



# Characterization of the effects of isoprostanes on platelet aggregation in human whole blood

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**1** We tested the effects of 11 commercially-available isoprostanes on platelet aggregation directly or when triggered by the thromboxane receptor agonist U46619 or collagen in healthy human citrated blood using a whole blood aggregometer.

**2** None of the isoprostanes tested triggered aggregation alone, nor facilitated aggregation by a sub-threshold dose of U46619 or collagen. Five isoprostanes inhibited aggregation (rank order of potency 8-iso PGE<sub>1</sub> > 8-iso PGE<sub>2</sub> > 8-iso PGF<sub>2α</sub> > 8-iso PGF<sub>3α</sub> > 8-iso-13,14-dihydro-15-keto PGF<sub>2α</sub>).

**3** Blood incubated with LPS to induce a gross inflammatory response exhibited a time dependent (2–12 h) reduction in aggregation to U46619 but maintained a consistent response to collagen. Under these conditions, as in control blood, none of the isoprostanes tested induced aggregation. In fact, the inhibitory actions of isoprostanes on U46619-induced aggregation were enhanced in blood treated with LPS.

**4** L-NAME inhibited aggregation induced by U46619 in fresh blood and in blood treated with LPS. In the presence of L-NAME, (with or without LPS) none of the isoprostanes tested induced aggregation but retained their inhibitory action.

**5** Thus, in human whole blood the action of 8-iso PGE<sub>1</sub>, 8-iso PGE<sub>2</sub>, 8-iso PGF<sub>2α</sub>, 8-iso PGF<sub>3α</sub>, and 8-iso-13,14-dihydro-15-keto PGF<sub>2α</sub> is antiaggregatory. Moreover, this inhibitory capacity is still apparent and may be enhanced in blood subjected to inflammatory stimulation.

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**Keywords:** Isoprostanes; whole blood aggregometry; lipopolysaccharide

**Abbreviations:** COX, cyclo-oxygenase; DP, prostaglandin D; EP, prostaglandin E; LPS, lipopolysaccharide; NO, nitric oxide; NOS, nitric oxide synthase; PG, prostaglandin; TP, thromboxane

## Introduction

Isoprostanes are a family of prostaglandin (PG) isomers generated by free radical-catalysed peroxidation and then rearrangement of arachidonic acid (Morrow *et al.*, 1990; 1992). In addition, isoprostanes can be produced by cyclo-oxygenase (COX) dependent pathways (Pratico *et al.*, 1995; Klein *et al.*, 1997; Jourdan *et al.*, 1997a; 1999). Several cell types have been shown to release increased levels of the F<sub>2</sub> isoprostane, 8-iso PGF<sub>2α</sub>, when stimulated with inflammatory mediators or subjected to oxidative stress *in vitro* (Patrignani *et al.*, 1996; Vacchiano *et al.*, 1998; Jourdan *et al.*, 1999). Furthermore, 8-iso PGF<sub>2α</sub> levels are elevated in rats *in vivo* when prooxidant conditions are induced experimentally (Morrow *et al.*, 1992b; Dabbagh *et al.*, 1994; Awad *et al.*, 1994; Lynch *et al.*, 1996). Similarly, elevated isoprostane production has been demonstrated in clinical conditions characterized by oxidant stress including diabetes (Davi *et al.*, 1999), alcoholism (Alechnik *et al.*, 1998), paraquat poisoning (Delanty *et al.*, 1996), myocardial reperfusion (Reilly *et al.*, 1997), critical illness (Carpenter *et al.*, 1998), pre-eclampsia (Walsh *et al.*, 2000) and smoking (Morrow *et al.*, 1995). Consequently, the measurement of 8-iso PGF<sub>2α</sub> in plasma and urine has been proposed as a relevant and convenient

method to monitor oxidant stress in man (Roberts & Morrow, 2000).

