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Cholesteryl ester accumulation in macrophages incubated with low density lipoprotein pretreated with cigarette smoke extract

(cigarette smoking/atherosclerosis/foam cell transformation/apolipoprotein B fragmentation/superoxide dismutase)

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ABSTRACT **Although cigarette smoking is one of the major risk factors for atherosclerosis and coronary heart disease, the precise mechanisms of its adverse effects have not been fully elucidated. We incubated low density lipoprotein (LDL) with cigarette smoke (CS) extract and examined the incorporation of the lipoprotein by macrophages *in vitro*. When incubated with macrophages, LDL pretreated with CS extract (100 μg/ml) stimulated cholesteryl [^{14}C]oleate synthesis ≈12.5-fold that with unmodified LDL and transformed macrophages to cells rich in lipid droplets positively stained with oil red O. Enhancement in cholesteryl ester synthesis was dependent on the concentration of CS-modified LDL and exhibited saturation kinetics. When subjected to electrophoreses, CS-modified LDL migrated to a more anionic position than did unmodified LDL and showed extensive fragmentation of apolipoprotein B. This LDL modification depended upon the incubation time and concentration of the CS extract. Superoxide dismutase inhibited modification of LDL by 52%, suggesting that superoxide anion is, at least in part, involved. These results suggest that CS extract alters LDL into a form recognized and incorporated by macrophages. Such modification if it occurs *in vivo*, could explain the increased incidence of atherosclerosis and coronary heart disease in smokers.**

Cigarette smoking is accepted as a major risk factor for ischemic heart disease (1). Epidemiological and pathological studies have accumulated strong evidence that smoking is a risk factor for coronary sclerosis (2, 3). However, the precise mechanisms whereby cigarette smoking exerts its adverse effects remain unclear (4). Several studies show that certain components of cigarette smoke (CS) play a role in atherogenesis. Among those substances, nicotine has been reported to accelerate development of coronary heart disease by increasing plasma catecholamine levels, leading to changes in cardiodynamics (5) and vascular tone (6). Another constituent of CS linked with atherogenesis is carbon monoxide, reported to be toxic for vessels by producing hypoxia (7). Clinical studies also indicate that cigarette smoking alters plasma lipoprotein levels, which might help explain the increased incidence of coronary heart disease in smokers (8). However, no analysis of the effects of cigarette smoking on molecular mechanisms of atheroma formation has been reported.

Recently the role of macrophages on atherogenesis has been emphasized (9–13); macrophages migrate to and accumulate in the subendothelial spaces, being subsequently transformed to lipid-laden foam cells. These lipid-laden foam cells have been suggested to represent the most early stage of atheroma. Macrophages, however, carry only low levels of low density lipoprotein (LDL) receptor, although they carry abundant scavenger receptors for anionic proteins. Via the scavenger receptor, macrophages efficiently incorporate modified LDL and then become foam cell-like *in vitro* (9). The first example of such modified LDL was acetylated LDL (acetyl-LDL) (14), but this chemically modified LDL has not been found in the body. Another example of modified LDL is oxidized LDL. For example, incubation of LDL with redox-active metal ions such as Cu^{2+} causes peroxidation of LDL and modification of apolipoprotein B (15–17). This modified LDL (Cu^{2+}-oxidized LDL) is also incorporated efficiently by macrophages via the scavenger receptor *in vitro* (15–19). Recent studies from several laboratories, including our own, suggested that oxidation is actually involved in LDL modification in the body (12, 18) and focused attention on physiological processes of oxidative LDL modification. Therefore, in this study we investigated whether CS alters LDL and consequently causes accumulation of cholesteryl ester in macrophages.

We show that a CS extract soluble in phosphate buffer changes LDL into a form that is recognized and incorporated via a receptor-mediated pathway in macrophages to transform macrophages into foam cells; this modification of LDL may result, at least in part, from superoxide anion generated from CS.

