

Urinary isoprostane excretion is not confounded by the lipid content of the diet

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Abstract This study aims to determine if isoprostanes accurately reflect *in vivo* lipid peroxidation or whether they are influenced by the lipid content of the diet. Isoprostanes were measured in urine of healthy subjects under different conditions of lipid intake and under conditions of oxidative stress (fasting). We found that isoprostanes were not influenced by the lipid content of the diet: the urinary level remained constant over 24 h as well as over 4 consecutive days when switching from high to low lipid intake. Urinary isoprostane excretion was increased by 40% following a 24 h fast. We concluded that urinary isoprostane excretion reflects endogenous lipid peroxidation *in vivo*.

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Key words: Metabolism; Isoprostane; 8-iso-Prostaglandin $F_2\alpha$; Lipid peroxidation; Malondialdehyde

1. Introduction

The detection and measurement of lipid peroxidation has been most frequently used to support the involvement of free radical reactions in pathophysiological conditions. Lipid peroxidation involves a cascade of events. The oxidation process of polyunsaturated fatty acids (PUFAs) is characterized by several parameters such as the disappearance of PUFAs, the appearance of radicals within the PUFAs, the formation of conjugated dienes and lipid peroxides which are rather unstable molecules or the formation of aldehydes or alkanes. A number of methods have been developed to measure these compounds [1]. However, each method has its limitations and pitfalls and is often not applicable to all circumstances, especially when applied to human samples. Indeed, these methods are often neither specific nor sensitive enough to monitor small changes in free radical status and lipid peroxidation *in vivo*. Therefore, the development of more specific and sensitive assays to detect and better represent *in vivo* rates of lipid peroxidation as an index of free radical generation and oxidative stress is desirable [2].

In recent years, the measurement of isoprostanes, products of arachidonic acid formed by free radical-catalyzed reactions, appears to be a promising assay that might be specific and sensitive enough to detect *in vivo* lipid peroxidation [3]. The isoprostanes are a series of prostaglandin-like compounds,

which are formed from oxidation of arachidonic acid present in phospholipids by free radical-mediated reactions [4], but mainly independent of cyclooxygenase enzymes. In 1990, Roberts and co-workers found that arachidonyl containing phospholipids in plasma can undergo autooxidation upon storage, yielding isoprostanes [5]. Later work established that these compounds could be detected in human plasma, in urine and in tissues [3]. Morrow et al. reported the detection of one class of these compounds *in vivo*, the F_2 -isoprostanes, using gas chromatography/mass spectrometry (GC/MS) [3]. F_2 -isoprostanes, in particular 8-iso-prostaglandin $F_2\alpha$ (8-iso-PGF $_2\alpha$) [3], are some of the most abundant forms of isoprostanes. Other isoprostanes, such as D_2 and E_2 -isoprostanes, are formed by the same non-cyclooxygenase pathway [6].

Malondialdehyde (MDA), the classical marker of lipid peroxidation, was demonstrated to vary in response to absorption of oxidized lipids present in the diet [7,8]. Therefore, MDA loses its usefulness as candidate of choice to assess the *in vivo* lipid peroxidation in situations where the diet is changed. In the case of isoprostanes, accumulated evidence indicates that isoprostanes are produced in *in vivo* situations associated with oxidative stress and free radical production such as in the hepatorenal syndrome [9], in scleroderma [10], in smokers [11], in interstitial lung disease [12], in ozone exposure [13], in aging [14], in diabetes [15] and in kidney disease [16]. The level of isoprostanes may be down-regulated with the administration of antioxidants [17–19]. However, the impact of a diet on isoprostane excretion is not yet known. Therefore, the aim of this study is to evaluate, in healthy subjects, the urinary excretion of isoprostanes in response to intake of a low fat diet and after 24 h of fasting. We demonstrate here that the measurement of urinary isoprostane levels provides a non-invasive *in vivo* index of free radical generation that appears not to be confounded by changes in the diet.

