Aspirin protects low density lipoprotein from oxidative modification

Keith A Steer, Tara M Wallace, Colin H Bolton, Martin Hartog

Abstract

Objective—To examine the effects of aspirin on the potential for oxidative modification of low density lipoprotein (LDL).

Design—Before and after trial.

Setting—University department of medicine within a district general hospital campus.

Patients—Ten healthy normolipidaemic volunteers drawn from laboratory and medical staff.

Interventions—Aspirin (enteric coated) 300 mg daily for two weeks.

Main outcome measures—In vitro oxidation of LDL following ultraviolet C (UVC) irradiation with measurements made of malondialdehyde, conjugated dienes, and electrophoretic mobility.

Results—There was a significant decrease in malondialdehyde production from LDL modified by aspirin in vivo following exposure to UVC irradiation for 90 minutes, culminating in a 30% decrease by 240 minutes (mean (SD) 64·2 (9·12) v 89·6 (11·6) nmol/mg LDL protein, P = 0·029). These observations were borne out using LDL modified by aspirin in vitro. The UVC induced increase in relative electrophoretic mobility of LDL was also significantly reduced following aspirin treatment (mean (SD) 2·17 (0·16) v 2·66 (0·24), P = 0·012).

Conclusions—Aspirin, both in vivo and in vitro, protects LDL against subsequent oxidative modification, providing an additional mechanism whereby aspirin may protect against atherosclerosis.
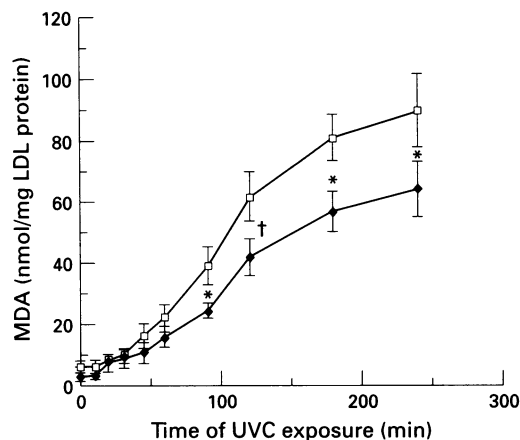
(*Heart* 1997;77:333–337)

Department of Medicine, University of Bristol, Bristol
K A Steer
T M Wallace
C H Bolton
M Hartog

Correspondence to:
Dr K A Steer, Department of Diabetes and Endocrinology, Northwick Park Hospital, Watford Road, Harrow, Middlesex HA1 3UJ, United Kingdom.

Accepted for publication 10 December 1996

Hypercholesterolaemia is well established as an important cause of coronary artery disease and the benefit of reducing blood cholesterol in both primary and secondary prevention of coronary artery disease is well established.[1][2] Hypercholesterolaemia per se is not the only causative factor, however, as at any blood cholesterol concentration there is considerable variation in the clinical expression of the disease.[3]

One basis for such variation rests with the complex nature of the biological response of arterial wall cells to the various postsecretory modifications in the structure of lipoproteins and in particular of low density lipoprotein (LDL). Over the past decade and a half, powerful evidence has accrued that modifications of LDL greatly increase its atherogenic potential, especially oxidative modification.[4]

Goldstein *et al*[5] were the first to show that modified LDL could be taken up by macrophages residing underneath the vascular endothelium, giving rise to foam cells and subsequently to the fatty streak, the earliest recognised gross lesion in atherogenesis. Chemical acetylation of LDL resulted in uptake at a rate 15 times greater than for unmodified (native) LDL. The reason for this was that such cells express few receptors for native LDL in vitro and in atherosclerotic lesions and these receptors are subject to downregulation.[5][6] This "scavenger" receptor system also recognises other chemically modified forms of LDL, including acetoacetyl LDL[7] and malondialdehyde conjugated LDL.[8]

Aspirin (acetylsalicylic acid) is capable of acetylating human serum proteins, both in vitro and in vivo.[9–12] In addition, there is evidence to suggest that aspirin can itself generate free radicals.[13][14]

Because of the known effects of chemical acetylation and free radical attack on LDL, both of which render it susceptible to catabolism by the macrophage scavenger receptor, we examined the effect of chemical modification of human LDL by aspirin, both in vivo and in vitro, on its potential for subsequent oxidative modification and hence its atherogenic potential.

Methods

Experiments were performed on normolipidaemic healthy volunteers from laboratory and medical staff before and after a two week treatment period with aspirin 300 mg daily. Informed consent was obtained.

All chemicals were obtained from BDH and Sigma Chemical Company and were of the highest grade and purity.

