Induction of cystine transport activity in mouse peritoneal macrophages by bacterial lipopolysaccharide

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The transport of cystine has been investigated in mouse peritoneal macrophages cultured *in vitro*. The transport activity for cystine was very low in freshly isolated macrophages but was potently induced during culture in the presence of bacterial lipopolysaccharide (LPS) at concentrations as low as 0.1 ng/ml. The transport activity for cystine was enhanced when the cells were incubated with tumour necrosis factor- α (TNF- α), but not with interferon- γ (IFN- γ) or interleukin-1. IFN- γ was rather repressive in the induction of the activity by LPS or TNF- α . The

transport activity for cystine induced by LPS has been characterized. Cystine was transported mainly by a Na⁺-independent system and the uptake of cystine was inhibited by extracellular glutamate and homocysteate, but not by aspartate, indicating that the transport of cystine in macrophages treated with LPS is mediated by System x_c⁻. Glutathione content of the macrophages increased when they were exposed to LPS, and this increase was, at least in part, attributable to the induced activity of the cystine transport.

INTRODUCTION

Transport of amino acids across the plasma membrane is performed by several systems with different substrate specificities [1]. We have described in cultured human fibroblasts and mouse peritoneal macrophages a Na⁺-independent, anionic amino acid transport system highly specific for cystine and glutamate [2,3]. This system, designated as System x_c⁻, is an exchange agency and cystine is transported in an anionic form in exchange for glutamate [4]. Cystine, once it enters the cell, is rapidly reduced to cysteine. The uptake of cystine mediated by this system is important in maintaining intracellular glutathione (GSH) levels, because cystine/cysteine is the rate-limiting precursor amino acid in GSH synthesis [5]. This transport activity is enhanced under various kinds of stress in many types of cell, including macrophages [6].

Macrophages are actively involved in the inflammatory and immune responses of the host as a result of their extensive endocytic capacity and various secretory products [7]. Recently, we have investigated the characteristics of the cationic amino acid transport in mouse peritoneal macrophages, and demonstrated that the transport activity of cationic amino acids is strongly induced by bacterial lipopolysaccharide (LPS) at a very low level [8,9]. LPS is an endotoxin which is a prototypical activator of cells of the immune and inflammatory systems. In this paper we report that LPS also induces the activity of cystine transport mediated by System x_c⁻ in the macrophages and by this induction the GSH content in the cells increases.

MATERIALS AND METHODS

Materials

L-[U-¹⁴C]Cystine and L-[3-³H]serine were obtained from Amersham International, Amersham, U.K. L-[U-¹⁴C]Glutamic acid was obtained from Du Pont–New England Nuclear, Boston, MA, U.S.A. Thioglycollate broth (Brewer's formula) and Bacto lipopolysaccharide (*Salmonella typhosa* 0901) were from Difco Laboratories, Detroit, MI, U.S.A. Recombinant mouse inter-

feron- γ (IFN- γ) and interleukin 1 β (IL-1 β) were from Boehringer Mannheim, Japan. Recombinant human tumour necrosis factor- α (TNF- α) was a kind gift from Suntory Biomedical Research Laboratory, Osaka, Japan. Fetal bovine sera were obtained from M. A. Bioproducts, Walkersville, MD, U.S.A. and from Filtron PTY, Victoria, Australia. A kit for *Limulus ameobocyte* lysate test was purchased from Seikagaku-kogyo, Tokyo, Japan.

Macrophage culture

Macrophages were collected by peritoneal lavage from female C57BL/6N mice, weighing 20–25 g, that 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 1×10^6 /35-mm-diam. plastic culture dish in RPMI 1640 containing 10% (v/v) fetal bovine serum, 50 units/ml penicillin and 50 μ g/ml streptomycin, and incubated at 37 °C in 5% CO₂/95% air. After 1 h the medium was renewed to remove non-adherent cells.

