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Impact of diabetic polyneuropathy and cardiovascular autonomic neuropathy on the excretion of urinary 8-epi-PGF$_{2\alpha}$ and its metabolites (2, 3-dinor and 2, 3-dinor-5, 6-dihydro)

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Abstract

The objective of this study was to establish if diabetes in the presence of polyneuropathy (PN) and/or cardiovascular autonomic neuropathy (CAN) is associated with alterations in the amounts of 8-epi-PGF$_{2\alpha}$ (IP) and its metabolites including 2, 3-dinor-8-epi-PGF$_{2\alpha}$ (dinor-IP) and 2, 3-dinor-5, 6 dihydro-8-epi-PGF$_{2\alpha}$ (dinor-dihydro-IP) in urine. Mass spectrometric separation showed that excretion of IP was similar in the PN + /CAN− and PN+/CAN+ groups but higher than in the PN−/CAN− group ($n = 103$, 22 and 60, respectively; $P < 0.05$). By contrast, excretion of dinor-IP or dinor-dihydro-IP were similar in the PN−/CAN− and PN+/CAN− groups but higher than in PN+/CAN+ group. Correlations were obtained between IP and dinor-IP or dinor-dihydro-IP ($r = 0.30$; $P < 0.001$ and $r = 0.31$; $P < 0.001$, respectively). A significant association was also observed between dinor-IP and dinor-dihydro-IP ($r = 0.48$; $P < 0.001$). In conclusion, these biomarkers should prove useful in studies evaluating the impact of therapeutic drugs or antioxidant interventions aimed at delaying the onset of diabetic complications.

Keywords: *Diabetes, Neuropathy, Cardiovascular autonomic neuropathy, Oxidative stress, Isoprostanes*

Introduction

A growing body of evidence supports the theory that oxidative stress represents a biochemical trigger for neural dysfunction [1]. In animal models of experimental diabetes, it has been proposed that this is due to reduced endoneural blood flow [2]. Lipid peroxidation products such as malondialdehyde, 4-hydroxyalkenals and conjugated dienes are elevated in sciatic nerves from diabetic rats [3–7]. Diminished glutathione, vitamin E and ascorbic acid concentrations, and increased ratios of oxidized to reduced glutathione and dehydroascorbate to ascorbate have been observed in nerves from diabetic animals [8–11]. Superoxide dismutase (Cu/Zn SOD), calatase, glutathione peroxidase and quinone reductase activities are also reduced in sciatic nerves in diabetic rats [12,13]. Treatment of diabetic rats with insulin or antioxidants is associated with improved neural function [14–16].

Despite the evidence for increased lipid peroxidation products in animal models of diabetic neuropathy [3–7], data from patients with diabetic neuropathies is lacking. We have recently shown that plasma 8-epi-PGF$_{2\alpha}$ (IP) levels are increased in diabetic patients without polynueropathy (PN) and autonomic cardiovascular neuropathy (PN−/CAN−) compared to age-matched control subjects [17]. This finding was in agreement with those previously reported other investigators, employing similar GC-MS based assays [18,19]. However, no differences in plasma IP levels

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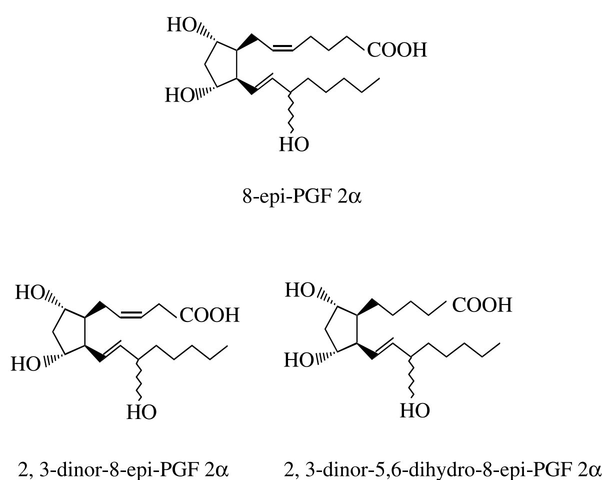

