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CORRELATION BETWEEN OXIDATION OF LOW DENSITY LIPOPROTEINS AND PROSTACYCLIN SYNTHESIS IN CULTURED SMOOTH MUSCLE CELLS

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Abstract—The effect of antioxidants on the oxidation of low density lipoproteins in relation to prostacyclin synthesis was investigated in the prescence of rabbit smooth muscle cells (SMC) and Fe-containing culture medium. The lipid peroxidation of low density lipoproteins (LDL) assayed as thiobarbituric acid reactive substances was increased from 0.5 to 1.4 nmol malondialdehyde/mL by the presence of smooth muscle cells. Two potent antioxidants, nordihydroguairetic acid (NDGA) and butylated hydroxytoluene (BHT), inhibited lipoprotein oxidation by IC_{50} values of 0.2 and 0.8 μM, respectively. Inhibition of lipoprotein oxidation was associated with an increased prostacyclin synthesis by the SMC, the effect being more pronounced with nordihydroguairetic acid than with butylated hydroxytoluene. The stable metabolite of the lipid hydroperoxide, 15-hydroxyeicosatetraenoic acid (15-HETE), formed in the 15-lipoxygenase pathway was measured following antioxidant treatment and found to be eliminated or greatly reduced by both antioxidants. The results presented show that lipid hydroperoxides, formed as a consequence of lipoprotein oxidation and promoted by the smooth muscle cells through a lipoxygenase reaction, may regulate prostacyclin synthase, a process which may be influenced by the addition of antioxidants.

High levels of lipid peroxides generated from the oxidation of polyunsaturated fatty acids in membrane phospholipids and a subsequent formation of lipid radicals may be associated with atherosclerosis and cellular dysfunction [1, 2]. This is supported by experiments in which lipid peroxidation caused oxidative modification of low density lipoproteins (LDL) in the presence of macrophages, smooth muscle cells and endothelial cells [3–5].

Lipid peroxidation appears to be closely related to prostaglandin biosynthesis by the regulation of cyclooxygenase activity by prostaglandin peroxidase. In this pathway, cyclooxygenase is activated by low levels of peroxides and inactivated by high levels of peroxides [6, 7]. It is known that hydroperoxidase utilizes the peroxide substrates prostaglandin-G_2 (PGG_2) in a peroxidase reaction which generates both prostaglandin-H_2 (PGH_2) and free radicals. PGH_2 then forms physiologically important prostaglandins, such as thromboxane A_2 (TXA_2) and prostacyclin (PGI_2). Several studies suggest that the balance between these two latter products, and thus an altered thrombotic status, may be influenced by lipid peroxidation [8, 9]. A second regulator of PGI_2 is the level of lipid hydroperoxides formed in the lipoxygenase pathways [10], where it has been shown that relatively low concentrations (3–15 μM) of lipid hydroperoxides inactivate prostacyclin synthase [11].

Inhibition of lipoxygenases may exert their action via antioxidant mechanisms by an inhibition of nonenzymatic lipid peroxidation processes, but more specific antioxidants are required to inhibit specific lipoxygenases [12, 13]. One example is nordihydroguairetic acid (NDGA), which may be considered as a lipoxygenase inhibitor, *in vitro* [13] which has IC_{50} values for the inhibition of 5-lipoxygenase in A12387-activated neutrophils of 0.1 μM and for the inhibition of 12-lipoxygenase and cyclooxygenase in AA-activated platelets of 10 and 6.3 μM, respectively [14].

In the present investigation, with the use of antioxidants having lipoxygenase inhibiting-properties, the inhibition of smooth muscle cell-catalysed peroxidative modification of LDL occurred together with the stimulation of PGI_2 synthesis. The less specific antioxidant, butylated hydroxytoluene (BHT) as a more general scavenger, also inhibited peroxidation, but caused less pronounced stimulation of PGI_2 synthesis. We suggest that the balance between lipid peroxidation and PGI_2 synthesis may be influenced therapeutically with suitable antioxidants.

MATERIALS AND METHODS

Materials. F12 medium, RPMI medium and cell culture plates (Linbro) were obtained from Flow Laboratories (Herts, U.K.). 15-HETE were obtained from the Caymen Chemical Co. (Ann Arbor, U.S.A.). All other compounds were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.).

