Aspirin Promotes Low Density Lipoprotein Susceptibility to Oxidative Modification in Healthy Volunteers

Matti Waterman MD¹, Bianca Fuhrman DSc², Shlomo Keidar MD¹,² and Tony Hayek MD²

¹Department of Internal Medicine A and ²Lipid Research Laboratory, Rambam Health Care Campus, and Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel

ABSTRACT: Background: Low density lipoprotein oxidation is a major pathogenic pathway in atherosclerosis. Previous studies suggested that aspirin, a commonly prescribed drug in patients with atherosclerosis, when given in a dose of 300 mg/day may decrease LDL susceptibility to oxidative modification. However, the effect of the more common lower dose aspirin on LDL oxidation is not known.

Objectives: To examine the effect of aspirin administration (low dosage) on the susceptibility of LDL to oxidative modification in healthy volunteers.

Methods: Aspirin 75 mg was administered daily for 2 weeks to 10 healthy volunteers selected from the medical staff and students at the faculty of medicine. The main outcome measure was ex vivo oxidation of LDL by ultraviolet C irradiation or by peroxy free radicals generated by AAPH (2,2’-azobis 2-aminodipropyl propane hydrochloride). The extent of LDL oxidation was determined by measuring the formed amounts of thiobarbituric acid-reactive substances, lipid peroxides and conjugated dienes.

Results: Following exposure to UVC irradiation there was a significant (P < 0.01) increase (10.8%) in TBARS concentrations and a significant (P < 0.05) increase (5.4%) in PD concentrations in LDL withdrawn after aspirin treatment as compared to LDL withdrawn before aspirin treatment. Following incubation with AAPH there was a significant (P < 0.05) increase (15%) in PD concentrations and a significant (P < 0.05) reduction (10%) of the LDL oxidation lag time in LDL withdrawn after aspirin intake as compared to LDL withdrawn before aspirin treatment.

Conclusions: Aspirin treatment given to healthy volunteers at a dose of 75 mg/day increased the susceptibility of their plasma LDL to oxidative modification ex vivo. Our study provides, for the first time, in vivo evidence of pro-oxidative properties of aspirin already suggested by previous in vitro trials.

KEY WORDS: low density lipoprotein, low density lipoprotein oxidation, aspirin

CARDIOVASCULAR DISEASE, WHICH INCLUDES CORONARY ARTERY DISEASE, STROKE, AND PERIPHERAL VASCULAR DISEASE, IS THE LEADING CAUSE OF DEATH IN THE UNITED STATES AND MOST DEVELOPED COUNTRIES [1]. Coronary artery disease develops as a result of various risk factors, including increased plasma low density lipoprotein levels as well as LDL oxidative modifications [2]. LDL oxidation is thought to play a key role in early atherogenesis [3]. Oxidized LDL is taken up by macrophages at an enhanced rate via their scavenger receptor, leading to formation of lipid-laden foam cells, the hallmark of early atherogenesis [4]. Furthermore, oxidized LDL has been shown to enhance atherogenesis by other mechanisms, such as cytotoxicity towards endothelial cells and macrophages and stimulation of thrombotic and inflammatory events [3]. It was previously shown that the use of hypocholesterolemic drugs, such as statins, to treat hypercholesterolemic patients results also in reduced susceptibility of the patients’ LDL to oxidation [5].

Aspirin is currently used for the prevention of cardiovascular events [1]. Aspirin, through inactivation of platelet prostaglandin G/H synthase and loss of cyclooxygenase activity, reduces the incidence of occlusive cardiovascular events [6]. However, the evidence accumulated to date regarding the effect of aspirin on LDL is conflicting. A preliminary study showed in vitro that aspirin-treated LDL altered biochemical properties and was more efficiently recognized by the apo B/E receptor of fibroblasts than native LDL [7]. Other studies investigated the antioxidative effect of aspirin. Combined treatment of aspirin and copper increased the antioxidative activity in the plasma of animals [8], and another study [9] demonstrated that aspirin protected endothelial cells against oxidative stress. Furthermore, aspirin was found to induce the synthesis of ferritin, an antioxidiant protein, by endothelial cells [10]. On the other hand, it was found that aspirin and salicylate were not able to counteract copper-driven LDL oxidation [11], and it had no effect on the modification of LDL induced by endothelial cells [12] or by human hepatoblastoma cell line [13]. Moreover, salicylate, the pharmacological active metabolite of aspirin, could act as a catalyst of myeloperoxidase-initiated LDL oxidation [14].

