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The effects of oxidation products of arachidonic acid and n3 fatty acids on vascular and platelet function

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Abstract

15-F_{2t}-isoprostane (15-F_{2t}-IsoP), an oxidation product of arachidonic acid (AA), affects vascular and platelet function; however, the bioactivity of other fatty acids oxidation products is unknown. This paper studied rat aortic vascular reactivity and human platelet aggregation in response to 14 oxidation products of AA, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and α-linolenic acid (ALA) compared with 15-F_{2t}-IsoP. It also compared the F_{2t}-IsoPs profile in human platelets. EPA-derived 15-F_{3t}-IsoP constricted rat aorta less than 15-F_{2t}-IsoP, but none of the other oxidation products affected vascular reactivity. Only 15-F_{2t}-IsoP (10^{-4} M) directly affected platelet aggregation. 15-F_{3t}-IsoP, ent-16-F_1-phytoprostane (from ALA) and isofurans A and B (from AA) inhibited reversible aggregation to U46619. Unlike plasma, the platelet profile of F_2-IsoP showed that 8-F_{2t}-IsoP were higher than 15-F_{2t}-IsoP. Unlike 15-F_{2t}-IsoP, the test compounds derived from fatty acids oxidation did not affect vascular or platelet function. Elevated platelet 8-F_{2t}-IsoP could limit 15-F_{2t}-IsoP-induced aggregation under conditions of oxidant stress.

Keywords: *Isoprostanes, phytoprostanes, isofurans, neuroprostanes, platelet aggregation, vascular reactivity*

Background

Oxidation of biomolecules including lipids has been implicated in a diverse range of diseases including cardiovascular disease, cancer, neurodegenerative disease and lung disease. Enhanced oxidant stress is characterized by an imbalance of increased free radicals principally derived from oxygen and antioxidant defenses. This process can occur either locally in the arterial wall or systemically and it is hypothesized to contribute to the development and progression of atherosclerosis [1]. Free radicals can be generated endogenously by the mitochondria or as a result of oxidative bursts during phagocytosis or from exogenous sources such as environmental or chemical toxins.

Lipids that undergo peroxidation are major targets of free radical attack. F_2–Isoprostanes (F_2–IsoP) are prostaglandin-like products of free radical attack on arachidonic acid (AA) [2,3]. They differ structurally from prostaglandins and are pre-formed esterified to phospholipids and released by phospholipases. There are four possible F_2-IsoP regioisomers giving rise to the 15-, 8-, 12- and 5- series F_2-IsoP. Their measurement provides a reliable assessment of *in vivo* lipid peroxidation [3]. Other fatty acids can also undergo peroxidation. α-linolenic acid (ALA) forms the 9-and 16-series, F_1-IsoP, also called F_1-phytoprostanes (F_1–PhytoP) [4]. The long chain omega-3 fatty acid eicosapentaenoic acid (EPA) forms six regioisomers

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of IsoP called F_3-isoprostanes (F_3–IsoP) [5] and docosahexaenoic acid (DHA) forms eight regioisomers of IsoP, also known as F_4-neuroprostanes (F_4-NeuroP) [6]. The IsoP are thought to be formed mainly under conditions of low oxygen tension. Under high oxygen tension arachidonic acid is preferentially channelled to form the isofurans (IsoF) that have a substituted tetrahydrofuran ring [7]. It has been suggested that assessment of both IsoP and IsoF will provide a better index of lipid peroxidation under all circumstances [8].

As the IsoP, IsoF, NeuroP and PhytoP are structurally related to prostaglandins there is a need to assess their biological activity, in particular with respect to their effects on vascular and platelet function. Research to date has shown that several of the 15-series F_2-IsoP, in particular 15-F_{2t}-IsoP, 9-*epi*-15-F_{2t}-IsoP and 15-*epi*-15-F_{2t}-IsoP, are vasoconstrictors [9,10]. 12-F_{2t}-IsoP constricts retinal and brain microvessels [11]. In contrast, two of the 5-series F_2-IsoP have no vasomotor effects [12]. In addition, 15-F_{2t}-IsoP is anti-aggregatory in human whole blood [13], but promoted platelet aggregation in platelet rich plasma in the presence of ADP [14].

The aim of this study was to examine the biological activity, particularly effects on vascular reactivity and platelet function, of a number of PhytoP, IsoP, NeuroP and IsoF (Figure 1), which to date have not been described.