Uptake of amino acid

Amino acid uptake was measured by techniques described previously [2]. The cells in the dish were rinsed three times in warmed PBSG [10 mM phosphate-buffered saline (137 mM NaCl, 3 mM KCl), pH 7.4, containing 0.01% CaCl₂, 0.01% MgCl₂, 6 H₂O 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 a labelled amino acid (0.1 μ Ci/0.5 ml for [¹⁴C]cystine and [¹⁴C]glutamate, or 1 μ Ci/0.5 ml for [³H]serine). When Na⁺-dependency of the uptake was examined, the uptake of the amino acid was measured in a Na⁺-free medium, where Na⁺ was replaced by choline. The uptake was terminated by rapidly rinsing the dish three times with ice-cold PBS and then the radioactivity in the cells was measured as described [2]. The rates of uptake were determined under conditions approaching the initial uptake rates, i.e. by

Abbreviations used: LPS, lipopolysaccharide; TNF- α , tumour necrosis factor α ; IFN- γ , interferon γ ; IL-1, interleukin 1; PMA, phorbol myristate acetate.

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taking the values for the 2 min uptake of cystine or glutamate and for 0.5 min uptake of serine. The uptake of cystine or glutamate increased with time linearly up to at least 2 min and the uptake of serine did up to 0.5 min.

Determination of glutamate and GSH levels

The cellular content of glutamate was measured as described previously [4]. Briefly, trichloroacetic acid-extract of the cells was treated with ether to remove the acid and the aqueous solution was evaporated, dissolved in Li⁺-citrate buffer and analysed by amino acid analyser. Norleucine was used as an internal standard. Glutathione (GSH and GSSG) was extracted with 5% trichloroacetic acid. The glutathione content in the cells was measured using an enzymic method described previously [10], which is based on the catalytic action of GSH in the reduction of 5,5'-dithiobis (2-nitrobenzoic acid) by the GSH reductase system [11]. The glutathione extracted from the cells was mostly GSH and the content of the oxidized form, GSSG, was negligibly low throughout the experiments in this study.

RESULTS

The initial rate of uptake of cystine was measured in macrophages cultured with or without LPS (Figure 1). The rate was very low in the freshly prepared macrophages and increased by severalfold during culture without LPS. However, it was greatly augmented in the cells incubated with 1 ng/ml LPS. The increase of the activity of cystine uptake was detectable even at 0.01 ng/ml LPS and became maximal at 1 ng/ml (Figure 2). In the presence of 0.1 µg/ml actinomycin D or 0.25 µg/ml cycloheximide, the enhancement by 1 ng/ml LPS was reduced by 84% or 95%, respectively, after 11 h in culture. Thus the enhancement of the cystine transport activity provoked by LPS depends on RNA and protein synthesis. Since System x_c⁻ is the exchange system and the uptake of cystine is associated with the efflux of glutamate from the cells, it is important to see whether LPS affects the cellular content of glutamate. We measured glutamate contents of macrophages at 11 h incubation with and without LPS. In the absence of LPS the glutamate content was 43.5 ± 4.0 nmol/mg of protein (mean ± S.D., *n* = 6), and no significant difference was found in macrophages cultured with 1 ng/ml LPS. Thus the transport activity for cystine or glutamate was enhanced without a change in the cellular content of glutamate. The above results indicate that LPS induces transcriptionally the synthesis of the transporter protein(s) for System x_c⁻.

Effects of LPS on the transport of other amino acids were examined. Serine is a typical substrate of the neutral-amino-acid transport system in macrophages [12], and its transport activity (0.05 mM [³H]serine) was increased only slightly (about 20%) by 1 ng/ml LPS after 11 h incubation. In contrast, the transport activity of glutamate (0.05 mM [¹⁴C]glutamate), another substrate of System x_c⁻, was increased greatly by LPS: after 11 h of incubation the activity was 0.15 nmol/min per mg of protein in the absence of LPS and 2.35 nmol/min per mg of protein in the presence of 1 ng/ml LPS.

