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Induction of cationic amino acid transport activity in mouse peritoneal macrophages by lipopolysaccharide

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The transport of cationic amino acids has been investigated in mouse peritoneal macrophages cultured in vitro. The transport activity for lysine was rather low in cells cultured for 1 h and increased slightly in cells cultured for 12 h. This increase varied with the serum lot used in the culture medium and was suppressed by polymyxin B, suggesting that the transport activity is induced by endotoxins in the serum. When the macrophages were cultured in the medium containing 1 ng/ml lipopolysaccharide, the transport activity for lysine increased by more than 10-fold. The transport activity for lysine induced by lipopolysaccharide has been characterized. Lysine was transported mainly by a Na^+-independent, saturable system. The uptake of lysine was potently inhibited by extracellular cationic amino acids, but not by neutral amino acids tested. In addition, transport of lysine showed *trans*-stimulation. From these results, we have concluded that the transport activity for cationic amino acids is potently induced by lipopolysaccharide and that the characteristics of the induced activity is consistent with those of system y^+.

Introduction

Amino acid transport systems in the plasma membrane have been studied in a variety of mammalian cells. In most cell types, major components, including systems A, ASC and L, mediate the uptake of neutral amino acids [1]. For anionic amino acids, some transport systems have been described, e.g., system X^-_{AG} and system x^-_c. Cationic amino acid such as lysine, arginine and ornithine are the ordinary substrates for system y^+, which is found in many types of cell, including Ehrlich cells [2], rabbit reticulocytes [3], cultured human fibroblasts [4], hepatoma cells [5] and murine P388 leukemia cells [6]. This system is Na^+-independent and subject to *trans*-stimulation, defined as the stimulation of the amino acid influx by the substrates inside the cell. System y^+ usually transports cationic amino acids with a relatively high affinity and mediates both influx and efflux of substrates across the plasma membrane [7].

We have previously reported that in mouse peritoneal macrophages anionic amino acids are transported through system x^-_c [8], a Na^+-independent system highly specific for cystine and glutamate, and that most neutral amino acids are taken up mainly through a common transport system with some resemblance to system L [9]. However, there have been no reports on cationic amino acid transport in macrophages.

Macrophages are known to play a central and essential part in specific and nonspecific immune responses [10]. It has been reported that cationic amino acids are involved in such functions as tumoricidal activity in macrophages [11–15]. In the present study, we have investigated cationic amino acid transport in macrophages stimulated by lipopolysaccharide (LPS). LPS is composed of a lipid moiety termed lipid A and a polysaccharide moiety and is a protein-free endotoxin which is an integral component of the outer membrane of Gram-negative bacteria [16]. The results demonstrate that the activity of the transport system for cationic amino acids is induced by treating the cells with LPS and that the induced transport activity is identified with that of system y^+.

Materials and Methods

Materials

L-[4,5-^{3}H(N)]Lysine and L-[4,5-^{3}H]leucine were obtained from Du Pont-New England Nuclear, Boston,

Abbreviations: LPS, lipopolysaccharide; BCH, 2-aminobicyclo-[2.2.1]-heptane-2-carboxylic acid.

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MA. Thioglycollate broth (Brewer's formula) and Bacto lipopolysaccharide were from Difco Laboratories, Detroit, MI. BCH was from Behring Diagnostics, La Jolla, CA. Unlabeled amino acids were from Sigma, St. Louis, MO. Culture media were from Gibco, Chagrin Falls, OH. Female C57BL/6N mice were purchased from Doken, Ibaraki, Japan. Fetal bovine serum was obtained from M.A. Bioproducts, Walkersville, MD. In some experiments, fetal bovine sera obtained from Hyclone Laboratories, Logan, UT, Filtron PTY, Victoria, Australia and Cwlth Serum Laboratories, Victoria, Australia, were used. A kit for *Limulus amebocyte* lysate test was purchased from Seikagakukogyo, Tokyo, Japan.

