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F_2-ISOPROSTANES BIOMARKERS OF LIPID PEROXIDATION: THEIR UTILITY IN EVALUATION OF OXIDATIVE STRESS INDUCED BY TOXIC AGENTS

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Abstract. Isoprostanes are prostaglandin-like compounds that are produced by free radical mediated peroxidation of polyunsaturated fatty acids. There is a direct evidence showing that F2-isoprostanes can be utilized as a marker of lipid peroxidation due to the mechanism of their formation (nonenzymatic oxidation of arachidonic acid), chemical stability, sensitive and non-invasive methods of their estimation. An altered generation of F2-isoprostanes has been found in a variety of pathological syndromes associated with oxidative stress. Their quantification allows to elucidate the role of free radicals in oxidative injury. This paper reviews briefly the recent data on isoprostanes: biochemical mechanisms of their formation, methods of their measurement, and the possibilities of their utilization as a quantitative/qualitative marker of oxidative stress *in vivo.*

Key words:
Isoprostanes, Oxidative stress, Lipid peroxidation, Biological markers, Reactive oxygen species

INTRODUCTION

Numerous pathological processes involve free radical mediated oxidative stress. The elaboration of reliable, and non-invasive methods for the assessment of oxidative stress in human body is one of the most important steps towards recognizing the variety of oxidative syndromes presumably produced by reactive oxygen species (ROS). Lipid peroxidation is one of the most common features associated with oxidative stress, and the measurement of lipid peroxidation products has been used to evaluate oxidative stress in *in vivo* conditions [1]. The assessment of primary end-products involves the measurement of conjugated dienes and lipid hydroperoxide, while the quantification of secondary end-products includes thiobarbituric-reactive substances, gaseous alkanes and prostaglandin F_2-like products, termed F_2-isoprostanes (F2-iPs) [2,3,4]. Recently, F_2-iPs have been regarded as the most valuable, accurate and reliable marker of oxidative stress *in vivo* and their quantification is recommended for assessing oxidant injuries in humans.

The purpose of this paper is to provide some information on biochemistry of isoprostanes and their utilization as a marker of oxidative stress.

A NOMENCLATURE SYSTEM FOR ISOPROSTANES

An increased interest in biological activity of isoprostanes and in their role as a possible marker of oxidative stress, as well as the first attempts to synthesize them chemically have created the need to introduce a new, clear nomenclature. Rokach et al. [5] proposed to use iP as a symbol assigned to isoprostanes, and the letters D,E,F,G and H,

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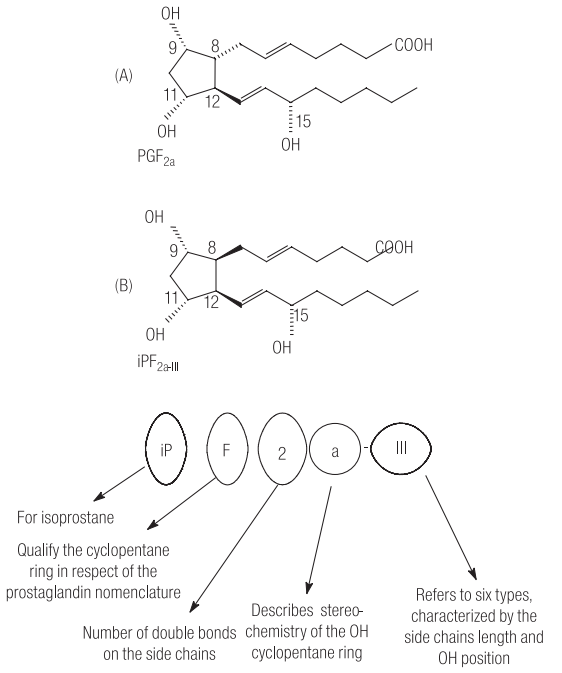

Fig. 1. Molecular structures of prostaglandin F_2 and corresponding isoprostane (A) and their nomenclature (B) [5].