Methods

Materials

15-F_{2t}-IsoP, 15-F_{3t}-IsoP, 8-F_{2t}-IsoP and 2,3-dinor-15-F_{2t}-IsoP were obtained from Cayman Chemical Co. (Ann Arbor, MI, USA) 9-F_{1t}-PhytoP, 9-epi-9-F_{1t}-PhytoP, ent-9-F_{1t}-PhytoP, 9-epi-ent-9-F_{1t}-PhytoP, 16-F_{1t}-PhytoP, 16-epi-16-F_{1t}-PhytoP, ent-16-F_{1t}-PhytoP, 16-epi-ent-16-F_{1t}-PhytoP and 4(RS)-4F_{4t}-NeuroP were synthesized according to the published procedure [15–17]. 8-epi-SC-Δ^{13}-9-IsoF (IsoF-A) and 8,15 diepi-SC-Δ^{13}-9-IsoF (IsoF-B) [18] were synthesized according to the method of Taber and Zhang [19] and provided by Professor L. J. Roberts II. The above compounds were stored under nitrogen to prevent oxidation and kept at –20°C until the day of platelet aggregation and vascular reactivity experiments.

Vascular reactivity studies in rat isolated aorta

This study was passed by the University of Western Australia Animal Ethics Committee. Male Wistar rats (Animal Resources Centre, Murdoch, Australia) were euthanized at 8 weeks of age (pentobarbitone sodium, 160 mg/kg i.p.). The thoracic aorta was quickly excised, cleaned of adhering connective tissue and cut into 3 mm long segments. Individual segments were suspended under a resting tension of 2 g in tissue baths containing 2 ml Krebs bicarbonate solution maintained at 37°C and bubbled with 5% CO_2 in O_2. Following a 45-min equilibration period, the integrity of the aorta was tested by pre-contracting with KCl (200 mM). Endothelial responses were subsequently tested using 1 μM acetylcholine after pre-contraction with phenylephrine (200 nM). Dilator and constrictor responses to the test compounds were then evaluated at a concentration of 10^{-9}–10^{-4} M after pre-contraction with phenylephrine (200 nM). The test compounds were dissolved in a small volume of ethanol and vasodilator and constrictor responses were compared with a reagent control of the same volume of ethanol.

Human platelet aggregation studies

Whole blood (20 ml) was collected into 3.8% trisodium citrate and centrifuged at 190 g for 10 min to give platelet-rich plasma (PRP). After removal of PRP, blood was further centrifuged at 2000 g for 10 min to provide platelet-poor plasma (PPP). The platelets were counted using a haemocytometer and were diluted in PPP to a final concentration of 2×10^8 platelets/ml. Aggregation was measured as percentage of light transmission at 5 min following the addition of the aggregant. Compounds 1–8 and 10–15 were tested at concentrations of 10^{-6} M and 10^{-4} M compared with the following tests compounds, collagen (1 μg/ml), U46619 (1 μM) and 15-F_{2t}-IsoP (10^{-4}M) [20]. The effect of test compounds on reversible aggregation to the thromboxane A_2 mimetic, U46619 (1.6×10^{-7} M) was determined by pre-incubating the platelets with the test compounds at 10^{-4} M for 5 min prior to challenge with U46619.

Measurement of platelet F_2-isoprostanes

Blood samples were taken from 36 fasting healthy men aged between 20–65 years recruited by advertisement from the general population. The study was passed by the University of Western Australia Human Ethics committee. All participants gave written informed consent to participate. The blood (20 ml) was collected into tubes containing EDTA, reduced glutathione and butylated hydroxytoluene. The blood was centrifuged immediately at 200 g to give platelet-rich plasma (PRP). The PRP was centrifuged and the platelet pellet was washed with 10 ml of 0.9% saline containing EDTA and BHT. Platelets were re-suspended in Hepes buffered Hanks (1 ml), counted using a haemocytometer, and diluted to 1×10^9 platelets/ml in Hepes buffered Hanks 0.1% BSA (HBSSB). Aliquots of 2×10^8 cells were incubated with saline (control) or calcium ionophore (2.5 μg/ml) for 15 min at 37°C. The incubates were placed on ice and centrifuged at 4°C for 10 min at 2000 g.