Lipoproteins. LDL was prepared from human plasma from normolipidaemic donors in EDTA-containing Vacutainer tubes by zonal ultracentrifugation [15]. The LDL fraction dissolved in NaCl and KBr was extensively dialysed (more than 72 hr), a process which included four changes of PBS buffer (NaCl 274 mM, KCl 27 mM, KH_2PO_4

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15 mM, Na_2HPO_4 16 mM, $MgCl_2$ 0.5 mM, $CaCl_2$ 1 mM, EDTA 0.03 mM, pH 7.2). The LDL was dialysed with PBS buffer without EDTA 24 hr prior to each experiment and the amount of LDL added to the culture medium was expressed as μg LDL protein/mL.

Preparation of cells. The rabbit aorta from arch to bifurcation of the femoral artery was removed under aseptic conditions, thoroughly dissected free of fat and connective tissue and cut into small pieces. The intima-media was dispersed in collagenase (Type 1, 2 mg/mL) and elastase (2 mg/mL) for 1 hr each, and with elastase and collagenase (final concentration 2 mg/mL) for a further 2–3 hr. The collagenase and elastase were dissolved in F12 medium supplemented with glutamine (1%), ascorbic acid (0.05 mg/mL) and gentamycin (0.05 mg/mL). The dispersed cells were washed three times in the medium as supplemented according to the above, with the last washing also containing 10% fetal calf serum (FCS, not heat inactivated). The cells were seeded in cell culture flasks at a concentration of 1×10^5 cells/cm^2. They were then grown to confluence and subcultured by using trypsin (0.05%) and EDTA (0.2 mg/mL). The cells were grown in flat bottomed, multi-well culture plates for approximately 24 hr. FCS, gentamycin and ascorbic acid were removed during the experiment and only F12 medium supplemented with glutamine was used. Each experiment started by adding medium containing LDL or medium containing LDL with antoxidant.

Assays. Kinetic experiments for the determination of LDL oxidation were performed in the presence of 200 μg LDL protein/mL. The effects of antioxidants (NDGA and BHT) were tested at concentrations ranging from 0.01 to 10 μM. Both antioxidants were dissolved in absolute ethanol and further dissolved in medium, giving a final concentration of 0.1% ethanol in each assay.

Analysis. Lipid peroxidation was assayed with thiobarbituric acid and indicated as "thiobarbituric acid reactive substances" (TBARS), a determination of the stable complex formed between malondialdehyde (MDA) and thiobarbituric acid [16]. Protein was determined by using the method of Lowry *et al.* [17]. The radioimmunoassay for the estimation of 6-keto$PGF_{1\alpha}$ was used for determining PGI_2 synthesis. The metabolized product of the lipid hydroperoxide formed in the 15-lipoxygenase pathway, 15-hydroxyeicosatetraenoic acid (15-HETE), was analysed by high performance liquid chromatography with a nucleosil C_{18} (5 μm, 150 × 4.6 mm) column using methanol/water/acetic acid (75/25/0.1%, by vol.) as eluant. The column was monitored and 15-HETE was detected at 235 nm and washed after each sample with methanol/water/acetic acid (90/10/0.1%, by vol.). The detected peaks from SMC were compared with 15-HETE obtained from soy bean lipoxygenase and arachidonic acid, purified by HPLC. Retention times were compared with those of authentic 15-HETE.

Calculations. Cell-induced oxidation of LDL was determined by subtracting the LDL oxidation products observed in cell-free dishes from the total LDL oxidation found in the presence of cells. The IC_{50} values of the antioxidants were determined from the inhibition curves of cell-induced modification of LDL. Results are presented as mean values ±SD.

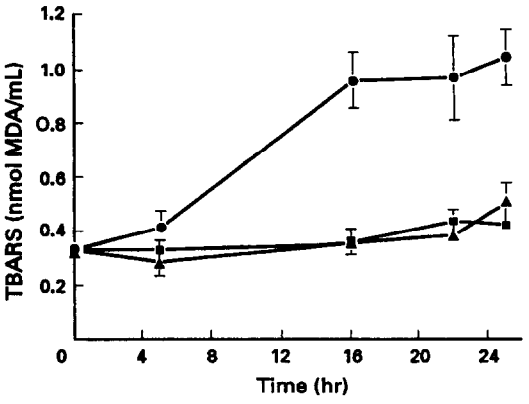

Fig. 1. Time-dependent increase in TBARS following incubation of LDL (200 μg LDL/mg protein) with SMC. TBARS in control cells (●) and in the presence of NDGA, 1.0 μM (▲) or BHT, 10.0 μM (■) are shown. Mean values ± SD, N = 3.