which correspond with the prostaglandin (PG) nomenclature, as indicative of the type of cyclopentane ring. In further description, they suggested to use: the numbers: 1, 2, 3, 4 or 5 written in subscript to correspond with the number of double bounds; prefix α or β to indicate the spherical localization of hydroxyl groups in cyclopentane ring; and Roman numerals from I–VI referring to the six types of isoprostanes derived from eicosapentaenoic acid and four types from arachidonic acid (AA) (III–VI) (Fig. 1). For example, for two most often investigated isoprostanes, previously named 8-epi$PGF_{2\alpha}$ or 8-iso$PGF_{2\alpha}$ and $iPF_{2\alpha}$-I, the new nomenclature reads as follows: $iPF_{2\alpha}$-III and $iPF_{2\alpha}$-VI.

THE MECHANISM OF ISOPROSTANES FORMATION

As mentioned earlier, polyunsaturated fatty acids (PUFA) in the presence of ROS are the main source of isoprostanes. Peroxidation of AA leads to formation of 4 regioisomers of F_2-iPs [6]; eicosapentaenoic acid is predicted to lead to the generation of 6 regioisomers of F_3-iPs [7], α-linolenic and γ-linolenic acids to two regioisomers of E_1- and F_1-iPs, respectively [8,9]; and decosahexaenoic acid to 8 regioisomers of D_4-iPs and 8 regioisomers of E_4-iPs [10,11]. Each regioisomer comprises 8 racemic diastereomers, thus providing a large number of forms of these compounds.

Most of the published data on iPs concerns in particular $PGF_{2\alpha}$-derived F_2-iPs. The mechanism of their formation involves AA peroxidation, leading to the formation of bicycloendoperoxide, and subsequently to its reduction, yielding finally F_2-iPs. Depending on which of the labile hydrogen atoms of AA is detached from the molecule as a first after the action of ROS, 64 different isoforms of F_2-iPs are formed. Due to their structural similarities, they were grouped into 4 types of regioisomers. The structure shows that two alkile chains are always bound to F-ring in *cis* position, whereas in prostanoids they occupy *trans* position [12] (Fig. 2).

The formation of D- and E-iPs has already been confirmed also in *in vivo* conditions where they may accumulate, reaching the levels found for $iPF_{2\alpha}$–III [11].

Over many years it has been considered that F_2-iPs are formed independently from cyclooxygenase (COX). However, not long ago it was shown that COX may participate in the formation of the F_2-iPs isoforms – $iPF_{2\alpha}$–III [13] (Fig. 2).

Cyclooxygenase is the first enzyme in the pathway that leads to the formation of prostanoids and thromboxanes

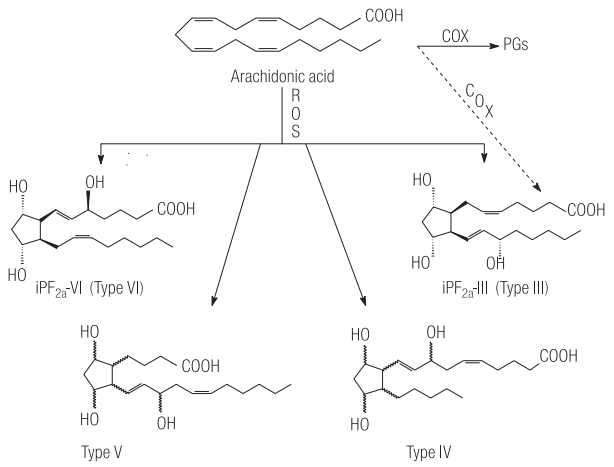

Fig. 2. Free radical and cyclooxygenase catalysed metabolism of AA to prostaglandins and F_2-isoprostanes, respectively.

from AA [14]. There are two isoforms of this enzyme, COX-1 – present in a majority of cell types, and COX-2 – a restriction enzyme, present in tissues at very low levels. COX-2 is easily inducible by mitogens or during inflammation [15]. Recently, it has been shown that (contrary to other iPs) $iPF_{2\alpha}$–III may be formed by activation with collagen, thrombin, or arachidonate of platelet COX-1 [13]. *In vivo* studies show however, that this biosynthesis pathway does not play an important role in the formation of $iPF_{2\alpha}$–III since nonsteroid anti-inflammatory drugs do not lower the level of $iPF_{2\alpha}$–III, as measured in the urine of healthy subjects [16]. Other authors [17] showed that $iPF_{2\alpha}$–III may be formed in activated monocytes by COX-2. Thus the hypothesis that formation of $iPF_{2\alpha}$–III *in vivo* is the result of nonenzymatic peroxidation of AA needs further studies [17].