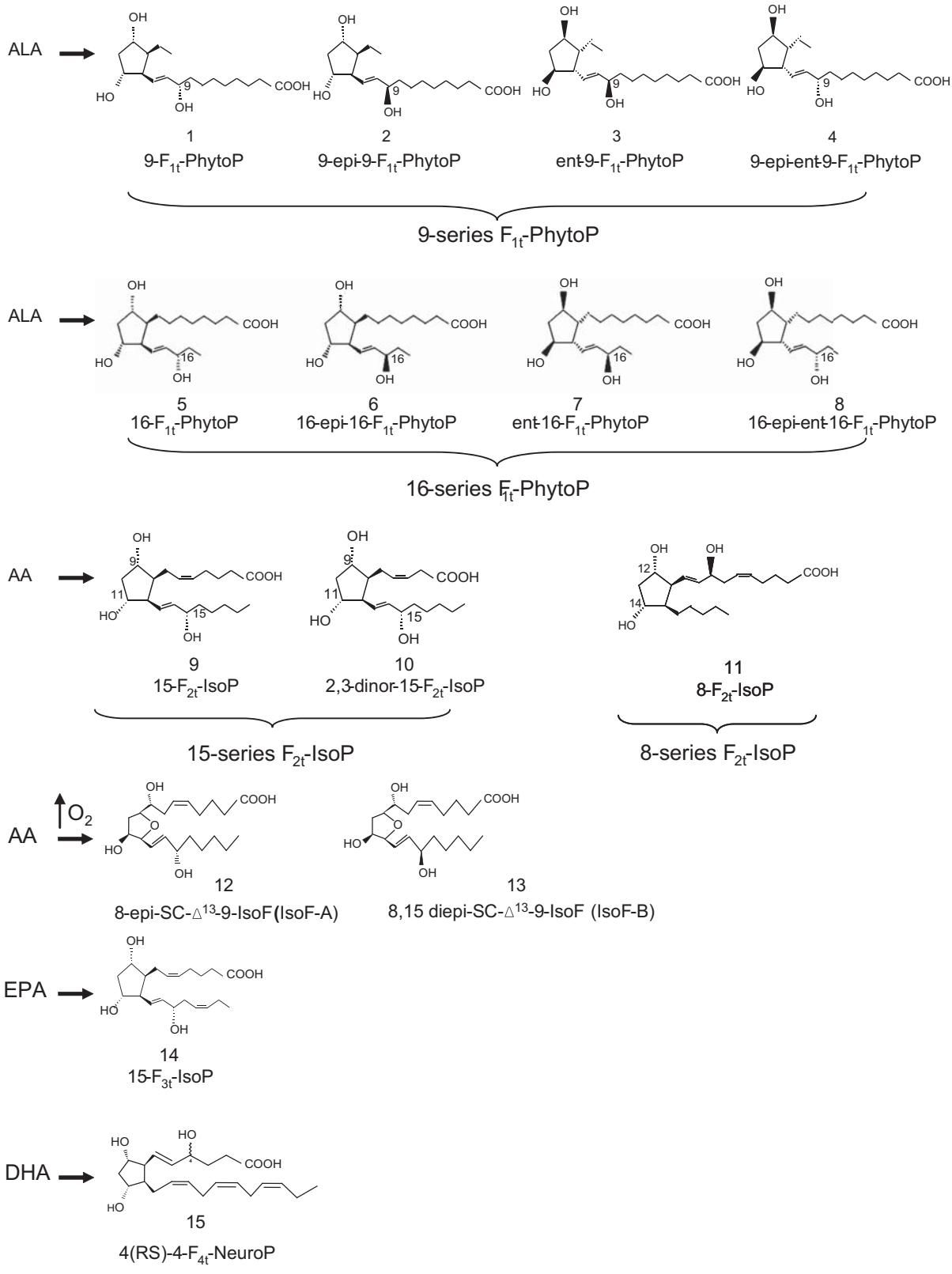

Figure 1. Structures of the 15 test compounds used for vascular reactivity and platelet aggregation studies. Products derived from α linolenic acid (ALA): (9-series F_{1t}-Phytoprostanes):- 9-F_{1t}-Phytoprostane (9-F_{1t}-PhytoP), 9-epi-9-F_{1t}-Phytoprostane (9-epi-9-F_{1t}-PhytoP), ent-9-F_{1t}-Phytoprostane (ent-9-F_{1t}-PhytoP), 9-epi-ent-9-F_{1t}-Phytoprostane (9-epi-ent-9-F_{1t}-PhytoP); (16-series F_{1t}-Phytoprostanes):- 16-F_{1t}-Phytoprostane (16-F_{1t}-PhytoP), 16-epi-16-F_{1t}-Phytoprostane (16-epi-16-F_{1t}-PhytoP), ent-16-F_{1t}-Phytoprostane (ent-16-F_{1t}-PhytoP), 16-epi-ent-16-F_{1t}-Phytoprostane (16-epi-ent-16-F_{1t}-PhytoP). Products derived from arachidonic acid (AA): (15-series F_{2t}-isoprostanes):- 15-F_{2t}-Isoprostane (15-F_{2t}-IsoP), 2,3-dinor-15-F_{2t}-Isoprostane (2,3-dinor-15-F_{2t}-IsoP), (8-series F_{2t}-isoprostanes):- 8-F_{2t}-Isoprostane (8-F_{2t}-IsoP). Derived from AA under high O_2 tension: 8-epi-SC-Δ^{13}-9-IsoF (IsoF-A) and 8,15 diepi-SC-Δ^{13}-9-IsoF (IsoF-B). Derived from EPA: 15-F_{3t}-Isoprostane (15-F_{3t}-IsoP). Derived from DHA: 4(RS)-4-F_{4t}-Neuroprostane (4(RS)-4-F_{4t}-NeuroP).