RESULTS

Incubation of human LDL (200 μg LDL/mL) for 24 hr in Ham's F12 medium with 3 μM Fe^{2+} increased the TBARS of the lipoprotein from 0.26 ± 0.10, N = 3, nmol MDA/mL to 0.5 ± 0.1, N = 3, nmol MDA/mL in the absence of cells. In the presence of SMC, the TBARS further increased to 1.02 ± 0.04, N = 3 nmol MDA/mL. The values for 6-keto$PGF_{1\alpha}$ were similar before, 1190 ± 245 pg/mL, N = 3, and after, 1475 ± 524 pg/mL, N = 9, incubation with LDL for 24 hr in Ham's media with 3 μM Fe. A complete inhibition of lipid peroxidation as measured by TBARS was achieved with 1 μM NDGA and 10 μM BHT (Fig. 1). As shown in Figs 2a and 3a, the antioxidants NDGA and BHT both reduced the formation of LDL oxidation products. This effect was also seen in the absence of SMC, suggesting a partial prevention of Fe-mediated TBARS formation in LDL. IC_{50} values for inhibition of lipid peroxidation for NDGA were approximately 0.24 ± 0.13 μM, N = 4 and 0.75 ± 0.1 μM, N = 3 for BHT. In order to show that iron was the major source for the peroxidation process, Fe-containing F12 medium was exchanged for Fe-deficient RPMI medium, which almost completely inhibited lipid peroxidation. A change back to F12 medium increased TBARS formation (data not shown).

Antioxidants were administered to study their possible effect on PGI_2 synthesis. This was followed by a parallel determination of lipid peroxidation and 6-keto$PGF_{1\alpha}$ (Figs 2 and 3a,b). In the presence of 0.1 μM NDGA, a concentration sufficiently high to reduce TBARS, PGI_2 synthesis increased 2–3-fold (Fig. 2b) with a less pronounced effect in the presence of BHT (Fig. 3b). When mean values from four experiments were compared the formation of 6-keto$PGF_{1\alpha}$ increased from 1605 ± 335 to 3296 ± 570 pg/mL ($P < 0.05$) in the presence of 0.1

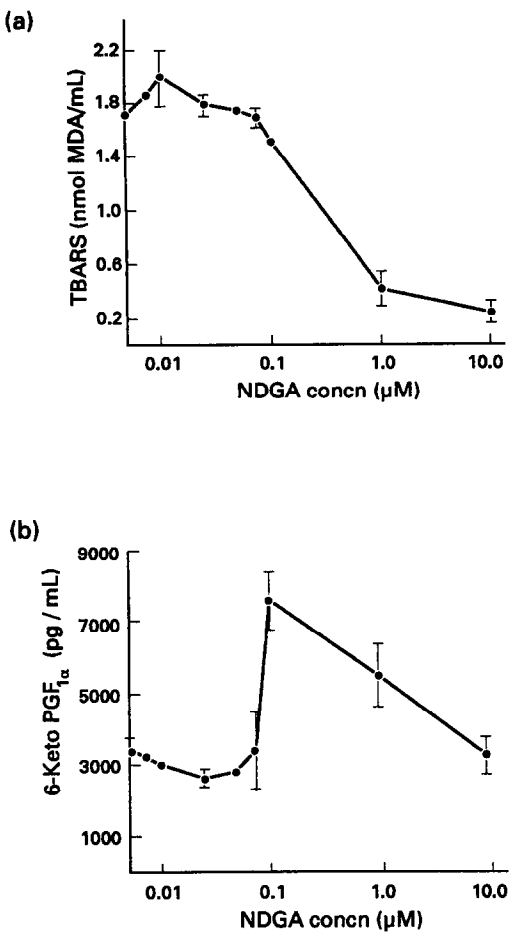

Fig. 2. Effect of NDGA on formation of TBARS (a) and on formation of 6-ketoPGF$_{1\alpha}$ (b) after 24 hr incubation with LDL (200 μg LDL/mg protein). Values are taken from one representative experiment in which each value represents triplicate determinations. Mean values ± SD are shown.