Figure 1. Structural differences between 8-epi-$PGF_{2\alpha}$ (IP), 2, 3-dinor-8-epi-$PGF_{2\alpha}$ (dinor-IP) and 2, 3-dinor-5, 6-dihydro-8-epi-$PGF_{2\alpha}$ (dinor-dihydro-IP).

were found in patients assigned to PN+/CAN− or PN+/CAN+ groups. A possible explanation for the absence of any differences in plasma IP concentrations, in the PN+/CAN− or PN+/CAN+ groups compared to the PN−/CAN− group could be the short half-life (about 16 min) of IP in blood with the result that the measurement of IP in plasma will only provide information regarding a discrete point in time [20].

The quantification of urinary of IP and its endogenous β-oxidation metabolites including 2, 3-dinor-8-epi-$PGF_{2\alpha}$ (dinor-IP) and 2, 3-dinor-5, 6-dihydro-$PGF_{2\alpha}$ (dinor-dihydro-IP) has been proposed as being superior to that of circulating IP as urinary levels represent an integrated index of systemic non-enzymatic lipid peroxidation [21,22]. Figure 1 shows structural differences between IP and its metabolites. In a previous study from this laboratory, it has been established that simultaneous measurement of urinary IP and its metabolites is achieved by a stable isotope-dilution gas-chromatographic-mass spectrometric procedure [23]. However, to date no information is available on the simultaneous measurement of urinary of IP, dinor-IP and dinor-dihydro-IP in diabetic patients or any other clinical condition associated with oxidative stress.

The objective of the present study was to examine impact of the presence or absence of PN and/or CAN on urinary excretion of IP, dinor-IP and dinor-dihydro-IP in diabetic patients.

Material and Methods

Reagents

Authentic 9α, 11α-PGF_2, 9α,11β PGF_2, 9β,11α PGF_2, 9α,11α-8epi-PGF_2, 9α,11α-15*R*-8epi-PGF_2, 9α,11α -15*R*-trihydroxy-2, 3-dinor-8-epi-prosta-13*E*-en-1-oic acid (2, 3-dinor-8-epi PGF_2), 3,3′,4,4′tetradeuterated 9α,11α-PGF_2 (PGF_2-d_4) and 3,3′,4,4′tetradeuterated 9α,11α-15*S*-8epi PGF_2 (8-epi-PGF_2-d_4) were obtained from SPI Bio (Massy Cedex, France). *N*,*O*-*bis*(trimethylsilyl)trifluoroacetamide (BSTFA), pentafluorobenzyl-bromide (PFB-Br), diisopropylethylamine (DIPEA) and butylated hydroxytoluene (BHT) were purchased from Sigma-Alderich Chemical Company (Poole, Dorset, UK). Aminopropyl (NH_2) and Silica (Si) cartridges (500 mg) were from Waters Corporation (Milford, MA, USA). 9α,11α-15*R*-trihydroxy-$^{5,6}\Delta$-dihydro-2,3-dinor-8-epi-prosta-13*E*-en-1-oic acid (2, 3-dinor-5, 6-dihydro-8-epi-PGF_2) was a gift from Dr Thierry Durand (Department of Pharmacy, University of Montpellier, Montpellier, France). All other general-purpose chemicals and organic solvents were of analytical grade and were from VWR International Ltd (Poole, Dorset, UK).

Studied population

Diabetes was classified according to the World Health Organisation/American Diabetes Association [24]. Inclusion criteria were type 1 or 2 diabetes and age >18 years. Exclusion criteria were: (1) neuropathy other than that of diabetic origin; (2) smokers or ex-smokers <1 year; (3) use of antioxidants (vitamin C, vitamin E, lipoic acid, β-carotene, probucol) or iron supplementation within the last 3 months; (4) peripheral arterial disease (intermittent claudication or non-palpable foot pulse); (5) history of coronary heart disease, myocardial infarction and heart failure; and (6) any medication that might adversely influence autonomic function. Patients were interviewed to collect data on demographics, diabetes type, diabetes duration, insulin treatment, medication, smoking habits and past history of neurological symptoms. Criteria for the diagnosis and staging of neuropathy and cardiovascular autonomic neuropathy were as described previously [25–27]. This study was conducted according to the principles of the Declaration of Helsinki as revised in 2000 and all patients provided informed written consent.

