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Cellular cysteine generation does not contribute to the initiation of LDL oxidation

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Abstract It has been suggested that the generation of cysteine (Cys-SH) by cells may play a role in the initiation of oxidation of low density lipoprotein (LDL). Cysteine has long been considered as an antioxidant. We studied the effect of Cys-SH on the oxidation of LDL by copper. The presence of Cys-SH had a profound inhibitory effect on the formation of conjugated dienes when fresh LDL was used. However, when we used LDL samples that were subjected to pre-incubation with copper, a progressive decrease in the inhibition and an actual enhancement of oxidation by Cys-SH could be demonstrated. The oxidation of freshly prepared LDL by RAW macrophages as compared to older LDL was considerably less. The addition of Cys-SH inhibited the oxidation of LDL by cells. In contrast, the addition of cystine (Cys-S-S) enhanced the oxidation of older LDL preparations while such additions had no effect on the oxidation of freshly prepared LDL. When pre-incubated LDL was subjected to oxidation by cells an enhancement of oxidation by Cys-S-S could be noted. ■ These results demonstrate that the role of Cys-SH generated as a result of cellular recycling of Cys-S-S in the oxidation of LDL may not relate to the initiation of oxidation reactions. However, Cys-SH may enhance the rate of oxidation of LDL that may contain peroxides.—**Santanam, N., and S. Parthasarathy.** Cellular cysteine generation does not contribute to the initiation of LDL oxidation. *J. Lipid Res.* 1995. **36:** 2203–2211.

Supplementary key words cystine • atherosclerosis • thiol-recycling • macrophage • lipid peroxidation

A plethora of evidence suggests that oxidation of low density lipoprotein generates potent pro-atherogenic mediators (1–4). Lipoprotein oxidation is presumed to occur in the artery and the specific cell type(s) or mechanism(s) that may be involved are still unclear. It appears that any mechanism that may generate superoxide radicals, hydrogen peroxide (H_2O_2) or lipid peroxide (LOOH) outside the cell may contribute to the oxidation of LDL. One such mechanism involves the generation of superoxide radicals from the oxidation of extracellularly generated Cys-SH (5–7). It is suggested that cells may take up Cys-S-S from the medium and intracellularly reduce it and release it as Cys-SH. Evidence for such a mechanism was provided by the findings that *a)* the addition of Cys-SH enhanced the oxidation of LDL in Ham's F-10 medium even in the absence of added copper (8), *b)* very little oxidation occurred when LDL was exposed to cells in Cys-S-S-free medium but the addition of Cys-S-S resulted in enhanced oxidations under such conditions (5–7), and *c)* the addition of thiol-binding agents or competitors of Cys-S-S transporter, such as glutamate (glu), prevented the oxidation of LDL (6).

Cys-SH, glutathione (GSH) and other thiols are traditionally viewed as antioxidants (9) and the putative role of the transporter is to provide cells with Cys-SH for the synthesis of the GSH. In fact, several studies have shown that cells exposed to oxidative stress or even to oxidized LDL (Ox-LDL) respond by an increased transporter activity (10–12). This might suggest that the increased availability of intracellular Cys-SH and GSH may actually be an adaptive response to counteract the toxicity of oxidant. Accordingly, Kuzuya et al. (13) and Gotoh et al. (14) have documented that GSH-enriched cells are resistant to the toxic effects of oxidized LDL. As the LDL preparations used in some of the studies on the oxidative mechanism(s) of LDL appear to contain appreciable levels of pre-formed peroxides, as judged by the large accumulation of thiobarbituric acid reactive substances (TBARS) after incubation without cells (Figs. 1 and 3 of ref. 6), we considered the possibility that Cys-S-S-dependent mechanism may not represent an initiating event but may suggest a mechanism by which the amino acid may aid in propagation. In fact, results presented in the studies of Sparrow and Olszewski (6) show that LDL was readily oxidized by cells, generating over 20

Abbreviations: LDL, low density lipoprotein; Ox-LDL, oxidized LDL; Cys-S-S, cysteine; Cys-SH, cysteine; GSH, glutathione; Glu, glutamic acid; HRP, horseradish peroxidase; MPO, myeloperoxidase; BSA, bovine serum albumin; PBS, phosphate-buffered saline; TBARS, thiobarbituric acid reactive substances; LOOH, lipid hydroperoxide; SLO, soybean lipoxygenase; NFκB, nuclear factor kappa B.

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nmols of TBARS in the absence of Cys-S-S and the addition of the amino acid only caused a further enhancement of oxidation (Figs. 1 and 3 of ref. 6). We, therefore, performed the following studies using cell-free and cell-dependent oxidation systems. The results of this study suggest that Cys-SH is a potent antioxidant and the ability of Cys-S-S to enhance the cell-mediated oxidation of LDL may rest on the nature of the LDL presented to cells. We also show that the use of older LDL preparations may account for the observed pro-oxidant effects of Cys-S-S and Cys-SH.

MATERIALS AND METHODS

Materials

Tissue culture supplies F-10, cysteine-free RPMI and other cell culture supplies were obtained from GIBCO (Grand Island, NY). Cys-SH, Cys-S-S, GSH, H_2O_2, horseradish peroxidase (HRP), myeloperoxidase (MPO), and amino acids were purchased from Sigma Chemical Co. (St. Louis, MO).

