F₂-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 F₂-like products, termed F₂-isoprostanes (F₂-iPs) [2,3,4]. Recently, F₂-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.
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 $\alpha$ or $\beta$ to indicate the spher- 
al 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 iso- 
prostanes, previously named 8-epiPGF$_2\alpha$ or 8-isoPGF$_2\alpha$ 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 iso-
prostanes. 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], $\alpha$-linolenic and $\gamma$-linolenic acids to two regio-

isomers of E$_1^{-}$- and F$_1$-iPs, respectively [8,9]; and docosa-

hexaenoic acid to 8 regioisomers of D$_4$-iPs and 8 regio-

isomers 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 isomers of F$_2$-iPs are formed. Due to their structural similarities, they were grouped into 4 types of regioisomers. The structure shows that two alkyl chains are always bound to F-ring in cis position, whereas in prostanoids they occupy trans position [12] (Fig. 2).

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late, reaching the levels found for iPF$_{2\alpha}$-III [11].

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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 malondialdehyde. 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-amidino propane. 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\textsubscript{2}-iPs esters in the rat liver was observed during the first hour after the challenge with hepatotoxic dose of CCl\textsubscript{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\textsubscript{4} and are dose-dependent [27].

The increased F\textsubscript{2}-iPs was observed after the administration of agents, such as isoniazid or phenobarbital compounds that induce microsomal enzymes, which in turn enhance the metabolic rate of CCl\textsubscript{4} and decrease the glutathion deposits [27]. Antioxidants, like lazaroid U78517 or cytochrome P-450 inhibitor (4-methylpyrazole or SKF525A) inhibit CCl\textsubscript{4}-induced synthesis of F\textsubscript{2}-iPs [27,28].

Utilizing a similar experimental model, the levels of malondialdehyde in the liver were estimated. An eighty-fold increase in F\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{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 iP\textsubscript{F\textsubscript{2}-}\textsubscript{α}-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\textsubscript{2α-III}, -VI and 2,3-dinor-5,6-dihydro-iPF\textsubscript{2α-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\textsubscript{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\textsubscript{2}-iPs in blood plasma of smokers. The decrease in blood plasma levels of iP\textsubscript{F\textsubscript{2α-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 paracetamol is responsible for the liver and kidney injury by a free radical mechanism. Circulating plasma concentrations of F\textsubscript{2}-iPs due to prooxidative processes induced by paracetamol were 8-times above the normal level. The authors suggest that the kidney injury in paracetamol-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 nucleophilic molecules and lead to decreased intracellular concentrations of thiol groups and thus to alterations in activity of glutathione peroxidase and accumulation of H\textsubscript{2}O\textsubscript{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\textsubscript{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 iP\textsubscript{F}\textsubscript{2},\textsubscript{α}-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 iP\textsubscript{F}\textsubscript{2},\textsubscript{α}-III on kidney vasculature [44]. iP\textsubscript{F}\textsubscript{2},\textsubscript{α}-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\textsubscript{2} \textalpha but less than U46619 [47]. In guinea pigs, iP\textsubscript{F}\textsubscript{2},\textsubscript{α}-III administered intracheally was shown to induce dose-dependent airflow obstruction and airway plasma exudation [48]. It was also revealed that iP\textsubscript{F}\textsubscript{2},\textsubscript{α}-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 iP\textsubscript{F}\textsubscript{2},\textsubscript{α}-III may exert its effect through this receptor. However, the studies on direct binding of iP\textsubscript{F}\textsubscript{2},\textsubscript{α}-III to thromboxane receptor did not confirm this effect [51]. In human platelets, iP\textsubscript{F}\textsubscript{2},\textsubscript{α}-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, iP\textsubscript{F}\textsubscript{2},\textsubscript{α}-III in the presence of sub-threshold 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 iP\textsubscript{F}\textsubscript{2},\textsubscript{α}-III coincide. It has also been observed that iP\textsubscript{F}\textsubscript{2},\textsubscript{α}-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\textsubscript{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\textsubscript{2}-iPs. Samples of blood plasma immediately frozen in liquid nitrogen and stored at –80\degreeC 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\textsubscript{2}-iPs are not changed if the samples are left at room temperature for 7 days [53]. The measurement of F\textsubscript{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 immunexchange 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.

REFERENCES