Clinical laboratory measurements

Glycosylated haemoglobin (HbA_{1c}) was measured using the high performance liquid chromatography (HPLC) technique (Diamat, Bio-Rad, Munich, Germany). Urinary albumin excretion rate was determined from 12-h samples using the immunonephelometric technique (Array Protein System, Beckman, Fullerton, CA, USA). Blood glucose was measured by a hexokinase-based method. Uricase based assay was employed for the determination of plasma uric acid (Boehringer Mannheim GmbH; Diagnostica & Biochemicals). Plasma and urinary creatinine were measured using a creatininase-based test (Boehringer Mannheim GmbH; Diagnostica & Biochemicals). Total plasma cholesterol and HDL cholesterol were measured using the Cholesterol-C

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high performance CHOD-PAP method (Boehringer Mannheim GmbH; Diagnostica & Biochemicals). Triglycerides were analysed by a GPO-PAP high-performance enzymatic colorimetric test (Boehringer Mannheim GmbH; Diagnostica & Biochemicals). LDL was calculated from total plasma cholesterol, triglycerides and HDL using the Friedewald formula as follows:

$$\begin{aligned} \text{LDL cholesterol (mmol/l)} \\ &= \text{Total cholesterol (mmol/l)} \\ &\quad - (\text{Triglyceride (mmol/l)}/2.19) \\ &\quad - \text{HDL} - \text{cholesterol (mmol/l)} \end{aligned}$$

Sample collection

Twelve-hour urine samples were collected in polyethylene bottles. Aliquots (10 ml) removed and stored at $-85^{\circ}C$ until analysed.

Isoprostane analysis

Urinary IP, dinor-IP and dinor-dihydro-IP excretions were analysed stable isotope dilution gas chromatography-mass spectrometry (GC-MS) as described previously [23]. Briefly, samples (2 ml) were mixed with 8-epi-$PGF_{2\alpha}$-d_4 (2.5 ng) as the internal standard and total lipids were partitioned with ethyl acetate (10 ml). The total lipid extracts were applied to NH_2 cartridges (500 mg) and isoprostanes eluted by washing the column with 5 ml of ethyl acetate/methanol/acetic acid (10/85/5, v/v/v). The final extracts from the NH_2 chromatography step were converted to pentaflourobenzyl (PFB) ester derivatives. Samples from the PFB-ester derivatisation step were applied to Si cartridges and isoprostanes eluted by washing the cartridge with 5 ml of ethyl acetate/methanol (95/5, v/v). Final determination was carried out by GC-MS using the negative ion chemical ionisation (NICI) with ammonia as reagent gas. Quantitative analysis IP and its metabolites as PFB-ester/TMS ether derivatives were performed using selected ion monitoring (SIM) of the carboxylate anion $[M-PBF]^-$ at m/z 541, 543, 569 and 573 for dinor-dihydro-IP, dinor-IP, IP and IP-d_4, respectively. Inter- and intra assay coefficients of variation for urinary IP were 5 and 7%, respectively.

Statistical analysis

Continuous data were expressed by the arithmetical mean ± SEM. Differences between groups were analyzed using parametric or non-parametric according to their distribution. Linear regression analysis was used to study associations between variables. The level of significance was set to $\alpha = 0.05$. Analyses were carried out using the SPSS for Windows (version 11) software package.

Results

Table I shows the demographic and clinical details of diabetic patients classified according to the presence or absence of PN and/or CAN. The mean of age of PN−/CAN− group was lower and diabetes duration shorter compared with PN+/CAN− group or PN+/CAN+ group. In addition, triglyceride levels were lower in the PN−/CAN− group. PN−/CAN− group also had a trend for slightly lower glucose and better glycaemic control (HbA_{1c}) than those with PN+/CAN− or PN+/CAN+ but these differences did not achieve statistical significance. Plasma creatinine concentrations were similar in the PN+/CAN− and PN+/CAN+ groups but slightly higher than in the PN−/CAN− group.

Table I. Clinical characteristic of diabetic patients segregated according to presence or absence of polyneuropathy (PN) and/or cardiovascular autonomic neuropathy (CAN).