However, beyond their role as oxidant markers, isoprostanes have demonstrable biological actions in some tissues. 8-iso PGF<sub>2α</sub> causes contraction of isolated blood vessels, (Kromer & Tippins, 1996; Jourdan *et al.*, 1997b; Gardan *et al.*, 2000; Oliveira *et al.*, 2000), lymphatics, (Sinzing *et al.*, 1997) and airway (Kawikova *et al.*, 1996) and myometrial smooth muscle (Crankshaw, 1995). Where studied, smooth muscle contraction appears to be mediated in part *via* thromboxane TP receptors (Fukunaga *et al.*, 1993; Kromer & Tippins, 1996; Jourdan *et al.*, 1997b; Gardan *et al.*, 2000; Oliveira *et al.*, 2000). However, the effects of 8-iso PGF<sub>2α</sub> on smooth muscle function may be altered in pathophysiological settings. Thus, its vasoconstrictor effect is increased after ischaemia (Kromer & Tippins, 1996; 1999), endothelial damage and nitric oxide synthase (NOS) inhibition (Jourdan *et al.*, 1997b; Sametz *et al.*, 1999).

8-iso PGF<sub>2α</sub> also has actions on isolated platelets where, in contrast to smooth muscle preparations, it may not apparently fully activate platelet TP receptors to cause aggregation (Pratico *et al.*, 1996; Habib *et al.*, 1999) and instead inhibits the proaggregatory effects of the TP ligand U46619 (Morrow *et al.*, 1992c; Yin *et al.*, 1994). In addition, although the E<sub>2</sub> isoprostane, 8-iso PGE<sub>2</sub>, causes aggregation

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of platelets in a sub-population of human volunteers, it also inhibits U46619-induced aggregation (Longmire *et al.*, 1994). Whether or not pathophysiological events alter platelet responses to isoprostanes is not known.

In addition to 8-iso PGF<sub>2α</sub> and 8-iso PGE<sub>2</sub>, a range of synthetic isoprostanes is now available, the majority of which have not been tested in either smooth muscle or platelet preparations. The aims of this study were therefore firstly, to investigate the effects of a range of isoprostanes on aggregation of human whole blood and secondly, to assess the effects of a gross inflammatory stimulus induced by bacterial lipopolysaccharide (LPS), on the response of whole blood to isoprostanes.

## Methods

The study protocol was approved by the Ethics Committee of the Royal Brompton and Harefield NHS Trust.

### *Measurement of aggregation in human whole blood*

Venous blood was taken in glass tubes containing sodium citrate ( $1.05 \times 10^{-1}$  M) from 20 healthy female and 20 male volunteers aged 20–40 years none of whom were taking medication. Volunteers who had taken any non-steroidal anti-inflammatory drug within 1 month were excluded from the study. Blood was rolled gently at 37°C whilst awaiting analysis. In preliminary experiments, aggregation induced by U46619 decreased significantly in blood maintained as above for more than 4 h and therefore samples were analysed within this time except where stated. Whole blood impedance aggregometry of 1 ml aliquots was performed in a CHRONO-LOG 560 aggregometer (Chronolog Corporation, Haverton, PA, U.S.A.) with a stir bar revolution rate of 8000 r.p.m. Aggregatory responses were monitored for 15 min after addition of stimuli and the maximum response in this time recorded.

### *Measurement of the proaggregatory effects of U46619, collagen or isoprostanes on aggregation in human blood*

U46619 ( $10^{-8}$ – $10^{-5}$  M), collagen ( $1$ – $10 \mu\text{g ml}^{-1}$ ) or isoprostanes ( $10^{-7}$ – $10^{-5}$  M) were added to blood samples in 5  $\mu\text{l}$  aliquots and aggregation monitored as above. The vehicles used, ethanol or methylacetate at 0.125% (by volume) or less, had no effect on aggregation. In separate experiments a sub-threshold concentration of U46619, which was independently determined for each blood sample (range  $1$ – $4 \times 10^{-7}$  M), was added 1 min after the addition of isoprostane and aggregation monitored as above.

### *Measurement of antiaggregatory effects of isoprostanes or the TP-receptor antagonists, SQ29458 on U46619 and collagen*

To assess their inhibitory capacity, blood was incubated with isoprostanes ( $10^{-5}$  M) for 15–30 min before stimulation by U46619 ( $10^{-6}$  M) or collagen ( $5 \mu\text{g ml}^{-1}$ ). Where an inhibitory effect of isoprostanes ( $10^{-5}$  M) was observed further experiments were conducted using a concentration range of  $10^{-9}$ – $10^{-5}$  M. In separate experiments, the TP

receptor antagonist SQ29458, ( $10^{-9}$ – $10^{-7}$  M) was added to blood samples for 15–30 min, before the addition of U46619. Aggregation was then monitored for a further 15 min. The vehicles had no inhibitory effect on aggregation in any of the protocols used.