2. Materials and methods

2.1. Materials

9 α , 11 α -8 isoprostane and 3,3',4,4'-tetradeuterated 9 α , 11 α -prostaglandin F_2 (PGF $_2$ -d4) were obtained from Cayman (MI, USA) and 1,3-diethyl-2-thiobarbituric acid was obtained from Sigma (St. Louis, MO, USA). C $_{18}$ Bakerbond solid phase extraction (SPE) and a silica Bakerbond SPE were purchased from Baker (The Netherlands) and the LK6D silica gel thin layer chromatography (TLC) plates from Whatman (Clifton, NJ, USA). All commercially available chemicals and reagents were of analytical grade or greater purity.

2.2. Study design

Four normolipidemic healthy males (age 35 $\pm$ 1 years, weight 73 $\pm$ 10 kg, BMI 23 $\pm$ 3 kg/m 2 ; mean $\pm$ S.D.) participated in this study. They fulfilled the following criteria: normal physical examination, no

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Abbreviations: PUFA, polyunsaturated fatty acid; GC, gas chromatography; MS, mass spectrometry; MDA, malondialdehyde; SPE, solid phase extraction; PFB, pentafluorobenzyl; TLC, thin layer chromatography; TMS, trimethylsilyl

metabolic disorder, fasting plasma triglycerides below 150 mg/dl, fasting plasma cholesterol below 5.19 mM. The volunteers received no medication from at least 2 weeks before the beginning of the study until the end of the study. The protocol was approved by the Ethical Committee of Nestlé. All volunteers signed informed consent before entering the study. Body weight, dietary habits and physical activity remained constant throughout the study period.

Each volunteer participated in two studies.

Effect of dietary lipid intake. The first study was conducted on four consecutive days, the volunteers ate their usual diet on day 1, a standardized low fat diet on day 2 and day 3 and then returned to their regular diet on day 4. The calory supply of the standardized regimen was designed to be similar to the normal meal (2000 calories/day). The major modification was a reduction of the total dietary fat content from 35 to 5% of total energy, for regular vs. standardized diet, respectively. The latter was achieved by replacing fat with carbohydrate.

Effect of fasting. In the second study, the volunteers ate their regular diet on day 1, went through a 24 h fasting on day 2 and returned to their regular diet on day 3.

Urine was collected over 24 h in five different containers according to time (fraction 1: 7–10 h, fraction 2: 10–14 h, fraction 3: 14–18 h, fraction 4: 18–22 h, fraction 5: 22–7 h). The volume of each fraction was recorded and an aliquot of 25 ml was stored at -80°C until analysis. The reconstitution of the 24 h urine sample was performed by pooling 10% of each of the five urine samples collected over the day. This 24 h reconstituted urine was then stored at -80°C until analysis.

2.3. Analytical determination

MDA was measured as the MDA-diethylthiobarbituric acid complex [20]. The complex was separated on a 30×3.9 mm I.D. Nova-Pak C_{18} column from Millipore (Volketswil, Switzerland) and quantified with a Waters 490E multiwavelength detector (Millipore, Milford, MA, USA) at 539 nm.