Although mechanisms of F_2-iPs release from cell membranes are not understood well enough, it is already known that they are formed at the site of inflammation where ROS affect AA before its release from ester bindings of phospholipid membrane. Subsequently, in response to cell activation presumably by phospholipase(s), F_2-iPs are released to circulation and ultimately secreted with urine [18].

METABOLISM OF ISOPROSTANES

The information about F_2-iPs metabolism is scarce. Studies of rats showed that halftime elimination of $iPF_{2\alpha}$–III from plasma was 16 min. Twenty percent of radioactivity was detected in urine after administration of radioisotope labeled $iPF_{2\alpha}$–III to monkeys or human volunteers. This radioactivity was associated with the metabolite of $iPF_{2\alpha}$–III – 2,3-dinor-5,6-dihydro-$iPF_{2\alpha}$–III [6]. There was linear correlation between concentrations of the metabolite and the levels of $iPF_{2\alpha}$–III in plasma [6]. Further studies by Chiabrando et al. [19] also showed the presence of 2,3-dinor- $iPF_{2\alpha}$–III metabolite. The proximate estimations of the concentrations of both metabolites in urine showed the correlation with the levels of $iPF_{2\alpha}$–III in plasma.

Thus the measurement of metabolites of $iPF_{2\alpha}$-III in urine could be regarded as a method for the estimation of total levels of endogenic $iPF_{2\alpha}$-III in human subjects. However, the lack of scientific data on whether F_2-iPs in urine comes only from filtrated blood plasma and not from other sources prevents the correct interpretation of data obtained with this method. Data from the studies of PG biosynthesis shows, for example, that unmetabolised eicosanoids present in urine are synthesized in kidneys [20,21].

F_2-ISOPROSTANES – A BIOACTIVE PRODUCT OF LIPID PEROXIDATION USED AS A MARKER OF OXIDATIVE STRESS

Numerous studies have shown that quantitative measurement of iPs formed during oxidative stress can be used as a significant marker of prooxidative status during pathogenesis of different diseases and in response to toxic substances exposure.

Evidence from *in vitro* studies. The evidence that isoprostanes can be utilized as a marker of lipid peroxidation were shown in several different *in vitro* experiments.

Lipid peroxidation induced in the system dependent on Fe/ADP/ascorbate in microsomal fraction of the rat liver results in formation of $iPF_{2\alpha}$-III and malondialadehyde. The increased concentration of these two metabolites during peroxidation correlates with decreased AA levels and the increased oxygen [22]. The role of $iPF_{2\alpha}$ -III as a marker of oxidation was also shown in the studies, in which blood plasma low density lipoproteins (LDL) were oxidized with Cu^{2+}, or the water soluble oxidizing agent 2,2-azo-bis-2-amidinopropane. This peroxidation resulted in increased levels of $iPF_{2\alpha}$-III and lipid hydroperoxide. However, this effect occurred only at low levels of ascorbate and ubiquinol-10, known antioxidant factors [23]. In conditions that resemble cellular inflammatory reactions, Pratico et al. [24] observed significant increase in $iPF_{2\alpha}$-III concentrations after LDL exposure to zymosan-stimulated macrophages. A dose-dependent increase in levels of $iPF_{2\alpha}$-III were also observed after LDL exposure to

peroxynitrite. It also correlated with the increased electrophoretic mobility of LDL fractions [25].

