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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.

2.4. Cell treatment with LPC

Subconfluent endothelial cells were cultured in RPMI1640 medium in the absence or presence of LPC (3 μmol/L) for 48 h as control and LPC treated groups respectively. Each group included three subgroups of untransfected cells, pcDNA3.1-hDDAH2-transfected cells, and empty vector pcDNA3.1-transfected cells. The cells without supplementation of LPC got the same concentration of the vehicle (dehydrated ethanol, 1:1000) as solvent control. The dose of LPC used in this study was chosen according to our previous studies showing that incubation of isolated rat aortic ring with 3 μmol/L LPC resulted in a significant impairment of endothelium-dependent relaxation (Deng and Xiong, 2005). Cells from all groups were harvested and stored in −70 °C for assays of transcriptions of DDAH1 and DDAH2 as well as activities of DDAH and NOS. The medium was collected and stored in −70 °C for measurements of dimethylarginines and nitrite/nitrate concentrations.

2.5. Assays of DDAH1 and DDAH2 transcriptions

Total RNA was extracted from cells exposed to LPC and reversely transcribed into cDNA. Primers and PCR procedures for DDAH2 have been described above. Primers for DDAH1 are forward 5′-GCAACTTTAGATGGCGGAGA-3′ and reverse 5′-CCAGTTCAGACATGCTCACG-3′. PCR amplification for DDAH1 was performed as follows: an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of 94 °C for 45 s, 55 °C for 45 s, 72 °C for 45 s, and a final extension at 72 °C for 10 min. The final PCR product is 427 bp and the transcription of DDAH1 was standardized with the corresponding β-actin as an internal control.

2.6. Measurements of DDAH and NOS activities

The cells were sonicated after being resuspended in 1 mL sodium phosphate buffer (0.1 mol/L, pH 6.5) and centrifuged at 3500 g for 30 min. Supernatant was used to assay DDAH activity by the conversion of ADMA to L-citrulline as previously described (Fu et al., 2005). One unit of the enzyme was defined as the amount that catalyzed formation of 1 μmol L-citrulline from ADMA per min at 37 °C.

The NOS activity was assayed by the conversion of L-arginine to NO with a commercial kit according to the manufacturer's instruction (Nanjing Jiancheng Biological Medical Engineering Institute, Jiangsu, China). Briefly, supernatant was incubated with 1 mmol/L reduced nicotinamide-adenine dinucleotide phosphate (NADPH), 1 μmol/L flavin adenine dinucleotide (FAD), 1 μmol/L flavin mononucleotide (FMN), 3 μmol/L tetrahydrobiopterin, 0.6 mmol/L $CaCl_2$, and 100 nmol/L arginine. Reactions were conducted at 37 °C for 15 min and then terminated by a stop buffer from the kit. NO in oxygen-containing solutions is chemically unstable and undergoes rapid oxidation to nitrite/nitrate (stable NO metabolites). To reflect the activity of NOS, the produced nitrite/nitrate was measured using the Griess reagent and a spectrophotometer at 530 nm as previously described (Yin et al., 2007). One unit of the enzyme activity was defined as the amount that catalyzed formation of 1 nmol NO from L-arginine per min at 37 °C. The protein content of the supernatant was measured by Coomassie brilliant blue methods (Barsanti and Duncan, 1977) and used to normalize the activities of DDAH and NOS.

2.7. Assays of ADMA and nitrite/nitrate concentrations

After adding 1.0 mL conditioned medium into a tube containing 5-sulfosalicylic acid (20 mg), the mixture was kept at 4 °C for 10 min. The precipitated protein was removed by centrifugation at 2500 g for 15 min (4 °C), and the supernatant was separated for measurement of ADMA by high-performance liquid chromatography (HPLC) method as previously described (Fu et al., 2005).

The stable end products of NO, nitrite/nitrate, in conditioned media were assayed to reflect NO synthesis by endothelial cells as previously described (Tarpey et al., 2001). Briefly, nitrate was converted to nitrite with aspergillus nitrite reductase, and the total nitrite was measured with the Griess reagent. The absorbance was determined at 550 nm with a spectrophotometer.