Lipoprotein

LDL was isolated from heparinized plasma of normal human donors using a table-top Beckman TL-100 ultracentrifuge and a TLA-100.4 rotor (15). A single-spin gradient was used and the isolated LDL was respun at d = 1.063 g/ml to concentrate and purify LDL from albumin contamination. The isolation was complete within 3 h of obtaining the plasma (15). The isolated LDL was dialyzed against phosphate-buffered saline (PBS) at 4°C (200 × volumes) for 6 h. The purity of the isolated LDL was confirmed by the presence of a single band on agarose gel electrophoresis and of intact apolipoprotein B on sodium dodecylsulfate polyacrylamide gel electrophoresis.

Cells and cell culture

We used the RAW line of macrophages. Cells were seeded in 6-well plates in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and allowed to reach confluence prior to use.

Oxidation of LDL

Cell-independent oxidations. Freshly prepared LDL (100 μg/ml protein) was incubated in phosphate-buffered saline (PBS) with or without 5 μM copper. The formation of conjugated dienes at OD 234 was measured continuously in a SLM-AMINCO DB-3500 spectrophotometer equipped with a 12-position sample changer (15). At the end of the incubation, TBARS (16) and fluorescence (Ex: 330–360 nm; Em: 390–500 nm) (17) were routinely measured.

Peroxidase-catalyzed oxidation of LDL was measured as follows. LDL (100 μg/ml) was incubated with 1 unit of HRP or 0.1 U of MPO in the presence of 50 μM H_2O_2 in 1 ml of PBS (15). The increase in O.D. 234 nm was followed as a measure of the formation of conjugated dienes. In some preliminary experiments, LDL (100 μg/ml) was incubated in unsupplemented Ham's F-10 medium in the presence of 100 μg/ml Cys-SH as described (8). Continuous formation of conjugated dienes and the formation of TBARS at the end of the experiment (usually 20 h) were measured.

Cell incubations. Confluent RAW cells in 6-well plates were washed thrice in serum-free Hank's buffered salt solution and then incubated in Ham's F-10 with LDL at 100 μg/ml concentration. After 24 h incubation at 37°C, the medium was removed and the fluorescence was measured at excitation wavelength 330–360 and emission between 390 and 500 nm. An aliquot was then taken for the measurements of TBARS.

Linoleic acid (200 nmols) was oxidized to 13-hydroperoxy linoleic acid using soy bean lipoxygenase (10 units) as described (17) and was added to LDL. Peroxide levels in LDL were measured using a leukomethylene blue-based assay as described by Auerbach, Kiely, and Cornicelli (18).

RESULTS

Previous studies have shown that addition of Cys-SH or other free thiols at millimolar concentrations to LDL in Ham's F-10 medium resulted in the generation of large amounts of TBARS and the formation of an Ox-LDL that was avidly degraded by macrophages (8). No additions of copper or other oxidants were necessary. We tested whether the freshly and rapidly isolated LDL is similarly oxidized in the presence of Cys-SH in the absence of metal or other oxidants. In over 12 fresh LDL preparations, there was no oxidation of LDL by Cys-SH at any concentrations tested (1 μM to 1 mM) in Ham's F-10 medium in a 24-h period. In fact, there was actually an inhibition of the basal level of oxidation observed in Ham's F-10 medium (results not given). We, therefore, tested whether Cys-SH may also decrease the oxidation of LDL induced by copper. The results are shown in **Figs. 1, A and B.** LDL was readily oxidized by copper in PBS (Fig. 1A) as well as in F-10 medium (Fig. 1B) as seen by the increase in the formation of conjugated dienes. The lag time observed was lower in PBS as compared to that in F-10 medium suggesting that components in F-10 may retard the oxidative process. The inclusion of as little as 10 μM of Cys-SH increased the lag time and higher concentrations of Cys-SH (50–100 μM) further protected the LDL from oxidation. The net increase in

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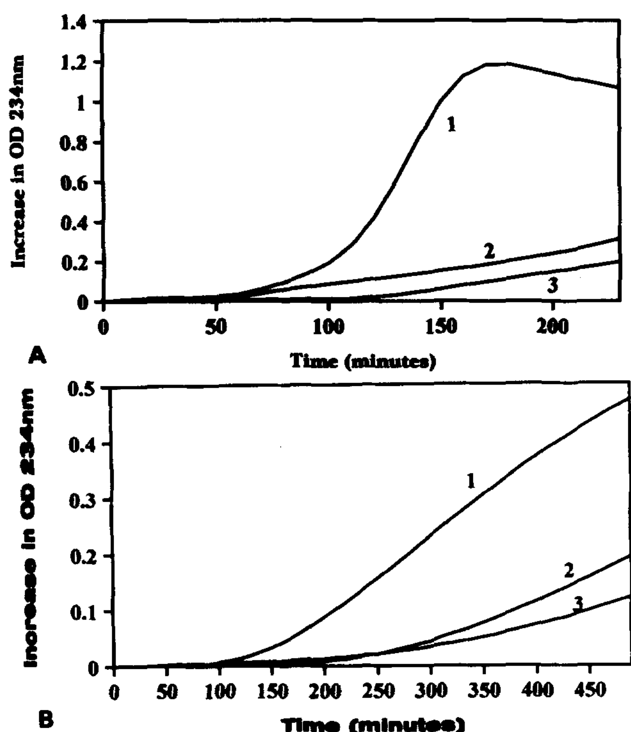

