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Lysophosphatidylcholine-induced elevation of asymmetric dimethylarginine level by the NADPH oxidase pathway in endothelial cells

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Abstract

Recent studies have suggested that endothelium is a main source of reactive oxygen species (ROS) and the major source was via NADPH oxidase pathway. Various stimuli including lysophosphatidylcholine (LPC), a major component of oxidized low-density lipoprotein (ox-LDL), can enhance the activity of NADPH oxidase and lead to a marked ROS generation. Asymmetric dimethylarginine (ADMA) is an endogenous nitric oxide (NO) synthase (NOS) inhibitor, which is synthesized by protein arginine methyltransferase I (PRMT I) and degraded by dimethylarginine dimethylaminohydrolase (DDAH) in endothelial cells. Much evidence showed that ADMA was closely related to endothelial dysfunction. Our previous study showed that LPC elevated ADMA level in endothelial cells via increasing oxidative stress, but the precise cellular mechanism is not defined yet. The present study was to explore the mechanism of NADPH oxidase in LPC-induced elevation of ADMA. In LPC-treated endothelial cells, the ROS production, cell viability, ADMA and NO levels, the activity of DDAH and expression of PRMT I were detected. Treatment with LPC (10 μg/ml) for 24 h markedly increased intracellular ROS production, the expression of PRMT I, level of ADMA, decreased the concentration of NO and the activity of DDAH. These effects were attenuated by diphenyliodonium, the NADPH oxidase inhibitor. In summary, the present results suggested that LPC-induced elevation of ADMA was due to reduction of DDAH activity and the up-regulation of PRMT expression by stimulation of ROS production via NADPH oxidase pathway.
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Keywords: NADPH oxidase; Lysophosphatidylcholine; Asymmetrical dimethylarginine; Reactive oxygen species; Endothelium

1. Introduction

Endothelial dysfunction is the foundation and initial step of numerous cardiovascular diseases. However, the mechanism responsible for it is not clearly understood yet. A growing body of evidence has suggested that endothelial dysfunction is closely related to oxidative stress induced by increased production of reactive oxygen species (ROS), including superoxide anion ($^{\cdot}O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl ($^{\cdot}OH$) (Kalinowski and Malinski, 2004). It has been indicated that cells in all layers of blood vessels produce ROS including $^{\cdot}O_2^-$ and the endothelium has been found to be a main source of oxidative stress (Cai and Harrison, 2000; Mohazzab et al., 1994). The potential sources of endothelial $^{\cdot}O_2^-$ production include xanthine oxidase, uncoupled NO synthase (NOS) and NADPH oxidase (Kalinowski and Malinski, 2004). A few recent studies in the literature have showed that NADPH oxidase pathway is a major source of $^{\cdot}O_2^-$ production in endothelial cells (Cai and Harrison, 2000; Mohazzab et al., 1994). It has been shown that NADPH oxidase is involved in multiple pathophysiological processes such as cell proliferation and apoptosis, NO-mediated vasorelaxation and anoxia/reoxygenation injury in endothelial cells (Abid et al., 2000; Brown and Goldstein, 1983; Heinloth et al., 2000; Rupin et al., 2004; Guzik et al., 2000).

Recent studies have suggested that asymmetric dimethylarginine (ADMA), an endogenous NOS inhibitor, can competitively inhibit the activity of NOS and decrease the synthesis of NO (Cooke, 2000). There is growing evidence that endogenous ADMA is a key factor contributing to endothelial dysfunction and is associated with the development of some cardiovascular diseases, such as hypercholesterolemia, peripheral vascular disease and hypertension (Cooke, 2000; Yu et al., 1994). It is known that ADMA is synthesized by PRMTs, which utilizes *S*-adenosylmethionine methyl group donor, and degraded by

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dimethylarginine dimethylaminohydrolase (DDAH) (Ito et al., 1999). It was reported that some oxidants such as ox-LDL could induce an increase in the level of ADMA by increasing PRMT I expression and (or) by decreasing DDAH activity (Böger et al., 2000; Jiang et al., 2003a,b). More recently, LPC, the major component of ox-LDL, has been found to induce oxidant stress by the activation of NADPH oxidase (Abid et al., 2000; Takeshita et al., 2000; Inoue et al., 2001). In the present study, therefore, we tested whether the increased level of ADMA induced by LPC is due to the reduction of DDAH activity and up-regulation of PRMT expression by the NADPH oxidase pathway.