Because LPS enhanced the activity of cystine uptake in nanogram or picogram quantities in the medium, it is highly likely that the activity of cystine uptake is influenced by the endotoxin or the endotoxin-like substance(s) which may be present in the fetal bovine serum used in the macrophage culture. We measured the rate of uptake of cystine in macrophages incubated for 12 h in medium containing different lots of serum. The activity of cystine uptake apparently varied with the serum lots used and lot A serum was least effective in the induction (Table 1). Actually 0.3 ng/ml endotoxin was detected in lot A

Figure 1 Changes in the activity of the uptake of cystine by macrophages incubated with LPS

Macrophages collected by peritoneal lavage were incubated for 1 h and the medium was replaced by a fresh medium with 1 ng/ml LPS (●) or without LPS (○). The rate of uptake of 0.05 mM [¹⁴C]cystine was measured at the time indicated. Each point represents the means ± S.D. (*n* = 4–6).

Figure 2 Effect of LPS on the activity of the uptake of cystine in macrophages

Macrophages collected by peritoneal lavage were incubated for 1 h and the medium was replaced by a fresh medium with the indicated amounts of LPS. The cells were incubated for 11 h and the rate of uptake of 0.05 mM [¹⁴C]cystine was measured. Each point represents the means ± S.D. (*n* = 4–6).

Table 1 The activity of cystine uptake in macrophages incubated with various lots of serum

The rate of uptake of 0.05 mM L-[¹⁴C]cystine was measured in the macrophages incubated for 12 h in medium containing one of the lots (A–E) of serum. Values for the uptake are means ± S.D. (*n* = 6–8). Values for the endotoxin content are means ± S.D. (*n* = 3). Abbreviation: nd, not determined. *Significantly greater than A (*P* < 0.01).

Serum lot	Rate of cystine uptake (nmol/min per mg of protein)	Endotoxin contents (ng/ml)
A	0.14 ± 0.10	0.3 ± 0.1
B	0.45 ± 0.16	nd
C	0.80 ± 0.13*	4.3 ± 0.5*
D	0.51 ± 0.14	nd
E	1.10 ± 0.22*	3.4 ± 0.5*

Table 2 Comparison of the inhibitory potential of various amino acids on the cystine uptake

In the macrophages incubated with 1 ng/ml LPS for 11 h, the rate of uptake of 0.05 mM L-[¹⁴C]cystine was measured in the absence of Na⁺ (— Na⁺), or in the presence of Na⁺ (+ Na⁺) and various inhibitor amino acids. Values are means $\pm$ S.D. ($n = 3$). *Significantly below control, $P < 0.01$; ** $P < 0.05$.

Amino acid (2.5 mM)	Rate of cystine uptake (nmol/min per mg of protein)	
	+ Na ⁺	— Na ⁺
None (control)	1.06 $\pm$ 0.26	1.17 $\pm$ 0.21
L-Arginine	1.17 $\pm$ 0.08	
L-Aspartate	0.81 $\pm$ 0.16	
L-Serine	0.57 $\pm$ 0.04**	
L-Glutamate	0.13 $\pm$ 0.14*	
L-Homocysteate	0.10 $\pm$ 0.06*	

serum by the *Limulus ameobocyte* lysate test using the endotoxin derived from *Escherichia coli* 0111:B4 as a standard. On the other hand, much more endotoxin was detected in lot C and E sera. Since culture media contain 10 % (v/v) serum, the endotoxin

Table 3 Effect of zymosan, PMA and some cytokines on the uptake of cystine

The rate of uptake of 0.05 mM L-[¹⁴C]cystine was measured in the macrophages incubated for 11 h under the conditions indicated. Values are the means $\pm$ S.D. ($n = 4-6$). *Significantly different from controls, $P < 0.01$; ** $P < 0.05$. § $P < 0.01$ in comparison with TNF- α (1000 units/ml) alone; §§ $P < 0.05$.

