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Participation of peroxynitrite in oxidative modification of LDL by aqueous extracts of cigarette smoke

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Abstract Aqueous extracts of cigarette smoke (CSE) can oxidatively modify plasma low-density lipoprotein (LDL). The aim of the present study was to elucidate the participation of peroxynitrite in LDL oxidation. When LDL was incubated with CSE, its oxidative modification was dependent on time and concentration. It could be effectively prevented by vitamin E, partially by superoxide dismutase, but hardly by catalase, mannitol and metal chelators. CSE also increased the 3-nitrotyrosine content in LDL. A similar increase of 3-nitrotyrosine occurred after incubation of LDL with a peroxynitrite generating agent, 3-morpholinosydnonimine, thus suggesting that prominent pro-oxidants in CSE are peroxynitrite-generating species.

Key words: Cigarette smoking; Cigarette smoke–low-density lipoprotein; Peroxynitrite; 3-Nitrotyrosine; Pro-oxidant; Atherosclerosis

1. Introduction

Cigarette smoking is believed to be a major risk factor in the development of atherosclerosis and related diseases, but the exact mechanisms underlying this association remain to be determined. Yokode et al. [1] have demonstrated that aqueous extracts of cigarette smoke (CSE) can modify low-density lipoprotein (LDL) in vitro and that the modified LDL can be taken up efficiently by macrophages to form foam cells. They also showed that these changes can be prevented by superoxide dismutase (SOD). Other reports have indicated that the plasma in cigarette smokers is in an oxidative stress condition [2–4] and has increased levels of antibody against oxidized LDL [5,6]. Our previous paper reported an increase in oxidatively modified LDL as well as lipid peroxides in the plasma of Watanabe heritable hyperlipidemic (WHHL) rabbits injected with CSE [7]. These reports suggest that cigarette smoke can generate oxidized LDL in vivo, as well as lead to the development of atherosclerosis, because oxidized LDL has been recognized as playing an important role in the development of atherosclerosis [8,9].

Cigarette smoke is known to produce a high concentration of free radicals such as superoxide anion, nitrogen oxides, reactive aldehyde species, carbon monoxide, nitric oxide and peroxynitrite [10]. These radicals in gas-phase cigarette smoke are very short-lived in aqueous solution, but some active oxidants or pro-oxidants capable of oxidizing LDL are likely to remain. The possible active pro-oxidants in CSE have been previously reported to be peroxynitrite-generating species having an effect similar to 3-morpholinosydnonimine (SIN-1) [11].

In the present study, we attempted to identify the relatively stable pro-oxidants in CSE, which can modify LDL. The chief focus was to ascertain the presence of peroxynitrite or its pro-oxidants by examining the presence of 3-nitrotyrosine (3-NT) in apolipoprotein, apolipoprotein B, of modified LDL by liquid chromatography-tandem mass spectrometry (LC-MS-MS).

2. Materials and methods

2.1. Materials

The cigarettes used were the Frontier Lights brand (JT, Tokyo, Japan) containing 1 mg of tar and 0.1 mg of nicotine per cigarette. Cambridge filters (Borgwaldt, Germany) were used to remove 99.9% of all particles and nicotine from the cigarette smoke. Cholesterol oxidase (EC 1.1.3.6; 18 U/mg) from *Streptomyces* sp., peroxidase (EC 1.11.1.7; 134 U/mg) from horseradish, and cholesteryl ester hydrolase (EC 3.1.1.13; 144 U/mg) from *Pseudomonas* sp. were purchased from Toyobo (Osaka, Japan), and homovanillic acid from Tokyo Kasei (Tokyo, Japan). Vitamin E, vitamin C, D-mannitol, uric acid, deferoxamine and D-(–)-penicillamine were purchased from Nacalai Tesque (Kyoto, Japan), 2-(4-trimethylammoniophenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide chloride (TMA-PTIO), SIN-1 and ethylenediamine-*N*,*N*,*N*,*N*-tetraacetic acid disodium salt (EDTA) from Dojindo Lab. (Kumamoto, Japan), sodium *N*,*N*-diethyl-dithiocarbamate (DDC) from Tokyo Kasei (Tokyo, Japan), SOD and catalase from Toyobo (Osaka, Japan), Dulbecco's phosphate-buffered saline (PBS) from Nissui Pharmaceutical (Tokyo, Japan) and authentic 3-NT from Sigma Chemical (St. Louis, MO, USA). Other chemicals of analytical reagent grade were purchased from Nacalai Tesque (Kyoto, Japan) and used without further purification. Water was purified with a Milli Q Jr. (Millipore, Tokyo, Japan) and used to prepare eluents for the LC method.