2. Materials and methods

2.1. Reagents

Lysophosphatidylcholine (LPC), diphenyliodonium (DPI), asymmetric dimethylarginine standard (ADMA), MTT, dimethyl sulfoxide (DMSO), allopurinol and monoclonal anti-PRMT I were obtained from Sigma. DMEM was obtained from Gibco. Western blot kit was purchased from KPL. NO assay kits were provided by Juli Biological Medical Engineering Institute (Nanjing, China). ROS detection kit was purchased from Beyotime Company (Jiangsu, China).

2.2. Cell culture

HUVEC-12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin and 100 U/ml streptomycin. Cells were passaged into 6-well culture dishes or 24-well culture dishes and were serum-starved for 24 h in DMEM containing 1% FBS when the cells had reached subconfluence. Then cells were treated with LPC (10 μg/ml) for 24 h in the presence or absence of NADPH oxidase inhibitor, DPI (100 or 300 μM), or the xanthine oxidase inhibitor, allopurinol (100 μM).

2.3. Determination of ROS generation

Changes in intracellular ROS levels were determined by measuring the oxidative conversion of cell permeable 2′,7′-dichlorofluorescein diacetate (DCFH-DA) to fluorescent dichlorofluorescein (DCF) in fluorospectro-photometer (F4000, Japan). Cells in 6-well culture dishes were incubated with control media or 10 μg/ml LPC for 4 h in the absence or presence of DPI (100 or 300 μM) or allopurinol (100 μM). The cells were washed with D-Hank's and incubated with DCFH-DA at 37 °C for 20 min. Then DCF fluorescence distribution of 20 000 cells was detected by fluorospectrophotometer analysis at an excitation wavelength of 488 nm and at an emission wavelength of 535 nm.

2.4. Cell viability assay

Cell viability was determined by the tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (Mosmann, 1983). Endothelial cells were planted into 96-well culture plates at an optimal density of 5×10^3 cells/ml with 200 μl culture medium per well. After 3–4 days of culture to 90% confluence, 20 μl assay medium containing 5 mg/ml MTT was added to each well and incubated at 37 °C for 4 h. The medium was gently aspirated, and then 150 μl DMSO was added to each well to solubilize the formazan crystals. The optical density of each sample was immediately measured in an ELISA microplate reader (ELX800, USA) at 490 nm.

2.5. Determination of nitrite/nitrate concentration

The level of NO in the conditioned medium was determined indirectly as the content of nitrite and nitrate. The level of nitrite/nitrate in the conditioned medium was measured as described previously (Feng et al., 2001). Briefly, nitrate was converted to nitrite with aspergillus nitrite reductase, and the total nitrite was measured with the Griess regent. The absorbance was determined at 540 nm with a spectrophotometer (Shanghai, China).

2.6. Determination of ADMA concentration

The proteins in the conditioned medium were removed using 5-sulfosalicylic acid (5-SSA). The levels of ADMA were measured by high-performance liquid chromatography (HPLC) as described previously with some modification (Chen et al., 1997). HPLC was carried out using a Shimadzu LC-6A liquid chromatograph with Shimadzu SCL-6A system controller and Shimadzu SIC-6A autosampler. *O*-Phthaldialdehyde adducts of methylated amino acids and internal standard ADMA produced by precolumn mixing were monitored using a model RF 530 fluorescence detector set at $\lambda^{ex}=338$ and $\lambda^{em}=425$ nm on a Resolve C_{18} column. Samples were eluted from the column using a linear gradient containing mobile phase A composed of 0.05 M (pH 6.8) sodium acetate–methanol–tetrahydrofuran (81:18:1 v/v/v) and mobile phase B composed of 0.05 mM sodium acetate–methanol–tetrahydrofuran (22:77:1 v/v/v) at a flow-rate of 1 ml/min.

2.7. DDAH activity assay

The activity of DDAH in endothelial cells was estimated by directly measuring the amount of ADMA metabolized by the enzyme (Lin et al., 2002). In an ice bath, cell lysates were divided into 2 groups, and ADMA was added (final concentration 1 mM). To inactivate DDAH, 30% sulfurosalicylic acid was immediately added to 1 experimental group. This group provided a baseline of 0% DDAH activity. The other lysate was incubated at 37 °C for 2 h before the addition of 30% sulfurosalicylic acid. The ADMA level in each group was measured by HPLC as described above. The difference in ADMA concentration between two groups reflected the DDAH activity. For every experiment, DDAH activity of endothelial cells exposed to normal conditioned medium is defined as 100%, and DDAH activity in other conditions is expressed as

percentages of the ADMA metabolized compared with the control.