### *Effect of LPS treatment on whole blood aggregation responses to isoprostanes*

Blood was incubated with or without LPS ( $10 \mu\text{g ml}^{-1}$ ) for 2, 4, 6, 8 and 12 h prior to stimulation with a sub-maximal concentration ( $5 \times 10^{-7}$  M) of U46619 or collagen ( $5 \mu\text{g ml}^{-1}$ ). To verify that cellular components of the blood did not decrease significantly over the incubation period, samples were analysed for red cell, white cell and platelet numbers  $\mu\text{l}^{-1}$  before each experiment in a clinical blood cell counter (Hematology System Advia 120, Bayer, Newbury, Berkshire, U.K.). Aggregatory and inhibitory effects of isoprostanes ( $10^{-6}$  M) were re-examined in untreated and LPS-treated blood at a time (approximately 6 h) when LPS treatment induced maximal changes in blood aggregation.

### *Measurement of the effect of L-NAME on whole blood responses to isoprostanes*

The effect of NOS inhibition on the ability of blood to aggregate in response to U46619 ( $5 \times 10^{-7}$  M) was assessed using the inhibitor N $\omega$ -nitro-L-arginine methyl ester (L-NAME) ( $10^{-4}$ – $10^{-2}$  M). Non-specific effects of this compound were determined by comparing its responses with those of the inactive analogue, N $\omega$ -nitro-D-arginine methyl ester (D-NAME) over the same concentration range. L-NAME and D-NAME were added 30 min before the addition of isoprostanes, to fresh whole blood or blood incubated for 6 h in the presence or absence of LPS.

### *Materials*

Isoprostanes U46619 (11 $\alpha$ , 9 $\alpha$ , epoxyethano PGH<sub>2</sub>) and SQ29458 (1S-[1 $\alpha$ ,2 $\beta$  (5Z),3 $\beta$ ,4 $\alpha$ ]-7-[3-2[[phenylamino] carbonylhydrazino] methyl]-7-oxobicyclo [2.2.1]-hept-2-yl]-5-heptenoic acid) (Cayman Chemicals, Ann Arbor, MI, U.S.A.) were dissolved in ethanol (Sigma, Poole, Dorset, U.K.) or methylacetate (Fisher Scientific U.K., Loughborough, Leicestershire, U.K.) if provided in this solvent (8-iso PGF<sub>3 $\alpha$</sub>  and 8-iso 13,14-dihydro-15-keto-PGF<sub>2 $\alpha$</sub> ) and diluted with distilled water before experiments. N $\omega$ -nitro-L-arginine methyl ester, N $\omega$ -nitro-D-arginine methyl ester and lipopolysaccharide from *E. coli* 0127:B8 were dissolved in phosphate buffered saline (all from Sigma, Poole, Dorset, U.K.) Equine collagen solution was purchased from the Chronolog Corporation (Chronolog Corporation, Haverton, PA, U.S.A.)

### *Statistical tests*

Results were analysed using a statistical package from GraphPAD Instat software and presented using GraphPAD PRISM (GraphPAD Software, San Antonio, CA, U.S.A.) The relevant test used is described in each figure legend.

Results are presented as means  $\pm$  s.e.m. and  $P$  values  $<0.05$  were considered to be statistically significant.

## Results

### Effects of U46619, collagen or isoprostanes on aggregation in human whole blood

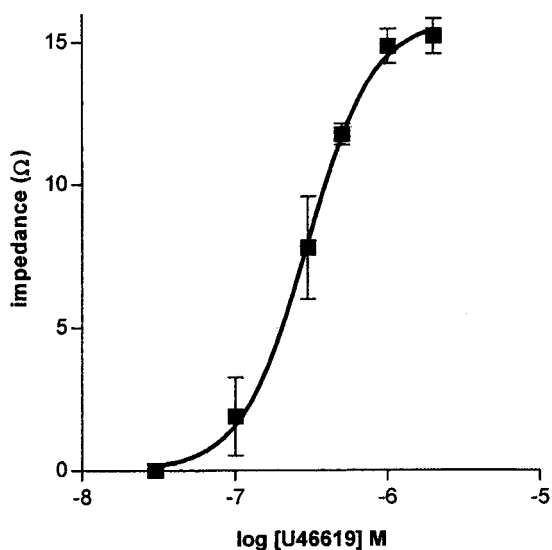
U46619 induced concentration-dependent aggregation of human whole blood with a threshold concentration of  $10^{-7}$  M, an  $EC_{50}$  of approximately  $3 \times 10^{-7}$  M and a maximum response of  $15.2 \pm 0.6 \Omega$  (Figure 1). Collagen also induced aggregation with a maximal effect observed at  $5 \mu\text{g ml}^{-1}$  of  $21 \pm 0.8 \Omega$ . By contrast, none of the isoprostanes tested over the concentration range  $10^{-7}$ – $10^{-5}$  M induced detectable aggregation. In addition, when the isoprostanes ( $10^{-6}$  M) were added immediately before a sub-threshold concentration of U46619 no proaggregatory response was observed (Table 1).