2.2. Preparation of CSE

CSE was prepared by modification of the technique by previous reports [1,12]. Briefly, CSE was prepared by bubbling into PBS (1 ml/three cigarettes) a stream of smoke from which tars and nicotine had been removed by gentle aspiration through a Cambridge filter; it took approximately 5 min to consume one cigarette. The CSE solution, with a pH of approximately 7.0, was sterilized through a 0.22-μm filter (Millipore, Tokyo, Japan). This CSE solution was regarded as the highest concentration (100%) and was diluted to various concentrations with PBS when necessary. The quality of the CSE

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solution prepared was assessed by measuring the absorbance at 302 nm, which is the specific absorption spectrum of peroxynitrite, and solutions with an O.D. value of more than 3.0 were used.

2.3. Animals

Male WHHL rabbits (7 months old, 2.5–2.8 kg) were obtained from our colony bred by mating pairs of homozygous WHHL rabbits kindly supplied by Dr. T. Kita (Department of Geriatric Medicine, Faculty of Medicine, Kyoto University, Japan), originally obtained from Dr. Y. Watanabe (Institute for Experimental Animals, School of Medicine, Kobe University, Japan).

2.4. Preparation of LDL

Blood was drawn from WHHL rabbits into tubes containing EDTA at a final concentration of 1 mg/ml for anticoagulation and prevention of autoxidation of lipoproteins. The plasma was separated by centrifugation ($1500\times g$ for 10 min) from the blood, and stored at 4°C. Native LDL (N-LDL, $d=1.019$–1.063 g/ml) was sequentially isolated by ultracentrifugation from the plasma samples [13]. A Beckman TL-100E ultracentrifuge was used to separate N-LDL with a TLA 110.3 fixed-angle rotor at $40\,000\times g$ for 330 min at 4°C. The isolated N-LDL was dialyzed against PBS at 4°C for removal of EDTA and used as experimental samples. The protein contents in LDLs were measured by the method of Lowry et al. [14].

2.5. Oxidative modification of LDL by CSE

N-LDL (200 μg of protein/300 μl) was incubated with 30% concentration of CSE in the absence or presence of various concentrations of antioxidants, reactive oxygen scavengers and metal chelators at 37°C for 24 h. The agents used were vitamin E (10^{-6}–10^{-4} M) as a lipid-soluble antioxidant, vitamin C (10^{-6}–10^{-4} M) as a water-soluble antioxidant, SOD (100–1000 U/ml) as a superoxide scavenger, catalase (75–750 U/ml) as a hydrogen peroxide scavenger, TMA-PTIO (10^{-6}–10^{-4} M) as a nitric oxide scavenger, D-mannitol (10^{-6}–10^{-4} M) as a hydroxyl radical scavenger, uric acid (10^{-6}–10^{-4} M) as a singlet oxygen scavenger, EDTA (10^{-6}–10^{-4} M) as a non-selective metal chelator, deferoxamine as an iron chelator, DDC (10^{-6}–10^{-4} M) as a copper chelator, and D-(–)-penicillamine (10^{-6}–10^{-4} M) as a copper chelator. After incubation, an aliquot (40 μl) of each reaction medium was immediately used to assess modified LDL by an LDL-subfraction assay method using anion-exchange LC (AE-LC) and another aliquot (150 μl) of the medium was frozen at −20°C for measurement of thiobarbituric acid-reactive substances (TBARS) by a fluorometric method according to Yagi [15].