2.8. Assessment of cell viability

Lactate dehydrogenase (LDH) generally exists in the cytoplasm of living mammalian cells. When cells are damaged, a large amount of LDH will leak into extracellular space. So the level of LDH activity in culture medium may reflect the cell viability or degree of cell injury. In the present study, LDH released into the culture medium of endothelial cells was measured spectrophotometrically. In brief, after untransfected, pcDNA3.1-DDAH2-transfected, and empty vector pcDNA3.1-transfected ECV304 endothelial cells were treated with 3 μmol/L LPC for 48 h, the conditional medium was collected for the measurement of LDH activity by the conversion of lactic acid to pyruvic acid as previously described (Jiang et al., 2003) with a commercial assay kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu China). The amount of the resultant with pyruvic acid was determined by reading the absorbance at 440 nm. One unit of the enzyme activity was defined as the amount that catalyzed formation of 1 mmol/L pyruvic acid per min at 37 °C and normalized to the cell number.

2.9. Reagents

The human umbilical vein endothelial cell line (ECV304) was purchased from America Type Culture Collection (ATCC, Manassas, USA). LPC, ADMA, antipyrine, diacetyl monoxime were obtained from Sigma (St Louis, MO, USA). LPC was dissolved in ethanol dehydrated ethanol to be made the stock solution of 3 mmol/L LPC. RPMI1640, fetal bovine serum, TRIzol were purchased from Gibco (Gaithersburg, MD, USA). AMV reverse-transcription reagents were purchased from Promega (Madison, WI, USA). Restriction endonucleases and

other reagents used for molecular cloning were from MBI (Vilnius, Lithuania). Specific PCR primers for DDAH1, DDAH2, and GAPDH were synthesized by BioAsia Biotechnologies (Shanghai, China). NOS, nitrite/nitrate, LDH and Coomassie brilliant blue assay kits were obtained from Nanjing Jiancheng Biological Medical Engineering Institute (Jiangsu, China).

2.10. Statistical analysis

Data are expressed as mean±S.E.M. The significance of differences between groups was tested by ANOVA followed by the Newman–Keuls test. $P<0.05$ was considered as statistically significant.

3. Results

3.1. Identification of DDAH2 gene overexpression cells

An 881 bp cDNA fragment was obtained by PCR using human DDAH2 gene specific primers. The cDNA encoding the full-length-hDDAH2 was inserted into the multiple cloning site of pcDNA3.1 plasmid. BLAST search confirmed no insertion or deletion mutations in our cloned hDDAH2 cDNA. Successfully transfected cells were identified by RT-PCR analysis as shown by a higher level of DDAH2 mRNA (881 bp) than untransfected or empty plasmid transfected cells (Fig. 1A). However, DDAH2 gene transfer had no effect on DDAH1 transcription (Fig. 1B).

3.2. LPC treatment blunts DDAH2 gene transcription

?twb=.27w?>Exposure of untransfected, pcDNA3.1-hDDAH2-transfected, and empty vector-transfected endothelial cells to 3 μmol/L LPC significantly inhibited DDAH2 transcription compared with respective control group (Fig. 2A and C, all $P<0.05$). DDAH2 gene transfer markedly attenuated the suppression of DDAH2 transcription induced by LPC (Fig. 2A and C, $P<0.05$). In contrast, LPC treatment did not affect DDAH1 transcription in these cells (Fig. 2B and D, all $P>0.05$).

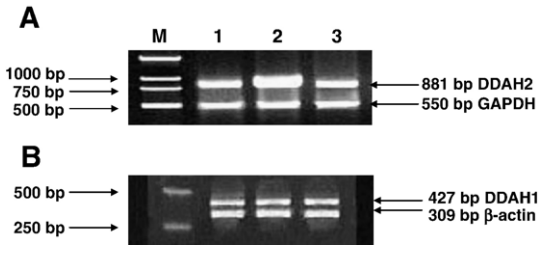

Fig. 1. Expression of DDAH2 and DDAH1 mRNA in cultured endothelial cells. Total RNA was extracted from untransfected (Lane 1), pcDNA3.1-hDDAH2-transfected (Lane 2), and empty vector pcDNA3.1-transfected (Lane 3) endothelial cells, respectively. RT-PCR was performed using DDAH1 or DDAH2 specific primers. A shows the higher level of DDAH2 mRNA expression in DDAH2-transfected cells compared to other groups. B shows no difference in the expression of DDAH1 mRNA among groups.

