High-Affinity Arginine Transport of Bovine Aortic Endothelial Cells Is Impaired by Lysophosphatidylcholine

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Abstract—The mechanisms of endothelial dysfunction characterized by the impaired nitric oxide (NO) release have not yet been clarified. Because the phenomenon is mimicked in vitro by the application of oxidized LDL and its major lipid constituent, lysophosphatidylcholine (LPC), we analyzed their effects on the arginine-NO system, especially on the arginine transport system. LPC inhibited NO release induced by ADP in cultured bovine aortic endothelial cells. The inhibition was attenuated by the excess amount of extracellular arginine. LPC was found to inhibit the arginine transport in bovine aortic endothelial cells, which is mediated by high- and low-affinity components. LPC predominantly impaired the high-affinity component. In the presence of a high concentration of arginine, LPC showed apparently no inhibition of arginine transport, because the low-affinity transporter compensated for the activity. Taken together, the impairment of the high-affinity transport system might account for the inhibition of NO release by LPC. LPC also inhibited arginine transport in the intima of intact bovine aorta. Furthermore, LPC inhibited the activity of the high-affinity arginine transporter in endothelial cells, in the cationic amino acid transporter-1 expressed in COS-7 cells. The activity of cationic amino acid transporter-1 might be important for the prevention of endothelial dysfunction. (Circ Res. 1998;83:1088-1096.)

Key Words: lysophosphatidylcholine ■ arginine transporter ■ nitric oxide ■ endothelium ■ lipoprotein, oxidized low-density

Numerous recent studies demonstrated that nitric oxide (NO) has a variety of antiatherogenic actions on the vascular system, such as inhibitory effects on the growth of vascular smooth muscle cells, platelet aggregation, and leukocyte adhesion to the endothelium. It is likely that the failure of NO release causes significant changes in vascular homeostasis and promotes atherogenesis.1 The vascular endothelium in atherosclerosis fails to release NO, in contrast to that in the normal vascular bed.2 In vivo studies have revealed that arginine supplementation improved NO release from the endothelium both in hypercholesterolemic animals and in humans.3–5

Significant alleviation of the formation and progression of atherosclerosis by arginine supplementation has been reported.6 These results suggest that the availability of arginine in the cytoplasm of endothelial cells is reduced in the state of hypercholesterolemia, and it is highly probable that an exogenous supply of arginine could ameliorate the impaired NO release and suppress atherogenesis.

Evidence has accumulated that oxidized LDL (OxLDL) or its lipid constituent, lysophosphatidylcholine (LPC), plays a critical role in atherogenesis.7,8 Immunohistochemical studies of atherosclerotic lesions have revealed the deposition of OxLDL, which is taken up by monocytes/macrophages.9 LPC is formed in the process of oxidative modifications of LDL. It is a major deleterious component of OxLDL, constituting up to 40% of the total lipid content of OxLDL. LPC, like OxLDL, shows a special atherogenic capacity including the induction of growth/chemotactic factors, induction of leukocyte adhesion molecule, and inhibition of the NO release from endothelium.8,10,11

To clarify the change of arginine availability, we focused on the relationships between arginine transport and NO production in endothelial cells treated with LPC. The arginine uptake in endothelial cells is mainly mediated by system y⁻, a sodium-independent transport system of cationic amino acids. The 4 members of the system y⁻ transporter family identified to date are cationic amino acid transporters (CAT1, CAT2a, CAT2b, and CAT3).12,13 According to their different affinities to arginine, 3 of these transport systems (CAT1, CAT2b, and CAT3) are classified as high-affinity ($K_d = 10^{-4}$ mol/L) transporters, whereas CAT2a, an alternatively spliced isoform of CAT2b, is classified as a low-affinity ($K_d = 10^{-3}$ or more mol/L) transporter.

The present results revealed that (1) the decrease in NO production in endothelial cells treated with LPC was reversed
by high dose of arginine; (2) arginine uptake of endothelial cells was mediated by the 2 pathways, high- and low-affinity arginine transport systems; and (3) LPC selectively inhibited the high-affinity system. Our findings unveil one of the molecular mechanisms underlying the in vivo observations that high-dose arginine attenuates atherogenesis by increasing endothelium-derived NO release.