Variables	PN−/CAN−	PN+/CAN−	PN+/CAN+
Counts	60	103	22
Gender (m/f)	21/39	58/44	12/10
BMI (kg/m^2)	27.68 ± 0.66	28.29 ± 0.51	26.70 ± 0.98
Age (Years)	42.60 ± 1.98	58.56 ± 1.13*	54.36 ± 2.86†
Diabetes duration (Years)	7.30 ± 0.88	12.04 ± 1.00*	18.52 ± 2.00†,‡
Glucose (mmol/l)	9.98 ± 0.36	10.72 ± 0.27	11.01 ± 0.61
HbA1c (%)	9.09 ± 0.25	9.67 ± 0.18	9.77 ± 0.33
Type 1/Type 2	30/30	23/80	8/13
Triglycerides (mmol/l)	1.85 ± 0.33	1.99 ± 0.11*	2.24 ± 0.31†
Cholesterol (mmol/l)	5.25 ± 0.15	5.80 ± 0.11*	5.54 ± 0.22
HDL-cholesterol (mmol/l)	1.33 ± 0.04	1.27 ± 0.05	1.28 ± 0.12
LDL-cholesterol (mmol/l)	3.14 ± 0.14	3.62 ± 0.10*	3.23 ± 0.27
Plasma creatinine (μmol/l)	65.12 ± 1.79	71.84 ± 2.26*	74.74 ± 10.05
Urinary creatinine (mmol/l)	6.42 ± 0.61	5.57 ± 0.39	5.11 ± 0.92
Uric acid (μmol/l)	294 ± 11.36	315 ± 9.61	321 ± 20.26
Albuminuria (no/yes)	49/10	60/40	9/12
Hypertension (no/yes)	42/15	40/61	10/12

Values represent mean ± SEM.
* PN−/CAN− vs PN+/CAN−; $p < 0.05$. † PN−/CAN− vs PN+/CAN+; $p < 0.05$. ‡ PN+/CAN− vs PN+/CAN+; $p < 0.05$.

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The differences, however, failed to reach statistical significance. On the contrary, microalbuminuria was more frequent in the PN+/CAN and PN+/CAN+ groups than in the PN−/CAN− group.

Figure 2 shows a typical $[M-PFB]^-$ chromatogram of urinary IP and its metabolites following GC-MS analysis. The traces at *m*/*z* 541, 543 and 569 represent dinor-dihydro-IP, dinor-8-IP and IP, respectively. The chromatogram at *m*/*z* 573 represents the tetraduerated 8-epi-$PGF_{2\alpha}$ (IP-d_4) as the internal standard. Identification of the components in the samples were based on comparison of relative

