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Regulated expression of endothelial lipase in atherosclerosis

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ABSTRACT

Endothelial lipase (EL) is a major determinant of HDL metabolism and associated with the development of atherosclerosis, however the regulated expression of EL in atherosclerosis is unclear. In this study, we investigated EL expression in rat atherosclerosis and explored the potential mechanisms regulating EL expression by employing LPS on Raw264.7 cells in vitro. Rat atherosclerosis model was established fed on high-cholesterol diet (HCD) combined with vitamin D_2 (VD). Western blotting and immunochemistry staining revealed that EL expression was increased in the aorta, especially the atherosclerotic lesions in HCD rats. LPS increased EL expression in a time and dose dependent manner in Raw264.7 cells and NFκB inhibitor, PDTC attenuated the effects of LPS on EL. EMSA revealed that LPS stimulated NFκB binding to EL promoter. In summary, EL was upregulated in rat atherosclerosis and LPS stimulates EL expression in vitro through NFκB activation.

1. Introduction

Plasma concentrations of high-density lipoprotein (HDL) cholesterol are inversely correlated with the risk of atherosclerotic cardiovascular disease (Gordon and Rifkind, 1989; Linsel-Nitschke and Tall, 2005). HDL cholesterol is low in patients with metabolic disorders, such as obesity, insulin resistance and diabetes (Krauss, 2004; Reaven, 2004). However, the mechanism contributing to variation of HDL cholesterol levels in humans remains to be elucidated. It is generally postulated that variations in plasma HDL cholesterol levels are determined by both the rate at which HDL cholesterol is produced and the rate of catabolism of HDL particles (Ma et al., 2003). Recently, endothelial lipase (EL) has been recognized as a key enzyme that modulates HDL metabolism (Hirata et al., 1999; Jaye et al., 1999). EL is a new member of the triglyceride lipase gene family, with highest identity (45%) to lipoprotein lipase (LPL) followed by hepatic lipase (HL, 40%) and pancreatic lipase (31%) (Hirata et al., 1999; Jaye et al., 1999; Choi et al., 2002). In contrast to LPL or HL, however, EL acts primarily as a phospholipase with HDL as a preferred substrate. Overexpression of EL in mice resulted in reduced plasma HDL levels and similar results were subsequently reported in EL transgenic mice (Ishida et al., 2003; Maugeais et al., 2003). Oppositely, in mice injected with EL-neutralizing antibodies and in EL knockout mice, HDL levels were significantly elevated compared with control (Ishida et al., 2003; Jin et al., 2003a,b). In addition to its lipolytic enzyme properties in HDL metabolism, there is accumulating evidences that EL serves as a bridge between proteoglycans, circulating lipoproteins and endothelial cells (Strauss et al., 2002; Broedl et al., 2004). The non-enzymatic bridging functions of EL can increase cellular lipoprotein uptake and monocyte recruitment (Broedl et al., 2004). HDL of the EL and apo-E double knockout mice was higher than that of apo-E knockout mice and there was a significant 70% decrease of atherosclerotic lesion area in the double knockout mice (Ishida et al., 2004). These findings suggested that EL may have indirect or direct atherogenic actions in vivo.

EL is unique in that it is synthesized by endothelial cells (Jin et al., 2003a,b), however it is also expressed to a lesser extent by non-endothelial cells such as macrophages, smooth muscle cells, hepatocytes and a variety of tissues, although its regulation and function in atherosclerosis are poorly understood (Hirata et al., 1999; Jaye et al., 1999). Atherosclerosis is now widely recognized as an inflammatory disease and hypercholesterolemia plays an important role in the initiation and progression of atherosclerosis. It was reported that proinflammatory cytokines such as tumor necrosis factor-α and interleukin (IL)-1β, have been shown to upregulate EL expression in endothelial cells and smooth muscle cells (Hirata et al., 2000; Jin et al., 2003a,b). However, there is relative paucity of knowledge regarding regulation of EL expression in macrophages which can transform into foam cells and play a pivotal role in the development and progression of atherosclerosis. Lipolysaccharide (LPS) is one of the major inflammatory stimuli which can initiate and promote atherosclerosis in animals and humans. In the present study, therefore, we aimed to explore the expression of EL in rat model of atherosclerosis, further to illuminate the regulation and possible mechanism of EL expression in macrophages, particularly

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under conditions of inflammatory stimulation caused by LPS. Our results revealed that EL expression is increased in the aorta of rats fed on high-cholesterol diet and LPS upregulates EL expression via NFκB pathway in macrophages.

