

High affinity arginine transport of bovine aortic endothelial cells is impaired by lysophosphatidylcholine.

(リゾフォスファチジルコリンはウシ大動脈血管内皮細胞における高親和型アルギニン輸送体を阻害する。)

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HIGH AFFINITY ARGININE TRANSPORT OF BOVINE AORTIC ENDOTHELIAL
CELLS IS IMPAIRED BY LYSOPHOSPHATIDYLCHOLINE.

Running Title : LPC and Arginine Transporter

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Abstract

The mechanisms of endothelial dysfunction characterized by the impaired nitric oxide (NO)-release have not yet been clarified. Since the phenomenon is mimicked by the application of oxidized LDL and, its major lipid constituent, lysophosphatidylcholine (LPC) *in vitro*, we analysed their effects on the arginine-NO system, especially on the arginine transport system. LPC inhibited NO-release induced by ADP in cultured bovine endothelial cells (BAE). The inhibition was attenuated by the excess amount of extracellular arginine. LPC was found to inhibit the arginine transport in BAE, 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, since 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, cationic amino acid transporter-1 (CAT-1) expressed in COS7 cells. The activity of CAT-1 might be important for the prevention of endothelial dysfunction.

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

Introduction

Numerous recent studies demonstrated that NO has a variety of anti-atherogenic 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 atherogenesis¹. Indeed, the vascular endothelium in atherosclerosis fails to release NO in contrast to that in the normal vascular bed².

In vivo studies have revealed that arginine supplement improved NO-release from the endothelium both in hypercholesterolemic animals and humans³⁻⁵. Moreover, significant alleviation of the formation and progression of atherosclerosis by arginine supplement has been reported⁶. 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.

However, evidence has accumulated that oxidized low density lipoprotein (OxLDL) or its lipid constituent, 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/macrophages⁹. 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 four members identified to date of the system y⁺ transporter family are cationic amino acid

transporter (CAT)1, CAT2a, CAT2b and CAT3^{12, 13}. According to their different affinities to arginine, three of these transport systems (CAT1, CAT2b and CAT3) are classified as high affinity ($K_d \sim 10^{-4} \text{M}$) transporters, whereas, CAT2a, an alternatively spliced isoform of CAT2b, is classified as a low affinity ($K_d > \sim 10^{-3} \text{M}$) transporter.

The present results revealed that: (i) the decrease in NO production in endothelial cells treated with LPC was reversed by high dose of arginine, (ii) arginine uptake of endothelial cells were mediated by the two pathways, high and low affinity arginine transport systems, and (iii) 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 cells (BAE) were maintained in Dalbecco's minimum essential medium (DMEM) supplemented with 10% fetal calf serum, 100 U/ml of ampicillin, 0.1 mg/ml of streptomycin, and 250 ng/ml of 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 an LDH-Cytotoxic test kit (Wako).

Preparation of low density-lipoprotein (LDL).

Human plasma LDL ($d=1.019-1.063$) was isolated by sequential ultracentrifugation, and oxidative modification of LDL was carried out with cupric ion *in vitro*, as described previously¹⁴. Oxidation was monitored by measuring the amount of thiobarbituric acid-reactive substances (around 10 nmol

malondialdehyde equivalent/mg protein in OxLDL). Agarose gel electrophoresis revealed an increase in electrophoretic mobility and minimal aggregation of OxLDL particles.

Bioassay of NO-release from BAE.

BAE seeded for the experiments were cultured for 16h in Eagle's minimum essential medium lacking arginine (Gibco). The cells were harvested by trypsinization, and then incubated for 2h at 37°C in Hepes-buffered saline (HBS) (10 mM Hepes, 5 mM glucose, 150 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, pH7.5), 0.3% bovine serum albumin (BSA) containing LPC or OxLDL at the concentrations indicated in the figures. After washing twice with HBS, BAE were used for the measurement of antiaggregatory effects.

Into a tube containing 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 two weeks. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were obtained as supernatants following centrifugation for 10 min at 160 x g and 1500 x g, respectively¹⁵. Two hundred μ l of PRP prewarmed at 37°C was set in an aggregometer (Hema tracer 601, NBC, Japan) with continuous stirring at 1000 rpm. Twenty μ l of the suspension of BAE, prepared as described above, was added to the PRP and incubated for 1 min. Addition of endothelial cells (5×10^4 - 1.5×10^5 cells) did not significantly affect the light transmission. Then, test reagents were added and incubated for 1 min. ADP was added to initiate platelet aggregation and to stimulate endothelial cells simultaneously. The numbers of BAE and the concentrations of ADP or test reagents used are shown in the figures. Changes in light transmission were recorded for 4 min after the stimulation, calibrated with the light transmission of PPP taken as the 100 % transparent control and that of PRP as the 0 % reference¹⁶.

Measurement of transient Ca²⁺-change induced by ADP.

BAE pretreated with Eagle's minimum essential medium without arginine (Gibco) were harvested by trypsinization and incubated for 1.5 h at 37°C in Ca²⁺ Loading Buffer (5 mM Hepes, 1 mM Na₂HPO₄, 5 mM glucose, 140 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 0.3% BSA, pH7.4) containing LPC or phosphatidylcholine (PC). Then, the suspension was added by Fluo-3/AM and further incubated for 30 min, as described elsewhere ¹⁷. The change of intracellular Ca²⁺ evoked by 10⁻⁵ M ADP was monitored by a fluorescence spectrophotometer (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 on 6 well-plates were cultured in Eagle's minimum essential medium(MEM) without arginine (Gibco) for 16 h before the experiments. The cells were washed twice with HBS and incubated at 37°C for 2 h with HBS, 0.3% BSA containing LPC or reagents indicated in the text. The media were then replaced with HBS and 0.3% BSA containing 10⁻⁷ M of [³H]arginine or [³H]leucine (222 GBq/mmol) to be taken up by the cells. The uptake was stopped by washing the cells three times with ice-cold HBS and 0.3 % BSA, and the cells were lysed with 0.2 M 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 endothelium of intact bovine aortas

Bovine aortas freshly obtained from a local slaughter house were cut longitudinally and incubated at 37°C for 2 h with HBS, 0.3% BSA with or without 100 μM LPC. The media were changed with HBS containing [³H]arginine to allow the aortas to incorporate arginine for 3 min. After being washed with ice-cold HBS, the endothelium were scraped off with cover slips and lysed with 0.2 M NaOH and 1% SDS. Then, the radioactivity and protein concentration of the lysate were

determined. The scraped cells were identified as vascular endothelial cells by the further culture of the cells in parallel experiments.

cDNA Cloning of cationic amino acid transporter-1 (CAT1)

cDNA libraries from human lung and brain constructed in λ gt10 vector were screened by the standard plaque hybridization method as described¹³. 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 CAT-1 cDNA.