Evidence from *in vivo* animal studies. Several *in vivo* studies show clear evidence of the role of iPs as an indicator of oxydo-redox reactions. In some of these studies a significantly increased concentration of F_2-iPs esters in the rat liver was observed during the first hour after the challenge with hepatotoxic dose of CCL_4. The increase in this metabolite showed incremental tendency for the next 24 h [26,27]. Blood plasma concentrations of esters and iPs reach maximal levels 4-8 h after the administration of CCl_4 and are dose-dependent [27].

The increased F_2-iPs was observed after the administration of agents, such as izoniazid or phenobarbital compounds that induce microsomal enzymes, which in turn enhance the metabolic rate of CCL_4 and decrease the glutathion deposits [27]. Antioxidants, like lazaroid U78517 or cytochrome P-450 inhibitor (4-methylpyrazole or SKF525A) inhibit CCL_4-induced synthesis of F_2-iPs [27,28].

Utilizing a similar experimental model, the levels of malondialdehyde in the liver were estimated. An eighty-fold increase in F_2-iPs was observed, while malondialdehyde concentration was elevated by only 2.5 times [22].

Deleterious effects of dipyridil herbicides such as paraquat or diquat are related to their metabolism. These compounds enter the metabolic redox cycles and produce large amounts of ROS. In consequence, the rats suffer from the liver and kidney damage, which is more severe in animals deficient in selenium (Se), an element essential for glutathione peroxidase and other anti-oxidant proteins [29]. The administration of diquat to Se deficient rats caused 100–200-fold increase in the liver and kidney derived F_2-iPs formation [29].

Rats fed with food lacking in Se and vitamin E lowered their body weight and often died because of massive liver necrosis. The *in vitro* studies showed the role of vitamin E in inhibition of lipid peroxidation, which suggested that this process may be the main cause of massive liver necrosis. The levels of F_2-iPs in the blood plasma and tissues of these animals were 6-fold higher than those in control group. In the liver, lungs, kidneys, heart and skeletal muscles there were found significantly increased levels of esterified form of F_2-iPs [26,30].

The role of oxidative stress in the ethanol-induced liver injury was shown both in humans [31] and in experimental animals [32]. The induction of isoenzyme CYP4502EI by ethanol leads to the formation of ROS and increased lipid peroxidation [32]. The causative relation between enhanced lipid peroxidation and liver damage was confirmed in the animal model studies by Nanji and French [8]. Lipid peroxidation was estimated using the method of "conjugated dienes" measurement [33], or by F_2-iPs estimation in plasma and tissues [34–37].

Evidence from *in vivo* human studies. The increased levels of iPs in urine were observed in subjects with chronic liver injury due to ethyl alcohol consumption. The levels of $iPF_{2\alpha}$-III were significantly higher in subjects with liver cirrhosis induced by former alcohol consumption than in subjects suffering from this disease induced by Hepatitis C virus infection [31]. Furthermore, increased levels of iPs released to urine ($iPF_{2\alpha}$-III, -VI and 2,3-dinor-5,6-dihydro-$iPF_{2\alpha}$-III metabolite) correlated with the severity of alcohol-induced liver disease and tended to increase in dose-dependent manner in healthy subjects [31].

Tobacco smoke contains large amounts of ROS able to induce oxidative damage of many important biomolecules [38]. Oxidative modifications of deoxyribonucleic acids, as well as low density lipoproteins can lead to tumor or atherosclerosis. To investigate the effect of tobacco smoke on the induction of oxidative stress, Morrow et al. [39] measured the levels of free and lipid ester bound forms of F_2–iPs in blood plasma and urine. The levels of both forms of iPs in blood plasma were significantly increased in smokers and they correlated with concentrations of metabolites in urine. Two weeks without smoking decreased the levels of free and lipid ester bound forms of F_2–iPs in blood plasma of smokers. The decrease in blood plasma levels of $iPF_{2\alpha}$-III in smokers was also observed after vitamin C intake [40]. The studies suggest that the measurement of iPs in blood plasma and their metabolites in urine can be utilized as a good marker of oxidative processes induced by tobacco smoke.