### Apparent role of TP receptors in aggregation induced by U46619 or collagen

The proaggregatory effects of U46619 ( $10^{-6}$  M) were completely blocked by pretreatment with SQ29548 ( $10^{-7}$  M). By contrast, the proaggregatory effects of collagen ( $5 \mu\text{g ml}^{-1}$ ) were reduced (by  $50\% \pm 10\%$ ;  $n=5$ ), but not blocked, by SQ29548.

### Effect of isoprostanes on aggregation in human whole blood induced by U46619 or collagen

8-iso  $\text{PGF}_{2\alpha}$ , 8-iso  $\text{PGE}_1$ , 8-iso  $\text{PGE}_2$  and 8-iso  $\text{PGF}_{3\alpha}$  induced consistent, concentration-dependent inhibition of U46619 ( $10^{-6}$  M) (Figure 2). In addition to a concentration inhibition of aggregation induced by U46619 these compounds also inhibited collagen ( $5 \mu\text{g ml}^{-1}$ ) induced aggrega-

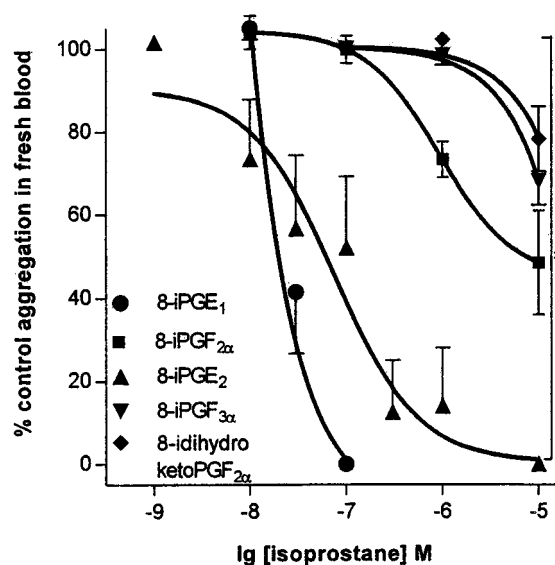


**Figure 1** Concentration-aggregation response curve of whole blood aggregation to U46619 in fresh blood of  $n=9$  donors. Each determination was performed in duplicate.

**Table 1** Effects of commercially available isoprostanes ( $10^{-7}$ – $10^{-5}$  M) on aggregation of whole blood

Isoprostane	Response
8-iso $\text{PGF}_{2\alpha}$	no aggregation
8-iso $\text{PGE}_1$	no aggregation
8-iso $\text{PGF}_{2\beta}$	no aggregation
8-iso $\text{PGA}_1$	no aggregation
8-iso $\text{PGF}_{3\alpha}$	no aggregation
8-iso $\text{PGF}_{1\alpha}$	no aggregation
8-iso-15(R)- $\text{PGF}_{2\alpha}$	no aggregation
8-iso-13,14-dihydro-15-keto $\text{PGF}_{2\alpha}$	no aggregation
8-iso $\text{PGF}_{1\beta}$	no aggregation
8-iso $\text{PGE}_2$	no aggregation
8-iso-15-keto $\text{PGF}_{2\alpha}$	no aggregation

The data represent observations made in blood donated by  $n=3-5$  separate individuals. In each case responses were performed in duplicate.



