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Attenuation of diet-induced atherosclerosis in rabbits with a highly selective 15-lipoxygenase inhibitor lacking significant antioxidant properties

*Sandra M. Sendobry, ‡Joseph A. Cornicelli, ‡Kathryn Welch, ‡Thomas Bocan, §Bradley Tait, §Bharat K. Trivedi, §Norman Colbry, ¶Richard D. Dyer, #Steven J. Feinmark & *,†,[1]Alan Daugherty

Cardiovascular Division, *Department of Medicine and †Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis MO 63110; ‡Departments of Artherosclerosis Therapeutics, ¶Biochemistry and §Chemistry Parke-Davis, Ann Arbor MI 48105 and #Department of Pharmacology, Columbia University, New York, NY 10032, U.S.A.

1 15-Lipoxygenase (15-LO) has been implicated in the pathogenesis of atherosclerosis because of its localization in lesions and the many biological activities exhibited by its products. To provide further evidence for a role of 15-LO, the effects of PD 146176 on the development of atherosclerosis in cholesterol-fed rabbits were assessed. This novel drug is a specific inhibitor of the enzyme *in vitro* and lacks significant non specific antioxidant properties.

2 PD 146176 inhibited rabbit reticulocyte 15-LO through a mixed noncompetitive mode with a K_i of 197 nM. The drug had minimal effects on either copper or 2,2′-azobis(2-amidinopropane)hydrochloride (ABAP) induced oxidation of LDL except at concentrations 2 orders higher than the K_i.

3 Control New Zealand rabbits were fed a high-fat diet containing 0.25% wt./wt. cholesterol; treated animals received inhibitor in this diet (175 mg kg^{-1}, b.i.d.). Plasma concentrations of inhibitor were similar to the estimated K_i (197 nM). During the 12 week study, there were no significant differences in weight gain, haematocrit, plasma total cholesterol concentrations, or distribution of lipoprotein cholesterol.

4 The drug plasma concentrations achieved *in vivo* did not inhibit low-density lipoprotein (LDL) oxidation *in vitro*. Furthermore, LDL isolated from PD 146176-treated animals was as susceptible as that from controls to oxidation *ex vivo* by either copper or ABAP.

5 PD 146176 was very effective in suppressing atherogenesis, especially in the aortic arch where lesion coverage diminished from 15 ± 4 to 0% ($P < 0.02$); esterified cholesterol content was reduced from 2.1 ± 0.7 to 0 μg mg^{-1} ($P < 0.02$) in this region. Immunostainable lipid-laden macrophages present in aortic intima of control animals were totally absent in the drug-treated group.

6 Results of these studies are consistent with a role for 15-LO in atherogenesis.

Keywords: Atherosclerosis; oxidation; lipoprotein; macrophages; endothelium; lipoxygenase

Introduction

Atherosclerotic lesions are characterized by excessive intracellular lipid deposition in macrophages, leading to formation of foam cells. There is extensive evidence that formation of oxidized low-density lipoprotein (LDL) within arterial tissue is required for this unregulated lipid accumulation (Steinberg *et al*., 1989; Daugherty & Roselaar, 1995). There are several lines of evidence that oxidation promotes the atherogenic process. First, oxidized LDL induces a wide array of biological activities in cultured cells that are considered to be atherogenic (Steinberg *et al*., 1989). Also, the presence of oxidized lipoproteins in atherosclerotic lesions has been demonstrated in several studies, both by characterization of isolated LDL from vessels (Daugherty *et al*., 1988; Palinski *et al*., 1989; Ylä-Herttuala *et al*., 1989) and immunocytochemical localization of oxidation-specific epitopes (Haberland *et al*., 1988; Rosenfeld *et al*., 1990). Finally, several synthetic antioxidants attenuate the development of atherosclerotic lesions (Kita *et al*., 1987; Carew *et al*., 1987; Daugherty *et al*., 1989; 1991; Björkhem *et al*., 1991; Sparrow *et al*., 1992). While there is consensus that LDL oxidation occurs in atherosclerotic lesions and contributes to disease development, the specific mechanism of modification has not been defined. Potential mediators of LDL oxidation may include myeloperoxidase (Daugherty *et al*., 1994), transition metal ions (Heinecke *et al*., 1984), ceruloplasmin (Ehrenwald *et al*., 1994) and 15-lipoxygenase (15-LO; Harats *et al*., 1995a).

15-LO was initially hypothesized to have a role in LDL oxidation because it provides a source of peroxyl radicals. Supporting this hypothesis, 15-LO inhibitors have been shown to reduce endothelial cell and macrophage-mediated oxidation of LDL (Parthasarathy *et al*., 1989; McNally *et al*., 1990; Rankin *et al*., 1991). LO isolated from soybean oxidatively modifies LDL, although this requires simultaneous incubation with phospholipase A_2 (Sparrow *et al*., 1988). Subsequent isolation of mammalian 15-LO led to the observation that the enzyme alone is capable of executing oxidative modifications (Belkner *et al*., 1993). Also, cells stably transfected with 15-LO have an increased ability to oxidize LDL compared with cells transfected with a control gene (Benz *et al*., 1995). However, the role of this enzyme in initiating LDL oxidation is controversial. Sparrow and Olszewski (1992) have attributed the ability of 15-LO inhibitors to block LDL modification to nonspecific antioxidant effects rather than direct effects on the enzyme. Furthermore, a dichotomy has been observed between the magnitude of cellular 15-LO activity and the extent of modification of LDL. Despite these conflicting findings with regard to a role in LDL oxidation, products of 15-LO have many other known effects that may influence the atherogenic process (Ford-Hutchinson, 1991).

[1]Author for correspondence at: Cardiovascular Division, Box 8086, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110, U.S.A.

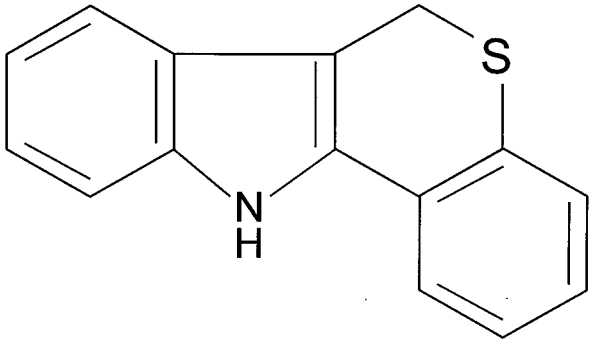

Figure 1 Chemical structure of PD 146176.

The importance of 15-LO in the atherogenic process is also implied by the presence of both the mRNA and protein in atherosclerotic lesions from Watanabe heritable hyperlipidemic rabbits (Ylä-Herttuala *et al*., 1990; 1991). The localization of the enzyme corresponds to the distribution of oxidation epitopes. Stereospecific oxygenated lipids produced by 15-LO have been detected in atherosclerotic tissue (Kühn *et al*., 1994b; Folcik *et al*., 1995). Also, gene transfer of 15-LO into arterial tissue promotes formation of oxidized epitopes at sites of protein expression (Ylä-Herttuala *et al*., 1995). In an attempt to resolve the controversy over the involvement of 15-LO in the disease process, we have studied the effects of a novel, highly selective pharmacological inhibitor of the enzyme, on the development of atherosclerotic lesions in cholesterol-fed rabbits. The compound was found by screening the Parke-Davis Pharmaceutical Research compound collection for drugs that inhibited rabbit reticulocyte 15-LO as described previously (Auerbach *et al*., 1996). Entities that had inhibitory activity were characterized further by quantifying products of rabbit 15-LO with high performance liquid chromatography (h.p.l.c.). PD 146176 (6,11-dihydro-5-thia-11-aza-benzo[a]-fluorene; Figure 1) was synthesized based on the structure of the prototype compound (8,9,10,11-tetrahydro-13H-indolo[2,3-d]naphtho[2,3-b]thiopyrylium chloride). We found that this novel 15-LO inhibitor, which lacks significant nonspecific antioxidant activity, has a dramatic effect in reducing atherogenesis.