Materials and Methods

Culture

Bovine aortic endothelial (BAE) cells were maintained in DMEM supplemented with 10% FCS, 100 U/mL of ampicillin, 0.1 mg/mL of streptomycin, and 250 ng/mL of amphotericin B (Fungizone; Gibco) at 37°C under humidified atmosphere containing 5% CO2. Cell viability was determined using the trypan blue exclusion assay and by measurement of the activity of lactate dehydrogenase leaked from dead cells into the culture medium using a lactate dehydrogenase-cytotoxic test kit (Wako).

Preparation of LDL

Human plasma LDL (density=1.019 to 1.063) was isolated by sequential ultracentrifugation, and oxidative modification of LDL was carried out with copper ion in vitro, as described previously.14 Oxidation was monitored by measuring the amount of thiobarbituric acid-reactive substances (≈10 nmol malondialdehyde equivalent/mg protein in OxLDL). Agarose gel electrophoresis revealed an increase in electrophoretic mobility and minimal aggregation of OxLDL particles.

Bioassy of NO Release From BAE Cells

BAE cells seeded for the experiments were cultured for 16 hours in Eagle’s MEM lacking arginine (Gibco). The cells were harvested by trypsinization and then incubated for 2 hours at 37°C in HEPESTM-buffered saline (HBS) (10 mmol/L HEPES, 5 mmol/L glucose, 150 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L CaCl2, 1 mmol/L MgCl2 [pH 7.5], and 0.3% BSA) containing LPC or OxLDL at the concentrations indicated in the figures. After washing twice with HBS, BAE cells were used for the measurement of antiaggregational effects.

Into a tube containing a 1:9 volume of 3.8% sodium citrate solution as an anticoagulant, peripheral venous blood was withdrawn from healthy volunteers who had not taken any drugs for at least 2 weeks. Platelet-rich plasma (PRP) and platelet-poor plasma were obtained as supernatants after centrifugation for 10 minutes at 160 and 1500g, respectively.15 Two hundred microliters of PRP pre-warmed at 37°C was set in an aggregometer (Heam Tracer 601; Niko Bioscience) with continuous stirring at 1000 rpm. Twenty microliters of warmed at 37°C was set in an aggregometer (Heam Tracer 601; Niko Bioscience) with continuous stirring at 1000 rpm. Twenty microliters of the suspension of BAE cells, prepared as described above, was added to the PRP and incubated for 1 minute. Addition of endothelial cells (5×10^4 to 1.5×10^5 cells) did not significantly affect the light transmission. Test reagents were added and incubated for 1 minute. ADP was added to initiate platelet aggregation and to stimulate endothelial cells simultaneously. The numbers of BAE cells and the concentrations of ADP or test reagents used are shown in the figures. Changes in light transmission were recorded for 4 minutes after the stimulation. The light transmission was calibrated with that of platelet-poor plasma taken as the 100% transparent control and that of PRP as the 0% reference.16

Measurement of Transient Ca2+ Change Induced by ADP

BAE cells pretreated with MEM without arginine (Gibco) were harvested by trypsinization and incubated for 1.5 hours at 37°C in Ca2+ loading buffer (5 mmol/L HEPES, 1 mmol/L NaHPO4, 5 mmol/L glucose, 140 mmol/L NaCl, 4 mmol/L KCl, 1 mmol/L MgCl2, and 0.3% BSA [pH 7.4]) containing LPC or phosphatidylcholine (PC). The suspension was added by fluo-3 bis[(acetyloxy)-methyl]ester and further incubated for 30 minutes, as described elsewhere.17 The change of intracellular Ca2+ evoked by 10^-3 mol/L ADP was monitored by a fluorescence spectrophotometer (model CAF-110; JASCO) with excitation at 490 nm and emission at 540 nm.

