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Smoking and oxidant stress: assay of isoprostane in human urine by gas chromatography–mass spectrometry

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Abstract

Isoprostane (8-epi-prostaglandin F2a) is synthesized non-enzymatically from arachidonate and active oxygen. We examined the relationship of smoking and excretion of isoprostane in urine with gas chromatography–mass spectrometry selected ion monitoring assay and the stable isotope dilution method. Urine isoprostane concentrations were significantly higher in smokers ($n=81$, 605.24 ± 59.01 ng/mg creatinine) than in non-smokers ($n=39$, 424.07 ± 70.37 ng/mg creatinine), but concentrations in ex-smokers ($n=21$, 487.27 ± 98.48 ng/mg creatinine) did not differ significantly from those in the other groups. In smokers, age, the duration of smoking, and the number of cigarettes per day were not correlated with urine isoprostane concentrations. However, urine isoprostane concentrations were negatively correlated with time since quitting in ex-smokers and with age in non-smokers. These results indicate that smoking increases isoprostane concentration in urine and suggest that smoking causes lipid peroxidation by oxidant stress.

Keywords: Isoprostane

1. Introduction

The production of isoprostane in stored medical specimens through non-cylooxygenase oxidation has been reported by Morrow et al. [1]. However, free radicals may also induce generation of isoprostanes in vivo [2]. Arachidonate easily accepts the free radical or superoxide, resulting in lipid peroxidation [3] and increased levels of isoprostane [4]. We have reported that mice exposed to very low doses of radiation have increased isoprostane levels in plasma [5]. This finding suggests that isoprostane might be an indicator of free radical or oxidant stress [6]. F2-type isoprostane, 8-epi PGF2α, mimics the action of thromboxane A2 by stimulating the thromboxane A2 receptor [7,8]. Oxidant stress, which induces isoprostane to be continuously generated or to reach excessive levels or both, should therefore affect the thromboxane receptor.

We examined the relationship of smoking to

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isoprostane generation by assaying the concentration of isoprostane in the urine of smokers, non-smokers and ex-smokers.

2. Method

Sample preparation and derivatization were modified for simultaneous analysis of prostaglandins by gas chromatography–mass spectrometry (GC–MS) as described by Nagakura et al. [9] and Obata et al. [10].

2.1. Materials

All organic solvents (Nacalai Tesque, Kyoto, Japan) were of high-performance liquid chromatography or analytical-reagent grade. Dimethyl isopropylsilyl imidazole was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). An ethereal solution of diazomethane was prepared from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Tokyo Kasei Kogyo). Deuterated 8-epi PGF2α was purchased from Cayman (Ann Arbor, MI, USA). A Chem-tube hydromatrix (purified Keisel gel, 10-ml capacity) was purchased from Varian (Harbor City, CA, USA). Cartridges of octadecyl silica (Sep-Pak C_{18}, 500 mg per cartridge) were purchased from Waters (Milford, MA, USA). Silica gel (Silica gel 60 extrapure, 70–230 mesh, E. Merck, Darmstadt, Germany) was purchased from Nacalai Tesque. Other reagents were of analytical-reagent grade and commercially available.

2.2. Sample collection

Urine was collected with informed consent from volunteers who had answered questionnaires about smoking habits (smoking history; duration of smoking, number of cigarettes smoked per day), age, and sex. Collected urine was stored at −40°C. Within 2 weeks, the urine was assayed for isoprostane and creatinine content. The urine isoprostane content was examined in 141 men which included smokers (n=81), non-smokers (n=39), and ex-smokers (n=21).

