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Vinpocetine attenuates lipid accumulation and atherosclerosis formation

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ABSTRACT

Atherosclerosis, the major cause of myocardial infarction and stroke, is a chronic arterial disease characterized by lipid deposition and inflammation in the vessel wall. Cholesterol, in low-density lipoprotein (LDL), plays a critical role in the pathogenesis of atherosclerosis. Vinpocetine, a derivative of the alkaloid vincamine, has long been used as a cerebral blood flow enhancer for treating cognitive impairment. Recent study indicated that vinpocetine is a potent anti-inflammatory agent. However, its role in the pathogenesis of atherosclerosis remains unexplored. In the present study, we show that vinpocetine significantly reduced atherosclerotic lesion formation in ApoE knockout mice fed with a high-fat diet. In cultured murine macrophage RAW264.7 cells, vinpocetine markedly attenuated oxidized LDL (ox-LDL) uptake and foam cell formation. Moreover, vinpocetine greatly blocked the induction of ox-LDL receptor 1 (LOX-1) in cultured macrophages as well as in the LOX-1 level in atherosclerotic lesions. Taken together, our data reveal a novel role of vinpocetine in reduction of pathogenesis of atherosclerosis, at least partially through suppressing LOX-1 signaling pathway. Given the excellent safety profile of vinpocetine, this study suggests vinpocetine may be a therapeutic candidate for treating atherosclerosis.

1. Introduction

Atherosclerosis is the major trigger of myocardial infarction and stroke, the leading causes of morbidity and mortality in the developed countries. Cholesterol deposition in the artery wall plays a critical role in atherosclerosis [1–3]. Elevated plasma lipid, particular the low density lipoprotein (LDL) cholesterol, is an important risk factor of atherosclerosis [1,4], and the clinical benefit of statins in atherosclerosis management is primarily dependent on the cholesterol lowering effect [5]. LDL can pass through endothelium of vessel wall and reside in sub-endothelial space, where LDL can be oxidatively modified to become oxidized LDL (ox-LDL). ox-LDL is the primary trigger of endothelial dysfunction and vascular inflammation, playing a key role in the development and progression of atherosclerosis [2,6]. Scavenger receptor such as lectin-like oxidized LDL receptor-1 (LOX-1) is a major ox-LDL receptor [6,7]. LOX-1 is expressed at low levels in healthy vascular cells and up-regulated by many pro-atherogenic stimuli and its agonist ox-LDL [6,7]. Macrophages in the subendothelial space ingest ox-LDL, become foam cells, and cause fatty streak formation, which represents major pathological characteristics at the early stage of atherogenesis [2,6].

Vinpocetine is produced by slightly altering the vincamine molecule, an alkaloid extracted from the periwinkle plant, Vinca minor. Vinpocetine was originally discovered and marketed in 1978 under the trade name Cavinton (Hungary). Since then, vinpocetine has been widely used in many countries for the prevention of cerebrovascular disorders and cognitive impairment, including stroke, senile dementia, and memory disturbances [8]. For instance, different types of vinpocetine-containing memory enhancers (Intelectol in Europe and Memolead in Japan) are currently used as dietary supplements worldwide. To date, there have been no reports of its significant side effects, toxicity, or contraindications at the reported therapeutic doses [9]. Vinpocetine is a cerebral vasodilator that improves brain blood flow [10], and a cerebral metabolic enhancer by enhancing oxygen and glucose uptake and increasing neuronal ATP production [11]. Vinpocetine appears to have multiple cellular targets, including cyclical nucleotide phosphodiesterase (PDE1), and voltage-dependent Na^+ channels and Ca^{2+} channels [12]. We have recently found that IKK is a new cellular target of vinpocetine and vinpocetine attenuates TNF-α-induced NF-κB activation and the subsequent induction of proinflammatory mediators in multiple cell types, including vascular smooth muscle cells, endothelial cells and macrophages [13]. More recently, we have demonstrated that vinpocetine is able to suppress intimal hyperplasia by inhibiting vascular smooth muscle cell proliferation and migration [14]. In the present study, we provide evidence to demonstrate that vinpocetine attenuates lipid deposition and atherosclerotic lesion development in a mouse model of

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atherosclerosis, at least partially through down-regulating LOX-1 expression, inhibiting ox-LDL uptake, and preventing macrophage foam cell formation.

2. Materials and methods

2.1. Animals

All animals were used in accordance with the guidelines of the National Institutes of Health and American Heart Association for the care and use of laboratory animals. The procedures were performed in accordance with experimental protocols that were approved by the University Committee on Animal Resources at the University of Rochester. Male C57BL/6J ApoE knockout mice (Jackson Laboratories) at age of 8 weeks were fed with the normal chow diet or high-fat diet containing 1.25% cholesterol (Research Diets D12108C) for 16 weeks. Vinpocetine (5 mg/kg of body weight) or same volume of vehicle was administrated via an i.p. route once every other day for 16 weeks as previously described [14].