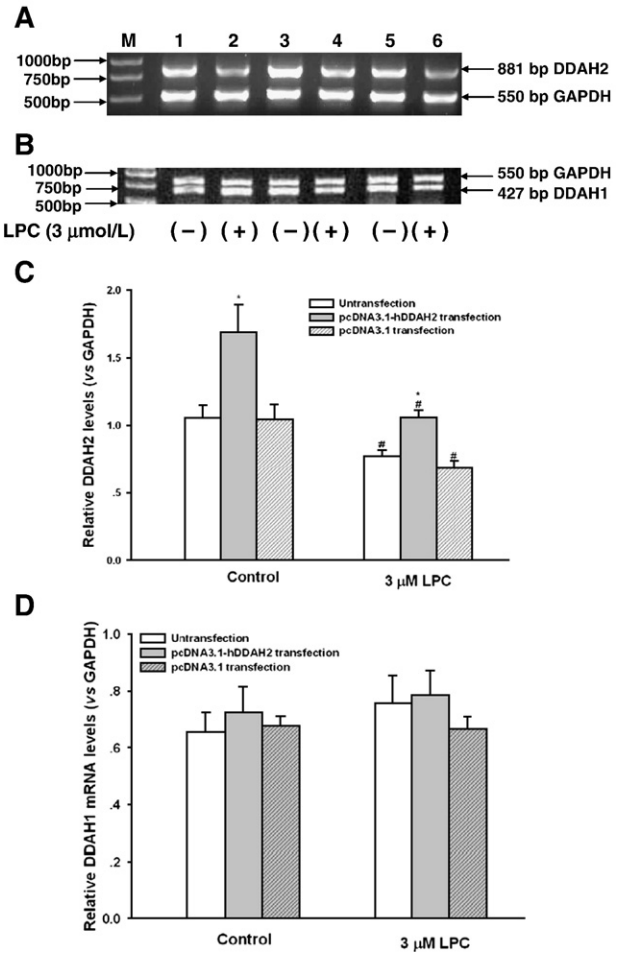

Fig. 2. Effects of DDAH2 gene transfer on DDAH1 and DDAH2 transcription in endothelial cells treated with LPC. The transcriptions of DDAH1 and DDAH2 were analyzed by RT-PCR in untransfected, pcDNA3.1-hDDAH2-transfected, and pcDNA3.1-transfected endothelial cells after treatment with or without 3 μmol/L lysophosphatidylcholine (LPC) for 48 h. A shows transcriptions of DDAH2 and GAPDH; B shows transcriptions of DDAH1 and GAPDH. Lanes 1–2 represent untransfected cells; Lanes 3–4 represent hDDAH2-transfected cells; and Lanes 5–6 represent empty vector-transfected cells. Lanes 1, 3, and 5 are control cells treated with vehicle, Lanes 2, 4, and 6 are the cells treated with 3 μmol/L lysophosphatidylcholine (LPC). C is the quantification of relative DDAH2 mRNA levels, and D is the quantification of relative DDAH1 mRNA levels, which were normalized to GAPDH gene expression and expressed as mean±S.E.M. from 3 independent experiments. #$P<0.05$ compared with the respective control group. *$P<0.05$ compared with the group-matched untransfected or pcDNA3.1-transfected cells.

3.3. DDAH2 gene transfer attenuated the suppression of DDAH activity due to LPC

As shown in Fig. 3, treatment with LPC remarkably suppresses DDAH activity among the three groups of untransfected, pcDNA3.1-hDDAH2-transfected, and empty vector-transfected group compared with their respective control group. However, DDAH2 transfection not only significantly increased the basal DDAH activity in endothelial cell compared to untransfected control cells or empty vector-transfected control cells (1.02±0.04 *vs* 0.71±0.02 or 0.66±0.03 U/g protein; $P<0.01$; Fig. 3), but also attenuated the suppression of DDAH

