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The Impact of Isoprostane Metabolism on the Assessment of Oxidative Stress

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Introduction

Isoprostanes, a group of 64 prostaglandin-like compounds, are derived primarily by free radical-mediated peroxidation of free or esterified arachidonic acid (1-3). Based on many comprehensive studies, the level of one representative isoprostane, 15-F_{2t}-isoprostane (formerly denoted 8-iso-prostaglandin $F_{2\alpha}$), in blood or urine is widely regarded as the "gold standard" biomarker for the assessment of oxidative stress (4-7).

Isoprostane Quantification

Several methods have been developed for the quantification of 15-F_{2t}-isoprostane, including GC/MS (8,9), LC/MS (10,11), RIA (12,13) and ELISA (14-16), and all of these methods are widely employed for the assessment of systemic oxidant stress. However, in contrast to enzymatically-derived eicosanoids, 15-F_{2t}-isoprostane concentrations determined by GC/MS or LC/MS do not always correlate well with those obtained using immunoassay methods. Some studies have reported good correlation among these methods (14,15), whereas others have not (17). Further, there is limited but clear evidence that isoprostanes are rapidly and extensively metabolized in humans (18-20). Given the need to rapidly clear these potentially toxic substances from the body, this is not unexpected. Although one metabolite has been identified and can be independently quantified (21), there appear to be multiple metabolic mechanisms for isoprostane metabolism. Since even relatively small elevations in isoprostane levels have been reported to be a significant risk indicator for cardiovascular disease (22), consideration of the

impact of isoprostane metabolism on results obtained by various assay methods is important (*see* figure 1).

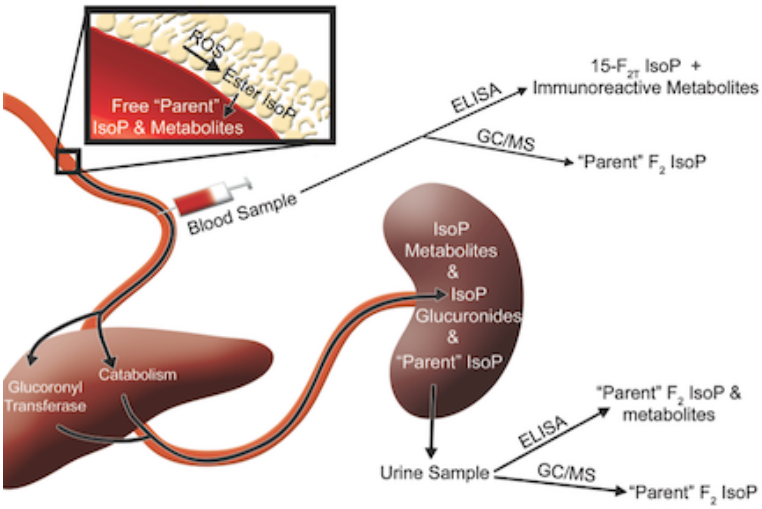

Fig. 1. Schematic representation of pathways for the formation, metabolism and excretion of isoprostanes in vertebrates. Multiple pathways for IsoP metabolism have been identified, including β-oxidation, glucuronidation and the formation of glutathione adducts. Analysis of IsoP-M and/or pretreatment of urine samples with β-glucuronidase can provide insights into variations in metabolism among individuals due to multiple factors, including genetics, diet, lifestyle, environment and disease

Methodological Considerations

- GC/MS and LC/MS methods typically quantify 15-F_{2t}-isoprostane, and some metabolites. However, the values obtained can be dependent on the sample preparation protocol (23). For example, four separate isoprostanes contribute to the GC/MS peak if solid phase extraction and TLC are employed for sample preparation. However, if immunoaffinity chromatography is employed to remove interfering substances, then only 15-F_{2t}-isoprostane is present in the GC/MS peak. A simple one-step method for

immunoaffinity purification of 15-F_{2t}-isoprostane, which can then be analyzed by GC/MS, LC/MS or ELISA has recently been published (10).

