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Apolipoprotein B of low-density lipoprotein impairs nitric oxide-mediated endothelium-dependent relaxation in rat mesenteric arteries

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indomethacin (PubChem CID: 3715)
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apamin (PubChem CID: 16129677)
N_ω-nitro-L-arginine methyl ester
(PubChem CID: 39836)

ABSTRACT

Apolipoprotein B (ApoB) of low-density lipoprotein (LDL) causes endothelial dysfunction in the initial stage of atherogenesis. The present study was designed to explore the underlying molecular mechanisms involved. Rat mesenteric arteries were organ cultured in the presence of different concentrations of ApoB or LDL. Vasodilation induced by acetylcholine was monitored by a sensitive myograph. Nitric oxide (NO), endothelium-dependent hyperpolarizing factor (EDHF) and prostacyclin (PGI_2) pathways were characterized by using specific pathway inhibitors. Real-time PCR and immunohistochemistry with confocal microscopy were used to examine alteration of mRNA and protein expressions for NO synthases (eNOS and iNOS) and cycloxygenase (COX), respectively. Lipid peroxidation was measured by thiobarbituric acid reactive substances. In the presence of either LDL or ApoB for 24 h concentration-dependently attenuated the endothelium-dependent vasodilation. Immunohistochemistry staining of endothelial cell marker CD31 was weaker in the presence of LDL, indicating that LDL induced damage to the endothelium. Using the pathway specific inhibitors demonstrated that LDL-induced impairing vasodilation was mainly due to attenuation of NO pathway. This was supported by decreasing mRNA (real-time PCR) and protein expression (immunohistochemistry) for eNOS and iNOS, but not COX, in the presence of LDL. In addition, the levels of lipid peroxidation significantly increased in the presence of LDL for 24 h. In conclusion, ApoB of LDL impairs vasodilation with damaging the endothelium and attenuating the NO-mediated endothelium-dependent relaxation, which might associate with lipid peroxidation and contribute to the development of cardiovascular disease.

1. Introduction

Endothelium-derived nitric oxide (NO) is a paracrine factor that controls vascular tone, inhibits platelet function, prevents adhesion of leukocytes and reduces proliferation of the intima (Forstermann, 2010). Impaired endothelium-dependent vasodilation represents an early manifestation of atherosclerosis and ischemic vascular disease (Egashira, 2002). Normal vasodilation is mediated by a number of endothelial systems including NO, prostaglandins (PGI_2 and PGE_2), and a family of endothelial-derived hyperpolarizing factors (EDHF) (Giles et al., 2012). NO is produced by nitric oxide synthase (NOS) in endothelial cells and relaxes smooth muscle cells by activating guanylate cyclase in the vascular smooth muscle cells. This enzyme can be inhibited by the NOS inhibitors such as N_ω-nitro-L-arginine methyl ester (L-NAME) (Moncada et al., 1991). Prostacyclin (PGI_2) is formed by cyclooxygenase (COX) from arachidonic acid in endothelial cells. It relaxes smooth muscle cells by activating adenylate cyclase. Formation of PGI_2 can be inhibited by indomethacin, a general COX inhibitor (Moncada, 1982). EDHF is an endothelium-derived vasodilation mediator, distinct from NO and PGI_2, which hyperpolarizes vascular smooth muscle cells. A combination of the potassium channel inhibitors, charybdotoxin and apamin, inhibits both EDHF-mediated vascular smooth muscle cell hyperpolarization and the subsequence of vasodilation (Chataigneau et al., 1998; Doughty et al., 1999).

Endothelial cells are the first cells to experience the impact of hyperlipidemia in lesion-prone areas (Ivan and Antohe, 2010). Abundant data have confirmed the exacerbating effects of low-density lipoprotein (LDL) and its modified forms on endothelial dysfunction in cells (Ji et al., 2006), animals (Dai et al., 2004) and human (Cosentino et al., 2008). LDL is mainly oxidized in the subendothelial layer of the vascular wall (Zhang et al., 2010). LDL

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oxidation is the key event in hyperlipidemia-induced dysfunction of endothelium and vascular damage, which includes attenuation of endothelium-dependent vasodilation (Hein et al., 2000), increased production of intracellular reactive oxygen species, and activation of transcription factor nuclear factor-kappaB (NF-κB) in cultured human endothelial cells (Matsunaga et al., 2003). However, exposure to LDL for only few hours has not seen such effects on vascular endothelial function (Galle and Bassenge, 1991), suggesting that longer time exposure is needed for oxidation of LDL and dysfunction of endothelium.

We have developed an organ culture model for studying functions of intact arterial ring segments (Adner et al., 1996), which provides the possibility for evaluating the effects of cardiovascular risk factors on pathophysiological changes at both functional and molecular levels. This model has been frequently used to study increased vasocontractility by cardiovascular risk factors such as smoke particles (Sandhu et al., 2010) and minimally modified LDL (Li et al., 2012, 2013). In addition to this, dysfunction of the endothelium and reduced endothelium-dependent relaxation also contribute to the increased vasocontractility, while this is relatively less studied.

