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8-Epi $PGF_{2\alpha}$: specific analysis of an isoeicosanoid as an index of oxidant stress *in vivo*

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1 Excessive free radical generation is thought to contribute to tissue injury in a broad spectrum of diseases. A particular constraint in addressing this hypothesis has been the inability to assess free radical generation *in vivo* and the lack of information on drugs or vitamins which act as effective antioxidants *in vivo*.

2 Traditional approaches have relied upon measures of substrate oxidizability or spin trapping of free radical adducts *ex vivo*. It is unknown how these measurements might relate, in a quantitative fashion, to the generation of reactive oxygen species *in vivo*. Isoeicosanoids are free radical catalyzed products of arachidonic acid. One of these compounds, 8-epi prostaglandin $F_{2\alpha}$ (8-epi $PGF_{2\alpha}$) exhibits biological activity and may function as an autacoid. Specific analysis of this 8-epi $PGF_{2\alpha}$ isomer indicates that it is elevated in certain syndromes thought to be associated with oxidant stress. These include vascular reperfusion, paracetamol poisoning and liver cirrhosis. Apparently healthy individuals who smoke cigarettes or consume alcohol exhibit dose dependent increments in excretion of 8-epi $PGF_{2\alpha}$. Excretion is depressed by antioxidant vitamins, although not by the nonspecific cyclooxygenase (COX) inhibitor, aspirin, even though 8-epi $PGF_{2\alpha}$ may be formed by either COX-1 or COX-2.

3 Specific analysis of this and other isoeicosanoids may afford an opportunity to evaluate the effects of antioxidant interventions in human diseases characterized by excessive lipid peroxidation *in vivo*.

Keywords isoeicosanoid isoprostane 8-epi $PGF_{2\alpha}$ free radical reperfusion smoking paracetamol prostaglandin cyclooxygenase aspirin

Free radical catalysed tissue injury is thought to play a fundamental role in human disease. Evidence consistent with this hypothesis may be derived from animal models of dysregulated antioxidant defense [1, 2] and, by implication, from epidemiological studies which relate inversely the incidence of certain diseases to estimates of antioxidant vitamin intake [3, 4]. However, direct measurement of free radical generation *in vivo* has proven problematic. The conventional approach has been to measure the oxidizability of low density lipoprotein (LDL), usually as induced by copper *ex vivo*. Although there are reports of indices of such oxidizability (e.g. the lag time) being modified in settings putatively associated with oxidant stress *in vivo* [5], it is unknown how LDL oxidizability *ex vivo* actually relates to the oxidation of LDL (or other substrates) *in vivo*. Similar approaches, such as detection of spin traps of adducts formed *ex vivo* by electron spin resonance are likewise constrained [5, 6]. The most commonly applied *in vivo* approach relies upon measurement of thiobarbituric reactive substances (TBARs), which are largely reflective of levels of malondialdehyde (MDA). However, MDA is a by product of cyclooxygenase (COX) turnover in activated platelets and platelet activation is a common feature of syndromes of oxidant stress, such as vascular reperfusion [7] and cigarette smoking [8].

These limitations result in a poor understanding of which conditions are actually characterized by excessive free radical formation in humans. Similarly, despite

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expensive, large scale prospective studies of vitamin therapy, we do not actually know what are effective antioxidant doses of these supplements or, for that matter, of other putative antioxidant drugs in humans. Thus, we cannot design studies with mechanistically effective interventions in syndromes of oxidant stress to address the functional importance of this process in human disease.

Isoeicosanoids

These compounds are free radical catalysed products of arachidonic acid. Initially described as being formed *in vitro*, Morrow and Roberts determined that one class of these compounds, the F_2 isoprostanes, circulate and are excreted in animal models of oxidant stress and in the urine of humans [9, 10]. Using gas chromatography/mass spectrometry (GC/MS) and an internal standard for the enzymatically formed prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), they established that these compounds were formed initially *in situ* in the cell membrane phospholipids, from which they were cleaved, presumptively by phospholipase(s) A_2 [11, 12]. Since then, families of free radical catalysed E_2 and D_2 isoprostanes, isothromboxanes and isoleukotrienes have been described [13–15]. These compounds may exert biological effects in the extracellular compartment or as intracellular signals of lipid peroxidation.

