Aqueous extracts of cigarette smoke promote the oxidation of low density lipoprotein by peroxidases

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Abstract Oxidation of low density lipoprotein (LDL) by cigarette smoke has been considered a potential mechanism by which smoking may promote atherosclerosis. We report in this study that cigarette smoke extract (CSE) inhibited copper-induced oxidation of LDL suggesting the presence of antioxidants in CSE. It is currently believed that peroxidases may oxidize LDL in vivo and during such oxidations antioxidants become pro-oxidants. Accordingly, when LDL was oxidized by peroxidase in the presence of CSE there was an increase in the oxidation of LDL. This is the first study suggesting that smoking may promote atherosclerosis by enhancing peroxidase-catalyzed lipid peroxidation.

Key words: Smoking; Atherosclerosis; Cardiovascular disease; Oxidized low density lipoprotein

1. Introduction

Oxidized low density lipoprotein (Ox-LDL) is considered to be atherogenic [1,2]. Cigarette smoking is a recognized risk factor for cardiovascular disease [3]. The exact mechanism by which smoking promotes atherosclerosis is not known. On the basis of the finding that cigarette smoke contains a number of different types of free radicals [4] it is widely believed that cigarette smoke may directly or indirectly influence the oxidation of LDL. Studies by Yokode et al. demonstrated that direct interaction of cigarette smoke with LDL resulted in the generation of changes in LDL that were attributed to oxidation [5,6]. Studies by Harats et al. showed that there was no difference in the thiobarbituric acid substances (TBARS) associated with the LDL of smokers and non-smokers, but the LDL isolated from smokers was more readily oxidized in vitro as compared to LDL from non-smokers [7]. A study by Morrow et al. [8] had shown increased levels of F_2-isoprostanes (another marker for peroxidation) in the plasma of smokers compared to non-smokers. Frei et al. demonstrated that plasma exposed to gas phase smoke was depleted of antioxidants, particularly water soluble antioxidants, and that LDL from such plasma showed evidence of oxidation [9]. However, there was no fragmentation of the apoprotein. These studies were interpreted to suggest that antioxidant vitamin E was oxidized in the LDL of smokers and that vitamin C regenerated the tocopherol. However, the tocopherol concentration remained unaffected after vitamin C feeding [10–13].

Cigarette smoke contains a number of components such as polyphenols which are potent antioxidants [4]. We have observed for over a decade that it was practically impossible to oxidize LDL by direct action of cigarette smoke and in fact, the addition of cigarette smoke extract (CSE) actually inhibited the oxidation of LDL by metal ions (S. Parthasarathy, unpublished observations). In a more recent study, a direct antioxidant effect of CSE on copper induced oxidation of LDL was shown [14]. It is currently believed that peroxidases, such as myeloperoxidase (MPO), may be involved in the oxidation of LDL in vivo [15]. Such oxidations required the participation of vitamin E radical, tyrosine or protein radicals as intermediates [16–18]. Smoking has been associated with increased neutrophil activity in the lung and increased plasma MPO activity [19]. We therefore considered the possibility that the 'antioxidant' component of CSE may actually act as 'pro-oxidant' and promote lipid peroxidation. The results presented in this study provide experimental support for the pro-oxidant effects of CSE in generating an oxidized LDL particle in the presence of peroxidases.

2. Materials and methods

Commercial cigarettes were used in this study with class A filters. Horseradish peroxidase (HRP, type X, 250 U/mg) and MPO (M-6908 from human leukocytes) were purchased from Sigma chemical Co. (St. Louis, MO).

2.1. LDL isolation

LDL was isolated from heparinized plasma of normal human donors using a TL-100 table top ultracentrifuge [18]. Briefly, plasma density was adjusted to 1.21 with KBr and a single spin gradient isolation was used and spun at 100 000 rpm for 1 h. The isolated LDL fraction was respun after adjusting the density to concentrate and purify the LDL from any albumin contamination. The isolation was carried out without any EDTA (to avoid any effect of EDTA) and was complete in < 3 h. The isolated LDL was dialyzed against phosphate buffered saline (PBS) at 4°C (100 volumes) for 6 h. The purity of the LDL fraction was confirmed by a single band on agarose gel electrophoresis and an intact apolipoprotein B band on SDS-PAGE. Most of the diene conjugation experiments described in this study were performed immediately after isolation.

2.2. Preparation of CSE

Smoke from one cigarette was collected in 20 ml PBS into a Buchner flask kept on ice. The side arm of the flask was connected to gentle suction and the lighted cigarette was inserted into a rubber cork attached to the mouth of the flask via a short polythene tube containing the filter. The short tube minimized condensation of the cigarette extract in the tube. One part of the CSE was directly used in the oxidation studies and the other part was extracted with ethyl ether. The washed ether extract was then transferred into a pre-weighed screw cap tube, dried under nitrogen, and the weight of the

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Abbreviations: CSE, cigarette smoke extract; LDL, low density lipoprotein; Ox-LDL, oxidized low density lipoprotein; MPO, myeloperoxidase; HRP, horseradish peroxidase; H_2O_2, hydrogen peroxide; DPPD, diphenyl phenylenediamine

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ether-soluble CSE was determined. The final extract was suspended in 1 ml of alcohol and was used in the oxidation study.