The only in vivo trial in humans that examined the effect of aspirin therapy on LDL oxidation was performed...
in healthy volunteers who took 300 mg of aspirin/day for 2 weeks [15]. The aim of our study was to examine the effect of 75 mg aspirin/day on the susceptibility of LDL to oxidative modification in healthy volunteers; this dosage is commonly used in patients with coronary artery disease [16].

**SUBJECTS AND METHODS**

Ten normolipidemic healthy, non-smoking male volunteers who were students and medical staff at the Rambam Medical Center and the Technion Faculty of Medicine, Haifa, Israel were administered aspirin, 75 mg/day (Microspirin 75®, Dexel Co. Ltd, Dexxon group, Or Akiva, Israel), for 2 weeks. Compliance with this regimen in all subjects was satisfactory, as assessed by daily contact with the subjects. Blood samples were drawn after 12 hours of fast before study entry, and after a 2 week course of aspirin treatment. Informed consent was obtained from all subjects, according to the Helsinki Committee of the Rambam Medical Center and the Israel Ministry of Health (No. 907).

**LDL ISOLATION**

Plasma was separated from 20 ml fresh blood that was drawn in EDTA (Sigma Chemical Co., St. Louis, USA) from the subjects before the study and after 2 weeks of the aspirin course. All samples drawn at the beginning of the study were stored at 4°C for 2 weeks until the second samples were drawn. LDL was then isolated from all plasma samples by discontinuous density gradient ultracentrifugation as previously described [17]. The LDL samples were washed at a density of 1.063 kg/L and dialyzed against 150 mmol NaCl/L and 1 mmol Na₂EDTA/L (pH 7.4) at 4°C and stored in the dark and at 4°C until oxidation was performed within 2 days from separation. The LDL protein concentration was measured with the Folin phenol reagent [18]. Before oxidation, LDL was dialyzed at 4°C (pH 7.4) against EDTA-free Dulbecco’s phosphate – buffered saline solution (Biological Industries, Beit HaEmek, Israel).

**LDL OXIDATION**

- **Ultraviolet radiation-induced LDL oxidation**

  Two milliliters of LDL solution (100 µg of LDL protein/ml) were placed in 35 mm diameter glass tubes that were positioned directly under a 325 nm ultraviolet lamp (Philips T.U.V 30W/G30 TB, Holland). All samples to be irradiated were held at a fixed distance of 9 cm from the light source for 4 hours at room temperature. Due to evaporation during the prolonged oxidation, the sample volume was reconstituted to 2 ml with PBS solution.

- **Free radicals-induced LDL oxidation**

  One milliliter of LDL solution (100 µg LDL protein/ml) was incubated in the absence or presence of 100 mmol/L of the free radical generator AAPH (2,2’-azobis 2-amidinopropane hydrochloride) (Wako Chemical Industries Ltd, Osaka, Japan) for 4 hours at 37°C. AAPH is a water-soluble azo compound that thermally decomposes to produce peroxy radicals at a constant rate [19].