Macrophage cultures

Macrophages were collected by peritoneal lavage from female C57BL/6N mice, weighing 20–25 g, who had received 4 days previously an intraperitoneal injection of 2 ml of 4% thioglycollate broth. The lavage medium was RPMI 1640 containing 10 units/ml heparin. The cells were washed twice with RPMI 1640, plated at $1 \cdot 10^6$/35 mm plastic culture dish or $2.5 \cdot 10^6$/60 mm dish in RPMI 1640 containing 10% fetal bovine serum, 50 units/ml penicillin and 50 μg/ml streptomycin, and incubated at 37 °C in 5% CO_2, 95% air. After 1 h the medium was renewed to remove nonadherent cells.

Uptake of amino acid

Amino acid uptake was measured by techniques described previously [17]. After culture, the cells were rinsed three times in warmed PBSG (10 mM phosphate-buffered saline (137 mM NaCl, 3 mM KCl), pH 7.4 containing 0.01% $CaCl_2$, 0.01% $MgCl_2 \cdot 6H_2O$ and 0.1% glucose). They were then incubated in 0.5 ml of the warmed uptake medium for specified time periods at 37 °C. The uptake medium was PBSG containing the ^{3}H-labeled lysine. When we measured Na^+-dependency of the uptake of lysine, the uptake of the amino acid was measured in a Na^+-free medium, where Na^+ was replaced by choline. However, in all other experiments the uptake was determined in the Na^+-containing buffer. The uptake was terminated by rapidly rinsing the dish three times with ice-cold phosphate-buffered saline and then the radioactivity in the cell was measured. The rates of uptake were determined under conditions approaching the initial uptake rates, i.e., by taking the values for the 30 s uptake of lysine.

Kinetic parameters of lysine uptake were determined by nonlinear regression analysis. The equation used was:

$$v = \frac{V_{max} \cdot [S]}{K_m + [S]} + K_d \cdot [S] \quad (1)$$

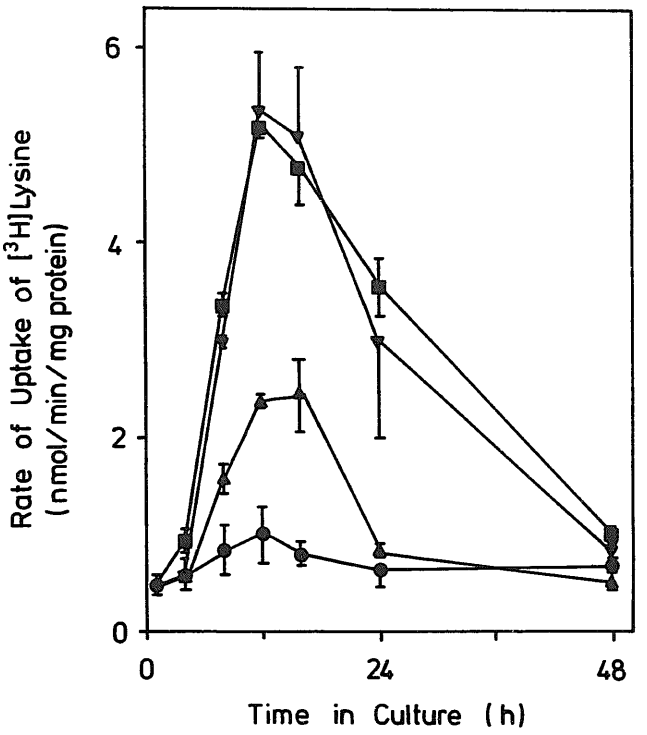

Fig. 1. Changes in the rates of the uptake of lysine by macrophages cultured with LPS. Macrophages collected by peritoneal lavage were cultured and after 1 h the medium was replaced by a fresh medium with or without LPS. The rates of the uptake of 0.05 mM L-[^{3}H]lysine were measured at the times indicated. The cells were cultured in the normal medium (●); with 0.1 ng/ml LPS (▲); with 1 ng/ml LPS (▼); with 10 ng/ml LPS (■). Each point represents the means ± S.D. of more than four determinations from two to six separate experiments.