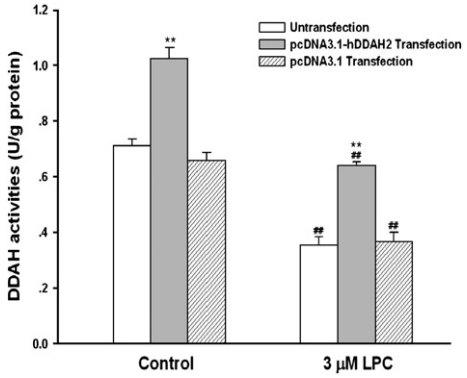

Fig. 3. Effect of DDAH2 gene transfer on the inhibition of DDAH activity induced by LPC in endothelial cells. DDAH activity was determined by the conversion of ADMA to L-citrulline in untransfected, pcDNA3.1-hDDAH2-transfected, and pcDNA3.1-transfected endothelial cells in the presence or absence of 3 μmol/L lysophosphatidylcholine (LPC) for 48 h. Data are expressed as mean±S.E.M. from 3 independent experiments. ##$P<0.01$ compared with the respective control group. **$P<0.01$ compared with the group-matched untransfected or pcDNA3.1-transfected cells.

activity induced by LPC compared to untransfected cells or vector-transfected cells with LPC treatment (0.64±0.01 *vs* 0.35±0.03 *vs* 0.37±0.03 U/g protein; $P<0.01$, Fig. 3). Empty plasmid transfection did not possess such effects as DDAH2 gene transfection (Fig. 3).

3.4. DDAH2 gene transfer decreased LPC-induced accumulation of endogenous ADMA

Treatment of endothelial cell with LPC significantly enhanced the concentrations of ADMA in the culture medium *vs* its corresponding control group (1.45±0.05 *vs* 0.42±0.07 μmol/L/10^6 cells; $P<0.01$; Fig. 4). DDAH2 gene transfer significantly attenuated the accumulation of endogenous ADMA either under static condition (0.15±0.02 *vs* 0.42±0.07 μmol/L/10^6 cells; $P<0.05$; Fig. 4) or under LPC challenge (0.70±0.08 *vs* 1.45±0.05 μmol/L/10^6 cells; $P<0.01$; Fig. 4) compared with untransfected cells. The empty vector transfection had no similar effect to DDAH2 transfection (Fig. 4).

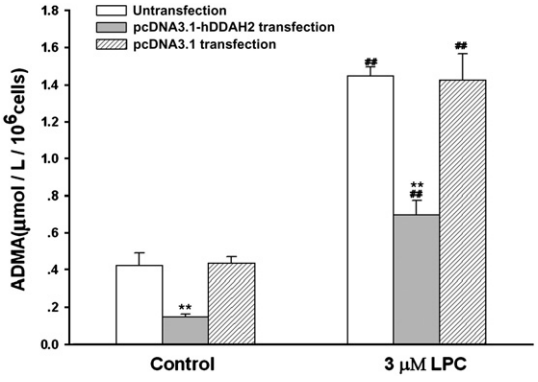

Fig. 4. Effect of DDAH2 gene transfer on the accumulation of ADMA in endothelial cells induced by LPC. Concentrations of endogenous ADMA were measured by high-performance liquid chromatography in the medium of untransfected, pcDNA3.1-hDDAH2-transfected, and pcDNA3.1-transfected endothelial cells treated with or without 3 μmol/L lysophosphatidylcholine (LPC) for 48 h. Data are expressed as mean±S.E.M. from 3 independent experiments. ##$P<0.01$ compared with the respective control group. **$P<0.01$ compared with the group-matched untransfected or pcDNA3.1-transfected cells.

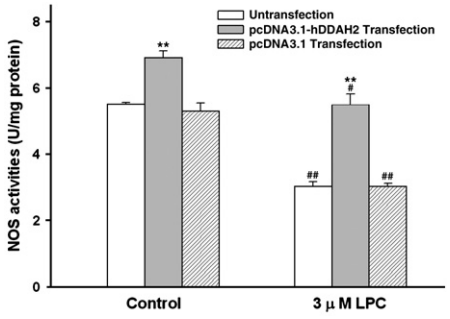

Fig. 5. Effect of DDAH2 gene transfer on the inhibition of NOS activity due to LPC in endothelial cells. The activity of NOS was determined by the conversion of L-arginine to NO in untransfected, pcDNA3.1-hDDAH2-transfected, and pcDNA3.1-transfected endothelial cells treated with or without 3 μmol/L lysophosphatidylcholine (LPC) for 48 h. Data are expressed as mean±S.E.M. from 3 independent experiments. ##$P<0.01$ compared with the respective control group. **$P<0.05$ compared with the group-matched untransfected or pcDNA3.1-transfected cells.