2.8. Expression of PRMT I

Cells were lysed in SDS sample buffer. Proteins of equal concentration were separated by 12% SDS–PAGE and electrophoretically transferred to nitrocellulose membranes. The membranes were blocked for 1 h with 1% blocker milk. After blocking, the membranes were incubated in the primary monoclonal–PRMT I antibody (1:1000) at 4 °C overnight. Membranes were washed in TBST for 1 h before incubation for 1 h in anti-mouse secondary antibody (1:1000). Then membranes were washed for 1 h in TBST and developed with enhanced chemiluminescence system (kit).

2.9. Statistical analysis

Results are expressed as means ± S.E.M. All data were analyzed by ANOVA followed by unpaired Student's *t*-test for multiple comparisons. The significance level was chosen as $P < 0.05$.

3. Results

3.1. Concentrations of ROS

LPC (10 μg/ml) significantly increased the intracellular level of ROS. Pretreatment with DPI (100 or 300 μM) significantly inhibited the elevated intracellular concentration of ROS by LPC. However, the xanthine oxidase inhibitor, allopurinol, had no effects. DPI or allopurinol itself had no effect on the intracellular level of ROS (Fig. 1).

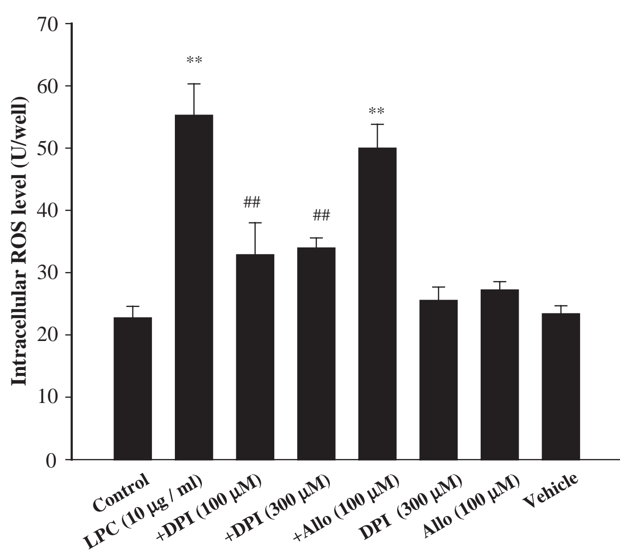

Fig. 1. Effect of diphenyliodonium on the intracellular ROS production of endothelial cells cultured with LPC. Endothelial cells were treated with LPC at the dose of 10 μg/ml for 4 h. Data are expressed as means ± S.E.M. $n = 4\sim6$. Compared with control, $^{**}p < 0.01$; compared with LPC group, $^{\#\#}p < 0.01$. +DPI = LPC + diphenyliodonium; +Allo = LPC + allopurinol; DPI = diphenyliodonium; Allo = allopurinol.

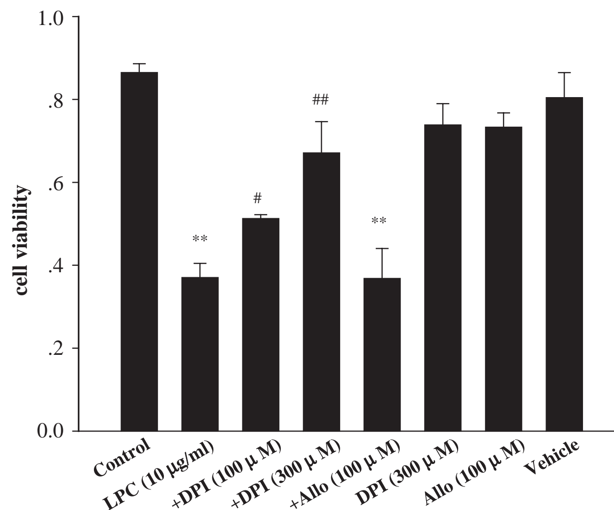

Fig. 2. Effects of diphenyliodonium on cell viability cultured with LPC. Endothelial cells were treated with LPC at the dose of 10 μg/ml for 24 h. Data are expressed as means ± S.E.M. $n = 4\sim6$. Compared with control, $^{**}p < 0.01$; compared with LPC group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$. +DPI = LPC + diphenyliodonium; +Allo = LPC + diphenyliodonium; DPI = diphenyliodonium; Allo = allopurinol.