Additions	Rate of cystine uptake (nmol/min per mg of protein)	
	— LPS	+ LPS (1 ng/ml)
None (control)	0.13 $\pm$ 0.03	1.27 $\pm$ 0.15
Zymosan		
(10 μ g/ml)	0.29 $\pm$ 0.02*	
(50 μ g/ml)	0.43 $\pm$ 0.03*	1.22 $\pm$ 0.07
(100 μ g/ml)	0.49 $\pm$ 0.02*	
PMA		
(10 ⁻⁸ M)	0.20 $\pm$ 0.01*	
(10 ⁻⁷ M)	0.26 $\pm$ 0.02*	1.24 $\pm$ 0.13
(10 ⁻⁶ M)	0.29 $\pm$ 0.02*	
IFN- γ		
(100 U/ml)	0.16 $\pm$ 0.02	
(1000 U/ml)	0.18 $\pm$ 0.02**	0.57 $\pm$ 0.04*
IL-1		
(100 U/ml)	0.15 $\pm$ 0.03	
(1000 U/ml)	0.15 $\pm$ 0.01	1.29 $\pm$ 0.12
TNF- α		
(100 U/ml)	0.21 $\pm$ 0.01*	
(500 U/ml)	0.43 $\pm$ 0.04*	
(1000 U/ml)	0.52 $\pm$ 0.07*	1.48 $\pm$ 0.12**
(5000 U/ml)	0.57 $\pm$ 0.10*	
TNF- α (1000 U/ml) + IL-1 (1000 U/ml)	0.54 $\pm$ 0.05	
TNF- α (1000 U/ml) + IFN- γ (1000 U/ml)	0.35 $\pm$ 0.07§§	
IL-1 (1000 U/ml) + IFN- γ (1000 U/ml)	0.21 $\pm$ 0.02	
TNF- α (1000 U/ml) + IFN- γ (1000 U/ml) + IL-1 (1000 U/ml)	0.34 $\pm$ 0.04§	

Table 4 Additive effect of diethylmaleate and LPS on the uptake of cystine

The rate of uptake of 0.05 mM L-[¹⁴C]cystine was measured in the macrophages incubated for 11 h with diethylmaleate, LPS, or both. Values are the means $\pm$ S.D. ($n = 4-6$). *Significantly greater than control, $P < 0.01$. § $P < 0.05$ in comparison with LPS alone.

Conditions	Rate of cystine uptake (nmol/min per mg of protein)
Control	0.16 $\pm$ 0.09
Diethylmaleate	
(0.01 mM)	0.66 $\pm$ 0.02*
(0.1 mM)	0.86 $\pm$ 0.07*
(0.2 mM)	0.88 $\pm$ 0.04*
LPS (1 ng/ml)	1.28 $\pm$ 0.36
Diethylmaleate (0.1 mM) + LPS (1 ng/ml)	1.79 $\pm$ 0.26§

concentrations of the culture media were in the range of 0.03–0.43 ng/ml. This may account for the different activities of the cystine uptake shown in Table 1. In the present study lot A serum was used throughout the experiments.

The activity of cystine uptake by the macrophages incubated for 11 h with 1 ng/ml LPS was measured in the medium containing or not containing Na⁺. As shown in Table 2, the uptake of cystine was almost entirely Na⁺-independent. The activity of the cystine uptake was measured in the presence of excess amounts of various amino acids (Table 2). The cystine uptake was strongly inhibited by glutamate and homocysteate. On the other hand, aspartate inhibited the cystine uptake to a much lesser extent. A cationic amino acid, arginine had no effect on the cystine uptake, whereas serine, a neutral amino acid, inhibited the uptake by about 40 %. From these results it is concluded that the induced activity of cystine transport is mediated by System x_c⁻.