2.3. Extraction from urine

Urine was thawed and divided into aliquots for isoprostane and creatinine assays. Isoprostane extraction and derivatization for GC–MS were performed with the modified method of Nagakura et al. [9]. Briefly, urine specimens (2.5 to 5 ml) were diluted to 10 ml with distilled water. An internal standard (d4-8-epi PGF2α, 1 ng per sample) was added, and the pH was adjusted to 3.0 with 2 *M* HCl. Diluted samples were applied to a Chem-tube hydromatrix (purified Kiesel gel) column to absorb the aqueous solution, and isoprostane was eluted with ethyl acetate (30 ml). The organic solvent was evaporated in a centrifugal concentrator in vacuo. The precipitate of the extract was dissolved in 5 ml of 15% (v/v) ethanol and applied to a SepPak C_{18} mini-column. The column was washed with 10 ml of 15% (v/v) ethanol and light petroleum, and isoprostane was collected with 8 ml of ethyl acetate elution. The organic solvent was evaporated in a centrifugal concentrator in vacuo.

2.4. Clean-up and derivatization

Samples were purified and derivatized with the modified method of Nagakura et al. [9].

Isoprostane was derivatized to the methyl ester of the dimethyl isopropylsilyl imidazole ether form and purified with a silica gel column as described previously.

2.5. GC–MS conditions

A JMS-DX 303 GC–MS system (JEOL, Tokyo, Japan) equipped with a JMA-DA 5000 data processing system was used. The column was a 30 m×0.317 mm I.D. fused-silica capillary (DB-1; film thickness, 0.1 μm; J&W Scientific, Folsom, CA, USA). The temperature of the column oven was programmed in a two-step gradient. In the first step, the temperature was 100°C for 1 min, then raised to 220°C at 3°C per min; in the second step, the temperature was raised from 220 to 300°C at 4°C per min. An all-glass solventless injector was mounted horizontally in the injection block of the gas chromatograph. Helium was used as the carrier gas at a linear velocity of 21 cm/s. The temperature of the injection port and the

separator block was 300°C, and that of the ionization source was maintained at 220°C. The ionization energy was 70 eV. The selected ions monitored are described in the text at a mass spectral resolution of 3000. The selected ions monitored were m/z 625.41 for 8-epi PGF2α and 629.44 for d4-8-epi PGF2α, respectively [10].

2.6. Creatinine content

The creatinine content was assayed with the colorimetric method and the Jaffe reaction [11]. After deproteination with sodium tungstenate and sulfuric acid, the creatinine was reacted with picric acid under alkaline conditions. The colorimetric assay of optical density 515 was estimated with a calibration curve created with commercial creatinine as a standard.

2.7. Data analysis

Concentrations of the isoprostane in urine are expressed in nanograms per milligram of creatinine.

Differences in results between smoking and non-smoking subjects were tested for statistical significance with the unpaired t-test. The factor analysis between the isoprostane content and other factors was performed with simple regression analysis.

3. Results

F-type isoprostane, 8-epi PGF2α, is the epimer form of PGF2α. Although other F-type prostaglandins have similar molecular formulae, 8-epi PGF2α can be isolated with GC (Fig. 1).

We recently reported that the same internal standard (penta deutrated PGF2α [d5-PGF2α]) can be used for assays of 9α,11β-PGF2 and PGF2α and for separating 9α,11β-PGF2 and PGF2α with GC. Although 8-epi PGF2α, 9α,11β-PGF2 and PGF2α have similar molecular masses and the same fragment [M−43] of the same derivative can be used for GC–MS-selected ion monitoring (SIM), we have used another internal standard, tetra deuterated 8-epi PGF2α (d4-8-epi PGF2α), because isoprostane is separated far from d5-PGF2α, 20 s earlier (Fig. 1). The calibration curve is linear from 10 pg to 100 ng

