Plasma 8-epi-$PGF_{2\alpha}$ levels are elevated in individuals with non-insulin dependent diabetes mellitus

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Abstract This study reports plasma levels of a specific non-enzymatic peroxidation product of arachidonic acid, esterified 8-epi-$PGF_{2\alpha}$, from healthy- and NIDDM individuals as an index of oxidative stress in vivo. Plasma 8-epi-$PGF_{2\alpha}$ was isolated by solid-phase extraction on a C_{18} followed by an NH_2 cartridge and analyzed by GC-MS/NICI as PFB-ester/TMS-ether derivative. We found that the average concentration of esterified 8-epi-$PGF_{2\alpha}$ among NIDDM subjects (0.93 ± 0.07 nM, n = 39) was higher (P < 0.0001, Mann-Whitney test) than in healthy individuals (0.28 ± 0.04 nM, n = 15). These data indicate that NIDDM is associated with increased plasma lipid peroxidation.

Key words: Prostaglandin; F_2-isoprostane; Lipid peroxidation; 8-Epi-$PGF_{2\alpha}$; Diabetes

1. Introduction

F_2-isoprostanes consists of a series of prostaglandin F_2-like compounds formed during peroxidation of arachidonic acid by a mechanism independent of the cyclooxgenase pathway [1]. In theory, F_2-isoprostanes can be grouped into four subtypes (Fig. 1). Of these, 8-epi-$PGF_{2\alpha}$ has received considerable attention because of its role as a pharmacological mediator in vivo [2].

Elevated plasma levels of 8-epi-$PGF_{2\alpha}$ have been reported in two animal models of free radical injury and lipid peroxidation [1,3]. Information in the literature concerning the levels of 8-epi-$PGF_{2\alpha}$ in human plasma, however, is limited . Morrow et al. [1] reported the presence of 0.01–0.11 nM of presumably free (unesterified) 8-epi-$PGF_{2\alpha}$ in plasma from healthy donors (n = 5). Their estimation was downgraded to 0.05 ± 0.02 nM in a recent publication [4]. Lynch et al. [5] detected 0.02 nM and 0.10 nM of free and esterified F_2-isoprostanes, respectively, in plasma from a normolipidemic individual. The 8-epi-$PGF_{2\alpha}$ is initially formed esterified to phospholipids and then released by the action of a phospholipase to become biologically active [6].

We have recently developed a rapid and reliable SPE/GC-MS assay for the measurement of plasma 8-epi-$PGF_{2\alpha}$, a specific non-enzymatic peroxidation product of arachidonic acid [7]. In this study, we: (a) investigated the influence of cyclooxygenase inhibitors on the formation of 8-epi-$PGF_{2\alpha}$ in plasma, both in vivo and ex vivo; and (b) analysed plasma from healthy and NIDDM individuals for the content of free (unesterified) as well as total (sum of free and esterified) 8-epi-$PGF_{2\alpha}$ as an index of oxidative stress in vivo.

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Abbreviations: SPE, solid-phase extraction; GC-MS/NICI, gas chromatography-mass spectrometry/negative ion chemical ionisation; PFB, pentafluorobenzyl; TMS, trimethylsilyl; BSTFA, *N,O-bis*(trimethyl)-trifluoroacetamide; BHT, butylated hydroxytoluene; DIPEA, diisopropylethylamine; NIDDM, non-insulin-dependent diabetes mellitus.

2. Materials and methods

2.1. Materials

Prostaglandin F_2 (9,11,15-trihydroxy-5,11-eicosadienoic acid) standards including 9α,11α-, 9α,11β-, 9β,11α-, 8-epi- and 3,3′,4,4′-tetradeuterated 9α,11α-PGF_2 (PGF_2-d_4) were obtained from Cascade Biochem (Reading, UK). Sep-Pak C_{18} (500 mg) cartridges were obtained from Millipore Inc. (Millford, MA, USA). Aminopropyl (NH_2) cartridges (Supelclean LC-NH_2, 500 mg) were supplied by Supelco (Bellefonte, PA, USA). BSTFA was purchased from Pierce Chemical Co. (Rockford, IL, USA). Indomethacin, BHT, PFB and DIPEA were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All commercially available chemicals and reagents were of analytical grade or greater purity.