Fig. 1. Effect of Cys-SH on the oxidation of LDL by copper. Freshly isolated LDL (100 µg/ml) was incubated in spectrophotometric cuvettes in PBS (A) or in Ham's F-10 medium (B) containing 5 µM copper in a total volume of 1 ml. The formation of conjugated dienes was measured by measuring the increase in absorbance at 234 nm continuously in an SLM-AMINCO DB-3500 spectrophotometer equipped with a 12-position sample changer. Cys-SH solution was freshly prepared and added to LDL samples at the beginning of the experiment. A: Line 1, LDL only; line 2, LDL plus 10 µM Cys-SH; line 3, LDL plus 50 µM Cys-SH; B: Line 1, LDL only; line 2, LDL plus 1 µM Cys-SH; line 3, LDL plus 10 µM Cys-SH. Figure given represents one of six individual experiments.

the amount of conjugated dienes formed at the end of the incubation (usually 20 h), in the absence or presence of Cys-SH, was comparable, indicating that the inhibitory effect could not be attributed to any interference in the assay method (data not shown in these figures). Accordingly, TBARS or fluorescence, measured at the end of the incubations, showed no difference. This is the first evidence of the antioxidant effect of Cys-SH in the oxidation of LDL and, to our knowledge, no studies have described the inhibitory effect of Cys-SH on the formation of conjugated dienes during the oxidation of LDL. Earlier studies, which measured the terminal effects of thiols by measuring the formation of TBARS or macrophage degradation, would have missed such effects. The antioxidant effect was not restricted to Cys-SH alone. Other thiols, such as GSH, gave very similar results and inhibited the oxidation of LDL in a concentration-dependent manner (**Fig. 2**). Again, 10–100 µM concentrations of GSH markedly inhibited the formation of conjugated dienes.

In support for the cellular thiol recycling hypothesis Sparrow and Olszewski (6) demonstrated that millimolar amounts of glu, a competitor for Cys-S-S transporter in cells (19), suppressed the oxidation of LDL by cells in the presence of Cys-S-S. We present evidence in **Fig. 3A** that glu and a number of other amino acids (Fig. 3B) inhibit the oxidation of LDL even in cell-free systems. The results presented show that glu was an effective inhibitor of the oxidation of LDL by copper even at micromolar concentrations. Fifty to 1000 µM glu progressively inhibited the formation of conjugated dienes with a corresponding increase in lag time from about 100 min in the control samples to over 300 min in the presence of 500 µM glu. When tested at 100 µM concentration, lysine, glycine, alanine, methionine, serine, histidine, and Cys-S-S were all able to retard the formation of conjugated dienes. In fact, Cys-S-S was one of the most effective inhibitors of the oxidation of LDL by copper. These findings may even suggest why copper-mediated oxidations in a cell-free system, are slower in Ham's F-10 which contains amino acids in contrast to simple PBS.

The oxidation of LDL by copper differs from that catalyzed by peroxidases (15). We and others have suggested that phenols such as vitamin E or tyrosine may act as substrates for the peroxidases and initiate oxidation (15, 20, 21). Because of the possibility that antioxidants may become pro-oxidants (15) under these conditions, we tested the effect of Cys-SH in the oxidation of LDL by peroxidases. We incubated 100 µg/ml of LDL protein with HRP or MPO and measured the formation of conjugated dienes. As can be seen in **Figs. 4A and B** the peroxidase-catalyzed oxidation of LDL is usually represented by a shorter lag time, perhaps indicative of the utilization of the intrinsic antioxidants for the initiation of oxidation (15). When increasing amounts of Cys-SH was present in the incubations, the lag time progressively increased, suggesting an inhibition of oxidation by the amino acid. These results again suggest

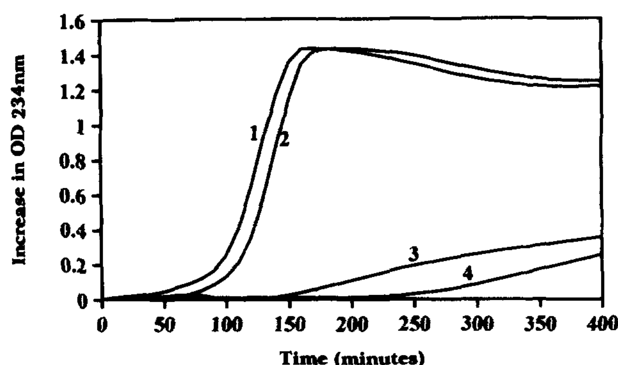

Fig. 2. Effect of GSH on the oxidation of LDL. LDL (100 µg/ml) was incubated in spectrophotometric cuvettes in PBS containing 5 µM copper in the presence or absence of GSH as described for Fig. 1. Line 1, LDL only; line 2, LDL plus 10 µM GSH; line 3, LDL plus 50 µM GSH; line 4, LDL plus 100 µM GSH. Results are from one of three experiments.

