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Gene transfer of dimethylarginine dimethylaminohydrolase-2 improves the impairments of DDAH/ADMA/NOS/NO pathway in endothelial cells induced by lysophosphatidylcholine

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Abstract

Dimethylarginine dimethylaminohydrolase (DDAH) is a key enzyme responsible for the metabolism of nitric oxide (NO) synthase (NOS) inhibitor asymmetric dimethylarginine (ADMA), and DDAH2 is the predominant isoform in vascular endothelium. Lysophosphatidylcholine (LPC) and ADMA are implicated in endothelial dysfunction of atherosclerosis. This study was to examine changes in DDAH/ADMA/NOS/NO pathway in endothelial cells after exposure to LPC and investigate whether DDAH2 gene transfer could reverse LPC-induced changes. Human endothelial cell line ECV304 cells were transfected with recombinant pcDNA3.1-hDDAH2 plasmid and incubated with 3 μmol/L LPC for 48 h. Cells were harvested for assays of DDAH transcription, DDAH and NOS activities. The culture medium was collected for measurements of ADMA and nitrite/nitrate concentrations. LPC treatment suppressed DDAH2 transcription and DDAH activity in parallel with increased ADMA concentration, inhibited NOS activity and decreased NO metabolites content. DDAH2 gene transfer not only prevented the suppression of DDAH activity and the elevation of endogenous ADMA, but also attenuated the inhibition of NOS activity and the reduction of NO level induced by LPC in endothelial cells. These results suggest that LPC induces impairments of DDAH/ADMA/NOS/NO pathway, and DDAH2 gene transfer could improve the LPC-elicited impairments in endothelial cells.

Keywords: Dimethylarginine dimethylaminohydrolase; Asymmetric dimethylarginine; Nitric oxide synthase; Nitric oxide; Lysophosphatidylcholine

1. Introduction

Nitric oxide (NO) is synthesized from L-arginine by NO synthase (NOS) in endothelial cells, and plays an important role in regulating vascular homeostasis (Cooke and Dzau, 1997). Endothelial dysfunction, characterized by reduced NO synthesis and impaired NO-mediated endothelium-dependent vasodilatation, is an initial factor predisposing to the development of atherosclerosis. It has been generally recognized that asymmetric dimethylarginine (ADMA) is an endogenous competitive NOS inhibitor (Vallance et al., 1992) and its elevation may account for reduced NO generation observed in numerous disorders associated with atherosclerosis such as hypercholesterolemia (Yu et al., 1994), hyperhomocysteinemia (Fu et al., 2005), diabetes (Xiong et al., 1997; Xiong et al., 2005), and aging (Xiong et al., 2001). We previously demonstrated that serum ADMA levels were increased in high cholesterol-fed rabbits (Yu et al., 1994), which was associated with impaired endothelium-dependent vasodilatation (Xiong et al., 1996). Similar results were observed by Böger et al. in monkeys (Böger et al., 2000a,b) and humans (Böger et al., 1998) with hypercholesterolemia. Therefore, ADMA has emerged as a novel risk factor for endothelial dysfunction.

Numerous studies demonstrated that oxidized low-density lipoprotein (ox-LDL) and its major lipid constituent lysophosphatidylcholine (LPC) to be implicated in the initiation and progression of endothelial dysfunction associated with atherosclerosis (Yla-Herttuala et al., 1989; Kugiyama et al., 1990).

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Exposure of normal blood vessels to LPC *in vitro* can mimic the inhibitory effect of ox-LDL on endothelium-dependent relaxation, which is similar to the impairment of endothelium-dependent relaxation observed in atherosclerosis (Kugiyama et al., 1990; Mangin et al., 1993; Deng and Xiong, 2005). Previous studies showed that LPC increased the oxidative degradation of NO (Ohara et al., 1994), and inhibited the high-affinity arginine transporter with a subsequent reduction in NO production (Kikuta et al., 1998), thereby leading to endothelial dysfunction. Recent investigation demonstrated that administration of exogenous LDL to normal rats also caused a significant elevation of serum ADMA levels and a corresponding impairment of endothelium-dependent relaxation (Jiang et al., 2002). Similarly, exposure of cultured human endothelial cells to ox-LDL (Ito et al., 1999) or LPC (Jiang et al., 2003) also increased ADMA contents in culture medium, simultaneously decreased NO production. Taken together, these studies support the proposal that the elevated endogenous ADMA is a novel mechanism for ox-LDL or LPC-elicited NO deficiency or endothelial dysfunction.