Methods

Animals, diet and drug administration

Specific pathogen-free New Zealand White rabbits ($\approx$2.5 kg) were obtained from Myrtle's Rabbitry (Thompson Station, TN, U.S.A.). The animals were fed a standard laboratory diet (Ralston Purina, St. Louis, MO, U.S.A.) and were allowed to acclimatize for 7 days before initiation of the study, at which time two groups of rabbits ($n=10$ in each group) were started on a diet enriched with cholesterol (0.25% wt./wt.), peanut oil (3% wt./wt.), and coconut oil (3% wt./wt.), with a small amount of apple sauce mixed into the food to enhance palatability. The control group received no additional treatment. The drug-treated group received 350 mg of PD 146176 kg^{-1} body weight day^{-1} in their food. Rabbits were permitted access to 40 g of food at $\approx$12 h intervals via automated feeders and diet intake was monitored every day such that the animals received 175 mg kg^{-1} b.i.d. Water was available *ad libitum*. Body weights were measured at weekly intervals throughout the 12 week study. Blood samples were obtained at the indicated intervals for determination of haematocrit and plasma lipid concentrations. All protocols were approved by the Washington University Animal Studies Committee.

Characteristics and specificity of 15-LO inhibition by PD 146176

15-LO was isolated from reticulocytes harvested from phenylhydrazine-treated rabbits (Sloane *et al*., 1990). Enzyme (820 pmol) was incubated for 20 min at 4°C in Dulbecco's phosphate-buffered saline (pH 7.4) without calcium containing HEPES (10 mM), ovalbumin (1 mg ml^{-1}), glycerol (10% v/v), $MgCl_2$ (5 mM), sodium cholate (4.4 mM) DMSO (5% v/v), and the required concentration of PD 146176 for 15 min. The reaction was initiated by addition of 13-hydroperoxyoctadecadienoate (13-HPODE) and [^{14}C]-linoleic acid (1.5 to 48 μM) at 4°C. Aliquots of the reaction mixture were withdrawn at selected intervals and quenched by addition of methanol and triphenylphosphine (100 μg). The production of [^{14}C]-13-HODE in the presence of increasing concentrations of PD 146176 was monitored by reverse-phase (r.p.) h.p.l.c. The concentration of drug causing 50% inhibition (IC_{50}) was determined by use of KaleidaGraph, version 3.0.1 (Synergy Software, Reading, Pennsylvania, U.S.A.). Per cent inhibition versus inhibitor concentration data were fit to the two-parameter equation [% inhibition $= 100/(1+(\mu M/IC_{50})\text{slope})$] and best fits for the IC_{50} and the slope coefficients were estimated by least-squares analysis. Reaction velocities were calculated by linear regression of product values versus reaction time for each incubation. These data were fitted to classical rapid equilibrium models by use of hyperbolic weighting (Segel, 1975; Cleland, 1979).

The effect of PD 146176 on a number of closely related enzymes was examined, including (1) 5-LO from a 20,000 *g* supernatant of lysed rat basophilic leukemia cells (ATCC, Rockville, MD, U.S.A.); (2) 12-LO in 30,000 *g* supernatants from lysed human platelets (Carter *et al*., 1991); (3) ovine cyclo-oxygenase I from ram seminal vesicles (Cayman Chemical, Ann Arbor, MI, U.S.A.); and (4) human recombinant cyclo-oxygenase II expressed in a baculovirus system from a cDNA with the sequence described by Hla and Neilson (1992). The ability of PD 146176 to inhibit the enzymatic conversion of [^{14}C]-arachidonic acid to specific products was monitored by r.p.-h.p.l.c.

Plasma drug concentrations

Plasma concentrations of PD 146176 were determined by h.p.l.c. Plasma from treated and control animals was incubated with NaOH and extracted into pentane/methyl t-butyl ether (3 : 2) after addition of a known amount of a structural analogue of PD 146176 (6,11-dihydro-8-methoxy-[1]benzothiopyrano(4,3-b)indole), which served as an internal standard. The organic layer was recovered, dried under nitrogen, and reconstituted with methanol/water (4 : 1) and applied to a Zorbax SB-C18 column (250 mm). Elution was achieved with acetonitrile in 25 mM ammonium phosphate.

Plasma lipid and lipoprotein characterization

Plasma lipid concentrations were determined with commercially available enzymatic kits (Wako Chemical Company, Richmond, VA, U.S.A.). Distribution of lipoproteins in plasma obtained after 4, 8 and 12 weeks of feeding the modified diet was resolved by fast protein liquid chromotography (f.p.l.c.) and cholesterol content of fractions was determined enzymatically as described previously (Daugherty & Rateri, 1994). Since this technique did not result in baseline separation of major lipoprotein fractions, the authentic cholesterol distributions were resolved into individual fractions with PeakFit (Jandel Scientific, San Rafael, CA, U.S.A.). These analyses demonstrated that profiles generated by f.p.l.c. could not be described by three normally distributed fractions corresponding to very low-density lipoproteins (VLDL), low density lipoproteins (LDL), and high-density lipoproteins (HDL), but rather are described by four normally distributed fractions.

The additional fraction eluted in a manner consistent with VLDL remnants.

Measurement of antioxidant activity of PD 146176

The ability of PD 146176 to scavenge free radicals was measured with *in vitro* model systems in which linoleate containing SDS micelles or human LDL (density of 1.006 to 1.063 mg ml^{-1}) were used as the lipid substrate for peroxidation, as described by Pryor *et al*. (1993). These systems assess the ability of a given compound to inhibit lipid peroxidation mediated by thermal decomposition of 2,2′-azobis (2-amidinopropane) hydrochloride (ABAP; 5 mM; Wako Chemical Company). In tests involving linoleic acid as the substrate, PD 146176 was tested at a concentration of 2.5 μM. When human LDL was used as a target lipid, the drug was present at concentrations comparable to and exceeding those achieved *in vivo*. Lipid peroxidation was monitored continuously at 234 nm to detect formation of conjugated dienes. The length of time that lipid peroxidation was inhibited (linoleate substrate) or delayed (LDL substrate) was measured in the presence of PD 146176 and compared with inhibition in the presence of α-tocopherol and probucol.

Since it is possible that PD 146176 might accumulate within LDL particles or be metabolized to an active antioxidant *in vivo*, LDL derived from rabbits treated with the drug was tested for its susceptibility to *ex vivo* oxidation. Oxidation was induced by incubating rabbit LDL (density of 1.019 to 1.063 mg ml^{-1}, 100 μg ml^{-1}) in the presence of either ABAP (5 mM) or copper (5 μM). Incubation with copper was performed in the absence of EDTA. The formation of conjugated dienes was monitored continuously at 234 nm as a measure of lipid peroxidation. The lag phase for induction of lipid peroxidation was calculated by the intersection of least squares analysis of the induction and propagation phases of the reaction.