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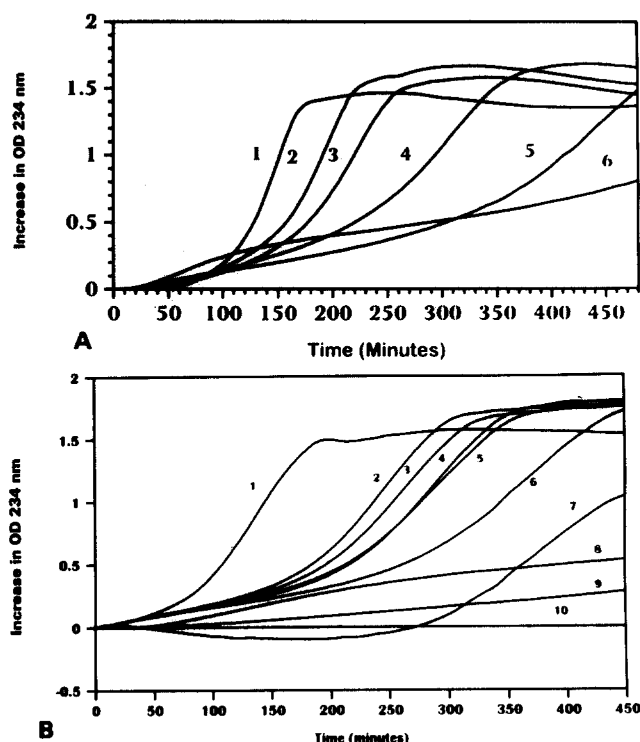

Fig. 3. Effect of glu and amino acids on the oxidation of LDL by copper. LDL (100 μg/ml) was incubated in spectrophotometric cuvettes in PBS containing 5 μM copper in the presence or absence of increasing concentrations of glu (A) or various amino acids (100 μM) (B) as described for Fig. 1. A: Line 1, LDL only; line 2, LDL plus 50 μM glu; line 3, LDL plus 100 μM glu; line 4, LDL plus 250 μM glu; line 5, LDL plus 500 μM glu; line 6, LDL plus 1 mM glu. B: Line 1, LDL only; line 2, alanine; line 3, glycine; line 4, methionine; line 5, lysine; line 6, serine; line 7, glu; line 8, Cys-SH; line 9, histidine; line 10, Cys-S-S. Results are from one of three experiments.

the conclusion that Cys-SH may act as an antioxidant even in model systems that may mimic cell-mediated oxidation.

As mentioned earlier in the Results section, the addition of Cys-SH to LDL either in PBS or in Ham's F-10 medium did not increase the formation of conjugated dienes or TBARS. This is in direct contrast to earlier reports (8, 22). As LDL preparations may differ in their initial LOOH content, we supplemented LDL with LOOH (generated by the action of soybean lipoxygenase on linoleic acid) (17), and tested the rate of oxidation by 5 μM copper in the presence and absence of Cys-SH. As seen in **Fig. 5A,** the addition of 10 μM LOOH to the system enhanced the rate of oxidation and the lag time was greatly diminished. However, the addition of 100 μM Cys-SH was able to increase the lag time regardless of the presence of peroxides.

In the above experiment, it can be argued that the added LOOH did not equilibrate with the lipoprotein and may not thus be amenable to activation by Cys-SH. We therefore conducted the following experiment. LDL (100 μg/ml) in PBS was pre-incubated with 5 μM copper for 0, 30, or 60 min and then the formation of conjugated diene was followed. As seen in Fig. 5B there was a progressive shortening of lag time with increasing pre-incubation time (lines 5, 3, and 1). The addition of Cys-SH to the control sample (0 time) resulted in an inhibition of oxidation as noted by a prolongation of lag time (line 6). Similar increase in lag time was also noted in sample that was incubated for 30 min. However, Cys-SH not only failed to inhibit the oxidation of LDL that was preincubated for 60 min but actually enhanced the rate of oxidation. This is clearly illustrated in Fig. 5C which shows that the addition of even 1 μM Cys-SH to LDL that was preincubated at 37°C for 60 min resulted in an enhancement of the rate of oxidation. In a separate trial, we subjected fresh and 2-week-old LDL to oxidation in Ham's F-10 medium without added copper. Fresh LDL sample generated, on the average, 3.84 and 4.35 nmols of TBARS/mg LDL protein without and with 100 μg/ml Cys-SH (n = 2), respectively, and the old LDL generated 3.6, 11.6, and 14.6 nmols of TBARS/mg LDL protein in the presence of 0,100 μg/ml, and 200 μg/ml Cys-SH, respectively (n = 3).