**Figure 2** Effects of isoprostanes on aggregation induced by U46619 ( $10^{-6}$  M). The data represents single responses in blood donated by  $n$  of five to nine separate individuals. \*Illustrates a statistical difference between the effects of either 8-iso (8-i)  $\text{PGE}_1$  or 8-iso (8-i)  $\text{PGE}_2$  and the other isoprostanes shown, calculated by two-way ANOVA. A  $P$  value of less than 0.05 was taken to be significant.

tion (Table 2). By contrast, 8-iso-13,14-dihydro-15-keto  $\text{PGF}_{2\alpha}$  produced weak inhibition of aggregation induced by U46619 or collagen and caused no reduction in aggregation in two out of five donors. No other isoprostane tested produced significant inhibition of aggregation (Table 2).

### Effect of LPS on aggregatory responses to U46619 or collagen in human whole blood

Erythrocyte ( $\mu\text{l}^{-1}$ ), white cell ( $\mu\text{l}^{-1}$ ) and platelet counts ( $\mu\text{l}^{-1}$ ) were not significantly affected by incubation at  $37^\circ\text{C}$  over 12 h with or without LPS ( $10 \mu\text{g ml}^{-1}$ ) (one way ANOVA  $P>0.05$ ). Equally there was no difference between cell counts in control blood and blood incubated with LPS at 2, 4, 6, 8 and 12 h (two-way ANOVA  $P>0.05$ ;  $n=5$  donors; single

**Table 2** Effects of isoprostanes ( $10^{-5}$  M) on whole blood aggregation induced by collagen ( $5 \mu\text{g ml}^{-1}$ ) or U46619 ( $10^{-6}$  M)

Isoprostane	Collagen-induced aggregation (percentage control)	s.e.m.	One sample t-test	U46619-induced aggregation (percentage control)	s.e.m.	One sample t-test
8-iso PGF <sub>2β</sub>	117	6	ns	92	3	ns
8-iso PGF <sub>1α</sub>	101	9	ns	97	16	ns
8-iso PGA <sub>1</sub>	108	6	ns	83	11	ns
8-iso 15-keto PGF <sub>2α</sub>	85	14	ns	80	26	ns
8-iso PGF <sub>1β</sub>	107	2	ns	81	7	ns
8-iso 15(R)-PGF <sub>2α</sub>	116	5	ns	91	2	ns
8-iso-13,14-dihydro-15-keto PGF <sub>2α</sub>	77	12	ns	78	8	$P < 0.05$
8-iso PGE <sub>2</sub>	50	5	$P < 0.05$	0	0	$P < 0.05$
8-iso PGE <sub>1</sub>	15	7	$P < 0.05$	0	0	$P < 0.05$
8-iso PGF <sub>2α</sub>	50	9	$P < 0.05$	48	12	$P < 0.05$
8-iso PGF <sub>3α</sub>	51	3	$P < 0.05$	68	6	$P < 0.05$

The data shown represent responses obtained in blood donated by  $n=3-5$  separate individuals. In each case, determinations were performed in duplicate. The effect of each isoprostane on aggregation induced by either collagen or U46619 was calculated as a percentage of the respective control response, performed in paired experiments. Statistical differences were calculated using the one-sample *t*-test for normalized data and a *P* value of less than 0.05 was considered significant. ns=not significant.

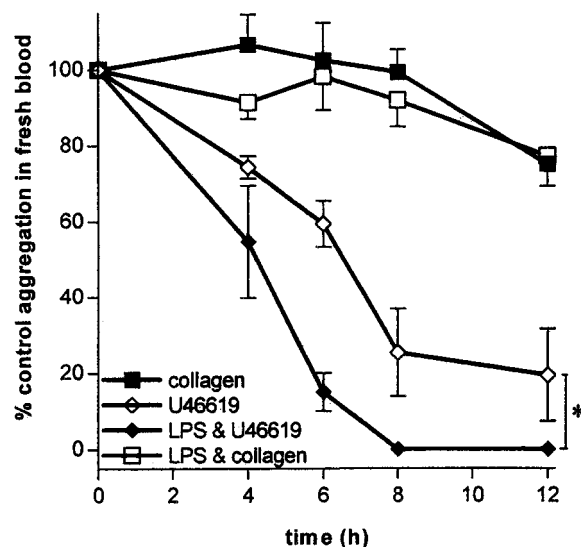
experiments; data not shown). However, when blood was incubated at 37°C there was a time dependent (2–12 h) reduction in the proaggregatory responses induced by U46619 ( $10^{-6}$  M), an effect that was enhanced by LPS ( $10 \mu\text{g ml}^{-1}$ ) (Figure 3). The greatest effect on aggregation induced by LPS was observed at 6 h of incubation. By contrast, the proaggregatory effects of collagen ( $5 \mu\text{g ml}^{-1}$ ) were not modified by time of incubation (up to 12 h), either in the presence or absence of LPS.