2. Materials and methods

2.1. Chemicals and reagents

Kits for total cholesterol (TC), free cholesterol (FC), low density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) were purchased from Beijing Zhongsheng Bioengineering Company, China. Vitamin D_2, β-glycerophosphate were obtained from Sigma; RPMI-1640 Medium and Trizol were from Invitrogen; fetal bovine serum (FBS) was from Gibco; dNTP, AMV, Taq, RNAsin and oligo(dT) were from Takala. LPS from Escherichia coli serotype 026:B6 (tissue culture-tested, L-2654) was purchased from Sigma. PDTC was purchased from Sigma. Other chemicals and reagents were of analytical grade.

2.2. Animals and atherosclerosis model in rats

Male Sprague–Dawley rats (weighing 190–220 g) were purchased from the Experimental Animal Center, Sun yat-sen University in China, and were housed under standard conditions (room temperature 20 ± 1 °C, humidity 60 ± 10%, lights from 6 a.m. to 6 p.m.) and given water freely. All experimental procedures were performed in accordance with the Guidelines of Animal Experiments from the Committee of Medical Ethics, National Health Department of China (1998).

Rats were randomly divided into 2 groups of 10 each: Control group (CTL), and high-cholesterol diet group (HCD). The preparation for high-cholesterol diet induced atherosclerosis was as described by Kitagawa et al. (1992) and Tang et al. (2006). In short, rats in CTL group were fed with standard chow; rats in HCD group were orally administered with 300,000 IU/kg/day vitamin D_2 for 4 consecutive days followed by consuming HCD for 12 weeks. The composition (%) of high-cholesterol diet (HCD): standard chow (94.3), cholesterol (2), lard (3), cholic acid (0.5), propylthiouracil (0.2).

2.3. Biochemical parameters in serum

At the end of the 12th week, blood samples for biochemical analysis were collected from fasting animals (12 h) by puncture in the retro-orbital sinus under light ether anesthesia. Serum concentration of TC was assayed enzymatically by using commercial kits and serum HDL-C and LDL-C were determined by precipitation with phosphotungstic acid/magnesium chloride or with heparin/sodium citrate, respectively followed by the assay same as TC (Tang et al., 2007).

2.4. HE and immunohistochemistry staining

Specimens of aortic arch in rats were fixed in 10% buffered formalin. After being post-fixed for about half an hour in Carnoy's fluid, samples were embedded in paraffin. Blocks were sectioned and stained with hematoxylin and eosin as described (Tang et al., 2006).

For immunohistochemistry staining, after removing paraffin and rehydrating, sections were incubated in 3% H_2O_2 for 30 min at room temperature (RT), washed with double distilled water and PBS, and heated in 1% citric acid to 199 F for 25 min. Sections were blocked with 10% goat serum (Vector Laboratories, Burlingame, CA) for 30 min and then incubated with rabbit anti-EL (1:1000 dilution, Cayman Chemical) over night. After washing, sections were incubated with a biotinylated goat anti-rabbit secondary antibody (Vector Laboratories) at RT for 40 min. After washing, sections were incubated with the ABCElite reagent (Vector Laboratories) for 10 min. The immunostaining was visualized using a peroxidase substrate kit (Vector Laboratories) according to the manufacturer's protocol. The sections were also counterstained with hematoxylin, dehydrated in alcohol, cleared with Histo-clear, and examined by light microscopy. For a negative control, the same protocol was used with antigen dilution reagent instead of the primary antibody.

2.5. Cell culture

The murine RAW264.7 macrophage-like cell line was routinely cultured in RPMI-1640 Medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and and 100 μg/ml streptomycin at 37 °C and humidified 95% air/5% CO_2.