Transient expression of CAT1 in COS-7 cells

The *Eco*RI 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 μ g of pMEhCAT1 or with the vector (for basal uptake) using LipofectAMINETM (Life Technologies, Inc.) according to the manufacture's instructions. Twenty-four hours later, the cells were transferred to 12-well plates and the medium was changed with arginine-free MEM. Twenty-four hours later, the cells were used for the determination of arginine-uptake¹³.

Nothern blotting

Poly(A)⁺ RNA (10 μ g per lane), prepared from BAE, was separated by a formaldehyde/1.1% agarose gel electrophoresis, and transferred to a nylon membrane (Gene Screen Plus, DuPont). The membrane was hybridized with the probe, prepared by labeling the *Eco*RI fragment of pMEhCAT1 with [³²P]dCTP (110 TBq/mmol, Amersham). Blotting procedures were as described¹⁸. The blot was visualized for radioactivity with a BAS2000 image analyser (Fujifilm Co. Ltd., Tokyo, Japan)

Results

To measure the amount of NO released from BAE, we used a standard bioassay based on the anti-aggregatory effect of NO as previously described¹⁶. PRP was used to analyze the reaction under conditions similar to those *in vivo*. ADP was chosen for initiating platelet-aggregation, since it simultaneously activates endothelial cells to release NO. ADP induced platelet aggregation in PRP in a dose-dependent manner (Fig. 1A). As expected, sodium nitroprusside, a NO-donor, inhibited the ADP (10^{-5} M)-induced platelet aggregation in a dose-dependent manner (Fig. 1B). Similarly, addition of BAE to PRP also inhibited ADP-stimulated platelet aggregation (Fig. 1C). The inhibitory effect of BAE was dependent on the number of BAE and was inhibited by L-nitroarginine-methylester (NAME), a NO synthase (NOS) inhibitor which has little effect on arginine transport (Fig. 1D)¹⁹. Although the inhibition by NAME was to a moderate extent, it may be because the serum contained about 0.1 mM arginine, one-tenth of the concentration of NAME used. Without addition of BAE, 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 (Fig. 2). The inhibited NO-release was restored by the addition of 10 mM arginine, but not by 10 mM lysine, which is also a dibasic amino acid but not a substrate of NOS (Fig. 3). In addition, the NO-release from untreated BAE was not affected by the addition of 10 mM arginine or lysine (data not shown). The findings also demonstrated that BAE 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, since the

concentration of serum arginine is about 0.1 mM, 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 in the presence of 100 nM of [^3H] arginine. As expected, the transport was significantly inhibited by the addition of LPC or OxLDL but not by PC or native LDL (Fig. 4A). The inhibitory effect of LPC reached the maximum within one hour of incubation, and continued for at least 5 hours (Fig. 4B, Fig. 5A). This inhibitory effect was dependent on the concentration of LPC (Fig. 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 (Fig. 5C).

To exclude the possibility that these changes in arginine transport were due to non-specific detergent-like effects of LPC, the viability of BAE was confirmed using the trypan blue exclusion assay and by measurement of the activity of lactate dehydrogenase released from dead BAE in the culture media. The results disclosed that BAE were still viable even in the presence of 100 μM LPC in our culture system. The concentration of LPC in vessel wall of atherosclerotic lesion, according to the report by Portman and Alexander, was about 1 mM, ten times higher than the maximum concentration used in the present study²⁰. Why the detergent-like effects do not occur *in vivo* even at the highest concentration of LPC is probably because most of LPC present was in a protein-bound form. In this sense, LPC was used in combination with 0.3 % albumine as a carrier protein, as described previously^{21, 22}.

Then, we examined whether the restoration of NO-release by the administration of arginine at high concentrations was due to 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 (Fig. 6A). The addition of LPC reduced the transport particularly at low concentrations of extracellular arginine, but not at the highest concentration (3 mM),

suggesting a decrease in the affinity of the transport system to arginine. The maximum activity of arginine transport in LPC-treated BAE was not significantly different from that in untreated BAE.

Analysis by the Eadie-Hoffstee plot (Fig. 6B) confirmed that there are two components of arginine transport with different affinities to arginine in BAE, as reported previously²³. 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 high concentration of extracellular arginine was predominantly due to the low affinity arginine transporter which was relatively insensitive to LPC.

Then, we confirmed the expression of CAT1, a ubiquitous and major high affinity transporter, in the endothelial cells using northern blot analysis (Fig. 7A). We, therefore, examined if 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 comparable dose of LPC, directly demonstrating the involvement of CAT1 in the LPC-mediated inhibition of high affinity transport of arginine (Fig. 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 amount of LPC from luminal side significantly decreased activity of arginine transport in endothelial cells (Fig. 8), suggesting that LPC inhibits arginine transporter not only in the cultured endothelial cells, but also *in vivo* in atherosclerotic lesions.

In addition, we examined whether LPC affects the Ca^{2+} -transient in BAE induced by ADP stimulation in our system, since it is controversial among the reports^{24, 25}. In the present study, LPC did not reveal significant change in the Ca^{2+} -transient (Fig. 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 lesions². Many reports indicate the amelioration of endothelial dysfunction and retardation in atherogenesis by *in vivo* arginine supplement^{3-6, 26-29}, 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 is close to *in vivo* conditions. While 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 being overlooked. Experiments using this method confirmed the reduction of NO-release from endothelial cells in the presence of LPC. Despite of the existence of about 0.1 mM arginine in the medium, supplements of extracellular arginine (10 mM) reversed the reduction, indicating that LPC possibly decreased the availability of extracellular arginine for NO synthase. In contrast, the NO-release from the BAE untreated 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 NO synthase, since the extracellular concentration of L-arginine (about 0.1 mM) is higher than the K_m value of endothelial NOS (23, 24). Indeed, the total arginine transport activity was reduced. The reduction was due to 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 mM) 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 the uptake of arginine by CAT1, which mediates high affinity transport, was several times higher than that by CAT2, which mediates the low affinity transport of arginine, at physiological concentrations using an expression system in *Xenopus oocytes*³⁰.

The mechanism of selective inhibition of the high affinity arginine transporter

The detailed mechanisms of selective inhibition of the high affinity arginine transporter are unclear (Fig. 7). Closs *et al.* reported that CAT1 requires cationic amino acids on the *trans* side of the plasma membrane for its transporter activity, but CAT2 does not³¹. Accordingly, if LPC reduces the intracellular arginine level, the activity of the high affinity arginine transporter decreases. Since 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 in the absence of extracellular arginine, the existence of some mechanism for specifically reducing the available intracellular arginine is suggested³².