#### Effect of isoprostanes on aggregation in human whole blood stimulated with LPS

After 6 h incubation with LPS, none of the isoprostanes were proaggregatory in whole blood, nor did they enhance the proaggregatory effects of a sub-maximal concentration of U46619 ( $n=3-5$  donors; duplicate determinations; data not shown). In fact, under these conditions, as with non-LPS-stimulated blood, 8-iso PGF<sub>2α</sub>, ( $10^{-6}$  M) (Figure 4) as well as 8-iso PGE<sub>1</sub>, 8-iso PGE<sub>2</sub>, 8-iso PGF<sub>3α</sub>, and 8-iso-13,14-dihydro-15-keto PGF<sub>2α</sub> inhibited aggregation induced by U46619 ( $n=3-5$  donors; single determinations paired with non-LPS-stimulated blood; data not shown). Interestingly, the inhibitory effect of 8-iso PGF<sub>2α</sub> ( $10^{-6}$  M) (Figure 4), and the other isoprostanes (data not shown) was enhanced by stimulation of blood with LPS.

#### Effect of L-NAME on human whole blood

L-NAME ( $10^{-4}-10^{-2}$  M) had an inhibitory effect on aggregation induced by U46619 ( $5 \times 10^{-7}$  M) in fresh whole blood and blood incubated with LPS ( $10 \mu\text{g ml}^{-1}$ ) (data not shown). D-NAME was also inhibitory at  $10^{-2}$  M, but inhibition was specific to L-NAME at concentrations between  $10^{-4}$  and  $10^{-3}$  M. Further, neither 8-iso PGF<sub>2α</sub>, 8-iso PGE<sub>1</sub>, 8-iso PGE<sub>2</sub>, 8-iso PGF<sub>3α</sub>, nor 8-iso-13,14-dihydro-15-keto PGF<sub>2α</sub> ( $10^{-6}$  M), induced aggregation in the presence or absence of a sub-threshold concentration of U46619 in blood containing L-NAME ( $10^{-3}$  M) nor in blood incubated with

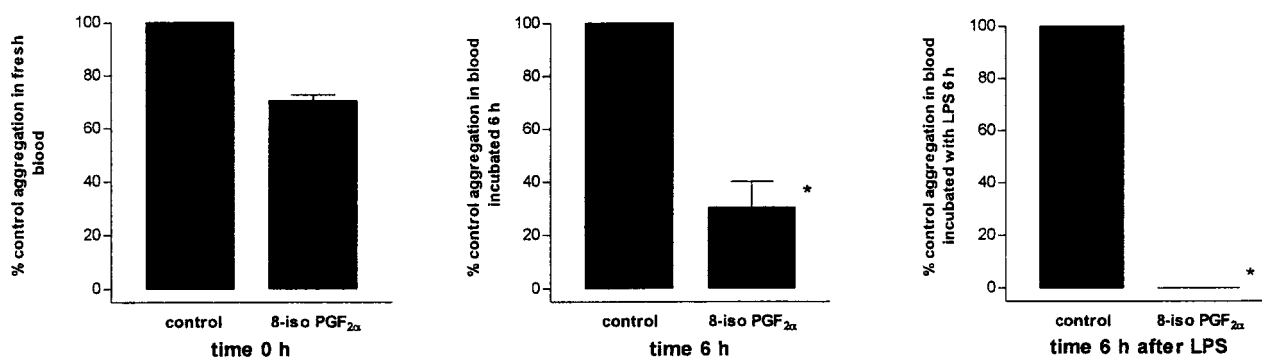


**Figure 3** Effects of incubation time (2–12 h) with LPS on aggregation of whole blood induced by collagen ( $5 \mu\text{g ml}^{-1}$ ) or U46619 ( $5 \times 10^{-7}$  M). The data shows responses, performed in parallel, of blood incubated with or without LPS and then stimulated with aggregatory agent. Each set of single responses was performed using blood donated by *n* of five separate individuals. \*Illustrates a statistical difference in the responses of blood treated with LPS compared to control calculated using two-way ANOVA. A *P* value of less than 0.05 was taken to be significant.