2.2. Subjects

Individuals with NIDDM (n = 39) were recruited from the University College London Hospital (UCL) outpatient clinic services. The control group comprised of 15 healthy volunteers from the staff of the department of Medicine, UCL. An additional group of healthy volunteers (n = 6) from the staff of UCL was used to study the effect of cyclooxygenase inhibitors on plasma 8-epi-$PGF_{2\alpha}$ levels.

2.3. Sample collection and plasma preparation

(a) Effect of cyclooxygenase inhibition on plasma levels of 8-epi-$PGF_{2\alpha}$. Aspirin and indomethacin were used to study cyclooxygenase inhibition in vivo and ex vivo, respectively. For the in vivo study, blood (20 ml) was collected in 3.8% trisodium citrate (blood/anticoagulant ratio 9:1) before and after administration of three doses of 600 mg aspirin (12, 4 and 2 h prior to sample collection). Platelet-poor plasma was prepared by centrifugation at 2400 × g at 4°C for 15 min. Aliquots (1 ml) of the plasma samples were transferred to Eppendorf tubes and BHT was added (final concentration 20 μM) as a chain breaking antioxidant. The samples were then stored at −70°C and analysed within 2 weeks. For the evaluation of ex vivo generation of 8-epi-$PGF_{2\alpha}$, indomethacin in 5% sodium bicarbonate was added to the blood samples immediately after collection (final concentration 14 μM). The samples were allowed to stand for 45 min at 4°C to achieve complete inhibition of the cyclooxgenase enzymes. Platelet-poor plasma was then isolated and stored as described above.

(b) Measurement of 8-epi-$PGF_{2\alpha}$ as an index of oxidative stress. Blood (10 ml) was drawn from control and NIDDM subjects into lithium heparin Vacutainers. Platelet-poor plasma was obtained by centrifugation (as above) and aliquots of the plasma (1 ml) were transferred to Eppendorf tubes and stored at −70°C until analysis.

2.4. Clinical measurements

Analysis of plasma triglycerides, total cholesterol, fasting glucose and glycosylated haemoglobin A (HbA_{1c}) were carried out at the UCL

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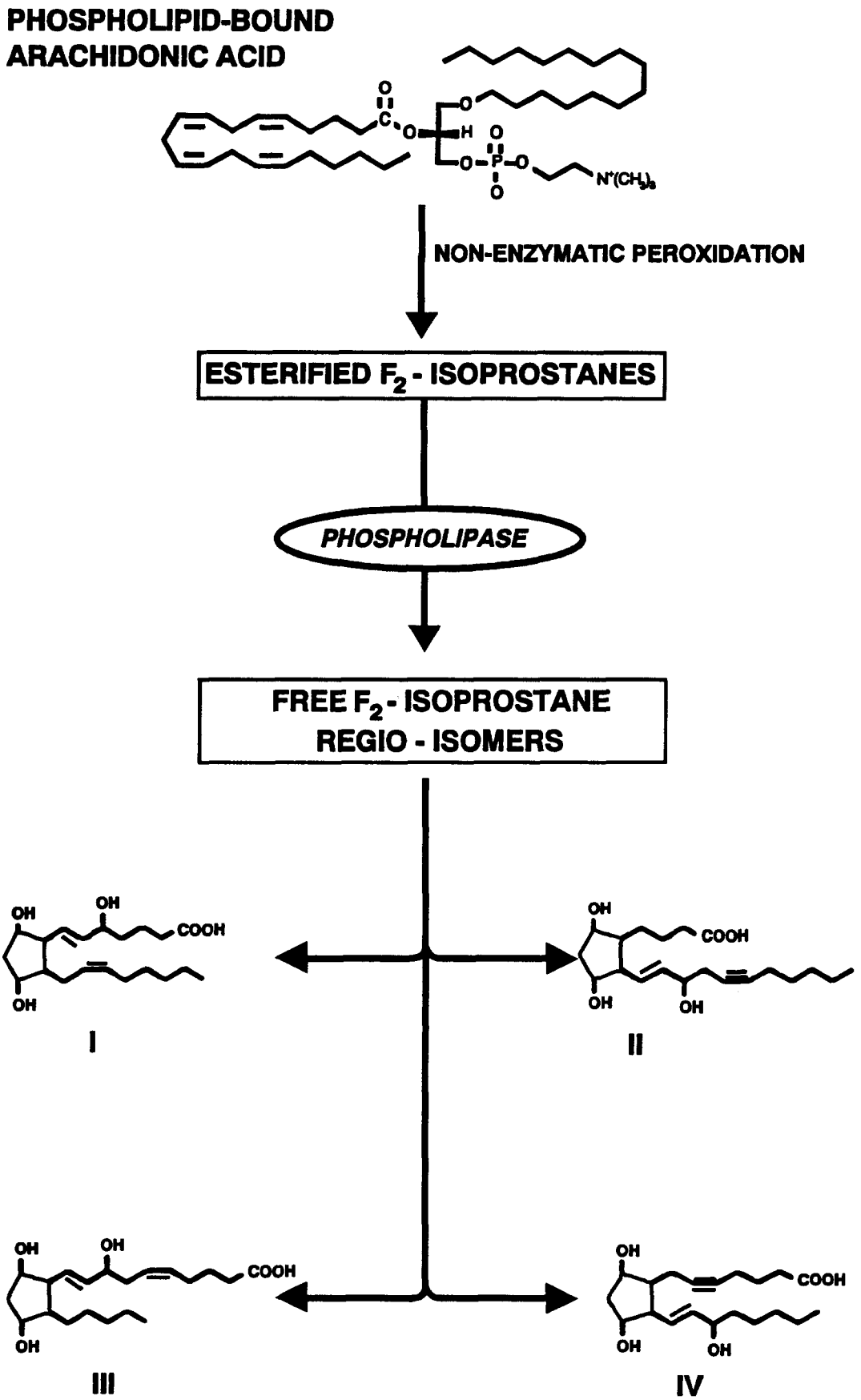