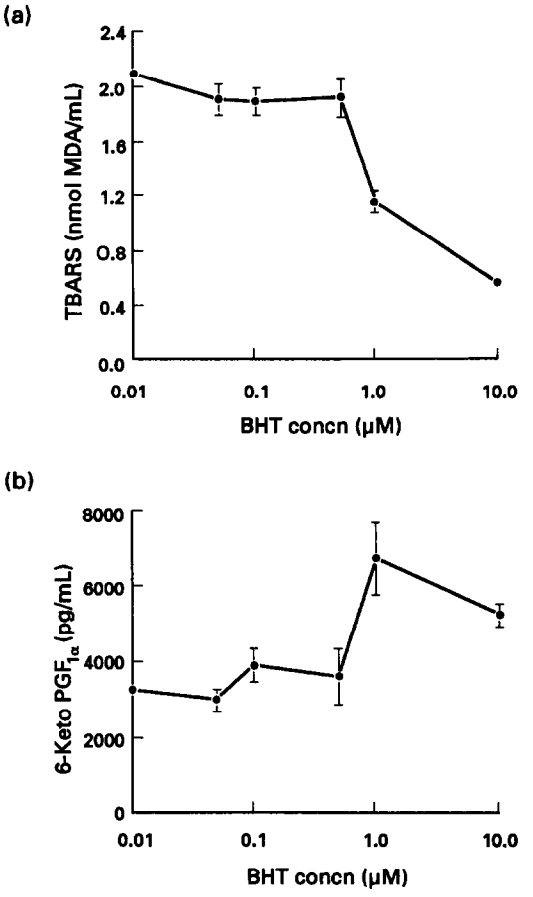

Fig. 3. Effect of BHT on formation of TBARS (a) and on formation of 6-ketoPGF$_{1\alpha}$ (b) after 24 hr incubation with LDL (200 μg LDL/mg protein). Values are taken from one representative experiment in which each value represents triplicate determination. Mean values ± SD are shown.

μM NDGA and to 3098 ± 562 pg/mL ($P < 0.05$) in the presence of 1.0 μM NDGA. Regarding the effect of BHT, mean values from four experiments showed an increase in 6-ketoPGF$_{1\alpha}$ from 1605 ± 335 to 2291 ± 419 pg/mL (not significant) in the presence of 1.0 μM BHT and to 1886 ± 289 pg/mL (not significant) in the presence of 10.0 μM BHT. Both 0.1 μM NDGA and 1.0 μM BHT were below the concentrations required for a complete inhibition of lipid peroxidation, which was approximately 3 μM for NDGA and 10 μM for BHT. In high concentrations, both antioxidants showed an inhibitory effect on PGI_2 synthesis, probably owing to a reduction in cyclooxygenase activity (Figs 2b and 3b). The cell protein was not changed by any of the concentrations of antioxidants used. The possibility that PGI_2 generation was the source of MDA was tested by the addition of phorbol 12-myristate 13-acetate (PMA). In the presence of PMA, the formation of 6-ketoPGF$_{1\alpha}$ was increased from 1811 ± 386 to 6658 ± 1564 pg/mL without affecting the TBARS.

The amount of 15-HETE was determined to explore the possibility that inhibition of lipid hydroperoxide formation, derived from the lipoxygenase pathway, was responsible for the increase in PGI_2 synthesis. A clearly detectable peak representing 15-HETE was observed in the absence of antioxidants. This was completely abolished in the presence of 1 μM NDGA and largely reduced in the presence of 1 μM BHT (Fig. 4). In the absence of LDL, the Ham medium gave very low lipid peroxidation values, and almost no 15-HETE was detected (Fig. 4).

