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INCREASE IN CYSTINE TRANSPORT ACTIVITY AND GLUTATHIONE LEVEL IN MOUSE PERITONEAL MACROPHAGES EXPOSED TO OXIDIZED LOW-DENSITY LIPOPROTEIN

Hideyo Sato, Yoko Takenaka, Kyoko Fujiwara,
Mineko Yamaguchi, Kiyoshi Abe, and Shiro Bannai*

Department of Biochemistry, Institute of Basic Medical Sciences,
University of Tsukuba, Tsukuba, Ibaraki 305, Japan

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Summary: The transport of cystine has been investigated in mouse peritoneal macrophages incubated with oxidized low-density lipoprotein (oxi-LDL; low-density lipoprotein, LDL). The transport activity for cystine was potently induced by oxi-LDL but not by native or acetylated LDL. The response of the cells to oxi-LDL was dependent on the extent of oxidative modification of LDL. The transport activity for other amino acids was not induced by oxi-LDL. GSH content increased in macrophages incubated with oxi-LDL and this increase was accounted for by the induction of the cystine transport activity because the increase was completely blocked by glutamate or homocysteate which shared the transport system for cystine and thus inhibited the uptake of cystine competitively.

The internalization of oxi-LDL by macrophages is hypothesized to contribute to foam cell formation and eventually to atherosclerotic lesion formation (1). The oxidation of LDL under certain conditions can modify LDL in a way that leads to uptake by the scavenger receptor. Oxidation of the lipids within the LDL particle leads to the decomposition of polyunsaturated fatty acids followed by the formation of a variety of reactive aldehyde products that are capable of attaching covalently to protein (2). It is highly likely that macrophages are exposed to a severe oxidative stress when they take up oxi-LDL. We have reported that in mouse peritoneal macrophages oxi-LDL induces synthesis of some proteins (3), which are known as stress proteins inducible by some oxidative stress such as hydrogen peroxide (4).

Recently Darley-Usmar, et al. have reported that the intracellular GSH level increases in the macrophages exposed to oxi-LDL (5) and that the inhibi-

*To whom correspondence should be addressed. Fax: 81-298-53-3039.

Abbreviations used: LDL, low-density lipoprotein; oxi-LDL, oxidized low-density lipoprotein; PBS, phosphate-buffered saline.

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tion of GSH synthesis enhances the toxicity of oxi-LDL for human monocytes and macrophages (6). GSH is the most abundant non-protein sulfhydryl compound in the cell and protects cells against a wide range of insulting agents. Elevated GSH in the cell may contribute to increase of resistance to the oxidative stress.

In this paper we provide evidence showing that the enhancement of GSH level in the macrophage exposed to oxi-LDL results from the induction of the activity of cystine uptake.

MATERIALS AND METHODS

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 x 10^6/35 mm plastic culture 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.

LDL was isolated from human plasma by differential ultracentrifugation using the method described in (7). After dialysis against PBS (phosphate-buffered saline) containing 10 µM EDTA, the LDL was sterilized by filtration and stored at 4 °C before use. Amount of LDL in the present experiments was expressed as amount of LDL protein, which was determined according to Lowry et al (8). Before oxidation or acetylation LDL was dialyzed against PBS for 12-24 h at 4 °C to remove EDTA. Oxidation was initiated in 3-4 mg protein/ml solution of LDL by the addition of $CuSO_4$ (final 10 µM) followed by incubation at 37 °C for 12 h (9). In some experiments LDL incubated with $CuSO_4$ for 0.5-48 h was used. LDL was acetylated with repeated additions of acetic anhydride as described (10). Oxidized or acetylated LDL was used after dialysis against PBS containing 10 µM EDTA for 24 h at 4 °C.

Uptake of amino acid was measured by techniques described previously (11). After the cells were incubated with the modified or native forms of LDL for a period of time indicated, the cells were rinsed three times in warmed PBSG (PBS 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 a specified time periods at 37 °C. The uptake medium was PBSG containing the ^{14}C-labeled cystine or ^{3}H-labeled arginine or serine. In some experiments the uptake of cystine 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 cell was measured. The rate of uptake was determined under conditions approaching the initial uptake rates, i.e., by taking the values for the 2 min uptake of cystine or for the 0.5 min uptake of arginine and serine.

Total glutathione (GSH and GSSG) in the cell was extracted with 5% trichloroacetic acid. The GSH content was measured using an enzymatic method (12). The glutathione extracted from the cells was mostly GSH and the content of the oxidized form, GSSG, was negligibly low throughout this study.