Characterization of atherosclerotic lesions

Rabbits were euthanized by an overdose of sodium pentobarbitone (150 mg kg^{-1}) and exsanguinated via the abdominal aorta. Aortae were removed from the valve to the ileal bifurcation, opened to expose the intima, and photographed with a Polaroid camera. By use of these photographs, the areas of grossly discernible atherosclerosis were manually integrated on a digitizing pad and calculated with SigmaScan (Jandel Scientific). Aortae were visually subdivided into three areas: arch (aortic valve to first intercostal), thoracic aorta (first intercostal to diaphragm area), and abdominal aorta (diaphragm to ileal bifurcation).

Immunocytochemical analyses

Aortic sections from the arch, thoracic and abdominal regions were placed into freshly prepared paraformaldehyde (4% wt./v) and paraffin embedded. Immunocytochemical analyses were performed as described previously (Daugherty & Rateri, 1994) with Vector ABC kits (Vector Laboratories, Burlingame, CA, U.S.A.). Macrophages were detected with RAM-11 and smooth muscle cells by HHF-35 (both obtained from Dako, Carpinatina, CA, U.S.A.) at the indicated dilutions. 15-LO was detected with sheep antibody directed against the rabbit reticulocyte enzyme.

Determination of cholesterol esters and unesterified cholesterol content

Weighed segments of each aortic region (arch, thoracic and abdominal) were extracted by the technique described by Bligh and Dyer (1959). Esterified and unesterified cholesterol contents of aortic tissue were determined by gas chromatography, with the use of 5-α-cholestane as an internal standard (Ishikawa *et al*., 1974).

Measurement of products of 15-lipoxygenase in vivo

Enantiomeric analysis of 13-hydroxyoctadecadienoate (13-HODE), a product of 15-LO, was performed on total lipid extracts of aortic tissue as described by Kühn *et al*. (1994a), with the use of 15(**S**)-hydroxyeicosadienoic acid (BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA, U.S.A.) as an internal standard.

Statistical analyses

Analyses of data for determination of statistical significance were performed with SigmaStat for Windows (Jandel). Data for lipid and lipoprotein concentrations and extent of atherosclerosis that passed the appropriate constraints of equivalent variances and normal distribution were analysed with the Student's *t* test (two-tailed), while other data were analysed by the Mann-Whitney rank-sum test. For studies on rate of oxidation the complete data set was initially analysed by ANOVA, followed by a *post-hoc* Fisher's protected least significant difference test to identify the specific treatments that were significant. A probability value of less than 0.05 was considered indicative of a statistically significant difference. Results are expressed as means $\pm$ s.e.means.

Results

Determination of inhibitory potency, specificity and lack of nonspecific antioxidant properties

Enzyme analyses were performed to determine the nature of the interaction of the drug with 15-LO and to quantitate the inhibitory potency. The rate of formation of products (13-HODE) after incubation with increasing concentrations of linoleic acid was analyzed by r.p.-h.p.l.c. and data are represented by a Lineweaver-Burke plot (Figure 2). These analyses provided a K_m of 2.87 μM and a V_{max} of 546 nmol min^{-1}. This plot also demonstrated that PD 146176 inhibited 15-LO through a mixed noncompetitive mode. Fitting the model for a mixed noncompetitive mode of inhibition implies at least two affinities for the inhibitor-enzyme interaction ranging from 197 nM (K_i) to 579 nM (αK_i).

To demonstrate the specificity of PD 146176 for inhibiting 15-LO, its activity was examined against a panel of closely related enzymes. PD 146176 had no demonstrable effect on

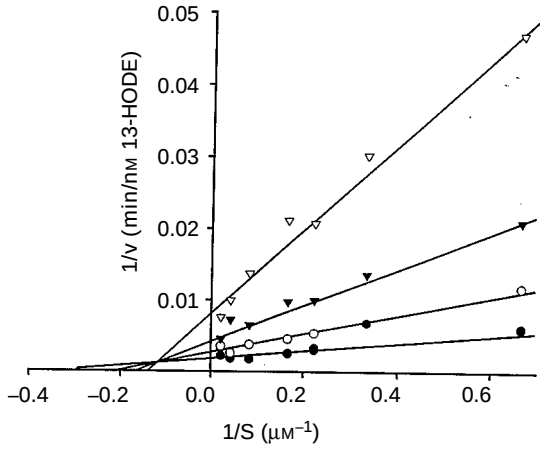

Figure 2 Lineweaver-Burke plot demonstrating the effects of PD 146176 on rabbit 15-lipoxygenase (15-LO) when incubated with arachidonic acid. In the absence of drug, K_m was 2.87 μM and V_{max} was 546 nmol min^{-1}. Incubations were repeated in the presence of indicated concentrations of PD 146176 (μM: 0 (●), 0.28 (○), 0.75 (▼), 2.0 (▽)). Analyses of these data yielded a K_i of 197 nM and demonstrated that the drug was a mixed noncompetitive inhibitor of 15-LO.

either 5- or 12-LO at the highest concentration studied (10 μM). Furthermore, the drug had only slight inhibitory effects on ovine cyclo-oxygenase I and human recombinant cyclo-oxygenase II even at concentrations several orders above that needed to inhibit 15-LO (Table 1). These findings provide the basis for the definition of PD 146176 as a specific inhibitor of 15-LO.

The intrinsic antioxidant properties of the compound were tested by a number of methods. PD 146176 failed to reduce 13-HPODE, the product of linoleate incubated with 15-LO, to the corresponding hydroxy fatty acid. In an assay system designed to detect antioxidant potential with linoleate-containing SDS micelles the drug was inactive, while both α-tocopherol and probucol provided significant inhibitory activity (induction periods of 75 and 104 min, respectively). PD 146176 provided no significant protection of human LDL against ABAP-initiated lipid peroxidation at concentrations achievable *in vivo* (10 and 100 ng ml^{-1}, Table 2). At concentrations (1,000 ng ml^{-1}) considerably above those achieved in plasma ($\approx$45 ng ml^{-1}), the drug produced a significant increase in lag time for LDL oxidation.

General effects of drug administration

To assess the role of 15-LO in atherosclerosis, PD 146176 was administered to rabbits on a high-cholesterol diet for 12 weeks. All rabbits were fed the same mass of food (80 g) that was routinely eaten by both the control and drug-treated groups. The drug was well tolerated and no overt clinical toxicity was observed. Weight gain was equivalent in the control and drug-treated groups (2.88$\pm$0.36 kg and 2.84$\pm$0.21 kg at the end of the study for control and drug treated, respectively). Drug dose was selected based on preliminary experiments to determine the amount necessary to obtain plasma concentrations 2 to 4 h after initiation of feeding consistent with the estimated K_i. Administration of 175 mg kg^{-1}, b.i.d., resulted in plasma concentrations that were consistently close to the estimated K_i throughout the course of the study (Figure 3). In both groups there was a modest drop in haematocrit within 2 weeks of initiating the cholesterol-enriched, high-fat diet (44$\pm$1% to 38$\pm$1% and 43$\pm$2% to 37$\pm$1%, for control and drug-treated group, respectively). However, there were no significant differences in haematocrit between the control and drug-treated groups.