**DETERMINATION OF LDL OXIDATION**

LDL oxidation was determined immediately by measuring the amount of thiobarbituric acid-reactive substance formed [20]. The formation of conjugated dienes was continuously monitored by measuring the increase in absorbance at 234 nm [20]. Incubations were carried out in a spectrophotometer cuvette at 37°C in a temperature-controlled six-cell holder (Ultrospec 3000 spectrophotometer, Pharmacia, Biotech). The reference cell contained PBS. The initial background of the different samples ranged between 0.1 and 0.2 optical density as recorded at 234 nm. After recording the initial absorbance, it was set to zero against blank, and the increase in absorbance during LDL oxidation was recorded over time every 10 minutes. The lag time required for the initiation of LDL oxidation was calculated from the oxidation curve. LDL-associated lipid peroxide formation was determined with a cholesterol color reagent as previously described [20]. This assay is based on the oxidative activity of lipid peroxides that convert iodide to iodine, which is then measured spectrophotometrically at 365 nm.

**STATISTICAL ANALYSIS**

Statistical analysis was performed using Student’s paired t-tests. Results are given as mean ± SEM. The computer software program STATEASE (version 1.00, Data Plus Systems Inc, New York) was used for computation.

**RESULTS**

Ten healthy male volunteers were studied (mean age 29.3 years). Blood serum urea nitrogen, glucose and electrolytes were within normal reference ranges and were unchanged following aspirin treatment. Baseline total cholesterol and high density lipoprotein were 164 ± 43.3 mg/dl, respectively, and these values did not change significantly after aspirin treatment. There was no significant change in LDL cholesterol concentrations before and after aspirin treatment (115.1 mg/dl before vs. 110 mg/dl after treatment).

**EFFECT OF ASPIRIN THERAPY ON LDL OXIDATION**

- **UVC irradiation-induced LDL oxidation**

  The susceptibility of LDL to UVC-induced oxidation increased after aspirin treatment, as demonstrated by a significant
effect was further evidenced by a significant \((P < 0.05)\) shortening (by 10%) of the lag time required for the initiation of LDL oxidation [Figure 3].

**DISCUSSION**

This study showed that aspirin treatment given to healthy volunteers at a common dosage of 75 mg/day increased the susceptibility of their plasma LDL to oxidative modification \textit{ex vivo}. The ability of aspirin to enhance oxidation of LDL was demonstrated
in two different oxidative systems, including free radical generators and UV irradiation. Using more than one assay (TBARS, lipid peroxides, and conjugated dienes formation) we showed that LDL susceptibility to oxidation ex vivo following aspirin treatment was mildly, but significantly, enhanced.

Our results are in contradiction to previously presented evidence by Steer et al. [15] of an inhibitory effect of aspirin on oxidation of LDL. The difference in results may stem from the large discrepancy in aspirin dosage used in both studies. In their study, Steer et al. used a high dose of 300 mg/day, whereas we used a much lower dose (75 mg/day). The lower dose has been proven to be an effective regimen with fewer adverse events than higher dosages [6].

The antioxidative properties of aspirin, in vitro as well as in vivo, are under debate. Several studies indicated a possible antioxidative effect of aspirin [8-10]. Aspirin has been shown to chelate iron ions in the cytosol and enhance synthesis of ferritin, a cytoprotective protein that sequesters free cytosolic iron, the main catalyst of oxygen radical formation. These studies suggest an important role of aspirin in preventing iron-catalyzed cellular injury and lipid peroxidation [21]. Moreover, Hermann et al. [14] suggested that long-term aspirin treatment might have an inhibitory effect on LDL oxidation through gentisic acid, a radical scavenger that is the main metabolite of salicylic acid, the pharmacological active derivative of aspirin. The role of gentisic acid in the prevention of LDL oxidation was also demonstrated in another study, where the addition of gentisic acid to human plasma samples prevented ex vivo LDL oxidation [22].

However, other studies have not been able to demonstrate antioxidative properties of aspirin. Lapenna and collaborators [11] showed that aspirin that was added to LDL in vitro could not inhibit AAPH or copper ion-induced oxidation. Other studies showed that aspirin was not able to inhibit in vitro oxidation of LDL by endothelial cells [12] or by the human hepatoblastoma cell line, HepG2 [13]. Another study found that neither aspirin nor salicylate, the principal metabolite of aspirin, was able to inhibit LDL oxidation by glucose-derived radicals [23]. Similarly, Sobal and team [24] have shown in an in vitro study that aspirin could inhibit native LDL and glycated LDL oxidation only in supratherapeutic concentrations [24]. Moreover, Hermann et al. [14] reported that salicylate could promote LDL oxidation induced by the myeloperoxidase cell-free system and by activated human neutrophil leukocytes. Our study provides, for the first time, in vivo evidence of a possible pro-oxidative effect of aspirin on lipid peroxidation.

