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Review

Isoprostanes: Novel Bioactive Products of Lipid Peroxidation

SAMAR BASU*

Section of Geriatrics and Clinical Nutrition Research, Faculty of Medicine, Uppsala University, Box 609, SE-751 25 Uppsala, Sweden

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Isoprostanes, are a novel group of prostaglandin-like compounds that are biosynthesised from esterified polyunsaturated fatty acid (PUFA) through a non-enzymatic free radical-catalysed reaction. Several of these compounds possess potent biological activity, as evidenced mainly through their pulmonary and renal vasoconstrictive effects, and have short half-lives. It has been shown that isoprostanes act as full or partial agonists through thromboxane receptors. Both human and experimental studies have indicated associations of isoprostanes and severe inflammatory conditions, ischemia-reperfusion, diabetes and atherosclerosis. Reports have shown that F_2-isoprostanes are authentic biomarkers of lipid peroxidation and can be used as potential *in vivo* indicators of oxidant stress in various clinical conditions, as well as in evaluations of anti-oxidants or drugs for their free radical-scavenging properties.

Higher levels of F_2-isoprostanes have been found in the normal human pregnancy compared to non-pregnancy, but their physiological role has not been well studied so far. Since bioactive F_2-isoprostanes are continuously formed in various tissues and large amounts of these potent compounds are found unmetabolised in their free acid form in the urine in normal basal conditions with a wide inter-individual variation, their role in the regulation of normal physiological functions could be of further biological interest, but has yet to be disclosed. Their potent biological activity has attracted great attention among scientists, since these compounds are found in humans and animals in both physiological and pathological conditions and can be used as reliable biomarkers of lipid peroxidation.

Keywords: Isoprostanes; Free radical; Oxidative stress; Lipid peroxidation; Diseases; Diet

BIOSYNTHESIS

Early evidence of formation of non-enzymatically auto-oxidised prostaglandin-like compounds from fatty acids was first produced *in vitro* by Nugteren and others about three decades ago.[1–3] It has also long been well known that uncautious storage of unsaturated fatty acids leads to the formation of several degraded prostaglandin-like compounds. However, the biological relevance of these arbitrarily formed non-enzymatic prostaglandin derivatives *in vivo* was not disclosed until 1990, when the characterisation of other $PGF_{2\alpha}$ isomers provided such novel information.[4] The identification of isoprostanes, a family of prostaglandin-like compounds generated *in vivo* by non-enzymatic free radical catalysed-peroxidation of arachidonic acid, opened a new era of detection of non-enzymatic lipid peroxidation products and discovery of their importance.[4–7] Unlike primary prostaglandins, isoprostanes do not require microsomal cyclooxygenases for their biosynthesis. Another structural distinction between free radical-mediated isoprostanes and COX-mediated prostaglandins is that in the former, *cis* side chains to the cyclopentane ring are predominant, as compared to *trans* orientation in the latter.[8]

The mechanism of biosynthesis of isoprostanes from the precursor arachidonic acid is shown in Fig. 1. Upon abstraction of a bis-allylic labile hydrogen atom (step 1) and addition of an oxygen molecule to arachidonic acid, four positional peroxyl radicals are formed (step 2). Further, endocyclisation

*Tel.: +46-18-6117958. Fax: +46-18-6117976. E-mail: samar.basu@pubcare.uu.se

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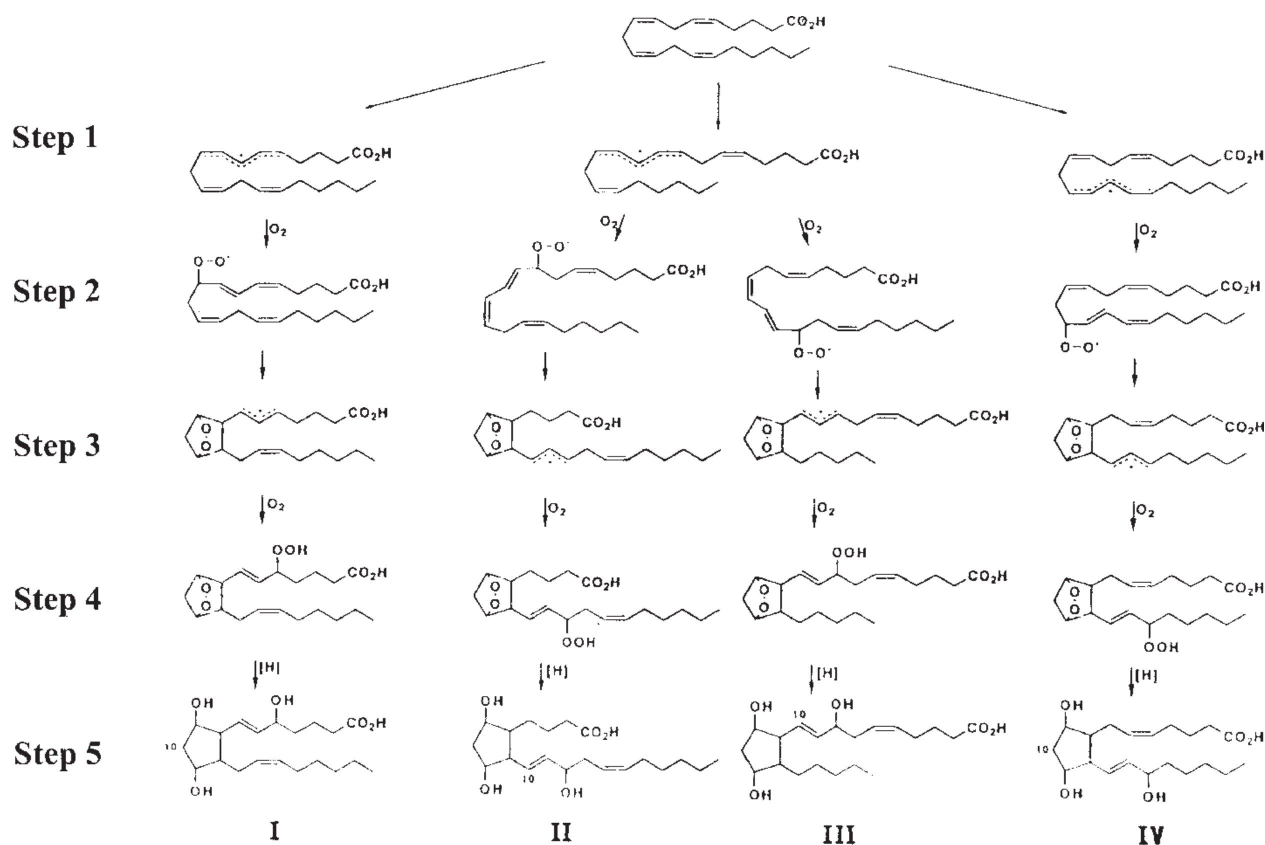

FIGURE 1 Mechanism of formation of isoprostanes acid by free radical catalysis of arachidonic acid. (Reprinted with kind permission from Ref. [5].)