Although the array of potential products is mesmerizing, it appeared that F_2 isoprostane excretion in urine might provide a chemically stable class of analytes, which might, when measured specifically in urine, afford a non-invasive, quantitative index of oxidant stress *in vivo*. Given that the relative amounts of various isomers might alter in different settings, we decided initially to focus in depth on one of these compounds, 8-epi $PGF_{2\alpha}$, a relatively abundant member of this family of isomers in human urine.

Formation and excretion of 8-Epi $PGF_{2\alpha}$

Aside from its relative abundance, an additional reason to focus on this compound was its biological activity. Thus, 8-epi $PGF_{2\alpha}$ is a vasoconstrictor and a mitogen in several cell types, including vascular smooth muscle cells [16, 17]. These effects are prevented by pharmacological antagonists of the thromboxane receptor [16]. Despite this observation, it is likely that they are mediated by a related but distinct receptor. For example, unlike analogs of thromboxane or its prostaglandin endoperoxide precursor, PGH_2, which also activates thromboxane receptors, 8-epi $PGF_{2\alpha}$ induces shape change, but not irreversible aggregation, in human platelets [18–20]. Interestingly, we have found that 8-epi $PGF_{2\alpha}$ does cause irreversible aggregation when combined with concentrations of platelet agonists which themselves do not activate platelets [21]. Additionally, 8-epi $PGF_{2\alpha}$ stimulates inositol phosphate generation more potently than do the other analogues in both vascular smooth muscle cells and platelets [17, 21]. Finally, we have found [21] that 8-epi $PGF_{2\alpha}$ differs from the other analogues in that it fails to displace radiolabeled thromboxane mimetics from either of the two recognized carboxy terminal splice variants of the human thromboxane receptor [22].

We developed a stable isotope dilution assay for 8-epi $PGF_{2\alpha}$ using GC/MS. More recently, an immunoassay for this compound has been developed [39]. Enhanced excretion has been demonstrated in a variety of syndromes putatively associated with oxidant stress. For example, urinary levels of 8-epi $PGF_{2\alpha}$ were 397 ± 114 pmol mmol^{-1} creatinine in the first 24 h after paracetamol (acetaminophen) overdose in nine female patients compared with 78 ± 8 in 15 age and gender matched controls ($P < 0.005$) (Figure 1). In two cases of paraquat overdose, levels were 378 and 220 pmol mmol^{-1} creatinine in the first 24 h. In the latter case, urinary 8-epi $PGF_{2\alpha}$ excretion correlated with urinary paraquat levels over time. During the 48 h for presentation, urinary 8-epi $PGF_{2\alpha}$ excretion fell from 220 to 73 pmol mmol^{-1} creatinine while the urinary paraquat level fell from 22 mg l^{-1} to 1 mg l^{-1} (Figure 2). The deleterious effects of poisoning with both paracetamol and paraquat are believed to be due to oxidative damage [23, 24]. In paracetamol overdose, the main conjugation pathway of excretion becomes saturated and large amounts of the drug are metabolized via the cytochrome P450 system to *N*-acetyl-*p*-benzoquinoneimine (NABQI), a highly reactive oxidizing agent [24]. Under normal circumstances or when a limited quantity of NABQI is formed, it is conjugated to glutathione, rendered inactive and is excreted. However, as liver glutathione stores are depleted, unconjugated NABQI accumulates. This leads to the oxidation of free thiol groups resulting in the formation of disulphide bonds in key proteins and thus, enzyme inactivation. In