3.2. Cell viability

Exposure of endothelial cells to LPC at the dose of 10 μg/ml for 24 h significantly decreased cell viability. Pretreatment with DPI (100 or 300 μM) attenuated the reduction of cell viability by LPC. However, the xanthine oxidase inhibitor, allopurinol, had no effects on cell viability. DPI or allopurinol itself had no effect on cell viability (Fig. 2).

3.3. Concentrations of nitrite/nitrate

Treatment with LPC (10 μg/ml) for 24 h significantly decreased the level of NO in the medium. DPI (100 or 300 μM) significantly inhibited the decreased concentration of NO by LPC. However, the xanthine oxidase inhibitor, allopurinol, had no effects. DPI or allopurinol itself had no effect on the level of NO (Fig. 3A).

3.4. Concentration of ADMA

Treatment with LPC (10 μg/ml) for 24 h significantly increased the level of ADMA in the medium. DPI (100 or 300 μM) significantly inhibited the elevated concentration of ADMA by LPC. However, the xanthine oxidase inhibitor, allopurinol, had no effects on intracellular level of ROS. DPI or allopurinol itself had no effect on the level of ADMA (Fig. 3B).

3.5. Activity of DDAH

DDAH activity was significantly decreased in the endothelial cells treated with LPC (10 μg/ml) for 24 h. DPI (100 or 300 μM) significantly improved the reduced activity of DDAH by LPC. However, the xanthine oxidase inhibitor, allopurinol, had

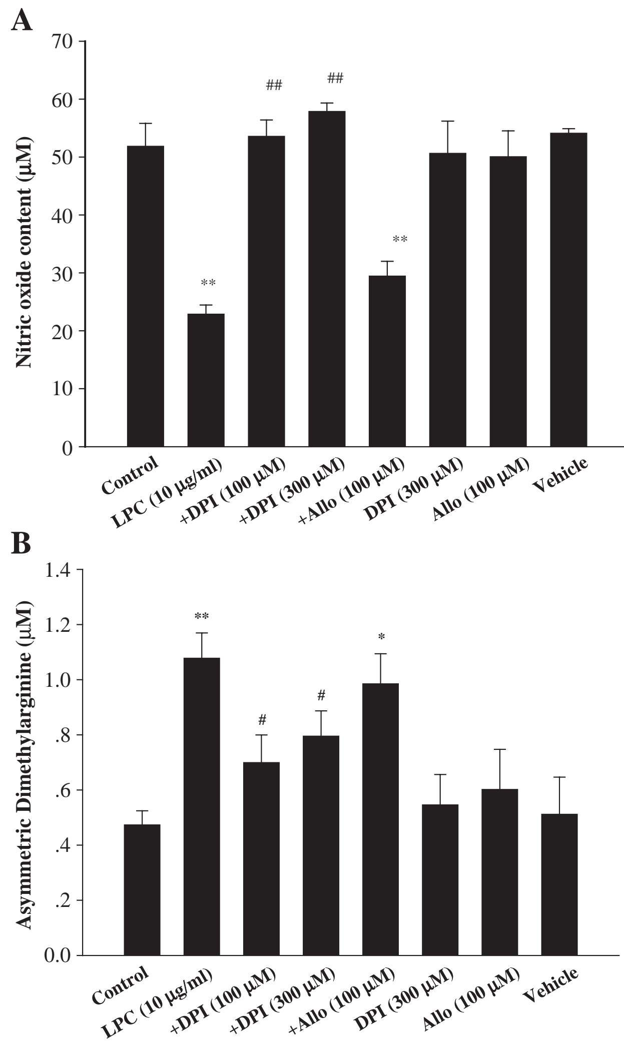

Fig. 3. Effect of diphenyliodonium on concentrations of nitrite/nitrate (A) and ADMA (B) in the conditioned medium. Endothelial cells were treated with LPC at the dose of 10 μg/ml for 24 h. Data are expressed as means ± S.E.M. n = 4~6. Compared with control, *p < 0.05, **p < 0.01; compared with LPC group, $^{\#}p$ < 0.05, $^{\#\#}p$ < 0.01. +DPI = LPC + diphenyliodonium; +Allo = LPC + diphenyliodonium; DPI = diphenyliodonium; Allo = allopurinol.

no effects on it. DPI or allopurinol itself had no effect on activity of DDAH (Fig. 4).