2.3. Conjugated diene formation

The formation of conjugated dienes was measured in a spectrophotometer (model DB-3500; SLM-Amico, Urbana, IL) equipped with a 12 position sample changer. Ten samples and two references were measured continuously for periods of up to 4–6 h. Typically 100 μg/ml of LDL was incubated in PBS with (1 U HRP or 0.1 U MPO) in the presence of 50 μM H_2O_2. For copper mediated oxidation, 100 μg/ml LDL was incubated with 5 μM copper sulfate solution.

3. Results and discussion

Studies by Chen and Loo [14] have shown that CSE inhibited the copper and AAPH mediated oxidative modification of LDL. This was attributed to the high levels of phenols in the tar fraction of the cigarette smoke which may have antioxidant properties. Results presented in Fig. 1A,B confirm and extend these findings. Addition of CSE or the ether extract of CSE inhibited the oxidation of LDL mediated by 5 μM copper. The ability of the ether extract to inhibit oxidation suggests that stable non-gaseous components of cigarette smoke were responsible for the observed inhibition.

The oxidation of LDL by peroxidases has been reported to be enhanced by tocopherol, presumably via the formation and participation of tocopheroxy radicals [18,20]. The paradoxical pro-oxidant nature of vitamin E is also shown by non-phenolic antioxidants such as diphenyl phenylenediamine (DPPD). We show in this study that the addition of a 1/25, 1/50 and 1/100 dilution of the CSE potentiated the oxidation

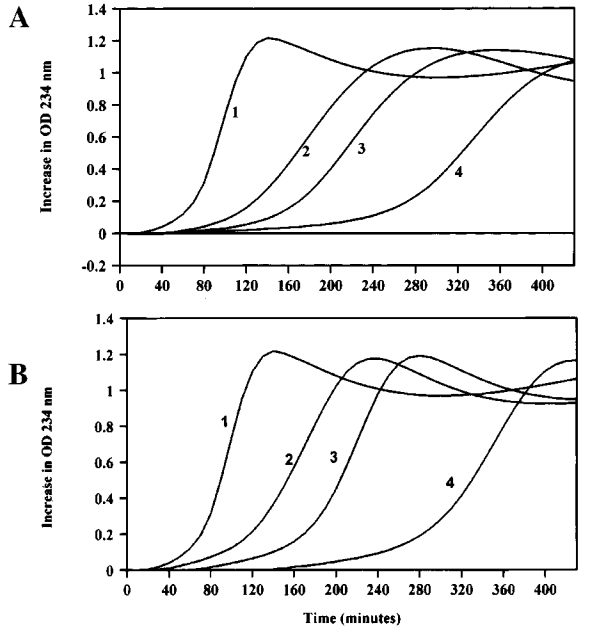

Fig. 1. A: Effect of CSE on LDL oxidation by copper. 100 μg LDL was incubated with 5 μM copper sulfate solution in a total volume of 1 ml PBS. Conjugated diene formed was measured continuously at $OD_{234\ nm}$ in a spectrophotometer. The figure represents a typical experiment from over four individual experiments. 1: Control LDL; 2: LDL+1/100 dilution of CSE; 3: LDL+1/50 dilution of CSE; 4: LDL+1/25 dilution of CSE. B: Effect of ether extract of CSE on LDL oxidation by copper. The conditions were as described in A. The figure represents a typical experiment from over four individual experiments. 1: Control LDL; 2: LDL+1 μl ether extract; 3: LDL+5 μl ether extract; 4: LDL+10 μl ether extract.