PREPARATION OF LDL

Plasma was separated from 10 ml fresh EDTA blood and LDL was isolated by the short run density gradient ultracentrifugation procedure developed by Kleinfeld *et al*.[15] The isolated LDL was diluted with phosphate buffer, pH 7·0, to a protein concentration of 50 μg/ml as assayed by Coomassie Blue protein reagent (Pierce and Warriner Chemical Company, United Kingdom). This was stored at 4°C

under nitrogen and used within one or two days.

LDL modified by aspirin in vitro was prepared by a modification of the method of Basu et al.[16] LDL was incubated at 37°C for 24 hours with acetylsalicylic acid to final concentration 1 mM.

OXIDATION OF LDL
LDL was oxidised using ultraviolet C (UVC) irradiation. In vitro oxidation induced by copper ion was not used in this series of experiments in view of the known effects of aspirin on copper chelation and subsequent chelate induced lipid peroxidation.[14 17 18] LDL was divided into 100 μl aliquots in silica glass tubes (40 × 10 mm internal diameter) according to the number of samples to be irradiated. Irradiation was carried out using a standard ultraviolet lamp (G8T5, 254 nm, 8 W) in a Camag lamp holder, such that the silica tubes were positioned directly under the lamp, parallel to the light source. The LDL samples were therefore at a fixed distance of 10 cm from the source where the power level was 0·9 mW/cm² as measured by an ultraviolet meter (Blak-Ray, UVP, California, USA). The tubes containing irradiated LDL were removed from the rack at specified time intervals (0, 10, 20, 30, 45, 60, 90, 120, 180, and 240 minutes). Samples were stored at 4°C before analysis (usually the same day).

MEASUREMENT OF LDL OXIDATION
Malondialdehyde
Malondialdehyde was assayed by high performance liquid chromatography (HPLC) analysis following complexing to thiobarbituric acid according to the method of Young and Trimble.[19]

Conjugated dienes
Samples were examined for the development of conjugated dienes by following the increase in absorption at 234 nm. This was done in 1 cm silica cells and absorption was measured in a Phillips PU8600 UV-VIS spectrophotometer.

LDL electrophoresis
This was performed on Sebia Hydragel Lipo + Lp(a) gels, using a Sebia power supply and chamber. Electrophoresis was carried out at a constant voltage of 50 mV for 90 minutes and the gels were stained using Sudan black according to the supplier's instructions.

The contents of LDL vitamins A, E (α tocopherol), and β carotene were determined by HPLC analysis according to the method of Arnaud et al.[20] Extraction and analysis of LDL fatty acids by HPLC was based on the method of Iverson et al.[21]

STATISTICAL ANALYSIS
Descriptive statistics were arithmetic means (standard deviation) and statistical analysis was based upon comparisons of variables made before and after aspirin treatment using paired *t* tests. Values are given as mean (SD).

Results
Ten subjects were studied (five male, five female; age 29·5 (1·93) years). Lipid profiles were unchanged following aspirin treatment (before aspirin treatment: serum cholesterol 4·87 mmol/l, triglyceride 1·11 mmol/l, and high density lipoprotein (HDL) cholesterol 1·75 mmol/l). Serum urea, creatinine, electrolytes, and albumin were within normal adult age matched reference ranges and were unchanged following aspirin treatment.
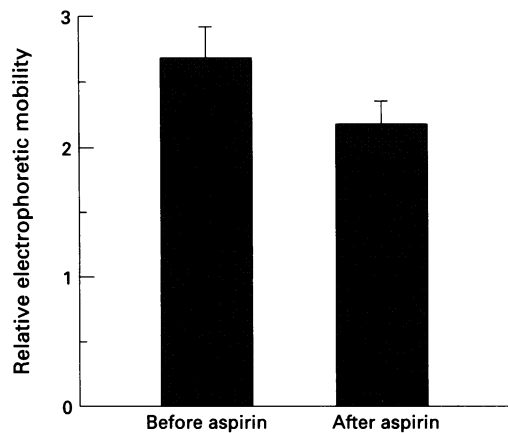
EFFECT OF ASPIRIN IN VIVO ON LDL
Formation of malondialdehyde following UVC irradiation
Malondialdehyde concentrations changed with time in a triphasic manner. After an initial period of approximately 30 minutes with little malondialdehyde production (lag phase), the concentration then rose for approximately three hours (propagation phase), after which it levelled off (decomposition phase). Basal malondialdehyde concentrations were 48% less after aspirin treatment (3·48 (1·5) *v* 6·64 (1·94) nmol/mg LDL protein) though—because of the wide range—this did not reach statistical significance. The lag phase was not affected by aspirin treatment. The rate of malondialdehyde production during the propagation phase was substantially reduced by treatment with aspirin. By 240 minutes 30% less malondialdehyde (64·2 (9·12) *v* 89·6 (11·6) nmol/mg LDL protein, P = 0·029) was formed from the aspirin modified LDL compared with control (fig 1).