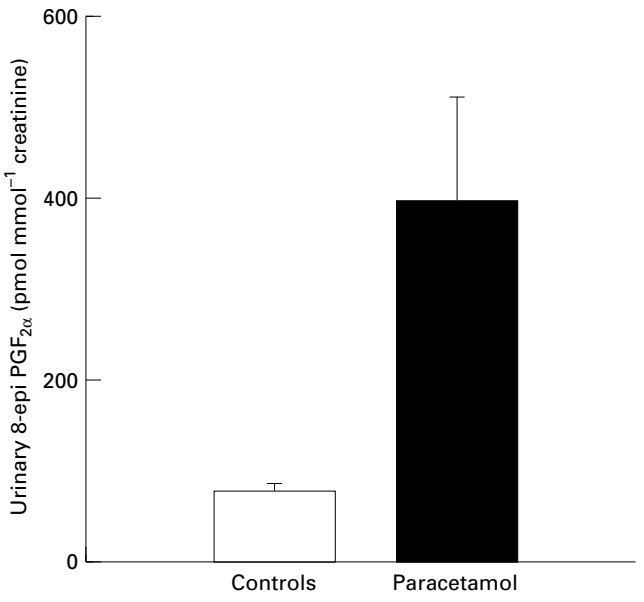

Figure 1 Urinary 8-epi $PGF_{2\alpha}$ was significantly ($P < 0.005$) elevated in patients ($n = 9$) presenting with paracetamol (acetaminophen) poisoning compared with age and gender matched healthy controls ($n = 9$).

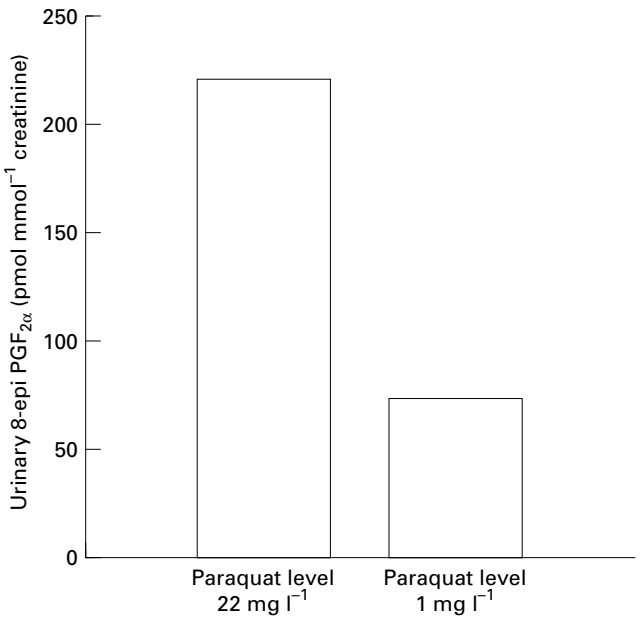

Figure 2 Urinary 8-epi $PGF_{2\alpha}$ in a patient with paraquat poisoning; paraquat levels were obtained at presentation and 48 h thereafter. Urine collections were based on 0–12 h and 42–44 h after presentation (time = 0).

addition, peroxidation of phospholipids with disruption of cell membranes may also contribute to cell death. Acetylcysteine, an antidote presently used in paracetamol poisoning, acts as a precursor of reduced glutathione, thus repleting its levels and promoting further conjugation of NABQI. Furthermore, glutathione may also have more direct antioxidant effects and may help prevent lipid peroxidation.

Paraquat is a dipyridylium compound that accepts electrons from cellular electron transport systems and donates them to oxygen to form the superoxide anion. The generation of this and other free radicals is believed to be responsible for the toxicity of paraquat [23]. Our observation of increased urinary excretion of 8-epi $PGF_{2\alpha}$ in paraquat poisoning is consistent with a significant role for lipid peroxidation in this setting. Indeed, urinary 8-epi $PGF_{2\alpha}$ and paraquat levels were related over time in a patient with paraquat overdose, consistent with a quantitative relationship between paraquat toxicity and this marker of lipid peroxidation. Thus, the measurement of urinary 8-epi $PGF_{2\alpha}$ in patients with paracetamol and paraquat overdoses, may offer a useful index of the extent of lipid peroxidation and provide a tool for the assessment of antioxidant therapeutic interventions.