Table 1 Effect of PD 146176 (10 μM) on the enzyme activity of rabbit 15-lipoxygenase (15-LO) and closely related enzymes

Enzyme	*Inhibitory effect*
Rabbit reticulocyte 15-LO	100%
Human platelet 12-LO	0%
Rat basophil leukocyte 5-LO	0%
Ovine cyclo-oxygenase I	26%
Recombinant human cyclo-oxygenase II	16%

Table 2 Effects of exogenously added PD 146176 on ABAP-induced conjugated diene formation in human low density lipoprotein (LDL)

Drug	*Concentration* (ng ml^{-1})	*Lag phase* (min)
None	0	50$\pm$2
PD 146176	10	46$\pm$2
	100	58$\pm$2
	1000	123$\pm$11*

Lag phases were calculated as described in the Methods. Values represent means$\pm$s.e.mean of 10 observations. *Denotes a significant difference, $P<0.01$, when compared to lag phases determined in the absence of drug.

Plasma lipid and lipoprotein concentrations

Plasma concentrations of total cholesterol increased during the first month and then plateaued in both groups (15.89$\pm$2.60 mM for control, 14.72$\pm$1.37 mM for treated). At none of the intervals examined did the drug cause a statistically significant change in plasma cholesterol concentrations, nor was there a significant change in plasma phospholipid concentrations (6.97$\pm$0.82 mM for control, 6.21$\pm$1.21 mM for treated). Nonfasting plasma triglyceride concentrations were increased 0.90$\pm$0.19 mM for control, 2.65$\pm$0.27 mM for treated; $P<0.01$). Lipoprotein-cholesterol distribution was defined by f.p.l.c. and analysed by nonlinear curve-fitting techniques. Lipoprotein analyses were performed 4, 8 and 12 weeks after initiation of the modified diet, with similar results being obtained at each interval. These analyses revealed that the distribution of cholesterol into three major subfractions did not permit an optimal fit of the data; the four fractions required for an optimal fit were designated VLDL, VLDL remnants, LDL and HDL. There were no statistically significant differences in concentrations of VLDL, LDL or HDL between the control and drug-treated group (Table 3). Concentrations of the fraction designated VLDL remnants was significantly increased in the PD 146176-treated group.

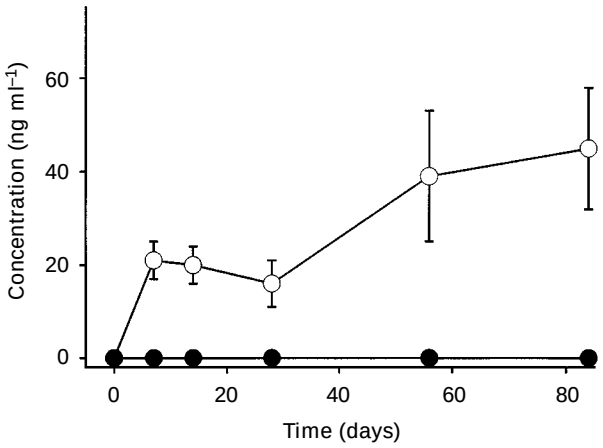

Figure 3 Plasma drug concentrations over the course of the study. Concentrations of drug were determined at the indicated intervals by h.p.l.c. as described in Methods. Data derived from PD 146176-treated animals are represented by (○) and those from control animals by (●). Points represent the mean of determinations from individual animals ($n=10$ per group) and vertical lines represent s.e.mean.

Table 3 Distribution of cholesterol among lipoproteins as percentages of total cholesterol in each fraction

Lipoprotein fraction	*VLDL*	*VLDL remnants*	*LDL*	*HDL*
Control	14$\pm$6	31$\pm$8	37$\pm$6	16$\pm$4
Drug-treated	9$\pm$4[1]	42$\pm$6[2]	36$\pm$6[1]	11$\pm$4[1,3]

Lipoproteins were fractionated by f.p.l.c. and area under the curves was calculated by use of nonlinear fitting parameters (PeakFit) as described in the Methods. Values represent the mean$\pm$s.e.mean from individual animals ($n=7$ per group). [1]Not significantly different from controls. [2]$P=0.02$. [3]Data failed normality testing and therefore were analysed by a nonparametric Mann-Whitney rank-sum test. Other groups were analysed by Student's t test. Data represent analysis of lipoproteins after 12 weeks of feeding the modified diet. No significant differences from these values at 12 weeks were observed in lipoprotein fractions characterized at 4 and 8 weeks. VLDL, very low density lipoprotein; LDL, low density lipoprotein and HDL, high density lipoprotein.

LDL oxidation susceptibility following chronic administration of PD 146176

To determine whether PD 146176 or any of its metabolites exerted any definable nonspecific antioxidant effect *in vivo*, LDL ($d = 1.019 - 1.063$ mg ml^{-1}) was isolated from control and drug-treated rabbits. The lag phases calculated for conjugated diene formation were not significantly different between the two groups for either of the oxidant stresses (Table 4).

Quantification of atherosclerosis

While intimal atherosclerotic lesions were readily discernible in control animals, there was a striking reduction in the extent of lesion coverage in the treated group, with virtually no atherosclerosis observed in any of the 10 animals studied (Figure 4a). In agreement with the data on intimal atherosclerotic lesion coverage, the increase in cholesterol ester content of the aorta was obliterated by the administration of the drug (Figure 4b). Furthermore, unesterified cholesterol content of aortic arch regions was significantly less in drug-treated than in control animals (Figure 4c).

Characteristics of atherosclerotic lesions

Atherosclerotic lesions formed in aortic arches of control animals under these conditions were predominantly macrophage laden, as defined by immunostaining with the monoclonal antibody RAM-11 (Figure 5a). Negligible immunostaining for smooth muscle cells was observed in all lesions of the control animals. In drug-treated animals, no atherosclerotic lesions were present and consequently, no immunostaining was observed for either macrophages or smooth muscle cells in the intima (Figure 5d). Aortic sections were immunostained for the presence of 15-LO with a monospecific polyclonal antibody. 15-LO was present diffusely in macrophages in the luminal aspect of atherosclerotic lesions. In atherosclerotic lesions from control animals endothelial cells exhibited the most intense immunostaining, with more diffuse immunostaining in intimal macrophages (Figure 5b). The immunostained 15-LO was restricted to the endothelium of aortae from PD 146176-treated rabbits; this was the only major cell type present in the intima of these animals (Figure 5e).

Detection of products formed by the action of 15-LO

Chiral analyses were performed to determine whether stereospecific products of 15-LO could be identified in atherosclerotic tissue and whether this was influenced by the presence of the drug. 13-HODE (the linoleate-derived 15-LO product) was detected as a racemic mixture in the control animals. Furthermore, since there were such dramatic differences in the morphological characteristics of aortae from animals in the two groups, chiral analyses were not useful in defining whether or not pathophysiologically significant inhibition of 15-LO was achieved by the drug.

Discussion

This study demonstrated that administration of a highly selective inhibitor of 15-LO has a profound protective effect against the development of atherosclerosis in cholesterol-fed rabbits. This protection occurred in the absence of significant effects on plasma cholesterol concentrations or pronounced changes in the distribution of lipoprotein cholesterol. Furthermore, these changes were produced by a 15-LO inhibitor that exhibited only relatively slight nonspecific antioxidant activity, even at concentrations far above those attained in plasma of treated rabbits.

