Review

Isoprostanes as potential cerebral vasospasm biomarkers

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A B S T R A C T

Despite enormous progress in medicine, symptomatic cerebral vasospasm (CVS), remains an unexplained clinical problem, which leaves both physicians and patients helpless and relying on chance, due to the lack of specific marker indicative of imminent danger as well as the lack of specific treatment. In our opinion CVS occurrence depends on dynamic disbalance between free radicals’ formation (oxidative stress) and antioxidant activity.

Isoprostanes are products of free-radical peroxidation of polyunsaturated fatty acids, and seem to mark a promising path for the research aiming to unravel its possible mechanism. Not only are they the biomarkers of oxidative stress in vivo and in vitro, but also have manifold biological effects (including vasoactive, inflammatory and mitogenic) via activation of the thromboxane A2 receptor (TXA2R), both in physiological and pathophysiological processes. This review addresses the importance of isoprostanes in CVS in quest of appropriate biomarkers.

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1. Introduction

Based on the recent literature, there is growing evidence supporting the clinical association between lifestyle/environmental risk factors, oxidative stress (OS) and endothelial dysfunction [1,2]. An unbalanced production of toxic oxygen species, reactive oxygen species (ROS) and endogenous antioxidants, may be the cause of a disease [3]. ROS can activate the transcription factor NF-kappaB, which has many inflammatory cytokines genes among its target genes [4]. The identification of cytokines as mediators of inflammation led to “cytokine theory of disease” [5]. Additionally, inflammation as consequence of chemical, physical or psychological changes, can induce OS [6].
It is very difficult to study OS in disease. ROS are the molecules effector, which have short half-lives, ranging from nanoseconds to milliseconds [7]. This makes it impossible to measure ROS in biological samples and we must rely on signs, chemicals that are produced by the interaction of ROS with various cellular molecules [8]. They take part in the peroxidation of membrane lipids of endothelial cells. Thus, recently F2-isoprostanes (F2-IsoPs, IsoPs) have been demonstrated to be the most specific markers of lipid peroxidation in vivo. They are considered oxidative stress markers, as their concentration directly reflects the free radical content. F2-IsoPs are compounds similar to prostaglandins, generated via nonenzymatic, free radical peroxidation of polyunsaturated fatty acids, in particular, arachidonic acid [9].

An inflammatory response often results in tissue damage (e.g. rheumatoid arthritis) and loss of function. Endothelial dysfunction is clearly associated with oxidative stress, and is also the first clinical correlate of atherosclerosis that can be diagnosed [10,11].

Our aim was to review the current state of knowledge but also to provide new concepts to understand the vital crosstalk of the different systems (stress response pathways and redox signalling machinery) involved in the CVS pathophysiological processes and to reveal probable disease pathways that may explain vasoconstrictive effect observed in CVS.

In this revision, we focused on the role of F2-IsoPs as a biomarker of oxidative stress in CVS after aneurysmal subarachnoid haemorrhage (aSAH).

1.1. Evidence acquisition

We conducted a review of the pertinent literature paying careful attention to large animal laboratory research and clinical studies. Unfortunately, there are no randomized controlled prospective clinical studies on F2-IsoPs. After a brief summary on oxidative stress and biological properties of IsoPs, we discussed the role of F2-IsoPs in CVS.

2. Clinical implications

2.1. Oxidative stress

OS is a disorder of homeostasis leading to an increase in the concentration of ROS. This tips the peroxidation – antioxidant balance towards the oxidation reaction and leads to disruption of redox signalling (RS) and physiological function [3].

The main endogenous sources of primary type of ROS, superoxide anion radical (O2−), are mitochondria. They contain electron-transportation chain (ETC) which major function is ATP production through the process of oxidative phosphorylation, while ROS are by-products of ETC functioning. ETC transfers high-energy electrons from NADH or FADH2 to the final electrons acceptor – O2−. Another source of O2− are NADPH-oxidases of Nox family enzymes that produce ROS as a primary product. Phagocytic NADPH-oxidase (NOX2) is the first identified ROS-generating enzyme. It has been documented that O2− production in neutrophils and macrophages participates in antibacterial signal transduction [12]. ROS production is controlled by endogenous antioxidants like glutathione and reactions catalyzed by peroxiredoxins and glutathione peroxidase.