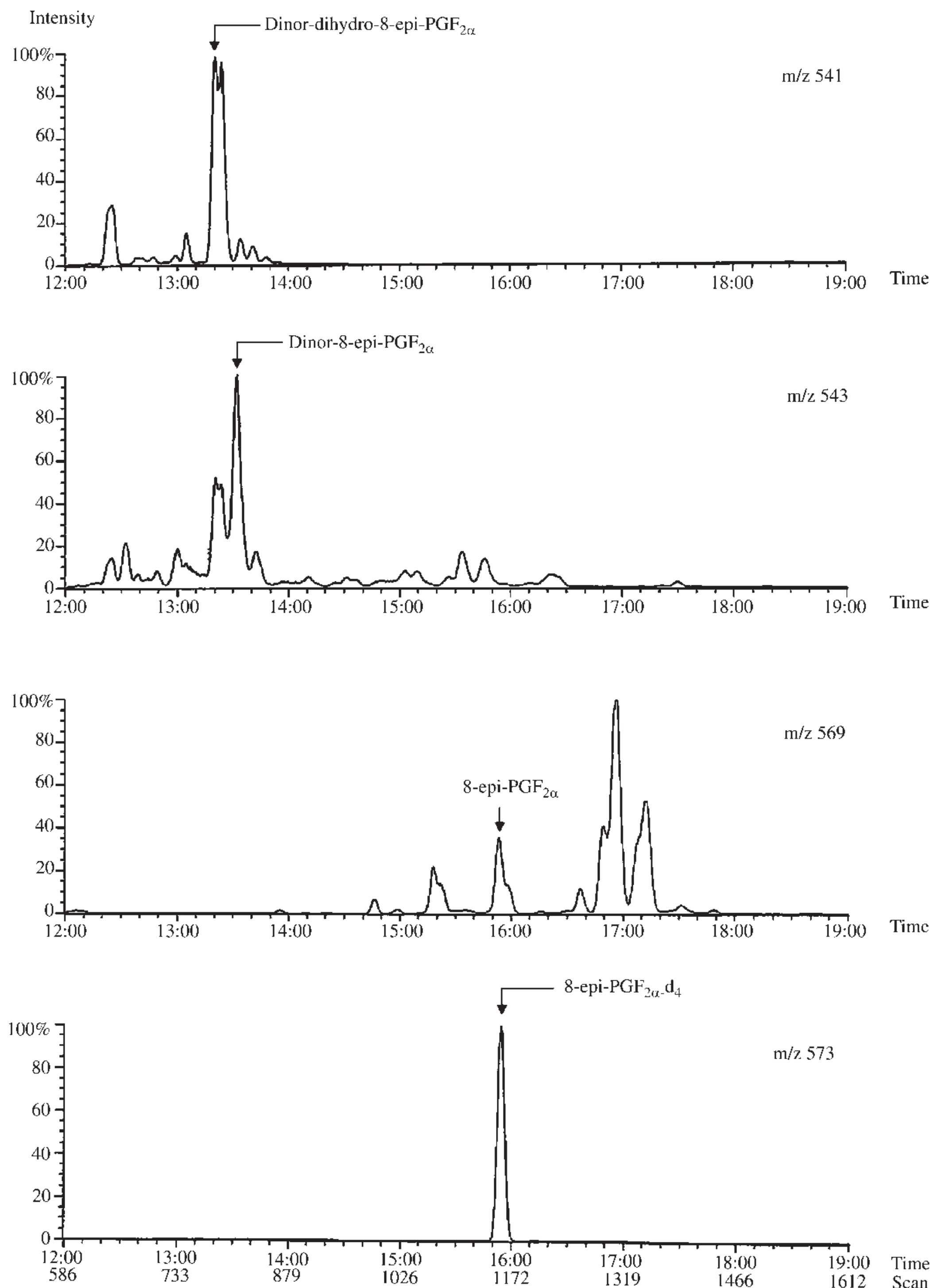

Figure 2. Gas chromatographic separation of urinary PGF_2-like compounds as the PBF-ester/TMS ether derivatives following total lipid with ethyl acetate and chromatography on aminopropy (NH_2) and silica (Si) cartridges. First trace at *m*/*z* (541), second (*m*/*z* 543), third (*m*/*z* 569) and fourth (*m*/*z* 573) represent 2, 3-dinor-5, 6-dihydro-8-epi-$PGF_{2\alpha}$, 2, 3-dinor-8-epi-$PGF_{2\alpha}$, 8-epi-$PGF_{2\alpha}$ and tetradeutrated 8-epi-$PGF_{2\alpha}$ as the internal standard, respectively.

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retention times relative to that of an internal standard as well as using a variety of chemical approaches as previously described [28].

Urinary excretion of IP was similar in the PN+/CAN− and PN+/CAN+ groups but higher than in the PN−/CAN− group (0.26 ± 0.06 nmol/mmol creatinine and 0.29 ± 0.08 nmol/mmol creatinine vs 0.16 ± 0.02 nmol/mmol creatinine; $P < 0.05$). There was no difference in dinor-IP excretion between patients assigned to PN+/CAN− and PN−/CAN− groups (4.98 ± 1.05 nmol/mmol creatinine and 4.85 ± 0.74 nmol/mmol creatinine). On the other hand, the PN+/CAN+ groups yielded a value of 2.41 ± 0.53 nmol/mmol creatinine. In the case of dinor-dihydro-IP, no difference was seen between PN+/CAN− and PN−/CAN− groups (1.38 ± 0.20 nmol/mmol creatinine and 1.38 ± 0.32 nmol/mmol creatinine). The PN+/CAN+ group produced a value of 0.86 ± 0.24 nmol/mmol creatinine. Figure 3 shows the excretion of IP and it metabolites in diabetic patients classified according to the presence of PN and/or CAN.