Much of the interest in a role for 15-LO in the development of atherosclerosis stems from the observations that this enzyme participates in the oxidation of LDL (Harats *et al*., 1995a). However, Sparrow & Olszewski (1992) have pointed out that many of the 15-LO inhibitors used in studies that support a role for the enzyme in atherogenesis are also potent general antioxidants. Many of these compounds inhibit LDL oxidation by free transition metal ions, making interpretation of mechanisms difficult. Consequently, the 15-LO inhibitor used

Table 4 Effects of ABAP and copper on oxidation of low density lipoprotein (LDL) from rabbits administered PD 146176

	Lag time (min)	
Group	*Copper*	*ABAP*
Control	96 ± 18	33 ± 5
Drug-treated	90 ± 6	40 ± 5

LDL ($d = 1.019 - 1.063$ mg ml^{-1}, 100 μg protein) from control rabbits and PD 146176-treated animals was incubated with either copper (5 μM) or ABAP (5 mM) and lag time for oxidation was determined as dscribed in the Methods. Means ± s.e.means are from assay of oxidation susceptibility of LDL from 5 animals. The calculated lag times were not statistically significantly different between the two groups for either of the oxidant stresses.

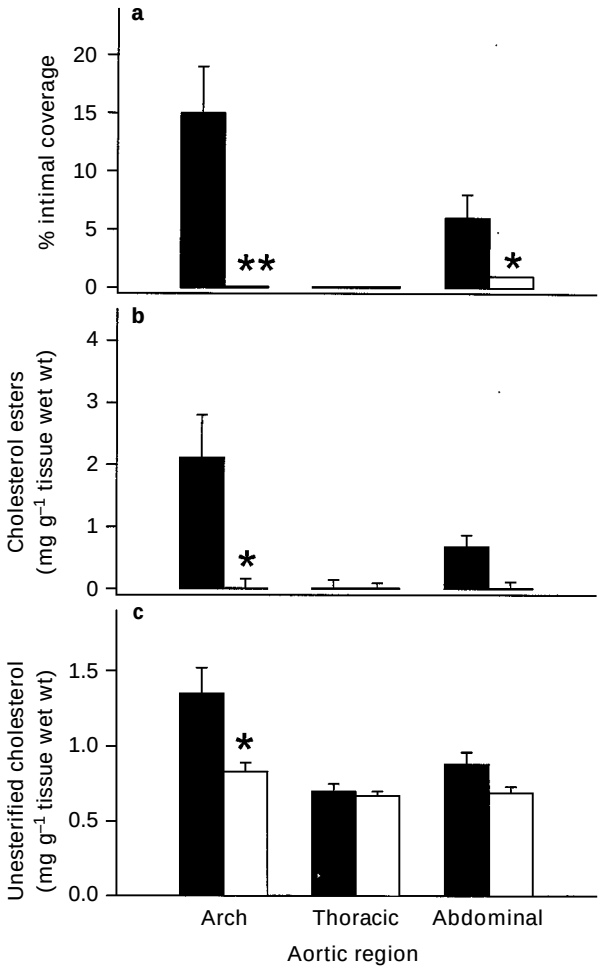

Figure 4 Extent of atherosclerosis and deposition of unesterified cholesterol and cholesterol esters. Data from control animals (solid columns) and PD 146176-treated animals (open columns) for the arch, thoracic and abdominal regions of the aortae were characterized for extent of intimal surface covered by grossly discernible atherosclerotic lesions (a), cholesterol ester content (b) and unesterified cholesterol content (c). Columns represent the mean of observations from individual animals ($n = 10$ per group) and vertical lines represent s.e.mean. Statistically significant differences determined by Mann Whitney rank-sum tests are denoted by $^{*}P < 0.05$, $^{**}P < 0.02$.

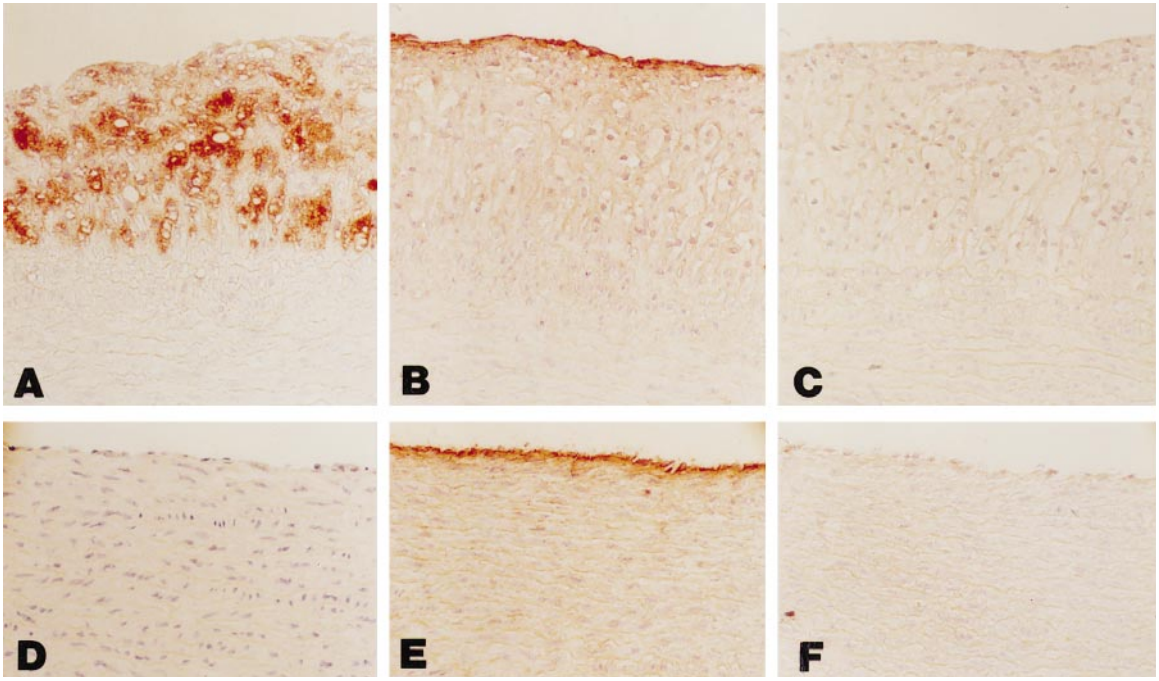

Figure 5 Immunolocalization of macrophages and 15-lipoxygenase (15-LO) in aortic tissue from control and drug-treated animals. Tissue sections from a representative control animal are represented in (a), (b) and (c), whereas sections from a PD 146176-treated animal are represented in (d), (e) and (f). Macrophages were immunostained with RAM-11 (a and d). 15-LO was immunostained with a monospecific sheep antiserum (b and e) and compared with the same dilution of a nonimmune sheep serum (c and f). Magnification for all panels is 400×.

in the present study was carefully tested for nonspecific antioxidant activity. The susceptibility of LDL to oxidation was determined by the rate of conjugated diene formation during incubation with either copper or ABAP since these promote oxidation by different mechanisms (Lynch & Frei, 1993). PD 146176 did not quench free radicals *in vitro* and *ex vivo* systems. Addition of the drug to isolated LDL, even in concentrations over one order of magnitude greater than those found in plasma, had only minor effects on the rate of conjugated diene formation induced by ABAP. However, even this change was very modest compared with that observed after administration of antioxidants such as probucol at concentrations required to attenuate atherogenesis (Demacker *et al*., 1993; Fruebis *et al*., 1994; Kleinveld *et al*., 1994; Morel *et al*., 1994). Furthermore, there was no evidence that LDL isolated from PD 146176-treated animals was protected against either copper or ABAP-induced oxidation, except at very high concentrations of the drug.