RS works through discrete, reversible modifications/changes like cysteine residues include S-nitrosylation/S-nitrosoation, sulphenylation, disulfide bonds and S-glutathionylation [13,14]. Under physiological condition RS, through ROS and reactive nitrogen species (RNS), induces changes in activities of proteins which are elements of complex networks of kinases, phosphatases, ion channels, apoptotic cascades and can cause changes in transcriptional activity [13,15,16]. RS regulates cell-signalling, vascular tension, homeostatic control, immunological response and pregnancy [17,18]. Even slight variations of lipid oxidation in vivo are vital to correct functioning of an organism. On the other hand, a rapid accumulation of biologically active oxidation by-products which overcomes the cellular reductive capability, results in DNA, protein and lipid damage, thus disrupting the cell environment and finally leading to cell’s death. Most importantly, damaged cells also produce reactive oxygen species creating a self-perpetuating vicious cycle which, if not interrupted, culminates in injury and disease.

The main problem arises from the evaluation of highly reactive and unstable free radicals. However, the recent discovery of isoprostanes has opened up new possibilities for the research.

2.2. Isoprostanes as oxidative stress markers

OS is induced for research purposes with carbon tetrachloride using animal models [19]. Such models were also employed for establishing F2-isoprostanes as oxidative stress markers [20].

Isoprostanes are compounds similar to prostaglandins, generated during non-enzymatic, free-radical peroxidation of multi-unsaturated fatty acids, mainly arachidonic acid [9]. They are considered oxidative stress markers, since their concentration directly correlates with the concentration of free oxygen species. The analysis of isoprostanes broadened the understanding of the role of free radicals in human physiology and pathophysiology, especially with reference to vascular diseases. Isoprostanes are found in urine, blood, cerebrospinal fluid, and also exhaled air. Their main metabolite, 2,3-dinor-5,6-dihydro-8-iso-PGF2α, is mostly expelled by kidneys. Importantly, there are same data which suggest that F2-IsoPs are conjugated with glucuronide and excreted to the urine. One should keep in mind another route of F2-IsoP metabolism when measuring these compounds in urine [21]. The half-life of isoprostanes is approximately 16 min. Plasma concentration of free 8-iso-PGF2α varies from 10 to 80 pg/ml (average 28 pg/ml), depending on the time of day [22].

The level of isoprostanes increases in various diseases: neurodegenerative (AD, HD, CJD, and MS), cirrhosis, atherosclerosis (hypercholesterolemia, hyperhomocysteinemia), diabetes mellitus, heart diseases, severe cholestasis, allergic asthma, adult respiratory distress syndrome, kidney distress syndrome, retinopathy and chronic obstructive pulmonary disease [23].

Appropriate collection and storage procedures are the key to repeatable results. Artefactual oxidation of lipids during storage could occur due to haemolysis of blood samples. To minimize this effect blood should be collected to tubes
containing anticoagulants such as EDTA (but not heparin), and the centrifugation should be performed as soon as possible (by that time it should be kept chilled). Plasma should be aliquoted and stored at −80 °C not −20 °C, because autoxidation can occur at −20 °C leading to artefactual generation of F2-IsoPs ex vivo. Tissue samples should be flash frozen immediately after collection and also stored at −80 °C. The antioxidants butylated hydroxytoluene to minimize artefactual elevation of F2-IsoPs should be used, especially when samples cannot be immediately centrifuged and snap frozen in liquid nitrogen [24].

Currently, three techniques are used to measure F2-IsoPs: gas chromatography–mass spectrometry (GC/MS), liquid chromatography–mass spectrometry (LC/MS) and immunoassays (ELISAs). Mass spectrometric-based assays are widely accepted as the most accurate methodologies, especially GC/MS, because of its greater sensitivity [25]. The use of ELISA to quantify urine F2-IsoPs, despite its simplicity and cost-effectiveness, may be associated with cross-reactivity with other isoprostane isomers [26].

