

Formation of F₂-isoprostanes during aortic endothelial cell-mediated oxidation of low density lipoprotein

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Abstract

We investigated the formation of F₂-isoprostanes produced by non-enzymatic peroxidation of arachidonic acid during rabbit aortic endothelial cell-mediated oxidation of low density lipoprotein (LDL). Free and total (sum of free and esterified) levels of F₂-isoprostanes were measured using a solid-phase extraction procedure and gas chromatography-mass spectrometry. Free levels of F₂-isoprostanes in native LDL were 0.06 ± 0.03 ng/mg protein ($n = 4$), whereas total levels were 0.28 ± 0.09 ng/mg protein ($n = 4$). Both free and total levels of the isoprostanes were found to increase during the oxidation. 8-epi-PGF_{2 α} was the major isoprostane formed (free and total concentrations after 24 h, 2.50 ± 0.24 and 6.42 ± 1.36 ng/mg protein ($n = 4$), respectively). The release of F₂-isoprostanes during aortic endothelial cell-induced oxidation of LDL could be a contributory factor in the development of atherosclerosis.

Key words: F₂-isoprostane; LDL; Cell-mediated oxidation; Lipid peroxidation; Atherosclerosis

1. Introduction

Free radical-mediated oxidation of low density lipoprotein (LDL) is an important factor in the development of atherosclerosis. It has been shown that several cell types of the vascular system (endothelial cells, smooth muscle cells, fibroblasts, monocytes and macrophages) can oxidise LDL in culture [1–4]. It is also known that a number of oxidative mechanisms cause native LDL to become minimally modified in vivo [5–7]. 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 to the formation of fatty streaks which characterise early atherosclerotic lesions.

In the presence of endothelial cells, smooth muscle cells or macrophages, the oxidation of LDL is believed to proceed via three major mechanisms which probably operate simultaneously [8]. These cells can catalyse the oxidative process by: (i) producing and subsequently releasing highly active oxygen species into the immediate environment (superoxide or hydrogen peroxide); (ii) generating lipid peroxides, which are then transferred to the LDL; and (iii) releasing enzymes which act directly on

lipid molecules. Cellular sources of reactive oxygen species and enzymes responsible for catalysing lipid peroxidation include xanthine oxidase, NADPH, mitochondrial electron transport systems, cyclooxygenase and lipoxygenase. Additional non-enzymatic systems which can enhance LDL oxidation have also been shown to occur in cells [9].

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. For routine biochemical analysis, the formation of conjugated dienes, lipid hydroperoxides, thiobarbituric acid reactive substances (TBARS) or the depletion of polyunsaturated fatty acids and vitamin E is measured [10]. Of these, the TBARS assay is the most frequently used because of its simplicity. This assay is also the most criticised one because of its lack of specificity.

Recently, it has been shown that a series of prostaglandin F₂-like compounds (F₂-isoprostanes) are produced in vivo by free radical-catalysed peroxidation of arachidonic acid independent of the cyclooxygenase enzyme [11]. Of these, 8-epi-PGF_{2 α} was found to be the major product and was shown to be a potent renal and pulmonary vasoconstrictor [11–13].

We have developed a new method based on a solid-phase extraction procedure and GC-MS for the isolation and quantitation of F₂-isoprostanes in biological fluids [14]. This method was applied to monitor the formation of F₂-isoprostanes during metal-catalysed oxidation of LDL [15]. In this study, we investigated the formation of F₂-isoprostanes during cell-mediated LDL oxidation and examined the possibility of using the formation of

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Abbreviations: LDL, low density lipoprotein; NADPH, reduced nicotinamide adenine dinucleotide phosphate oxidase; BHT, butylated hydroxytoluene; SPE, solid-phase extraction; C₁₈, NH₂, octadecylsilane; aminopropyl; PFB, pentafluorobenzylbromide; TMS, trimethylsilyl, TBARS, thiobarbituric acid reactive substances; FOX 2, ferrous oxidation/xylenol orange.