The platelet pellet and supernatant were stored frozen with 800 μg of BHT at −80°C until assay. Platelet content and release of F_2-IsoPs eluting with the 15-F_{2t}-IsoP and 8-F_{2t}-IsoP peaks were assayed in the platelet pellet and supernatant after base hydrolysis using deuterated internal standards for 15-F_{2t}-IsoP and 8-F_{2t}-IsoP as previously described [21].

Statistics

Differences in vascular response to the lipid oxidation products compared to the reagent control were assessed with a Dunnett's test. Values are presented as mean and SEM. Differences in aggregation response to U46619 before and after incubation with each lipid oxidation product were assessed by comparing the area under the curve for each aggregation. Unstimulated and calcium ionophore stimulated platelet content and release of F_2-IsoP identified by 8-F_{2t}-IsoP and 15-F_{2t}-IsoP internal standards were assessed using a paired *t*-test after log transformation. Values are geometric mean and 95% CI.

Results

Vascular reactivity studies in rat isolated aorta

None of the compounds tested (#1–15, Figure 1) had vasodilator activity when tested in a rat aorta preparation pre-contracted with phenylephrine. When vasoconstrictor responses were examined in the rat aorta, significant changes in tension were only observed at a concentration of 10^{-4}M. A comparison of compounds at 10^{-4}M showed that 15-F_{2t}-IsoP (#9) produced a significant change in tension of 1.4 ± 0.24 g ($p < 0.05$) and 15-F_{3t}-IsoP (#14) produced a change of 0.757 ± 0.2 g ($p < 0.05$) when compared with the ethanol control (Figure 2). Responses to 2,3-dinor-15-F_{2t}-IsoP (#10, Figure 1), 4(RS)-4-F_{4t}-NeuroP (#15, Figure 1), 16-epi-ent-16-F_{1t}-PhytoP (#8, Figure 1), ent-16-F_{1t}-PhytoP (#7, Figure 1), 16-epi-16-F_{1t}-PhytoP (#6, Figure 1), 16-F_{1t}-PhytoP (#5, Figure 1), 9-epi-ent-9-F_{1t}-PhytoP (#4, Figure 1), ent-9-F_{1t}-PhytoP (#3, Figure 1), 9-epi-9-F_{1t}-PhytoP (#2, Figure 1), 9-F_{1t}-PhytoP (#1, Figure 1), 8-epi-SC-Δ^{13}-9-IsoF (IsoF-A) (#12, Figure 1), 8,15 diepi-SC-Δ^{13}-9-IsoF (IsoF-B) (#13, Figure 1) and 8-F_{2t}-IsoP (#11, Figure 1) were not significantly different from the vehicle control.

Platelet aggregation studies

None of test compounds #1–8 or #10–15 caused aggregation or reversible aggregation at doses up to 10^{-4} M. We confirmed previous reports that 15-F_{2t}-IsoP (#9) caused reversible aggregation at a dose of 10^{-4} M, and inhibited U46619 induced reversible aggregation. We showed that pre-incubation of platelets with

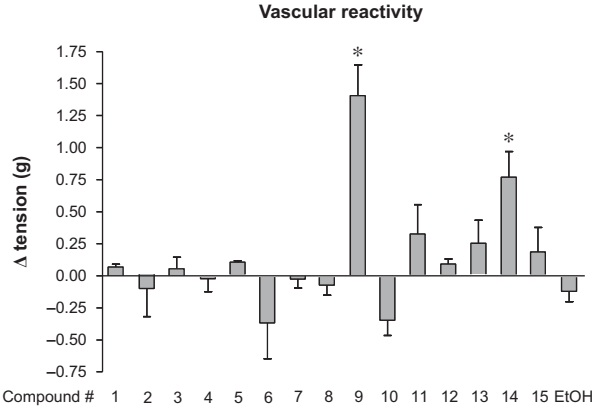

Figure 2. Change in tension of rat isolated aorta preparations in response to each of the test compounds at dose of 10^{-4} M. $^{*}p < 0.05$, compared with the ethanol control Dunnett's test. Shown is the mean response (± SEM) obtained in four separate aortic segments.