2.6. LDL-subfraction assay method using AE-LC

The LDL-subfraction assay method by AE-LC was used to assess the modification of LDL, as reported in previous papers [12,16]. Briefly, the pumps (all from Japan Spectroscopic, Tokyo, Japan) used were two PU-980 intelligent pumps for delivering eluents and two 880-PU intelligent pumps for delivering cholesterol reagent and sodium hydroxide solution. ProtEX-DEAE (particle diameter 5 μm; column size 50×4.6 mm I.D.) columns, which are anion-exchange columns having DEAE groups, were kindly donated by Mitsubishi (Tokyo, Japan). The eluents used were as follows: eluent A, 20 mM sodium phosphate buffer (pH 7.0) containing 1×10^{-3} M EDTA; eluent B, 500 mM sodium chloride containing 1×10^{-3} M EDTA. The flow rate was maintained at 1.0 ml/min. The separation was carried out at 25°C using a water bath (Thermo Minder Lt-100, Taitec, Saitama, Japan). The modified LDLs were separated by stepwise elution with 43, 52, 60 and 100% eluent B. Those fractions were labeled as LDL1, LDL2 and LDL3, respectively. The total cholesterol level of each subfraction was evaluated by enzymatic postcolumn reaction with the cholesterol reagent described below. Lipoproteins were detected with an excitation wavelength of 325 nm and an emission wavelength of 420 nm using an RF-535 spectrofluorimeter (Shimadzu, Kyoto, Japan). Chromatographic data were collected with a CBM-10A interface, transmitted to COMPAQ computer, and integrated using CLASS-LC10 software version 1.41 (Shimazu, Kyoto, Japan).

For the enzymatic postcolumn reaction, the cholesterol reagent, which includes cholesteryl ester hydrolase (5 μg/ml, 0.7 U/ml), cholesterol oxidase (20 μg/ml, 0.4 U/ml), peroxidase (50 μg/ml, 7 U/ml) and homovanillic acid (500 mg/ml), was dissolved in 20 mM sodium phosphate buffer (pH 7.0) containing 0.2% Triton X-100, and delivered at a flow rate of 0.5 ml/min. A knitted reaction coil (15 m×0.5 mm I.D.) immersed in the water bath (Thermo Minder, Lt-100) at 45°C was used as the enzymatic reactor. After the enzymatic reaction, a 0.1-M sodium hydroxide solution was delivered into a knitted reaction coil (0.5 m×0.5 mm I.D.) at a flow rate of 0.5 ml/min to alkalize the effluent, because the stable and higher fluorescence can be detected at more than pH 10.

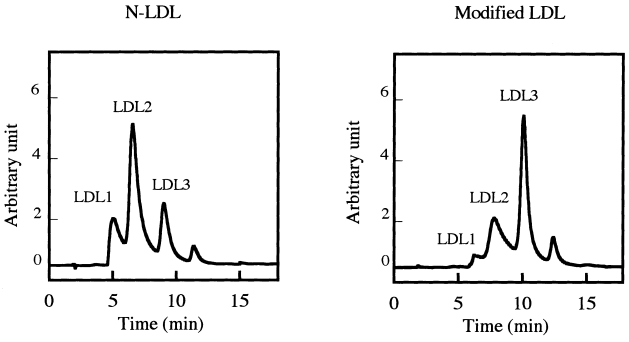

Fig. 1. Chromatograms of N-LDL and modified LDL incubated with CSE (30%, 24 h) from LDL-subfraction assay using AE-LC method. LDL1, LDL2, and LDL3 represent subfractions separated by this method. The increase in the ratio of LDL3 to LDL2 was used as an indicator of oxidative modification of LDL. The LC conditions are described in Section 2.