F2-IsoPs in plasma mostly are quantified as free fatty acids. Since these compounds are found esterified in phospholipids in the plasma, base hydrolysis should be performed to analyze total F2-IsoPs in the plasma. F2-IsoPs can be directly excreted into the urine without metabolism, so that if renal disease coexists with oxidative damage, their urine levels are falsified and actual changes in total body lipid peroxidation should be checked.

Establishing specific thresholds of the level of isoprostanes may be helpful in early detection and prevention of distinct disorders. Moreover, such analysis may offer a non-invasive way of monitoring the course of disease.

With regard to CVS, a biological marker of the developing pathology may help in recognizing the impending exacerbation and enable an early management.

2.3. **Biological properties of isoprostanes**

The formation of prostaglandin-like compounds (IsoPs) as products of free-radical peroxidation of arachidonic acid was first described by Pryor and Godber in 1991. These structures were defined as F2-IsoP due to their prostantanoid 1,3-dihydroxy-cyclopentane ring (ring F), analogous to prostaglandins F (PGF2α). F2-IsoPs are the structural isomers of prostaglandins, which consists of a cyclopentane ring and two side chains with double bonds and a hydroxyl group. The distinguishing feature is the conformation of the side chains in relation to ring F: F2-isoprostane is a cis-, while PGF2α a trans-conformer [27].

The isoprostanes forming mechanism is associated with the presence of ROS (such as HO, RO, ROO, O2) which detach the hydrogen atom from polyunsaturated fatty acids and propagate a chain reaction. This reaction occurs at positions C-13, C-10 and C-7 of arachidonic acid and may result in three radical forms, exposed to further internal cyclization. Through reduction, each of them gives rise to four regioisomers with ring F, which, in turn, may form eight racemic diastereomers each. Thus, 96 different compounds are generated in different amounts [28]. The formation of isoprostane may occur via two peroxidation routes [29,30] — by endoperoxide and dioxygenate mechanisms.

Bioactive isoprostanes (mainly 8-iso-PGF2α and 8-iso-PGE2α) are formed in various tissues, while their unmetabolized portion can be found in plasma. Local hormones reach their biological properties in tissues with low or high concentrations in plasma and reduce them after esterification. Tissue hydrolases revert the esterified IsoPs into potent mediators which affect the cellular membrane integrity and fluidity, exacerbating the oxidative stress reaction. These compounds are mainly formed in tissues in esterified forms, which act as substrates for the hydrolytic activation by specific enzymes [31,32]. Then in the free-acid form isoprostanes are released into blood. Nonetheless, their physiological function and pharmacokinetics are not well-understood.

The most extensively analyzed isoprostane, 8-iso-PGF2α, causes vasoconstriction in renal and pulmonary circulatory systems, as well as induces DNA synthesis in smooth muscle cells [34]. The 8-iso-PGF2a stimulate endothelial cell and smooth muscle cell proliferation. [35]. Still, their mechanisms of action are vaguely defined. Mitogenesis and stimulation of platelet aggregation are achieved via the thromboxane receptor [33,34], however, several reports indicate the existence of a specific receptor for isoprostanes [35,36]. In addition, other F2-isoP, 12epi-PGF2, stimulate fibroblast proliferation through PGF2α receptors [37]. Vasoconstriction is a well-known effect of isoprostanes, particularly 8-iso-PGF2α observed in various organs, including brain, kidney, lung, pulmonary arteries and retinal vessels [38,39]. It depends on the concentration of extracellular calcium ions and, possibly, of protein kinase C [34,40]. In addition, 8-iso-PGF2α induces the formation of a cyclooxygenase-dependent prostacyclin PGF2α [41] as well as thromboxane in the endothelium [39].

### 3. Future directions

#### 3.1. Isoprostanes in cerebral vasospasm after aneurysmal subarachnoid haemorrhage

The most common and fatal, yet the least understood complication of aSAH is CVS, as it leads to delayed ischaemic neurologic deficit resulting from irreversible vasoconstriction caused by proliferative angiopathy [42,43]. CVS is always taken into consideration in aSAH patients presenting with symptoms of confusion or decreased level of consciousness with or without focal neurologic deficits (speech or motor).