METHODS

Materials. Sodium [^{125}I]iodide (17.4 Ci/mg); 1 Ci = 37 GBq and [1-^{14}C]oleic acid (52.6 mCi/mmol) were obtained from New England Nuclear. Fetal calf serum (FCS) was from M.A. Bioproducts (Walkersville, MD) and was heat inactivated at 56°C for 30 min before use. Dulbecco's modified Eagle's medium (DMEM) and Dulbecco's phosphate-buffered saline (PBS) were purchased from Nissui Seiyaku (Tokyo). L-Glutamine and penicillin/streptomycin were obtained from Flow Laboratories and GIBCO, respectively. Superoxide dismutase (SOD) (3200 units/mg of protein) and catalase (58,000 units/mg of protein) were from Sigma. Oil red O and EDTA were from Nakarai Chemicals (Kyoto, Japan). Commercial cigarettes without filters were purchased from Japan Tobacco Incorporation (Tokyo). Plastic tubes and Petri dishes came from Falcon and Nunc, respectively. Lipoperoxide test kits for thiobarbituric acid-reactive substances were from Wako Pure Chemical (Osaka, Japan). All other chemicals used were of reagent grade.

Macrophages. Peritoneal cells were harvested from unstimulated female DDY mice (25–30 g) in PBS as described by Edelson *et al.* (20) and Kita *et al.* (13). The peritoneal perfusates from 20 to 40 mice were pooled, and the cells (3–6 × 10^6 cells per mouse) were collected by centrifugation at 400 × *g* for 10 min at 4°C. After being washed once with 30

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; LDL, low density lipoprotein(s); CS, cigarette smoke; SOD, superoxide dismutase.

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ml of PBS, cells were resuspended in the culture medium DMEM containing 10% (vol/vol) FCS, penicillin (100 units/ml) and streptomycin (100 μg/ml) at a final density of 4.5×10^6 cells per ml. Aliquots (1 ml) were dispersed onto plastic Petri dishes (35 × 10 mm), and the cells were cultured in humidified air containing 5% CO_2 at 37°C. After 2 hr, each dish was washed twice with 2 ml of DMEM without serum to remove nonadherent cells. After cells were cultured for another 18 hr at 37°C in 1 ml of culture medium, they were washed with 1 ml of DMEM without FCS. The experiment was initiated by adding various amounts of lipoproteins in 0.9 ml of the culture medium without serum, and incubation occurred in humidified air containing 5% CO_2 at 37°C.

Preparation of LDL and LDL Modification with CS Extract. Blood from fasting Japanese White rabbits (3.0–3.5 kg) was treated with EDTA as an anticoagulant (13), and LDL (density = 1.019–1.063 g/ml) was isolated from the plasma by ultracentrifugation (21). LDL was then dialyzed at 4°C against two changes of at least 500-fold volumes of 10 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl and stored at 4°C. The thiobarbituric acid-reactive substance level in LDL at isolation was 1.3 ± 0.65 nmol of malondialdehyde per mg, and no increase in thiobarbituric acid-reactive substances was detected during dialysis and storage. In all experiments, LDL was used within 14 days of isolation.

Modification of LDL with CS extract was done as follows. CS was passed through 1 ml of PBS, pH 7.4, at 4°C by aspiration at constant pressure of ≈2.5 cm of H_2O (1 inch H_2O at 39° = 249 Pa) below atmospheric pressure; it took ≈5 min to consume one cigarette. Final pH of the bubbled solution was 6.6–6.7. LDL (2 mg) was added to 1 ml of the CS extract, which was immediately filtered through a 0.45-μm filter (Millipore). Recovery of protein during filtration was ≈85%. The LDL suspension was then incubated with or without a scavenger of active oxygens in a sterile plastic tube (15 ml) at 37°C. After incubation, the LDL suspension was applied to a PD-10 column (Pharmacia) for gel filtration at 4°C with PBS as eluent. LDL was collected in 2.5–4.0 ml of eluent and separated by this procedure from the CS extract eluting in 7.0–9.0 ml with yellowish color.