3.6. Expression of PRMT I

Treatment with LPC (10 μg/ml) for 24 h significantly increased the protein expression of PRMT I in endothelial cells. DPI (100 or 300 μM) significantly inhibited the elevated protein expression of PRMT I by LPC. However, the xanthine oxidase inhibitor, allopurinol, had no effects on the expression of PRMT I. DPI or allopurinol itself had no effect on the expression of PRMT I (Fig. 5A, B).

4. Discussion

The major findings in the present study were: (1) LPC-induced elevation of ADMA level is related to the reduction of

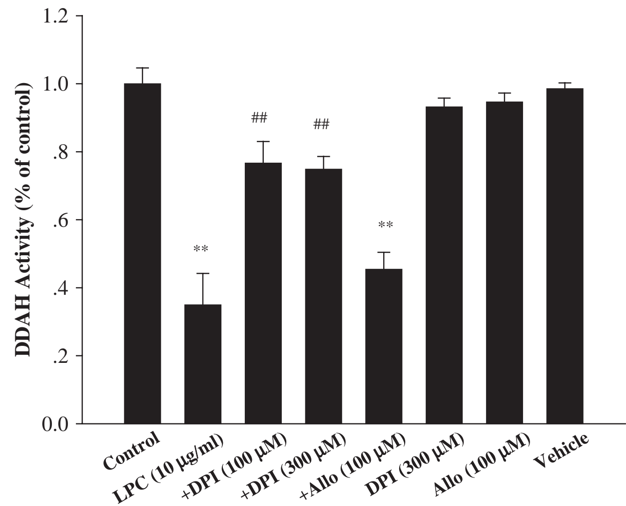

Fig. 4. Effect of diphenyliodonium on DDAH activity. Endothelial cells were treated with LPC at the dose of 10 μg/ml for 24 h. Data are expressed as means ± S.E.M. n = 4~6. Compared with control, **p < 0.01; compared with LPC group, ##p < 0.01. +DPI = LPC + diphenyliodonium; +Allo = LPC + diphenyliodonium; DPI = diphenyliodonium; Allo = allopurinol.

DDAH activity and up-regulation of PRMT expression; and (2) these effects of LPC are mediated by the stimulation of ROS production via the NADPH oxidase pathway.

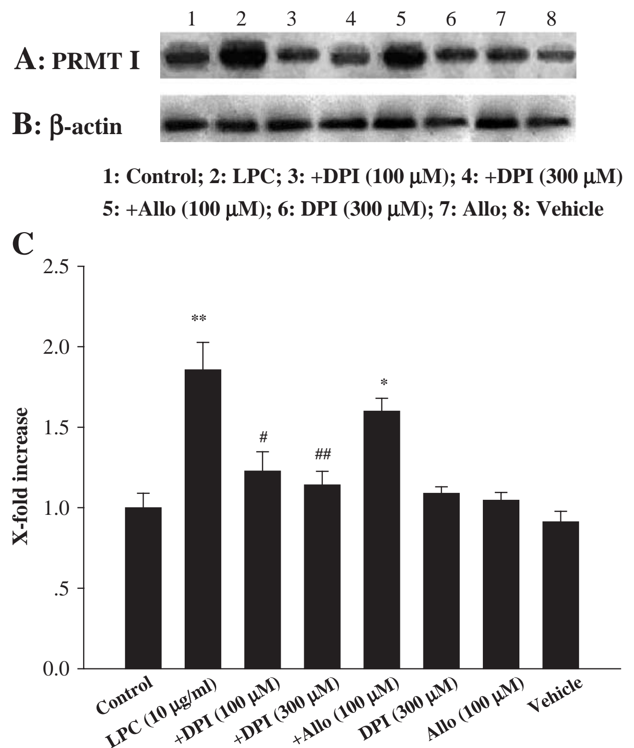

Fig. 5. Effects of diphenyliodonium on the protein expression of PRMT I (Western blot bands of PRMT-1 (A) and β-actin (B); (C) the statistic analysis results of the folds increase of PRMT I compared to β-actin). Endothelial cells were treated with LPC at the dose of 10 μg/ml for 24 h. Data are expressed as means ± S.E.M. n = 3. Compared with control, *p < 0.05, **p < 0.01; compared with LPC group, $^{\#}p$ < 0.05, $^{\#\#}p$ < 0.01. +DPI = LPC + diphenyliodonium; +Allo = LPC + diphenyliodonium; DPI = diphenyliodonium; Allo = allopurinol.