Similarly, chronic cigarette smokers exhibit dose dependent increments in excretion of 8-epi $PGF_{2\alpha}$ [25]. Cigarette smoke is an abundant source of free radicals and increased urinary excretion of 8-oxoDg, an index of free radical catalysed DNA adduct formation has been described in apparently healthy chronic cigarette smokers [26]. Increased excretion of total F_2 isoprostanes has also been described in this group [27]. Interestingly, we found that short term therapy with vitamin C (2600 mg), an endogenous antioxidant deficient in smokers [28], depressed 8-epi $PGF_{2\alpha}$ excretion, as did switching from cigarettes to nicotine patches [25].

Excretion of 8-epi $PGF_{2\alpha}$ is also enhanced in patients undergoing coronary reperfusion with thrombolytic drugs. Similarly, excretion is enhanced coincident with clamp release in patients undergoing coronary artery bypass grafting and in an animal model of thrombosis coincident with reperfusion induced by thrombolytic drugs [29]. Levels are increased in a dose-dependent fashion by alcohol intake in volunteers and in patients with cirrhosis, particularly those with alcohol induced liver disease [30, 31].

To explore the formation of 8-epi $PGF_{2\alpha}$ in more detail, we focused initially upon human platelets. To our surprise, we found that 8-epi $PGF_{2\alpha}$, but not other F_2 isoprostanes, could be formed as a minor product of the COX enzyme in platelets [40]. This was a potentially important observation, because, as mentioned previously, platelet activation is often a feature of syndromes putatively associated with oxidant stress. However, administration of aspirin, at doses which completely inhibited platelet thromboxane formation, did not suppress urinary 8-epi $PGF_{2\alpha}$ excretion in chronic smokers. The same regimen significantly suppressed excretion of the 11-dehydro metabolite of thromboxane B_2 in these patients (Figure 3). Thus, the capacity of platelets to form 8-epi $PGF_{2\alpha}$ in a COX dependent manner did not contribute, in a measurable way, to the urinary index of overall biosynthesis, even in the setting of moderate COX activation (heavy smokers).

Platelets contain COX-1 [32]. However, COX-2 is the more readily induced form of the two enzymes [33] and its active site appears to be more accommodating than that of COX-1. Thus, coincident with inhibition of prostaglandin production by aspirin, 15-HETE is formed by COX-2, but not COX-1. Mutational analysis infers that this reflects further penetration of the substrate a larger active site in COX-2 [34]. Human monocytes contain both enzymes and we have recently shown that they exhibit the capacity to form 8-epi $PGF_{2\alpha}$ by a COX-2 dependent mechanism [35]. It is unknown to what extent, if any, that this pathway might contribute to the actions of 8-epi $PGF_{2\alpha}$ in settings of inflammation or cellular proliferation, in which COX-2 induction might be anticipated. Monocytes may also form 8-epi $PGF_{2\alpha}$ as an isoprostane when stimulated in the presence of an LDL substrate *in vitro* [35]. We have identified 8-epi $PGF_{2\alpha}$ in atherosclerotic plaque and its excretion is elevated in patients with familial hyperlipidaemia. The ready measurement of this compound in mouse urine allows exploration of its utility as a marker of atherogenesis *in vivo*.