Measurement of Amino Acid Uptake by Culture Cells

Confluent monolayers of BAE cells on 6-well plates were cultured in MEM without arginine (Gibco) for 16 hours before the experiments. The cells were washed twice with HBS and incubated at 37°C for 2 hours with HBS, 0.3% BSA containing LPC, or reagents indicated in the figure legends. The media were then replaced with HBS and 0.3% BSA containing 10^-3 mol/L of [3H]arginine or [3H]leucine (222 GBq/mmol) to be taken up by the cells. The uptake was stopped by washing the cells 3 times with ice-cold HBS and 0.3% BSA, and the cells were lysed with 0.2 mol/L NaOH and 1% SDS. The incorporated radioactivity was determined with a solid scintillation counter and calibrated with the amount of protein determined by Micro BCA assay (Pierce).

Measurement of Arginine Uptake by Endothelia of Intact Bovine Aortas

Bovine aortas freshly obtained from a local slaughterhouse were cut longitudinally and incubated at 37°C for 2 hours with HBS, 0.3% BSA with or without 100 μmol/L LPC. The media were changed with HBS containing [3H]arginine to allow the aortas to incorporate arginine for 3 minutes. After being washed with ice-cold HBS, the endothelia were scraped off with coverslips and lysed with 0.2 mol/L NaOH and 1% SDS. The radioactivity and protein concentration of the lysate were determined. The scraped cells were identified as vascular endothelial cells by the further culturing of the cells in parallel experiments.

cDNA Cloning of CAT1

cDNA libraries from human lung and brain constructed in the Agt10 vector were screened by the standard plaque hybridization method as described.13 The insert of a positive clone (hCAT1) was subcloned into pBluescript II SK- (Stratagene) to give pBShCAT1. It was confirmed by sequencing that hCAT1 covered the whole coding region of human CAT1 cDNA.

Transient Expression of CAT1 in COS-7 cells

The EcoRI fragment of hCAT1 was subcloned into a mammalian expression vector pME18S to give pMEhCAT1. COS-7 cells were maintained in DMEM supplemented with 10% FBS. The COS-7 cells to be transfected were seeded on 10-cm dishes the night before to give 50% confluence at the time of transfection. The cells were transfected with 10 μg of pMEhCAT1 or with the vector (for basal uptake) using LipofectAMINE (Life Technologies, Inc) according to the instructions of the manufacturer. Twenty-four hours later, the cells were transferred to 12-well plates and the medium was changed with arginine-free MEM. Twenty-four hours after this, the cells were used for the determination of arginine uptake.15

Northern Blotting

Poly(A)+ RNA (10 μg per lane), prepared from BAE cells, was separated by formaldehyde/1% agarose gel electrophoresis and transferred to a nylon membrane (Gene Screen Plus; Du Pont). The membrane was hybridized with the probe, which was prepared by labeling the EcoRI fragment of pMEhCAT1 with [32P]dCTP (110 TBq/mmol; Amersham). Blotting procedures were as described.18 The blot was visualized for radioactivity with a BAS2000 image analyzer (FujiFilm Co Ltd).

Results

To measure the amount of NO released from BAE cells, we used a standard bioassay based on the antiaggregational effect of NO, as previously described.16 PRP was used to analyze
the reaction under conditions similar to those in vivo. ADP was chosen for initiating platelet aggregation, because it simultaneously activates endothelial cells to release NO. ADP induced platelet aggregation in PRP in a dose-dependent manner (Figure 1A). As expected, sodium nitroprusside, a NO donor, inhibited the ADP (10^{-5} to 10^{-6} mol/L)-induced platelet aggregation in a dose-dependent manner (Figure 1B). Similarly, the addition of BAE cells to PRP also inhibited ADP-stimulated platelet aggregation (Figure 1C). The inhibitory effect of BAE cells was dependent on the number of BAE cells and was inhibited by L-nitroarginine-methylester (NAME), a NO synthase (NOS) inhibitor that has little effect on arginine transport (Figure 1D). Although the inhibition by NAME was moderate, the reason may be that the serum contained about 0.1 mmol/L arginine, one tenth of the concentration of NAME used. Without addition of BAE cells, NAME did not affect platelet aggregation. Thus, this bioassay system is an appropriate method for the measurement of NO release from endothelial cells.