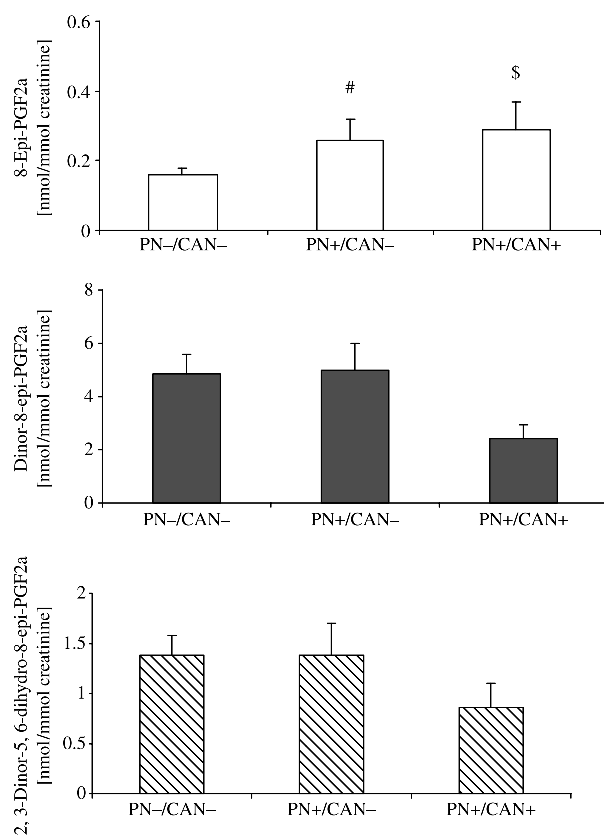

Figure 3. Excretion rates for dinor-8-epi-PGF$_{2\alpha}$, 2, 3-dinor-5, 6-dihydro-8-epi-PGF$_{2\alpha}$ and their precursor 8-epi-PGF$_{2\alpha}$ in diabetic patients classified according to the presence and absence of PN and/or CAN. The PN−/CAN−, PN+/CAN− and PN+/CAN+ groups comprised 60, 103 and 22 patients, respectively. Data are presented as the mean ± S.E. $ PN−/CAN− vs PN+/CAN−; $p < 0.05$. # PN−/CAN− vs PN+/CAN+; $p < 0.05$.

No correlations were seen between IP, dinor-IP or dinor-dihydro-IP with body mass index, age, duration of diabetes, glucose, HbA$_{1c}$, total cholesterol, LDL-cholesterol, triglycerides or plasma creatinine. However, significant correlations were obtained between IP and dinor-IP with HDL-cholesterol levels ($r = 0.191$; $P < 0.01$ and $r = 0.153$; $P < 0.05$). Significant correlations were also found between IP and dinor-IP or dinor-dihydro-IP ($r = 0.296$; $P < 0.001$ and $r = 0.308$; $P < 0.001$). Furthermore, there was a correlation between dinor-IP and dinor-dihydro-IP ($r = 0.477$; $P < 0.001$).

Discussion

Accurate methods for the assessment of oxidative stress *in vivo* are prerequisite for examining the relationship between measures of oxidative stress and diabetic complications. In this study, a reliable and sensitive stable isotope dilution GC-MS procedure has been employed for the simultaneous measurement of urinary IP, dinor-IP and dinor-dihydro-IP as an index of oxidative stress in diabetic patients with neuropathies.

Products of lipid peroxidation exert adverse effects on a variety of processes such as inhibiting anti-thrombin III activity, producing procoagulant activity, enhancing platelet aggregation, modulating vascular responses and acting as mitogens [29]. Increased formation of lipid peroxidation products is shown to be associated with neuronal damage in experimental diabetic neuropathy [30]. In the present study, it was found that dinor-IP was the major metabolite of IP whilst the parent compound 8-epi-PGF$_{2\alpha}$ was only a minor component. This confirms previous data obtained from healthy control subjects [23] and that of Chiabrandos et al. [31] that dinor-IP is the major urinary metabolite of IP in humans.