This equation represents the total influx as the sum of a single saturable system plus a non-saturable component.

Results

Effect of LPS on the activity of lysine uptake during culture

The initial rate of lysine uptake was measured in macrophages cultured with and without LPS (Fig. 1). The activity was relatively low in early time in culture. It slightly increased and reached maximum at 12 h in culture without LPS. The rate of lysine uptake, however, was greatly augmented in macrophages incubated with LPS. The increase of the rate of the uptake was observed at 0.1 ng/ml LPS and became maximum at 1 ng/ml. The decline of the activity of lysine uptake was observed after 16 h in culture (Fig. 1). A re-enhancement of the activity was observed by the addition of LPS at a similar quantity to the cells of which the activity of lysine uptake had been decreased (data not shown). This suggests that the decline of the transport activity was caused by the reduction of LPS in the medium, e.g., by the uptake of LPS into the cells [18].

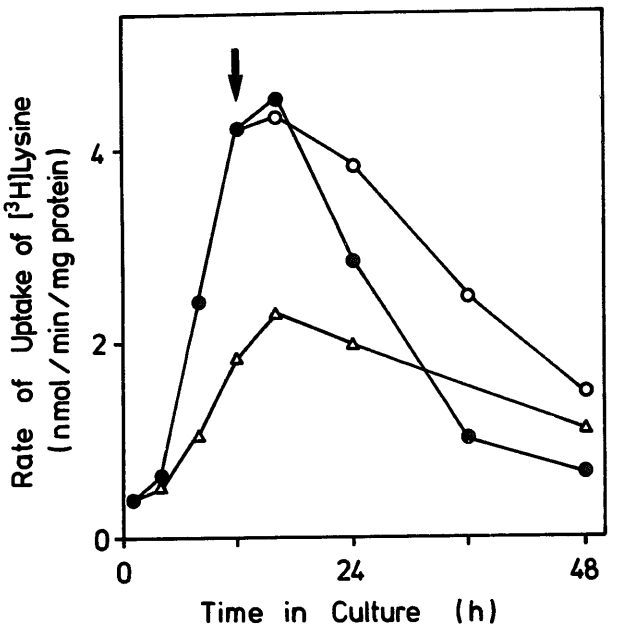

Fig. 2. Effect of cycloheximide on the rate of the uptake of lysine. Macrophages were cultured with 1 ng/ml LPS (●) or 1 ng/ml LPS plus 0.25 μg/ml cycloheximide (△) and the rates of the uptake of 0.05 mM L-[^{3}H]lysine were measured at the time indicated. (○), macrophages were incubated with 1 ng/ml LPS and after 12 h as indicated by the arrow 0.25 μg/ml cycloheximide was added and then the rates of the uptake of lysine were measured. The data points are the means of duplicate determinations from one experiment typical of two similar experiments.

In the presence of 0.25 μg/ml cycloheximide, the enhancement by 1 ng/ml LPS was reduced by about 65% at 12 h in culture (Fig. 2). Cycloheximide at this concentration inhibited about 75% of total protein synthesis, as determined by radiolabeled leucine incorporation into an acid-insoluble fraction. These results suggest that the increase of the rate of uptake depends on the protein synthesis. The addition of cycloheximide delayed the decline of the activity of lysine uptake. Fig. 2 also shows the delay of the decline of the activity when 0.25 μg/ml cycloheximide was added after 12 h in culture. These results suggest that cycloheximide inhibits not only the synthesis of carrier protein but also the degradation. Because the addition of more than 0.25 μg/ml cycloheximide caused cell injury during the culture with 1 ng/ml LPS, we could not carry out an experiment in which the protein synthesis was inhibited by cycloheximide more thoroughly.