Using this assay system, we confirmed that LPC dose-dependently inhibits NO release from BAE cells (Figure 2). The inhibited NO release was restored by the addition of 10 mmol/L arginine, but not by 10 mmol/L lysine, which is also a dibasic amino acid but not a substrate of NOS (Figure 3). In addition, the NO release from untreated BAE cells was not affected by the addition of 10 mmol/L arginine or lysine (data not shown). The findings also demonstrated that BAE cells treated with LPC required more extracellular arginine than the physiological concentration to produce an equivalent amount of NO as in the normal condition. These findings suggest that the arginine uptake by endothelial cells would be inhibited by the LPC treatment because the concentration of serum arginine is about 0.1 mmol/L, which is far higher than the $K_m$ value of NOS for arginine.

To test this hypothesis, we measured the uptake of extracellular arginine into BAE cells in the presence of 100 nmol/L of [H]arginine. As expected, the transport was significantly inhibited by the addition of LPC or OxLDL but not by PC or native LDL (Figure 4A). The inhibitory effect of LPC reached the maximum within 1 hour of incubation and continued for at least 5 hours (Figures 4B and 5A). This inhibitory effect was dependent on the concentration of LPC (Figure 5B). In contrast to that of arginine, the transport of leucine, which is carried out by
another transport system, was not inhibited even at the maximum dose of LPC (Figure 5C).

To exclude the possibility that these changes in arginine transport were due to nonspecific detergent-like effects of LPC, the viability of BAE cells was confirmed using the trypan blue exclusion assay and by measurement of the activity of lactate dehydrogenase released from dead BAE cells in the culture media. The results disclosed that BAE

Figure 2. LPC dose-dependently impaired the antiaggregational effect of BAE cells in PRP. BAE cells were pretreated with LPC at the concentrations indicated. Then, the antiaggregational effect of BAE cells (7.5×10⁴ cells) was measured after removal of LPC.

Figure 3. Supplementation with arginine restored the antiaggregational effect of BAE cells impaired by LPC. BAE cells (7.5×10⁴ cells) were pretreated with 100 μmol/L LPC. Antiaggregational effects of BAE cells (7.5×10⁴ cells) stimulated with 10⁻⁵ mol/L ADP were measured in PRP supplemented with or without arginine or lysine (10 mmol/L). Neither arginine nor lysine affected the platelet aggregation without BAE cells (data not shown).

Figure 4. Atherogenic lipid and lipoprotein inhibited the uptake of arginine by BAE cells. A, BAE cells were pretreated with native LDL or OxLDL at 100 mg/mL and PC or LPC at 100 μmol/L for 2 hours at 37°C. After removal of these lipids and lipoproteins, the uptake of 0.1 μmol/L [³H]arginine by BAE cells in 3 minutes was determined. B, Time course of the uptake of [³H]arginine by BAE cells with (▲) or without (○) pretreatment with LPC (100 μmol/L). Values in panels A and B are mean±SE of triplicate determinations.

cells were still viable even in the presence of 100 μmol/L LPC in our culture system. The concentration of LPC in the vessel wall of an atherosclerotic lesion, according to the report by Portman and Alexander, was about 1 mmol/L, 10 times higher than the maximum concentration used in the present study. The detergent-like effects do not occur in vivo even at the highest concentration of LPC, probably because most of the LPC present was in a protein-bound form. In this sense, LPC was used in combination with 0.3% albumin as a carrier protein, as described previously.
We examined whether the restoration of NO release by the administration of arginine at high concentrations was caused by the change in the activity of arginine transport. We analyzed the transport of arginine in the presence of extracellular arginine at various concentrations. Increase in the concentration of extracellular arginine facilitated the transport of arginine in a dose-dependent manner (Figure 6A). The addition of LPC reduced the transport particularly at low concentrations of extracellular arginine but not at the highest concentration (3 mmol/L), suggesting a decrease in the affinity of the transport system to arginine. The maximum activity of arginine transport in LPC-treated BAE cells was not significantly different from that in untreated BAE cells. Analysis by the Eadie-Hoffstee plot (Figure 6B) confirmed that there are 2 components of arginine transport with different affinities to arginine in BAE cells, as reported previously.23 The plot further revealed that the inhibition with LPC was highly selective to the high-affinity component of arginine transport, indicating that the restored activity at a high concentration of extracellular arginine was predominantly caused by the low-affinity arginine transporter that was relatively insensitive to LPC.