15-F_{3t}-IsoP (#14, Figure 1), ent-16-F_{1t}-PhytoP (#7, Figure 1), IsoF-A (#12, Figure 1) and IsoF-B (#13, Figure 1) at a concentration of 10^{-4} M for 5 min significantly inhibited subsequent reversible aggregation to U46619 (Figure 3). None of the other compounds tested had any effect on U46619 reversible aggregation.

Comparison of plasma and platelet F_2-isoprostanes

Using deuterated standards and gas chromatography mass spectrometry, we identified F_2-IsoPs in peaks corresponding to 15-F_{2t}-IsoP and 8-F_{2t}-IsoP internal standards. Both of these F_2-IsoPs were quantified in human platelets and plasma. In contrast to plasma where 15-F_{2t}-IsoP is found in large quantities, in platelets 8-F_{2t}-IsoP is present in much larger amounts than 15-F_{2t}-IsoP (Figure 4).

Basal and Ca ionophore stimulated platelet 15-F_{2t}-IsoP and 8-F_{2t}-IsoP

In platelets the levels of 8-F_{2t}-IsoPs were 6-fold higher (295, CI 170-582 fmol) than those of 15-F_{2t}-IsoP (43, CI 30-60 fmol), (Figures 5A and B). The levels of 15-F_{2t} and 8-F_{2t}-IsoPs released into the medium under basal conditions were similar to those found in the platelet pellet (Figures 5C and D). Stimulation of platelets with Ca ionophore resulted in a 5-fold increase in platelet content of 8-F_{2t}-IsoP ($p < 0.01$) and a 3-fold increase in 15-F_{2t}-IsoP ($p < 0.01$) (Figures 5A and B). Release of 15-F_{2t} and 8-F_{2t}-IsoPs into the medium after Ca ionophore were also significantly increased (Figures 5C and D).

Discussion

This study examined the effects of previously untested oxidation products of ALA, AA, EPA and DHA on