Electrophoreses of LDL. Agarose gel electrophoresis of LDL was done as described by Nobel (22). $NaDodSO_4$ gel electrophoresis of LDL was done as described by Laemmli (23). In brief, LDL (100 μg) was delipidated by 20 volumes of ethanol/ethyl ether, 3:1 (vol/vol) and treated with 10% trichloroacetic acid. After centrifugation at 10,000 × *g* for 20 min, the pellet was dissolved in 100 μl of buffer composed of 62.5 mM Tris chloride, pH 6.8/2% $NaDodSO_4$/20% glycerol (vol/vol)/0.1% bromophenol blue/0.25% 2-mercaptoethanol by incubating in a boiling water bath for 4 min. Electrophoresis was done using 3–15% gradient gels at a constant current of 25 mA. Gels were stained with Coomassie blue.

Assays. Reacylation of cholesterol was measured as an incorporation of [1-^{14}C]oleate (5 mCi/mmol) into cellular cholesteryl oleate by cell monolayers as described by Brown *et al.* (24) except that culture was done without FCS (13). The degradation of ^{125}I-labeled lipoproteins by macrophage monolayer was measured as described by Goldstein *et al.* (14) and Kita *et al.* (13). Lipid peroxide contents in lipoprotein preparation were estimated as thiobarbituric acid-reactive substances using lipoperoxide test kits according to the methods of Yagi (25) and Heinecke *et al.* (26) with a slight modification (12, 19).

Macrophage monolayers on Petri dishes after the indicated incubation were fixed with 6% formaldehyde in 100 mM sodium phosphate buffer, pH 7.3, for 30 min at room temperature. The monolayers were then stained with oil red O (27) and counterstained with Gill's double-strength hematoxylin for 15 min.

Protein contents of lipoproteins and cells were determined by the method of Lowry *et al.* (28). The values shown represent mean ± SEM, and statistical significance was determined by Student's *t* test.

RESULTS

Incubation of macrophages with LDL pretreated with CS extract (CS-modified LDL) caused massive accumulation of lipid droplets in their cell bodies. Fig. 1*A* shows that after incubation with CS-modified LDL (100 μg/ml) for 12 hr, macrophages had numerous lipid droplets, which were positively stained with oil red O, in their cell bodies. On the other hand, in macrophages incubated with unmodified LDL, no such droplet was detected (Fig. 1*B*). Thus, incubation with CS-modified LDL transformed macrophages to foam cell-like appearance.

We next followed lipid accumulation in the cells by measuring conversion of [^{14}C]oleate into cholesteryl [^{14}C]-oleate. When macrophages were incubated with increasing concentrations of CS-modified LDL for 6 hr with [^{14}C]oleate bound to bovine serum albumin, formation of cholesteryl [^{14}C]oleate was stimulated in a dose-dependent manner to a saturation concentration of 100 μg of protein per ml (Fig. 2*A*). Synthesis of cholesteryl oleate in macrophages incubated with CS-modified LDL (100 μg protein per ml) was

