Local Amplification of Platelet Function by 8-Epi Prostaglandin F$_2$\alpha Is Not Mediated by Thromboxane Receptor Isoforms*

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8-epi-Prostaglandin (PG) F$_2$\alpha may be formed by cyclooxygenases 1 and 2 or by a free radical catalyzed process as an isoprostane. Concentrations of 8-epi-PGF$_2$\alpha, in the range 1 nM to 1 \( \mu \)M induce a dose-dependent increase in platelet shape change, in calcium release from intracellular stores (Ca$^{2+}$), and in inositol phosphates; it also causes irreversible platelet aggregation, dependent on thromboxane generation, when incubated with subthreshold concentrations of ADP, thrombin, collagen, and arachidonic acid. Much higher concentrations of 8-epi-PGF$_2$\alpha (10–20 \( \mu \)M) alone induce weak, reversible aggregation.

Although these effects are prevented by pharmacological thromboxane receptor antagonists, they are unlikely to be mediated by thromboxane receptors. Thus, 8-epi-PGF$_2$\alpha does not compete for binding at the stably expressed placental or endothelial isoforms of the thromboxane receptor or for binding of thromboxane ligands to human platelets. Furthermore, the response to 8-epi PGF$_2$\alpha exhibits structural specificity versus 8-epi PGF$_3$\alpha and PGF$_2$\beta. Concentrations in the range that evoke its effects on platelets do not desensitize the aggregation response stimulated by thromboxane or PGH$_2$ analogs. Unlike primary prostaglandins, which are rapidly metabolized to inactive products, 8-epi PGF$_2$\alpha circulates in plasma. However, the systemic concentrations found in healthy volunteers (median 48 pmol/liter) and in patients with hepatic cirrhosis (median 147 pmol/liter), a syndrome of oxidant stress in vivo, fall well below those which modulate platelet function.

8-Epi PGF$_2$\alpha may amplify the response to platelet agonists in syndromes where oxidant stress and platelet activation coincide. Despite blockade by thromboxane antagonists, 8-epi PGF$_2$\alpha does not activate either of the thromboxane receptor isoforms described in platelets. Activation of a distinct receptor would be consistent with the enzymatic formation of 8-epi PGF$_2$\alpha by cyclooxygenases. However, incidental activation of such a receptor by systemic concentrations of 8-epi PGF$_2$\alpha is unlikely to occur, even in syndromes of excessive free radical generation in vivo.

F$_2$ isoprostanes are free radical catalyzed products of arachidonic acid (1, 2). They are members of a growing family of isoeicosanoids, which includes isoleukotrienes and isomers of thromboxanes and prostaglandins (PGs)$^3$ of the E and D series (3–5). One of the F$_2$ isoprostanes, 8-epi PGF$_2$\alpha, has been shown to exhibit biological activity in vitro (6) and has been postulated to function as an autacoid. It is a vasoconstrictor and a mitogen in vascular smooth muscle cells (7, 8); both effects are prevented by thromboxane receptor antagonists. 8-Epi PGF$_2$\alpha also modulates the function of human platelets, although its effects are distinct from those elicited by analogs of thromboxane A$_2$ or its prostaglandin endoperoxide precursors, which also activate thromboxane receptors (9). Both of these classes of compounds induce platelet shape change, followed by irreversible aggregation at high concentrations or to prevent aggregation induced by thromboxane or prostaglandin endoperoxide analogs and other platelet agonists (12, 13). Similar effects have been ascribed to 8-epi PGF$_2$\epsilon (14).

We have recently developed a stable isotope dilution assay for 8-epi PGF$_2$\alpha, using gas chromatography/mass spectrometry (15). We have demonstrated the potential utility of measuring this compound in human urine as an index of oxidant stress in vivo (16, 17). Interestingly, we have found that 8-epi PGF$_2$\alpha, unlike other F$_2$ isoprostanes, can be formed in a free radical or cyclooxygenase (COX)-dependent manner. It is a minor product of the COX-1 enzyme in human platelets (18) and a somewhat more abundant product of COX-2 in human monocytes (19). Despite these observations, enzymatic pathways account for a trivial component of overall formation of 8-epi PGF$_2$\alpha in vivo, even in syndromes of COX activation (16, 20). However, such a mechanism of formation might be consistent with a local autacoidal or systemic hormonal function, mediated via distinct receptors.

The present study was designed to address the hypothesis that 8-epi PGF$_2$\alpha might exert biological effects at the concentrations which circulate in vivo. We decided to explore its effects on platelets in particular, as reports of its effects on this cell type have been conflicting. The platelet, which possesses the ability to form the compound enzymatically, might be a likely target for a receptor mediated activation in vivo, either in the local microenvironment or in the systemic circulation. Finally, we wished to determine if 8-epi PGF$_2$\alpha was likely to exert its effects on platelets and, by implication, on other cells, via a newly described (21) splice variant of the thromboxane receptor.