Electrophoretic mobility of LDL
There was no significant change in electrophoretic mobility of LDL following aspirin treatment. A highly significant increase in electrophoretic mobility following UVC irradiation (for four hours) was observed both before and after aspirin treatment. Importantly, the increase in relative electrophoretic mobility (migration distance of oxidised to native LDL) of LDL following UVC irradiation was significantly reduced following aspirin treatment (mean (SD) after aspirin, 2·17 (0·16) *v* before aspirin, 2·66 (0·24), P = 0·012 (fig 2)) and this is in keeping with the observation that significantly less malondialdehyde was detected

Figure 2 Effect of aspirin in vivo on relative electrophoretic mobility of LDL following UVC oxidation. Mean of 10 values, error bars = SEM. Difference between before and after treatments P = 0·012.

prolonged UVC exposure (unpublished observations) and this was seen here in the case of native LDL. The aspirin modified LDL did not, however, show this phenomenon and malondialdehyde concentrations were slightly higher at eight than at four hours.

Discussion

This study provides evidence that LDL modified by aspirin, both in vitro and in vivo, is protected against subsequent oxidative modification.

Oxidatively modified LDL can affect the atherosclerotic process in various ways. It can directly injure endothelial cells.[22] Furthermore, oxidised LDL can attract circulating monocytes into the subendothelial space and inhibit their subsequent mobility.[23 24] One of the most interesting facets is its capacity for rapid uptake into tissue macrophages which subsequently form foam cells.

Most of the cholesterol entering the macrophage is derived from serum lipoproteins, especially LDL. The macrophage, however, expresses few receptors for native (unmodified) LDL, and hence uptake is slow. In direct contrast, chemically modified LDL (acetylated, acetoacetylated, or malondialdehyde conjugated) is taken up rapidly by an alternative receptor, the so called scavenger receptor.[5 7 8]

The apoprotein B epitopes which bind to the scavenger receptor are formed almost exclusively during the period when lipid hydroperoxides decompose to secondary oxidative products.[25] In the case of LDL oxidised in vitro, this corresponds to the decomposition phase which follows oxidation,[26] seen as a flattening off of the curve in fig 1.

Oxidation of aspirin modified LDL by ultraviolet C irradiation was retarded to a highly significant degree in these experiments. The malondialdehyde concentrations at the start of the decomposition phase (four hours of UVC irradiation) were 30% less in the LDL modified by aspirin in vivo (64·2 versus 89·6 nmol/mg LDL protein) and 65% less at the same time in the LDL modified by aspirin in vitro (33·4 versus 95·2 nmol/mg LDL protein). Thus the inherent capacity of LDL to undergo oxidative modification to a degree which renders it susceptible to uptake by the scavenger receptor is markedly reduced by aspirin, both in vivo and in vitro. Basal levels of malondialdehyde were also reduced by both in vitro and in vivo aspirin modification (24% and 48%, respectively), though this was only significant in the in vitro modified group. This suggests that aspirin is also able to reduce the inherent capacity of LDL to undergo oxidation.

As LDL becomes oxidised it undergoes a series of changes both in its physical and chemical properties. The malondialdehyde produced results in an increase in the net negative charge on the LDL molecule and renders it more mobile in an electrical field. The mechanism may involve both the binding of aldehyde to the free amino groups of lysine,

at this time. The net negative charge on the LDL molecule, and hence its mobility, would have therefore been correspondingly reduced.

LDL antioxidants
Vitamin A and β carotene were below detectable limits. Vitamin E concentrations were not changed significantly by aspirin treatment (mean (SD) before aspirin 12·4 (12) v after aspirin, 17·8 (17·2) nmol/mg LDL protein).
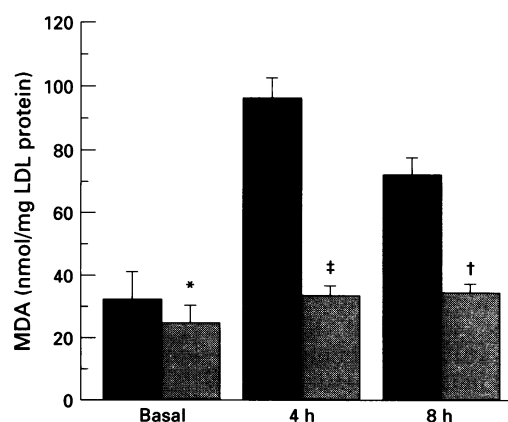
LDL fatty acids and conjugated dienes
Conjugated dienes were not affected by aspirin treatment (mean (SD) before aspirin, 0·028 (0·012) v after aspirin, 0·030 (0·012) nmol/mg LDL protein) and similarly there was no effect on pentaenoic, linolenic, hexaenoic, arachidonic, or linoleic fatty acid content. There was a significant increase in oleic acid content following aspirin treatment (mean (SD) before aspirin, 2·76 (1·85) v after aspirin, 4·85 (3·12) nmol per mg LDL protein, P = 0·024).