Secondly, LPC is assumed to inactivate the arginine transporter with translocation of the transporter from caveolae to some other portion, as observed in endothelial NOS³³⁻³⁵. Endothelial NOS reportedly localizes in caveolae, specialized structures in the plasma membrane. Disruption of the localization by a point mutation of endothelial NOS significantly decreased the activity^{34, 36, 37}, suggesting that the microenvironment around caveolae is important in constitutive production of NO, and that the arginine concentration in the cytoplasm might be heterogeneous and concentrated around caveolae. According to the recent report of McDonald *et al.*, CAT1 and endothelial NOS are co-localize in caveolae in endothelial cells³⁵. 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^{38, 39}. Since the response following transient Ca^{2+} change induced by A23187 was preserved, the upstream mechanism of the transient Ca^{2+} change in response to agonists has been focused on as the cause of the impaired NO-release^{40, 41}. Inoue *et al.* reported that LPC inhibited bradykinin-induced Ca^{2+} -transients²⁴. The phenomenon observed seems to be a heterologous desensitization, because LPC itself induced Ca^{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^{2+} -transient in response to ADP as analyzed with Fluo-3 (Fig. 9). Shin *et al.* also reported that LPC inhibited NO-release induced by ATP without decreasing Ca^{2+} -transient of endothelial cells²⁵. These results suggest that the impairment of NO-release can be induced solely by the reduction of arginine availability, although the Ca^{2+} -dependent mechanism may also work under some conditions. Impairment of basal release of NO, which is

independent of the intracellular Ca^{2+} -transient, may also be explained by the change of arginine availability. It is noteworthy that arginine transport is also stimulated by the agonists of endothelial cells such as bradykinin and ATP⁴². 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 NO synthase^{43, 44}. Durante *et al.* reported that the transport of cationic amino acid in vascular smooth muscle cells is inhibited in the short term but enhanced in long term by the treatment of LPC⁴⁵. The enhancement of the transport was not observed in the present study, this may be due to the difference in the origin and the metabolism of the cells. They observed the change in the amount of mRNA for CAT-1 and -2, but for 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. They also suggested that the polyamine metabolism might be important for the inhibition of NO-production by *inducible NO synthase* in vascular smooth muscle cells. The inducible NO synthase 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 transport appears to constitute, at least in part, the impairment of NO-release by LPC in endothelial cells.

Therapeutic possibilities

Finally, evidence has accumulated that *in vivo* administration of arginine improves atherosclerosis by increasing NO-release from endothelial cells⁶. 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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Footnotes

Abbreviations used in this paper:

NO, nitric oxide; LDL, low density lipoprotein; OxLDL, oxidized LDL; PC, phosphatidylcholine; LPC, lysophosphatidylcholine; NOS, nitric oxide synthase; BAE, bovine aortic endothelial cells.

Figure Legends

Fig. 1.

Measurement of NO released from BAE by its anti-aggregatory effects in platelet-rich plasma. The platelet aggregation was measured by the increase of light-transmission.

A, Dose-dependent aggregation of platelets. Platelet aggregation was induced by the application of ADP (10^{-5} ~ 10^{-6} M).

B, Dose-dependent inhibition of ADP-induced platelet aggregation by a NO donor, sodium nitroprusside (10^{-8} ~ 10^{-4} M).

C, Dose-dependent inhibition of ADP-induced platelet aggregation by BAE (5×10^4 ~ 1.5×10^5 cells).

D, Inhibition of anti-aggregatory effects of BAE by 1mM NAME. NAME alone did not affect the platelet aggregation in the absence of BAE.

Fig. 2.

LPC dose-dependently impaired the anti-aggregatory effect of BAE in PRP.

BAE were pretreated with LPC at the concentrations indicated. Then, the anti-aggregatory effect of BAE (7.5×10^4 cells) was measured after removal of LPC.

Fig. 3.

Supplement with arginine restored the anti-aggregatory effect of BAE impaired by LPC.

BAE (7.5×10^4 cells) were pretreated with $100 \mu\text{M}$ LPC. Anti-aggregatory effects of BAE (7.5×10^4 cells) stimulated with 10^{-5} M ADP were measured in PRP supplemented with or without arginine or lysine (10 mM). Neither arginine nor lysine affected the platelet aggregation without BAE (data not shown).

Fig. 4.

Atherogenic lipid and lipoprotein inhibited the uptake of arginine by BAE.

A, BAE were pretreated with native LDL or OxLDL at 100 mg/ml and PC or LPC at 100 μ M for 2h at 37°C. After removal of these lipids and lipoproteins, the uptake of 0.1 μ M [3 H]arginine by BAE in 3 min was determined.

B, Time course of the uptake of [3 H]arginine by BAE with (closed triangle) or without (open circle) pretreatment with LPC (100 μ M).

Values shown in both A and B are the means $\pm$ S.E. of triplicate determinations.

Fig. 5.

A, Effects of preincubation time on LPC treatment. BAE were treated with 100 μ M LPC for the indicated periods. Then, the uptake of [3 H]arginine was determined as described in "Materials and methods."

B, Dose-dependent inhibition by LPC of arginine uptake by BAE. BAE was treated with the indicated amount of LPC for 2 h at 37°C. Then, the uptake of [3 H]arginine was determined.

C, Uptake of leucine was not inhibited by LPC even at the highest concentration. BAE were treated with the indicated amount of LPC for 2h at 37°C. Then, the uptake of leucine was determined using the same procedure for arginine.

All the values shown are the means $\pm$ S.E. of triplicate determinations.

Fig. 6.

LPC selectively inhibited the high affinity arginine-transport system of BAE.

A, Effects of concentration of extracellular arginine on the uptake by BAE with (closed triangle) or without (open circle) pretreatment with LPC (100 μ M). The velocity of arginine uptake by BAE treated with or without LPC were determined in the presence of different amounts of arginine in the medium.

B, Eadie-Hoffstee plot of arginine uptake by BAE. The data in A are rearranged in the Eadie-Hoffstee plot. The arginine uptake by BAE consists of high and low affinity transport systems. The K_d of the high and low affinity arginine transport

systems of BAE were 68 and 847 μM , respectively. Those of BAE treated with LPC were 69 and 1130 μM , respectively. The V_{max} of the high and low affinity arginine transport systems of BAE were 8.07 and 21.71 nmol/min/mg protein, respectively, and these changed to 1.42 and 14.67 nmol/min/mg protein, respectively after LPC treatment.

