Clin Investig (1992) 70:263–268

Original Article

The Clinical Investigator

Smoking influences the atherogenic potential of low-density lipoprotein

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Summary. The possible influence of smoking on the low-density lipoprotein (LDL) and its biological activity was investigated. Plasma LDL was prepared from healthy male smokers and nonsmokers, and oxidized with Cu (II) as prooxidant. Oxidized LDL from smokers generated significantly more lipidperoxidation products, so-called thiobarbituric acid reactive substances (TBARS), when compared to oxidized nonsmoker LDL. Analysis of vitamin E levels in LDL obtained from both smokers and nonsmokers revealed that the vitamin E content of smoker LDL was significantly less than that of nonsmoker LDL. The amounts of cholesteryl esters formed in cultured P388. D.1 macrophages were greater in the presence of smoker LDL than with nonsmoker LDL. The data suggest that some of the proatherogenic effects of smoking may be related to oxidative modification of LDL and alteration of its biological activity.

Key words: Low-density lipoprotein – Vitamin E – Smoking – Lipid peroxidation – Atherosclerosis

Smoking and elevated plasma low-density lipoprotein (LDL) levels are well-established risk factor for premature atherosclerosis [22]. However, little is known about the atherogenic mechanisms of smoking or whether it influences the atherogenicity of LDL. Moreover, there is no integrated view about the role of the well-known risk factors in the pathogenesis of premature atherosclerosis. Interestingly, evidence is emerging that oxidation of the LDL particles occurs in the arterial wall. There is rather good evidence that oxidative modification of the LDL particle causes shunting of LDL cholesterol into macrophages, converting them to foam cells in the early lesions [14, 25, 41, 46]. LDL is rich in polyunsaturated fatty acids (PUFAs) and particularly sensitive to oxidation [7]. Indeed, the atherogenicity of LDL could be related to its oxidation in the arterial wall, since treatment with the antioxidant probucol has shown highly antiatherosclerotic effects in spontaneously hypercholesterolemic rabbits [4].

As new technologies became available in recent years, specific experiments to approach the pathogenesis of atherosclerosis became possible. Infiltration of the subendothelial space by monocytes as early as a few days after the rise of plasma cholesterol was observed by electronmicroscopical and immunochemical analyses [9, 10, 32]. Increased influx of LDL into the arterial wall and prolonged residence time of LDL in the arterial wall in response to hypercholesterolemia at the most susceptible sites could be documented [36, 37, 40]. Modified LDL was extracted from the arterial wall and tested. Arterial wall LDL stimulated cholesteryl ester accumulation in cultured macrophages and their conversion to foam cells [12, 46]. Chemotaxis of monocytes was increased by lysolecithin generated and released by phospholipase A2 activity in the oxidizing LDL particle [30, 31]. Oxidatively modified LDL is avidly degraded by the macrophagocytic foam cells through scavenger pathways, involving a scavenger receptor [26] or another specific receptor system for oxidized LDL [39], or IgG receptors that recognize oxidized LDL complexed with specific autoantibodies [29]. Thus,

Abbreviations: CE = cholesteryl ester; FC = free cholesterol; FCS = fetal calf serum; HPLC = high performance liquid chromatography; LDL = low-density lipoprotein; MDA = malondialdehyde; PUFA = polyunsaturated fatty acid; SD = standard deviation; TBA = thiobarbituric acid; TBARS = thiobarbituric acid reactive substances; TLC = thin-layer chromatography

the extent of oxidative LDL modification in the arterial wall could correlate with its atherogenicity. LDL oxidation was discussed as a key process in atheromata formation [25, 41].

In analyzing the possible relationship between smoking and LDL modification, we speculated that in a smoker a decrement of antioxidant protection might render the LDL more sensitive to oxidation. Cigarette smoke contains oxidants such as nitrogen oxides [15] and organic hydroperoxides [45] which might damage LDL directly if their half-lives are long enough to affect the LDL particles through the circulation. The oxidants of the cigarette smoke, however, could also induce activation of cells in the airway system and increase their production of endogenous oxidants [42]. Such a mechanism could stimulate an oxidative modification of LDL.