2.6. RNA extraction and reverse transcription-PCR

Total RNA was extracted by using the TRIzol method (Invitrogen). Purified RNA (1 μg) was reversely transcripted using the RNA PCR Kit (TaKaRa) according to the manufacturer's instructions. The cDNAs were amplified as follows: 94 °C for 5 min, then different cycles consisting of 45 s at 95 °C, 30 s at different T_m and, 1 min at 72 °C, and 72 °C for 10 min. PCR products (10 μl) were electrophoresed on a 2% agarose gel. For the semi-quantification, an image of the gel was captured and the intensity of bands was analyzed by Labworks software (UVP, Upland, CA, USA). 18 s was the internal control to normalize the RNA loading. The set of primers used for mouse EL was: forward, 5′-TGA GTG GCA TGT TTG AGA GC-3′, and reverse, 5′-CAG CCT TCT GTT GAT GTC CA-3′. For mouse 18 s amplification primers used were: forward, 5′-GTC CCC CAA CTT CTT AGA G-3′, and reverse, 5′-CAC CTA CGG AAA CCT TGT TAC-3′.

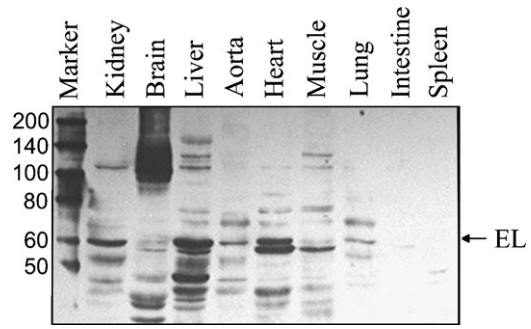

Fig. 1. EL protein expression in different tissues of rats assessed by Western blotting.

2.7. Western blot analysis

For EL protein detection, cells or aorta tissue samples were lysed in RIPA buffer (25 mM Tris–HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS). For NFκB detection, nuclear protein was extracted with the CelLytic NuCLEAR Extraction Kit (sigma). Protein concentrations were determined by the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Equal amounts of protein were loaded onto 10% SDS-polyacrylamide gel, separated by electrophoresis and transferred onto PVDF membranes (Biorad, Hercules, CA, USA). After blocked with 5% skim milk for 1 h at room temperature membranes were incubated with rabbit anti-EL (1:1000 dilution, Cayman Chemical), rabbit anti-IκBα (1:1000 dilution, Santa Cruz) or mouse anti-NFκB (1:10,000 Santa Cruz) overnight at 4 °C and with anti-α-tubulin (1:10,000 dilution, Sigma) for 1 h at room temperature. Then horseradish peroxidase (HRP)-labeled second antibodies (1:2000 dilution, Cell Signaling) were added for 1 h at room temperature. Immunoreactive bands were detected with the Super-Signal West Pico Chemiluminescent Substrate (Pierce). The intensity of protein bands was analyzed by Labworks software.

2.8. Electrophoresis mobility shift assay

Raw264.7 macrophages were treated with or without 100 ng/ml LPS for 0–2 h. Nuclei protein was extracted using CelLytic NuCLEAR Extraction Kit (sigma) according to the manufacturer's instructions. Nuclear extracts (10 μg of protein) were then used to assess DNA binding activity by electrophoresis mobility shift assay using a biotin-labeled oligonucleotide probe containing the sequence of the NFκB binding site in the EL promoter, 5′-cca ggg cgt ggc cag tag agc-3′, and, 5′-gct cta ctg gcc acg ccc tgg-3′.

2.9. Statistics

Results are presented as mean ± SE. Statistical analysis was performed by Student's *t*-test and one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test when appropriate. The SPSS 13.0 program was used for the calculations. $P < 0.05$ was considered statistically significant.

3. Result

3.1. Expression of EL in different tissues of rats

The expression of EL in rats is not fully understood; especially it is not clear about the regulation of EL in the atherosclerosis model of rats. To investigate EL expression in the normal rats, we extracted total protein from different tissues and performed Western blotting assay. As shown in Fig. 1, we can see that EL is highly expressed in the liver, kidney and heart; lower in the aorta and lung; there is almost no EL tested in the brain, muscle, spleen and intestine.

3.2. Plasma lipids profile

Table 1 shows plasma lipid level in the rats of CTL and HCD groups at the end of 12 week. There was no significant differ-