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occurs (step 3) and an additional oxygen molecule is eventually added to form four unstable PGG_2-like bicyclic endoperoxide intermediates (step 4). These intermediates are then reduced by the presence of glutathione to parent isoprostanes of various series (step 5). Depending on the mechanism of formation, four F-ring isoprostane regioisomers are formed, which are designated as either 5-, 12-, 8- or 15-series regioisomers on the basis of the carbon atom to which the side-chain hydroxyl group is located.[9] Since F-ring compounds are structurally isomeric to the COX-derived primary $PGF_{2\alpha}$, these compounds are collectively termed as F_2-isoprostanes and are abundant in the tissues.[10] After rearrangement of these bicyclic endoperoxide intermediates, other isoprostanes of type E_2/ D_2, A_2 and J_2 and isothromboxanes can be formed.[11] Further, these unstable endoperoxide intermediates may also form highly reactive acyclic γ-ketoaldehydes, designated isolevuglandins.[12] Unlike COX-derived primary prostaglandins, isoprostanes have been shown to be formed *in situ* in esterified form to tissue phospholipids and subsequently released in free acid form after de-esterification of the ester moiety, mainly by phospholipases.[6] Recently, various other isoprostane-like compounds biosynthesised from eicosapentanoic acid have been identified.[13] Several other isoprostane-like compounds, named neuroprostanes and isoketal-like compounds designated neuroketals, are formed through peroxidation of docosahexaenoic acid, which is predominantly found in the brain.[11,14]

OCCURRENCES

Isoprostanes are detectable in various tissues including the lung, liver, kidney, heart, vascular tissues, fat, muscle, brain and stomach. Tissue that does not contain isoprostanes is yet to be reported. They have also been found in measurable quantities in most of the biological fluid analysed, including plasma, urine, synovial fluid, bronchoalveolar fluid, bile, lymph, microdialysis fluid from various organs, and amniotic, pericardial and seminal fluid. However, the basal level in plasma and urine varies widely between species, and also between individuals, indicating ongoing lipid peroxidation of varying extent. This observed variation in isoprostane concentration is assumed to be due to the variation in the rate of formation and/or metabolism. The basal plasma level of free F_2-isoprostanes (8-iso-$PGF_{2\alpha}$) in healthy humans has been reported to be 25 pg/ml (range 15–50 pg/ml), which is about 10 times higher than that of the COX-mediated $PGF_{2\alpha}$ and somewhat lower than that

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of the 15-keto-dihydro-$PGF_{2\alpha}$, the major plasma metabolite of $PGF_{2\alpha}$ in the human circulation.[15–17] The concentration of 8-iso-$PGF_{2\alpha}$ in urine from healthy subjects is about 30–40 times higher than the plasma concentration.[16] A similar relationship has been found in other species, e.g. pigs, rabbits, rats and mice.

ABSORPTION, DISTRIBUTION, METABOLISM AND EXCRETION

Knowledge of the absorption, distribution, metabolism and excretion (ADME) of a bioactive compound is critical to the understanding of the properties of a particular compound, and in qualitative and quantitative assays of the compound or its end-products. At an infant stage of discovery of COX-mediated prostaglandins, ADME studies played a key role in the quantitative determination of very low levels (picogram/ml) of prostaglandins or their metabolites of interest *in vivo*. The same can be said for F_2-isoprostanes, mainly 8-iso-$PGF_{2\alpha}$. The isoprostanes that are produced *in situ*, preferentially in their esterified form in the tissues, bioconvert first to their free acid form. In the tissues they are distributed in both the esterified and free acid form.[6,43] It is well known that various hydrolytic enzymes that are ubiquitous in the body are primarily responsible for the formation of free isoprostanes from their esterified moiety in the tissues. This rapid de-esterification phase might possibly be regarded as one of the rate-limiting steps for the release of free isoprostanes in the tissues and their further availability in the peripheral circulation. This reaction is the first step of enzymatic degradation of esterified isoprostanes leading to the presence of free isoprostanes in the tissues, which later secrete effectively into the peripheral circulation. This step is unlike the events in the enzymatically derived prostaglandins, which biosynthesise in free form from arachidonic acid in the tissue phospholipids.

When tritium-labelled 8-iso-$PGF_{2\alpha}$ was infused over 1 h into a male subject, 75% of the infused compound was excreted into the urine during the following 4.5 h.[18] On characterization, 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ has been identified in human urine which represented for 29% of the total radioactivity. This is the major urinary metabolite of 8-iso-$PGF_{2\alpha}$ in humans, degraded through one step of β-oxidation.[18,19] Another major metabolite of 8-iso-$PGF_{2\alpha}$ in human was found to be 2,3-dinor-8-iso-$PGF_{2\alpha}$.[19] The half-life of 8-iso-$PGF_{2\alpha}$ has been found to be about 16 min in humans.

In a pharmacokinetic and metabolic study, in which tritium-labelled 8-iso-$PGF_{2\alpha}$ was administered intravenously in rabbits, the total radioactivity appeared instantly in the blood, and disappeared quickly from the circulation.[20] About 80% of the total radioactivity was found in the urine within 4 h. The plasma half-life of 8-iso-$PGF_{2\alpha}$ in the rabbit was found to be 1 min in the distribution phase. The half-life in the terminal elimination phase was about 4 min which is rather shorter than in humans. Several polar β-oxidised metabolites appeared in the plasma within 2 min and finally they were effectively excreted in the urine. α-Tetranor-15-keto-13,14-dihydro-8-iso-$PGF_{2\alpha}$ was identified as a major urinary metabolite in the rabbits along with several other β-oxidised products. The metabolism of 8-iso-$PGF_{2\alpha}$ to α-tetranor-15-keto-13,14-dihydro-8-iso-$PGF_{2\alpha}$ and other β-oxidised products occurs in several steps in the rabbit,[20] a process which resembles the COX-mediated primary $PGF_{2\alpha}$ metabolism in this species.[21,22] The amount of β-oxidised metabolites of 8-iso-$PGF_{2\alpha}$ in the plasma was increased within 5 min after administration of radiolabelled 8-iso-$PGF_{2\alpha}$, compared to the parent compound.[20] A tentative metabolic pathway of 8-iso-$PGF_{2\alpha}$ in the rabbit is shown in Fig. 2.

Studies *in vitro* with various tissue enzymes[23] and experiments *in vivo* showed that oxidation of

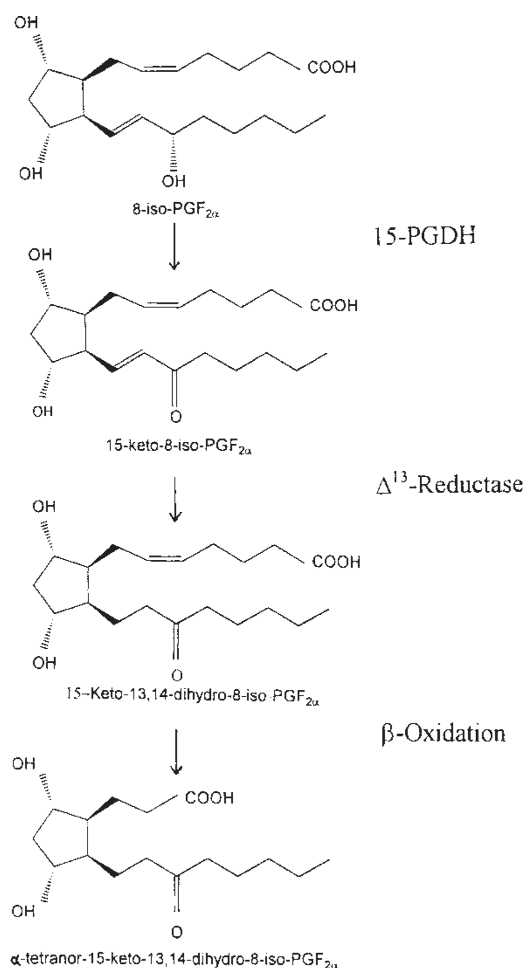

FIGURE 2 Metabolism of 8-iso-prostaglandin $F_{2\alpha}$ in the rabbit. (Reprinted with kind permission from Ref. [20]).