Values shown in both A and B are the means $\pm$ S.E. of triplicate determinations.

Fig.7.

Expression of CAT1 in endothelial cells and inhibition of CAT1 by LPC.

A. Northern blot analysis of CAT1 in BAE.

B. Inhibition by LPC of the arginine transport activity of CAT1 expressed in COS-7 cells. COS-7 cells transfected with CAT1 cDNA or vector were treated with the indicated reagents for 2h at 37°C. Then, the activity of arginine uptake were determined. The CAT1 mediated activity was determined as the difference between the values from CAT1 transfected cells and that from mock transfected cells. Values shown are the means $\pm$ S.E. of triplicate determinations.

Fig.8

Inhibition by LPC of arginine transport activity of endothelial cells in intact bovine aortas. The intimal side of freshly separated aortas were incubated with the indicated reagents for 2 h at 37°C. After the removal of reagents, the activity of arginine uptake were determined. Values shown are the means $\pm$ S.E. of triplicate determinations.

Fig.9

Intracellular Ca^{2+} -transient induced by ADP was not affected by LPC
BAE were pretreated with (left) or without (right) 100 μM LPC for 2h. Fluo-3 was loaded into the cells in suspension. Then, the cells were stimulated with 10 μM ADP. Intracellular Ca^{2+} -transient measured by the change in fluorescence at 540

nm is displayed. There was no significant change in Ca^{2+} -transient between LPC treated and untreated cells.

Fig.10

Schematic representation of arginine transport systems in endothelial cells.