These modest antioxidant effects of PD 146176 were in contrast to the pronounced effects of probucol on these same oxidant parameters. Recently, Fruebis *et al.* (1994) demonstrated that a probucol analogue (BM15.0639) was not antiatherogenic despite the fact that it had a marked effect on *ex vivo* oxidation of LDL. It was proposed that protection against oxidation had to reach a certain threshold to provide an antiatherogenic effect. Based on the findings of Fruebis *et al.* (1994) and the lack of significant antioxidant properties of PD 146176, except at concentrations almost two orders above those obtained in plasma, it is unlikely that nonspecific antioxidant characterstics contributed to the antiatherosclerotic properties of the drug.

There are several lines of evidence that 15-LO is present in atherosclerotic lesions. 15-LO mRNA and protein are present in macrophages of such lesions (Ylä-Herttuala *et al*., 1989). While 15-LO is not present in monocytes, incubation of this cell type with interleukin-4 leads to the biosynthesis of the enzyme (Conrad *et al*., 1992; Cornicelli *et al*., 1996). Interleukin-4 is a product of activated lymphocytes that have been detected in human atherosclerotic lesions (Hansson *et al*., 1989). Irrespective of the mode in which enzyme biosynthesis is initiated, atherosclerotic aortae from cholesterol-fed and Watanabe heritable hyperlipidaemic rabbits synthesize products consistent with 15-LO activity *ex vivo* (Simon *et al*., 1989a, b). Furthermore, products of 15-LO have been detected in atherosclerotic lesions from man and cholesterol-fed rabbits (Kühn *et al*., 1994b; Folcik *et al*., 1995). In the present study, we did not detect 15-LO protein throughout the lesion, but rather found it restricted to diffuse immunostaining in macrophages in the abluminal region of lesions. Therefore, there is heterogeniety of 15-LO expression within lesion macrophages. The strongest immunostaining was noted in the aortic endothelium for both groups and, as may be expected, the expression of the protein was not influenced by drug administration. The mechanisms that control 15-LO synthesis *in vivo* have not been fully defined and therefore the reason for the varied content in differing macrophage populations and for the relatively low content of macrophages compared to endothelium are unknown.

In interpreting the results of the present study, we assumed that long-term inhibition of 15-LO activity was achieved. The dose of PD 146176 selected was sufficient to induce plasma concentrations similar to those needed to inhibit the isolated enzyme. The data illustrated in Figure 3 demonstrate that spacing the delivery of the food at 12 h intervals resulted in appropriate plasma concentrations of the drug throughout the 12 week study period. As a potential index of 15-LO inhibition in drug-treated animals, enantiomeric analyses for 13-HODE in total lipid extracts from aortic segments were conducted. However, we detected racemic 13-HODE in extracts from control animals so that chiral analysis of reactions products in the arterial tissue could not be used to define enzyme inhibition at this locus. Previous studies have demonstrated that 15-LO-catalysed oxidation of linoleate esterified in complex lipids and lipoproteins is far less stereospecific than the same reaction in the free fatty acid (Kühn & Brash, 1990). Also, the degree of positional and stereochemical specificity of enzymatic oxidation of esterified linoleate decreases substantially with increasing concentrations of substrate (Kühn *et al*., 1990; 1994a). Furthermore, it seems likely that induction of 15-LO during atherogenesis contributes to the transient generation of a po-

tent metabolite that is not reflected in the measurements of the accumulated mass or enantiomeric composition of the bulk 13-HODE esterified in aortic lipids. Based on the known features of the enzyme-substrate interactions, it is unlikely that chiral analysis of reaction products *in vivo* would provide a quantitive index of 15-LO inhibition. This is borne out by the idiosyncratic presence of isomeric forms of 13-HODE in atherosclerotic lesions from both rabbits (Kühn *et al*., 1994b) and man (Folcik *et al*., 1995).

As mentioned earlier, the initial stimulus for exploration of the involvement of 15-LO in atherogenesis was its putative role in LDL oxidation. Oxidation of LDL has a number of potentially atherogenic sequelae, including stimulation of intracellular macrophage lipid deposition, monocyte and lymphocyte recruitment, monocyte adhesion to endothelium, macrophage stasis and cytotoxicity (Daugherty & Roselaar, 1995). Therefore, one potential mechanism by which the 15-LO inhibitor we studied may have reduced atherogenesis is via the reduction of LDL oxidation at the locus of the arterial wall. In addition, the products of this enzyme promote a number of cellular processes unrelated to lipoprotein oxidation that may influence the atherogenic process (Benz *et al*., 1995). These include changes in expression of oxidant genes by the hydroperoxides generated and the many cellular effects of 15-LO products on endothelial and smooth muscle cells (Setty *et al*., 1987; Graeber *et al*., 1990; Brinkman *et al*., 1991; Khan *et al*., 1995). Therefore, determination of the mechanism underlying the protective effect of PD 146176 against atherogenesis will require elucidation of the many potential contributions of 15-LO to disease development.

In conclusion, we have determined that administration of a highly specific 15-LO inhibitor that lacks appreciable nonspecific antioxidant potency has a profound inhibitory effect on the development of macrophage-rich lesions in cholesterol-fed rabbits. Further evidence to support a role of 15-LO in the development of atherosclerosis will be supplied by the production of mice in which genetic engineering results in increased activity of the enzyme (Harats *et al*., 1995b). These studies, combined with other pharmacological approaches, should reveal the relative importance of 15-LO in the initiation of lesions and the progression through specific stages of atherosclerosis. Furthermore, they will define the specific mechanism through which 15-LO exerts proatherogenic effects.

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References

AUERBACH, B.J., BISGAIER, C.L., WOLLE, J. & SAXENA, U. (1996). Oxidation of low density lipoproteins greatly enhances their association with lipoprotein lipase anchored to endothelial cell matrix. *J. Biol. Chem*., **271,** 1329 – 1335.

BELKNER, J., WIESNER, R., RATHMAN, J., BARNETT, J., SIGAL, E. & KÜHN, H. (1993). Oxygenation of lipoproteins by mammalian lipoxygenases. *Eur. J. Biochem*., **213,** 251 – 261.

BENZ, D.J., MOL, M., EZAKI, M., MORITO, N., ZELAN, I., MIYANOHARA, A., FRIEDMANN, T., PARTHASARATHY, S., STEINBERG, D. & WITZTUM, J.L. (1995). Enhanced levels of lipoperoxides in low density lipoprotein incubated with murine fibroblasts expressing high levels of human 15-lipoxygenase. *J. Biol. Chem*., **270,** 5191 – 5197.

BJÖRKHEM, I., HENRIKSSON-FREYSCHUSS, A., BREUER, O., DICZFALUSY, U., BERGLUND, L. & HENRIKSSON, P. (1991). The antioxidant butylated hydroxytoluene protects against atherosclerosis. *Arterioscler. Thromb*., **11,** 15 – 22.

BLIGH, E.G. & DYER, W.J. (1959). A rapid method of total lipid extraction and characterization. *Can. J. Physiol*., **37,** 911 – 916.

BRINKMAN, H.J.M., BUUL-WORTELBOER, M.F. & VAN MOURIK, J.A. (1991). Selective conversion and esterification of monohydroxyeicosatetranoic acids: Relevance to smooth muscle cell proliferation. *Exp. Cell. Res*., **192,** 87 – 92.