DISCUSSION

This study showed that NDGA is a better inhibitor of LDL oxidation than BHT. Both compounds are lipid-soluble, allowing possibilities for scavenging radicals in both the aqueous region and the hydrophobic lipid region of the cell membrane. Radicals generated from redox reactions involving transition metals or from enzyme catalyses are chiefly organic free radical species. These are generated

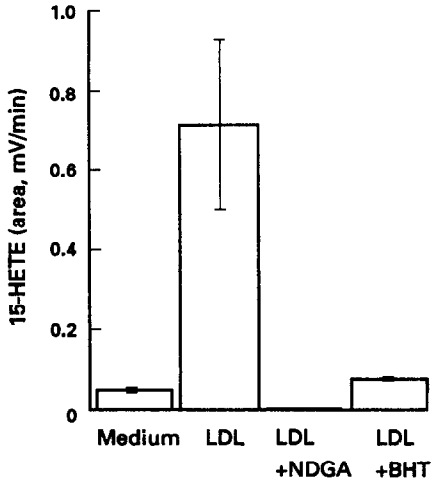

Fig. 4. Effect of NDGA and BHT on 15-HETE production. The first bar represents SMC cultured for 24 hr, the second in the presence of LDL, the third LDL and NDGA (1 μM) and the fourth LDL and BHT (1 μM). 15-HETE was determined in a reversed phase HPLC system, see methods. Peak content represents the area under the 15-HETE curve.

from a reaction between hydroxyl radicals and membrane phospholipid polyunsaturated fatty acids, generating lipid peroxidation and other more complex free radical peroxides. Such radicals are primarily scavenged by chain breaking antioxidants.

The balance between the production and removal of lipid peroxides has been discussed by Hemler *et al.* [6], who showed that a variety of lipid peroxides can trigger and accelerate the activity of cyclooxygenase. It has also been shown in greater detail that high tissue concentrations of lipid peroxides not only inactivate prostacyclin synthesis [18] but also regulate cyclooxygenase activity [19], and that concentrations of 3–15 μM of lipid hydroperoxides inactivate PGH synthase [11]. In vascular segments, lipid peroxide concentrations of 1 μM reduced the formation of PGI_2 from PGH_2 by approximately 50% [20, 21] and, in endothelial cells, 12-HETE reduces prostacyclin production [22]. The present study demonstrates that the antioxidative effect of NDGA and BHT appears to be partly related to an inhibition of lipoxygenase activity, reducing the formation of 15-HETE. In our studies, LDL oxidation for 24 hr was not associated with a reduced prostacyclin formation which may be an indicator of optimal cyclooxygenase activity. This finding may be crucial for the increased formation of PGI_2 in the presence of antioxidants, as reduced cyclooxygenase activity should reduce the possibility for picking up an interaction with the PGI_2 synthase. As relates tc the levels of lipid peroxides, it is quite possible that lipid peroxides, as measured with TBARS, are much lower than the total concentration of lipid peroxides, and that lipid peroxidation in cell cultures is higher than that found in isolated organs. Previous studies using aortic slices have shown that an increased production of lipid peroxides caused by the oxidation of LDL is accompanied by a decrease in PGI_2 formation [19]. These findings were suggested as an effect dependent upon the inactivation of arterial PGI_2 synthase by lipid hydroperoxides. The proposal that the atherogenic properties of LDL are linked to an inactivation of PGI_2 synthase owing to high levels of tissue lipid peroxides may be supported by our own results, and the increased formation of 15-HETE found in both hyperlipidemic and cholesterol-fed rabbits may point to the connection between high levels of LDL and increased plaque formation [23].

The present study shows a reduction in the formation of 15-HETE in concentrations of NDGA and BHT which increased the formation of PGI_2. As has been found in other studies, the increased formation of PGI_2 found in the presence of NDGA and BHT may reflect an activation of PGI_2 synthase owing to an inhibition of 15-lipoxygenase activity. However, this may also be dependent upon a general lowering of the tissue lipid peroxide concentration.

The present results may be taken as evidence for a coupling between the inhibition of lipid hydroperoxide formation and an increase in PGI_2 synthesis, probably associated with an inhibition of lipoxygenase activity. It is therefore suggested that antioxidants/lipoxygenase inhibitors may be beneficial in cases of vascular damage via a reduction of both the nonenzymatic and the enzymatic lipid peroxidation.