- Isoprostane Metabolism: Given the large number of isoprostanes generated from the action of reactive oxygen species on arachidonic acid *in vivo*, and the likelihood that multiple 15-F_{2t}-isoprostane metabolites multiple isoprostanes, and isoprostane metabolites undoubtedly contribute to "15-F_{2t}-isoprostane" values determined by immunoassays, with differences among immunoassays expected based on the relative specificity of the antibodies employed (20). The antibody employed in our ELISA kit has been extensively characterized so that 15-F_{2t}-isoprostane results obtained by ELISA for serum samples following established solid phase extraction protocols – or following immunoaffinity isolation of 15-F_{2t}-isoprostane - correlate very well with those results obtained by GC/MS (14,15).
- Indeed, given the rapid and extensive metabolism of isoprostanes *in vivo*, including β-oxidation, glucuronidation and other pathways (18,24), and the well documented inter-individual differences that have been reported for at least some of these pathways (see below), it is actually pretty amazing that isoprostane assays have emerged as a "gold standard" for oxidative stress. Further complicating comparison of isoprostane vales obtained by different analytical techniques are the significant differences among the methods for sample preparation (e.g. multiple Sep-Pak + TLC versus immunoaffinity).
- Assays have been developed to quantify one major 15-F_{2t}-isoprostane metabolite, 2,3-dinor-8-iso-$PGF_{2\alpha}$ (15, 18), affording the opportunity to evaluate and factor in inter-

individual differences in metabolism by one pathway of this isoprostane.

- In addition, the rapid and extensive metabolism of 15-F_{2t}-isoprostane, suggests that elevated isoprostane levels best serve as biomarkers for acute oxidative stress. In animal models, the pronounced elevation of 15-F_{2t}-isoprostane in response to oxidative stress returns to baseline values within 24 hours (2,9). The strong correlations reported between 15-F2t-isoprostane levels and conditions such as cardiovascular disease in humans is presumably due to chronic oxidative stress to replenish the rapidly metabolized 15-F2t-isoprostane.
- Although GC/MS or LC/MS may more reliably quantify levels of a specific isoprostane, e.g. 15-F_{2t}-isoprostane, the ability of immunoassays to detect isoprostane metabolites (19), and the more robust assessment of isoprostane production that can be obtained by pretreatment of urine with β–glucuronidase (see below) may further improve the utility of "IsoP" as a biomarker for oxidative stress by making the measurements more independent of variations in metabolism.

Glucuronidation is a Major Pathway for Isoprostane Metabolism

With the exception of 20-HETE (25), glucuronidation is not an important pathway for the excretion of enzymatically-derived eicosanoids. Based on the significant differences between the stereochemistry of isoprostanes and prostaglandins, and on the observation that approximately 50% of radiolabeled 15-F_{2t}-isoprostane elutes with the aqueous fraction during solid phase extraction of human urine (18), we investigated the extent to

which isoprostanes are excreted as glucuronic acid conjugates in humans. Whether quantified by GC/MS, RIA or ELISA (24,26), pretreatment of human urine samples with b-glucuronidase increased the 15-F2t-Isoprostane levels by an average of ~100%, indicating that glucuronidation is an important pathway for 15-F_{2t}-isoprostane elimination (figure 2).