Endogenous ADMA is constantly produced in the course of normal protein turnover in many tissues, including vascular endothelial cells (Böger et al., 2000a,b). The major pathway for ADMA clearance is hydrolysis by dimethylarginine dimethylaminohydrolase (DDAH) to L-citrulline and dimethylamines (MacAllister et al., 1994). Pharmacological inhibition of DDAH led to local accumulation of ADMA, inhibition of NOS activity in endothelial cells, and impairment of endothelium-dependent relaxation in isolated blood vessels (MacAllister et al., 1996). Conversely, up-regulation of DDAH expression in endothelial cells reduced ADMA levels and increased NO synthesis (Achan et al., 2002). Therefore, DDAH plays a crucial role in the regulation of NO synthesis via modulating endogenous ADMA levels. Up to date, two distinct human DDAH isoforms have been identified, and DDAH1 is typically found in tissues of expressing neuronal NOS, whereas DDAH2 predominates in tissues of expressing endothelial NOS (Leiper et al., 1999). Recently, Chen et al. confirmed that DDAH1 is strongly expressed in coronary endothelium (Chen et al., 2005). Although DDAH1 overexpression could increase NOS activity in cultured endothelial cells and transgenic animals (Dayoub et al., 2003), it has been shown that the elevation of ADMA induced by ox-LDL was secondary to the decrease of DDAH activity, whereas DDAH1 protein expression was unchanged under this condition (Ito et al., 1999). Achan et al. also reported that the increase of DDAH activity elicited by all-*trans*-retinoic acid was only related to the up-regulation of DDAH2 expression in endothelial cells (Achan et al., 2002). In view of these reports, we speculated that DDAH2 may play a crucial role in the regulation of endogenous ADMA levels in endothelial cells. Accordingly, the present study was designed to determine whether LPC could impair the NO synthesis in endothelial cells by inhibition of DDAH2 transcription or DDAH activity and subsequent elevation of endogenous ADMA, and further investigate whether DDAH2 gene transfer could reverse these changes in DDAH/ADMA/NOS/NO pathway induced by LPC in endothelial cells.

2. Materials and methods

2.1. Cell culture

Spontaneously transformed human umbilical vein endothelial cells (ECV304, ATCC, Manassas, USA) were grown in RPMI1640 medium supplemented with 10% fetal bovine serum and incubated at 37 °C in a humidified atmosphere of 5% CO_2. This cell line retains many characteristics of primary endothelial cells, including the expression of DDAH and NOS as well as the synthesis of NO (Ito et al., 1999; Achan et al., 2002).

2.2. Construction of hDDAH2 expression vector

Since DDAH2 is the predominant isoform expressed in cardiovascular system (Böger et al., 2000a,b; Achan et al., 2002) and hypercholesterolemia impaired endothelial DDAH activity while DDAH1 protein expression remained unchanged (Ito et al., 1999), DDAH2 was chosen as the target of gene transfer in the present study. Full-length cDNA of human DDAH2 was obtained by reverse transcription-polymerase chain reaction (RT-PCR). In briefly, total RNA was extracted from endothelial cells using TRIzol reagent according to the manufacturer's recommendation. The obtained total RNA was used for synthesis of cDNA with AMV reverse transcriptase kit. Then the resulting cDNA sample was PCR amplified with hDDAH2 specific primers: 5′-GATCGAATTCAGGATGGGGACGCCGGGG-3′ (sense) and reverse 5′-GATCTCTAGATCGCTGTGGGGGCGTGTG-3′ (antisense) at below conditions: an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 5 min. The final PCR product of 881 bp was ligated into pGEM-T Easy cloning vector (Invitrogen, CA, USA). The recombinant pEGM-hDDAH2 plasmid was digested with restrictive endonuclease Not I, and the obtained fragment of hDDAH2 PCR product was gel-purified, then cloned into Not I-digested, dephosphorylated mammalian expression vector pcDNA3.1 (Invitrogen, CA, USA). Ligation product was transformed into *Eacherichia coli* JM109. Positive clones were identified and sequenced to verify successful insertion in the sense direction.

2.3. Cell transfection with hDDAH2 expressive vector

Endothelial cells were transfected with pcDNA3.1-hDDAH2 expression plasmid, or empty plasmid pcDNA3.1 as transfection control using FuGENE 6 transfection reagent (Roche, Basel, Switzerland) according to the manufacture's instructions. After 24 h, cells were kept in medium containing 800 μg/mL G418 for selection of stably transfected cells. Approximately 14 days after G418 incubation, individual clones resistant to G418 became visible. Single clone was picked up and transferred to culture flasks. Cells with over-transcription of DDAH2 were screen by RT-PCR and normalized to the transcription of glyceraldehydes-3-phosphoate dehydrogenase (GAPDH) as an internal control.