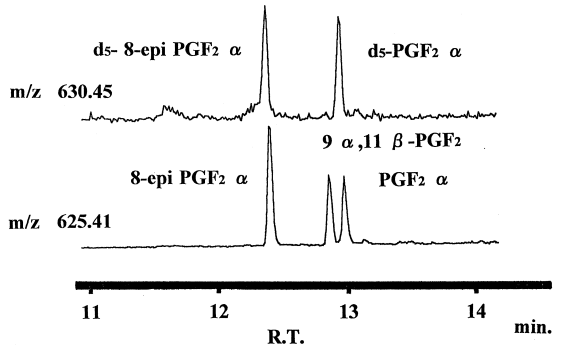

Fig. 1. GC with selected ion monitoring of standard F-type isoprostane. PGF2α, 9α,11β-PGF2 and 8-epi PGF2α with deuterated internal standard are derivatized and assayed (GC–MS-SIM) (see Methods). Commercial tetra deuterated 8-epi PGF2α contained two-thirds penta deuterated form. This chromatography was monitored at [M−43] of penta deuterated PGF2α and 8-epi PGF2α (m/z 630.45) and authentic prostaglandins (m/z 625.41).

($y=0.4137x+0.1950$, $r=0.9987$). Typical GC runs with SIM of 8-epi PGF2α in urine and its internal standard are shown in Fig. 2.

Urine isoprostane content was measured in 141 men (Fig. 3). Current smokers ($n=81$; age, 37.6±11.1 years) and persons who had never smoked (non-smokers; $n=39$; age, 38.6±10.9 years) were of similar age, but ex-smokers ($n=21$; age, 44.8±8.5 years) were older because they had quit smoking only after many years (time since quitting smoking, 9.05±7.53 years; range, 2 week to 25 years).

Isoprostane content differed significantly ($P<$

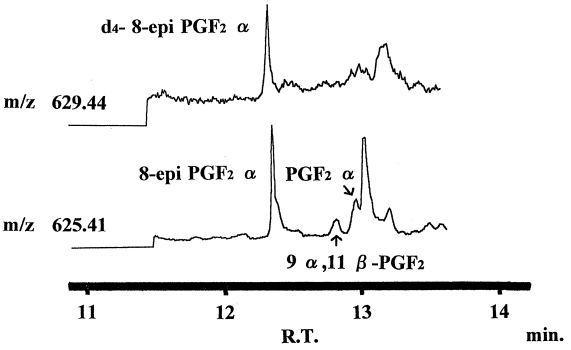

Fig. 2. Typical GC with selected ion monitoring of 8-epi PGF2α in urine sample. Urine sample was extracted, and derivatized as described in Methods, monitored using the [M−43] fragment with tetra deuterated 8-epi PGF2α.

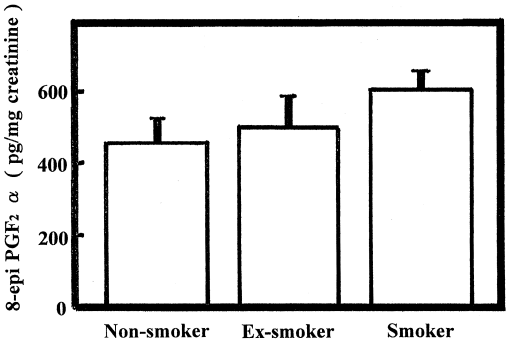

Fig. 3. Urine isoprostane content. Three groups (non-smoking, ex-smoking and smoking) were studied to calculate isoprostane content. Bar: SD.

0.05) between smokers (605.2±59.01 ng/mg creatinine) and non-smokers (424.0±70.37 ng/mg creatinine). However, isoprostane content in ex-smokers (487.2±98.48 ng/mg creatinine) did not differ significantly from that in smokers or non-smokers.

Factors related to urine isoprostane content was analyzed within each group. There are negative weak correlations between urine isoprostane content was weakly and negatively correlated with the age of non-smokers ($r=-0.23888$, Fig. 4) and time since quitting smoking in ex-smokers ($r=-0.35911$). Furthermore, urine isoprostane content was negatively correlated with the age of ex-smokers ($r=-0.24329$). However, isoprostane content was not correlated with the age of smokers ($r=-0.0153$, Fig. 5). Also, isoprostane content was not correlated with the duration of smoking (mean, 18.4±9.1 years; range, 1.5 to 35 years; $r=-0.10177$), and the number of cigarettes smoked per day (mean, 20.6±11.1; range, 1 to 40; $r=-0.10421$).