Although LPS has most often been used as a triggering agent in macrophage activation, some potential activating agents are known, which differed markedly in their ability to prime macrophages for, for example, nitric oxide release [13]. Table 3 shows the effect of zymosan, phorbol ester and some cytokines on the activity of cystine transport. In the absence of LPS, the cystine transport activity was significantly enhanced by challenge with zymosan, whereas it was less weakly enhanced by phorbol myristate acetate (PMA). IFN- γ and IL-1 have little, if any, or no effect on the cystine transport activity. However, TNF- α markedly enhanced the cystine transport activity. In the presence of LPS the effect of zymosan or PMA disappeared. When both LPS and TNF- α were present, the cystine transport activity was significantly greater than when only LPS was present. However, the effect of LPS and TNF- α was apparently not additive, suggesting that LPS and TNF- α have partly synergistic effects on the induction of the cystine transport activity. Of particular interest is that IFN- γ unequivocally diminished the cystine transport activity induced by LPS or by TNF- α .

The activity of System x_c⁻ has been shown to be induced by oxidative or sulphhydryl-reactive agents [6,10]. We investigated the effect of LPS and a sulphhydryl-reactive agent, diethylmaleate, when added simultaneously (Table 4). The effect of diethylmaleate alone on the induction was maximal at 0.1 mM. The activity was induced additively by LPS and diethylmaleate, suggesting that the effect of the sulphhydryl-reactive agent and that of LPS are independent. It should be noted that diethylmaleate induced the cystine transport activity in various types of

Table 5 Intracellular GSH levels in macrophages incubated with LPS

Macrophages were incubated for 11 h with or without 1 ng/ml LPS, then intracellular GSH levels were measured. In some experiments, the cells were incubated in the presence of homocysteate or aspartate. Values are means $\pm$ S.D. ($n = 4$). *Significantly below controls, $P < 0.01$; ** $P < 0.05$. § $P < 0.01$ in comparison with $-LPS$.

Homocysteate/aspartate added	Intracellular GSH level (nmol/mg of protein)	
	$-LPS$	$+LPS$
None (control)	13.7 ± 0.8	27.0 ± 2.0 §
+ Homocysteate (2.5 mM)	$10.9 \pm 1.0^*$	$18.7 \pm 0.6^*$
(10 mM)	$8.4 \pm 2.1^*$	$10.5 \pm 0.7^*$
+ Aspartate (2.5 mM)	14.0 ± 1.2	25.5 ± 0.6
(10 mM)	13.8 ± 0.4	$22.9 \pm 1.1^{**}$

cells including fibroblasts [10], whereas LPS could not induce the activity at all in human fibroblasts (results not shown).

We measured GSH content in the macrophages incubated for 11 h with or without LPS (Table 5). In the macrophages incubated with LPS, the intracellular GSH level increased markedly. However, the increase of GSH was blocked dose-dependently by homocysteate, which is one of the substrates for system x_c^- and which strongly inhibits the uptake of cystine. In contrast aspartate, which inhibits the uptake of cystine only slightly, blocked the increase of GSH by LPS to only a limited extent. The results suggest that the increase in GSH level in the cells results, at least in part, from the induction of cystine transport activity. It should be noted that in these experiments macrophages were cultured in RPMI 1640 supplemented with 10% (v/v) serum. Cystine concentration of this medium is 0.2 mM, much higher than the concentration employed for the measurement of cystine uptake. Since the cystine uptake was measured in PBS for a very short time, the relationship between the extent of the inhibition of the cystine uptake and that of the decrease of GSH by homocysteate or aspartate may be only qualitative.

DISCUSSION

The present experiments demonstrated that the activity of System x_c^- was strongly induced in the macrophages incubated with LPS at concentrations as low as 0.1 ng/ml. Previously we reported the induction of the cystine transport activity in the macrophages during culture without any additions [3]. However, it is very probable that the induction in the previous study was provoked by endotoxin-like substances which were contained in the fetal bovine serum used in those experiments. In the present study the serum lot with the low endotoxin content was selected, and it was found that the induction in the absence of added LPS was very weak when such serum was used (Figure 1). This limited induction is likely to be due to the endotoxin still present, and the cystine transport activity might not be induced at all if the culture medium were absolutely free from endotoxin. The activity of cystine uptake reached its maximum at 1 ng/ml LPS, and it slightly decreased at higher concentrations of LPS. The reason is unknown but it might be that metabolic changes occurring in macrophages in response to relatively high concentrations of LPS have this effect on the activity of cystine uptake. LPS has been reported to stimulate the activity of neutral- and cationic-amino-acid transport in pulmonary artery endothelial cells

[14,15]. However, the concentration of LPS employed was in the order of 1 μ g/ml, indicating the low sensitivity to LPS in the transport systems for these amino acids.

