Vol. 200, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1994 Pages 338-343

FORMATION OF PGF_2-ISOPROSTANES DURING THE OXIDATIVE MODIFICATION OF LOW DENSITY LIPOPROTEIN

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Received February 24, 1994

It has been recently reported that a series of prostaglandin F_2-like compounds (PGF_2-isoprostanes) are produced *in vivo* by non-enzymatic peroxidation of arachidonic acid. Of these, 8-epi-$PGF_{2\alpha}$ is the major component and has been shown to be a potent vasoconstrictor. Here we investigated the formation of PGF_2-isoprostanes during copper-mediated oxidation of low density lipoprotein (LDL). Free and total (sum of free and esterified) levels of PGF_2-isoprostanes were measured at times 0, 3, 6, 8 and 24 h, using a solid-phase extraction procedure and gas chromatography - mass spectrometry. In native LDL, free levels of PGF_2-isoprostanes were between 0.06 and 0.10 ng/mg protein (n = 4), and the total levels ranged from 0.027 to 0.057 ng/mg protein (n = 4). Free levels of the isoprostanes were found to increase throughout the oxidation, whereas total levels reached a maximum after 3 h then gradually decreased. 8-epi-$PGF_{2\alpha}$ was the major isoprostane formed (free concentration after 24 h 1.8 $\pm$ 0.1 ng/mg protein (n = 4); total concentrations of 8.8 $\pm$ 1.8 and 6.1 $\pm$ 3.6 ng/mg protein (n = 4) after 3 and 24 h, respectively). The levels of isoprostanes correlated well with other indices of lipid peroxidation (conjugated dienes, hydroperoxides, thiobarbituric reactive substances) measured at similar time points. The release of PGF_2-isoprostanes from oxidised LDL in macrophages could be a contributory factor in the development of atherosclerosis.

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Abbreviations : Low density lipoprotein (LDL), butylated hydroxytoluene (BHT), phosphate buffered saline (PBS, Ca^{2+} and Mg^{2+} free), solid-phase extraction (SPE), octadecylsilane (C_{18}), aminopropyl (NH_2), pentafluorobenzylbromide (PFB), diisopropylethylamine (DIPEA), trimethylsilyl (TMS), thiobarbituric reactive substances (TBARS), ferrous oxidation / xylenol orange (FOX 2), tetrahydrofuran (THF).

0006-291X/94 $5.00

Oxidation of low density lipoprotein (LDL) by free radicals is a major event in the development of atherosclerosis. A number of oxidative mechanisms cause native LDL to become minimally modified *in vivo* [1-3]. The appearance of oxidised LDL in the sub-endothelial space of the arterial wall is accompanied by the generation of chemotactic factors. These in turn, initiate a cascade of events leading eventually to the formation of typical fatty streaks which characterise early atherosclerotic lesions.

At several stages of the oxidation of LDL, the formation or depletion of certain molecules can be monitored and used as indices of lipid peroxidation. Thus, the formation of conjugated dienes and lipid hydroperoxides or the depletion of fatty acids and vitamin E during the initial stages of oxidation, can be measured [2]. Initial products of lipid hydroperoxide decomposition (hydroxy fatty acids) and secondary products, such as malondialdehyde (MDA), which are generated as the peroxidation process continues can also be measured [4]. Among the lipid-derived molecules produced during the oxidative modification of LDL, some derive from arachidonic acid and have been found to possess important pharmacological properties. Recently, it was reported that a series of prostaglandin F_2-like compounds (PGF_2-isoprostanes) are produced *in vivo* by free radical - catalysed peroxidation of arachidonic acid independent of the cyclooxygenase enzyme [5]. Of these, 8-epi-$PGF_{2\alpha}$ was found to be the major product and was shown to be a potent renal and pulmonary vasoconstrictor acting via thromboxane/endoperoxide receptors [5-7].

We have recently developed a new method based on a solid-phase extraction procedure and GC-MS, for the isolation and quantitation of PGF_2-isoprostanes in biological fluids [8]. Here we have investigated the formation of PGF_2-isoprostanes during copper-mediated LDL oxidation and have also examined the possibility that the formation of 8-epi-$PGF_{2\alpha}$ may be monitored and used as an additional index of the peroxidation process.

Materials and Methods

Materials

Chemicals were obtained from Sigma Chemical Co (Poole, UK) or Aldrich Chemical Co (Gillingham, UK). BSTFA (N,O-*bis*(Trimethylsilyl)-trifluoroacetamide) was obtained from Pierce & Warriner (Chester, UK). Prostaglandin standards 8-epi-$PGF_{2\alpha}$, 9β,11α-PGF_2, 9α,11β-PGF_2, 9α,11α-PGF_2 and the deuterated analogue of 9α,11α-PGF_2 ($PGF_{2\alpha}$ - 3, 3', 4, 4'-d_4) were purchased from Cascade Biochem Ltd (Reading, UK).