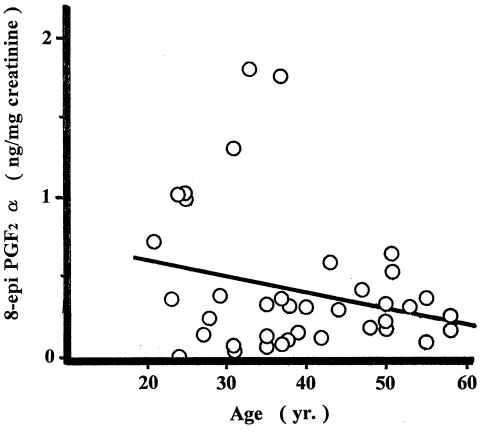

Fig. 4. The relationship between urine isoprostane content and age of non-smoking volunteer.

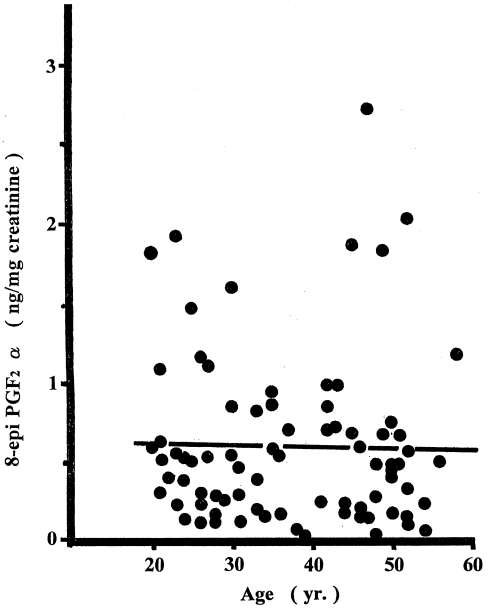

Fig. 5. The relationship between urine isoprostane content and age of smoking volunteer.

4. Discussion

The generation of isoprostane is not controlled by physiological, or homeostatic, regulation. Isoprostane is catalyzed from arachidonate by non-enzymatic lipid peroxidation with free radicals or active oxygen or both in vivo. Therefore, the amount of isoprostane should reflect the levels of oxidant stress and free radicals in vivo [6]. We have reported the sensitivity of prostanoid generation in mice to low-dose radiation [5]. Isoprostane, as 8-epi PGF2α concentration, was generated by a low-dose of radiation.

The purpose of this study is to identify the peroxidation level in the human body. Excreted isoprostane in urine may be the indicator of free-radical level and/or oxidant stress in the human body. An advantage of the urinary isoprostane assay is that urine samples are simpler and less difficult to

collect than serum samples. This study has demonstrated that the GC–MS-SIM assay is a useful tool for estimating the isoprostane content to assess human public health.

The urine isoprostane content differed between smokers and non-smokers. Although isoprostane content in non-smokers decreased with age, that in smokers was not correlated with age, number of cigarettes per day, or duration of smoking. This result indicates that the presence of the smoking habit increases isoprostane concentration in urine. This speculation is supported by the finding that isoprostane content in ex-smokers decreased with time since quitting and with age. Therefore, the habit of smoking increases oxidant stress in older persons, and the isoprostane generates high concentration in the local site related smoking.

Isoprostane has been reported to have several biological effects. The F2-type isoprostane, 8-epi PGF2α stimulates the thromboxane A2 receptor [7] and increases thromboxane A2-like activity [8]. Low but continuous exposure to 8-epi PGF2α should increase thromboxane activity or the sensitivity of thromboxane receptor or both. Further study is needed on the effects of 8-epi PGF2α under longer exposure to oxidant stress.

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