Fig. 1. Formation of F_2-isoprostane regio-isomers during non-enzymatic peroxidation of arachidonic acid. Regio-isomer IV represent the 8-epi-PGF_2 stereo-isomers.

Hospital blood analysis laboratory. Clinical data for the control and patient groups are listed in Table 1.

2.5. Solid-phase extraction procedure

(a) Free (non-esterified) F_2-isoprostanes. Plasma (1 ml) was spiked with PGF_2-d_4 (5 ng in 50 μl ethanol) as the internal standard. The sample was acidified using 2 ml of water (pH 3.0) and allowed to equilibrate at 4°C for 15 min. The sample was then applied to a C_{18} cartridge (500 mg) preconditioned with methanol and water (pH 3.0). The cartridge was sequentially washed with 10 ml of water (pH 3.0) and acetonitrile/water (15:85, v/v) to remove polar materials. Lipids were eluted by washing the cartridge with 5 ml of hexane/ethyl acetate/propan-2-ol (30:65:5, v/v). This eluate was then applied to an NH_2 cartridge (500 mg), preconditioned with hexane (5 ml). The NH_2 cartridge was sequentially washed with 10 ml of hexane/ethyl acetate (30:70, v/v), acetonitrile/water (9:1, v/v) and acetonitrile. F_2-isoprostanes were eluted from the NH_2 cartridge with 5 ml of ethyl acetate/methanol/acetic acid (10:85:5, v/v). The sample was immediately transferred into a screw-cap vial and the solvent was evaporated under nitrogen at room temperature.

(b) Total (sum of free and esterified) F_2-isoprostanes . Plasma (1 ml) was incubated with 1 ml of an aqueous solution of potassium hydroxide (1.0 M) at 40°C for 30 min to hydrolyse esterified F_2-isoprostanes. Thereafter, water (1 ml) was added and the pH was adjusted to 3.0 using concentrated HCl. PGF_2-d_4 (5 ng in 50 μl ethanol) was added as an internal standard and the sample was centrifuged at 2400 × *g* for 5 min. The supernatant was removed and applied to the solid-phase extraction procedure as described above.