Methods

Blood collection and isolation of LDL: Blood (100 mL per donor) was collected in flasks containing 3.8% trisodium citrate (blood / anticoagulant ratio of 9/1), indomethacin (14 μM) as cyclooxygenase inhibitor and BHT (20 μM) as free radical scavenger. Platelet-poor plasma was obtained by centrifugation at 2400 x g for 15 min at 4°C. LDL was isolated by sequential density ultracentrifugation (ρ = 1.019 - 1.063) in EDTA (final concentration 5 mM), using the method of Havel *et al* [9]. The LDL was dialysed extensively against 150 mM sodium chloride containing 0.01% EDTA (pH 7.4) at 4°C. Protein was determined by the method of Lowry [10] using bovine serum albumin as a standard.

Oxidation of LDL : LDL (600 μg protein/mL) was oxidised using Cu^{2+} (40 μM) in PBS at 37°C. Samples were collected at times 0, 3, 6, 8 and 24 h and stored at 4°C in the presence of BHT (final concentration 20 μM) until analysis.

Isolation of PGF_2-isoprostanes : PGF_2-isoprostanes were isolated using solid-phase extraction (SPE) [8]. For the determination of free levels, LDL (300 μg protein) was spiked with the internal standard $PGF_{2\alpha}$-d_4 (5 ng in 100 μL ethanol). The sample was acidified (pH 3) and was loaded on a C_{18} cartridge (Waters). After washing sequentially with 10 mL of water (pH 3) and acetonitrile / water (15 / 85), lipids were eluted with 5 mL of hexane / ethyl acetate / propan-2-ol (30 / 65 / 5). This eluate was then applied to an NH_2 cartridge (Supelco). The cartridge was sequentially washed with 10 mL of hexane / ethyl acetate (30 / 70), acetonitrile / water (90 / 10) and acetonitrile. The PGF_2-isoprostanes were then eluted with 5 mL of methanol / acetic acid / ethyl acetate (85 / 5 / 10) and the solvents evaporated under nitrogen at room temperature. For the analysis of total (esterified) PGF_2-isoprostane levels, 300 μg of each sample were hydrolysed with potassium hydroxide (1.0 M) for 30 min at 40°C prior to the SPE procedure.

Derivatisation : The pentafluorobenzyl ester was prepared by adding 40 μL of PFB and 20 μL of DIPEA (10% in acetonitrile, each) to the dried eluate and incubating at 40°C for 45 min. TMS ethers prepared by incubation with 50 μL of BSTFA and 5 μL of DIPEA (10% in acetonitrile) at 4°C overnight. After removing the solvent with nitrogen, the derivatised sample was reconstituted in 40 μL of iso-octane containing 10% BSTFA for analysis by GC-MS.

GC-MS analysis : Samples were analysed with a Hewlett Packard 5890 GC (Bracknell, UK) linked to a VG70SEQ MS (Fisons Instruments, Manchester, UK), using electron capture negative ion chemical ionisation (NICI) with ammonia reagent gas. PGF_2-isoprostanes were separated using an SPB-1701 column (30 m x 0.25 mm ID x 0.25 μm D_f , Supelco, PA, USA). Samples (2 μL) were injected into a temperature programmed Gerstel injector (Thames Chromatography, Maidenhead, UK). The GC was programmed from 175 to 270°C at a rate of 30°C/min. Quantitative analysis was carried out by selected ion monitoring (SIM) of the carboxylate anion $[M-181]^-$ at m/z 569 and 573 for the PGF_2-isoprostanes and $PGF_{2\alpha}$-d_4, respectively. With our method, inter- and intra-assay coefficients of variation are 12.5% and 7.1%, respectively, and the recovery of the PGF_2-isoprostanes is 65 ± 4% (n = 4). The lower limit of reliable detection (LRD) is approximately 10 pg/mL.

Additional lipid peroxidation measurements : Conjugated dienes were detected at 234 nm by UV spectrophotometry [11, 12]. Lipid hydroperoxides were measured using the FOX 2 assay at 560 nm [13] whereas TBARS were monitored at 532 nm [13]. For vitamin E analysis, an aliquot of the LDL sample (300 μL) was removed and the internal standard (+)-γ-tocopherol (50 ng in 500 μL ethanol) was added. The vitamin E was extracted with hexane (500 μL). The hexane layer was dried under a stream of nitrogen and the residue was reconstituted in acetonitrile (100 μL) and analysed by HPLC. Separation was carried out on a Hypersil ODS column (200 x 3 mm ID, particle size 5 μm, Chrompack, Middelburg, The Netherlands), using acetonitrile / THF / water (80 / 14 / 6) at a flow rate of 0.7 mL/min. The signal was monitored at λ_{EX} 295 nm and λ_{EM} 340 nm.