3.5. DDAH2 gene transfer preserved NOS activity from LPC impairment

Fig. 5 shows that LPC treatment suppressed the NOS activity in untransfected cells compared to untransfected cells without LPC treatment (3.04±0.13 *vs* 5.52±0.05 U/mg protein, $P<0.01$; Fig. 5). DDAH2 transfection could preserve NOS activity from LPC impairment, maintaining it nearly to the basal level (5.51±0.31 U/mg protein, $P>0.05$ *vs* untransfected group without LPC treatment; Fig. 5). Moreover, DDAH2 transfection also enhanced basal levels of NOS activity in endothelial cells compared to untransfected cells (6.94±0.18 *vs* 5.52±0.05 U/mg protein, $P<0.05$; Fig. 5), whereas empty vector transfection

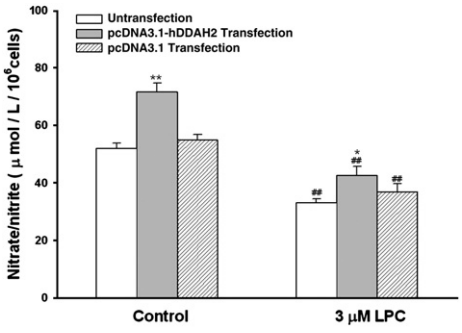

Fig. 6. Effect of DDAH2 gene transfer on the reduction of nitrate/nitrite contents in endothelial cells after exposure to LPC. The contents of nitrate/nitrite in the culture medium were determined to reflect NO synthesis in untransfected, pcDNA3.1-hDDAH2-transfected, and pcDNA3.1-transfected endothelial cells treated with or without 3 μmol/L lysophosphatidylcholine (LPC) for 48 h. Data are expressed as mean±S.E.M. from 3 independent experiments. ##$P<0.01$ compared with the respective control group. *$P<0.05$, **$P<0.01$ compared with the group-matched untransfected or pcDNA3.1-transfected cells.

did not significantly affect NOS activity in endothelial cells under basal state and LPC challenge (5.32±0.24 and 3.02±0.10 U/mg protein, both $P>0.05$ *vs* their group-matched untransfected cells; Fig. 5).

3.6. DDAH2 gene transfer improved the inhibition of NO synthesis induced by LPC

In parallel with the change in NOS activity, the nitrite/nitrate concentration was significantly reduced after LPC treatment in untransfected cells compared with untransfected control cells (51.83±1.85 *vs* 33.14±1.38 μmol/L/10^6 cells, $P<0.01$; Fig. 6). DDAH2 gene transfer improved this impairment induced by LPC as shown by enhancing the concentration of nitrite/nitrate in conditioned medium from 33.14±1.38 μmol/L/10^6 cells in untransfected cells plus LPC treatment to 42.63±3.03 μmol/L/10^6 cells in DDAH2-transfected cells with LPC treatment ($P<0.05$; Fig. 6). Moreover, DDAH2 gene transfer also raised basal level of nitrite/nitrate in DDAH2-transfected cells compared with untransfected cells (51.83±1.85 *vs* 71.65±3.12 μmol/L/10^6 cells, $P<0.01$; Fig. 5). In contrast, there was no significant difference in nitrite/nitrate concentrations when vector-transfected cells were compared with untransfected cells either in basal state or in LPC treatment (54.98±1.85 *vs* 51.83±1.85 μmol/L/10^6 cells in basal level and 36.90±3.01 *vs* 33.14±1.38 μmol/L/10^6 cells, both $P>0.05$; Fig. 6).