This study has revealed that the presence of PN in diabetic patients (i.e. PN+/CAN− group) was associated with a marked elevation (63%) in the excretion of IP when compared to those without PN (i.e. PN−/CAN−). Excretion of IP was, however, not altered by the additional occurrence of CAN (i.e. PN+/CAN+ group). The observed elevation in of urinary of IP in patients assigned to PN+/CAN− and PN+/CAN+ groups is unlikely to be explained by deterioration in renal function because only a slight increase (10%) was seen in plasma creatinine levels between the PN+/CAN− and PN−/CAN− groups and that the additional occurrence of CAN− was not associated with further changes in plasma creatinine levels (Table I). These data provide support for the notion that the observed elevation in of urinary of IP in patients assigned to PN+/CAN− and PN+/CAN+ groups may reflect increased oxidative stress.

In line with our findings, Davi et al. [32] reported that excretion rates of IP were similar in type 1 and 2 diabetic patients, despite the group type 2 diabetic

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patients having more individuals with hypertension and microvascular complications. A 37% reduction in the urinary excretion of IP in type 2 diabetic subjects following vitamin E supplementation (600 mg daily for 2 weeks) was also observed. On the other hand, Devaraj et al. [33] measured urinary IP in type 2 diabetics with and without macrovascular complications. In these patients, IP excretion rates were found to be higher in patients with macrovascular complications than in those without complications. Moreover, it was shown that dietary supplementation with α-tocopherol (1200 U/day) for 3 months led to a 50% reduction in IP concentrations. Taken together, these findings would imply that the measurement of urinary IP is a reliable marker of systemic non-enzymatic lipid peroxidation in human.

Another observation from this study is that excretion of dinor-IP and dinor-dihydro-IP was similar in the PN−/CAN− and PN+/CAN− groups. By contrast, the presence of CAN was linked to reductions in the excretion of dinor-IP and dinor-dihydro-IP (38 and 45%, respectively). The reductions in dinor-IP and dihydro-IP, however, did not achieve statistical significance possibly because of the considerable intra-individual variation in the values obtained for the three groups of diabetic patients. It is of note that large degrees of variance (up to 15-fold) in urinary excretion rates for various prostaglandin-metabolites have been reported by several investigators [34–42]. The observed decline in dinor-IP and dinor-hydro-IP in the PNP+/CAN+ group is not explained by impaired renal function as both subgroups, i.e PNP+/CAN− and PNP+/CAN+ were matched with respect to plasma creatinine concentrations as well as the prevalence of individuals with albuminuria and hypertension. These data suggest that the observed alterations in urinary dinor-IP and dinor-dihydro-IP in the PN+/CAN+ group reflect impaired degradation of the parent compound as a consequence of increased oxidative stress.

Moreover, variations in the ratios of dinor-dihydro-IP or dinor-IP to IP were seen in relation to the occurrence of PN and/or CAN. In the PN−/CAN− group, the ratio of dinor-dihydro-IP to IP was 8.6. This ratio declined to 5.3 in the presence of PN and was further reduced to 3.1 in the presence of additional CAN. The respective values for the ratio of dinor-IP to IP were 30.3, 18.7 and 9.3. These findings indicate that simultaneous measurement of dinor-IP and dinor-dihydro-IP and their precursor the 8-epi $PGF_{2\alpha}$ (i.e. IP) is required in order to obtain an accurate picture of the systemic non-enzymatic lipid peroxidation in clinical settings linked oxidant injury.

In summary, it has been established that dinor-IP is the predominant endogenous β-oxidation product derived from the 8-epi-$PGF_{2\alpha}$ in diabetic patients with and without neurological complications. Importantly, a divergence in the excretion of IP and its metabolites was observed, with increased excretion of IP in those patients with diabetic PN and/or CAN when compared to those without neurological complications, but reduced excretion of its metabolites in the PN+/CAN+ group than in those assigned to PN+/CAN− or PN−/CAN− groups. These biomarkers should prove useful in studies examining the role of oxidant injury in human disease.

Acknowledgements

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