Acute intoxication with paracethamol is responsible for the liver and kidney injury by a free radical mechanism. Circulating plasma concentrations of F_2-iPs due to prooxidative processes induced by paracethamol were 8-times above the normal level. The authors suggest that the kidney injury in paracethamol-intoxicated subjects may be the consequence of large amounts of iPs released from injured liver and subsequent early renal vasoconstriction [41,42].

According to the present evidence cisplatin injury in renal tubular epithelial cells is associated with lipid peroxidation and elevated production of isoprostanes. It has been revealed that platinum bound chlorine molecules in *cis* position readily exchange between nuclofilic molecules and lead to decreased intracellular concentrations of thiol groups and thus to alterations in activity of glutathione peroxidase and accumulation of H_2O_2. Further consequence of these disturbances could result in lipid peroxidation and iPs accumulation. Cisplatin-induced lipid peroxidation may contribute to renal dysfunction due to the potent renal vasoconstrictive action of isoprostanes [43]. It is confirmed in *in vitro* conditions that cisplatin induces F_2-iPs formation in concentration-dependent way, and in addition to the reaction of acetyl-cysteine (thiols donors) inhibits this effect [43].

BIOLOGICAL ACTIVITY OF ISOPROSTANES

Several isoprostanes have been found to exert potent biological effect. This involves a receptor mediated action, e.g. vasoconstriction or adduct formation, which is associated with their chemical property. Rats administered with $iPF_{2\alpha}$-III showed contraction of the kidney vein smooth muscles that was accompanied by reduced glomerular filtration and blood flow. However, these changes did not cause alterations in blood pressure, suggesting of selective effect of $iPF_{2\alpha}$-III on kidney vasculature [44]. $IPF_{2\alpha}$-III was also shown to constrict pulmonary artery in rabbits and rats [45,46], and to narrow porcine and bovine coronary arteries twice as much as $PGF_{2\alpha}$ but less than U46619 [47]. In guinea pigs, $iPF_{2\alpha}$-III administered intratracheally was shown to induce dose-dependent airflow obstruction and airway plasma exudation [48]. It was also revealed that $iPF_{2\alpha}$-III exerts a constrictor effect on cerebral arteries and retinal vessels [49,50]. All these effects are inhibited by thromboxane receptor antagonist – SQ29548, suggesting that $iPF_{2\alpha}$-III may exert its effect through this receptor. However, the studies on direct binding of $iPF_{2\alpha}$-III to thromboxane receptor did not confirm this effect [51]. In human platelets, $iPF_{2\alpha}$-III within the concentration range between 1 nmol/L – 1 μmol/L changes the cell shape, as well as the release of calcium ions from intracellular deposits and inositol phosphates [18,24]. Furthermore, $iPF_{2\alpha}$-III in the presence of subthreshold doses of platelet agonists induces dose-dependent platelet aggregation [24]. The ability of increased platelet aggregation may take place at the sites, where the increased activation and the production of $iPF_{2\alpha}$-III coincide. It has also been observed that $iPF_{2\alpha}$-III indirectly increases platelet adhesion by reduction of anti-adhesive and anti-aggregatory activity of nitric oxide [52]. However, in spite of the collected data, it is still unclear whether local *in vivo* concentrations of iPs can reach the levels that could significantly influence the processes of oxidative stress.

QUANTIFICATION OF ISOPROSTANES AS A MARKER OF OXIDATIVE STRESS

As indicated earlier, F_2-iPs are formed during ROS-mediated autooxidation of AA in *in vitro* conditions [3]. Thus the appropriate storage and assessment conditions have to be taken into consideration when measuring the levels of F_2-iPs. Samples of blood plasma immediately frozen in liquid nitrogen and stored at –80°C do not show autooxidation up to 8 months. Similar precautions have to be considered when handling the solid tissue samples. It has been shown that autooxidation processes, due to low levels of AA do not significantly affect the measurements made in urine samples; the concentrations of two different forms of F_2-iPs are not changed if the samples are left at room temperature for 7 days [53]. The measurement of F_2-iPs metabolites also prevents from obtaining false pos-

itive results that otherwise could be obtained by *ex vivo* created artifacts.