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the 15-hydroxy group at C-15 by 15-prostaglandin dehydrogenase (15-PGDH) is the first step of 8-iso-$PGF_{2\alpha}$ metabolism.[20] A reduction of C-13,14 double bond by Δ^{13}-reductase and formation of 15-keto-13, 14-dihydro-8-iso-$PGF_{2\alpha}$ occurred in the second step of metabolism. Thus, 15-PGDH and Δ^{13}-reductase are the key enzymes involved in the metabolism of 8-iso-$PGF_{2\alpha}$, and most possibly, they are also responsible for the metabolism of other isoprostanes. Since various hydrolytic, oxidative and reductive enzymes, including phopholipases, 15-PGDH and Δ^{13}-reductase, respectively are found in all parts of the body,[24–26] possible formation of F_2-isoprostanes in any tissue after an induction of oxidant stress would be followed by a rapid degradation and further release into the circulation unless there was a deficiency in the *de novo* metabolising enzyme systems. Since 15-PGDH and Δ^{13}-reductase are rich in lung, liver and kidney these organs are the major metabolism sites for isoprostanes.

Both β-oxidation and ω-oxidation are very common reactions in later steps of prostaglandin metabolism. It has been shown in the rabbits that 15-keto-13,14-dihydro-8-iso-$PGF_{2\alpha}$ rapidly metabolises through two steps of β-oxidation mainly to α-tetranor-15-keto-13,14-dihydro-8-iso-$PGF_{2\alpha}$ and also to several other β-oxidised metabolites.[20] Both the human and rabbit studies confirm that β-oxidation is the common degradation pathway in the later step of metabolism of 15-keto-13,14-dihydro-8-iso-$PGF_{2\alpha}$ (Fig. 2). Hence, this step of metabolism of F_2-isoprostanes is species-specific at least in regard to the formation of different structural end-products. Both the dinor and tetranor metabolites are found in plasma and urine in higher concentrations than their parent compounds.[20]

ISOPROSTANES AS INDICATORS OF LIPID PEROXIDATION AND OXIDANT STRESS

It is a long-known fact that polyunsaturated fatty acids (PUFA) readily undergo a peroxidation reaction in the presence of free radicals.[27] Excess production of many of these lipid peroxidation products may result in a condition that is frequently referred to as oxidant stress, which is supposed to be the major underlying cause of development of various diseases, including cancer and cardiovascular and neurological diseases.[28] However, a lack of reliable analytical method for the detection of lipid peroxidation *in vivo* or its end-products has hampered the progress in this field.[29]

During the last 13 years, a large number of studies have revealed several favourable properties of isoprostanes. As a result, measurement of F_2-isoprostanes is at present regarded as one of the major reliable approaches for the assessment of oxidant stress status or free radical mediated lipid peroxidation *in vivo*.[4,7,30,31,42] F_2-isoprostanes are chemically stable and specific bioactive products of free radical-catalysed lipid peroxidation. With the currently available assay methods (Table I), they are found in detectable amounts in many tissues and body fluids, even in the normal basal state, which allows scientists to assess any fluctuation in their levels following any degree of oxidant stress or lipid peroxidation *in vivo*. Most significantly in normal rats, concentrations of isoprostanes increase dramatically in the tissues, peripheral circulation and other body fluids, and are later found in the urine, in a step-wise kinetic pattern following administration of carbon tetrachloride (CCl_4), a classical model of oxidative stress induction.[16,32–34,42] Further, in

TABLE I F2-Isoprostane measurements by various methods

Methods	Selected references	Sample preparation
GC-MS	Morrow[5,176]	Extraction, TLC
	Morrow[177]	Solid phase extraction
	Adiyaman[184]	Solid-phase extraction, TLC
	Nourooz-Zadeh[178]	Solid-phase extraction
	Bessard[179]	Solid-phase extraction
	Walter[180]	HPLC
	Burke[181]	Solid-phase extraction, TLC
	Mori[182]	Reversed-phase extraction, HPLC
	Parker[183]	Extraction, TLC
	Proudfoot[185]	Solid-phase extraction, HPLC
LC-MS-MS/LC-MS	Li[186]	Solid-phase extraction, HPLC
	Liang[187]	Solid-phase extraction, LC
EIA	Wang[45]	Solid-phase extraction, TLC
	Sasaki[188]	Not known
RIA	Wang[45]	Solid-phase extraction, HPLC
	Basu[16]	No sample preparation

LC-MS = Liquid chromatography-mass spectrometry; EIA = Enzyme immunoassay; RIA = Radioimmunoassay.

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animal models of oxidant stress, the levels of these compounds have been shown not to be affected by the lipid content of the diet,[35,36] but are regulated by endogenous or exogenous antioxidants.[7,34,37]

Carbon Tetrachloride Induced Oxidative Injury Model

Carbon tetrachloride is a well known toxic compound that induces cirrhosis and oxidative injury in the liver through the formation of trichloromethyl radical ($^{\cdot}CCl_3$) or other radicals, and has been widely used in experimental models of oxidative stress and lipid peroxidation.[38–42] In a study a large amount of esterified 8-iso-$PGF_{2\alpha}$ was detected in the liver, plasma and urine in the rat, 2 h after oral administration of CCl_4 (2.5 ml/kg),[16,34,37] whereas the content of free 8-iso-$PGF_{2\alpha}$ levels in the liver tissue was quite low.[43] In another study in rats it was shown that compared to the basal levels, the level of free 8-iso-$PGF_{2\alpha}$ was increased 17-fold in the plasma and 53-fold in the urine 4 h after oral administration of CCl_4 (2 ml/kg).[33] After 6 h, when the animals were sacrificed, the free 8-iso-$PGF_{2\alpha}$ level in the plasma was increased 7-fold and in the urine 87-fold (Fig. 3). The highest level of free 8-iso-$PGF_{2\alpha}$ was seen in the peripheral circulation 4 h after administration of CCl_4.[33,34] The levels of F_2-isoprostanes were still significantly elevated 24 and 48 h after the administration of CCl_4 as compared to the baseline values.[32] The formation of isoprostanes preceded the appearance of biochemical markers (sGPT) of hepatic necrosis and it