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Fig. 1

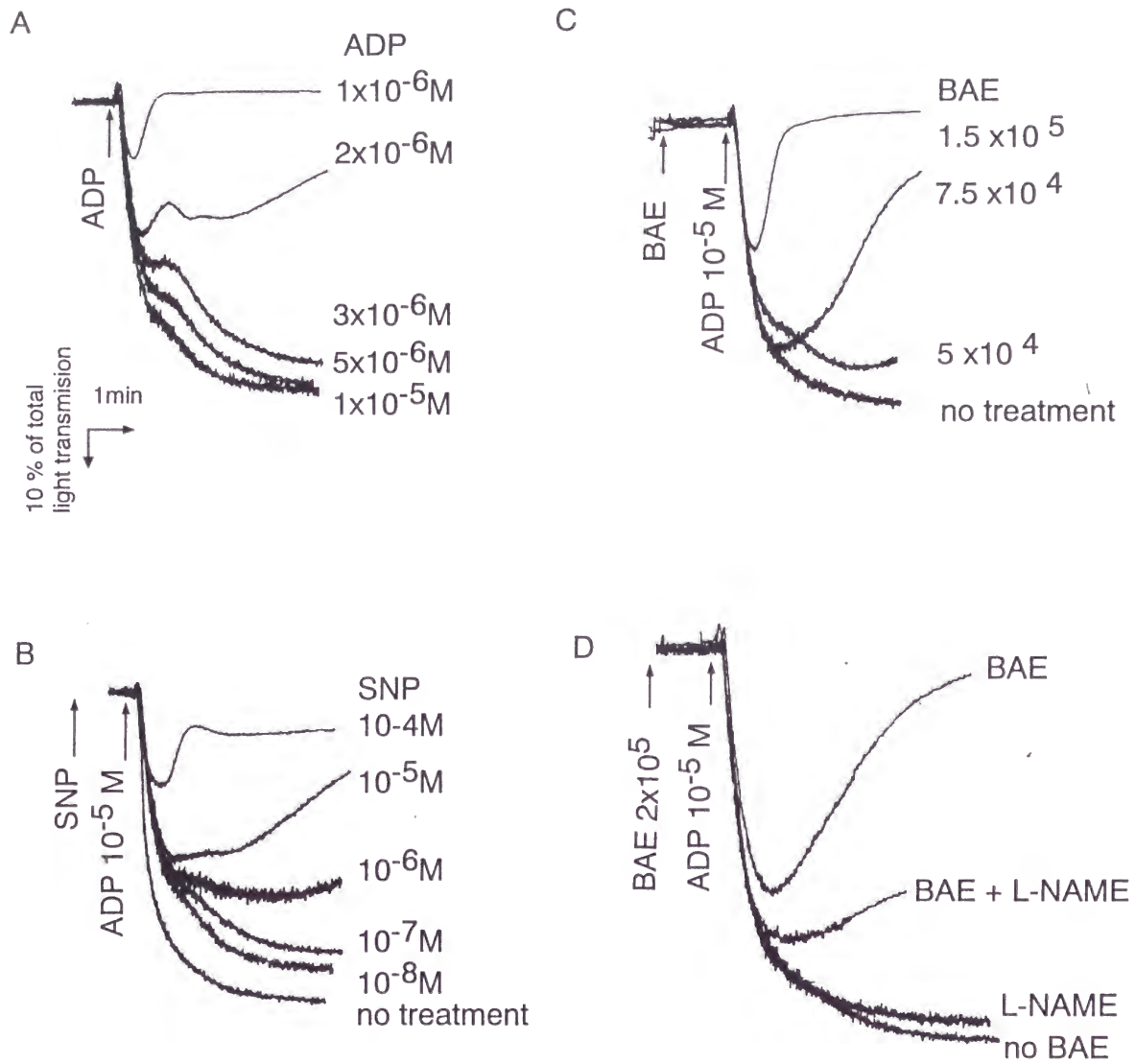


Fig. 2

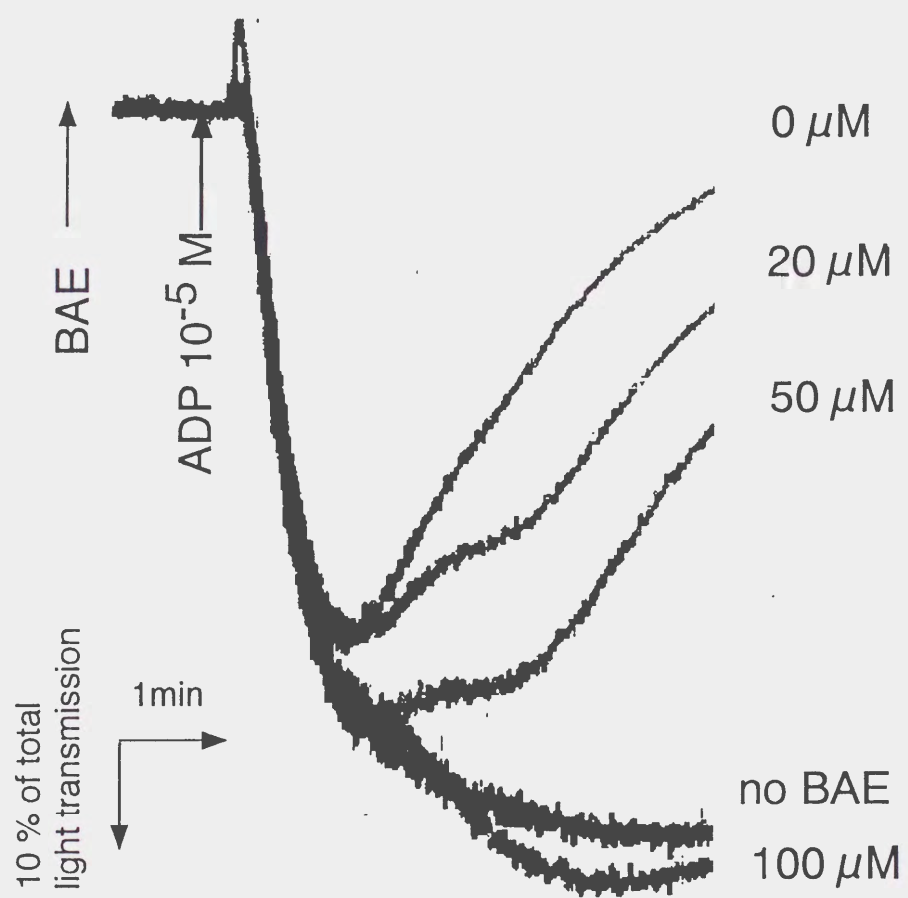


Fig. 3

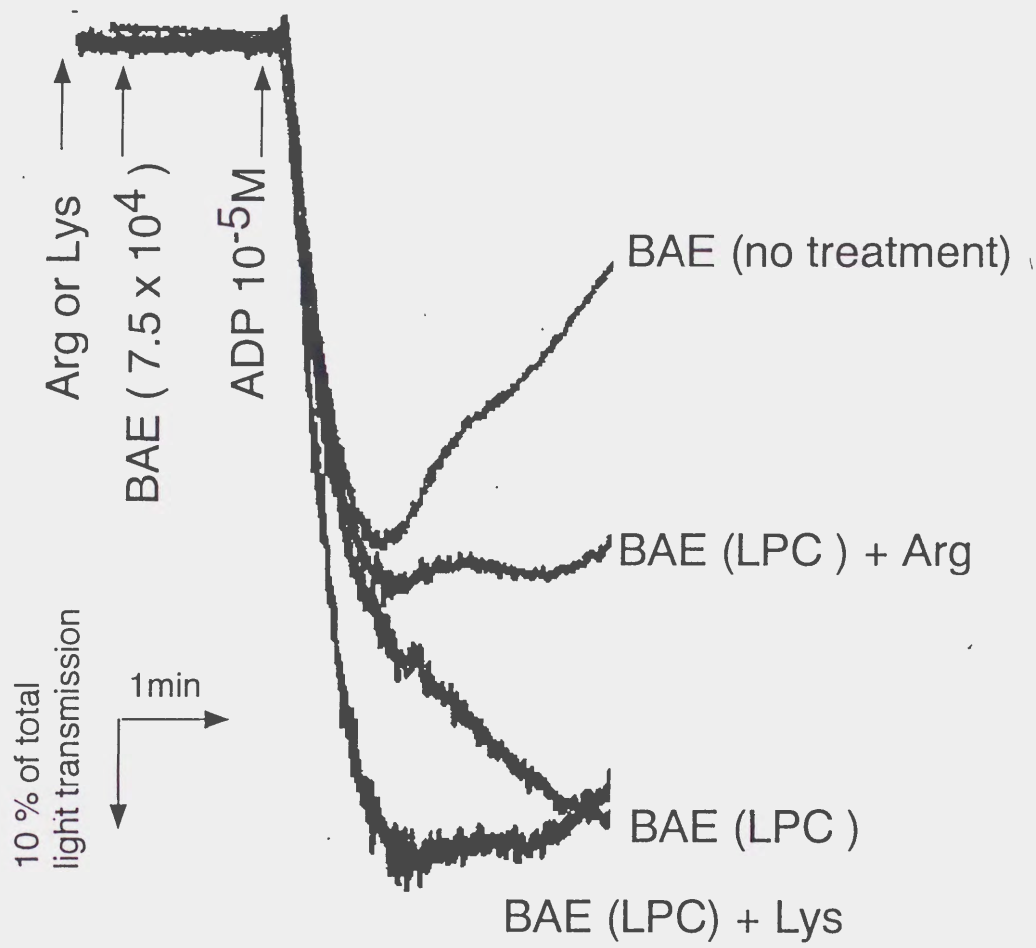