RESULTS

The initial rate of uptake of cystine was measured in macrophages incubated with or without LDL. As shown in Fig. 1, the rate of uptake of cystine increased when the cells were incubated with oxi-LDL. Native LDL had little effect though the rate of uptake increased slightly during culture. LDLs of

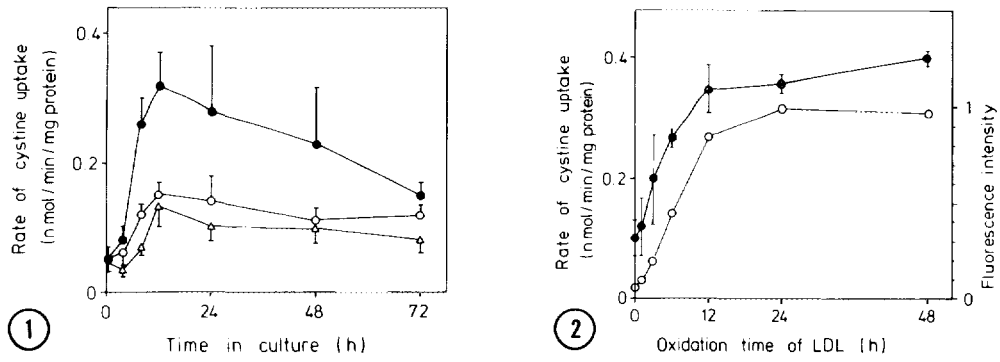

Fig. 1. Changes in the rate of uptake of cystine during culture: effect of native and oxi-LDL. Macrophages were incubated with 100 μg/ml native (○) or oxi-LDL (●), or without LDL(△). The rate of the uptake of 0.05 mM [^{14}C]cystine was measured at the time indicated. Values are the means ± S.D. (n = 4).

Fig. 2. Increase in the rate of uptake of cystine by LDLs of the different degrees of oxidative modification. LDL was incubated with $CuSO_4$ for the time periods indicated in the abscissa. The macrophages were incubated for 11 h with these LDLs at 100 μg/ml, and the rate of uptake of 0.05 mM [^{14}C]cystine was measured (●). Open circles (○) showed the relative fluorescence intensity of LDL at 440 nm with excitation of 365 nm. Values are the means ± S.D. (n = 4).

different oxidative modifications were prepared and their effect on the cystine uptake was investigated (Fig. 2). The oxidative modifications of LDL molecules was estimated by measuring the fluorescence at 440 nm with excitation at 365 nm (2). The increase in the rate of uptake of cystine was dependent on the extent of the oxidative modification of LDL, and reached maximum when the cells were exposed to the oxi-LDL modified for 12 h.

The rates of uptake of cystine, arginine, and serine were measured in the macrophages incubated for 11 h with native, oxidized, and acetylated LDL (Table 1). Oxi-LDL specifically enhanced the rate of uptake of cystine whereas treatment with native and acetylated LDL had little effects on the rates of the uptake of cystine, arginine, and serine.

Table 1 Effect of modified LDL on the rates of uptake of some amino acids*

Medium	Rate of uptake of cystine	arginine	serine
	(n mol/min/mg protein)		
Control	0.12 ± 0.03	1.01 ± 0.23	2.93 ± 0.44
+ native LDL	0.13 ± 0.06	1.05 ± 0.41	2.87 ± 0.60
+ acetylated LDL	0.12 ± 0.03	1.22 ± 0.05	2.55 ± 0.32
+ oxi-LDL	0.30 ± 0.04	1.04 ± 0.30	2.45 ± 0.10

*The macrophages were incubated for 11 h with and without (control) 100 μg/ml LDLs. The rates of the uptake of 0.05 mM [^{14}C]cystine, 0.05 mM [^{3}H]arginine, and 0.05 mM [^{3}H]serine were measured. Values are the means ± S.D. (n = 4).

We investigated the characteristics of the uptake of cystine enhanced by oxi-LDL. The rate of uptake of cystine in the macrophages incubated for 11 h with 100 μg/ml oxi-LDL 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 rate of the uptake of cystine was measured in the presence of the excess amount of various amino acids (Table 2). The cystine uptake was strongly inhibited by glutamate and homocysteate but not by aspartate. 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 in the macrophages the uptake of cystine is mediated by the transport system, named System x_c^- (13).