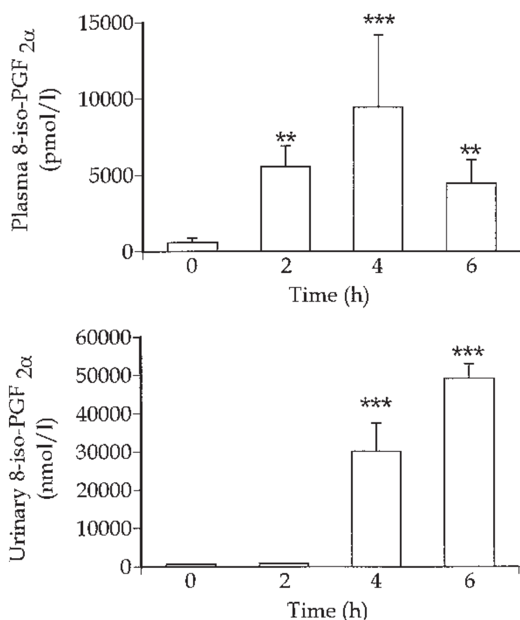

FIGURE 3 The levels of free 8-iso-$PGF_{2\alpha}$ in peripheral plasma and urine at different times following oral administration of CCl_4 to rats (2 ml/kg). (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). (Reprinted with kind permission from Ref. [33].)

was suggested that the isoprostane formation was not a non-specific consequence of cell death through necrosis but rather a manifestation of lipid peroxidation-induced oxidative stress. The findings suggest that hepatotoxicity caused by CCl_4 intoxication does not account to a significant extent for the elevated levels of plasma isoprostane observed 24–48 h after injury, as the elimination half-life of 8-iso-$PGF_{2\alpha}$ was not changed by devascularisation of the liver.[32] Thus, there is evidence that CCl_4-induced formation of F_2-isoprostanes is linked to lipid peroxidation rather than to hepatic necrosis. A major proportion of the esterified isoprostanes that are formed in the tissue is subsequently released into the peripheral circulation in free form, since the hydrolysis of the esterified compounds is an instantaneous process.

Other Oxidative Injury Models

In early experimental studies, administration of either diquat to selenium-deficient rats or CCl_4 to normal rats resulted in a dramatic increase in F_2-isoprostanes levels, which correlated with the tissue injury caused by the free radical-mediated oxidative stress.[4,33] Plasma levels of F_2-isoprostanes have been reported to correlate with the severity of alcohol-induced liver injury in different dietary models in rats.[112] However, isoprostane formation through other experimental-oxidative stress models are not common as CCl_4-induced oxidative stress model.

F_2-ISOPROSTANE ASSAYS

Studies have shown that for quantitation of lipid peroxidation, measurements of F_2-isoprostanes have a clear advantage over the currently available methods such as assay of malondialdehyde (MDA), thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides or conjugated diene, for example, which are hampered by various methodological limitations.[7,30,44] Although gas chromatography with mass spectrometry (GC-MS) was the first assay technique used in the discovery and quantitation of isoprostanes, this method is now accompanied by other assay methods such as liquid chromatography (LC)-mass spectrometry, GC-MS-MS, LC-MS-MS, radioimmunoassays and enzyme immunoassays (Table I).[5,16,45,46] The mass spectrometry based methods are expensive and relatively arduous to perform because of the formation of numerous isomers, which need extensive chromatographic purification steps prior to analysis. Further, mass spectrometrical methods require derivatisation procedures and have a comparatively lower sample analysing capacity, and are thus difficult to apply in

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large clinical, experimental and epidemiological studies. Even though mass spectrometry based methods are more specific than many others, they require well-skilled technical personnel and a high sensitivity to detect low levels of these compounds. Although immunoassays, on the other hand, are less specific or quantitative than GC-MS methods unless the antibodies are extensively tested for cross-reactivities and other accuracy tests are performed, have been found to be important tools for new discoveries in medical sciences for the last 30 years. Immunoassays have a large sample analysing capacity with a fairly low cost if the above requirements are fulfilled and if the results are verified through the experimental animal models of oxidant injury proving that an increase in the quantified compound is justified with the experimental protocol. An example of such an experiment is CCl_4-induced oxidant stress and isoprostane formation, where tissues, plasma and urine should be analysed and the results carefully correlated with the kinetics of formation.[5,16,20,34] Although immunoassays have been regarded as semi-quantitative on account of the accuracy problems availability of a well-validated method could be an important tool for evaluating free radicals-mediated reactions in clinical research, where a large number of samples need to be analysed at an affordable cost.

Several enzyme immunoassays that need extensive sample hydrolysis, extraction and purification procedures are now commercially available which require a careful evaluation. Recently, a radioimmunoassay has been developed by raising a specific antibody, where free 8-iso-$PGF_{2\alpha}$ can be measured fairly reliably in most of the body fluids without any extraction or hydrolysis procedures, which have been shown to be the major problem regarding the accuracy outcome of various isoprostane assays.[16] This method can also be used to measure total levels (esterified and free) of this compound in target sites of interest, e.g. tissues or biological fluid collected from certain key organs.[34,43] But the accuracy is lower in the tissue measurements than in assays in body fluids because of the need for extraction and hydrolysis of the tissue samples.

Most of the assays of isoprostanes to date have focused on assessment of 8-iso-$PGF_{2\alpha}$ in body fluids which is a major product of the total lipid peroxidation process *in vivo*. Several other F_2-isoprostanes of the $ipF_{2\alpha}$-IV series are also found in high concentrations in the urine and are considered to be reliable parameters of oxidant stress.[31] When measuring the urinary metabolites of 8-iso-$PGF_{2\alpha}$, the choice of appropriate parameter is of importance, since the metabolic profile and the appearance of the different metabolites of this compound at the later step of the metabolism differs between species.[18–20] Tetranor metabolites are the major urinary products of 8-iso-$PGF_{2\alpha}$ in rabbits, whereas the dinor metabolite is the dominant product in humans. Since the levels of isoprostanes differ between laboratories as a result of differences in the assay methods applied, and also for the reason that some assays measure total (esterified plus free) compound, and others only free compound, each laboratory should have at least its own basal levels calculated from various species to compare. Further the methods could be verified by a network study involving various laboratories where body fluids should be analysed for isoprostanes both at basal condition and after induction of oxidative stress. A study of this kind is ongoing at the NIH, USA.

Choice of Biological Fluid

Although isoprostanes have been detected in most of the body fluids (see above), plasma and urine samples are the biological fluids that have been commonly analysed. The choice of biological fluid and of sample collection regime for the assessment of isoprostanes is critically important because of the short half-lives of the compounds. Also, of great importance is the choice of study protocol. In acute experimental studies frequent plasma and/or continuous urine samples are preferable for observing changes over a certain period of time in the same individual, which are shown to be very informative both when studying pathophysiological changes and when evaluating a certain drug or compound of interest. In cross-sectional or longitudinal studies, urinary samples collected during 24-h or in the morning are more suitable, to get an integrated picture of the lipid peroxidation over a certain time-period. In a recent study, no statistically significant difference was seen in healthy people between 8-iso-$PGF_{2\alpha}$ levels in urine samples collected at any time of the day, in the morning urine and in 24-h urine [47,48]. For practical reason a morning urine sample would be a better choise than a 24-h urine sample. Furthermore, a morning sample reflects a time-period of 6–8 h and thus is more representative than a spot urine sample. However, among healthy individuals there is a wide inter-individual variation in the concentration of 8-iso-$PGF_{2\alpha}$ in the urine. In addition, urinary levels of 8-iso-$PGF_{2\alpha}$ collected on 10 consecutive days from the same individual showed a large inter-day variation.[49] This also provides further insight into the differential ongoing lipid peroxidation process in the normal state.