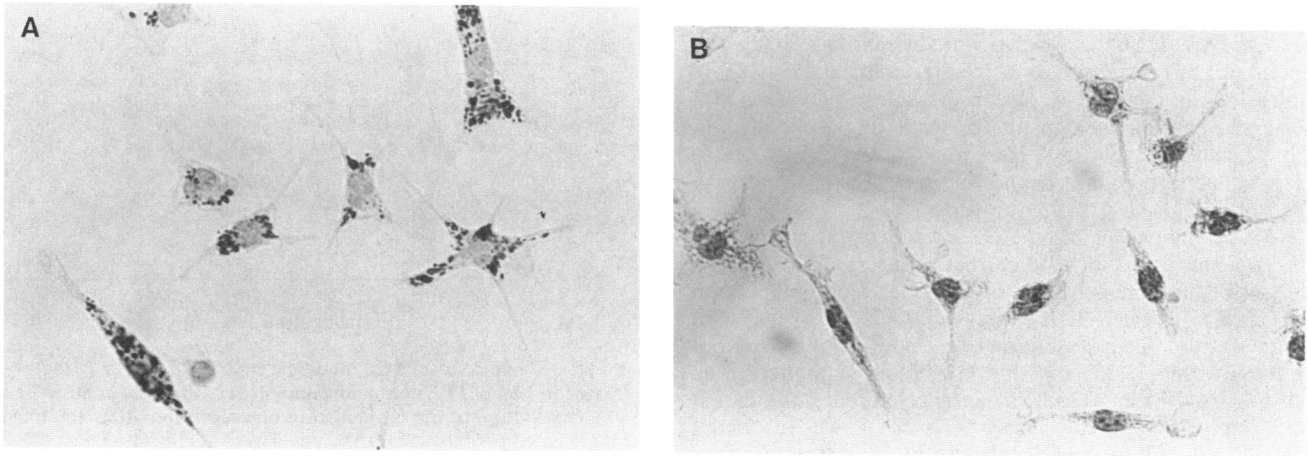

FIG. 1. Light microscope appearance of macrophages incubated with CS-modified LDL (*A*) and unmodified LDL (*B*). Cells (4.5×10^6) were dispersed onto a 35-mm dish. Each dish received 1.0 ml of DMEM containing either 100 μg of protein of CS-modified LDL or unmodified LDL. After incubation for 12 hr, monolayers were stained with oil red O and hematoxylin as described.