The mechanism whereby aspirin promotes LDL oxidation is still unclear. A possible explanation has been proposed by Hermann and co-researchers [14]; their study provided evidence that salicylate, a monophenolic compound, may form phenoxyl radicals in certain pro-oxidative conditions. The addition of ascorbic acid, a scavenger of phenoxyl radicals, was able to inhibit LDL oxidation. Other studies used electron spin resonance assays to show that salicylate can undergo phenoxyl radical formation under peroxidative conditions [25].

We cannot explain the ability of aspirin to inhibit LDL oxidation at one dosage (300 mg/day), as reported by Steer et al. [15], and to promote LDL oxidation at another dosage (75 mg/day), as our results demonstrate. It is possible that the plasma concentrations of gentisic acid, a metabolite of salicylate that has been shown to impair LDL oxidation in previous studies [14,22], were not sufficiently high at the aspirin dosage that was used in our study.

Further studies are needed to clarify the exact mechanism whereby aspirin treatment renders LDL to be less resistant to oxidative modification. This pro-oxidative effect must also be demonstrated in atherosclerotic patients. This study, performed in healthy volunteers, suggests that aspirin treatment at a daily dosage of 75 mg has an adverse effect that may increase the atherogenicity of LDL. Should this effect be found in atherosclerotic patients, it may put aspirin treatment in a new perspective.

Correspondence:
Dr. M. Waterman
Dept. of Medicine A, Rambam Medical Center, P.O. Box 9602, Haifa 31096, Israel
Phone: (972-4) 854-2518
Fax: (972-4) 854-2721
e-mail: m_waterman@rambam.health.gov.il

Figure 3. Effect of 2 weeks of aspirin treatment (75 mg/day) on the susceptibility of LDL to AAPH-induced oxidation ex vivo: kinetic study. LDL samples that were obtained from 10 healthy volunteers before and after aspirin treatment were incubated with 100 mmol/L of AAPH for 4 hours at 37°C. Formation of conjugated dienes was continuously monitored at 234 nm. Results are expressed as mean ± SEM (n=10) of the lag time needed until LDL lipid peroxidation was initiated. **P < 0.05 (after vs. before aspirin administration).
References


Capsule

A problem with anti-HIV antibodies

Despite significant efforts, an effective vaccine against the HIV-1 virus remains elusive. A site on the HIV-1 gp120 envelope glycoprotein that binds to the CD4 receptor on host cells is vulnerable to antibody, but only rarely are antibodies against this site broadly neutralizing. L. Chen et al. have determined crystal structures for two weakly neutralizing antibodies in complex with gp120. The epitopes recognized by these antibodies were similar to those bound by CD4 or a broadly neutralizing antibody. However, small differences in recognition induced conformational shifts in gp120 that were incompatible with formation of a functional viral spike. Thus, the antibody-vulnerable site on HIV-1 is protected by conformational constraints.

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Elon Israeli

Capsule

Nanobible presented to the Pope

Israeli President Shimon Peres presented Pope Benedict XVI with an Old Testament inscribed onto a pinhead-sized silicon chip. Nanotechnology experts at the Technion-Israel Institute of Technology engraved the 308,428 word text from the Hebrew Bible on a chip measuring 0.5 mm² and covered with a thin layer of gold (20 mm in thickness, or 0.0002 mm). The chip lies in a glass case decked with a magnifying glass along with technical explanations of the nano-Bible in Hebrew and English, and the first 13 verses of the Book of Genesis magnified 10,000 times.