Typical chromatograms of N-LDL and LDL modified by CSE (30%, 24 h) are shown in Fig. 1. The degree of modification in the LDL after incubation was evaluated by the increase in the ratio of LDL3 to LDL2.

2.7. Comparison of LDL modification by CSE and SIN-1

N-LDL (200 μg of protein/300 μl) was modified by incubation with various concentrations of CSE (15–85%) at 37°C for 24 h or SIN-1 (0.1–1.0 mM), a peroxynitrite generating agent, at 37°C for 6 h. Also, N-LDL (200 μg of protein/300 μl) was modified by incubation with CSE solution (30%) or SIN-1 (0.5 mM) at 37°C for various periods of time (0.5–24 h). After the incubation, an aliquot of the reaction medium was immediately assessed for the oxidative modification by the LDL-subfraction assay method and measured for TBARS levels. Another aliquot of the medium containing modified LDL was used for the lipid extraction with ethanol:ether (3:1, v/v), and the residue containing apolipoprotein B was hydrolyzed with 6 M hydrochloric acid for 4 h at 110°C. After removal of hydrochloric acid from the hydrolysate, the residue was dissolved in distilled water and used for identification and determination of 3-NT.

2.8. Identification of 3-NT in modified LDL

The identification of 3-NT in modified LDL was performed by LC-MS-MS with electrospray ionization. A Finningan TSQ 7000 mass spectrometer (Thermo Quest, Sydney, Australia) was coupled to an HP 1050 LC system (Agilent Technologies, Hachioji, Japan). The analytical column was an ODS-Hypersil (100×2.1 mm I.D., 3 μm, Agilent Technologies, Hachioji, Japan) at a flow rate of 0.2 ml/min and mobile phase was 10% (v/v) methanol in 0.1% (v/v) formic acid. The MS-MS conditions were as follows: capillary temperature, 250°C; sheath gas, 70 psi (20 U auxiliary gas); needle potential, 4.5 kV (negative); collision offset, 23 eV. Identification of the 3-NT in the sample was performed by comparison with authentic 3-NT.

2.9. Determination of 3-NT in modified LDL

The determination of 3-NT in modified LDL was performed by LC with coulometric electrochemical array detection (LC-ECD) (Coulochem-II, ESA, Chelmsford, MA, USA). The voltage of the guard cell, Model 5020, was set at 800 mV and those of the first and second electrodes of analytical cell, Model 5010, were set at 550 mV and at 750 mV. The analytical column was a 5-μm Cosmosil $5C_{18}$-AR-II (150 ×4.6 mm I.D.; Nacalai Tesque, Kyoto, Japan) equipped with a guard column (10×4.6 mm I.D.) and the mobile phase was 20 mM sodium phosphate buffer, pH 3.07, including 5% (v/v) methanol. The flow rate was 1 ml/min and the temperature was set at 25°C. Quantitation of the 3-NT was performed by comparison of the peak area to that of the standard, authentic 3-NT.

2.10. Statistics

The results are expressed as the mean ± S.D. of triplicate determinations in the experiment using a single CSE preparation and a single LDL preparation. For Table 1, statistical analysis was performed using unpaired Student's *t*-test between the data from the non-treated LDL and CSE-treated LDL (control), and then using Bonferroni multiple-range test among the data from the control and LDL treated with antioxidants at various concentrations. These statistical analyses were performed using the Stat View software package (SAS Institute Inc., Cary, NC, USA). A difference was considered significant when $P < 0.05$.