At the beginning of our studies our hypothesis was that the balance of oxidants and antioxidants in a biological system would be affected by smoking. We reasoned that damage of the antioxidant defense line by cigarette smoking could reach such an extent that the LDL particles rich in polyunsaturated fatty acids could be altered in their biological activity. We isolated plasma LDL from normolipidemic, healthy smokers and nonsmokers. To test our hypothesis we determined (1) the thiobarbituric acid reactive substances (TBARS) in native and oxidized LDL, (2) the vitamin E content of LDL, and (3) the cholesteryl ester formation in cultured macrophages incubated with both kinds of LDL. Some data from our initial pilot study have been reported recently [33].

Material and methods

Experimental procedures

Preparation and analysis of low-density lipoprotein. LDL was prepared from plasma obtained from normolipidemic, healthy, male, heavy smokers (> 20 cigarettes per day, 25–45 years old), and from a healthy group of nonsmokers of the same age. LDL was prepared by sequential ultracentrifugation at preselected densities as described [17]. The concentration of LDL was determined as described [24] with bovine serum albumin as standard. LDL was dialysed repetitively at 4° C against 20 mM Tris-Cl, pH 8 and 150 mM NaCl under nitrogen. TBARS in LDL samples were determined as described with malon(di)aldehyde as standard [38]. Vitamin E was estimated by the HPLC method with vitamin E acetate as internal standard added before the lipid extraction [43].

Tissue Culture and Quantification of Cholesteryl Ester in Cultured Cells. P 388 D.1 macrophagocytic cells were obtained from D.P. Via, Baylor College of Medicine, Houston, Texas. The cells were grown in RPMI-1640 with glutamin, penicillin, streptomycin, and 5% fetal calf serum (FCS) as described [44]. To test for LDL oxidation-dependent formation of cholesteryl esters, the P 388 D.1 cells were incubated for 18 h in F 10 medium (containing 2.2 μM Cu^{++}) + 5% FCS + 50 μg/ml conditioned LDL. LDL was conditioned by 5 h of preincubation in F 10 medium at 37° C.

Cholesterol and cholesteryl ester in macrophages were determined by thin-layer chromatography [34]. The CAMAG TLC scanner II was used for quantitative analysis of the chromatograms as described [35]. Reacylation of cholesterol in P 388 D.1 cells was measured as [1–14 C] oleate (≈ 5 mCi/mmol) incorporation into cholesteryl esters as described [44].

Results

TBARS in native LDL and oxidized LDL from smokers and nonsmokers

The thiobarbituric acid reaction (TBAR) detects malon(di)aldehyde and compounds that can be converted to malondialdehyde (MDA), e.g., lipid hydroperoxides, adducts of MDA with aminogroups [13]. The assay is a valuable test of lipid rancidity and is often used because of its procedural simplicity and high sensitivity. We used the TBA reactive substances (TBARS) as described in an earlier study by others [38] to estimate the products of lipid peroxidation in native LDL and to monitor for oxidant change in oxidizing LDL.

Fifty-six LDL samples were analyzed in two experimental series. The first series included 17 heavy smokers (>20 cigarettes/day) and 21 nonsmokers. The LDL samples were analyzed immediately after separation from plasma by ultracentrifugation and dialysis. The mean TBARS of native smokers and native nonsmoker were low (1 ± 0.03 nmol/ml MDA) and did not differ significantly between the two groups. With Cu (II) as prooxidant, production of TBARS in each sample increased sharply after ~60 min. The increase of TBARS was steeper in the smoker LDL sample, but after 3 h the TBARS reached a plateau, which was at 14.2 nmol/ml in the smoker LDLs, 1.94-fold above the TBARS in the nonsmoker LDLs. In the second study 18 samples were analyzed, 10 from smokers (>10 cigarettes/day) and 8 from nonsmokers. These samples were analyzed after one

Table 1. TBARS plateau values in smoker LDL and nonsmoker LDL after 6 h incubation under prooxidant conditions (presence of copper ions)[a]