Because LPS enhanced the activity of lysine uptake in nanogram or picogram quantities in the medium, it is highly likely that the activity of lysine uptake is influenced by the endotoxin or the endotoxin-like substance(s) contained in the fetal bovine serum used in the experiments. We have measured the uptake of lysine in cells cultured in media containing different lots of serum. The activity of lysine uptake apparently varied with the serum lots used (Table I). The effect of polymyxin B, which is known to neutralize the effect of

TABLE I

The rate of uptake of lysine in macrophages cultured with various lots of serum

The rates of the uptake of 0.05 mM L-[^{3}H]lysine were measured in the macrophages cultured for 12 h in medium containing one of several lots of serum. The results are the means ± S.D. of at least four determinations from two to four experiments with a duplicate assay for each.

Serum lot	Rate of uptake of lysine (nmol/min per mg protein)
A	0.86 ± 0.10
B	0.86 ± 0.12
C	3.06 ± 0.39
D	4.45 ± 0.54
E	2.36 ± 0.26
F	3.10 ± 0.51

the endotoxin [19], was tested on the activity of lysine uptake in the cells cultured in the medium containing one of the lots by which the activity was greatly increased. Table II shows that the induction of the transport activity was depressed by polymyxin B. These results support the view that the activity of lysine uptake in the macrophages is induced by LPS-like substance(s) contained in sera. In the present study lot B serum was used throughout the experiments unless otherwise stated, because it induced the transport activity to a lesser extent. Actually less than 0.3 ng/ml endotoxin was detected in lot B serum by the *Limulus amebocyte* lysate test using the endotoxin derived from *Escherichia coli* 0111:B4 as a standard. On the other hand, 2.1 ng/ml endotoxin was detected in lot C serum.

The identification of the transport system that mediates cationic amino acid uptake

The effect of several amino acids on the uptake of lysine was measured in 1 h-cultured macrophages (cul-

TABLE II

The effect of polymyxin B on the uptake of lysine enhanced by serum

The macrophages were cultured for 1 h in the medium containing the serum of lot C used in the Table I. Then the medium was renewed with the fresh medium containing the same lot of serum and the cells were cultured for further 11 h with polymyxin B. The rates of the uptake of 0.05 mM L-[^{3}H]lysine were measured. The data points are the means ± S.D. of four determinations from two separate experiments.

Polymyxin B (μg/ml)	Rate of uptake of lysine (nmol/min per mg protein)
0	2.84 ± 0.20
1	2.41 ± 0.28
10	1.87 ± 0.36
50	1.13 ± 0.04
100	1.07 ± 0.16