Endothelial dysfunction is the foundation and initial step of numerous cardiovascular diseases, yet the underlying molecular mechanisms remain poorly understood. It has been documented that the oxidative stress induced by dramatically increased production of $^{\cdot}O_2^-$ and other ROS have been implicated in the initiation and progression of endothelial dysfunction (Kalinowski and Malinski, 2004). Recently, it has been found that cells in all layers of blood vessels produce ROS including $^{\cdot}O_2^-$, and endothelium has been found to be a main source of $^{\cdot}O_2^-$ (Mohazzab et al., 1994; Guzik et al., 2002; Munzel et al., 1995). The potential endothelial ROS sources include xanthine oxidase, uncoupled NOS and NADPH oxidase, and among them, NADPH oxidase is the main and most important one.

NADPH oxidase is an inducible electron transport system, which transfers reducing equivalents from NADPH to molecular oxygen via flavins, resulting in $^{\cdot}O_2^-$ generation. Previous study showed that NADPH oxidase was present in neutrophils (Cox et al., 1985). Recent studies indicated that NADPH oxidase was also the major origin of ROS of some non-phagocytic cells, such as endothelial cells and smooth muscle cells (Meyer et al., 1999; Jones et al., 1996). It has been reported that the activity of NADPH oxidase was increased by up-regulation of gene expression and/or post-transcriptional expression in protein level, and the enzyme-dependent oxidative stress is thought to play a pivotal role in endothelial dysfunction of some cardiovascular diseases (Griendling et al., 1994; De Keulenaer et al., 1998; Al-Mehdi et al., 1998).

LDL, the main component of serum lipids, is a crucial risk factor of atherosclerosis and endothelial dysfunction. Numerous studies have suggested that NADPH oxidase plays an important role in LDL-induced oxidative stress, and ox-LDL and LPC can induce ROS generation by increasing NADPH oxidase activity in the endothelium (Holland et al., 1996; Heinloth et al., 2000). More significantly, the increase in ROS is both required and sufficient to generate the physiological changes that accompany the generation of an atherosclerotic endothelium (Morel and DiCorleto, 1984). There is evidence to suggest that the proliferation and migration of the endothelium induced by LPC are mediated by NADPH oxidase. Pretreatment with the NADPH oxidase inhibitor, diphenyliodonium, attenuated the effects of LPC (Abid et al., 2000). In the present study, we confirmed the previous studies that LPC significantly enhanced the production of intracellular ROS, an effect which was attenuated by diphenyliodonium. However, allopurinol, the xanthine oxidase inhibitor, had no effects. These results further support the hypothesis that the excessive ROS generation induced by LPC is derived from NADPH oxidase.

Recent studies have suggested that ADMA, an endogenous inhibitor of NOS, is an important marker of endothelial dysfunction. There is growing evidence that the elevated plasma level of ADMA is closely related to endothelial dysfunction in several cardiovascular diseases (Cooke, 2000). It is known that ADMA is synthesized by PRMTs and degraded by DDAH (Ito et al., 1999). Previous study has showed that ox-LDL increased the level of ADMA by reducing DDAH activity, but not expression (Böger et al., 2000; Jiang et al., 2003a). And it was recently reported that PRMT I, the subtype responsible for the synthesis of ADMA, is expressed in human endothelial cells under baseline conditions and that gene expression of PRMT I is markedly up-regulated in the presence of ox-LDL and shear stress (Ito et al., 1999; Osanai et al., 2003).

We and others have documented that LPC or ox-LDL induced the elevation of ADMA, and some antioxidants such as vitamin E, probucol and xanthones markedly decreased the elevated level of ADMA by ox-LDL or LPC (Jiang et al., 2003b; Jiang et al., 2002). Based on LPC-induced oxidative stress by the NADPH oxidase pathway in endothelial cells, we postulated that the increased level of ADMA might be secondary to changes of the activity of the enzymes involved in the regulation of ADMA. The results of the present study revealed that LPC significantly increased the levels of ADMA, accompanied by the reduction of the activity of DDAH and the up-regulation of the protein expression of PRMT I, which was reversed by diphenyliodonium, the NADPH oxidase inhibitor. Taken together, these results add to the existing evidence that the increased level of ADMA induced by LPC is due to the reduction of DDAH activity and up-regulation of PRMT expression by stimulation of ROS production via the NADPH oxidase pathway.

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