The materials used to measure F_2-iPs and their metabolites are: urine [53,54,55], blood plasma [39,55], cerebrospinal fluid [56], expiratory air condensate [57,58], bronchopulmonary lavage [59], tissues and blood lipid molecules [39]. The best material of choice for measuring the levels of F_2-iPs is urine due to uninvasive method of its collection, and the lack of artifacts and autooxidative processes. It is still unclear whether the formation of different regioisomers is specific and may be attributed to the specific oxidative processes induced by different compounds. Li et al. [60] utilizing liquid chromatography (LC) together with mass spectrometry (MS)-MS showed that concentrations of F_2-iPs–III and –VI in urine of patients suffering from inherited homozygotic hypercholesteremia were similar. The authors, however, did not find this correlation in patients with heart failure.

In spite of a large number of different forms of iPs, the investigators mainly focus on two of them: $iPF_{2\alpha}$-III and –VI [5,61–64]. Based on the measurements of these two forms in body fluids and in tissues one can notice disregulation of oxydo-reduction processes *in vivo*, which leads to lipid overperoxidation due to the exposure to different toxic substances [25,31,39–43]. Quite recently, the measurement of metabolite-2,3-dinor-5,6-dihydro-$iPF_{2\alpha}$-III has been often used as a marker of lipid peroxidation [55]. This metabolite is present in urine in higher concentrations than primary iP, and reflects general *in vivo* lipid peroxidation. The measurement of this metabolite prevents obtaining false positive results which could originate from COX-dependent lipid peroxidation in platelets [20].

The measurement of $iPF_{2\alpha}$-III in different research laboratories is carried out by employing complex procedures of gas chromatography/negative ion chemical ionisation/mass spectrometry (GC/NICI/MS) [13,16,50,65,66], or LC/MS [67] or GC/MS/MS [65,68] or LC/MS/MS [60]. In primary studies, Pratico [13] to measure the levels of $iPF_{2\alpha}$-III in blood plasma used GC-MS technique involving a standard radioisotope labeled $iPF_{2\alpha}$-III. Working together with Rokach et al. [5], he elaborated the methods for other isomers, mainly for $iPF_{2\alpha}$-VI. The measurement of the $iPF_{2\alpha}$-VI is superior to the measurement of other metabolites because it allows an easy conversion of this compound to cyclic lactone, and thus separation from other iPs. Furthermore, it shows better specificity towards detection of ROS-dependent oxidative processes because it is not formed in platelets or monocytes in the COX-dependent way [53]. Bachi et al. [66] have described new method for $iPF_{2\alpha}$-III isolation that utilizes immune-exchange columns. It facilitates isolation and purification of this iP from samples by GC/MS method [65]. However, columns need to be replaced with new antibodies and show restricted period of time in which they can be used. Procedures described above, due to their complexity and equipment required, can be performed only in specialized laboratories. Large scale clinical studies can employ only immunochemical methods [16,69].

Commercially available kits for the measurement of $iPF_{2\alpha}$-III or $iPF_{2\alpha}$-VI and their metabolites in urine include enzyme linked assays (EIA) [16,70,71] or radioisotope immuno assays (RIA) [72].

However, several articles report that the concentrations of iPs measured by means of immunochemical methods or GC/MS are different [16,73]. Investigations by Bessard et al. [74] confirm this observation, however, they indicate high values of correlation coefficient (0.863) and standard deviations, which suggests that none of these methods measured the same metabolites. The presence of a large number of regioisomers makes for the existence of cross-immunoreactivity of the antibodies utilized in EIA techniques and for yielding false results. As a consequence, comparison of clinical data using GC/MS and EIA should be avoided.

CONCLUSIONS

The discovery of iPs, nonenzymatic products of lipid peroxidation, provided a new possibility of assessing the role of ROS in human physiology and pathophysiology. The elaboration of credible methods for the quantitative measurement of iPs and their metabolites as a marker of *in vivo* prooxidative processes in easily accessible material

is a significant progress towards the recognition of the role of ROS in the pathogenesis of various diseases and the assessment of the effects of toxic substances on the human health.

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