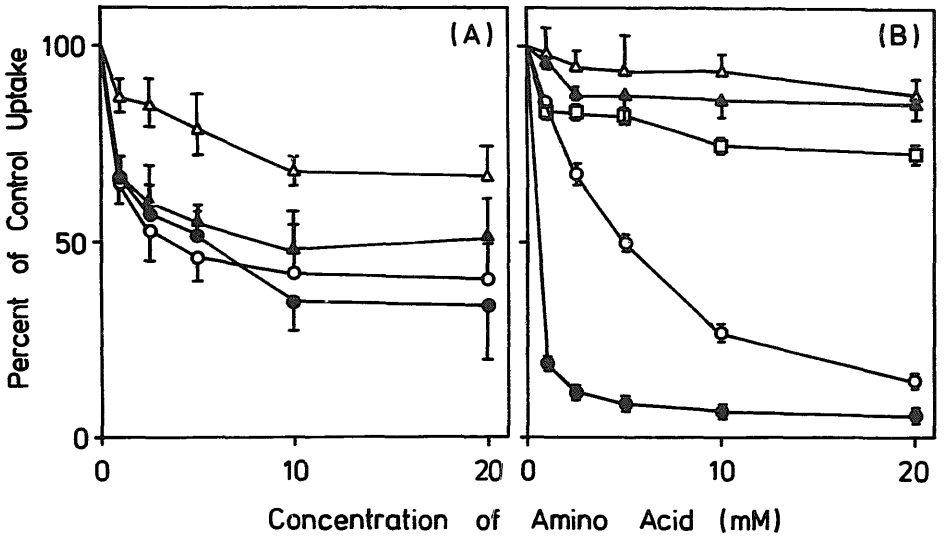

Fig. 3. Comparison of the inhibitory potential of various amino acids on the uptake of lysine. (A) In 1 h-cultured macrophages (cultured for 1 h after collecting from the peritoneal cavity), the rates of the uptake of 0.05 mM L-[^{3}H]lysine were measured in the presence of various concentrations of inhibitor amino acids. (B) In LPS-treated macrophages (cultured for 1 h after collecting and then cultured for further 11 h in the renewed medium containing 1 ng/ml LPS), the rates of the uptake of 0.05 mM L-[^{3}H]lysine were measured in the presence of various concentrations of inhibitor amino acids. In both cases, the data are expressed as the percent of the control uptake (uptake in the absence of an inhibitor) and the means ± S.D. of at least four determinations. (●), arginine; (○), histidine; (▲), glutamine; (△), serine; (□), leucine.

tured for 1 h after collecting from the peritoneal cavity) and LPS-treated macrophages (cultured for 1 h after collecting and then cultured for further 11 h in the renewed medium containing 1 ng/ml LPS). In 1 h-cultured macrophages, the uptake of lysine was inhibited by arginine, histidine and glutamine to the same extent (Fig. 3A), although the inhibition of the uptake by these amino acids was incomplete. Serine inhibited the uptake to a lesser extent. In LPS-treated cells, arginine and histidine almost completely inhibited the uptake of lysine although the inhibition by a low concentration of histidine was much weaker than that by the same concentration of arginine (Fig. 3B). On the other hand, neutral amino acids tested hardly inhibited the uptake.