3. Results

3.1. Effects of various antioxidants, reactive oxygen scavengers and metal chelators on oxidative modification of LDL by CSE

The degree of modification of LDL was estimated by the percentages of LDL2 and LDL3 separated by AE-LC and TBARS levels, as an index for lipid peroxidation (Table 1). In the control (LDL+CSE), where N-LDL was incubated with CSE without antioxidant at 37°C for 24 h, LDL2 significantly (12.0%) decreased and LDL3 significantly (39.6%) increased compared with those in the non-treated LDL, where N-LDL was incubated without CSE at 37°C for 24 h. The TBARS level in the control significantly (209%) increased compared with that in non-treated LDL. These changes in the control were significantly prevented by 59.6, 11.5 and 77.1% in LDL1, LDL2 and TBARS, $P < 0.05$, respectively, at 10^{-5} M and 78.8, 31.7, and 105.5% in LDL2, LDL3 and TBARS, $P < 0.05$, respectively, at 10^{-4} M of vitamin E, and by 57.7, 11.5 and 13.6% in LDL2, LDL3 and TBARS, $P < 0.05$, respectively, at 10^{-4} M of vitamin C. The reactive oxygen scavengers did not prevent the oxidative modification of LDL by CSE, except that SOD was significantly prevented at 1000 U/ml by 51.9 and 18.7% in LDL2 and LDL3, $P < 0.05$, respectively. No effect on the oxidative modification of LDL was found with various metal chelators, such as EDTA, deferoxamine, DDC and D-(–)-penicillamine (data not shown).

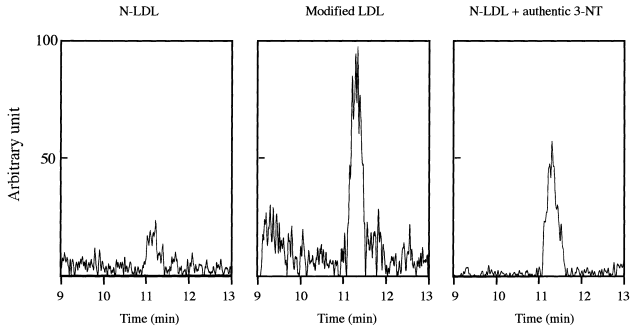

Fig. 2. Representative SRM ion chromatograms of the hydrolysates of apolipoprotein B in various LDLs by LC-MS-MS. Hydrolysate samples were prepared as described in Section 2. A 10-μl portion of a sample was injected onto the column. Modified LDL: N-LDL incubated with 85% CSE at 37°C for 24 h. N-LDL+authentic 3-NT: 2 μM authentic 3-NT was added in the hydrolysate sample of apolipoprotein B of N-LDL.

3.2. Identification of 3-NT in modified LDL

The production of 3-NT in LDL incubated with CSE was established by the method of LC-MS-MS. The mode examined was negative ionization with selective reaction monitoring (SRM). The parent ions (M–H) had a *m/z* 224.9. One daughter ion selected had a mass of *m/z* 163.1 and the other daughter ion selected had a mass of *m/z* 134.6, detection at *m/z* 163.1 was more sensitive. Representative SRM ion chromatograms of 3-NT in N-LDL incubated with or without CSE as well as authentic 3-NT are shown in Fig. 2. A single peak of 3-NT was detected only slightly, but markedly increased in LDL after incubation with CSE (85%, 24 h). As a further test of the selectivity of 3-NT detection by LC-MS-MS, authentic 3-NT was added to the N-LDL sample (Fig. 2). The

Table 1
Effect of antioxidants and reactive oxygen scavengers on oxidative modification of LDL by CSE