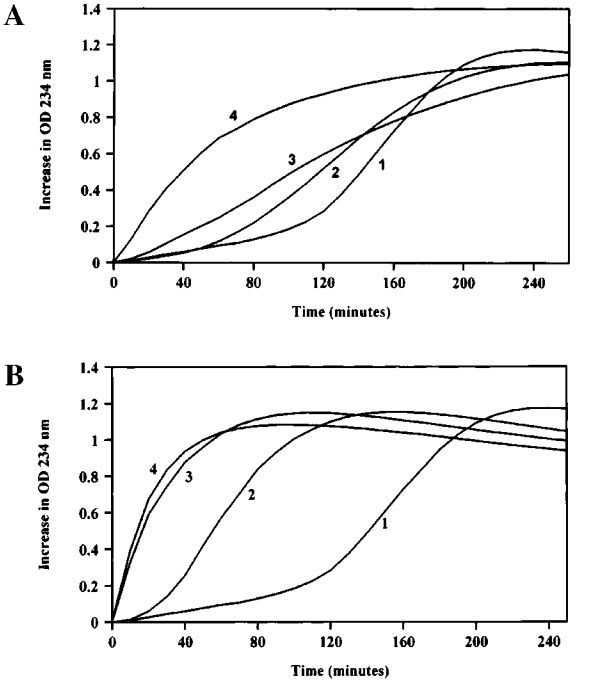

Fig. 2. A: Effect of CSE on LDL oxidation by H_2O_2/HRP. 100 μg LDL was incubated with 50 μM H_2O_2 and 1 U HRP in the presence of various concentrations of CSE in a total volume of 1 ml PBS. Conjugated diene formed was measured continuously at $OD_{234\ nm}$ in spectrophotometer. CSE was prepared as mentioned in Section 2. The figure represents a typical experiment from over four individual experiments. 1: Control LDL; 2: LDL+1/100 dilution of CSE; 3: LDL+1/50 dilution of CSE; 4: LDL+1/25 dilution of CSE. B: Effect of ether extract of CSE on LDL oxidation by H_2O_2/HRP. The conditions were as described in A. The figure represents a typical experiment from over four individual experiments. 1: Control LDL; 2: LDL+1 μl ether extract; 3: LDL+5 μl ether extract; 4: LDL+10 μl ether extract.

of LDL in the presence of HRP. There was a dose dependent increase in the formation of conjugated dienes as measured by the increase in OD at 234 nm (Fig. 2A). Similar results were obtained when the ether extract of CSE was included in the system (Fig. 2B).

HRP being a plant peroxidase, the study was repeated with a mammalian peroxidase such as MPO which has been implicated in the atherosclerotic process [16–18]. A very similar potentiation of oxidation of LDL was observed when MPO was used in the system, however, much higher dilutions (1/100 000, 1/10 000 and 1/1000), representing lower concentrations of the CSE were needed to have a pro-oxidant effect (Fig. 3). As the concentration of CSE increased, there was an increase in the inhibitory effect. This was very similar to our earlier observation [18] in the MPO mediated oxidation system, in which higher concentrations of vitamin E had an inhibitory effect. This again supports the hypothesis that depending on the concentration of the compound, there is a delicate balance between the pro-oxidant and antioxidant effect.

The gas phase of the cigarette smoke contains mostly nitric oxide, nitrogen dioxide, lipid radicals, lipid peroxy radical and several aldehydes and polyphenols [4]. The presence of these components may act as a substrate for a peroxidase mediated oxidation. However, the ability of the ether extract, which does not contain water soluble products, to promote oxida-

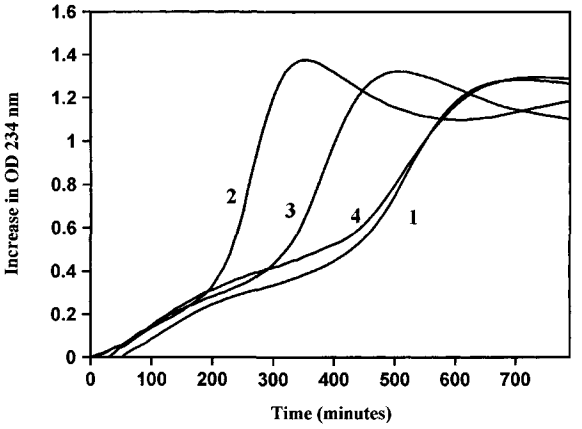

Fig. 3. Effect of CSE on LDL oxidation by H_2O_2/MPO. 100 μg LDL was incubated with 50 μM H_2O_2 and 0.1 U MPO in the presence of various concentrations of CSE in a total volume of 1 ml PBS. Conjugated diene formed was measured continuously at $OD_{234\ nm}$ in spectrophotometer. CSE was prepared as mentioned in Section 2. The figure represents a typical experiment from over four individual experiments. 1: Control LDL; 2: LDL+1/100 000 dilution of CSE; 3: LDL+1/10 000 dilution of CSE; 4: LDL+1/1000 dilution of CSE.

tion rules out the oxides of nitrogen as well as H_2O_2 as potential activators. Besides, the incubation system already contains substantial amounts of H_2O_2.

It is also well known that smokers have increased inflammatory leukocytes and alveolar macrophages [21,22]. These leukocytes and alveolar macrophages release increased amounts of superoxide and hydrogen peroxide in smokers [23]. Smokers have also been shown to have increased MPO activity compared to non-smokers [19]. These studies together with the results presented here would suggest that CSE may promote the oxidation of LDL not only by enhancing the availability of H_2O_2 and MPO but also by providing components that may form radical intermediates capable of abstracting hydrogen atoms from LDL associated lipids. This may be the first study to show a pro-atherogenic role of cigarette smoke extract.

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