The mechanism by which the activity of System x_c^- is induced by LPS is unclear at present. TNF- α is rapidly secreted by macrophages in response to activating agents and thus may serve as an autocrine regulator of macrophage function. Although TNF- α appears to induce the activity of System x_c^- , the induction was weak in comparison with that by LPS (Table 3). Therefore TNF- α may be involved little, if any, in the induction of the activity of System x_c^- by LPS. IFN- γ is the most effective agent for induction of NO synthesis and leishmanicidal activity, and several investigations have revealed that the activity of TNF- α is synergized with IFN- γ for the induction [16]. The present experiments showed that IFN- γ is repressive in the induction of the activity of System x_c^- by TNF- α or by LPS. The result may cause a controversy in the notion that TNF- γ generally promotes macrophage activation by LPS.

We have shown that in various cells the rate-limiting precursor for GSH synthesis is cystine in the culture medium and that the GSH level is regulated, at least in part, by the activity of System x_c^- [3,4]. GSH is known to protect cells against a wide range of insulting agents, including reactive oxygen species. Macrophages are activated by various substances such as LPS, and mediate anti-tumour or anti-microbial effects [17]. In this paper, it has been demonstrated that the level of GSH increased in the macrophages incubated with LPS. It is likely that the induction of the activity of System x_c^- by LPS is important for macrophages to protect themselves, because they could be exposed to oxidative stress such as superoxide anion at the sites where they are activated.

Previously we reported that the activity of cationic amino acid transport, mediated by System y^+ , was strongly induced by LPS at a similar concentration to that which provoked the induction of the System x_c^- activity shown here [8,9]. Nitric oxide produced by macrophages is shown to be one of the mediators involved in macrophage functions such as bactericidal activity [18]. Mann and co-workers demonstrated that the activity of System y^+ is important for the supply of arginine, which is the precursor of nitric oxide [19]. Induction of System y^+ activity by LPS may contribute to synthesis of nitric oxide by supplying the substrate arginine. GSH is assumed to be one of the cofactors required in nitric oxide production [20]. Furthermore, it is suggested that highly reactive nitric oxide is stabilized by the formation of a nitrosothiol compound with GSH [21]. This compound has a prolonged half-life *in vivo* and may serve as a stable intermediary. Taking this into consideration, the induction of System y^+ and that of System x_c^- by LPS are functionally correlated; the induction of System x_c^- , which leads to the elevated GSH in the cells, may be important for sustaining the cofactor GSH and for stabilizing nitric oxide.

Recently Piani and Fontana reported that LPS at 1 ng/ml enhanced the release of glutamate by mouse microglial cells [22]. System x_c^- is an exchange agency and under the routine culture conditions cystine enters the cell in exchange for glutamate [4]. Enhancement of the cystine uptake is associated with the enhanced release of glutamate. Their results are thus consistent with the present data and it should be noted that microglial cells are in the same cell lineage as the peritoneal macrophages.

Because the activity of cystine uptake in cultured macrophages is induced by LPS at very low concentrations, it is of interest to assume that the activity of System x_c^- in macrophages is induced *in vivo* by endotoxins occurring in the pathological state. The reported values of plasma endotoxin concentrations in patients with endotoxaemia or sepsis are in the range of 0.03–

1.00 ng/ml, with relatively large variations depending on the literature viewed [23,24]. The mechanism(s) by which LPS induces the activity of System x_c⁻ thus deserves further exploration.

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