Source of LDL	Mean ± SD (nmol/ml)	
Study 1		
Smokers ($n=17$)	14.2 ± 2.4	$P<0.05$
Nonsmokers ($n=21$)	7.3 ± 2.2	
Study 2		
Smokers ($n=10$)	12.0 ± 1.4	$P<0.05$
Nonsmokers ($n=8$)	9.8 ± 1.34	

[a] 2 ml samples of smoker LDL and nonsmoker LDL were incubated at 37° C (final concentrations: 125 µg/ml protein, 10 µM Cu^{++}, 20 mM Tris-Cl, pH 8, 150 mM NaCl). Aliquots were precipitated with 50% trichloroacetic acid. The precipitate was pelleted and the supernatant heated for 45 min at 95° C with 1 ml TBA (10 g/l). TBARS were determined at 532 nm

Table 2. Vitamin E/smoker LDL and vitamin E/nonsmoker LDL[a]

Source of LDL	Mean ± SD (µg/mg)	
Smokers ($n=17$)	2.65 ± 0.52	$P<0.05$
Nonsmokers ($n=14$)	3.22 ± 0.97	

[a] Vitamin E was extracted from LDL from subjects of Studies 1 and 2 and quantified as described by Thompson & Hatina [43]. Vitamin E acetate was added as internal standard before the extraction

week of storage at 4° C under nitrogen. As in the companion study, the mean TBARS in the native state did not differ between smoker and nonsmoker LDL. Oxidized smoker LDL contained 12 nmol/ml TBARS (SD 1.4, range 8.3–15.8 nmol/ml) and nonsmoker LDL, 9.8 nmol/ml (SD 1.34, range 6.93–10.6 nmol/ml). The difference between oxidized smoker and nonsmoker LDL was statistically significant ($p<0.05$, unpaired t-test, one-tail hypothesis). The results are summarized in Table 1.

Vitamin E/LDL in smokers and nonsmokers

Vitamin E is the major lipophilic antioxidant in plasma. It has a high affinity for plasma lipoproteins; in males, vitamin E is carried mainly by LDL [2]. Lipid peroxides and aldehydes in LDL under oxidant stress increase after consumption of vitamin E [1, 7, 8, 21].

Therefore, vitamin E/LDL ratios in smokers and nonsmokers were determined to evaluate the possible effect of smoking on LDL in our study group. Thirty-one samples from individuals in

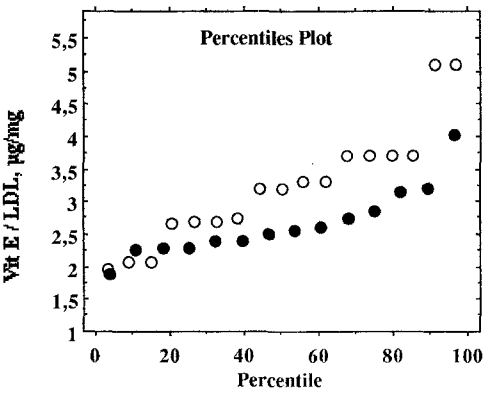

Fig. 1. Percentiles plot of Vitamin E/LDL protein from smokers and nonsmokers. ● Vitamin E smoker; ○ Vitamin E nonsmoker

Study 1 and 2 were analyzed; 14 smokers and 17 nonsmokers were compared (Table 2). Vitamin E/LDL was 3.22 µg/ml protein (SD 0.975 µg/mg) in the nonsmoker LDL and 2.65 µg/mg protein (SD 0.53 µg/mg) in the smoker LDL; smoking is associated with a significant decrease of vitamin E/LDL of 17.5% ($p<0.05$; unpaired t-test, one-tail hypothesis). Figure 1 shows these data in a percentile plot. Vitamin E/LDL ratios differ between smokers and nonsmokers in the range of the 30th–100th percentiles; below the 30th percentile there were samples with only minor differences.