Fig. 4

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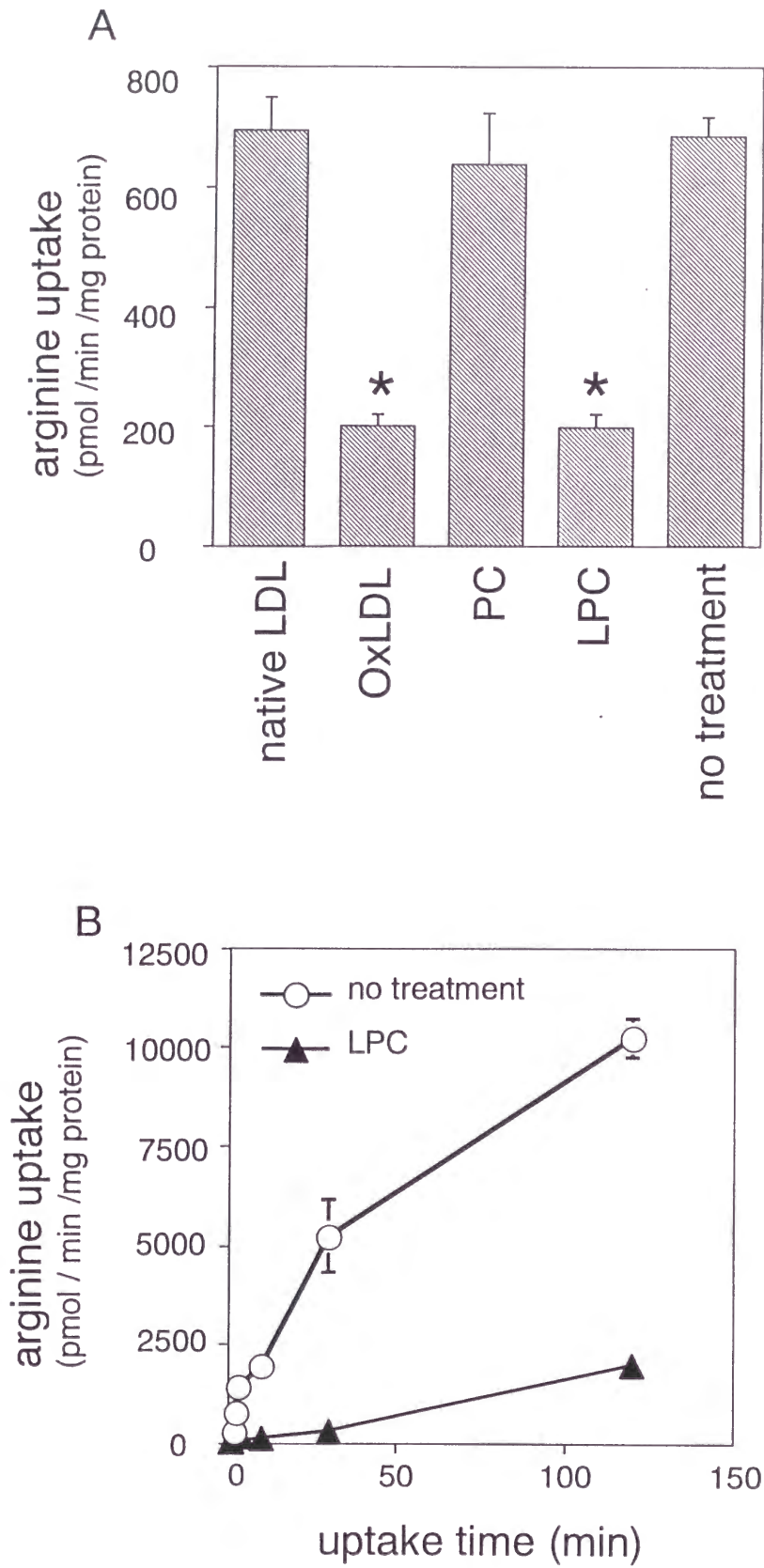


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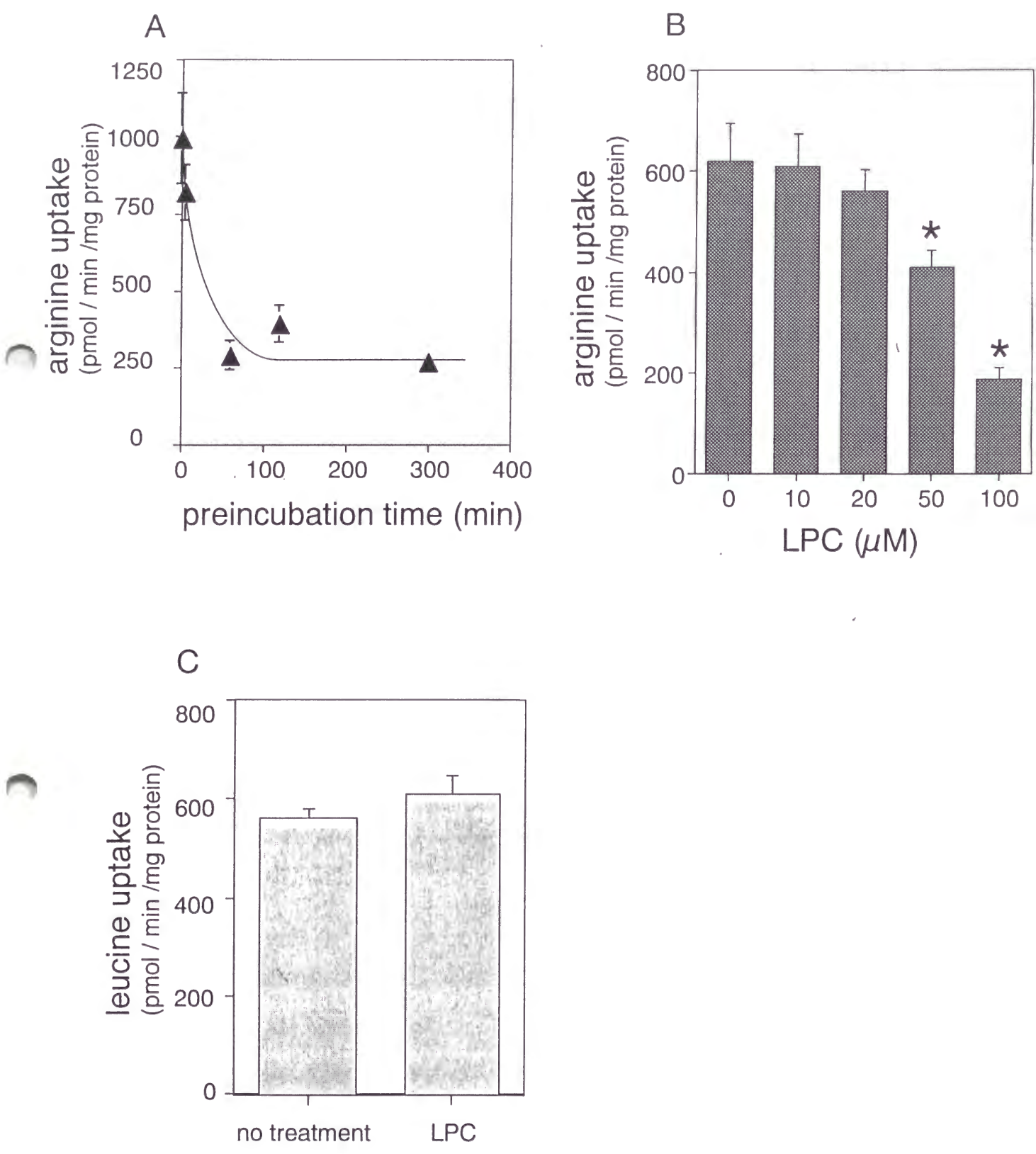