Isoprostane was determined by a method described by Morrow and Roberts [21] and Tsikas et al. [22] with some modifications. In a Pyrex tube, 2 ng of $[^2\text{H}_4]\text{PGF}_2\alpha$ was added as internal standard and mixed with 10 ml of water pH 3 and 5 ml of urine. The mixture was adjusted to pH 3 with 1 N hydrochloric acid. A C_{18} Bakerbond SPE was pre-conditioned with 5 ml of methanol and 5 ml of water pH 3. The sample was loaded on this column, rinsed with 10 ml water pH 3 and 10 ml heptane. The sample was eluted with 10 ml ethyl acetate/heptane (1/1) and dried over anhydrous sodium sulfate. A silica Bakerbond SPE was then pre-conditioned with 5 ml of ethyl acetate. The sample from the C_{18} column was then loaded on this silica column, rinsed with 5 ml ethyl acetate, eluted with 5 ml ethyl acetate/methanol (1/1) and then dried under a nitrogen stream. 8-iso- $\text{PGF}_2\alpha$ was derivatized to pentafluorobenzyl (PFB) esters by the addition of 40 μl 10% PFB bromide in acetonitrile and 20 μl 10% *N,N*-diisopropylethylamine in acetonitrile followed by a 20 min incubation at 37°C . The mixture was then dried under a nitrogen stream and this derivatization procedure was repeated. The residue was treated with 200 μl of water and extracted twice with 1 ml of diethyl ether. The organic phases were decanted, combined and the solvent was evaporated. The sample was resuspended in 50 μl of methanol/chloroform (3/2) and subjected to a TLC using Whatman LK6D silica gel TLC plates with the solvent

chloroform/ethanol (97/3). For the standard, 3–5 μg $\text{PGF}_2\alpha$ derivatized as PFB esters was spotted on a separate TLC plate and visualized by spraying with a 10% solution of phosphomolybdic acid in ethanol followed by heating. By comparison with the migration distance of the standard, compounds on the TLC plate containing the samples migrating in the region of $\text{PGF}_2\alpha$ (R_f about 0.23) and 1 cm on either side were scraped off and extracted twice with 1 ml ethyl acetate, centrifuged and dried under a nitrogen stream. The formation of trimethylsilyl (TMS) ether derivatives was performed by the addition of 20 μl *N,O*-bis(TMS)trifluoroacetamide and 10 μl dimethyl formamide, incubated at room temperature overnight. Then, the samples were dried under a nitrogen stream and finally reconstituted in 50 μl undecane.

The GC separations were performed using a HP-5890 gas chromatograph equipped with a HP-7673 autosampler (HP, Geneva, Switzerland). The column was a J&W Sci DB-1701 capillary column 30 m $\times$ 0.32 mm I.D. with a 0.25 μm film thickness (MSP-Friedli, Koeniz, Switzerland). The oven heating was performed as follows: 90°C (1 min), $30^{\circ}\text{C}/\text{min}$ up to 220°C , $2^{\circ}\text{C}/\text{min}$ up to 280°C , then, 15°C up to 300°C . Helium was used as carrier gas at a pressure of 10 psi. Samples (2–5 μl) were injected in a cold on-column injector.

The MS and MS/MS experiments were carried out using a Finnigan TSQ-700 mass spectrometer (Finnigan MAT, Bremen, Germany) working in negative ionization mode at 200 eV using ammonia as reagent gas and a source temperature set at 180°C . GC/MS analyses were done after selective monitoring of the $[\text{M}-181]^-$ of the derivatives of each compound. For the tandem mass spectrometric experiments (GC/MS/MS), argon was used as collision gas, set to between 1 and 1.5 mTorr. A collision energy of 30 eV in the laboratory frame was used. The transition $[\text{M}-181]^- \rightarrow [\text{M}-181-3 \times 90]^-$ was recorded for each compound of interest.

Creatinine was measured by the modified Jaffé reaction procedure using commercial kits (Sigma Diagnostics, Deisenhofen, Germany).

2.4. Statistical analysis

This is a pilot study with a low number of subjects involved ($n=4$). The data were analyzed by ANOVA with repeated measures using Systat v 7.0 (SPSS, Zürich, Switzerland).