We confirmed the expression of CAT1, a ubiquitous and major high-affinity transporter, in the endothelial cells using Northern blot analysis (Figure 7A). We examined whether the activity of CAT1 was inhibited by LPC using COS-7 cells transfected with CAT1 cDNA. As expected, the activity of CAT1 was inhibited by LPC in a comparable dose of LPC, directly demonstrating the involvement of CAT1 in the LPC-mediated inhibition of high-affinity transport of arginine (Figure 7B).

Finally, we examined whether the effect of LPC on the arginine transport would be seen in intact blood vessels. Treatment of freshly isolated intact bovine aortas with comparable amounts of LPC from the luminal side significantly decreased activity of arginine transport in endothelial cells (Figure 8), suggesting that LPC inhibits arginine transporter not only in the cultured endothelial cells, but also in vivo in atherosclerotic lesions.

We also examined whether LPC affects the Ca$^{2+}$ transient in BAE cells induced by ADP stimulation in our system, because it is controversial between other reports.24,25 In the present study, LPC did not reveal significant change in the Ca$^{2+}$ transient activity.
(Figure 9). The findings suggest that NO release can be impaired with the change in arginine transport but without the change in agonist-induced intracellular Ca\(^{2+}\) transient.

**Discussion**

The findings in the present study revealed a novel mechanism of the impairment of the arginine-NO pathway in endothelial cells by atherogenic lipid. The failure of NO release by endothelial cells in the basal state and/or in response to various stimuli is thought to be a major change in atherosclerotic lesions.\(^2\) Many reports indicate the amelioration of endothelial dysfunction and retardation in atherogenesis by in vivo arginine supplementation,\(^1\) suggesting that the amount of arginine available to NOS might be decreased in these endothelial cells.

**Extracellular Arginine and NO Production**

To investigate the potential mechanisms of the effect of LPC, we developed an assay system specifically to measure the amount of NO release from endothelial cells suspended in PRP. The advantage of this method is that the medium closely approximates in vivo conditions. Whereas other methods used for the measurement of NO were not sensitive enough, the method used in the present study permitted direct observation of the interaction between endothelial cells and platelets mediated by NO with enough sensitivity in real time to prevent the subtle changes of NO release from being overlooked. Experiments using this method confirmed the reduction of NO release from endothelial cells in the presence of LPC. Despite the existence of \(\approx 0.1\) mmol/L arginine in the medium, supplements of extracellular arginine (10 mmol/L) reversed the reduction, indicating that LPC possibly decreased the availability of extracellular arginine for NOS. In contrast, the NO release from the BAE cells not treated with LPC was not affected by high concentrations of arginine.
These findings suggested that incorporation of arginine from extracellular space would not work well in the LPC-treated cells, in combination with the intracellular arginine supply system for NOS, because the extracellular concentration of L-arginine (about 0.1 mmol/L) is higher than the \( K_m \) value of endothelial NOS. The total arginine transport activity was reduced. The reduction was caused by the decrease in the activity of the high-affinity transport system. This explains why the addition of excess amounts of arginine restores NO release in LPC-treated cells. With an increase in the extracellular arginine level, arginine can be carried by the low-affinity arginine transporter rather than the high-affinity transporter. The \( K_d \) value of high-affinity arginine transporter (about 0.1 mmol/L) was close to the concentration of arginine in normal plasma. Therefore, the change in the activity of the high-affinity arginine transporter markedly affected the total capacity of the arginine transport system in endothelial cells under physiological conditions. Indeed, Closs et al. reported that CAT1 requires cationic amino acids on the \textit{trans} side of the plasma membrane for its transporter activity, but CAT2 does not. Accordingly, if LPC reduces the intracellular arginine level, the activity of the high-affinity arginine transporter decreases. Because at normal physiological concentrations of arginine CAT1 is the predominant transport system in endothelial cells, this decrease results in the lowering of the intracellular arginine concentration and, in turn, a decrease in the arginine transport activity. Although its triggering mechanism is still unknown, this positive feedback loop may produce a deficiency of arginine in endothelial cells. Given that OxLDL readily evokes arginine deficiency in BAE cells in the absence of extracellular arginine, the existence of some mechanism for specifically reducing the available intracellular arginine is suggested.