Cys-SH and other amino acids can affect a number of

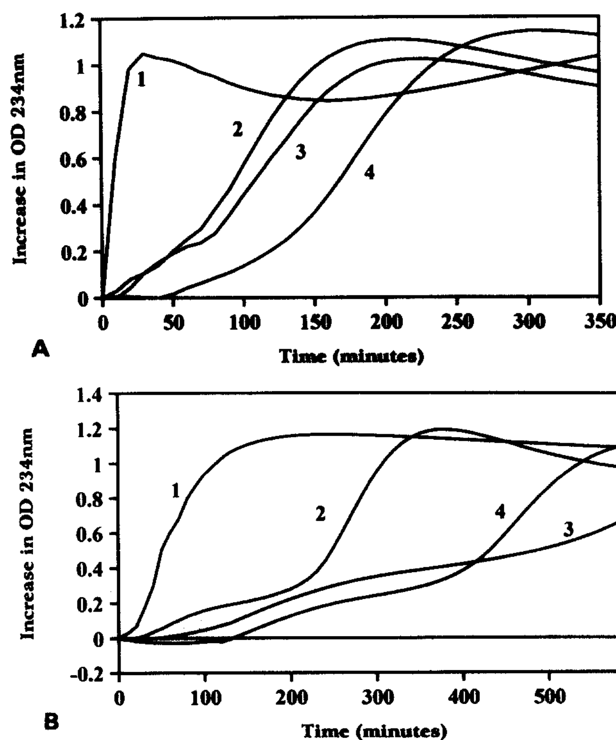

Fig. 4. Effect of Cys-SH on the oxidation of LDL by peroxidases. Freshly prepared LDL (100 μg/ml) was incubated in PBS containing 50 μM H_2O_2 with 1.0 U HRP (A) or 0.1 U MPO (B). Cys-SH was added from a freshly prepared solution. Figures represent one of four individual experiments. Line 1, LDL only; line 2, LDL plus 10 μM Cys-SH; line 3, LDL plus 50 μM Cys-SH; line 4, LDL plus 100 μM Cys-SH.

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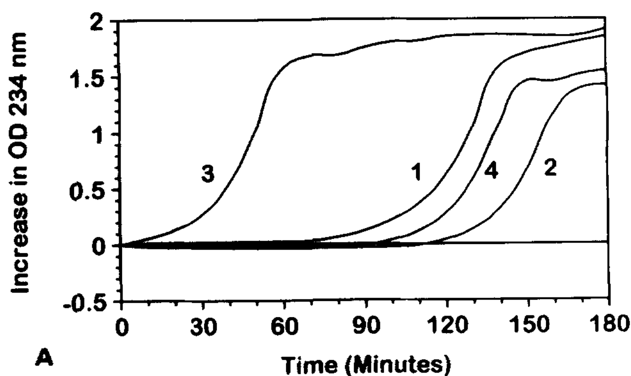

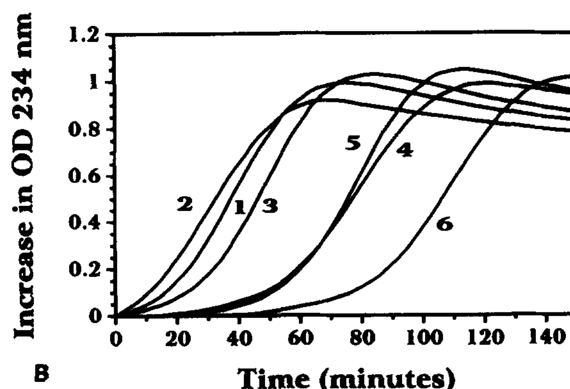

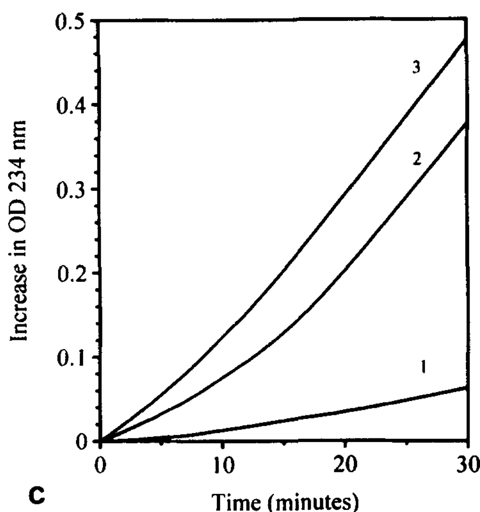

Fig. 5. A: Effect of LOOH on the inhibition of LDL oxidation by Cys-SH. Linoleoyl hydroperoxide was prepared by the oxidation of linoleic acid (200 nmol) with SLO (10 U). LDL (100 μg/ml) was incubated in spectrophotometric cuvettes in PBS containing 5 μM copper with and without the addition of 10 μM LOOH. Cys-SH was added from a freshly prepared solution after the addition of LOOH. Figure represents one of three independent experiments. Line 1, LDL only; line 2, LDL plus 100 μm Cys-SH; line 3, LDL plus 10 μM LOOH; line 4, LDL plus 10 μM LOOH plus 100 μM Cys-SH. B: Effect of Cys-SH on the oxidation of fresh and pre-incubated LDL by copper. LDL at 100 μg/ml was pre-incubated in PBS containing 5 μM copper for 30 and 60 min in spectrophotometric cuvettes maintained at 37°C. The formation of conjugated dienes was measured after the addition of Cys-SH to one set of duplicates and after adjustment of the initial optical density to zero. Figure represents one of three independent experiments. Line 1, 60 min LDL; line 2, 60 min LDL plus 5 μM Cys-SH; line 3, 30 min LDL; line 4, 30 min LDL plus 5 μM Cys-SH; line 5, 0 min LDL; line 6, 0 min LDL plus 5 μM Cys-SH. C: Effect of Cys-SH on the oxidation of fresh and pre-incubated LDL by copper. Conditions are the same as described for Fig. 5B. Line 1, 0 min control LDL; line 2, 60 min LDL; line 3, 60 min LDL plus 1 μM Cys-SH.