Results and Discussion

Measurements of PGF_2-isoprostanes : Figure 1A shows a SIM chromatogram of a mixture of authentic standards following solid-phase extraction on C_{18} and NH_2 cartridges. The formation of PGF_2-isoprostanes during copper-mediated oxidation of

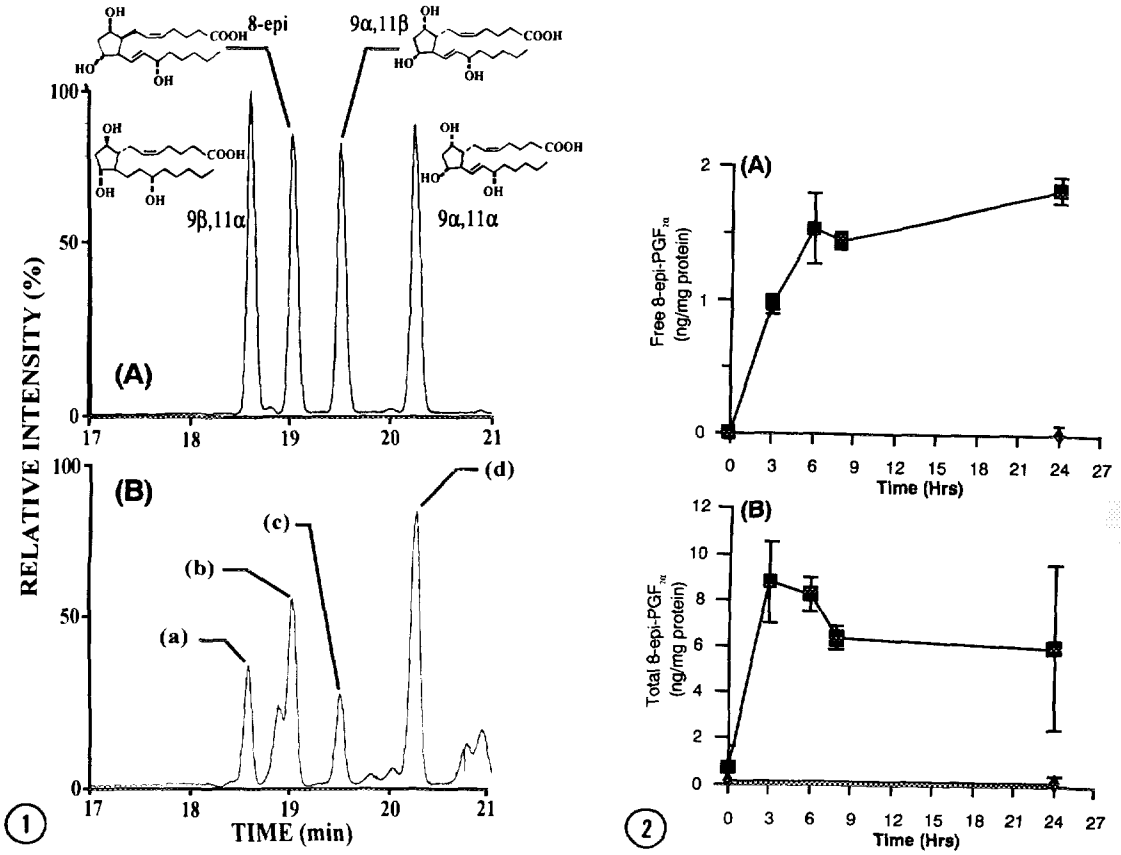

Figure 1. GC-MS/NICI selected ion monitoring chromatograms of (A) a mixture of authentic PGF_2 standards, and (B) PGF_2 compounds isolated from oxidised LDL. Peaks : (a) 9β,11α-PGF_2, (b) 8-epi-$PGF_{2\alpha}$, (c) 9α,11β-PGF_2 and (d) 9α,11α-PGF_2. *LDL (600 μg protein/mL) was incubated with Cu^{2+} (40 μM in PBS) at 37°C for 24 h.* Separation of the PGF_2-isoprostanes as PFB-ester, TMS-ether derivatives was carried out on an SPB-1701 column (30 m x 0.25 mm ID x 0.25 μm D_f) as described in Materials and Methods.

Figure 2. Time course for the formation of (A) free, and (B) total (sum of free and esterified) 8-epi-$PGF_{2\alpha}$ during copper-mediated oxidation of LDL. (■)Oxidised LDL, (◆) control. LDL (600 μg protein/mL) was incubated with Cu^{2+} (40 μM in PBS) at 37°C for 24 h. Data points are represented as the mean ± SD (n = 4). 8-epi-$PGF_{2\alpha}$ was isolated from samples by a solid-phase extraction procedure and was analysed as described in Materials and Methods.