Measurement of free isoprostanes in human urine samples has proved to be very useful, since the sampling is non-invasive and any artifactual formation that may occur in the plasma is minimal in urine samples. In most cases a morning urine sample representing 6–8 h of urine would be sufficient when a change in the basal level is anticipated.

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However, the creatinine or glomerular filtration rate adjustment is necessary to get an accurate picture of isoprostane excretion except in the case of extremely high concentration obtained, e.g. after CCl_4 treatment or experimental septic shock studies. If there is a probability of a large intra-day variation in the urine production, e.g. in certain diseases such as type 2 diabetes, a daily or morning urine sample would give a more integrated picture of the total F_2-isoprostane formation. Assessment of esterified F_2-isoprostanes and their free acid in key tissues of interest or by collection of microdialysis fluid from such organs would allow a direct assay of lipid oxidation at an oxidant injury site.[43,50] However, quantification of isoprostanes in tissues can be practically more difficult than that in body fluids in the view of the ethical problems in collection of tissues, and the difficulties associated with collection timing, as well as the rapid hydrolysis of the esterified isoprostanes and subsequent release in their free form.

Preservation

Sample storage is an important factor in the accuracy of measurements of isoprostanes *in vivo*. Since artifactual oxidative formation of F_2-isoprostanes may occur in the tissues or plasma samples, if the samples are not properly processed or preserved because of their high content of fatty acids, they should be stored at or below $-70°C$. However, no such *ex vivo* formation of F_2-isoprostanes seems to occur in the urinary samples, since the lipids are sparse in the urine. Urinary samples kept at room temperature for several days did not show any artifactual formation of F_2-isoprostanes.[46] Some investigators add antioxidants to store the sample which is unnecessary if the samples are handled with precaution according to our experiences.

PHARMACOLOGICAL ACTIONS AND MEDIATORS OF OXIDATIVE STRESS

Isoprostanes have been shown to exert potent biological activity in several biological systems. When esterified to phospholipids, these compounds might not be as active as those in their free acid form. In the presence of efficient hydrolysing enzymes in various tissues in the mammalian body, the esterified isoprostanes rapidly metabolise to their active free acid form. When they are available as free acid, they affect first the integrity and fluidity of the membranes and subsequently, the surrounding tissues causing oxidant stress. Isoprostanes exert potent vasocontrictive effects in a variety of organs, e.g. the lung,[51–53] aortic ring segments,[54] kidney,[4] retinal vessels[55] and brain.[56] Further, an inverse correlation has been found between plasma levels of 8-iso-$PGF_{2\alpha}$ and both endothelium-dependent and endothelium-independent vasodilation in healthy women following infusion of methacholine or sodium nitroprusside.[57] This indicates a possible association between circulatory 8-iso-$PGF_{2\alpha}$ and a impaired vasodilation in women. However, no conclusive data has yet been obtained on the relation between endothelial function and isoprostanes.

The biological action of 8-iso-$PGF_{2\alpha}$ has been shown to be mediated partly by interaction with the vascular TXA_2/PGH_2 receptors.[51] It is still not settled whether isoprostanes have any distinct receptors for their biological action, as some studies have suggested.[58] The contractile response to 8-iso-$PGF_{2\alpha}$ is reported to be dependent on extracellular Ca^{++} via both L- and T-type Ca^{++} channels, and perhaps also on protein kinase C.[54] The role of isoprostanes as mediators of oxidant stress and signals of other biochemical cascades *in vivo* is still uncertain. Recently, it was shown in rabbits that intravenous administration of 8-iso-$PGF_{2\alpha}$ induced COX-mediated $PGF_{2\alpha}$ formation which have shown to be related to inflammation.[59,17,50,120] Formation of COX-mediated $PGF_{2\alpha}$ has also been seen subsequent to CCl_4-induced F_2-isoprostane production in rats, showing that these two structurally closely related but biosynthetically distinct compounds have diviant kinetics of biosynthesis and release, and indirectly supports the view of activation of cyclooxygenases and inflammatory responses.[33,34] However, further research on the mechanism of $PGF_{2\alpha}$ formation induction by 8-iso-$PGF_{2\alpha}$ is needed to clarify this phenomenon further. In another recent study, 8-isoprostane induced IL-8 (Interleukin-8) expression in human macrophages, a chemokine involved in inflammation and atherogenesis through mitogen-activated protein kinases.[123] Together, these studies emphasize that F_2-isoprostane might be a mediator of inflammation, involving cyclo-oxygen and/or cytokines and a possible link between oxidative stress and inflammation.[33,59] Those isoprostanes or related compounds that are derived from eicosapentaenoic acid or docosahexaenoic acid seem to have only marginal or no biological effects.[13,60]

ISOPROSTANES IN HUMAN STUDIES

Alcohol can generate F_2-isoprostanes, leading to higher excretion in the urine.[61,62] Increased concentrations of F_2-isoprostanes in circulatory fluids, urine and exhaled breath condensate have been found among cigarette smokers, changes that are associated with free radical-elicited oxidant stress, possibly resulting from the large number of toxic components, including radical species, that are absorbed in the tissue vasculature following

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smoking.[63–66] These increased levels seem to return to normal, after 2–4 weeks abstinence from cigarette smoking.[63,65,67] On further resumption of smoking, plasma and urinary levels of F_2-isoprostanes increased.[68] In a recent study, it was found that post-menopausal women had higher urinary 8-iso-$PGF_{2\alpha}$ levels compared to pre-menopausal women.[69] Further, the urinary levels of 8-iso-$PGF_{2\alpha}$ were higher in young men than in pre-menopausal women.[70] In a recent study, higher levels of plasma and urinary 8-iso-$PGF_{2\alpha}$ were found in normal pregnant women compared to non-pregnant women with a regular menstruation cycle using no contraceptives, vitamins or NSAID.[95] Thus, in studies with post-menopausal women and pregnant women it is evident that isoprostane levels are regulated in normal physiological conditions. Urinary levels of 8-iso-$PGF_{2\alpha}$ have also been found to be related to the bone mineral density index in a healthy human population.[71] However, the role of this isoprostane in osteoporosis is still uncertain.