Drug	Concentration	LDL2 (% of TC)	LDL3 (% of TC)	TBARS (nmol/mg TC)
Non-treated LDL		41.3 ± 0.2	35.1 ± 0.2	0.113 ± 0.006
Control (LDL+CSE)		36.1 ± 0.2‡	49.0 ± 0.2‡	0.349 ± 0.004‡
Vitamin E	10^{-6} M	36.6 ± 0.4	49.5 ± 0.2	0.261 ± 0.004*
	10^{-5} M	39.2 ± 0.7*	47.4 ± 0.7*	0.167 ± 0.006*
	10^{-4} M	40.2 ± 1.1*	44.6 ± 1.0*	0.100 ± 0.007*
Vitamin C	10^{-6} M	37.5 ± 0.4	48.8 ± 0.3	0.377 ± 0.012
	10^{-5} M	38.4 ± 0.3*	48.1 ± 0.4	0.351 ± 0.006
	10^{-4} M	39.1 ± 0.3*	47.4 ± 0.1*	0.317 ± 0.007*
SOD	100 U/ml	36.6 ± 0.2	48.6 ± 0.4	0.346 ± 0.025
	500 U/ml	37.2 ± 0.6	48.1 ± 0.9	0.329 ± 0.002
	1000 U/ml	38.8 ± 0.1*	46.4 ± 0.3*	0.321 ± 0.012
Catalase	75 U/ml	36.1 ± 0.2	48.9 ± 0.3	0.341 ± 0.015
	375 U/ml	36.0 ± 0.1	49.4 ± 0.3	0.342 ± 0.017
	750 U/ml	35.9 ± 0.1	49.2 ± 0.2	0.340 ± 0.020
TMA-PTIO	10^{-6} M	36.2 ± 0.3	48.9 ± 0.3	0.338 ± 0.028
	10^{-5} M	36.1 ± 0.3	48.9 ± 0.3	0.352 ± 0.009
	10^{-4} M	36.1 ± 0.1	49.4 ± 0.3	0.335 ± 0.019
D-Mannitol	10^{-6} M	36.1 ± 0.2	49.2 ± 0.4	0.326 ± 0.005
	10^{-5} M	36.1 ± 0.3	49.2 ± 0.3	0.341 ± 0.012
	10^{-4} M	36.1 ± 0.3	49.3 ± 0.3	0.350 ± 0.014
Uric acid	10^{-6} M	36.2 ± 0.2	49.0 ± 0.6	0.341 ± 0.026
	10^{-5} M	36.0 ± 0.3	49.2 ± 0.2	0.345 ± 0.025
	10^{-4} M	36.1 ± 0.3	48.9 ± 0.2	0.348 ± 0.020

Each value represents the mean ± S.D. of triplicate determinations in the experiment using a single CSE preparation and a single LDL preparation. ‡$P < 0.05$, non-treated LDL and the control; *$P < 0.05$, control vs. agent-treated LDLs.

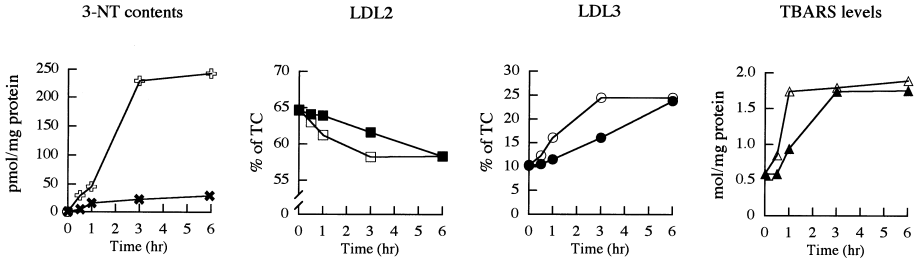

Fig. 3. Effect of incubation time on oxidative modification of LDL and 3-NT formation by CSE (closed symbols) and SIN-1 (open symbols). N-LDL (200 μg) was incubated with CSE (30%) or SIN-1 (0.5 mM) for various periods of time at 37°C in PBS, pH 7.4. The degree of oxidative modification of LDL was assessed by LDL-subfraction assay for the percentage of LDL2 (squares) and LDL3 (circles), and by the AE-LC method for TBARS levels (triangles). The 3-NT formation was assessed by 3-NT contents (crosses) in apolipoprotein B of LDL, measured by the LC-ECD method. Each data point represents the mean of triplicate determinations in the experiment using a single CSE preparation and a single LDL preparation.

peak identified as 3-NT in the modified LDL sample by CSE had the same retention time as the authentic 3-NT added to the N-LDL sample.