Table 3 shows the intracellular GSH level of the macrophages exposed to native, oxidized, and acetylated LDL. GSH contents increased markedly in macrophages incubated with oxi-LDL but not with native and acetylated LDL. We measured the intracellular GSH contents in the macrophages incubated for 11 h with oxi-LDL and various amino acids (Table 3). Addition of glutamate or homocysteate, which are the substrates of System x_c^- and inhibit the uptake of cystine, depressed the GSH levels. However, other amino acids had little effect on the GSH content in the macrophages incubated with oxi-LDL. Intracellular GSH level decreased drastically when the macrophages were incubated in cystine-free medium. These results strongly suggest that the uptake of cystine is pivotal in maintaining the GSH level and that the increase in the intracellular GSH content in the macrophages incubated with oxi-LDL is caused by the enhancement of the rate of uptake of cystine.

DISCUSSION

It has been reported that exposure of macrophages to oxidized LDL results in an initial oxidative stress causing the depletion of GSH, followed by

Table 2 Na^+-dependency and comparison of the inhibitory potentials of various amino acids on cystine uptake*

Amino acid (2.5 mM)	Rate of cystine uptake	
	+ Na^+	- Na^+
	(% of control)	
None	100	110 ± 2
Arginine	119 ± 17	
Aspartate	84 ± 17	
Serine	71 ± 6	
Glutamate	4 ± 1	
Homocysteate	11 ± 5	

*The macrophages were incubated for 11 h with 100 μg/ml oxi-LDL, and then the rate of the uptake of 0.05 mM [^{14}C]cystine was measured in the absence of Na^+ (-Na^+), or in the presence of Na^+ (+Na^+) and various amino acids. Values are the means ± S.D. (n = 4) and are expressed as the percent inhibition of the control uptake (uptake in the presence of Na^+ but without amino acid).

Table 3 Effect of modified LDL and various amino acids on intracellular GSH levels*

Medium	Intracellular GSH
	(n mol/mg protein)
Control	14.4 ± 1.4
+ native LDL	13.7 ± 2.6
+ acetylated LDL	12.6 ± 0.5
+ oxi-LDL	23.3 ± 2.7
+ oxi-LDL + glutamate	11.3 ± 2.1
+ oxi-LDL + aspartate	20.3 ± 3.9
+ oxi-LDL + homocysteate	9.7 ± 3.3
+ oxi-LDL + arginine	20.6 ± 4.9
+ oxi-LDL + serine	23.9 ± 3.3
Cystine-free	2.5 ± 0.8

*The macrophages were incubated for 11 h with 100 μg/ml native, acetylated, or oxi-LDL, and intracellular GSH levels were measured. In some experiments, the cells were incubated for 11 h in the medium containing oxi-LDL supplemented with 2.5 mM amino acids indicated, or they were incubated for 11 h in cystine-free medium. Values are the means ± S.D. (n = 4-8).

resynthesis and elevated levels of GSH after 24 h (5). In the present paper we have shown that the activity of cystine uptake was enhanced in the macrophages incubated with oxi-LDL, and that the elevation of GSH contents in the macrophages incubated with oxi-LDL was blocked by inhibiting the uptake of cystine. From these results, we have concluded that the elevation of GSH contents in the macrophages incubated with oxi-LDL is caused by the enhancement of the activity of cystine uptake.

We have shown that in various cells in culture the rate-limiting precursor for GSH synthesis is cystine in the medium and that the GSH level is regulated, at least in part, by the activity of the cystine transport system, System x_c^- (14). The activity of system x_c^- is induced by hydrogen peroxide which is neutralized by glutathione peroxidase, or by electrophilic agents which are detoxified through GSH-dependent pathway mediated by GSH transferases (15, 16). Since both hydrogen peroxide and electrophilic agents tend to dissipate GSH, the induction of the System x_c^- activity by these agents is regarded as adaptive.

Native LDL is not recognized by the macrophage scavenger receptor and will not be taken up by the cells whereas acetylated LDL can act as a ligand for the scavenger receptor. Acetyl LDL had little effect on the uptake of cystine and on intracellular GSH levels (Tables 1 and 3), suggesting LDL uptake and lipid loading by the macrophage is not sufficient in itself to promote the uptake of cystine. Oxi-LDL is a highly heterogeneous mixture of lipid and protein oxidation products (17). Oxidative modification of LDL *in vitro* involves lipid peroxidation and degradation to reactive aldehydes, with subsequent lysine residue modification in apolipoprotein B. The macropohages that sequester this form of LDL are subject to a severe oxidative stress, and

the increase in GSH could afford protections against it. Although the in vivo nature of such oxidative modification remains unequivocal identification, there is convincing evidence showing the presence of modified LDL, which resembles LDL oxidatively modified in vitro, in atherosclerotic lesions (18). In addition, the GSH levels and activity of glutathione peroxidase are markedly elevated in the atherosclerotic lesions of rabbits (19). Enhanced uptake of cystine may thus be involved in the metabolilc process leading to atherogenesis.

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