From the histopathological point of view, in the course of CVS, between day 1 and day 9, one observes increased percentage of inflammatory cells (lymphocytes, plasmocytes, and mast cells) in the adventitia, necrosis and the loss of elasticity in smooth muscles, as well as thickening, swelling, vacuolization and loosening of intracellular connections in the endothelium. Furthermore, between day 9 and day 60, proliferation of smooth muscles and irreversible thickening of the intermediate layer of the vessel are also observed [44,45].

Up to now, numerous theories aiming to explain the occurrence of CVS have been proposed, but its specific mechanism has not been defined, yet. The most plausible theory suggests that the extravasated blood acts as the nidus for inflammatory factors, which lead to CVS. Extravasated haemoglobin leads to ROS release and OS. This results in RS
changes which implicates peroxidation of membrane lipids of endothelial cells and perivascular adipose tissue, as well as proliferation of smooth muscle cells. The extravasated haemoglobin is bound to haptoglobin (HP), which may be found in two forms. HP 1-1 binds with a lower affinity [46,47], while HP 2-2 induces a cascade of inflammatory reactions, elevating the concentration of cytokines, leukocytes and cell adhesion molecules [46,48].

As aforementioned, RS which is modified by ROS, regulate the vascular tone in various mechanisms, called "redox switches" leading to endothelial dysfunction and vasospastic disorder. The first of "redox switches" involves endothelial NO synthase (eNOS). The mechanisms underlying eNOS uncoupling include S-glutathionylation of the eNOS reductase domain, eNOS phosphorylation (H2O2/ONOO− mediate activation of protein kinase C (PKC) and protein tyrosine kinase-2 (PYK-2) dependent eNOS phosphorylation). Furthermore, direct oxidation depletes eNOS cofactor — tetrahydrobiopterin, regulates dimethylarginine (ADMA) synthesis/break-down and disrupts the zinc-sulphur complex in the dimer binding interface. It is also known that cerebrospinal fluid (CSF) ADMA levels are closely associated with the degree and time course of vasospasm (when CSF ADMA levels decrease, vasospasm resolves) [49,50].

The other switches have to do with soluble guanylyl cyclase (sGC) which is a key enzyme of the nitric oxide (NO) signalling pathway. Upon binding of NO to a prosthetic heme group on sGC, the enzyme catalyses synthesis of the second messenger — cyclic guanosine monophosphate (cGMP), which produces vasorelaxation and inhibits smooth muscle proliferation, leucocyte recruitment and platelet aggregation through a number of downstream mechanisms. OS leads to an impaired NO/cGMP-signalling by oxidative inhibition of sGC via oxidation of Cys122, making the enzyme less sensitive to NO'. Additionally, oxidation promote depletion of the heme moiety [51–53].

The third switch comprises the endothelin-1 (ET-1) and NADPH oxidase system since oxidants can lead to increased expression of ET-1 and potentiate its vasconstrictory properties, which can be further self-amplified by ET-1 dependent activation of NADPH oxidase [54–56].

The last switch relates to prostanoid synthesis (PGIS). The key enzyme of PGIS, cyclooxygenase, is activated at low peroxide levels, thus oxidative stress promotes isoprostanes formation [57,58]. Reduced synthesis of prostacyclin in the vessel wall might also contribute to the cerebral vasospasm [59].

Activation of all of these redox switches by ROS (most likely hydrogen peroxide and superoxide) alters endothelial and vascular function, which will favour vasoconstriction. These switches are usually activated simultaneously and one finds eNOS uncoupling, sGC desensitization and PGIS synthesis/break-down along with isoprostane formation or up-regulated ET-1 signalling at the same time. It is worth mentioning that 8-iso-PGF2α has been shown to stimulate ET-1-release in a dose-dependent manner [60]. ET-1 has been well investigated as an important mediator of CVS [61] (Fig. 1).