LPS ( $10 \mu\text{g ml}^{-1}$ ) and then treated with L-NAME ( $10^{-3}$  M) (data not shown).

## Discussion

The isoprostanes are commonly described as inert markers of oxidative damage. However, their PG-like structure clearly implicates them as potential biological mediators of inflammatory responses. We have shown that a number of



**Figure 4** Effect of LPS ( $10 \mu\text{g ml}^{-1}$ ) on the antiaggregatory responses of 8-iso PGF<sub>2 $\alpha$</sub>  ( $10^{-6}$  M) in U46619 ( $5 \times 10^{-7}$  M) stimulated whole blood. Responses are shown in untreated blood after (a) collection without storage or (b) after storage for 6 h. For comparison, responses are shown in (c) in blood stimulated with LPS for 6 h. The data is represented as percentage of control response obtained in each condition in blood stimulated with U46619 alone. Each data point comprises data from *n* of five separate donors. Experiments illustrated in (a), (b) or (c) were performed in parallel. \*Illustrates a significant difference between aggregation in comparison to control response in non-LPS-treated blood using two-way ANOVA. A *P* value of less than 0.05 was taken to be significant.

structurally distinct isoprostanes inhibit human whole blood aggregation, an effect enhanced under proinflammatory conditions.

In the current study 8-iso PGF<sub>2 $\alpha$</sub>  inhibited whole blood aggregation stimulated by U46619 or collagen. This observation is in agreement with others showing that 8-iso PGF<sub>2 $\alpha$</sub>  inhibits platelet aggregation stimulated with TP-agonists (Yin *et al.*, 1994; Morrow *et al.*, 1992c). Indeed, there is a growing body of evidence that illustrates interactions between 8-iso PGF<sub>2 $\alpha$</sub>  and TP receptors. In human platelets 8-iso PGF<sub>2 $\alpha$</sub>  binds to TP receptors, although its interaction at these sites appears complex and has not been fully defined (Pratico *et al.*, 1996; Minuz *et al.*, 1998; Habib *et al.*, 1999). Using platelet-rich plasma, Pratico *et al.* (1996) have shown that 8-iso PGF<sub>2 $\alpha$</sub>  can activate human platelets to induce shape change and facilitate the proaggregatory actions of other full agonists but cannot itself induce full aggregation. Initially, this phenomenon could not be attributed to TP receptor activation. However, 8-iso PGF<sub>2 $\alpha$</sub>  was subsequently (Habib *et al.*, 1999) shown to bind a specific site on TP $\alpha$  receptors and to trigger signal transduction distinct from thromboxane (Takahara *et al.*, 1990). Using whole blood, we were not able to reliably determine effects on shape change. Furthermore, in our study 8-iso PGF<sub>2 $\alpha$</sub>  did not potentiate the proaggregatory effects of either U46619 or collagen. This apparent discrepancy between platelet stimulation in isolated preparations and whole blood may be attributable to proaggregatory alterations in platelets and plasma during separation (Nicholson *et al.*, 1998; Cox, 1998; Kulkarni *et al.*, 2000) and the removal of components of whole blood that inhibit platelet aggregation (Zatta *et al.*, 1990).

Of the 11 commercially-available isoprostanes tested in this study, as has been found with 8-iso PGF<sub>2 $\alpha$</sub>  (Yin *et al.*, 1994; Morrow *et al.*, 1992c), we found that, 8-iso PGE<sub>1</sub>, 8-iso PGE<sub>2</sub>, 8-iso PGF<sub>3 $\alpha$</sub> , and 8-iso-13,14-dihydro-15-keto PGF<sub>2 $\alpha$</sub>  all inhibited whole blood aggregation. However, others have suggested, again using isolated platelet preparations, that 8-iso PGE<sub>2</sub> can act as a full agonist in a small proportion of individuals and induce irreversible aggregation (Longmire *et al.*, 1994; Leitinger *et al.*, 1997; Kobzar *et al.*, 1997). The

proaggregatory effects of 8-iso PGE<sub>2</sub> on platelets are thought to be, like those of 8-iso PGF<sub>2 $\alpha$</sub> , mediated by interactions with TP receptors (Longmire *et al.*, 1994). The absence of this effect in whole blood may again be due to the factors mentioned above or related to the population of donors used in this study.