CAREW, T.E., SCHWENKE, D.C. & STEINBERG, D. (1987). Antiatherogenic effect of probucol unrelated to its hypocholesterolemic effect: Evidence that antioxidants in vivo can selectively inhibit low density lipoprotein degradation in macrophage-rich fatty streaks and slow the progression of atherosclerosis in the Watanabe heritable hyperlipidemic rabbit. *Proc. Natl. Acad. Sci. U.S.A*., **84,** 7725 – 7729.

CARTER, G.W., YOUNG, P.R., ALBERT, D.H., BOUSKA, J., DYER, R., BELL, R.L., SUMMERS, J.B. & BROOKS, D.W. (1991). The 5-lipoxygenase inhibition activity of zileuton. *J. Pharmacol. Exp. Ther*., **256,** 929 – 937.

CLELAND, W.W. (1979). Statistical analysis of enzyme kinetic data. *Meth. Enzym*. **63,** 103 – 138.

CONRAD, D.J., KÜHN, H., MULKINS, M., HIGHLAND, E. & SIGAL, E. (1992). Specific inflammatory cytokines regulate the expression of human monocyte 15-lipoxygenase. *Proc. Natl. Acad. Sci. U.S.A*., **89,** 217 – 221.

CORNICELLI, J.A., WELCH, K., AUERBACH, B. & DAUGHERTY, A. (1996). Interleukin 4 is not a necessary mediator of lipoxygenase expression in vivo. *Arterioscler. Thromb. Vasc. Biol*., **16,** 1488 – 1494.

DAUGHERTY, A., ZWEIFEL., B.S., SOBEL, B.E. & SCHONFELD, G. (1988). Isolation of low density lipoprotein from atherosclerotic vascular tissue of Watanabe heritable hyperlipidemic rabbits. *Arteriosclerosis*, **8,** 768 – 777.

DAUGHERTY, A., ZWEIFEL, B.S. & SCHONFELD, G. (1989). Probucol attenuates the development of aortic atherosclerosis in cholesterol-fed rabbits. *Br. J. Pharmacol*., **98,** 612 – 618.

DAUGHERTY, A., ZWEIFEL, B.S. & SCHONFELD, G. (1991). The effects of probucol on the progression of atherosclerosis in mature Watanabe heritable hyperlipidaemic rabbits. *Br. J. Pharmacol*., **103,** 1013 – 1018.

DAUGHERTY, A., DUNN, J.L., RATERI, D.L. & HEINECKE, J.W. (1994). Myeloperoxidase, a catalyst for lipoprotein oxidation, is expressed in human atherosclerotic lesions. *J. Clin. Invest*., **94,** 437 – 444.

DAUGHERTY, A. & RATERI, D.L. (1994). Presence of LDL receptor-related protein /α_2-macroglobulin receptors in macrophages of atherosclerotic lesions from cholesterol-fed New Zealand and heterozygous Watanabe heritable hyperlipidemic rabbits. *Arterioscler. Thromb*., **14,** 2017 – 2024.

DAUGHERTY, A. & ROSELAAR, S.E. (1995). Lipoprotein oxidation as a mediator of atherogenesis: Insights from pharmacological studies. *Cardiovasc. Res*., **29,** 297 – 311.

DEMACKER, P.N.M., HIJMANS, A.G.M., KLEINVELD, H.A. & STALENHOEF, A.F.H. (1993). Indications for the presence of circulating peroxidized low density lipoproteins in WHHL rabbits treated with antioxidants. *Atherosclerosis*, **102,** 69 – 77.

EHRENWALD, E., CHISOLM, G.M. & FOX, P.L. (1994). Intact human ceruloplasmin oxidatively modifies low density lipoprotein. *J. Clin. Invest*., **93,** 1493 – 1501.

FOLCIK, V.A., NIVARARISTY, R.A., KRAJEWSKI, L.P. & CATHCART, M.K. (1995). Lipoxygenase contributes to the oxidation of lipids in human atherosclerotic plaques. *J. Clin. Invest*., **96,** 504 – 510.

FORD-HUTCHINSON, A.W. (1991). Arachidonate 15-lipoxygenase - characteristics and potential biological significance. *Eicosanoids*, **4,** 65 – 74.

FRUEBIS, J., STEINBERG, D., DRESEL, H.A. & CAREW, T.E. (1994). A comparison of the antiatherogenic effects of probucol and of a structural analogue of probucol in low density lipoprotein receptor-deficient rabbits. *J. Clin. Invest*., **94,** 392 – 398.

GRAEBER, J.E., GLASER, B.M., SETTY, B.N.Y., JERDAN, J.A., WALENGA, R.W. & STUART, M.J. (1990). 15-Hydroxyeicosatetraenoic acid stimulates migration of human retinal endothelium in vitro and neovascularization in vivo. *Prostaglandins*, **39,** 665–673.

HABERLAND, M.E., FONG, D. & CHENG, L. (1988). Malondialdehyde-altered protein occurs in atheroma of Watanabe heritable hyperlipidemic rabbits. *Science*, **241,** 215–218.

HANSSON, G.K., HOLM, J. & JONASSON, L. (1989). Detection of activated T lymphocytes in the human atherosclerotic plaque. *Am. J. Pathol.*, **135,** 169–175.

HARATS, D., KURIHARA, H., BELLONI, P., OAKLEY, H., ZIOBER, A., ACKLEY, D., CAIN, G., KURIHARA, Y., LAWN, R. & SIGAL, E. (1995a). Targeting gene expression to the vascular wall in transgenic mice using the murine preproendothelin-1 promoter. *J. Clin. Invest.*, **95,** 1335–1344.

HARATS, D., MULKINS, M.A. & SIGAL, E. (1995b). A possible role for 15-lipoxygenase in atherogenesis. *Trends Cardiovasc. Med.*, **5,** 29–36.

HEINECKE, J.W., ROSEN, H. & CHAIT, A. (1984). Iron and copper promote modification of low density lipoprotein by human arterial smooth muscle cells in culture. *J. Clin. Invest.*, **74,** 1890–1894.

HLA, T. & NEILSON, K. (1992). Human cyclooxygenase-2 cDNA. *Proc. Natl. Acad. Sci. U.S.A.*, **89,** 7384–7388.

ISHIKAWA, T.T., MACGEE, J., MORRISON, J.A. & GLUECK, C.J. (1974). Quantitative analysis of cholesterol in 5 and 20 μl of plasma. *J. Lipid Res.*, **15,** 286–291.

KHAN, B.V., PARTHASARATHY, S.S., ALEXANDER, R.W. & MEDFORD, R.M. (1995). Modified low density lipoprotein and its constituents augment cytokine-activated vascular cell adhesion molecule-1 gene expression in human vascular endothelial cells. *J. Clin. Invest.*, **95,** 1262–1270.

KITA, T., NAGANO, Y., YOKODE, M., ISHI, K., KUME, N., OOSHIMA, A., YOSHIDA, H & KAWAI, C. (1987). Probucol prevents the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbit, an animal model for familial hypercholesterolemia. *Proc. Natl. Acad. Sci. U.S.A.*, **84,** 5928–5931.

KLEINVELD, H.A., DEMACKER, P.N.M. & STALENHOEF, A.F.H. (1994). Comparative study on the effect of low-dose vitamin E and probucol on the susceptibility of LDL to oxidation and the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbits. *Arterioscler. Thromb.*, **14,** 1386–1391.