The capacity of cells to form 8-epi $PGF_{2\alpha}$ enzymatically provides a rationale for the possibility that there is a distinct receptor for this compound. Were this to be the case, or indeed, if 8-epi $PGF_{2\alpha}$ were to activate a variant of the thromboxane receptor which has yet to be identified, an important distinction from primary prostaglandins must be born in mind. Thus, primary prostaglandins are rapidly metabolized to inactive compounds, a process which limits their biologically active concentrations to the immediate microenviron-

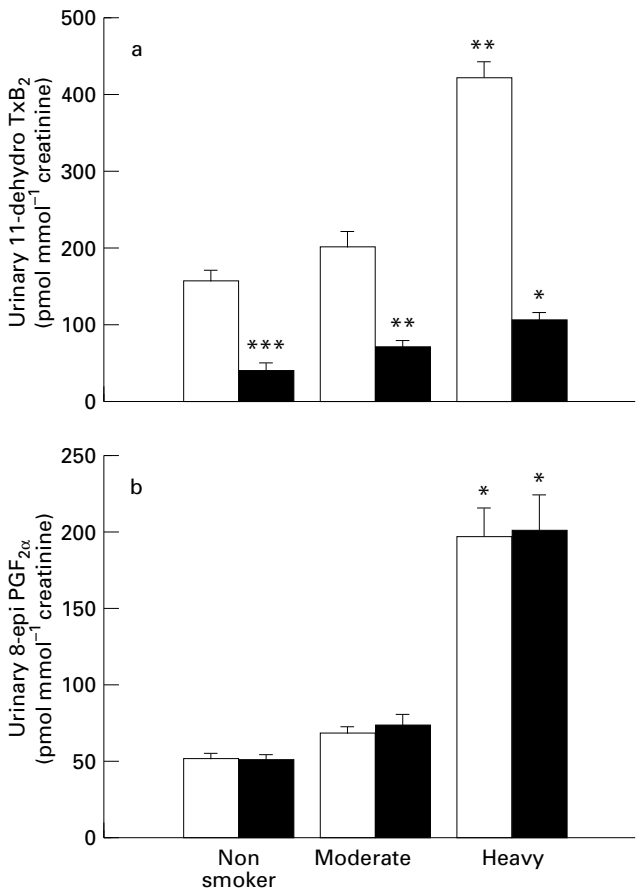

Figure 3 The effect of aspirin (pre (□); post (■)) in non smokers ($n=6$), moderate ($n=5$) and heavy smokers ($n=4$) on urinary 8-epi $PGF_{2\alpha}$ and 11-dehydro TxB_2. (a) Urinary 11-dehydro TxB_2 levels were significantly depressed by aspirin in all groups (** = $P<0.005$). (b) Aspirin failed to suppress urinary 8-epi $PGF_{2\alpha}$ excretion in any group. (Reproduced from reference [25]).

ment of their formation. Thus, in the case of prostacyclin, measurement of its metabolites in urine and of its hydrolysis product in plasma allowed Blair, Dollery and ourselves to concur that the compound did not function as a circulating hormone, despite its potent *in vitro* effects on platelet function and vascular tone [36, 37]. Although the disposition of 8-epi $PGF_{2\alpha}$ *in vivo* remains to be characterized, we do know that the active species circulates and that a fraction is excreted unchanged in human urine. However, at least in the case of platelets, the systemic concentrations attained in plasma, even in syndromes of oxidant stress, are far below those necessary to modulate platelet function [21]. Thus, while incidental receptor activation by 8-epi $PGF_{2\alpha}$ formed as an isoprostane, may serve to amplify the effects of conventional platelet agonists in syndromes where oxidant stress and platelet activation coincide, it must do so locally, as an autacoid, if at all.

Conclusion

Although enzymatic formation of 8-epi $PGF_{2\alpha}$ does not appear to confound its potential utility as an index of oxidant stress *in vivo*, development of the capability to measure specifically other isoprostanes could be most informative. Recently, we have developed methods for measurement of a second, even more abundant F_2 isoprostane, tentatively designated IPF-1, which is not formed enzymatically [38]. Paired analysis with 8-epi $PGF_{2\alpha}$ will allow us to clarify the relative importance of the enzymatic pathway with more precision than heretofore. Additionally, this approach will be helpful in studies of the effects of antioxidant drugs and vitamins and of the utility of isoeicosanoid analysis in clarifying the role of oxidant stress in human disease.

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