steps in the oxidation of LDL. It may react with the metal and reduce its effective concentration, or it may react with radical(s) generated during the oxidation or it may interact with products of oxidation and prevent their interaction with the apoprotein. Results presented in the previous paragraph provide evidence for the second possibility (as there are no added free metals in the peroxidase reaction). To test the third possibility, we used the reaction between pre-formed lipid peroxide and bovine serum albumin (BSA) (17) and tested the effect of added Cys-SH on the formation of fluorescent lipid–protein products. The results presented in **Fig. 6** show that cysteine was able to decrease the formation of fluorescent products in a concentration-dependent manner. These studies would suggest that Cys-SH may interact with pre-formed LOOH and prevent their interaction with lysine or other amino acid residues of apolipoprotein B-100. This finding is of no surprise and supports the well-established finding that aldehydes formed during the decomposition of LOOH also readily react with -SH groups (23).

The following experiments were performed to test the effect of Cys-SH and Cys-S-S on cell-mediated oxidation of LDL. We used unlabeled LDL (1 week old) and measured the formation of TBARS. Fluorescence of incubated LDL was also measured in some experiments after acidification of the medium to quench the color due to phenol red. **Figure 7** shows that added Cys-SH at as little as 5 μM levels decreased the formation of TBARS. The addition of Cys-SH at 100 μM concentration showed even greater degree of inhibition. The formation of oxidized LDL-associated fluorescence was also decreased by the inclusion of Cys-SH in the medium. We were concerned about the high TBAR values (over 60 nmol) generated after oxidation and wondered whether the LDL preparation contained substantial amounts of LOOH before it was added to cells. We therefore prepared LDL by rapid isolation techniques and the following experiments, unless otherwise specified, were performed using freshly isolated LDL and completed within 24 h after the collection of plasma.

Figure 8 shows the effect of Cys-SH and Cys-S-S on the oxidation of fresh LDL by RAW macrophages. Incubation of LDL alone with cells generated less than

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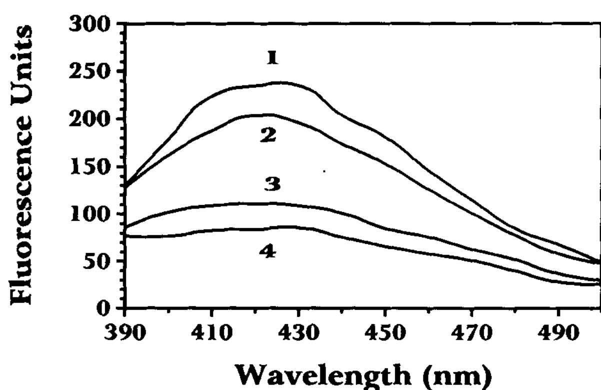

Fig. 6. Cys-SH inhibits the formation of fluorescent adducts from LOOH and BSA. LOOH (200 nmol) was incubated with 200 μg of BSA in the presence or absence of Cys-SH in 1 ml of PBS at 37°C for 72 h. The formation of fluorescent products was measured at excitation wavelength between 330 and 360 nm and emission wavelength between 390 and 500 nm. The results given are from one of three independent determinations. Line 1, control; line 2, plus 10 μM Cys-SH; line 3, plus 50 μM Cys-SH; line 4, plus 100 μM Cys-SH.

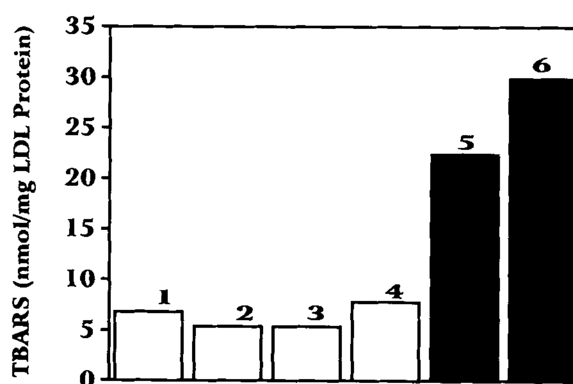

Fig. 8. Effect of Cys-S-S on the oxidation of fresh and old LDL by RAW macrophages. Fresh LDL and LDL (1 week old) at 100 μg/ml were incubated in Ham's F-10 medium with RAW macrophages in 6-well dishes for 24 h. TBARS was measured as described in the text. Results are averages from a duplicate set of determinations from a total of three separate experiments. 1, fresh LDL; 2, fresh LDL plus 100 μM Cys-SH; 3, fresh LDL plus 100 μM Cys-S-S; 4, fresh LDL plus 200 μM Cys-S-S; 5, old LDL; 6, old LDL plus 200 μM Cys-S-S.