2.2. Atherosclerotic lesion assessment

Atherosclerotic lesion area was quantified by *En face* Oil-red O staining as described previously [15]. Briefly, mice were anesthetized by i.p. injection with 80 mg kg^{-1} ketamine and 5 mg kg^{-1} xylazine, and perfused with saline and 10% neutral buffered formalin (10% NBF), then fixed overnight with 10% NBF. The heart and aortas were carefully removed and cleaned under a dissecting microscope. All peripheral fat and connective tissue was removed. The vessels were then cut open longitudinally in PBS. Aortas were rinsed with 60% isopropanol for 5 min and stained with Oil-red O solution for 15 min. Vessels were then rinsed with 60% isopropanol for 15 min, followed by several rinse with distilled water. After staining, vessels were precisely kept open with entire luminal surface area faced up on a microscope slide. Images were captured by MZ12.5 microscope (Leica) with a SPOT camera (SPOT Insight 4; Diagnostic Instruments, Inc.). The lesion area was quantified using Image-Pro 6.2 software (Media Cybernetics). For aortic valve lesion quantification, heart was fixed in 10% and incubated with two changes of 30% sucrose–PBS within 48 h at 4 °C and embedded into OCT. The cross cryostat sections (6 mm) were cut at 100 μm intervals. Slides were stained with hematoxylin and eosin (H&E) and Images were captured with microscope (BX41, Olympus) and with digital camera (Spot Insight 2, Diagnostic Instruments, Inc.). The lesion area (remodeling area) was quantified using Image-Pro 6.2 software (Media Cybernetics) and four sections from each animal were examined.

2.3. Blood pressure, serum cholesterol measurement

Blood pressures were measured using a non-invasive tail-cuff procedure and Visitech BP-2000 blood pressure analysis system as described previously [16]. The cholesterol of serum LDL and HDL was measured using HDL and LDL/VLDL Cholesterol Assay Kit (Abcam) according to the manufacturer's instructions.

2.4. Cell culture

Murine RAW264.7 macrophage cell line (ATCC, Rockville, MD) were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) in a humidified incubator (37 °C, 5% CO_2).

2.5. RNA isolation and RT-PCR

Total cellular RNA was isolated from RAW264.7 cells using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized by reverse transcription using Taqman reverse transcription kit (Applied Biosystems) following the manufacturer's instructions. The real time PCR was performed using iQ™ SYBR Green supermix (Bio-Rad) with LOX-1 primers: 5′-CAAGATGAAGCCTGCGAATGA (forward) and 5′-ACCTGGCGTAATTGTGTCCAC (reverse). The relative quantities of mRNAs were obtained by normalizing with glyceraldehydes-3-phosphate dehydrogenase (GAPDH).

2.6. Immunofluorescent staining

Immunostaining were performed as described previously [14]. Briefly, frozen sections were fixed with 4% paraformaldehyde and permeabilized in 0.2% Triton X-100. The sections were blocked with Dako serum-free blocking solution (M0841, Dako) and incubated with primary antibody. The primary antibodies were MAC-2 (CL8942AP, Cedarlane) and LOX-1 (sc-11653, Santa Cruz). The sections were then incubated with fluorescence conjugated secondary antibodies. The nuclear was stained with DAPI. Images were captured with an Olympus (BX-51) fluorescent microscope. The Oil-red O positive area, LOX-1 expression and Mac-2 positive area were quantified using Image-Pro 6.2 software (Media Cybernetics).

2.7. Ox-LDL accumulation

To measure fluorescence-labeled ox-LDL (Dil-ox-LDL) uptake and accumulation, RAW264.7 cells were pretreated with different doses of vinpocetine for 24 h in DMEM containing 0.1% FBS, then loaded with 10 μg/ml Dil-ox-LDL (Biomedical Technologies, Inc.) for an additional 4 h. Cells were then washed with PBS and fixed with formalin. Nuclear was stained with DAPI. Images were taken with a BX-51 Olympus fluorescent microscope. To measure regular ox-LDL uptake and accumulation, cells were treated with vinpocetine as described above, then loaded with 50 μg/ml ox-LDL (Biomedical Technologies, Inc.) for 24 h. Cells were then washed, fixed and stained with Oil-red O solution. The Dil-ox-LDL and Oil-red O staining intensities were quantified using Image-Pro 6.2 software (Media Cybernetics) and five fields were examined for each sample.

2.8. Statistical analysis

Quantitative results are expressed as mean ± SEM or mean ± SD as indicated. All results shown were confirmed by at least three independent experiments. Data were analyzed by one-way ANOVA. $^{*}P$-values < 0.05 was considered statistically significant.

3. Results

3.1. Vinpocetine ameliorates atherosclerosis formation

To assess effects of vinpocetine on atherosclerotic formation, we utilized hyperlipidemia-induced atherosclerosis in ApoE knockout mouse, a well-established mouse model of atherosclerosis [17]. ApoE−/− mice were fed with normal chow diet or Western high-fat diet for 16 weeks, and received 5 mg/kg vinpocetine or equal volume of vehicle once every two days via i.p., as previously described [14]. The atherosclerotic lesions were evaluated by morphometric analyses of *En face* arterial trees stained with Oil-red O for lipids. The lesion size was quantified by the percentage of

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