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REFERENCES

1. Parthasarathy S, Fong LG and Steinberg D, Oxidative modifications of low density lipoprotein and atherosclerosis: concept and consequences. In: *Lipid Peroxidation in Biological Systems* (Ed. Sevarian A), pp 225–235. American Oil Chemist Society, 1988.
2. Henning B and Chow CK, Lipid peroxidation and endothelial cell injury: implications in atherosclerosis. *Free Rad Biol Med* **4**: 99–106, 1988.
3. Henrikssen T, Mahoney EM and Steinberg D, Enhanced macrophage degradation of low density lipoprotein previously incubated with cultured endothelial cells: recognition by receptors for acetylated low density lipoproteins. *Proc Natl Acad Sci USA* **78**: 6499–6503, 1981.
4. Steinbrecher Urs P, Witzum JL, Parthasarathy S and Steinberg D, Decrease in reactive amino groups during oxidation or endothelial cell modification of LDL. *Arteriosclerosis* **7**: 135–143, 1987.
5. Morel DW, DiCorleto PE and Chisholm GM, Endothelial and smooth muscle cells alter low density lipoprotein *in vivo* by free radical oxidation. *Arteriosclerosis* **4**: 357–364, 1984.
6. Hemler M, Cook H and Lands W, Prostaglandin biosynthesis can be triggered by lipid peroxides. *Archs Biochem Biophys* **193**: 340–345, 1979.
7. Lands WEM, Interaction of lipid hydroperoxides with eicosanoid biosynthesis. *J Free Radicals Biol Med* **1**: 97–101, 1985.
8. Gryglewski R, Dembinska-Kiec Muda A and Gryglewska T, Prostacyclin and thromboxane A_2 biosynthesis capacities of heart, arteries and platelets at various stages of experimental atherosclerosis in rabbits. *Atherosclerosis* **31**: 385–394, 1978.

9. Gryglewski RJ, Prostacyclin and atherosclerosis. *TIPS* **1**: 164–166, 1980.
10. Davies JA and Menys VC, Effect of dazoxiben on platelet-vessel wall interaction. *Br J Clin Pharmacol* **15**: 45S–46S, 1983.
11. Siegel MI, McConell RI, Abrahams SL, Porter NA and Cuatrecasus P, Regulation of arachidonic metabolism via lipoxygenase and cyclooxygenase by 12-HPETE, the product of human platelet lipoxygenase. *Biochem Biophys Res Commun* **89**: 1273–1280, 1979.
12. Thody VE, Buckle DR and Foster KA, Studies on the antioxidant activity of 5-lipoxygenase inhibitors. *Biochem Soc Trans 621st Meeting* 416–417, 1987.
13. Moore PK, Effect of drugs in arachidonic acid metabolism. In: *Prostanoids: Pharmacological, Physiological and Clinical Relevance* (Ed. Moore PK), pp. 41–78. Cambridge University Press, Cambridge, 1984.
14. Falknäs A-K, Palmer M, Ek B and Rydström J, Effect of radical scavengers on superoxide and eicosanoid production in neutrophils. *633rd Biochem Soc Meeting, London,* 19–21 *Dec*, 1989. Abstract no. P24.
15. Patsch JR and Patsch W, Zonal ultracentrifugation. *Methods Enzymol* **129B**: 3–26, 1986.
16. Yagi K, A simple fluorometric assay for lipoperoxide in blood plasma. *Biochem Med* **15**: 212–216, 1976.
17. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
18. Hemler ME and Lands WEM, Evidence for a peroxide-initiated free radical mechanism of prostaglandin biosynthesis. *J Biol Chem* **193**: 340–345, 1980.
19. Szczeklik A and Gryglewski RJ, Low density lipoproteins (LDL) are carriers for lipid peroxides and inhibit prostacyclin (PGI_2) biosynthesis in arteries. *Artery* **7**: 488–495, 1980.
20. Moncada S, Gryglewski R, Bunting S and Vane JR, An enzyme isolated from arteries transforms prostaglandin endoperoxidases to an unstable substance that inhibit platelet aggregation. *Nature* **263**: 663–665, 1976.
21. Salomen JA, Smith DR, Flower RJ, Moncada S and Vane JR, Further studies on the enzymatic conversion of prostaglandin endoperoxide into prostacyclin by porcine aorta microsomes. *Biochim Biophys Acta* **523**: 250–262, 1978.
22. Hadjiagapiou C and Spector AA, 12-Hydroxyeicosa tetraenoic acid reduces prostacyclin production by endothelial cells. *Prostaglandins* **31**: 1135–1144, 1986.
23. Simon TC, Makheja AN and Bailey JM, Formation of 15-hydroxyeicosa tetraenoic acid (15-HETE) as the predominant eicosanoid in aortas from Watanabe heritable hyperlipedemic and cholesterol-fed rabbits. *Atherosclerosis* **75**: 31–38, 1989.