Induction of cholesteryl ester formation in cultured macrophages by LDL from smokers and nonsmokers

Cholesteryl ester-rich foam cells are characteristic of the early atherosclerotic lesion. They are derived from blood monocytes [9, 10, 32]. Recently is was reported that LDL isolated from an atherosclerotic lesion causes an increased cholesterol load and increased reacylation of cholesterol [46]. It was therefore of interest to study the formation of cholesteryl esters in macrophages incubated with LDL from smokers and nonsmokers. The less-resistant smoker LDLs should be modified faster and increase shunting of cholesterol into the macrophages, thereby causing accumulation of cholesteryl esters. We analyzed 16 individuals (selected from Study 1). The results (Tables 3 and 4) demonstrate that cholesteryl esters are 19% higher in macrophages incubated with smoker LDL than in those with non-smoker LDL. Smoker LDL was associated with an increased incorporation of oleate into the cholesteryl esters of the macrophages, 27% more than that observed with nonsmoker LDL.

Table 3. Cholesteryl ester/free cholesterol (EC/FC ratio) of P 388 D.1 macrophages incubated with smoker and nonsmoker LDL (subjects of study 1)[a]

Source of LDL	EC/FC (means)	
Smokers ($n=8$)	1.095	$P<0.05$
Nonsmokers ($n=8$)	0.92	

[a] P 388 D.1 macrophages (5×10^5/ml) were grown in the presence of conditioned LDL for 18 h. Conditioned LDL was obtained by preincubation of LDL in F 10 medium for 5 h at 37° C without cells.
Cholesterol and cholesteryl ester were extracted by the Folch procedure, separated by thin-layer chromatography, and quantified as described [34, 35]

Table 4. ^{14}C Oleate incorporation/cellular protein in P 388 D.1 macrophages incubated with LDL[a]

Source of LDL	nmol/mg × 5 h (means)	
Smokers ($n=6$)	8.37	$P<0.05$
Nonsmokers ($n=6$)	6.7	

[a] Reacylation of cholesterol was measured under the conditions of Via et al. [44], except that the macrophages were incubated in F 10 medium supplemented with 50 µg/ml conditioned LDL for 18 h

Discussion

Our results demonstrate that differences between plasma LDL from healthy male smokers and nonsmokers exist in vivo and are possibly related to increased lipid peroxidation in smokers and/or increased concentrations of destabilizing agents in smoker LDL.

The determination of malon(di)aldehyde is a useful indicator of lipid peroxidation [6, 13]. Malon(di)aldehyde is generated by the oxidative breakdown of the PUFAs or the degradation of already preexisting intermediates; it is reactive with TBA. Our experiments provide strong evidence that the TBARS content of oxidized smoker LDL is significantly higher than that of oxidized nonsmoker LDL. The underlying reasons are not easily understood. Differences in the resistance of LDL to oxidants have been observed in LDL particles which differ in the content of linoleic and oleic acids [28]. However, the probands of our studies were on a normal diet. It therefore appears unlikely that the increased formation of TBARS in the smoker LDL can be attributed to differences in their PUFA content. It seems more likely that lipophilic xenobiotics from cigarette smoke and/or hydroperoxides produced endogenously in response to smoking are carried by LDL, and that these factors destabilize domains on smoker-LDL particles rich in PUFAs. Some evidence for this possibility is provided by the studies of Esterbauer [7, 8], who noted that in LDL a portion of the PUFAs easily attacked by oxygen can be distinguished from PUFAs in a more resistant state. Interestingly, it was also observed that LDL PUFAs from various probands differed in the extent of their oxidation when the LDLs were autoxidized [7]. We do not know the real basis for the differences in stability of PUFAs in LDL. However, taking the findings of Esterbauer et al. into consideration, we think that the best suggestion for the increased production of malon(di)aldehyde in oxidized smoker LDL may be a reduced resistance of some LDL domains rich in PUFAs, causing the increased formation of malon(di)aldehyde in these smoker-LDL particles during their incubation in the presence of copper and, as discussed below, in the presence of cellular oxidants. This hypothesis should be considered in further studies.