3. Results and discussion

3.1. Effect of urine sampling

Urine collection is simpler than plasma sampling because it is a non-invasive procedure, sample handling and storage are easier, and there is no evidence of artefactual formation of isoprostanes during handling and storage, unlike plasma [5]. However, a 24 h urine collection required good compliance of the volunteers and cannot be performed in most clinical situations while a single sample of urine is usually easy to obtain. Isoprostane and MDA concentrations were determined in urine samples, either those which were collected in the morning or in the 24 h reconstituted samples. A good correlation between the two sets of urine samples was obtained for

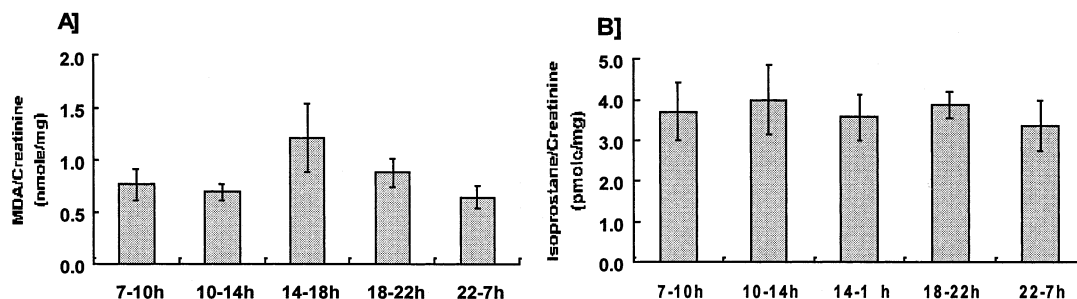


Fig. 1. MDA (A) and isoprostane (B) excretion in urine samples collected over 1 day. Urine was collected over 24 h in five different containers according to time: fraction 1: 7–10 h, fraction 2: 10–14 h, fraction 3: 14–18 h, fraction 4: 18–22 h, fraction 5: 22–7 h. Results are expressed as mean $\pm$ S.E.M.

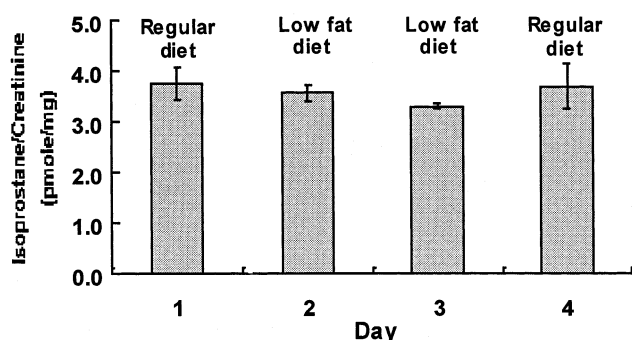


Fig. 2. Lack of an effect of low dietary lipid intake on urinary isoprostane excretion. The volunteers ate their regular diet (35% of total energy as lipids) on day 1, a standardized low fat diet (5% of total energy as lipids) on day 2 and day 3 and then returned to their regular diet on day 4. Results are expressed as mean $\pm$ S.E.M.

isoprostanes ($y = 0.82x + 0.67$, $r^2 = 0.89$, $n = 20$) and MDA ($y = 1.59x + 0.62$, $r^2 = 0.85$, $n = 27$). In this study, we provide strong evidence that isoprostanes determined in the urine collected in the morning of the day adequately represent the daily isoprostane excretion.

3.2. Effect of low lipid dietary intake

The assessment of *in vivo* lipid peroxidation is very problematic because the question arises whether the chosen biomarker reflects only the *in vivo* lipid peroxidation of the subject or whether it reflects also the absorption of oxidized lipid products produced in the gut and/or present in the diet [7,8]. We, therefore, evaluated the isoprostane concentration in different dietary conditions.

The urinary isoprostane excretion was determined in the five urine samples collected at different time intervals over 1 day (Fig. 1). When entering the study, subjects exhibited an isoprostane excretion of 3.428 ± 0.829 pmol/mg creatinine, which is in agreement with that previously described for healthy subjects [22]. The level of isoprostane excretion was similar in all these five consecutive urine samples collected over 24 h. In contrast, the level of MDA in the 14–18 h sample (corresponding to the post-lunch period) tended to be higher in comparison to the other four urine samples. The MDA data are in agreement with data previously described in the literature [7,8], showing that dietary MDA is

absorbed. These results demonstrate that daily isoprostane excretion is not affected by the dietary intake in contrast to MDA.