7 nmol of TBARS (no cell incubated LDL contained less than 2 nmol of TBARS) and the addition of Cys-SH had detectable but insignificant inhibitory effect on the generation of TBARS. Addition of 100 or 200 μM Cys-S-S had little or no effect on the generation of TBARS. In contrast, when 2-week-old LDL preparation was incubated with cells, over 20 nmol of TBARS was generated (no cell incubations generated 3.6 nmol of TBARS/mg LDL protein). The addition of Cys-S-S at 200 μM concentration further enhanced the formation of TBARS. These results suggested that older LDL preparations that may already have oxidized components may be responsible for increased oxidation by cells and enhanced response by Cys-S-S. To test this directly, we isolated fresh LDL and subjected it to in vitro incubation

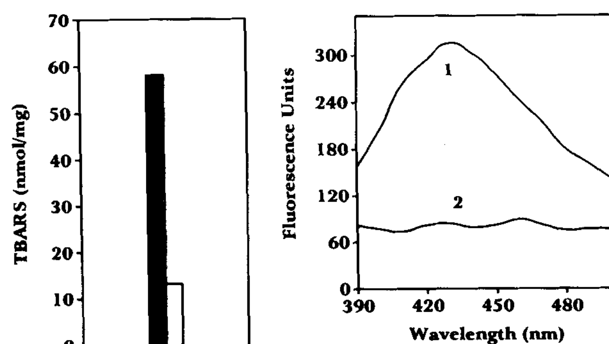

Fig. 7. Oxidation of 1-week-old LDL by RAW macrophages. LDL (1 week old; isolated in the absence of EDTA) at 100 μg/ml was incubated in Ham's F-10 medium with confluent cultures of RAW macrophages in 6-well dishes for 24 h. TBARS and fluorescence were determined as described in the text. Results are averages from a duplicate set of determinations from a total of three separate experiments. Left panel: TBARS values (■) LDL only; (□) LDL plus 5 μM Cys-SH. Right panel: fluorescence units; line 1, LDL only; line 2, LDL plus 5 μM Cys-SH.

for 4 h (in Ham's F-10 medium at 37°C). We then tested the effect of Cys-S-S on the oxidation of fresh and 4-h incubated LDL by cells. The results (**Fig. 9**) show that very low levels of TBARS were generated during the incubation of fresh LDL with cells and the addition of Cys-S-S had no effect on the production of TBARS. In contrast, the incubation of pre-incubated LDL with cells generated greater amounts of TBARS and the addition of Cys-S-S further enhanced the production of TBARS. The unincubated and 4-h incubated LDL preparations generated less than 1.5 nmol of TBARS in the absence of cells after 24-h incubation in Ham's F-10 medium.

DISCUSSION

Thiol compounds such as GSH and N-acetyl Cys-SH have long been considered as antioxidants (9). Thiols may also react with redox metals, H_2O_2 and a host of other chemicals and generate oxygen and thiyl radicals and there is no doubt that some of these radicals may promote lipid peroxidation under appropriate conditions (24, 25). However, there is a contradiction in the proposed radical generating role (5–8) for Cys-SH and Cys-S-S in the oxidation of LDL.

Ox-LDL and components of Ox-LDL affect a wide variety of cells and induce pro-atherogenic and pro-inflammatory responses from target cells (1–4, 26). In addition to their apparent interaction with scavenger receptor(s) on macrophages, their ability to induce chemotactic factors, adhesion molecules, and growth factors appears to rest on the presence of oxidized lipid components. Although the precise mechanism(s) is yet

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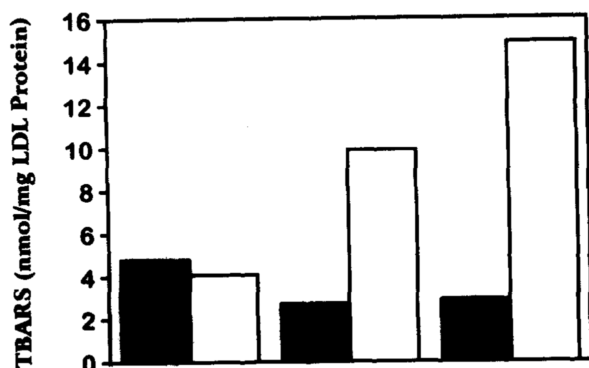

Fig. 9. Effect of cystine on the oxidation of pre-incubated LDL by RAW macrophages. Fresh LDL or LDL pre-incubated for 4 h in Ham's F-10 medium at 100 μg/ml was incubated with RAW macrophages in 6-well dishes for 24 h. TBARS was measured as described in the text. Results are averages from a duplicate set of determinations from a total of three separate experiments. From left to right, closed bars represent fresh LDL alone and in the presence of 100 and 200 μM Cys-S-S. From left to right, open bars represent F-10 incubated LDL alone with cells and in the presence of 100 and 200 μM Cys-S-S.

unknown, the activation of an important transcription factor, nuclear factor kappa B (NFκB) has been implicated in the activation of these genes (27–29). A variety of antioxidants including Cys-SH and other thiols decrease the activation of NFκB (30, 31). Thus, it is unlikely that thiols could act as prooxidants, as proposed earlier, and yet act as antioxidants under the same conditions in preventing the pro-atherogenic effects of Ox-LDL. If, during atherogenesis, thiol-derived radicals promote oxidation reactions, their potential antioxidant effects in inhibiting the activation of NFκB-mediated gene transcription is unlikely to occur. On the other hand, thiols as antioxidants, as proposed in these studies, would not only prevent the initiation of the oxidation of LDL but also would protect cells from the damaging effects of oxidants, as noted by others (13, 14).