The ability of 8-iso PGF<sub>2 $\alpha$</sub>  to inhibit whole blood aggregation in response to U46619 or collagen may be related to its effects as a partial or false agonist for TP receptors on platelets (Yin *et al.*, 1994). As with 8-iso PGF<sub>2 $\alpha$</sub> , we found that 8-iso PGE<sub>2</sub> inhibited the proaggregatory effects of U46619 and collagen. This observation is in agreement with others using isolated platelets, where the inhibitory effect of low concentrations of 8-iso PGE<sub>2</sub> were attributed to antagonism at the TP receptor (Longmire *et al.*, 1994). To our knowledge, we have shown here for the first time that 8-iso PGE<sub>1</sub> also inhibits TP-receptor and collagen-mediated platelet aggregation in whole blood. In fact, the rank order of potency for inhibition of U46619-induced aggregation was 8-iso PGE<sub>1</sub> > 8-iso PGE<sub>2</sub> > 8-iso PGF<sub>2 $\alpha$</sub>  > 8-iso PGF<sub>3 $\alpha$</sub>  > 8-iso-13,14-dihydro-15-keto PGF<sub>2 $\alpha$</sub> . Furthermore, 8-iso PGE<sub>1</sub> was able to inhibit completely collagen-induced aggregation, an effect not demonstrable by the potent thromboxane receptor antagonist SQ29548, nor any of the other isoprostanes tested. This suggests that 8-iso PGE<sub>1</sub> inhibits platelet aggregation, at least in part, independently of its actions on TP receptors. The mechanism of the additional effects of 8-iso PGE<sub>1</sub> on platelet aggregation in whole blood remains unclear. An action at IP receptors has been proposed (Leitinger *et al.*, 1997) but EP, DP or novel isoprostane receptors are other possible effector sites (Armstrong, 1996).

Inflammatory states are commonly associated with platelet abnormalities and both inappropriate aggregation and inadequate aggregation are features of clinical sepsis (Lundahl *et al.*, 1996; Mammen, 1998). Endotoxaemia results in increased production of reactive oxygen species (Blackwell *et al.*, 1996) and LPS induces isoprostane production *in vivo* (Mcadam *et al.*, 2000). As blood vessels alter their responses to 8-iso PGF<sub>2 $\alpha$</sub>  after exposure to oxidative (Kromer & Tippins, 1996) or inflammatory (Jourdan *et al.*, 1998) stress

we hypothesized that the effect of isoprostanes on platelet aggregation might also change in blood stimulated by LPS. LPS alone induced a time-dependent depression in the aggregatory response to U46619 but not to collagen. This observation is in agreement with other researchers (Sheu *et al.*, 1999) who have shown similar effects in isolated platelets stimulated with LPS. However, under the same conditions, in contrast to the reported effects on blood vessels, we did not reveal any enhancement of TP receptor-mediated effects by isoprostanes. In fact, the efficacy of the five inhibitory isoprostanes increased in part possibly through a rise in endogenous generation of isoprostanes in this setting.

However, it is interesting to note that the inhibitory effect of LPS on washed human platelets is affected by the presence of calcium-chelating citrate ions in the suspending medium (Baba, 1994). We cannot be sure that this is not relevant to our experiments. The role of raised cytoplasmic calcium in platelet activation is undisputed. However the relative importance to activation of the intracellular or extracellular mobilization of calcium ions is dependent on the agonist, with some weak agonists being more dependent on extracellular concentrations. Other strong agonists including collagen, are capable of inducing platelet aggregation by several mechanisms including thromboxane-independent paths, and dependence on extracellular calcium is less than for weak agonists (Holmsen, 1994). Nevertheless a significant proportion of the aggregatory effect of collagen is dependent on induced platelet thromboxane production (Blackmans *et al.*, 1995). Our experiments indicate that collagen-initiated aggregation remains wholly intact in blood incubated with LPS but incubation with LPS may have produced extracellular calcium dependence in U46619-initiated aggregation. However it is clear that a gross inflammatory insult does not reveal any masked proaggregatory function of the isoprostanes tested in a system where thromboxane-mediated responses are still apparent.

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