8-epi-PGF_{2α} as a specific marker of non-enzymatic lipid peroxidation. In addition, the quantitative relation between F₂-isoprostanes, hydroperoxides, conjugated dienes and TBARS is examined.

2. Materials and methods

2.1. 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 and Warriner (Chester, UK). Prostaglandin standards 8-epi-PGF_{2α}, 9β,11α-PGF₂, 9α,11β-PGF₂, 9α,11α-PGF₂ and the deuterated analogue of 9α,11α-PGF₂ (PGF_{2α}-3,3',4,4'-d₄) were purchased from Cascade Biochem Ltd (Reading, UK). Solvents were obtained from Rathburn Chemicals Ltd (Walkerburn, UK).

2.2. 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 × g for 15 min at 4°C. LDL was isolated by sequential density ultracentrifugation ($\rho = 1.019\text{--}1.063$) in EDTA (final concentration 5 mM), using the method of Havel et al. [16]. 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 Lowry method using bovine serum albumin as a standard [17].

2.3. Cells

Rabbit aortic endothelial cells were used from a line established and characterised by Buonassisi et al. [18]. The cells were grown in Ham's F-10 medium containing 15% fetal bovine serum, epidermal growth factor (50 ng/ml), endothelial cell growth supplement (75 μg/ml) and heparin (20 μg/ml). Six-well culture plates were seeded with approximately 10⁵ cells and were used at confluence.

2.4. Oxidation of LDL

LDL (200 μg protein/ml) was incubated with washed endothelial cells in 1 ml Ham's F-10 (with glutamine, without Phenol red) at 37°C in a humidified 5% CO₂ atmosphere. 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. Control plates were incubated under identical conditions without LDL.

2.5. Cell viability tests

Cell viability was determined by microscopic observation and cell protein measurements (Lowry method) of the endothelial cells before and after the 24 h incubation period.

2.6. Isolation of F₂-isoprostanes

F₂-isoprostanes were isolated using solid-phase extraction (SPE) [14]. For the determination of free levels, LDL (100 μg protein) was spiked with the internal standard PGF_{2α}-d₄ (5 ng in 100 μl ethanol). The sample was acidified (pH 3) and was loaded on a C₁₈ cartridge (Waters). After washing sequentially with 10 ml of water (pH 3) and acetonitrile/water (15:85), F₂-isoprostanes were eluted with 5 ml of hexane/ethyl acetate/propan-2-ol (30:65:5). This eluate was then applied to an NH₂ cartridge (Supelco). The cartridge was sequentially washed with 10 ml of hexane/ethyl acetate (30:70), acetonitrile/water (90:10) and acetonitrile. F₂-isoprostanes were then eluted with 5 ml of methanol/acetic acid/ethyl acetate (85:5:10). For the analysis of total (sum of free and esterified) F₂-isoprostanes, 100 μg of each sample were hydrolysed with potassium hydroxide (1.0 M) for 30 min at 40°C prior to the SPE procedure.

2.7. Derivatisation

F₂-isoprostanes collected in the final eluate from the NH₂ cartridge were derivatised to PFB esters and TMS ethers prior to GC-MS analysis, as described earlier [15].

2.8. GC-MS analysis

Samples were analysed on 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. F₂-isoprostanes were separated with an SPB-1701 column (30 m × 0.25 mm ID × 0.25 μm D_f, Supelco, PA, USA). Quantitative analysis was carried out by selected ion monitoring (SIM) of the carboxylate anion [M-181]⁻ at *m/z* 569 and 573 for the F₂-isoprostanes and PGF_{2α}-d₄, respectively [15].

2.9. Additional lipid peroxidation measurements

Conjugated dienes were detected by spectrophotometry at 234 nm [19]. Hydroperoxides were measured using the FOX 2 assay at 560 nm [20] and TBARS were monitored at 532 nm [20]. Vitamin E was analysed using HPLC and fluorescence detection as described earlier [15].

