrophoresis of ApoB fragmentation

After oxidation with or without capsaicin, samples were denatured with 3% SDS, 10% glycerol, and 5% 2-mercaptoethanol at 95 °C for 5 min. SDS polyacrylamide gel electrophoresis (7.5% SDS–PAGE, 100 V for 6 h) was performed to detect ApoB fragmentation. The gel was subsequently stained with Coomassie Brilliant blue R250 and dried [18].

2.9. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The DPPH assay was used to measure the anti-radical activity of capsaicin. Briefly, 0.1 mM DPPH solution was freshly prepared by dissolving DPPH powder in methanol. Then, capsicin and Trolox (as a standard) were added to DPPH solution to make indicated concentration. The absorbance was measured at 517 nm after 5 min of reaction at room temperature.