KÜHN, H. & BRASH, A.R. (1990). Occurrence of lipoxygenase products in membranes of rabbit reticulocytes. *J. Biol. Chem.*, **265,** 1454–1458.

KÜHN, H., BELKNER, R., WIESNER, R. & BRASH, A.R. (1990). Oxygenation of biological membranes by the reticulocyte lipoxygenase. *J. Biol. Chem.*, **265,** 18351–18361.

KÜHN, H., BELKNER, J., SUZUKI, H. & YAMAMOTO, S. (1994a). Oxidative modification of human lipoproteins by lipoxygenases of different positional specificities. *J. Lipid Res.*, **35,** 1749–1759.

KÜHN H., BELKNER, J., ZAISS, S., FAHRENKLEMPER, T. & WOHLFEIL, S. (1994b). Involvement of 15-lipoxygenase in early stages of atherogenesis. *J. Exp. Med.*, **179,** 1903–1911.

LYNCH, S.M. & FREI, B. (1993). Mechanisms of copper-dependent and iron-dependent oxidative modification of human low density lipoprotein. *J. Lipid Res.*, **34,** 1745–1753.

MCNALLY, A.K., CHISOLM, G.M., MOREL, D.W. & CATHCART, M.K. (1990). Activated human monocytes oxidize low density lipoprotein by a lipoxygenase dependent pathway. *J. Immunol.*, **145,** 254–259.

MOREL, D.W., DE LA LLERA-MOYA, M. & FRIDAY, K.E. (1994). Treatment of cholesterol-fed rabbits with dietary vitamins E and C inhibits lipoprotein oxidation but not development of atherosclerosis. *J. Nutr.*, **124,** 2123–2130.

PALINSKI, W., ROSENFELD, M.E., YLÄ-HERTTUALA, S., GURTNER, G.C., SOCHER, S.S., BUTLER, S.W., PARTHASARATHY, S., CAREW, T.E., STEINBERG, D. & WITZTUM, J.L. (1989). Low density lipoprotein undergoes oxidative modification in vivo. *Proc. Natl. Acad. Sci. U.S.A.*, **86,** 1372–1376.

PARTHASARATHY, S., WIELAND, E. & STEINBERG, D. (1989). A role for endothelial cell lipoxygenase in the oxidative modification of low density lipoprotein. *Proc. Natl. Acad. Sci. U.S.A.*, **86,** 1046–1050.

PRYOR, W.A., CORNICELLI, J.A., DEVALL, L.J., TAIT, B., TRIVEDI, B.K., WITIAK, D.T. & WU, M. (1993). A rapid screening test to determine the antioxidant potencies of natural and synthetic antioxidants. *J. Org. Chem.*, **58,** 3521–3532.

RANKIN, S.M., PARTHASARATHY, S., & STEINBERG, D. (1991). Evidence for a dominant role of lipoxygenase(s) in the oxidation of LDL by mouse peritoneal macrophages. *J. Lipid Res.*, **32,** 449–456.

ROSENFELD, M.E., PALINSKI, W., YLÄ-HERTTUALA, S., BUTLER, S. & WITZTUM, J.L. (1990). Distribution of oxidation specific lipid-protein adducts and apolipoprotein-B in atherosclerotic lesions of varying severity from WHHL rabbits. *Arteriosclerosis*, **10,** 336–349.

SEGEL, I.H. (1975). Rapid equilibrium, partial and mixed-type inhibitions. In *Enzyme Kinetics*: Behavior and Analysis of Rapid Equilibrium and Steady State Enzyme Systems. ed. Segel, I.H. pp. 170–202. New York: John Wiley & Sons.

SETTY, B.N., GRAEBER, J.E. & STUART, M.J. (1987). Mitogenic effect of 15- and 12-hydroxyeicosatetraenoic acid on endothelial cells may be mediated via diacylglycerol kinase inhibition. *J. Biol. Chem.*, **262,** 17613–17622.

SIMON, T.C., MAKHEJA, A.N. & BAILEY, J.M. (1989a). Formation of 15-hydroxyeicosatetraenoic acid (15-HETE) as the predominant eicosanoid in aortas from Watanabe Heritable Hyperlipidemic and cholesterol-fed rabbits. *Atherosclerosis*, **75,** 31–38.

SIMON, T.C., MAKHEJA, A.N. & BAILEY, J.M. (1989b). The induced lipoxygenase in atherosclerotic aorta converts linoleic acid to the platelet chemorepellant factor 13-HODE. *Thromb. Res.*, **55,** 171–178.

SLOANE, D.L., BROWNER, M.F., DAUTER, Z., WILSON, K., FLETTERICK, R.J. & SIGAL, E. (1990). Purification and crystallization of 15-lipoxygenase from rabbit reticulocytes. *Biochem. Biophys. Res. Commun.*, **173,** 507–513.

SPARROW, C.P., PARTHASARATHY, S. & STEINBERG, D. (1988). Enzymatic modification of low density lipoprotein by purified lipoxygenase plus phospholipase A_2 mimics cell-mediated oxidative modification. *J. Lipid Res.*, **29,** 745–753.

SPARROW, C.P. DOEBBER, T.W., OLSZEWSKI, J., WU, M.S., VENTRE, J., STEVENS, K.A. & CHAO, Y.S. (1992). Low density lipoprotein is protected from oxidation and the progression of atherosclerosis is slowed in cholesterol-fed rabbits by the antioxidant N,N′-diphenyl-phenylenediamine. *J. Clin. Invest.*, **89,** 1885–1891.

SPARROW, C.P. & OLSZEWSKI, J. (1992). Cellular oxidative modification of low density lipoprotein does not require lipoxygenases. *Proc. Natl. Acad. Sci. U.S.A.*, **89,** 128–131.

STEINBERG, D., PARTHASARATHY, S., CAREW, T.E., KHOO, J.C. & WITZTUM, J.L. (1989). Beyond cholesterol. Modifications of low density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.*, **320,** 915–924.

YLÄ-HERTTUALA, S., PALINSKI, W., ROSENFELD, M.E., PARTHASARATHY, S., CAREW, T.E., BUTLER, S., WITZTUM, J.L. & STEINBERG, D. (1989). Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. *J. Clin. Invest.*, **84,** 1086–1095.

YLÄ-HERTTUALA, S., ROSENFELD, M.E., PARTHASARATHY, S., GLASS, C.K., SIGAL, E., WITZTUM, J.L. & STEINBERG, D. (1990). Colocalization of 15-lipoxygenase messenger RNA and protein with epitopes of oxidized low density lipoprotein in macrophage-rich areas of atherosclerotic lesions. *Proc. Natl. Acad. Sci. U.S.A.*, **87,** 6959–6963.

YLÄ-HERTTUALA, S., ROSENFELD, M.E., PARTHASARATHY, S., SIGAL, E., SARKIOJA, T., WITZTUM, J.L. & STEINBERG, D. (1991). Gene expression in macrophage-rich human atherosclerotic lesions - 15-lipoxygenase and acetyl low density lipoprotein receptor messenger RNA colocalize with oxidation specific lipid-protein adducts. *J. Clin. Invest.*, **87,** 1146–1152.

YLÄ-HERTTUALA, S., LUOMA, J., VIITA, H., HILTUNEN, T., SISTO, T. & NIKKARI, T. (1995). Transfer of 15-lipoxygenase gene into rabbit iliac arteries results in the appearance of oxidation-specific lipid-protein adducts characteristic of oxidized low density lipoprotein. *J. Clin. Invest.*, **95,** 2692–2698.

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