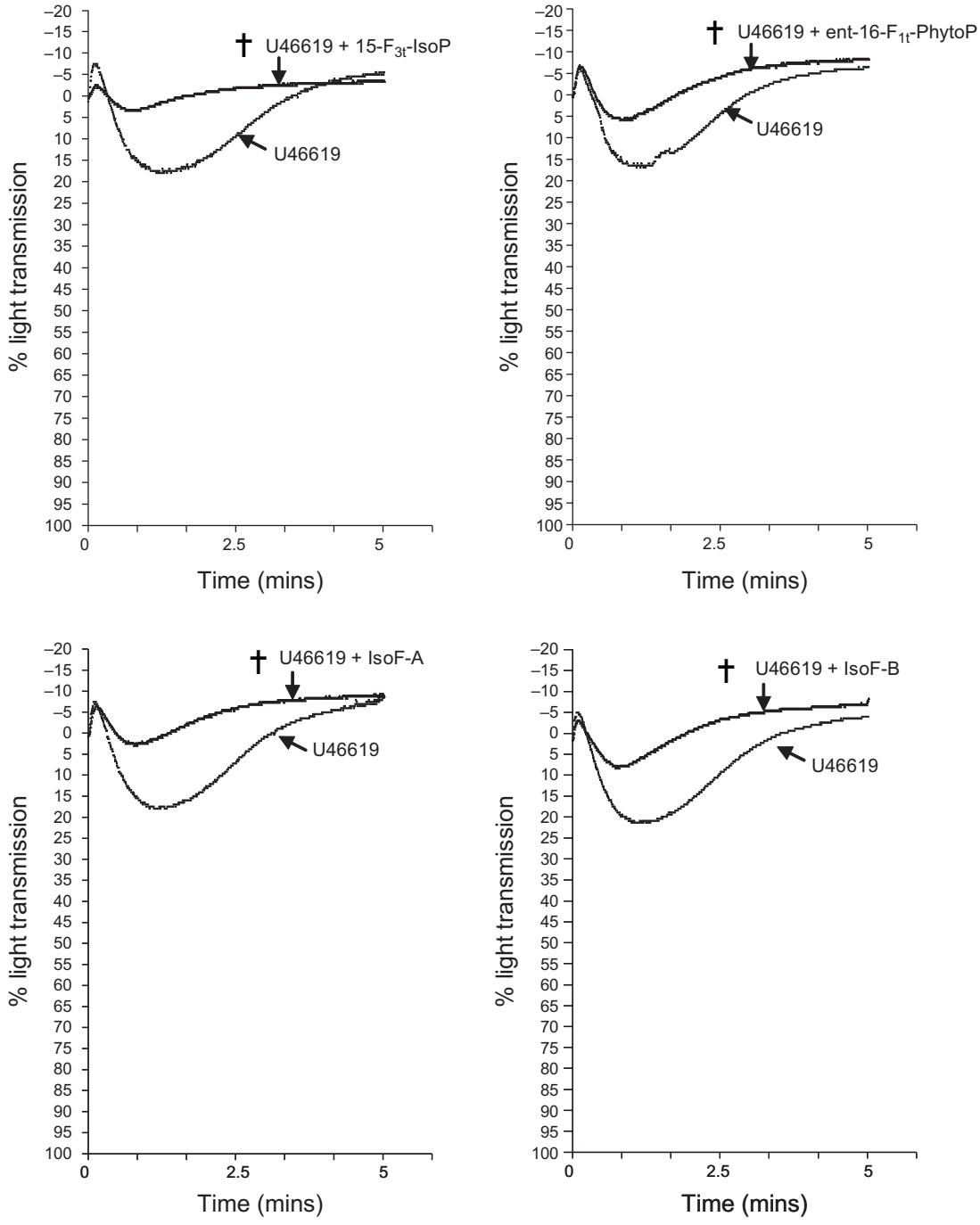

Figure 3. Change in light transmission during platelet aggregation in response to U46619 alone or after pre-incubation with the test compounds 15-F_{3t}-IsoP, ent-16-F_{1t}-PhytoP, IsoF-A and IsoF-B. $^{\dagger}p < 0.001$, for differences in area under the curve for incubation of each of the test compounds with U46619 compared with U46619 alone.

vascular reactivity and platelet function. None of the products tested was a vasodilator in rat aorta pre-contracted with phenylephrine. Our data confirm previous reports that 15-F_{2t}-IsoP possesses significant vasoconstrictor activity. We describe for the first time that 15-F_{3t}-IsoP derived from EPA also had significant vasoconstrictor activity in rat aorta, although the vasoconstrictor response induced by 15-F_{3t}-IsoP was less than that by 15-F_{2t}-IsoP. Other IsoP derived from oxidation of AA including 2,3-dinor-15F_{2t}-IsoP and 8-F_{2t}-IsoP had no detectable vasoconstrictor activity. The IsoF formed from AA under conditions of high oxygen tension and the oxidation products of ALA (F_1-PhytoP) and DHA (F_4-NeuroP) also had no detectable vasoconstrictor activity.

The effect of the compounds tested on platelet aggregation was examined in platelet-rich plasma. We confirmed that 15-F_{2t}-IsoP (10^{-4} M) caused reversible aggregation of platelets and inhibited reversible aggregation induced by the TXA_2 mimetic U46619 [20].