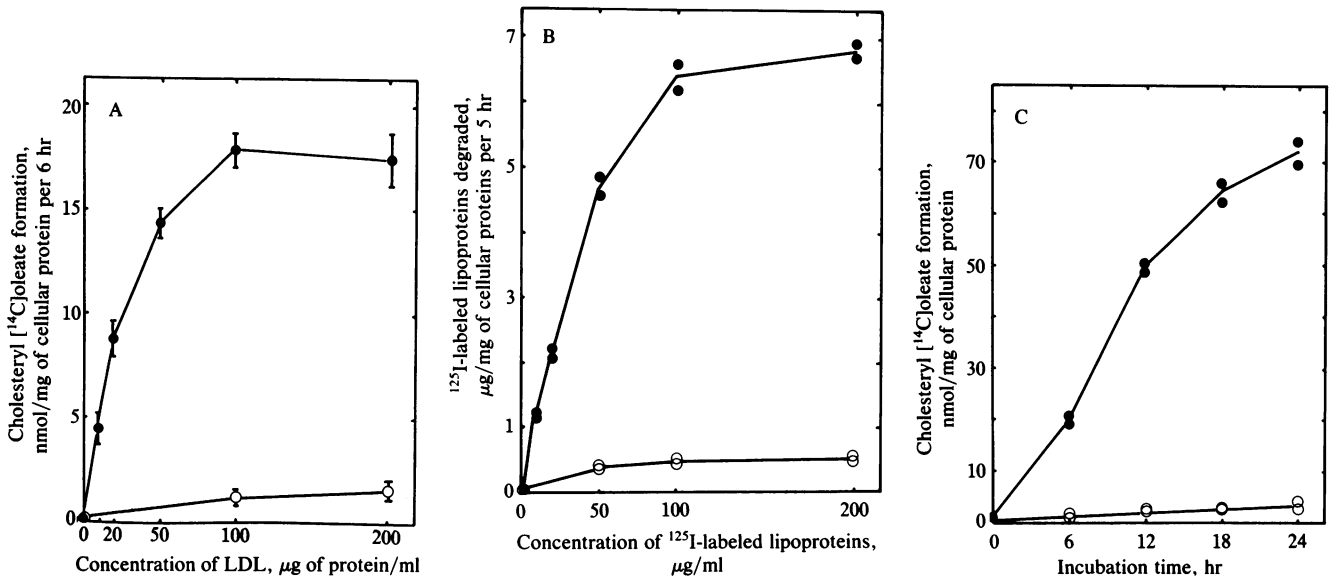

FIG. 2. (*A*) Dose–response effect of CS-modified LDL on cholesteryl ester formation in mouse peritoneal macrophages. To each monolayer (4.5×10^6 cells) was added 0.9 ml of DMEM containing 0.2 mM [^{14}C]oleate–albumin (2.4 mg/ml) and increasing concentrations of CS-modified LDL (●) or unmodified LDL (○). After 6-hr incubation cell cholesteryl [^{14}C]oleate was measured. Values are mean ± SEM from three separate experiments. (*B*) Degradation of ^{125}I-labeled CS-modified LDL by mouse peritoneal macrophages. To each monolayer (4.5×10^6 cells) was added 0.9 ml of DMEM containing the indicated concentrations of either ^{125}I-labeled CS-modified LDL (●; specific activity, 146 cpm/ng) or ^{125}I-labeled unmodified LDL (○; specific activity, 153 cpm/ng). After 5-hr incubation medium was collected, and the degradation products of ^{125}I-labeled lipoprotein were measured. Each data point represents the mean of duplicates from two separate experiments. (*C*) Time course of cholesteryl [^{14}C]oleate synthesis in macrophages incubated with CS-modified LDL. Each monolayer was incubated with DMEM containing either CS-modified LDL (●) or unmodified LDL (○), both at 100 μg/ml. After incubation for indicated periods, cholesteryl [^{14}C]oleate was determined. Values are from a representative experiment done in duplicate.

12.5-fold higher than that in cells incubated with the same concentration of unmodified LDL: the amounts of cholesteryl oleate synthesized in macrophages incubated with CS-modified LDL and unmodified LDL were 18.5 ± 2.4 and 1.48 ± 0.3 nmol per mg of protein per 6 hr, respectively. No enhanced formation of cholesteryl oleate was seen in macrophages incubated with LDL pretreated in PBS through which air had passed—i.e., 1.53 nmol per mg of protein per 6 hr. The incorporation of CS-modified LDL was also examined by measuring degradation of ^{125}I-labeled CS-modified LDL by macrophages. The degradation rate of CS-modified LDL increased in a dose-dependent manner and showed similar saturation kinetics reaching a plateau at the concentration of 100–200 μg/ml (Fig. 2*B*). The degradation rate of CS-modified LDL at 100 μg/ml was 6.51 μg/mg of protein per 5 hr, which was 10.5-fold higher than that of unmodified LDL. The incorporation of CS-modified LDL was also time dependent. Cholesteryl oleate that formed in macrophages incubated with CS-modified LDL (100 μg of protein per ml) increased to 24 hr after contact (Fig. 2*C*). In contrast, little increase was seen in cholesteryl oleate synthesis in macrophages incubated with unmodified LDL. These results clearly show that CS extract modifies LDL to a form that is recognized and incorporated efficiently by macrophages.

We next followed LDL modification over time with various concentrations of CS extract; this modification depended on the concentration of CS extract and incubation time (Fig. 3). When LDL was incubated with undiluted smoke extract from one cigarette, modification of LDL occurred within 12 hr, and the time course exhibited three phases—(*i*) a linear phase, (*ii*) an accelerated modification phase, and (*iii*) a plateau phase. When LDL was incubated with 80% dilution of the CS extract, the initial modification rate was ≈29% that with undiluted CS extract and did not plateau until 12–16 hr. When LDL was preincubated with 60% dilution of CS extract, modification proceeded more slowly and continued to rise gradually 33 hr after contact. To examine whether modification of LDL depended on temperature, we incubated LDL with CS extract at 4°C and examined LDL incorporation by macrophages. This preparation of LDL caused no increase in incorporation rate by macrophage—i.e., 1.65 nmol per mg of protein per 6 hr. We also incubated LDL in PBS with 3 mg/ml of nicotine, a major constituent of cigarette smoke at 37°C for 18 hr; no enhanced incorporation of LDL was found (1.80 nmol/mg of protein per 6 hr).

The chemical changes of LDL associated with this modification were then analyzed by two types of electrophoresis.