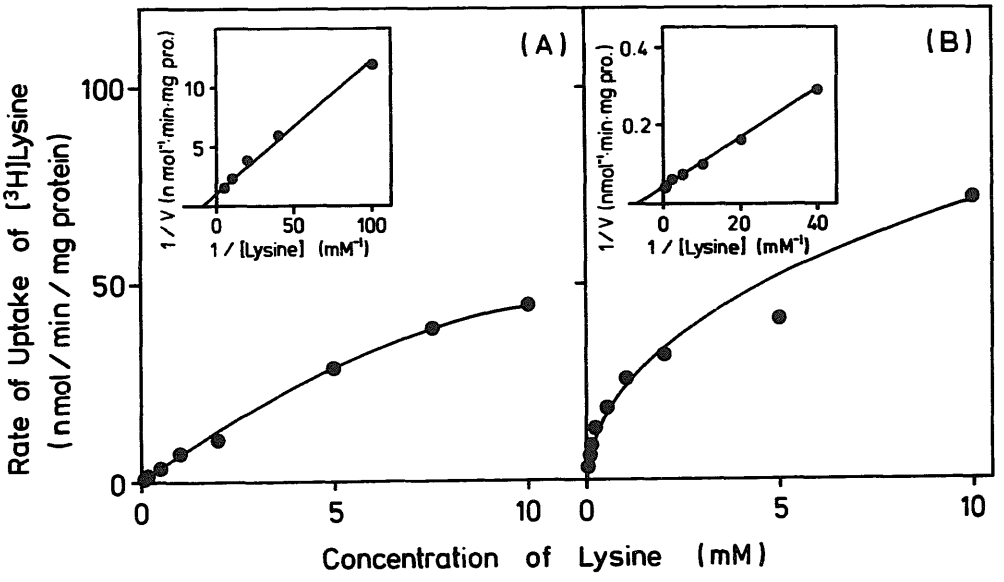

Fig. 4. Concentration dependence of the uptake of lysine. (A) The rates of the uptake of L-[^{3}H]lysine at the concentrations indicated were measured in 1 h-cultured macrophages. The double reciprocal plot (the inset) shows lysine uptake after subtraction of the non-saturable component. The non-saturable component was estimated as described in the text. (B) The rates of the uptake of L-[^{3}H]lysine at the concentrations indicated were measured in LPS-treated cells. The double reciprocal plot (the inset) shows lysine uptake after subtraction of the non-saturable component. The kinetic parameters were obtained by fitting the data to Eqn. 1 between 0.025 to 10 mM. In both cases, the data points are the means of duplicate determinations from one experiment typical of three similar experiments.

The rates of lysine uptake at various concentrations were measured in 1 h-cultured and LPS-treated macrophages (Fig. 4). The kinetic parameters for the uptake in LPS-treated cells were determined by fitting the data to Eqn. 1 described in Materials and Methods. The K_d value obtained in LPS-treated cells was 4.9 nmol/min per mg of cell protein per mM. Subtraction of the calculated non-saturable influx, represented by the product of the K_d and the concentration of the substrate, from the total uptake of lysine yields almost a straight line by graphing the data as a double reciprocal plot (Fig. 4B, inset). The K_m and V_{max} values obtained by fitting the data to the equation were 0.14 mM and 21.6 nmol/min per mg of cell protein, respectively. These data suggest the existence of a single, saturable transport component in LPS-treated cells. A similar K_d value (3–4 nmol/min per mg of cell protein per mM) was obtained in 1 h-cultured cells. However, since the activity of lysine uptake via the saturable component was very low in 1 h-cultured cells, its kinetical analysis at the concentrations higher than 0.2 mM was strongly obstructed by the non-saturable component. Therefore, the data of the activity of lysine uptake at the high concentrations was inappropriate for the kinetic analysis using Eqn. 1. From the result of Fig. 3B, about 35% of the total uptake of 0.05 mM lysine was not inhibited by the excess arginine. Measurement of these arginine-uninhibitable uptake of lysine at various concentrations resulted in the presence of non-saturable uptake with the K_d value of 4.0 nmol/min per mg of cell protein per mM (data not shown). The result of the subtraction analysis using the data of the activity of lysine uptake at the concentrations from 0.01 to 0.2 mM and the K_d value of 4.0 nmol/min per mg of cell protein per mM was shown as a double reciprocal plot in Fig. 4A, inset. The K_m and V_{max} values obtained from the graph were 0.19 mM and 1.6 nmol/min per mg of cell protein, respectively.

The Na^+ requirement for the uptake of 0.05 mM lysine was measured in 1 h-cultured and LPS-treated cells. About 90% of the total uptake of lysine was Na^+-independent in 1 h-cultured cells and about 75% of the total uptake was Na^+-independent in LPS-treated cells. In the later cells, the values of V_{max} obtained in the presence and the absence of Na^+ were not substantially different (data not shown). A hypothesis could be made that two distinct components, a Na^+-independent system and a Na^+-dependent system, additively contribute to the transport of lysine in LPS-treated cells. However, it is very probable that the saturable system is a single component, because the double-reciprocal plot of the saturable lysine uptake is linear (Fig. 4B) and the values of V_{max} in the presence and the absence of Na^+ are not substantially different.

We have investigated the more detailed characteristics of lysine uptake in LPS-treated cells. The inhibition of lysine uptake by arginine or ornithine was competitive (Fig. 5). The K_i values of arginine and ornithine obtained by graphing the data as the Dixon plots were 0.13 and 0.28 mM, respectively. These re-