3.7. DDAH2 gene transfer enhanced resistance of endothelial cells to LPC injury

Treatment of endothelial cells with 3 μmol/L LPC for 48 h significantly increased the level of LDH activity in the conditional medium of either untransfected endothelial cells or empty vector pcDNA3.1-transfected endothelial cells compared to their control group, respectively (0.136±0.002 *vs* 0.099±0.001 U/L/10^6 cells or 0.144±0.004 *vs* 0.103±0.005 U/L/10^6 cells; both $P<0.01$; Fig. 7), indicating that LPC treatment injures endothelial cells and decreases cell viability. DDAH2 gene transfer could prevent the increase of LDH levels in conditional medium under LPC challenge compared to both untransfected and empty vector pcDNA3.1-transfected endothelial cells (0.112±0.003 *vs* 0.136±0.002 or 0.144±0.004 U/L/10^6 cells; both $P<0.01$; Fig. 7) but did not affect LDH activity in conditional medium under static state compared with untransfected group or empty vector-transfected group (0.104±0.002 *vs* 0.099±0.001 or 0.103±0.005 U/L/10^6 cells; both $P>0.05$; Fig. 7), suggesting that DDAH2 gene transfer enhanced resistance of endothelial cells to LPC injury.

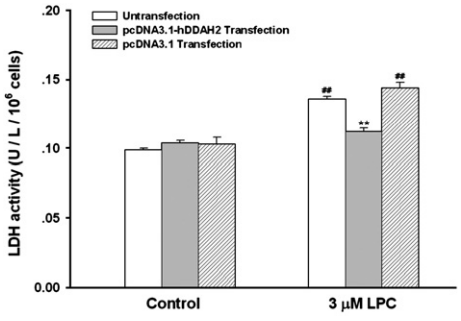

Fig. 7. Effect of DDAH2 gene transfer on the enhancement of LDH activity in culture medium of endothelial cells induced by LPC. The activity of lactate dehydrogenase (LDH) in the culture medium was determined to assess cell viability of untransfected, pcDNA3.1-hDDAH2-transfected, and pcDNA3.1-transfected endothelial cells in the presence or absence of 3 μmol/L lysophosphatidylcholine (LPC) for 48 h. Data are expressed as mean±S.E.M. from 3 independent experiments. ##$P<0.01$ compared with the respective control group. **$P<0.01$ compared with the group-matched untransfected or pcDNA3.1-transfected cells.

4. Discussion

Endothelial dysfunction plays an important role in the development of atherosclerosis. It is well documented that ox-LDL is a key risk factor responsible for the endothelial dysfunction in animal and patients with atherosclerosis (Yla-Herttuala et al., 1989; Ito et al., 1999). LPC is the major component of ox-LDL (Kugiyama et al., 1990). We and others have previously demonstrated that direct treatment of normal aortic rings with LPC mimicked the inhibition of endothelium-dependent relaxation observed in atherosclerosis (Deng and Xiong, 2005; Cowan and Steffen, 1995). Results from the present study further revealed that LPC could decrease NO synthesis in cultured endothelial cells (Fig. 6). This result provides the evidence at the cellular level that LPC plays an important role in the development of endothelial dysfunction associated with hypercholesterolemia or atherosclerosis.

The mechanisms for the decrease of NO biosynthesis in endothelial cells elicited by LPC are not fully understood. Results from the present study suggest that the inhibition of DDAH activity and subsequent accumulation of ADMA could be a critical abnormality implicated in this process. We found that DDAH activity was significantly inhibited after incubation of endothelial cells with LPC (Fig. 3), which was concomitant with a corresponding elevation of ADMA concentration (Fig. 4), inhibition of NOS activity (Fig. 5) and decrease of NO generation (Fig. 6). Similar results were observed in vessels from hypercholesterolemic rabbits and in cultured endothelial cells after incubation with ox-LDL (Ito et al., 1999). These results suggest that impaired DDAH activity contributes to the suppression of endothelium-derived NO synthesis due to LPC. However, the possible mechanisms for LPC to inhibit DDAH activity are poorly understood. Firstly, the present study provides the evidence that LPC significantly inhibited DDAH2 transcription but did not affect DDAH1 transcription in cultured endothelial cells (Fig. 2). This inhibition of DDAH2 transcription may account for the suppression of DDAH activity induced by LPC in endothelial cells. This result is similar to the recent reports that high glucose or homocysteine suppressed DDAH activity by means of down-regulating the expression of DDAH2 mRNA or protein in endothelial cells (Sorrenti et al., 2006; Tyagi et al., 2005) and previous reports that increased DDAH activity by estrogen was not related to the increase of DDAH1 expression in endothelial cells (Holden et al., 2003); and that up-regulation of DDAH2 expression but not DDAH1 mediated the increase of