3. Results

3.1. Measurement of F₂-isoprostanes

Fig. 1A shows a SIM/NICI chromatogram (*m/z* 569) of a mixture of authentic standards following solid-phase extraction on C₁₈ and NH₂ cartridges. A SIM/NICI

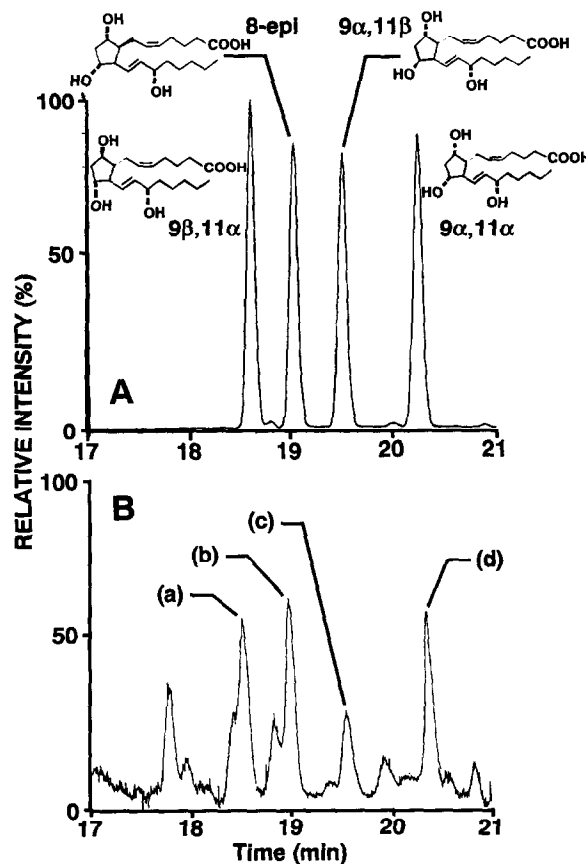


Fig. 1. GC-MS/NICI selected ion monitoring chromatograms of (A) a mixture of authentic PGF₂ standards, and (B) PGF₂ compounds isolated from cell-mediated oxidised LDL. Peaks: (a) 9β, 11α-PGF₂, (b) 8-epi-PGF_{2α}, (c) 9α, 11α-PGF₂ and (d) 9α, 11α-PGF₂. LDL (200 μg protein/ml) was incubated with rabbit aortic endothelial cells in Ham's F-19 at 37°C for 24 h. PGF₂ compounds were isolated by solid-phase extraction and were separated as PFB-ester, TMS-ether derivatives using an SPB-1701 column (30 m × 0.25 mm ID × 0.25 μm D_f) as described in section 2.

chromatogram of total (sum of free and esterified) F_2 -isoprostanes isolated by SPE following oxidation of LDL by rabbit aortic endothelial cells for 24 h is shown in Fig. 1B. Analysis of native LDL revealed the presence of low levels of free F_2 -isoprostanes (0.06 ± 0.03 ng/mg protein, $n = 4$). Total (sum of free and esterified) F_2 -isoprostanes were 0.28 ± 0.09 ng/mg protein ($n = 4$). Of these, 8-epi-PGF $_{2\alpha}$ was present in abundance. These findings suggest that F_2 -isoprostanes are present mainly in an esterified form in circulating lipoproteins.

In the presence of endothelial cells, no changes in the free and total levels of F_2 -isoprostanes were observed during the first 3 h of incubation. The levels then increased rapidly during the remaining time of the incubation. At $t = 24$ h, free and total levels of 8-epi-PGF $_{2\alpha}$ were 2.50 ± 0.24 and 6.42 ± 1.36 ng/mg

protein ($n = 4$), respectively. Fig. 2 illustrates the time course for the formation of free and total 8-epi-PGF $_{2\alpha}$ during cell-mediated oxidation of LDL.