3.3. Effects of incubation time on LDL modification and 3-NT production by CSE and SIN-1

Fig. 3 shows the time course of degree of oxidative modification and 3-NT contents in the LDL incubated with CSE (30%) or SIN-1 (0.5 mM) at 37°C. SIN-1 gradually increased the 3-NT contents as well as the degree of oxidative modification in the LDL, as assessed by the decreased LDL2 level and the increased LDL3 level. Both reactions were terminated after 3 h at the concentration of 0.5 mM. Similar changes were also detected in the case of CSE, but the reaction rate was much slower than that of SIN-1 and the reaction was terminated after 6 h at the concentration of 30%. However, the production of TBARS by CSE and SIN-1 was terminated after 3 and 1 h, respectively, somewhat faster than the changes in the LDL subfraction and nitration. The activities of nitration and oxidative modification were preserved in both CSE and SIN-1 solutions for 1 month or more at −20°C.

3.4. Effects of concentrations of CSE or SIN-1 on LDL modification and 3-NT production

Fig. 4 shows the concentration-related increase in 3-NT content and progress of oxidative modification in the LDL incubated with CSE or SIN-1 for 24 or 6 h, respectively. CSE and SIN-1 caused an increase in the 3-NT content, as well as a decrease in the LDL2 level and an increase in the LDL3 level in a concentration-dependent manner. Also, the TBARS levels linearly increased and reached a plateau at 50% CSE and 0.5 mM SIN-1, respectively. On the basis of the concentration–production curves of 3-NT, the nitration potency of CSE was calculated as being roughly equivalent to that of 0.2 mM SIN-1.

4. Discussion

The present study demonstrated that CSE can cause nitration of tyrosine residues in apolipoprotein B in LDL. The 3-NT produced is used as a biomarker of nitrative pathology caused by peroxynitrite and appears to be a stable and specific footprint, because peroxynitrite reacts with tyrosine to yield 3-

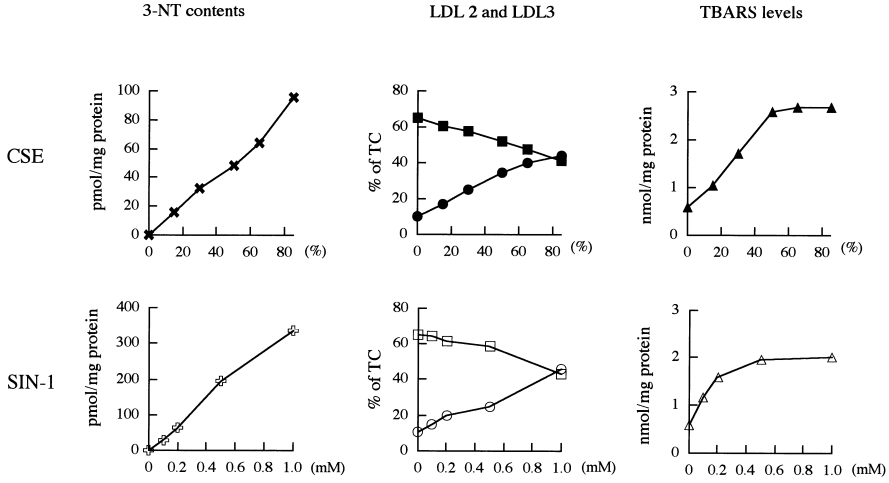

Fig. 4. Effect of concentration of CSE (closed symbols) and SIN-1 (open symbols) on oxidative modification of LDL and 3-NT formation. N-LDL (200 μg) was incubated with CSE (15–85%) for 24 h and SIN-1 (0.1–1.0 mM) for 6 h at 37°C in PBS, pH 7.4. The degree of oxidative modification of LDL was assessed by LDL-subfraction assay for the percentage of LDL2 (squares) and LDL3 (circles), and by AE-LC method for TBARS levels (triangles). The 3-NT formation was assessed by 3-NT contents (crosses) in apolipoprotein B of LDL measured by the LC-ECD method. Each data point represents the mean of triplicate determinations in the experiment using a single CSE preparation and a single LDL preparation.