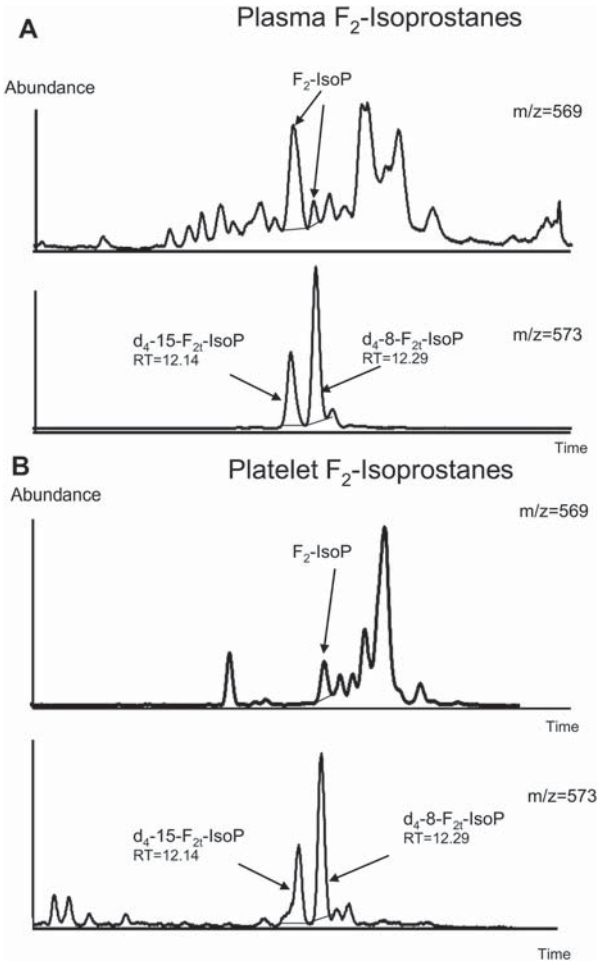

Figure 4. (A) Shows a typical ion chromatogram of F_2-IsoP in plasma (m/z = 569, top panel) corresponding to the retention time of the deuterated internal standards for 15-F_{2t}-IsoP and 8-F_{2t}-IsoP (m/z = 573, lower panel) and showing that the peak corresponding with 15-F_{2t}-IsoP internal standard is more abundant in plasma (B) a typical ion chromatogram for platelet F_2-IsoP (m/z = 569, top panel) corresponding to the retention time of the deuterated standards for 15-F_{2t}-IsoP and 8-F_{2t}-IsoP (m/z = 573, lower panel) showing that the peak corresponding to 8-F_{2t}-IsoP is more abundant in platelets.

None of the other compounds tested caused aggregation at doses up to 10^{-4} M. We report for the first time that under these conditions, 15-F_{3t}-IsoP inhibited reversible aggregation to U46619. In contrast, 8-F_{2t}-IsoP, 2,3-dinor-15-F_{2t}-IsoP and 4(RS)-4-F_{4t}-NeuroP did not significantly alter platelet aggregation in response to U46619. The PhytoP that are formed from the n3 fatty acid ALA did not affect platelet aggregation *per se*. However, of the PhytoP tested only ent-16-F_{1t}-PhytoP attenuated reversible aggregation to U46619.

Our data suggest that the lipid peroxidation products 15-F_{3t}-IsoP derived from the long chain n3 fatty acid EPA has attenuated constrictor action compared with 15-F_{2t}-IsoP, but is similar in that it inhibits reversible aggregation to a thromboxane A_2 mimetic. Recently two binding sites for 15-F_{2t}-IsoP (8-iso-$PGF_2\alpha$) have been identified. One pathway is a stimulatory pathway via the TXA_2 receptor whilst the other is an inhibitory pathway signalling via cAMP, the receptor for which has not been identified [22]. Further studies are required to characterize the platelet binding sites and signalling pathways for 15-F_{3t}-IsoP derived from EPA. In contrast, 4(RS)-4-F_{4t}-NeuroP from DHA did not show any vasoactive or platelet effects. Randomized controlled trials of dietary supplementation with fish, fish oil or purified EPA and DHA show that EPA and DHA are significantly incorporated into platelet membranes resulting in a significant attenuation of agonist-induced platelet aggregation [23–25]. Our findings in this study suggest that the reduced vasoconstrictor activity of 15-F_{3t}-IsoP in particular may in part contribute to the cardiovascular benefits of fish oil and n3 fatty acids. However, it should be noted that although a number of studies have shown reduced 15-F_{2t}-IsoP in human and animal tissue, plasma and urine after n3 fatty acid supplementation [26–29], most of the studies that have detected 15-F_{3t}-IsoPs and NeuroPs after fish oil have been *in vitro* or in small animals [30] or humans after an acute inflammatory challenge [31]. Therefore, the beneficial effects of n3 fatty acids are, in part, more likely due to a reduction in the biologically active 15-F_{2t}-IsoP rather than an increase in 15-F_{3t}-IsoP. Similarly the PhytoP had no detectable direct effects on vasoconstrictor or platelet aggregatory activity, suggesting that although they circulate in comparatively high concentrations in plasma [32] they are unlikely to affect vascular or platelet function.

The lipid peroxidation products formed under high oxygen tension, IsoF-A and IsoF-B both inhibited reversible aggregation to U46619, suggesting they may modulate platelet function under conditions of high oxygen tension. The only studies of platelet function under conditions of increased oxygen have been carried out in asphyxiated piglets that have been resuscitated with 21% and 100% oxygen [33]. In that study, high oxygen was associated with inhibition of platelet aggregation to collagen; however, the effect of high oxygen could not be dissociated from the effects of hypoxia. The effects of high blood oxygen levels on platelet aggregation and isofuran production need further investigation.