The vitamin E content of LDL particles is critical for their resistance to reactive oxygen [1, 7, 21]. Vitamin E was significantly lower in the LDL from our smoker group. We note that the differences between smoker LDL and nonsmoker LDL occur between the 30th and 100th percentile, and not lower than the 30th percentile. We do not know whether the observed difference between the vitamin E/LDL ratio in smokers and nonsmokers is a chance difference; others studied the vitamin E level in the whole plasma of five male smokers and eight female smokers and found the plasma vitamin E of the smokers to be not significantly different from that of 19 healthy nonsmokers [19]. The design of this study, however, was different from ours. Neither the plasma lipid levels nor the vitamin E/LDL ratios were reported. A possible difference in the vitamin E LDL ratio between the male smokers and nonsmokers and the female subjects was not considered by these authors despite previous reports that gender influences vitamin E transport by lipoproteins. It is known that in males LDL is the major vitamin E carrier, while in females more vitamin E is found in the HDL particle [2, 3]. Since we apparently found a significant difference, however, we speculate that smokers with lower vitamin E/LDL might be at increased risk. It is of interest to us that the ongoing WHO Monica project investigating the "antioxidant hypothesis" shows evidence for a strong correlation between the risk for ischemic heart disease and the vitamin E/plasma cholesterol ratio [11].

In the macrophage system we could define ex-

perimental conditions to differentiate the biological effects of smoker and nonsmoker LDL. In the presence of smoker LDL we observed increased formation of cholesteryl esters in the P 388 D.1 cells. To achieve this, we had to preincubate the LDL with low copper ion concentrations for 5 h. This condition does not cause measurable oxidation of LDL, but the procedure renders the LDL somehow. LDL particles are very sensitive to certain manipulations; e.g., Khoo et al. [23] observed that even short vortexing of LDL causes self-aggregation of the particles and increased uptake by cultured macrophages via LDL receptors. Our so-called preconditioned LDL is also shunted more rapidly into the macrophage than the native LDL, with the preconditioned smoker-LDL cholesterol being shunted at a slightly higher rate than the preconditioned nonsmokers LDL. We think that preconditioning of the lipoprotein particles makes them more prone to cellular modifications by the macrophage itself. We might induce a slight oxidative modification of LDL in the conditioning phase, and further damage by subsequent incubation with the macrophages, which finally results in an increased LDL-cholesterol uptake by the macrophages via scavenger receptors. We do not know to what extent the LDL modification is caused by superoxide anions, and to what extent cellular lipoxygenases are involved. Superoxide anions participate in a monocyte/macrophage-dependent LDL-oxidation system [5]. Heinecke et al. [18] observed oxidation of LDL in smooth muscle cell cultures in the presence of a redox metal. Inhibition of the cellular lipoxygenase system prevented LDL oxidation by cultured cells [27]. In any case, independent of our activity, others have demonstrated conditions for modification of smoker and nonsmoker LDL by cultured smooth muscle cells, which allowed them to show that their conditioned smoker LDL, modified by smooth muscle cells, has an enhanced metabolism when the LDL probes were subsequently tested in a macrophage culture system [16]. How extensive is the effect in our test system when we compare it with other conditions? The observed effects appear to be quite small (27%) compared with other conditions; for example, degradation of LDL particles isolated from atherosclerotic plaques by cultured mouse peritoneal macrophages was 14.6-fold higher than the degradation of native plasma LDL and resulted in a sixfold-increased incorporation of oleate into cholesteryl esters in the cells [46]. However, our rather small observed differences reach statistic significance.

In summary, the perspective of our experiments is that there is a simple approach to analyzing resistance of LDL against oxidative modification and its potential to induce cholesteryl ester formation under oxidative stress in macrophages. We used this strategy before to test estradiol in LDL particles [20]. In this study we show that we can also consider the relationship among the risk factors such as LDL cholesterol and smoking. These test systems might become a preferable technology for testing high-risk groups and even for characterizing the antiatherosclerotic activity of pharmaceuticals.

Acknowledgement. This work was supported by the German Research Council on Smoking and Health, Hamburg (Germany). We thank Professor G. Schettler (Heidelberger Akademie der Wissenschaften) for his stimulating discussions.

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Received: July 15, 1991
Returned for revision: August 29, 1991
Accepted: October 3, 1991

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