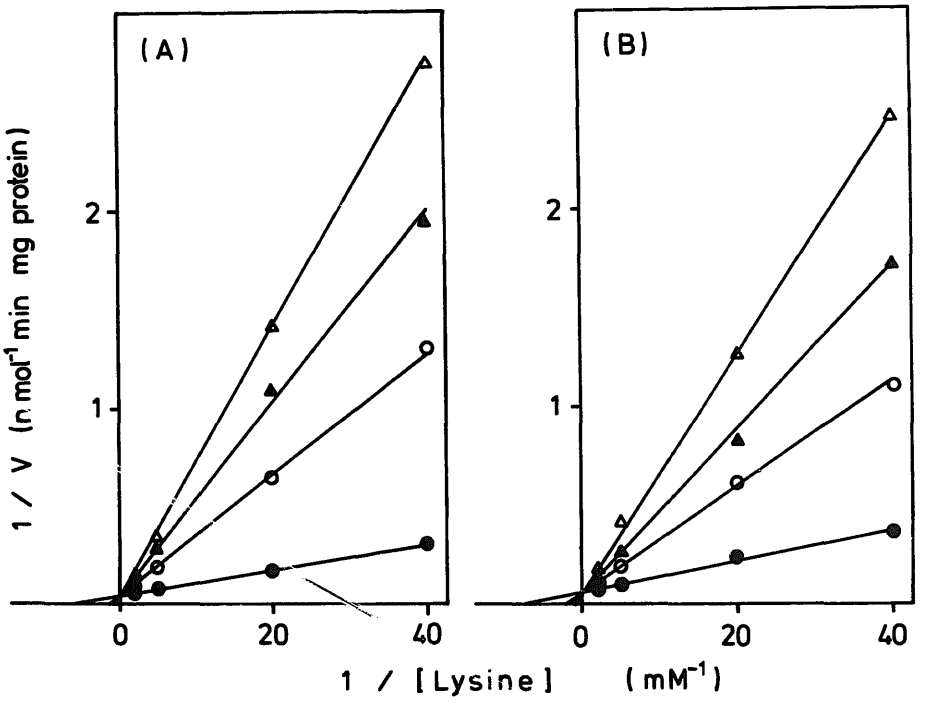

Fig. 5. Double-reciprocal plots of the inhibition of lysine uptake by arginine or ornithine. The rates of the uptake of L-[^{3}H]lysine at 0.025, 0.05, 0.2 and 0.5 mM was measured in the absence (●) or in the presence of 0.5 (○), 1 (▲) and 2 (△) mM arginine (A) or ornithine (B). The data points are the means of duplicate determinations from one experiment typical of two similar experiments.

TABLE III

trans-Stimulation of the uptake of lysine

LPS-treated macrophages (cultured for 1 h after collecting and then cultured for further 11 h in the renewed medium containing 1 ng/ml LPS) were used. The cells were preincubated in PBSG for 10 min and then fresh PBSG with or without 2.5 mM amino acid as indicated was added. The cells were incubated for 5 min and assayed for the uptake of 0.05 mM L-[^{3}H]lysine. The results are the means ± S.D. of four determinations.

Treatment of macrophages		Rate of uptake of L-lysine (0.05 mM) (nmol/min per mg protein)
preincubation in PBSG	amino acid loaded	
− (untreated control)		5.07 ± 0.25
+	none	2.73 ± 0.09
+	L-lysine	6.01 ± 0.07
+	L-arginine	6.21 ± 0.01
+	L-glutamate	2.51 ± 0.01
+	L-serine	2.36 ± 0.11

sults indicate that lysine, arginine and ornithine are transported by a common system.

We measured the uptake of lysine by cells preincubated in an amino acid-free medium to reduce the amino acid contents in the cells and then loaded with some amino acids (Table III). The rates of lysine uptake decreased by depletion of intracellular amino acids and then were restored by loading the cells with lysine or arginine. The rates of the uptake were not, however, restored by loading with serine or glutamate. The results suggest *trans*-stimulation in the influx of lysine into the cells.