Isoprostanes have been shown to be increased in a number of disorders that are possibly associated with oxidant stress (Table II). A study of isoprostanes in various rheumatic diseases showed that the systemic levels of 8-iso-$PGF_{2\alpha}$ were higher in patients with rheumatoid arthritis, psoriatic arthritis, reactive arthritis and osteoarthritis than in healthy controls.[72] A high level of 8-iso-$PGF_{2\alpha}$ in the synovial fluid was also found in these patients. In systemic sclerosis and other chronic inflammatory diseases, increased urinary levels of F_2-isoprostanes or their metabolites have been seen.[73–75] These results provide evidence that isoprostanes are involved in chronic inflammation both locally and systemically which is also supported by the experimental studies.[120–127]

In several neurodegenerative disorders such as Alzheimer's and Huntington's diseases, increased production of F_2-isoprostanes has been found specifically in the cerebrospinal fluid and brain tissues, and also in one study in the urine and plasma.[76–79,153] However, no such change was found in the plasma or urine in other studies.[80,81] The reason for this discrepancy is possibly because the samples are not taken at the same stage of the disease or various drugs that the patients use might have affected the outcome. Majority of the studies with Alzheimer's diseases have shown that isoprostanes are localized in the tissues or in the CSF. In a recent study in substance, Nigra of patients with Parkinson's disease and with levy body disease, an increase was seen only in the levels of isofurans but not in isoprostanes.[167] Patients with spinal cord injury have shown increased seminal fluid levels of F_2-isoprostanes.[82] But no difference in the urinary level of 8-iso-$PGF_{2\alpha}$ was seen in migraine patients when the samples were collected during migraine and migraine-free days.[69]

F_2-isoprostanes have been shown be to increased in various body fluids or exhaled breath condensate in several pulmonary diseases such as asthma,[83,84,161] interstitial lung disease,[85] cystic fibrosis,[86–88] pulmonary hypertension,[89] acute chest syndrome sickle cell disease[90] and ARDS.[158] This shows that isoprostanes are involved in pulmonary diseases (Table II). However, conflicting results have been obtained regarding F_2-isoprostanes in pre-eclamptic patients. Several studies have shown increased levels of plasma or placental isoprostanes,[91,92,165] but in others no such differences have been found in plasma or urine.[93,94,164] In a recent study it was shown that the level of plasma or urinary free 8-iso-$PGF_{2\alpha}$ in severely pre-eclampic patients did not differ from that in pregnant controls at the same stage.[95] However, the γ-tocopherol level was significantly lower in the pre-eclamptic patients than in normal pregnant and non-pregnant controls. The difference in the levels of isoprostanes in various studies might possibly be due to differences in the sampling regime, with sampling at different stages of the disease or isoprostanes are measured in various compartments.

There have been several reports of higher levels of F_2-isoprostanes in body fluids patients with cardiovascular diseases (Table II). Elevated concentrations of isoprostanes have been found in atherosclerotic lesions in different arteries,[96–98] but not in body fluids. Patients with unstable angina,[99] or heart failure,[104,105] have been reported to have higher levels of isoprostanes than healthy controls, whereas no increase in isoprostane levels were seen in patients with stable angina pectoris.[99,100] Furthermore, high levels of isoprostanes have been reported in several conditions related to reperfusion, including during and after cardiopulmonary bypass,[100,101] and reperfusion following myocardial infarction.[102,103] Patients with hypertension did not show any increase in urinary isoprostanes compared to the healthy controls.[175] However, hypertensive patients with renovascular disease showed significant increase in urinary isoprostanes compared to the healthy controls and patients with essential hypertension.[175]

Elevated levels of 8-iso-$PGF_{2\alpha}$ have been found in plasma or urinary samples from type 2 diabetic patients, compared to the non-diabetic controls (Table II).[106–108,173] In a large cross-sectional study in elderly men (77 years) it was shown that the 24-h urinary level of 8-iso-$PGF_{2\alpha}$ was significantly higher in men with type 2 diabetes ($n = 101$) than in the control men ($n = 585$) of the same age.[109] However, in a sub-group of these patients with disease duration <7 years, diagnosis did not show any

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TABLE II F2-Isoprostanes in common human disorders

Pathophysiological conditions	Selected studies	Biological fluids/Tissues	Observations
Cardiovascular diseases			
Atherosclerosis	Pratico[96]	Atherosclerotic lesion	Elevated
	Gniwotta[97]	Atherosclerotic lesion	Elevated
	Mehrabi[98]	Coronary artery	Elevated
Cardiopulmonary bypass	Ulus[101]	Plasma	Elevated
	Delanty[100]	Urine	Elevated
Angioplasty/PCI/ Coronary reperfusion	Reilly[102]	Urine	Elevated
	Berg[155]	Plasma	Elevated
	Iuliano[103]	Coronary sinus	Elevated
Angiography	Berg[155]	Plasma	Elevated
Heart failure	Crawcowski[105]	Urine	Elevated
	Mallat[104]	Pericardial fluid	Elevated
Hypertension	Crawcowski[172]	Urine	Not differed
Hypertension with RVD	Minuz[175]	Urine	Elevated
Diabetes			
Type 1 diabetes	Davi[107,162]	Urine	Elevated
	O'Byrne[111]	Urine	Not differed
	Vessby[110]	Plasma and urine	Not differed
	Hoeldtke[163]	Plasma	Not differed
Type 2 diabetes	Gopaul[106,173]	Plasma	Elevated
	Davi[107]	Urine	Elevated
	Murai[108]	Urine	Elevated
	Helmersson[109]	Urine	Elevated
Metabolic syndrome / Lipid metabolism			
Hypercholesterolemia	Davi[145]	Urine	Elevated
	Reilly[156]	Plasma	Elevated
	Roberts[144]	Plasma	Elevated
	Raal[154]	Plasma	Not differed
Type IIa hypercholesterolemia	Cracowski[157]	Urine	Not differed
Pulmonary diseases			
Asthma	Montuschi[83]	Exhaled breath condensate	Elevated
	Wood[84]	Plasma	Elevated
	Baraldi[161]	Exhaled breath condensate	Elevated
ARDS	Carpenter[158]	Exhaled breath condensate	Elevated
Cystic fibrosis	Collins[86]	Plasma	Elevated
	Ciabattoni[87]	Urine	Elevated
	Montuschi[88]	Exhaled breath condensate	Elevated
Pulmonary hypertension	Cracowski[89]	Urine	Elevated
Sickle cell disease	Kling[90]	Plasma	Elevated
Chronic obstructive pulmonary disease	Pratico[160]	Urine	Elevated
	Montuschi[159]	Exhaled breath condensate	Elevated
Interstitial lung disease	Montuschi[85]	Exhaled breath condensate	Elevated
Inflammatory diseases			
Rheumatoid arthritis	Basu[72]	Plasma/synovial fluid	Elevated
Psoriatic arthritis	Basu[72]	Plasma/synovial fluid	Elevated
Reactive arthritis	Basu[72]	Plasma/synovial fluid	Elevated
Osteoarthritis	Basu[72]	Plasma/synovial fluid	Elevated
Systemic sclerosis	Cracowski[73,74]	Urine	Elevated
	Stein[75]	Urine	Elevated
Reproductive diseases			
Pre-eclampsia	Morris[93]	Plasma	Not differed
	Basu[95]	Plasma, urine	Not differed
	Regan[94]	Urine	Not differed
	Walsh[92]	Placenta	Elevated
	Barden[91,165]	Plasma	Elevated
	McKinney[164]	Plasma	Elevated
		Urine and saliva	Not differed
Neurodegenerative diseases			
Alzheimer's disease	Montine[76,78]	CSF	Elevated
	Pratico[79]	Brain tissue	Elevated
	Feillet-Coudrey[80]	Plasma	Not differed
	Montine[81,166]	Plasma and urine	Not differed
Huntington's disease	Montine[81]	Plasma and Urine	Not differed
	Montine[77]	CSF	Elevated
Parkinson's disease	Fessel[167]	Brain tissue	Not differed
Dementia with Lewy body disease	Fessel[167]	Brain tissue	Not differed
Migraine	Helmersson[69]	Urine	Not differed
Spinal cord injury	Monga[82]	Semen	Elevated

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TABLE II – *continued*

Pathophysiological conditions	Selected studies	Biological fluids/Tissues	Observations
Other diseases			
Chronic hemodialysis	Handelman[169]	Plasma	Elevated
	Ikizler[170]	Plasma	Elevated
	Spittle[174]	Plasma	Elevated
Prostate Cancer	Camphausen[168]	Urine	Not differed
Kidney transplantation	Crawcowski[171]	Urine	Not differed

ARDS = Acute respiratory distress syndrome; RVD = Renovascular disease; PCI = Percutaneous coronary intervention.