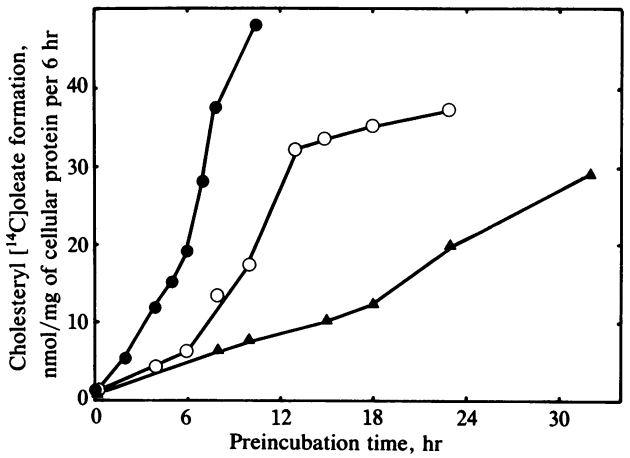

FIG. 3. Time course of CS modification of LDL. LDL (2 mg) was treated in 1 ml of PBS containing undiluted extract (●) or 80% (○) or 60% (▲) dilution of the extract from one cigarette. After treatment for indicated periods, each LDL underwent gel filtration on a PD-10 column, and an aliquot (100 μg of protein) was added to each macrophage monolayer. After 6-hr incubation cholesteryl [14C]-oleate was measured. Values are the means of duplicates from a representative experiment.

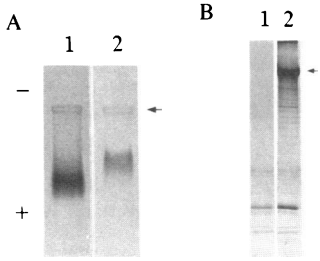

FIG. 4. Agarose and $NaDodSO_4$ gel electrophoreses of LDL. (*A*) Aliquots (50 μg of protein) of CS-modified LDL (lane 1) and unmodified LDL (lane 2) were subjected to 1% agarose gel electrophoresis and stained with fat red 7B. Application sites are indicated by arrow. (*B*) Aliquots (100 μg of protein) of CS-modified LDL (lane 1) and unmodified LDL (lane 2) were loaded on $NaDodSO_4$ electrophoresis. Position of apolipoprotein B is indicated by arrow.

When applied to agarose gel electrophoresis, CS-modified LDL migrated in a single band to a more anionic position than that of unmodified LDL (Fig. 4*A*). On $NaDodSO_4$ gel electrophoresis, CS-modified LDL showed no discrete protein band at the position of apolipoprotein B (Fig. 4*B*). These results suggested that this modification caused extensive fragmentation of apolipoprotein B and that apolipoprotein fragmentation was associated with an increase in net negative charge in LDL particles. We also measured thiobarbituric acid-reactive substances in CS-modified LDL as an index for lipid peroxidation. No increase in thiobarbituric acid-reactive substances was detected: thiobarbituric acid-reactive substances in CS-modified LDL and unmodified LDL were 1.3 and 1.4 nmol of malondialdehyde per mg of protein, respectively.

Because previous studies reported that substantial amounts of active oxygens such as superoxide anion ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) are produced in CS extract (29–31), effects of superoxide dismutase (SOD) and catalase on LDL modification were examined. We first incubated LDL with CS extract with various SOD concentrations. SOD partially inhibited LDL modification in a dose-dependent manner. SOD inhibited anionic migration of LDL on agarose gel electrophoresis and restored the apolipoprotein B band on $NaDodSO_4$ gel electrophoresis (Fig. 5 *A* and *B*). Consistent with these findings, SOD partially inhibited incorporation of LDL by macrophages. Inhibition depended on SOD amount, and ≈52% inhibition was achieved with the enzyme at 100 μg/ml (Fig. 5*C*). SOD added to macrophages after LDL modification caused no inhibition (data not shown). Nor did SOD inactivated by incubation at 120°C for 15 min inhibit CS modification of LDL (Fig. 5*C*). In contrast to SOD, catalase failed to inhibit this LDL modification (Fig. 5*C*). To exclude the involvement of divalent cations such as Cu^{2+} or Fe^{3+} in this CS modification, we treated LDL with CS extract in the presence of 500 μM EDTA. No significant inhibition of CS modification of LDL was found—i.e., <4% inhibition.