We have characterized the uptake of lysine in the macrophages cultured for 12 h in the medium containing lot C serum in Table I. The uptake of lysine was strongly enhanced without LPS in this medium. It was found that the Na^+-dependency, the inhibition by arginine and ornithine and the *trans*-stimulation of the activity of lysine uptake in these cells were identical with those of LPS-treated cells (data not shown).

Discussion

From the results presented here it is concluded that in the macrophages the transport activity for cationic amino acids is potently induced by treating the cells with LPS. The properties of the induced activity are consistent with those of system y^+ because the uptake of lysine is mainly mediated by a Na^+-independent and relatively high-affinity route, inhibited potently by arginine and ornithine and subject to *trans*-stimulation. System y^+ is known not to be coupled to a co-transport with Na^+. However, the rate of lysine and arginine influx via system y^+ is slightly decreased in the complete absence of Na^+ [7]. The degree of Na^+-independence for the lysine uptake into P388 leukemia cell line [6] and for the arginine uptake into Chinese hamster ovary cells [20] were reported to be about 70–80%. This is consistent with the present result that the degree of Na^+-independence for lysine uptake by the LPS-treated macrophages is about 75%.

In 1 h-cultured macrophages, the incomplete inhibition of lysine uptake by cationic amino acids (Fig. 3A) probably results from the participation of the non-saturable route. On the other hand, glutamine at the low concentration unexpectedly inhibited the uptake of lysine as effectively as arginine and the uptake was significantly inhibited by serine at the high concentrations. These results suggest that the saturable component which mediates the uptake of lysine at the physiological concentration in 1 h-cultured cells is somewhat different from the saturable process observed in LPS-treated cells with respect to substrate specificity. However, the former component might still be present in LPS-treated cell. Whether these two systems are quite distinctive from each other or a modification is made during the induction remains to be investigated.

Recently, some transport systems for cationic amino acids, systems $b^{0,+}$ and b^+ in mouse blastocysts and conceptuses and system c in lysosomes of human fibroblasts, have been reported [21–23]. The transport system in LPS-treated macrophages is different from system $b^{0,+}$, which preferably transports lysine and leucine in the absence of Na^+, because leucine was hardly interacted with lysine in LPS-treated cells (Fig. 3B). System b^+ interacts more strongly with arginine than with lysine. In the present experiment,arginine competitively inhibited the uptake of lysine and the K_i value of arginine was close to the K_m value of lysine (Fig. 5), suggesting that the system observed in LPS-treated cells is different from system b^+. System c is different from system y^+ in several ways [23]. One of the important differences between these systems is that the K_m value for cationic amino acid uptake of system c is much larger than that of system y^+. In this regard, the transport system of lysine in LPS-treated macrophages (K_m = 0.14 mM) is different from system c. On the other hand, it is possible that the saturable component in 1 h-cultured cells is similar to system $b^{0,+}$ since it reacts with both glutamine and arginine.

It has been reported that system y^+ operates as a facilitated diffusion system driven by the membrane potential [24] and that the activity of system y^+ is lowered by a decrease of the membrane potential [25]. It was reported that neither endotoxin nor lipid A, a component of LPS, induced depolarization of the monocyte's plasma membrane [26]. Recently, LPS has been reported to promote membrane depolarization in a murine B-cell line, resulting in reduction of membrane potential [27]. Therefore, it is unlikely that the increase of the activity of lysine uptake is due to the changes of the membrane potential by LPS.

It is notable that system y^+ activity is induced by treating the cells with LPS at 0.1 ng/ml, which is comparable to the concentration of endotoxins in serum and possibly occurs in pathological state in vivo. Probably in macrophages the transport system identified with system y^+ is little expressed in vivo and is induced by certain stimuli. There has been few report on the induction of the activity of system y^+ except that certain treatments lead to a increase in the activity of system y^+ in hepatocytes [7]. The present report is, as far as we know, the first that shows the prominent induction of the system y^+ activity by a simple stimulus in vitro. The role of system y^+ in the expression of macrophage functions such as cytostatic activity deserves further investigation.

Acknowledgement

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