DDAH activity elicited by all-*trans*-retinoic acid in endothelial cells (Achan et al., 2002). Secondly, LPC may directly inhibit DDAH activity by inducing oxidative stress to attack the sulfhydryl of cysteine residue in DDAH active site and stimulate the production of ADMA by up-regulation of *S*-adenosylmethionine-dependent methyltransferases (Böger et al., 2000b). Thirdly, our results also show that treatment with 3 μmol/L LPC for 48 h induced an injury on cell viability reflected by the increase of LDH leakage into culture medium from endothelial cells (Fig. 7). The decrease of cell viability may be one of the contributors to the inhibition of DDAH activity, and this result is consistent with Jiang's report (Jiang et al., 2003). Taken together, these results propose that DDAH2 is the predominant isoform for the maintenance of DDAH activity in endothelial cells.

Given that there is no effective pharmacological approach in increasing DDAH activity to resist the inhibitory effect of LPC, we postulate that gene transfer of DDAH to endothelial cells might have the potential to prevent the inhibition of LPC on NO synthesis. Since DDAH2 is the predominant isoform and plays a major role in maintaining DDAH activity in endothelial cells, we chose DDAH2 as the gene therapeutic target to investigate whether DDAH2 overexpression could improve LPC-induced impairments of ADMA/NOS/NO pathway in endothelial cells. The results from this study showed that basal DDAH2 transcription was apparently higher in DDAH2-transfected cells (Figs. 1A and 2A), accompanied by increased DDAH activity (Fig. 3) when compared with untransfected cells, suggesting that a DDAH2 gene overexpressing endothelial cell line was successfully constructed. With this cell model, we further revealed that DDAH2 gene transfer not only increased DDAH activity in basal level but also enhanced the resistance of endothelial cells to the adverse effects of LPC on DDAH activity (Fig. 3). These effects were specific for DDHA2 gene transfer because empty vector transfection had no similar effects (Fig. 3). Increased DDAH activity by DDAH2 gene transfer could accelerate ADMA degradation, thereby enhancing NOS activity and eventually augmenting NO synthesis, thus significantly improved the impairments of DDAH/ADMA/NOS pathway elicited by LPC in endothelial cells (Figs. 4–6). Furthermore, DDAH2 gene transfer could protect endothelial cells against LPC injury, remaining cell viability in normal state as shown by preventing enhancement of LDH activity in culture medium of endothelial cells after exposure to LPC (Fig. 7). This protection of DDAH gene transfer may be in favor of NO synthesis in endothelial cells though it is nonspecific for DDAH/ADMA/NOS/ NO pathway. It is well established that NO exerts pleiotropic antiatherosclerotic effects in the vascular wall. In addition to its vasodilator activity, NO inhibits key processes in atherogenesis, such as monocyte adhesion, platelet aggregation, and vascular smooth muscle proliferation (Napoli and Ignarro, 2001; Tsao et al., 1994; Miyazaki et al., 1999). Therefore, increased NO synthesis in endothelial cell could preserve vasculature from atherosclerosis.

In conclusion, the present study provides the first evidence that LPC inhibited DDAH2 transcription and DDAH activity, resulting in accumulation of endogenous ADMA, inhibition of NOS activity, and decrease of NO synthesis in cultured endothelial cells. More importantly, this study reveals that DDAH2 gene transfer can enhance the resistance of endothelial cells to LPC. These results suggest that DDAH2 plays a pivotal role in the regulation of NO synthesis in endothelial cells and that targeted up-regulation of DDAH2 in vasculature by gene or protein engineering even by drugs may represent a novel effective approach to the improvement of endothelial dysfunction associated with hypercholesterolemia and atherosclerosis.

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