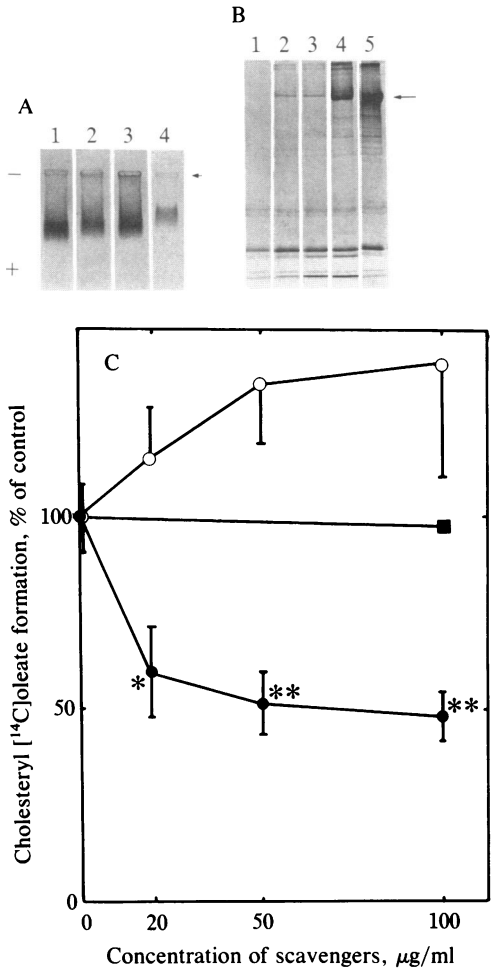

FIG. 5. (*A*) Agarose gel electrophoresis of LDL incubated with the CS extract with increasing concentrations of SOD. LDL (50 μg) of each preparation was applied to the gel. Lanes: 1, LDL incubated with CS extract without SOD; 2, LDL incubated with CS extract with SOD (50 μg/ml) 3, LDL incubated with CS extract with SOD (100 μg/ml) 4, unmodified LDL. Application sites are indicated by arrow. (*B*) $NaDodSO_4$ gel electrophoresis of LDL incubated with CS-extract with increasing concentrations of SOD. LDL (100 μg) of each preparation was applied to the gel. Lanes: 1, LDL incubated with CS extract without SOD; 2, LDL incubated with CS extract with SOD (20 μg/ml); 3, LDL incubated with CS extract with SOD (50 μg/ml); 4, LDL incubated with CS extract with SOD (100 μg/ml); 5, unmodified LDL. Position of apolipoprotein B is indicated by arrow. (*C*) Effect of SOD and catalase on CS modification of LDL assessed by cholesteryl ester formation in mouse peritoneal macrophages. Tobacco smoke from eight cigarettes was passed through 8 ml of PBS. To each 500 μl of this extract was added 1 mg of LDL and the indicated concentrations of either SOD (●), and heat-inactivated SOD (■), or catalase (○), and the mixture was incubated for 8 hr. SOD and catalase were added in 20 μl of PBS. In the control experiment 20 μl of PBS was added. After incubation, LDL underwent gel filtration as described and an aliquot (50 μg of protein) was added to the macrophage monolayer. After 6-hr incubation cholesteryl [^{14}C]oleate formation in cells was measured. Values are given as % cholesteryl [^{14}C]oleate formation in control and represent the mean ± SEM from four separate experiments. *, $P < 0.05$; **, $P < 0.01$ as compared with control values.