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difference between the type 2 diabetes patients and control subjects. Thus, type 2 diabetes seems to be associated with a higher isoprostane level only in patients with disease duration >7 years. However, this study confines only elderly patients of age 77. One report described a higher level of 8-iso-PGF$_{2\alpha}$ in urine from patients with type 1 diabetes.[107] However, several other reports showed no differences in isoprostane levels in patients with type 1 diabetes and controls (Table II).[110,111,163] Young Swedish type 1 diabetic patients had no increase in 8-iso-PGF$_{2\alpha}$ concentrations compared to those of matched controls.[110] Similarly, no increase in the urinary 2,3-dinor-5,6-dihydro metabolite of 8-iso-PGF$_{2\alpha}$ was found in type 1 diabetes.[111] The different results may be due to diverse degree of glycaemic control in these populations, in turn affecting the degree of lipid peroxidation and oxidative stress.

ISOPROSTANES IN ANIMAL STUDIES

A number of studies have shown that oxidative stress and higher lipid peroxidation play a major role in animal models of ischemia-reperfusion injury.[113,114] Rapid appearance of 8-iso-PGF$_{2\alpha}$ in the plasma and followed by urinary excretion of this compound was seen during ischemia-reperfusion in experimental spinal cord ischemia in pigs, compared to baseline values, and also compared to controls with no spinal cord ischemia.[115] An instant increase in 8-iso-PGF$_{2\alpha}$ was also seen both systemically and in jugular bulb plasma collected from the brain, concomitantly with an increase in the levels of PGF$_{2\alpha}$ metabolite, hypoxanthine and lactate, during the post-resuscitation period in a porcine model of cardiopulmonary resuscitation (CPR).[116–119] Further, the neurological outcome 24 h after experimental CPR was shown to be correlated with the cerebral plasma 8-iso-PGF$_{2\alpha}$ concentration after restoration of spontaneous circulation (ROSC).[118] Recently, it was also found that a time-dependent jugular bulb 8-iso-PGF$_{2\alpha}$ formation following ventricular fibrillation of various durations (2, 5, 8, 10 and 12 min) and CPR (5 and 8 min) occurred in conjunction with experimental cardiac arrest and ROSC as a cause of free radical-mediated brain damage.[117]

These studies evidence that biologically active isoprostanes are involved at an early stage of ischemia and reperfusion as has been shown for humans.

Both plasma and urinary 8-iso-PGF$_{2\alpha}$ increased dramatically in a well established porcine model of septic shock following intravenous administration of LPS/*E. coli*, and the survival of the pigs was dependent on the kinetics of formation and the levels of 8-iso-PGF$_{2\alpha}$ in the circulation, and there was an increase in arterial $PaCO_2$.[120,121] An inverse relationship was also seen between the plasma levels of 8-iso-PGF$_{2\alpha}$ and vitamin E in this septic shock model.[122] Thus, isoprostanes seem to be involved in acute inflammatory condition. Recently, an upregulation of inflammatory gene expression through activation of MAPKs pathway as shown by the induction of cytokine (IL-6) in human macrophages,[123] and prostaglandin F$_{2\alpha}$ formation[33,59] through COX pathway by F$_2$-isoprostanes are reported. The excretion of F$_2$-isoprostanes was related to the rate of atherogenesis and the high level of oxidised LDL in mice, but was not influenced by extra cellular-superoxide dismutase (EC-SOD) genotype mice.[124] This possibly indicates that isoprostane generation are not regulated by this antioxidant defence enzyme. Increased production of 8-iso-PGF$_{2\alpha}$ has been observed in acute coronary thrombolysis/reperfusion in an experimental canine model,[100] in streptozotocin-induced diabetic pregnancy,[125] in diquat induced hepatic and renal injury[126,127] and in oxidant-induced pulmonary lipid peroxidation.[128]

ISOPROSTANES IN DIETARY SUPPLEMENTATION STUDIES

There have been several reports on effects of different forms of dietary supplementation on isoprostane formation. No effect on the urinary levels of 8-iso-PGF$_{2\alpha}$ was observed after supplementation with tea extracts for four weeks in healthy subjects.[129] Neither there was any change in the urinary levels of 8-iso-PGF$_{2\alpha}$ after supplementation 25-day intake of

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fruit and vegetables,[130] and varying intake of vegetables, berries and apple combined with high intake of linoleic acid or oleic acid.[131] This might be due to the supplementation of various dietary products needs a longer exposure to affect or simply do not affect the isoprostane formation in these settings. A patient group with a higher level of isoprostanes could be a suitable group to determine the antioxidative effect of certain dietary component. However, reduced levels of F_2-isoprostanes were observed following supplementation with olive oil[132] or fish oil rich in eicosapentaenoic acid or docosahexaenoic acid.[133] Dietary fish decreased the urinary concentrations of F_2-isoprostanes in patients with non-insulin-dependent diabetes mellitus.[134] Intake of a high linoleic acid diet for four weeks increased the urinary excretion of 8-iso-$PGF_{2\alpha}$, but decreased the urinary levels of nitric oxide metabolites.[135] This evidence that PUFAs might affect isoprostane formation from arachidonic acid. Whether this is due to the competition of PUFAs to the arachidonic acid metabolism through free radical pathway needs to be further investigated. Theoretically PUFAs are not scavenger of free radical formation.

Conjugated linoleic acid (CLA) has been shown to have chemoprotective and anti-obesity properties in animal models, and it has been suggested that increased lipid oxidation may contribute to its anti-tumorigenic effects. A significant increase in 8-iso-$PGF_{2\alpha}$ in the plasma and/or urine was observed after one or three months of daily CLA intake (4.2 or 3.4 g/day) in healthy subjects or in men with abdominal obesity as compared to control subjects.[48,136–138] Conjugated linoleic acid had no or only a slight effect on the serum α-tocopherol levels. Further, supplementation with t10c12-isomer of CLA significantly increased the urinary concentration of 8-iso-$PGF_{2\alpha}$ (by 578%) and the plasma level of C-reactive proteins (by 110%) compared to placebo, independently of changes in hyperglycaemia and dyslipidaemia. The increase in the 8-iso-$PGF_{2\alpha}$ level was significantly and independently related to aggravated insulin resistance.[137] The latter suggests that 8-iso-$PGF_{2\alpha}$ formation seems to be closely related to induced insulin resistance. When vaccenic acid (*trans*-11-octadecaenoic acid), a trans fatty acid, was given to humans, conversion to *cis*-9, *trans*-11 octadecenoic acid and later to 8-iso-$PGF_{2\alpha}$ was found to occur.[139] However, the mechanism of formation of 8-iso-$PGF_{2\alpha}$ following supplementation of CLA is unclear at present.