The pro-oxidant nature of thiols may not be relevant in the initiation of oxidation of LDL in vivo. Thiol-mediated generation of oxidants (superoxide radicals, thiyl radicals, and others) again depends on the availability of free redox metals or the presence of H_2O_2. There is no reason to believe that large concentrations of iron or copper are present in the early lesions and if large amounts of H_2O_2 and iron are present in the extracellular milieu, there is no need for reduced thiols to generate reactive oxygen species. It also appears that thiyl radicals generated from Cys-SH are incapable of oxidizing LDL unless micromolar amounts of copper or iron are present (S. Parthasarathy and B. Kalyanaraman, unpublished results).

How do we reconcile our findings with those reported in literature (5–8)? First, there is a major difference in the method of preparation of LDL. Most of the oxidation studies on LDL have been performed using lipoproteins isolated in the presence of EDTA. It is arbitrarily assumed that EDTA will prevent oxidation. However, EDTA chelated iron (32) or chelated copper (33) are equally potent oxidants and EDTA used in isolation is routinely carried in the oxidation incubations to varying extents depending on the concentration of the LDL. In the current study we have used LDL isolated from heparinized plasma. Although LDL complexes with macromolecules have been reported to have altered susceptibility to oxidation (34), we would like to point out that we have not used LDL-heparin complexes and only a catalytic amount of heparin was present in the whole blood as an anticoagulant. Yet another major difference is the freshness of the LDL used. We observed that even in sealed ultracentrifuge tubes at 4°C there is a rapid deterioration of LDL with time. We have therefore used the rapid isolation technique (15) and most of the studies described in the study were performed within hours after the collection of the blood. On the other hand, most of the earlier studies on LDL oxidation were performed with LDL isolated by conventional isolation techniques that takes several days to complete. Furthermore, iodination of such LDL, as was used in the study of Sparrow and Olszewski (6), would impose additional dialysis steps and promote further oxidation.

The second reason why we did not observe an enhancement of oxidation may again rest on the initial oxidation status of the LDL. Two lines of evidence presented in the current study support the conclusion that the "older" the preparation of LDL the less the antioxidant effect of Cys-SH. The inhibitory effect of Cys-SH on the oxidation of LDL by copper was less pronounced when in vitro "aged" LDL preparations were used (Fig. 5b and c). Furthermore, the same concentration of Cys-SH became less and less effective as the pre-incubation time of LDL was increased. It is possible that very low concentration of Cys-SH could have actually enhanced the rate of oxidation. Support for such a conclusion comes from cell incubations. When fresh LDL was incubated with cells, very little oxidation was noticed and neither Cys-SH nor Cys-S-S had any noticeable effect. The low TBARS generated by cells in these experiments as compared to those reported in literature would suggest that the rate of initiation of oxidation or "seeding" of LDL with peroxides by cells is quite low. Consequently, even in Ham's F-10 medium, the propagation reactions are low and low levels of TBARS are generated. Besides, in these cultured macrophage cell lines the presence of enzymes that may be involved in initiation reactions (15-lipoxygenase, myeloperoxidase) have not been reported. When "aged" LDL preparations were used, higher levels

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of TBARS were generated; however, large concentrations of Cys-SH were still inhibitory but Cys-S-S showed a concentration-dependent activation. This cannot be attributed to a direct effect of Cys-S-S, as in cell-free systems Cys-S-S as well as several other amino acids inhibited the oxidation. Cys-S-S has been shown to be taken up by cells under these conditions (10, 11) for the synthesis of GSH and it is likely that nanomolar concentrations of GSH or Cys-SH released from cells enhanced the rate of oxidation. The mechanism(s) by which the continuous release of thiols may enhance the rate of oxidation might be similar to the one proposed by Fruebis, Parthasarathy, and Steinberg (17) as discussed for the enhancement of oxidation of LDL by aminoguanidine (35). Another possibility by which thiols may "enhance" oxidation is by protecting the aldehydes formed from further oxidation by the formation of hemithioacetals.

The results presented in this study seem to suggest that Cys-S-S or its reduced products derived from cells may not be involved in the initiation of oxidation of LDL. They may, however, enhance the rate of oxidation of LDL that are already moderately oxidized. Such a conclusion is well supported by the observations of Ishi et al. (36) who suggested that oxidative stress increased the transport of Cys-S-S, perhaps to enhance cellular defense.

The mechanism(s) of initiation of oxidation of LDL in vivo is yet to be established. Considering all the evidence (15, 20, 37, 38), it is likely that lipoxygenases and peroxidases may play a greater role in the initiation and propagation processes. Evidence presented here as well as from literature may suggest that depletion of cellular and extracellular thiols may adversely affect the redox status of the cells and may greatly enhance the atherogenic process. Conversely, increased extra- and intra-cellular thiols including GSH may not only afford protection against the generation of Ox-LDL but also may protect cells against oxidation-induced pro-atherogenic effects. ■

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