To evaluate the effect of dietary lipid intake on the excretion of isoprostanes, volunteers were switched from their regular diet containing 35% fat as energy to an isocaloric-controlled diet containing 5% fat as energy for 2 consecutive days. At the beginning of the study, the urinary isoprostane level was around 3.736 ± 0.314 pmol/mg creatinine (Fig. 2). Interestingly, the inter-subject variability of the isoprostane excretion was rather low, indicating that this group of four subjects was rather homogenous. When the subjects consumed the standardized low fat diet, their isoprostane excretion was not significantly modified. When subjects returned to their usual diet, the isoprostane level in urine remained stable. These results demonstrate that isoprostane excretion is not affected by a marked reduction of the dietary lipid intake.

3.3. Effect of 1 day fasting

Fasting over 1 day is associated with lipolysis in adipose tissue. After 1 day fasting, isoprostane excretion increased significantly to 4.409 ± 0.547 pmol/mg creatinine ($P < 0.005$) in all studied subjects (Fig. 3). On day 3, when the subjects consumed their regular diet, isoprostane excretion returned to the level observed on day 0. The urinary MDA excretion profile over the studied period mirrored that of isoprostanes. Indeed, MDA started at 0.9 mmol/mg creatinine on day 0, decreased by 50% on day 2 and returned back to initial values on day 3. The increase of isoprostanes during fasting could be due to the induction of an oxidative stress and/or due to the release of isoprostanes from adipose tissue.

4. Conclusions

Evidence is growing that isoprostanes may represent a reliable and sensitive marker of *in vivo* lipid peroxidation that can be assessed in plasma, urine and tissues. For the first time, we demonstrate one of the criteria needed to establish isoprostanes as a reliable biomarker for assessing *in vivo* lipid peroxidation because they are not affected by the lipid content of the diet. Therefore, isoprostanes may be used in the future to characterize the status of lipid peroxidation in different *in vivo* situations such as in premature infants, in exercising subjects, in ageing, in skin but also to evaluate the efficacy of anti-

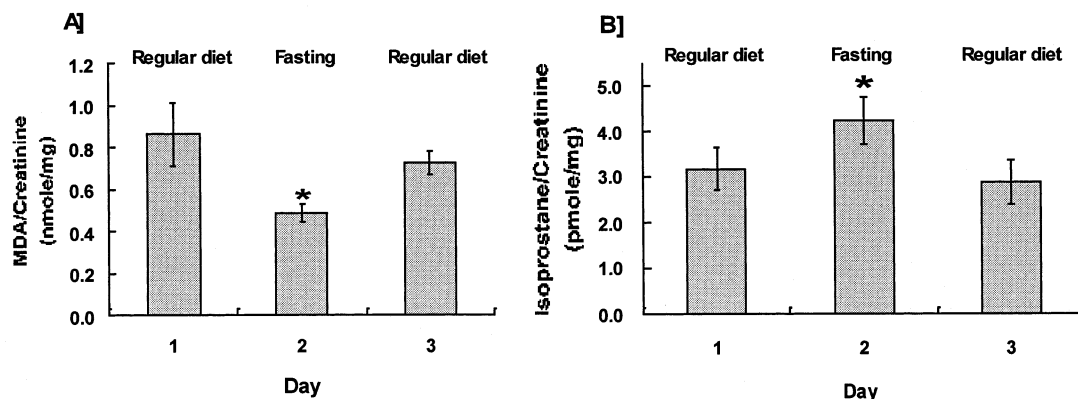


Fig. 3. Effect of 24 h fasting on urinary MDA (A) and isoprostane (B) excretion. The volunteers ate their regular diet on day 1, went through a 24 h fasting on day 2 and returned to their regular diet on day 3. Results are expressed as mean $\pm$ S.E.M.

oxidant molecules or cocktails to prevent or to reduce in vivo lipid peroxidation.

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