In controls (cells alone), 8-epi-PGF $_{2\alpha}$ (free or total) was not formed during the incubation. In additional controls (LDL in Ham's F-10 alone), the initial free 8-epi-PGF $_{2\alpha}$ levels remained unchanged after 24 h (Fig. 2A) whereas the total levels increased by 37.5% (Fig. 2B).

3.2. Additional lipid peroxidation measurements

No significant changes in the levels of conjugated dienes, lipid hydroperoxides and TBARS were observed during the first 3 h of oxidation. Conjugated dienes increased during the following 5 h of incubation (197 ± 39.6 nmol/mg protein at $t = 8$ h), then decreased gradually (Fig. 3). Lipid hydroperoxides (117 ± 14.2 nmol/mg protein at $t = 0$) were also found to increase to a maximum at $t = 8$ h, before decreasing (Fig. 3). TBARS reached a maximum of 38.3 ± 3.1 nmol/mg protein after 24 h (Fig. 3). Vitamin E (1800 ± 79.2 ng/mg protein at $t = 0$) was depleted within the first 6 h of the oxidation reaction. In controls, no significant changes were observed in these parameters during the incubation period.

3.3. Cell viability tests

Cell protein decreased by 7.8% after the 24 h incubation, indicating that the oxidation products generated were not exerting significant cytotoxicity. No significant morphological changes were apparent from microscopic observations of the cells.

4. Discussion

Recently, we reported the formation of F_2 -isoprostanes during copper-mediated LDL oxidation [15]. It was found that native LDL contains significant amounts of F_2 -isoprostanes in an esterified form and that their levels increase during metal-catalysed oxidation. We also found that 8-epi-PGF $_{2\alpha}$ was the most abundant isoprostane formed. The increase in free levels of 8-epi-PGF $_{2\alpha}$ was preceded by an increase in esterified levels. Previous studies have shown that rabbit aortic endothelial cells can oxidise LDL [21,22]. In this study, we investigated the formation of F_2 -isoprostanes during cell-mediated LDL oxidation and also examined the possibility that the formation of 8-epi-PGF $_{2\alpha}$ may be monitored and used as an additional index of the peroxidative process.

Our results show that 8-epi-PGF $_{2\alpha}$ was formed in substantial amounts during endothelial cell-induced oxidation of LDL in vitro (Fig. 1). Both free and total levels increased during the 24 h incubation and the final concentrations of total 8-epi-PGF $_{2\alpha}$ were about 2.5 times higher than the corresponding free levels (Fig. 2). The oxidation exhibited a lag phase of about 3 h, during which there were no significant changes in the levels of