NT. The nitration of tyrosine to 3-NT by peroxynitrite may be one of the important mechanisms regarding disease pathology. Many reports have demonstrated that increased 3-NT formation is associated with Alzheimer's disease [17], amyotrophic lateral sclerosis [18], Parkinson's disease [19], ischemia [20], as well as a number of atherosclerosis and relative diseases [21–24].

In order to know the property of oxidants in CSE, we first examined the effects of antioxidants, reactive oxygen scavengers and chelators on oxidative modification of LDL by CSE. We previously developed an LDL-subfraction assay method using AE-LC for measuring oxidized LDL [16] and used this method to estimate oxidatively modify LDL, which was prepared by incubation of N-LDL with CSE in vitro [12]. In the present study, metal chelators, such as EDTA, deferoxamine, DDC or D-(–)-penicillamine, could not prevent the oxidative modification of LDL by CSE at all, indicating that catalytic oxidation through metal ions such as iron or copper in CSE was not involved in the oxidative modification of LDL. Additionally, hydrogen peroxide, nitric oxide, hydroxyl radical and singlet oxygen do not appear to be involved in this oxidative modification by CSE, because the respective scavenger, catalase, TMA-PTIO, D-mannitol and uric acid, did not significantly prevent the oxidative modification, although SOD was slightly effective. From these results, we concluded that the oxidants in CSE are capable of not only oxidation but also nitration of LDL; that is, the property of peroxynitrite. Cigarette smoke has been shown to contain a considerable concentration of peroxynitrite that would appear to be produced by reaction of nitric oxide and superoxide in the gas phase [10]. Muller et al. [25] have also reported that peroxynitrite is formed in even aqueous CSE solution together with nicotine and tars, although this could not directly be certified. Peroxynitrite is converted within seconds to nitrite in aqueous solution at pH 7.4 [26]. Nevertheless, it has been demonstrated that the ability of CSE to modify LDL can be preserved for 1 month or more at −20°C [7]. Thus, the active oxidants in CSE seem to be relatively stable pro-oxidants, probably peroxynitrite-generating substances such as SIN-1. Both CSE and SIN-1 increased the 3-NT contents and the degree of modification in the LDL in a concentration-dependent manner. It has been known that a SIN-1 solution produces peroxynitrite via one-electron oxidation by molecular oxygen to produce peroxide and the subsequent release of nitric oxide [27] and its decomposition is essentially completed after 3 h [26]. In the present study, the nitration as well as modification of LDL by SIN-1 terminated after 3 h, as might have been anticipated, while the termination time of CSE was 6 h. These results suggested that CSE gradually releases peroxynitrite in a similar manner to SIN-1 but with a much slower reaction rate. Petruzzelli et al. [28] have reported the increased production of 3-NT in the plasma protein of cigarette smokers. However, there has been no evidence for the production of 3-NT in LDL treated with cigarette smoke in vitro and vivo. In the present study, the presence of 3-NT in apolipoprotein B of LDL due to CSE was identified by LC-MS-MS and measured by LC-ECD. The detection of 3-NT by LC-MS-MS is more selective but less sensitive than the detection by LC-ECD [29]. This is the first report showing the participation of peroxynitrite in the production of oxidatively modified LDL by CSE.

This work shows that peroxynitrite is a major active oxidant involved in LDL modification by CSE and that it arises from peroxynitrite-generating species soluble in CSE.

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