In our opinion isoprostanes play a vital role in CVS, disturbing endothelial integrity and leading to vasospastic disorder. These compounds inform as about the level of OS and indirectly about "redox switches" condition. They are crucial biomarkers of CVS. We have reported elevated urine F2-IsOPs levels in patients following aSAH compared with healthy controls, which may reflect the increased oxidative stress in patients after aSAH. We observed that increased F2-IsOPs on day 3 after surgery were associated with greater risk of CVS and worse long-term outcomes. Moreover, the ROC curves plotted for prediction of CVS and GOS score 4 at 4 months after surgery with day 3 F2-IsOPs had AUC >0.8, what indicates the potential clinical value of these markers. We also employed logistic regression models combining F2-IsOPs with clinical features to investigate whether such an approach might further improve the prediction rate. This analysis produced perfectly fitted models, which resulted from the small group size and the associated overfitting. To limit its impact, we performed the same analysis after resampling and stratified cross-validation, obtaining more adequate ROC curves. Thus, although both issues are associated, based on the perioperative condition and F2-IsOP levels the risk of CVS may be more effectively predicted than the long-term outcome. The latter is undoubtedly affected by a number of other factors. The association of the F2-IsOPs levels with risk of CVS may offer a new insight into the pathogenesis of this poorly understood condition [62]. Additionally, F2-IsOPs as biologically active chemicals, start cascade reactions leading to irreversible vasospasm.

These results, together with the various biological activities of 8-iso-PGF2α, suggested that 8-iso-PGF2α,
a free radical catalyzed peroxide independent of cyclooxygenase and lipoxgenase, could be assumed to be at least one of the causes of delayed cerebral vasospasm. We can differentiate two mechanism connected to IsoPs which are observed in CVS. The first, is a calcium-dependent process and the second are calcium-independent molecular events, both lead to smooth muscle contraction.

F2-IsoPs have probably slightly different calcium-dependent processes. 8-iso-PGF2 trigger an increase in intracellular Ca2+ that is dependent on extracellular Ca2+. Since nifedipine did not inhibit the effects of 8-iso-PGF2, possibly the L-type voltage-gated Ca2+ channels are not implicated in vasoconstriction. Other studies performed with Ca2+ entry blocker, including that by non-voltage-dependent Ca2+ channels showed that non-voltage-gated Ca2+ channels are involved in the vasoconstrictor action of 8-iso-PGF2 [63–65].

Calcium-independent smooth muscle contraction following SAH begins with PKC activation by the phospholipase C mediated release of diacylglycerol (DAG) and inositol-triphosphate (IP3) initiated by ligand binding to G-protein coupled membrane receptors [66]. PKC is activated as shown in the first “redox switch”, but it is worth mentioning that, 8-iso-PGF2 stimulates IP3 synthesis [67,68]. Activated PKC phosphorylates caldesmon and caldesmin resulting in a prolonged constriction. Furthermore, PKC alters several other intracellular signalling pathways including mitogen activated protein kinase, protein tyrosine kinase (PYK-2) and Rho kinase [66]. RhoA (Ras homolog gene family protein, member A) translocation activates its downstream target Rho kinase inhibiting myosin light chain kinase (MLC) phosphatase activity to increase the amount of phosphorylated MLC, leading to a prolonged contraction without Ca2+ elevation [69,70]. RhoA and Rho kinase messenger RNAs are up-regulated in sustained vasospasm, a RhoA/Rho kinase activation is found in canine basilar arteries, and vasospasm is partially reversed by the Rho antagonist [66,71].

It has also been shown that 8-iso-PGF2 constricts cerebral vessels, which can be inhibited by thromboxane receptor antagonists [72,73]. It raises suspicion that IsoPs bioactivity is achieved via the thromboxane receptor [74,75]. The biological actions of the thromboxane (TP) receptor are achieved through activation of one or both of its isoforms (TPα and TPβ) that are produced through differential splicing of a single gene product [76,77]. Both isoforms are members of the G-protein-coupled receptor superfamily that differ with regard to their C termini [78]. G-protein coupled receptors are associated with an intracellular calcium release via IP3 and DAG [79–81]. Stimulation of G-protein-coupled receptor results in activation of phospholipase C (PLC). This leads to hydrolysis of phosphatidylcholine 4,5-bisphosphate and generation of DAG and IP3, resulting in activation of PKC and Ca2+ release from intracellular stores. DAG stimulates PKC which activates phospholipase A2, an enzyme responsible for the release of arachidonic acid from membrane phospholipids [82] (Fig. 2).