We have identified for the first time that F_2-IsoP corresponding with the 8-F_{2t}-IsoP peak was the more abundant in platelets with basal levels 6-fold higher than that of 15-F_{2t}-IsoP. This contrasts with plasma and urine where 15-F_{2t}-IsoPs are more abundant. The reason for the increased platelet content of 8-F_{2t}-IsoP compared with 15-F_{2t}-IsoP is unclear, but given that 8-F_{2t}-IsoP did not affect platelet aggregation in contrast to 15-F_{2t}-IsoP that causes aggregation, it may be an adaptive mechanism to limit aggregatory responses. Stimulation of platelets with calcium led to a significant increase

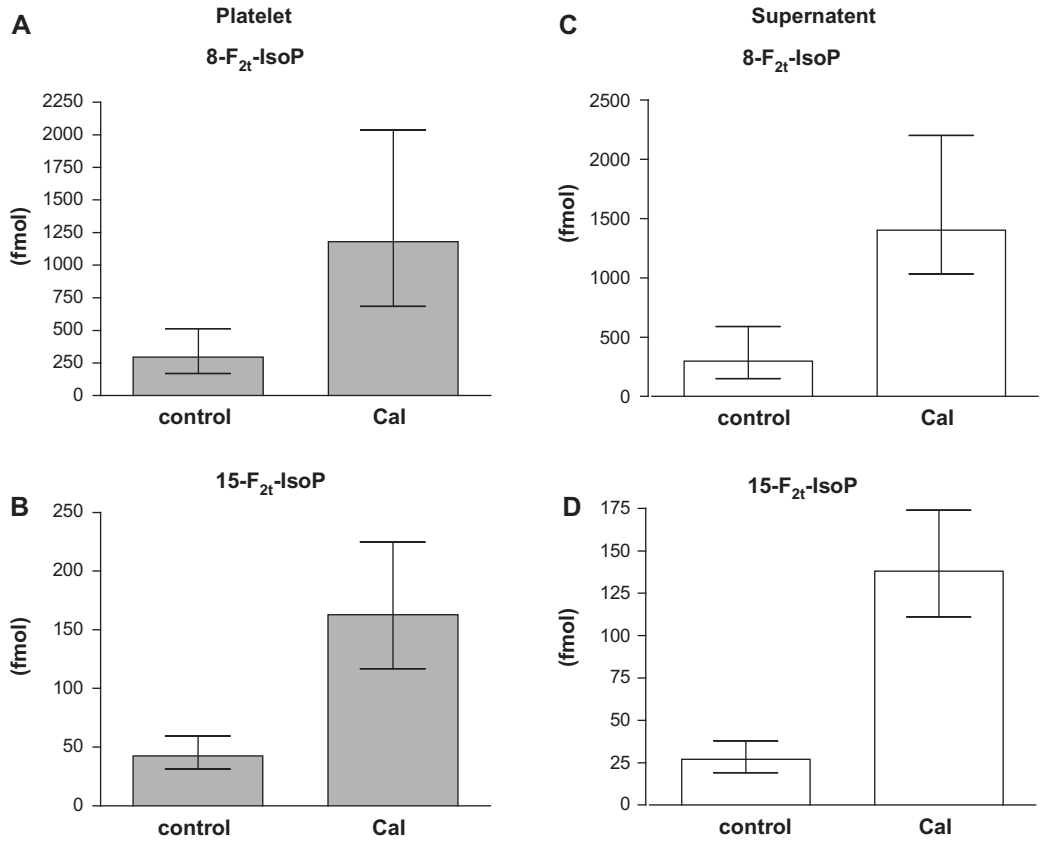

Figure 5. Basal and calcium ionophore stimulated platelets and supernatent levels of F_2-IsoP corresponding to the peaks co-eluting with 8-F_{2t}-IsoP (top panel, A and C), 15-F_{2t}-IsoP (bottom panel, B and D). $^{\dagger}p < 0.01$, calcium ionophore compared with control paired *t*-test.

in the platelet content and release of 8-F_{2t}-IsoPs and 15-F_{2t}-IsoPs. The ability of Ca ionophore to increase platelet content and release of both 8-F_{2t}-IsoP and 15-F_{2t}-IsoP suggests that it may stimulate oxidant stress in the platelet. As our studies were not conducted under conditions of cyclooxygenase inhibition we cannot exclude the possibility that the actions of Ca ionophore are in part due to free radical release, secondary to activation of cyclooxygenase.

We conclude that vascular and platelet function is not directly affected by the majority of the test compounds formed from free-radical attack on AA, EPA, DHA and ALA. The higher levels of 8-F_{2t}-IsoP in platelets could be a protective mechanism to limit 15-F_{2t}-IsoP induced aggregation under conditions of oxidant stress.

Declaration of interest

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