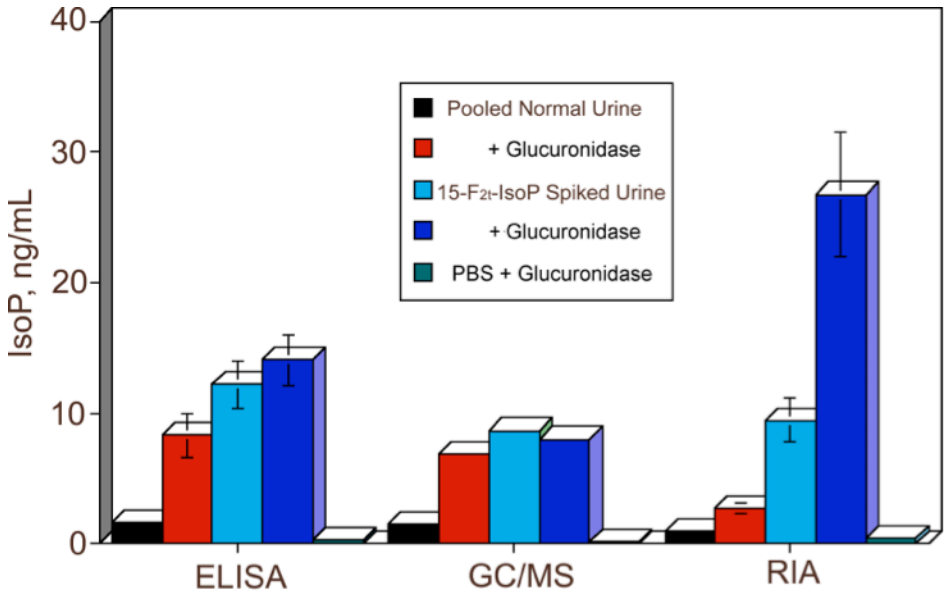

Fig 2. Effect of β-glucuronidase pretreatment on the concentration of F_2-isoprostanes in human urine. Urine from 6 normal individuals was pooled and ½ was spiked with 10 ng/mL of synthetic 15F_{2t}-IsoP. Samples were analyzed using standard protocols without or with (+G) pretreatment with β-glucuronidase. PBS = phosphate buffered saline preincubated with β-glucuronidase.

Moreover, the extent of glucuronidation ranged from 28% to 80% for the human urine specimens examined. This is not surprising based on well-known inter-individual differences in the expression of UDP-glucuronyl transferases and the impact of diet, lifestyle and other factors on the expression of these enzymes (27,28). Given the extent of and the wide variations observed for 15-F_{2t}-isoprostane glucuronidation, it is strongly recommended that urine specimens be pretreated with β-glucuronidase prior to isoprostane analysis to provide more accurate assessment of oxidative stress.

Additional Developments

Our purified antibodies to IsoP and IsoP-M can be used for immunohistochemical localization of these biomartkers for oxidative damage (see 26). Indeed, anti-IsoP was recently used to visualize the high concentrations of IsoP in the brain of Alzheimer's patients (figure 3). Since, as detailed above, the values obtained for the concentration of IsoP in serum or urine samples depend on (a) the method for sample preparation, (b) the analytic method, and (c) the rate of IsoP metabolism in the subjects. In order to assist investigators who wish to compare results obtained using different sample preparation and analytical methods, we have prepared a large pool of normal human urine and have obtained IsoP concentrations using SepPak isolation and GC/MS as well as by ELISA using a proprietary extraction-free method. Values were obtained ± β-glucuronidase pretreatment. A second pool of urine has been prepared by spiking with authentic 15-F_{2t}-isoprostane to provide an elevated calibrator. Additional efforts to further standardize and improve IsoP as a biomarker for oxidative stress are ongoing. However, it is critical that investigators in this field be cognizant of the impact of experimental methods and IsoP metabolism as the design and execute their studies.

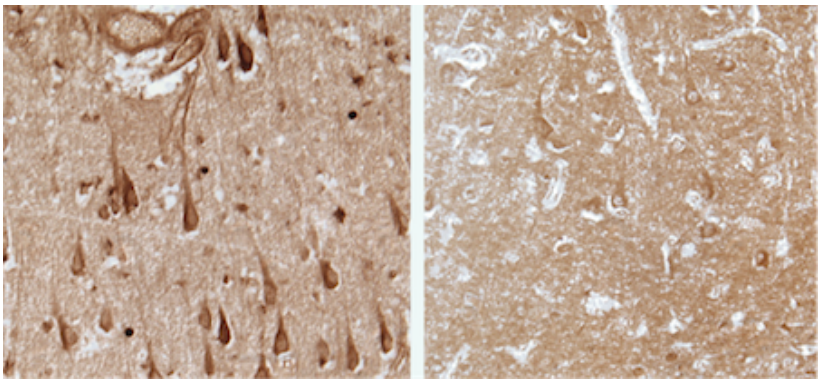

Fig 3. Isoprostanes localization in AD and control brain. In adjacent serial sections of hippocampus of AD cases, neurons are intensely labeled with antisera against 8-iso-PGF2α (Left). In adjacent serial sections from an age-matched control, neuronal levels of 8-iso-PGF2α are significantly lower. Tissue sections were immunostained using the peroxidase/anti-peroxidase method with 3-3'-diaminobenzidine.

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