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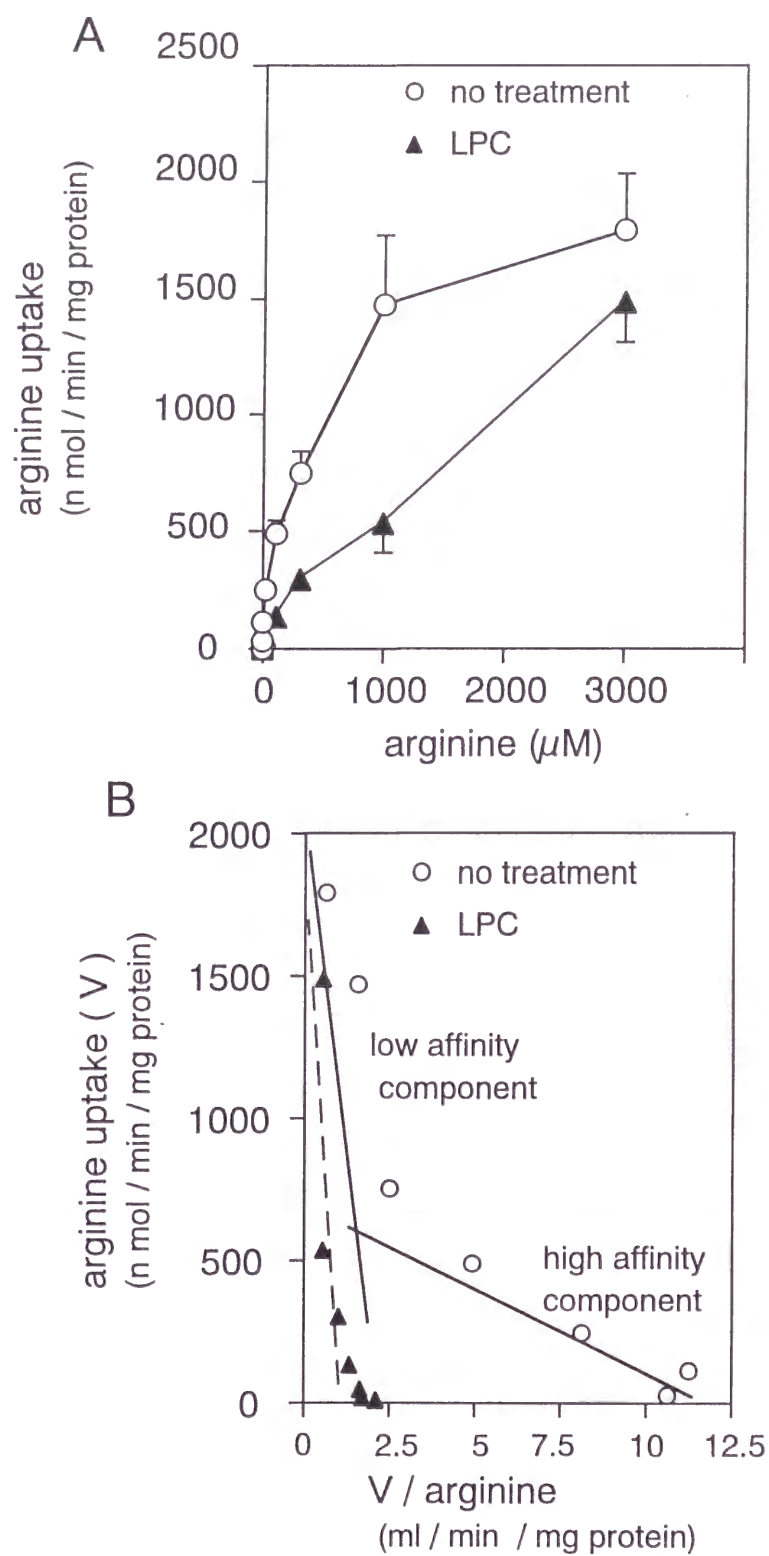


Fig. 7

Kikuta et al.