LPC is assumed to inactivate the arginine transporter with translocation of the transporter from caveolae to some other portion, as observed in endothelial NOS. Endothelial...
NOS reportedly localizes in the caveola, a specialized structure in the plasma membrane. Disruption of the localization by a point mutation of endothelial NOS significantly decreased the activity,\textsuperscript{34,36,37} suggesting that the microenvironment around caveola is important in constitutive production of NO and that the arginine concentration in the cytoplasm might be heterogeneous and concentrated around caveola. According to the recent report of McDonald et al.,\textsuperscript{38} CAT1 and endothelial NOS are colocalized in caveola in endothelial cells. Therefore, a decrease in the concentration of arginine around arginine transporters results in a decrease in the activity of the arginine transporter, as described above, and further decreases the amount of arginine supplied to NOS complexed with CAT1.

**Relationships to Other Proposed Mechanisms**

Several investigators reported that G protein–mediated signal transduction was impaired by OxLDL and LPC.\textsuperscript{39,39} Because the response after transient Ca\textsuperscript{2+} change induced by A23187 was preserved, the upstream mechanism of the transient Ca\textsuperscript{2+} change in response to agonists has been focused on as the cause of the impaired NO release.\textsuperscript{30,41} Inoue et al.\textsuperscript{24} reported that LPC inhibited bradykinin-induced Ca\textsuperscript{2+} transients. The phenomenon observed seems to be a heterologous desensitization, because LPC itself induced Ca\textsuperscript{2+} transients in endothelial cells. In contrast, in the present study, NO release was inhibited by LPC without any significant change in the intracellular Ca\textsuperscript{2+} transient in response to ADP as analyzed with fluo-3 (Figure 9). Shin et al.\textsuperscript{25} also reported that LPC inhibited NO release induced by ATP without decreasing the Ca\textsuperscript{2+} transient of endothelial cells. These results suggest that the impairment of NO release can be induced solely by the reduction of arginine availability, although the Ca\textsuperscript{2+}-dependent mechanism may also work under some conditions. Impairment of basal release of NO, which is independent of the intracellular Ca\textsuperscript{2+} transient, may also be explained by the change of arginine availability. Arginine transport is also stimulated by the agonists of endothelial cells (Figure 10) such as bradykinin and ATP.\textsuperscript{45} The process of receptor-mediated arginine transport may also be involved in the impairment of NO release.

It has been reported that interactions between arginine and glutamine change endothelial NO production and that arginine deficiency causes production of superoxide anions by neuronal NOS.\textsuperscript{43,44} Durante et al.\textsuperscript{45} reported that the transport of cationic amino acid in vascular smooth muscle cells is inhibited in the short term but enhanced in the long term by the treatment of LPC. The enhancement of the transport was not observed in the present study; this may be because of the difference in the origin and the metabolism of the cells. They observed the change in the amount of mRNA for CAT1 and CAT2, but for the short-term effect of LPC, the change does not seem to correlate with the transport activity. Therefore, the inhibitory effect should occur at the protein level of the transporters. There may be a common mechanism reducing the activity of CAT in endothelial cells. Durante et al.\textsuperscript{45} also suggested that the polyamine metabolism might be important for the inhibition of NO production by inducible NOS in vascular smooth muscle cells. The inducible NOS requires much larger amounts of arginine (and various factors should modulate the arginine availability) than is the case with endothelial NOS. Thus, the relationships between arginine metabolism and NO production may be more complicated than the system used in the present study. Our present findings, however, are still compatible with previous reports on other factors. The inhibition of the high-affinity arginine transporter seems to constitute, at least in part, the impairment of NO release by LPC in endothelial cells.

**Therapeutic Possibilities**

Evidence has accumulated that in vivo administration of arginine improves atherosclerosis by increasing NO release from endothelial cells.\textsuperscript{6} The present results suggest the potential of the arginine transporter for the gene therapy of atherogenesis. Two types of high-affinity arginine transporter, CAT1 and CAT3, would be good transgene candidates.

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**References**

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