DISCUSSION

Results indicate that CS extract modifies LDL to a form incorporated efficiently by macrophages. When incubated with macrophages, CS-modified LDL caused accumulation of oil red O-positive droplets and enhanced cholesteryl ester synthesis in macrophages (Figs. 1 and 2*A*). CS-modification was associated with an increase in negative charge (Fig. 4*A*). Increased negative charge is a feature common to other types of modified LDL, such as acetyl-LDL and Cu^{2+}-oxidized LDL, which are incorporated by macrophages via the scavenger receptor (9, 32). Saturation kinetics for incorporation of CS-modified LDL (Fig. 2 *A* and *B*) suggest that this modified LDL is also incorporated via a receptor-mediated pathway. Whether CS-modified LDL is incorporated via the scavenger receptors common to other modified forms of LDL remains undetermined, however. We also show that CS modification is associated with extensive fragmentation of apolipoprotein B (Fig. 4*B*). In some LDL modifications, fragmentation of apolipoprotein B is associated with lipid peroxidation (16, 33). However, we found no signs of lipid peroxidation in CS-modified LDL as far as

could be assessed by thiobarbituric acid-reactive substances. Thus, it is unlikely that CS modification of LDL is associated with lipid peroxidation.

We further show that CS modification depends on incubation time, concentration of CS extract, and incubation temperature (Fig. 3). Time course of modification consisted of three phases—the first linear phase with slow modification, an acceleration phase, and a plateau phase. This time course suggests that LDL modification is promoted by concurrent accumulation of certain compounds generated in the CS extract. Some active oxygen species, such as superoxide anion ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2), have already been found to be formed in CS extract, the amounts of which reach maximal value 2 hr after incubation (29–31). In our results, SOD inhibited modification of LDL with CS extract by 52% and partially restored apolipoprotein B. Because SOD is considered to be an $O_2^{\cdot -}$ scavenger, $O_2^{\cdot -}$, might be at least in part, responsible for this modification. What phase of modification in the above time course is prevented by SOD needs to be answered. In contrast to SOD, neither catalase, a scavenger of H_2O_2, or EDTA had any inhibitory effect on this modification. These results make an H_2O_2-dependent Haber–Weiss-type mechanism involving Cu^{2+} or Fe^{3+}-catalyzed hydroxyl-radical formation unlikely in this modification.

As discussed, thiobarbituric acid-reactive substances in CS modification of LDL did not increase. This contradicts previous results by Heinecke *et al.* (34) in which thiobarbituric acid-reactive substances in LDL increased during Cu^{2+}-catalyzed oxidation, an increase inhibited by SOD addition. However, several studies suggest that the coexistence of redox-active metal ions with $O_2^{\cdot -}$ is essential in evoking lipid peroxidation. For example, exposure of LDL (15, 26, 34) or phospholipid liposome (35) to $O_2^{\cdot -}$ in the absence of either Cu^{2+} or Fe^{3+} resulted in little increase in lipid peroxide. Our results suggest that superoxide anion modifies LDL by acting directly on the protein moiety of LDL and causing polypeptide break-down. A similar mechanism has been proposed for the single-strand break of DNA by CS extract (29). In addition to protection of peptide cleavage, SOD also inhibited anionic migration of LDL. In other types of modified LDL, derivatization of the ε-amino group of lysine residues mediates an increase in anionic charge (17, 36, 37). However, we were unable to determine the mechanism of anionic transformation in CS modification and how SOD could inhibit this conversion. Because SOD only partially inhibited this modification what other mechanism contributes to modification remains to be determined.

In our experiments we trapped in aqueous solution a component(s) of CS that causes LDL modification. How closely this method imitates events in human smoking is unclear. However, using a similar *in vitro* trapping method, Lange (38) showed that a peroxide-producing component(s) in smoke inactivated some enzymes *in vitro* and found that this component was removed by human inhalation, suggesting that the component could be trapped and retained *in vivo* in the lung. Then, possibly like nicotine (2), such a component(s) is absorbed from lung and distributed in the body. Should this distribution occur, such a component(s) may cause LDL modification *in vivo* and eventually lead to atheroma formation.

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