2.6. Derivatization

The PFB-ester was prepared by adding 40 μl of PFB (10% in acetonitrile) and 20 μl of DIPEA (10% in acetonitrile) to the dried eluate following SPE. The vial was sealed with a Teflon-lined cap and was incubated at 40°C for 45 min. The solvent was then removed under a stream of nitrogen. TMS-ethers were prepared by incubation of the dried sample with 50 μl BSTFA and 5 μl DIPEA (10% in acetonitrile) at 4°C overnight. After removing excess solvent with nitrogen, the derivatised sample was reconstituted in *iso*-octane (40 μl) containing 10% BSTFA and was analysed immediately by GC-MS.

2.7. Gas chromatography-mass spectrometry

GC-MS/NICI analysis was carried out using a Hewlett Packard 5890 GC (Bracknell, UK) linked to a VG70SEQ MS (Fisons Instruments, Manchester, UK), using ammonia as reagent gas. F_2-isoprostanes were separated on an SPB-1701 column (30 m × 0.25 mm i.d. × 0.25 μm D_f, Supelco, PA, USA). Samples (2 μl) were injected automatically into a temperature programmed Gerstel injector (Thames Chromatography, Maidenhead, UK). The GC oven was programmed from 175 to 270°C at a rate of 30°C/min. Quantitative analysis was performed using selected ion monitoring (SIM) of the carboxylate anion $[M-181]^-$ at m/z 569 for the F_2-isoprostanes and m/z 573 for PGF_2-d_4.

3. Results and discussion

3.1. Effect of cyclooxygenase inhibition

Recent reports concerning the influence of cyclooxygenase inhibitors on the endogenous production of 8-epi-$PGF_{2\alpha}$ are conflicting [1,8,9]. As an integral part of our study, we investigated the effect of aspirin and indomethacin on the plasma levels of 8-epi-$PGF_{2\alpha}$, both in vivo and ex vivo.

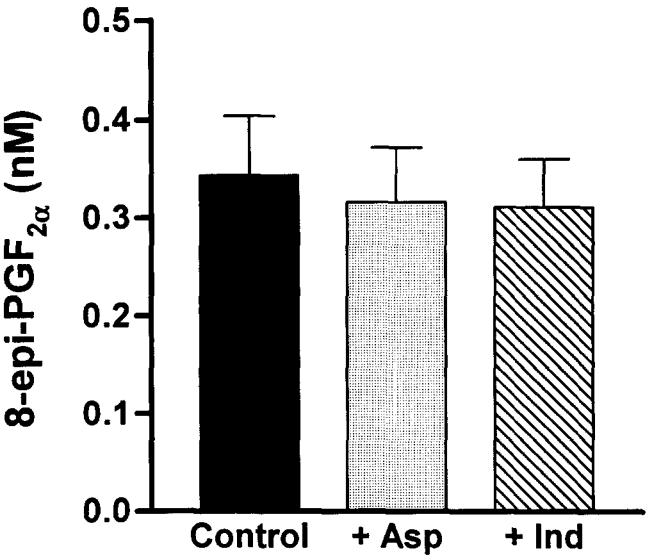

Fig. 2. Influence of cyclooxgenase inhibitors on the plasma levels of esterified 8-epi-$PGF_{2\alpha}$. Aspirin was used to examine the effect on in vivo production of 8-epi-$PGF_{2\alpha}$ in healthy volunteers (n = 6). Indomethacin was used to evaluate ex vivo generation of 8-epi-$PGF_{2\alpha}$ during blood collection (n = 6).

GS-MS/NICI analysis revealed no quantifiable levels of free 8-epi-$PGF_{2\alpha}$ in the plasma samples before and after aspirin treatment (n = 6) at a detection limit of 0.02 nM. However, plasma levels (0.02–0.25 nM) of a component with similar retention time to that of the cyclooxygenase derived 9α,11α-PGF_2 were found, regardless of aspirin treatment. In contrast, when analysed for total F_2-isoprostanes, the plasma samples were found to contain 0.10–0.46 nM of esterified 8-epi-$PGF_{2\alpha}$. As shown in Fig. 2, aspirin treatment had no statistically significant effect on the plasma levels of esterified 8-epi-$PGF_{2\alpha}$ (paired Student's t-test, P = 0.39).