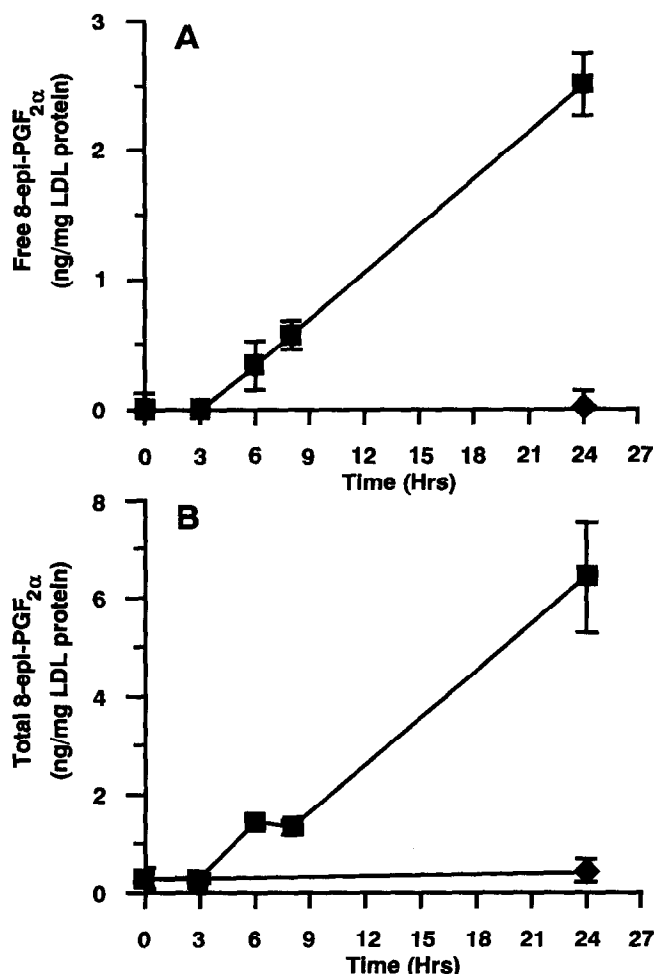


Fig. 2. Time course for the formation of (A) and (B) total (sum of free and esterified) 8-epi-PGF $_{2\alpha}$ during cell-mediated oxidation of LDL. (●) oxidised LDL, (●) control. LDL (200 μ g protein/ml) was incubated with rabbit aortic endothelial cells in Ham's F-10 at 37°C for 24 h. 8-epi-PGF $_{2\alpha}$ was isolated by a solid-phase extraction procedure and was analysed by GC-MS/NICI as described in section 2. For total measurements, the samples were hydrolysed with KOH (1.0 M) prior to solid-phase extraction. Data points are represented as the mean $\pm$ S.D. ($n = 4$).

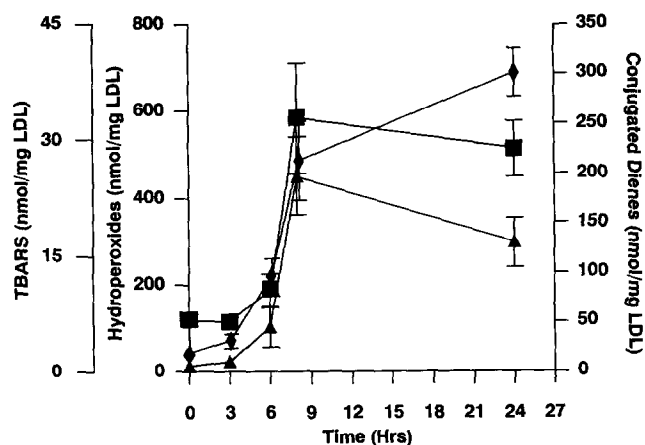


Fig. 3. Formation of (■) lipid hydroperoxides, (◆) TBARS and (▲) conjugated dienes during cell-mediated LDL oxidation. LDL (200 µg protein/ml) was incubated with rabbit aortic endothelial cells in Ham's F-10 at 37°C for 24 h. Hydroperoxides, TBARS and conjugated dienes were determined by UV/visible spectrophotometry as described in section 2.

8-epi-PGF_{2α} (free and total) as well as lipid hydroperoxides, conjugated dienes and TBARS (Figs. 2 and 3). Increased concentrations were found after the lag phase, which corresponded to a 47.8% decrease in endogenous vitamin E (939 ± 21.5 ng/mg protein at *t* = 3 h). Changes in the levels of 8-epi-PGF_{2α} were related to other indices of lipid peroxidation showing that its formation can be used as an additional index of lipid peroxidation. In contrast to copper-mediated LDL oxidation, we also found an increase in the formation of a component with similar retention time to the cyclooxygenase-derived prostaglandin 9α,11α-PGF₂ (total concentrations of 0.39 ± 0.04 and 10.6 ± 2.9 ng/mg protein (*n* = 4) at *t* = 0 and 24 h, respectively).

These results contribute to growing evidence that endothelial cells have the capacity to oxidise LDL by several mechanisms operating simultaneously and can generate a wide array of compounds, including some which are potent pharmacological mediators. Aortic endothelial cell-induced LDL oxidation could therefore contribute to the pathophysiology of atherosclerosis and other disease states where considerable oxidative stress may be involved.

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