A



B

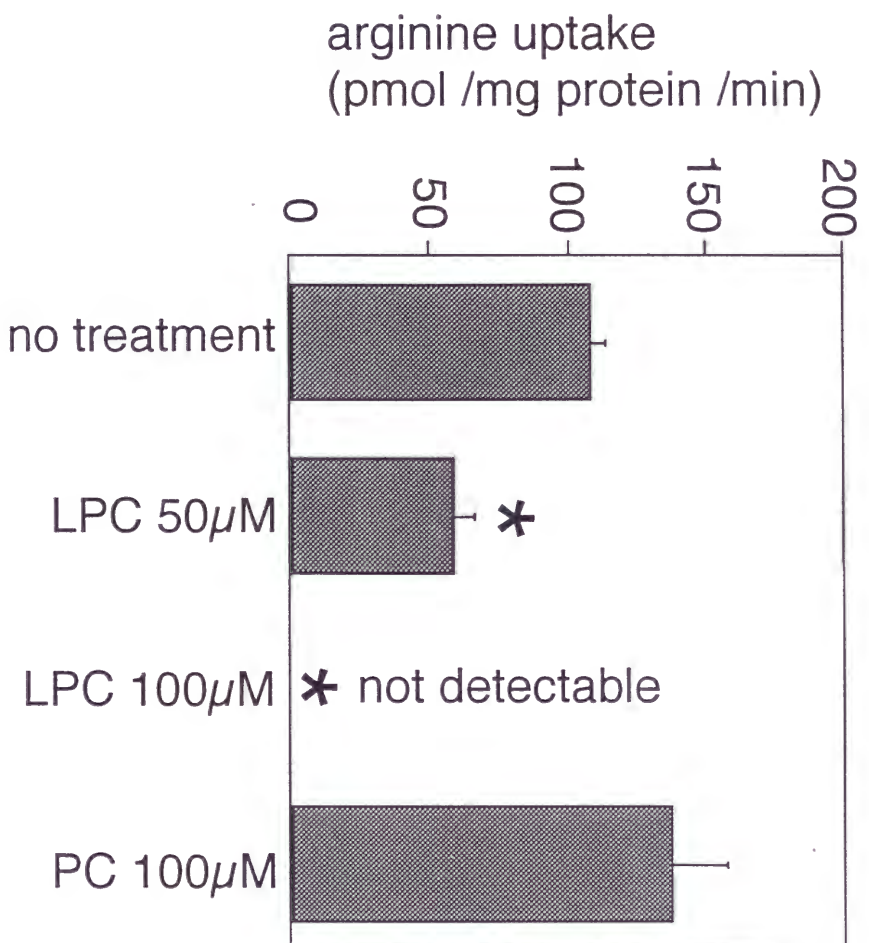


Fig. 8

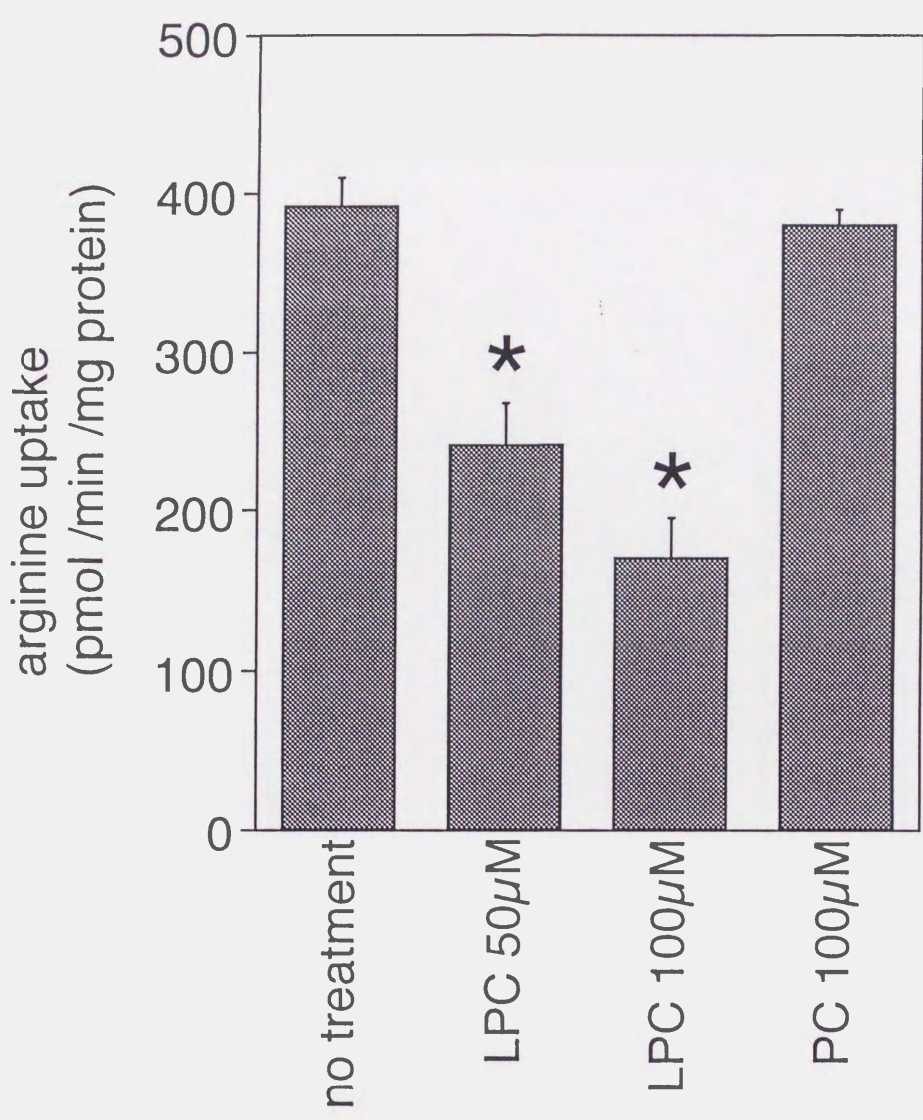


Fig. 9

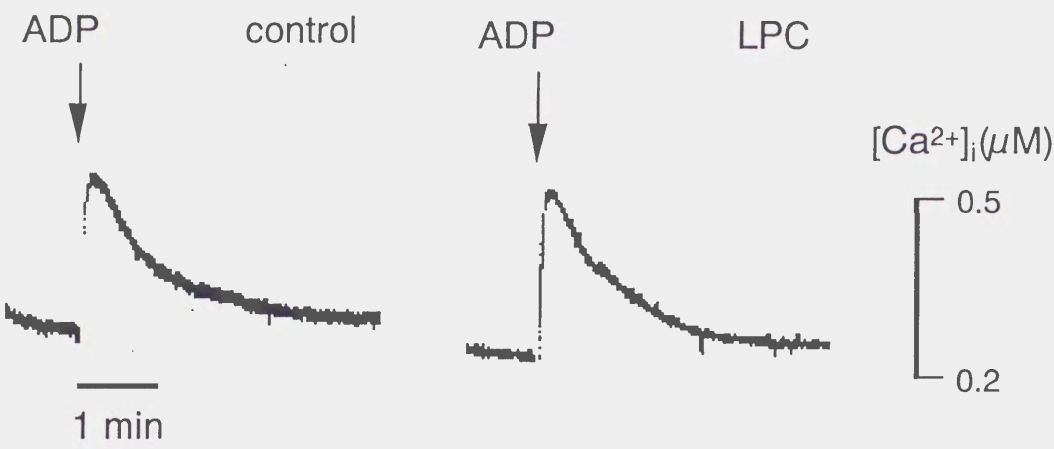
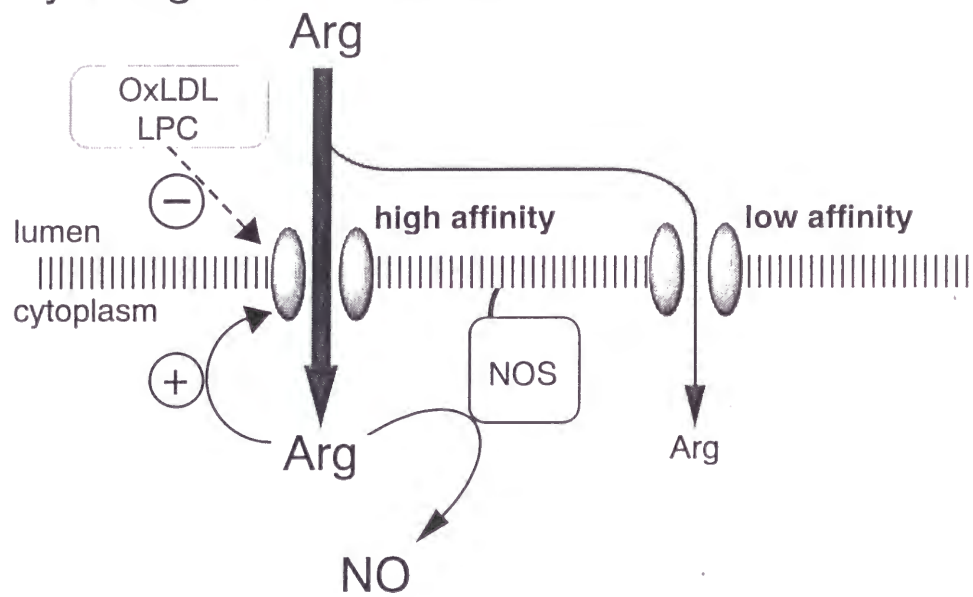


Fig. 10

A. physiological concentration



B. high concentration