Analysis of plasma samples obtained from indomethacin-treated blood (Fig. 2) showed that there was no significant effect on the esterified levels of 8-epi-$PGF_{2\alpha}$, compared to the controls (paired Student's t-test, P = 0.43). In contrast, free levels associated with the peak co-eluting with cyclooxygenase-derived 9α,11α-PGF_2 was completely suppressed. This indicated that the component co-eluting with 9α,11α-PGF_2 was generated ex vivo, possibly by platelet activation during the preparation of plasma. In agreement with earlier findings by Morrow et al. [1], we found that formation of endogenous

Table 1
Clinical parameters for control and NIDDM subjects

	Control	NIDDM	P	Normal range
Size (n)	15	39	–	–
Age (years)	33.4 ± 3.1	58.9 ± 2.3	<0.0001	–
Fasting glucose (mM)	4.93 ± 0.10	12.82 ± 0.09	<0.0001	3.3–5.6
HbA_{1c} (%)	–	11.17 ± 0.37	–	5.0–8.0
Triglycerides (mM)	1.02 ± 0.15	3.50 ± 0.60	0.0002	0.5–1.8
Cholesterol (mM)	5.34 ± 0.27	5.92 ± 1.11	0.61	2.3–6.9

Data are represented as the mean ± S.E.M.

8-epi-$PGF_{2\alpha}$ is independent of the cyclooxgenase pathway. These results also suggest that blood sampling for the routine analysis of 8-epi-$PGF_{2\alpha}$ can be done without the use of aspirin or indomethacin.

3.2. *Plasma 8-epi-$PGF_{2\alpha}$ as an index of oxidative stress in vivo*

Plasma from control and NIDDM subjects was analysed for the content of free as well as total of 8-epi-$PGF_{2\alpha}$ as an indicator of non-enzymatic lipid peroxidation. The analysis revealed no measureable levels of free 8-epi-$PGF_{2\alpha}$. The plasma samples, however, had substantial amounts of esterified 8-epi-$PGF_{2\alpha}$. The levels of 8-epi-$PGF_{2\alpha}$ in individuals with NIDDM (n = 39) varied from 0.49 to 2.16 nM whereas the corresponding levels for the controls (n = 16) ranged from 0.02 to 0.63 nM (Fig. 3). A representative GC-MS/NICI chromatogram of total F_2-isoprostanes isolated from plasma is shown in Fig. 4. Plasma levels of esterified 8-epi-$PGF_{2\alpha}$ among the diabetic subjects were significantly higher than those of the controls ($P < 0.0001$, Mann-Whitney test). Our data reveals that NIDDM is associated with increased lipid peroxidation.

Variation in age among the control and NIDDM individuals did not explain the differences in the levels of 8-epi-$PGF_{2\alpha}$ (Pearson's $r^2 = 0.0529$; $P = 0.19$ and $r^2 = 0.0144$; $P = 0.21$, for controls and NIDDM, respectively). Plasma 8-epi-$PGF_{2\alpha}$ in the diabetic group did not correlate with fasting glucose nor HbA_{1c} ($r^2 = 0.0064$; $P = 0.31$ and $r^2 = 0.0009$; $P = 0.43$, for controls and NIDDM, respectively). Moreover, there was no significant correlation between plasma 8-epi-$PGF_{2\alpha}$ and triglycerides or total cholesterol in the diabetic group ($r^2 = 0.0576$; $P = 0.24$ and $r^2 = 0.0169$; $P = 0.24$, respectively). These data suggest that increased plasma 8-epi-$PGF_{2\alpha}$ levels are not functions of hyperglycaemia or hyperlipidaemia.

F_2-isoprostanes may be generated in plasma which has undergone oxidative degradation during prolonged or improper storage. Morrow et al. showed that addition of 0.002% BHT inhibited the in vitro formation of F_2-isoprostanes in plasma by >90%, but did not suppress the levels originally present [1]. In this study, SPE and GC-MS analysis of plasma was carried out within 2 weeks following sample collection to minimise artefact formation during the storage of plasma at −70°C. The absence of free 8-epi-$PGF_{2\alpha}$ in the plasma of control and NIDDM individuals, and the fact that esterified plasma 8-epi-$PGF_{2\alpha}$ levels in the two groups of healthy subjects were not statistically different (0.34 ± 0.06 nM (n = 6) vs. 0.28 ± 0.04 nM (n = 15); $P = 0.43$) confirm the specificity, precision and reliability of our SPE/GC-MS assay.