Fig. 2 – OS, oxidative stress; IP3, inositol-triphosphate; DAG, diacylglycerol; PLA2, phospholipase A2; TRPCs, transient receptor potential proteins, non-voltage-gated Ca2+ channels; PKC, protein kinase C; PYK-2, protein tyrosine kinase-2; MAPK, mitogen activated protein kinase; MLC, myosin light chain kinase.
The transient receptor potential (TRP) proteins are a novel and rapidly expanding superfamily of non-voltage-gated Ca²⁺ channels. TRPs are coded by the TRPC 3,4,5,6,7 genes and are promising proteins for the 8-iso-PGF₂α receptor. Accumulated evidence strongly suggests that especially TRPC6 may serve as a major Ca²⁺ entry channel that integrates various mechanisms regulating the arterial tone [89], via DAG [81,90]. The scientific literature widely describes, that endothelin-1 activates TRPC6-like cationic currents to cause Ca²⁺ increase [91] (Fig. 2).

It is also now recognized that perivascular adipose tissue contributes to vascular homeostasis by producing vasoactive compounds such as adipokines, ROS, NO⁻ and promote endothelial dysfunction from outside the vessel, which answered the question, by which mechanism the perivascular blood causes endothelial dysfunction leading to CVS [92,93].

Literature data suggest, that the types of IsoPs that will be formed and in what ratio, depend on oxygen tension and glutathione concentration [94]. The role of antioxidants in patients with CVS remains unexplained. Some evidence suggests that their level is decreased after aSAH. Mean levels of retinol, β-γ-tocopherol, δ-tocopherol, β-carotene, lutein, and coenzyme Q10, but not α-tocopherol, in plasma were significantly lower in SAH patients than in controls [95]. On the other hand, it has been shown that oral coenzyme Q10 administration and intracisternal administration of α-tocopherol could suppress vasospasm and ischaemic brain damage in experimental SAH [96,97]. NRF2 is the main transcriptional regulator of cellular homeostasis and the protector against multiple stress conditions. Upon dimerization with small MAF proteins, it recognizes an enhancer in the promoter region of target genes, knowns as Antioxidant Response Element/ Electrophile Responsive Element genes. These accounts for about 1% of human genome and encode phase I, II and III detoxification enzymes; glutathione, peroxiredoxin [98].

In our opinion CVS depends on dynamic disbalance between free radicals’ formation (oxidative stress) and antioxidant activity. IsoPs start cascade reactions leading to arterial wall changes which in turn cause irreversible vasoconstriction as was shown in histopathological studies.

4. Conclusions

The specific mechanism of CVS has not been defined yet. The most plausible theory suggests that:

1. The extravasated haemoglobin leads to ROS release and OS.
2. This results in redox signalling changes, which implicate peroxidation of perivascular adipose tissue and endothelial cell membrane lipids, followed by the proliferation of smooth muscle cells.
3. Perivascular adipose tissue promotes endothelial dysfunction from the outside the vessel, which explains the link between perivascular blood and endothelial dysfunction.
4. Moreover, ROS-dependent redox signalling regulates the vascular tone in various mechanisms, called “redox switches”, leading to further endothelial dysfunction and vasoconstriction.
5. These switches are usually activated simultaneously and function in parallel. The key enzyme of PGIS synthesis, cyclooxygenase, is activated at low peroxide levels, while, in turn, oxidative stress promotes IsoPs formation.
6. IsoPs are considered as oxidative stress markers, since their concentration directly correlates with the concentration of free oxygen species.
7. Still, their mechanisms of action are vaguely defined. Stimulation of endothelial cell, smooth muscle cell and stimulation of platelet aggregation are achieved via the thromboxane receptor, however, several reports indicate the probable existence of a specific receptor for isoprostanes.
8. The vasoconstrictor action of IsoPs is not inhibited by a calcium channel blocker (nifedipine) because they work through a G-protein-coupled receptor (thromboxane receptor), which are associated with an intracellular calcium release via IP3 and DAG.
9. The types of IsoPs that will be formed and in what ratio, depend on oxygen tension and antioxidant mechanisms.

To sum up, it may be concluded that CVS is a dynamic disbalance between free radicals’ formation and antioxidant activity.

Conflict of interest

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