LDL particles have two major compartments, an apolar lipid core and an outer amphipathic shell. The shell composed of a phospholipid monolayer and one single copy of apolipoprotein B (ApoB) that is atherogenic (Prassl and Laggner, 2009). In contrast, high-density lipoproteins contain exchangeable apolipoproteins of the A, C and E families that are antiatherogenic (Lund-Katz and Phillips, 2010). Clinical studies demonstrate that the atherogenic lipoprotein can cause endothelial dysfunction in the initial stage of atherogenesis with impairing endothelium-dependent vasodilation (Kraml et al., 2004). The ApoB/A1 levels, but not LDL-cholesterol, were inversely related to endothelium-dependent vasodilation (Lind, 2007), indicating that the ApoB might be an early marker of structural vascular changes, whereas high-density lipoprotein specifically relates to endothelial vasodilatory function (Steer et al., 2002). However, the underlying molecular mechanisms involved in ApoB of LDL-impaired endothelium-dependent vasodilation are not fully understood. The present study was designed to explore how ApoB of LDL impairs vascular endothelium-dependent vasodilation. We have demonstrated that 24 h of exposure to ApoB and LDL induces damage to the endothelium and impairs endothelium-dependent vasodilation mainly by attenuating the NO-pathway. This might be relevant to the early stage of cardiovascular pathogenesis in clinical patients.

2. Material and methods

2.1. Drugs and reagents

Acetylcholine, 5-hydroxytryptamine (5-HT), indomethacin, charybdotoxin, apamin, N_ω-nitro-L-arginine methyl ester (L-NAME), LDL (lyophilized powder reconstituted with solution of 150 mM NaCl and 0.01% EDTA, pH 7.4) and ApoB (lyophilized powder) were purchased from Sigma-Aldrich (St. Louis, MO, USA). LDL and ApoB were diluted/dissolved in solution with 150 mM NaCl and 0.01% EDTA, pH 7.4, according to the manufacture's instruction. All other drugs were dissolved in double distilled water. The confection and storage of the reagents were according to the product information sheet and preparation guide.

2.2. Tissue preparation and organ culture procedure

Male Sprague-Dawley rats (weighting 300–350 g) were anaesthetized with CO_2 and exsanguinated. The vessels were cut into 1 mm long cylindrical segments and incubated at 37 °C in humified 5% CO_2 in air for different time periods. Culture was carried out in a 96-well plate, one segments in each well, containing 250 μL of Dulbecco's modified Eagle's medium (DMEM) supplying with L-glutamine (584 mg/L) and supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL). The organ culture experiments were performed with LDL or ApoB or vehicle (solution with 150 mM NaCl and 0.01% EDTA, pH 7.4). The experimental protocol was approved by Lund University Animal Ethics Committee.

2.3. In-vitro pharmacology

Following the incubation in DMEM, the vessel segments were transferred to buffer solution (without LDL or ApoB) for functional results. Arterial segments were immersed in temperature-controlled (37 °C) myographs (Organ Bath Model 700MO, J.P. Trading, Aarhus, Denmark) containing 5 ml bicarbonate buffer solution. The solution was continuously aerated with 5% CO_2 in O_2 resulting in a pH of 7.4. The arterial segments were mounted for continuous recording of isometric tension by the Chart software (ADInstruments, Hastings, UK). A resting tone of 2.5 mN was applied to each segment. The segments were allowed to stabilize at this tension for at least 1.5 h. After equilibration, the vessels were pre-constricted with 5-HT ($10^{-5.5}$ or $10^{-5.7}$ M). Once the sustained tension was obtained, acetylcholine (from 10^{-10} to 10^{-4} M) was cumulatively added to the tissue baths and the isometric tension was recorded. Vasodilation was expressed as percentage of pre-constriction with 5-HT (Alm et al., 2002).

2.4. Characterization of endothelium-dependent vasodilation

Acetylcholine-induced vasodilation mainly consists of NO-, PGI_2- and EDHF-mediated pathways. NO-mediated dilatation induced by acetylcholine was studied in the presence of indomethacin (10^{-5} M), charybdotoxin ($10^{-7.3}$ M) and apamin (10^{-6} M). EDHF was studied in the presence of L-NAME (10^{-4} M) and indomethacin. PGI_2 was studied in the presence of L-NAME, charybdotoxin and apamin. The inhibitors were added 20 min before administration of 5-HT pre-constriction as described before (Alm et al., 2002; Zhang et al., 2006).

2.5. Real-time PCR

Arterial segments were homogenized in 1 ml of the RNApro solution (Q-BIOgene, CA, USA) by using a FastPrep instrument (Q-BIOgene, CA, USA). The total RNA was extracted following a protocol from the FastRNA Pro kit (Q-BIOgene, CA, USA) supplier. Reverse transcription of total RNA to cDNA was carried out using TaqMan Reverse Transcription Reagents (PE Applied Biosystems, CA, USA). The real-time quantitative PCR was performed with the GeneAmp SYBR Green PCR kit in a GeneAmp 7500 sequence detection system (PE Applied Biosystems, CA, USA) (Xu et al., 2008). Specific primers for the rat cycloxygenase-1 (COX-1) mRNA, cycloxygenase-2 (COX-2) mRNA, endothelial NO synthase (eNOS) mRNA and inducible NO synthase (iNOS) mRNA were designed as follows, respectively:

COX-1	Forward: 5′-GTGAATGCCACCTTCATCCG-3′
	Reverse: 5′-CACCGTGAGTACCCAGCCC-3′
COX-2	Forward: 5′-CAGAGCAGAGAGATGAAATACCAGTC-3′
	Reverse: 5′-GAAGCGTTTGCGGTACTCATT-3′
eNOS	Forward: 5′-TCCTGGTGCGTCTGGACACT-3′
	Reverse: 5′-CTCCTGCTGTTCGCTGGACT-3′
iNOS	Forward: 5′-AACAGTGGCAACATCAGGTCG-3′
	Reverse: 5′-CATGCTTCCCATCGCTCC-3′