ISOPROSTANES IN ANTIOXIDANT OR DRUG EVALUATION

In the last two decades scientists have shown increasing interest in evaluating the pharmacological properties of various antioxidants, but the progress in this field has been partly hampered by limitations in the methods available for determining their activity. During the past years, measurements of isoprostanes have proved to be a valuable tool for determining the effect of various antioxidants and drugs with free radical-scavenging properties.

In healthy subjects supplementation with vitamin E in various doses (200–2000 IU/day for 8 weeks) did not affect the concentrations of F_2-isoprostanes in spite of a dose-dependent increase in circulating vitamin E.[140] Neither did vitamin E (200 IU/day for two weeks) have any effect on the basal F_2-isoprostane level in another study in the healthy subjects.[138] Vitamin E supplementation did not affect the levels of F_2-isoprostanes in moderate cigarette smokers.[142] In a study in which cigarette smokers consumed a diet high in polyunsaturated fat a pro-oxidant effect of supplementary vitamin E was observed.[143] When hypercholesterolaemic patients were treated with 800–3200 IU/day of vitamin E for 20 weeks, a significant decrease in the F_2-isoprostane level was noted after 16 weeks.[144] However, no such decrease was seen in patients given vitamin E doses of 100–400 IU/day for 20 weeks.[144] Vitamin E supplementation reduced the concentration of F_2-isoprostanes in patients with type 2 diabetes,[107] cystic fibrosis,[87] hypercholesterolaemia[145] and homozygous homocystinuria.[146] Vitamin E has been shown to suppress urinary F_2-isoprostane level in hepatic cirrhosis and alcoholic liver disease.[61,147] In a mechanistic study in which the formation of different isomers of CLA-induced F_2-isoprostanes was followed in healthy subjects, no decrease in the urinary 8-iso-$PGF_{2\alpha}$ level was seen during four weeks of supplementation with vitamin E (200 mg/day) or COX-2 inhibitor (Rofecoxib, 12.5 mg/day).[138] Together, these studies show that vitamin E supplementation has a varying antioxidative effect in studies with different patients or population groups depending on the basal lipid peroxidation process in these individuals. Which adds more complexity in evaluating the radical scavenging effect of vitamin E as known from the clinical trials of α-tocopherol in cardiovascular diseases. In one study vitamin C supplementation reduced the F_2-isoprostane level in patients with chronic alcoholic liver disease.[61] Vitamin C had no effect on the F_2-isoprostane levels in healthy women.[141]

In animal models vitamin E suppresses isoprostane generation *in vivo* and reduces atherosclerosis in ApoE-deficient mice.[148] Further, vitamin E deprivation has been found to be associated with increased levels of F_2-isoprostanes in rats.[126] Vitamin E supplementation decreased the basal levels of F_2-isoprostanes and prostaglandin $F_{2\alpha}$ in the rat.[37] In a recent study, a high dose of vitamin E

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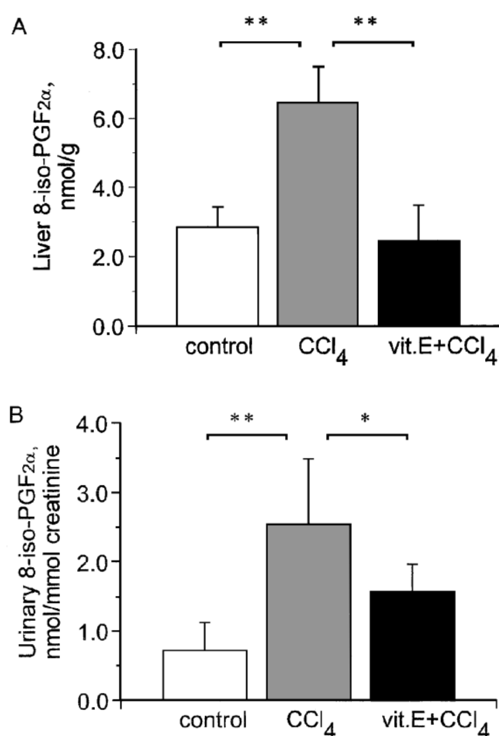

FIGURE 4 Effect of CCl_4 treatment (2.5 ml/kg) with and without prior supplementation with vitamin E daily for 3 weeks (2 g/kg) on the concentrations of 8-iso-$PGF_{2\alpha}$ in liver (A) and urine samples (B) in rats. Values are mean ± SD of controls ($n = 6$), CCl_4-treated rats ($n = 6$) and vitamin E-supplemented CCl_4-treated rats ($n = 8$). ($^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). (Reprinted with kind permission from Ref. [34].)

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(20 g/kg diet of all-rac-tocopheryl succinate for three weeks) was administered with the aim of counteracting non-enzymatic lipid peroxidation in rats with CCl_4-induced hepatotoxicity (2.5 ml/kg).[34] Non-enzymatic lipid peroxidation during experimental hepatic oxidative injury could be reduced by daily dietary supplementation with high doses of vitamin E. Rats that were given vitamin E prior to CCl_4 treatment had significantly lower levels of urinary and liver free 8-iso-$PGF_{2\alpha}$ than rats treated with CCl_4 alone (Fig. 4). Thus, it seems that supplementation with high doses of vitamin E might need to affect lipid peroxidation, as shown in the hypercholesterolaemic subjects.[144]

Very few studies have been addressed the question whether other antioxidant nutrients have any role on CCl_4-induced isoprostane formation *in vivo*. In a recent study, Lieber and his group showed that CCl_4-induced lipid peroxidation (as measured by F_2-isoprostanes and 4-hydroxynonenal) was attenuated by polyenylphosphatidylcholine, a mixture of phospholipids extracted from soybean, and the levels of F_2-isoprostanes and 4-hydroxynonenal paralleled liver fibrotic scores and collagen accumulation.[149] They suggested that the observed hepatic protective effect of polyenylphosphatidylcholine against CCl_4-induced lipid peroxidation might be partly exerted through its antioxidant activity via inhibition of lipid peroxidation. In an experimental study of LPS-induced septic shock in pigs, retinol palmitate counteracted oxidative stress by reducing plasma F_2-isoprostanes, which would explain some of the therapeutic effects of nutrients rich in carotene/retinols used in some clinical studies.[150] In a similar experimental septic shock study with LPS, propofol (Diprivan-EDTA), an anaesthetic and sedative agent reduced endotoxin-induced plasma F_2-isoprostanes to the basal levels and diminished the fall in arterial oxygen tension.[151] At present, propofol seems to be one of the best compound that inhibits the isoprostane formation *in vivo*. However, melagatran, a novel direct thrombin inhibitor, did not affect the F_2-isoprostanes level during LPS-induced experimental septic shock.[152]

SUMMARY

Isoprostanes are biologically potent free radical catalysed-compounds and reliable biomarkers of lipid peroxidation and possibly, a mediator of inflammation, involving cyclo-oxygenases and/or cytokines. Their role in medical science is of great importance especially in regard to various human diseases in which oxidative stress and inflammation are involved. Since bioactive F_2-isoprostanes are found in variable concentrations under normal basal conditions, their ongoing biosynthesis might have some vital as yet discovered role in signalling various biochemical cascades that are essential for maintenance of physiological functions and also normal ageing processes. Only a few antioxidants or radical scavengers have been shown to affect isoprostane formation *in vivo*.

Acknowledgements

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