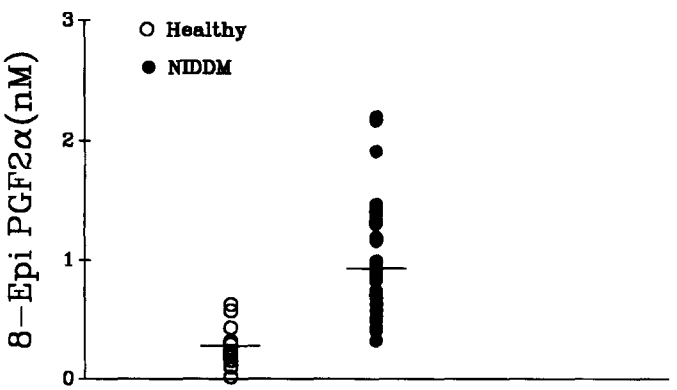

Fig. 3. Data distribution of total (sum of free and esterified) 8-epi-$PGF_{2\alpha}$ levels in plasma from controls (n = 15) and NIDDM (n = 39) subjects.

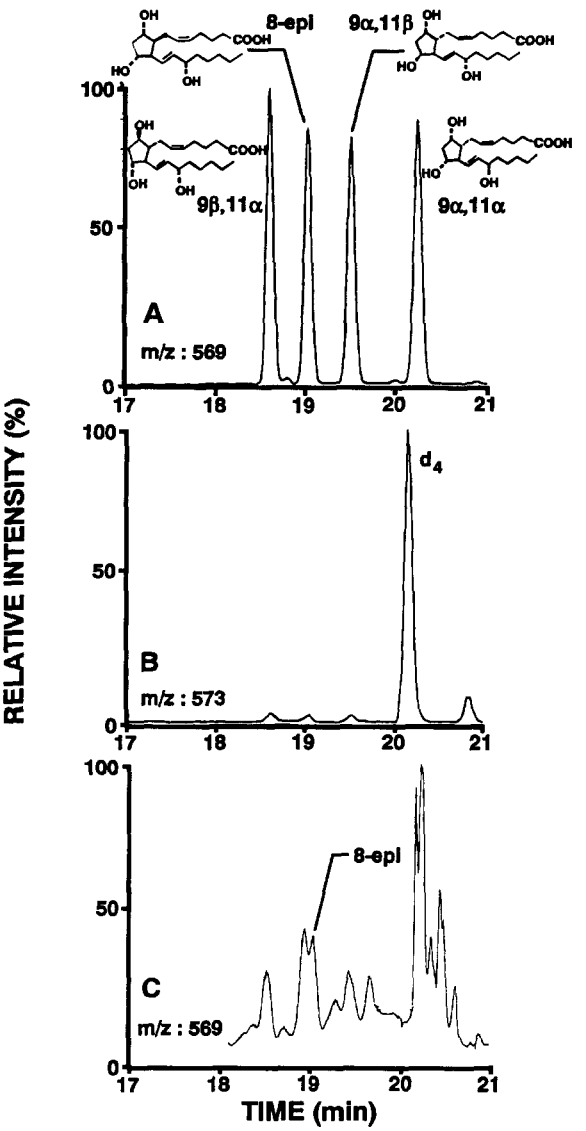

Fig. 4. GC-MS/NICI SIM chromatograms of (A) a standard mixture of PGF_2-like compounds; (B) the internal standard PGF_2-d_4 and (C) total (sum of free and esterified) F_2-isoprostanes isolated from an individual with NIDDM.

In conclusion, we have shown that the GC-MS/NICI analysis of esterified 8-epi-$PGF_{2\alpha}$ in plasma provides a specific and reliable marker of non-enzymatic peroxidation of arachidonic acid in vivo. We have also established that NIDDM is associated with increased plasma levels of esterified 8-epi-$PGF_{2\alpha}$. High levels of 8-epi-$PGF_{2\alpha}$ may contribute to the pathophysiology of NIDDM and may be related to long-term vascular changes, leading to further complications such as renal failure and accelerated atherosclerosis.

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