EFFECT OF ASPIRIN IN VITRO ON LDL
Formation of malondialdehyde following UVC oxidation
A comparison was made of the malondialdehyde concentrations generated by UVC irradiation for four and eight hours using LDL modified in vitro by aspirin. Basal malondialdehyde concentrations and those at four and eight hours of UVC exposure were all significantly reduced in the aspirin modified LDL (fig 3). We have previously observed a fall in malondialdehyde concentrations following

Figure 3 Effect of aspirin in vitro on malondialdehyde (MDA) production before and after four and eight hours of UVC oxidation. Mean of 10 observations, error bars = SEM. Differences between aspirin modified (grey bars) and unmodified (black bars) LDL achieved the following levels of significance: *P < 0·05; †P < 0·001; ‡P < 0·0001.

thus reducing the net positive charge, and free radical conversion of positively charged histidine and proline to the negatively charged aspartate and glutamate.[25]

Jurgens et al[26] showed a close linear relation between the relative electrophoretic mobility of modified LDL and its uptake into macrophages through the scavenger receptor. The scavenger receptor only recognises a certain pattern of negative charge on the apoprotein B molecule, with the density of charge at defined regions of the apoprotein B molecule being important. The relative electrophoretic mobility (REM) of the LDL following oxidation was significantly reduced by in vivo modification with aspirin (2·17 v 2·66), in keeping with the observation that significantly less malondialdehyde was produced (64·2 v 89·6 nmol/mg LDL protein).

The mechanism whereby aspirin affords LDL protection against oxidative modification is unclear, though there are several possible explanations.

Grootveld and Halliwell used aspirin to detect hydroxyl radical formation in vivo.[27] They showed that aspirin undergoes aromatic hydroxylation when exposed to hydroxyl radicals to form a specific set of hydroxylated products. With this in mind, it is possible that hydroxyl radicals formed when LDL undergoes oxidative modification, in this case following exposure to UVC irradiation, react with the aromatic nucleus of the salicylate molecule and are therefore neutralised.

Lysine residues in the LDL apoprotein B molecule are modified by malondialdehyde, rendering it susceptible to further oxidative modification and uptake by the scavenger system.[28] Aspirin too reacts by transferring its acetyl group to the lysine residues of human serum proteins, including LDL.[29 30] Malondialdehyde is also produced in human cataract as a byproduct of thromboxane synthesis. Aspirin inhibits the cross linking of human lens crystallin protein by malondialdehyde in vitro, by preventing access to the primary amino groups necessary for covalent cross linking and hence cataractogensis.[31] A similar action may operate with respect to LDL oxidation. The fall in malondialdehyde concentration which occurs during the later stage of UVC oxidation of native LDL may be caused by the malondialdehyde complex being converted from the reversible Schiff base to an irreversible Amadori product, which is inaccessible to the TBA reaction. It is possible that aspirin prevents this process as described above. Thus—as was shown in these experiments—in aspirin modified LDL the concentration of malondialdehyde did not fall during the later stages of oxidation.

Aspirin could also be exerting an effect by inhibiting the prostaglandin generating system. Free radicals are generated through the prostaglandin synthase reaction and these are capable of initiating lipid peroxidation.[32] Aspirin has been shown to inhibit this sequence in experimental studies examining free radical production and lipid peroxidation, including rat heart.[33] Against this would be the

observation that aspirin exerted an inhibitory effect on lipid peroxidation not only in vivo but also in vitro. Similarly, an increase in the LDL content of the monounsaturated fatty acid, oleic acid, which almost doubled following aspirin treatment in vivo, would reduce the oxidisability of LDL,[34] but again this would not account for the effect seen on LDL modified in vitro.

The in vivo and in vitro effects of aspirin on LDL oxidation seem to confer important benefit with regards to reducing its oxidisability, and hence its atherogenicity. However, modification of LDL by aspirin would at first sight be expected to render it more susceptible to macrophage uptake by virtue of its increased net negative charge and electrophoretic mobility.[26] Maziere et al did, however, show that such modified LDL is a poor candidate for uptake by the scavenger receptor, despite the electrochemical changes induced by aspirin derivatisation.[29]

In conclusion, aspirin modifies LDL, both in vivo and in vitro, to a form which resists both endogenous and UVC induced oxidation, and this is likely to result in a significant reduction in the atherogenic potential of LDL. This study provides evidence of a hitherto unrecognised mechanism whereby aspirin offers protection against vascular disease and raises the question as to whether aspirin should play a routine part in the management of patients with dyslipidaemia.

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