LDL is shown in Fig. 1B. The most abundant isoprostane formed during the oxidation was 8-epi-$PGF_{2\alpha}$. In native LDL, free isoprostane levels were between 0.06 - 0.10 ng/mg protein and were found to increase during the incubation period (Fig. 2A). Total (sum of free and esterified) levels in native LDL ranged between 0.027 - 0.057 ng/mg protein. The total levels of PGF_2-isoprostanes were found to increase to a maximum during the first 3 h of the oxidation, then gradually decreased (Fig. 2B). Our findings suggest that lipid peroxidation occurs on functional phospholipids in LDL.

Free concentrations of the components eluting at about 18.5 and 19.5 min in copper-oxidised samples at t = 24 h were about 43 and 74 times higher, respectively, than the controls. Total levels of these compounds, however, were around 26 and 30 times higher than controls, respectively. In contrast, total levels of PGF_2-isoprostanes eluting at about 19.0 and 20.3 min were found to be 220- and 130-fold greater, respectively, than controls. Morrow *et al* showed that increases in total levels of F_2-isoprostanes in livers of CCl_4-treated rats preceded the appearance of free compounds in the circulation [14]. Our results (Fig. 2) support these observations in that, increases in levels of free PGF_2-isoprostanes lag behind increases in total levels during the oxidation of LDL.

Additional lipid peroxidation measurements : Conjugated dienes increased throughout the incubation period (238 $\pm$ 21.9 and 962 $\pm$ 16.6 nmol/mg protein , at t = 0 and 24 h, respectively). Lipid hydroperoxides (239 $\pm$ 12.3 nmol/mg protein at t = 0) were found to increase rapidly, reaching a maximum after 3 h (1911 $\pm$ 30.0 nmol/mg protein). The levels then decreased to near starting values at t = 24 h (347 $\pm$ 11.7 nmol/mg protein) (Fig. 3). TBARS were also found to reach maximum levels at t = 3 h (201 $\pm$ 4.9 nmol/mg protein), but there was no significant decrease until the end of the incubation period (185 $\pm$ 12.7 nmol/mg protein at t = 24 h) (Fig. 3). Vitamin E (650 $\pm$ 3.2 ng/mg protein at t = 0) was depleted within the first 3 h of the oxidation reaction. In controls, no significant changes were observed in these parameters during the incubation period.

Our results show that PGF_2-isoprostanes are formed in substantial amounts during the oxidative modification of LDL *in vitro*. More importantly, we have found that 8-epi-$PGF_{2\alpha}$ is the major product of the oxidation process. Changes in the free levels of 8-epi-$PGF_{2\alpha}$ correlate well with other indices of lipid peroxidation, and therefore its

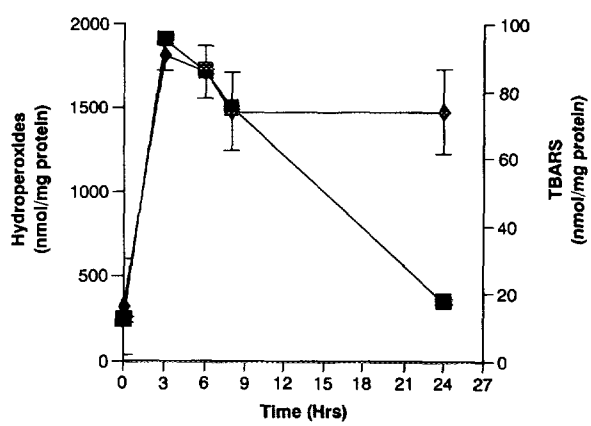

Figure 3. Formation of (■) lipid hydroperoxides and (◆) TBARS during LDL oxidation. LDL (600 μg protein/mL) was incubated with Cu^{2+} (40 μM in PBS) at 37°C for 24 h. Hydroperoxides and TBARS were determined by UV spectrophotometry as described in Materials and Methods.

formation can be used as an additional index of lipid peroxidation. The fact that the PGF_2-isoprostanes appear to be formed initially esterified in the LDL and are then gradually released into the surrounding medium, may have important consequences *in vivo* and may contribute to the pathophysiology of atherosclerosis. Investigations to monitor the formation of PGF_2-isoprostanes during cell-mediated LDL oxidation are being carried out. Experiments are also being done to determine the effect of specific inhibitors on the production of these isoprostanes.

Acknowledgments

This work was supported by grants from ONO Pharmaceutical Company (Osaka, Japan) and the British Heart Foundation.

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