*This work is supported by a Specialized Center for Research in Thrombosis Grant HL 54500. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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$^3$The abbreviations used are: PG, prostaglandin; COX, cyclooxygenase enzyme; PRP, platelet-rich plasma; WP, washed platelets; TP$_\alpha$, placental thromboxane receptor; TP$_\beta$, endothelial thromboxane receptor; HEK cells, human embryonic kidney cells; Tx, thromboxane; HBSS, Hanks' balanced salt solution.
Venous blood was collected from the antecubital veins of nonsmoking, healthy volunteers who had abstained from medications for at least 2 weeks prior to the study. Blood was collected into plastic syringes containing 3.8% sodium citrate as anticoagulant (ratio 9:1). Platelet-rich plasma was removed by centrifugation for 10 min at 200 g. Platelet-poor plasma was prepared by spinning the remaining blood at 900 × g for 10 min. WP were prepared from PRP after centrifugation and resuspended in calcium and magnesium-free HBSS at pH 7.4. Unless otherwise stated, platelet number was always adjusted at 3 × 10^8 platelet/ml with platelet-poor plasma or HBSS. Platelet aggregation was performed in 500-μl aliquots (Biodata PAP-4, Biodata Corp., Hatboro, PA) using threshold concentration of agonists. Threshold is defined as the lowest concentration that evokes receptor expression irreversibly and aggregation, with an amplitude between 65 and 85% of the potential maximum deflection in light transmission (22). Platelet aggregation was also studied using subthreshold concentrations of agonists. Subthreshold concentrations are defined as the highest dose that induces less than a 10% increase in light transmission (22).

**Platelet Functional Studies**

Venous blood was collected from the antecubital veins of nonsmoking, healthy volunteers who had abstained from medications for at least 2 weeks prior to the study. Blood was collected into plastic syringes containing 3.8% sodium citrate as anticoagulant (ratio 9:1). Platelet aggregation was examined in platelet-rich plasma (PRP) and in washed platelets (WP) by light transmission at 37°C, as described previously (18). Briefly, citrated whole blood was centrifuged at 160 × g for 10 min, and the PRP was removed. Platelet-poor plasma was prepared by spinning the remaining blood at 900 × g for 10 min. WP were prepared from PRP after centrifugation and resuspended in calcium and magnesium-free HBSS at pH 7.4. Unless otherwise stated, platelet number was always adjusted at 3 × 10^8 platelet/ml with platelet-poor plasma or HBSS. Platelet aggregation was performed in 500-μl aliquots (Biodata PAP-4, Biodata Corp., Hatboro, PA) using threshold concentration of agonists. Threshold is defined as the lowest concentration that evokes irreversible aggregation, with an amplitude between 65 and 85% of the potential maximum deflection in light transmission (22). Platelet aggregation was also studied using subthreshold concentrations of agonists. Subthreshold concentrations are defined as the highest dose that induces less than a 10% increase in light transmission (22).

**Platelet Biochemistry Studies**

**Pertussis Toxin Studies**—To address the possibility that 8-epi PGF2α stimulation of human platelets might involve a phospholipase coupled with a toxin-sensitive G protein, WP were incubated with saponin (15 μg/ml) and pertussis toxin (15 μg/ml) for 5 min at 37°C, then different stimuli added, as described previously (28).

**Intracellular Calcium—PRP was incubated with 2 μM Fura-2/AM at 37°C for 45 min, washed and then allowed to form plasma membrane vesicles (PMV) by using a scintillation counter (Beckman Instruments). The fluorescence signal was monitored at 510 nm with the excitation wavelength of 340 and 380 nm. The ratio of maximum and minimum fluorescence were determined by the addition of 250 μM digitonin in the presence of 1 mM CaCl₂ and in the presence of 10 μM EGTA (pH >8.5), respectively, as described previously (22).

**Inositol Phosphate Formation—Platelets were labeled with myo-[3H]inositol and the formed inositol phosphates were extracted from the cells by chloroform/methanol/HCl and separated by Dowex-1 anion exchange chromatography, as described previously (29). Briefly, WP were incubated with myo-[2-3H]inositol (50 μCi/ml) at 37°C for 3 h, then washed and resuspended in HBSS, pH 7.4, containing 10 mM LiCl. These conditions inhibit conversion of inositol phosphates to free inositol. The platelets were then incubated for 10 min at 37°C, prior to their stimulation by agonists. Total inositol phosphates were separated on a TSKgel column, eluting at 4 ml/min. The void volume contained 5 mFura-2/AM at 37°C for 45 min, washed and then allowed to form plasma membrane vesicles (PMV) by using a scintillation counter (Beckman Instruments). The fluorescence signal was monitored at 510 nm with the excitation wavelength of 340 and 380 nm. The ratio of maximum and minimum fluorescence were determined by the addition of 250 μM digitonin in the presence of 1 mM CaCl₂ and in the presence of 10 μM EGTA (pH >8.5), respectively, as described previously (22).

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10 healthy volunteers (30–55 years of age; six were men), and from eight patients with hepatic cirrhosis (40–60 years of age; five men). In all patients the diagnosis of cirrhosis was confirmed by histology. Their clinical characteristics are defined elsewhere (30).

Data Analyses

Data were subjected to analysis of variance prior to pairwise comparisons as appropriate. A nonparametric approach was employed to avoid assumptions as to the distribution of the parameters under study. The data are presented as the mean ± the standard error of the mean (S.E.).

RESULTS

Effects of 8-Epi PGF$_{2\alpha}$ on Human Platelets—Initially, we examined the effect of 8-ei PGF$_{2\alpha}$ on platelet shape change at various concentrations ranging from 1 nM to 10 M. 8-Epi PGF$_{2\alpha}$ induced a dose-dependent platelet shape change with an EC$_{50}$ of 0.2 ± 0.2 M (Fig. 1). SQ29,548 (1 M) completely inhibited the shape change induced by 8-epi PGF$_{2\alpha}$, in contrast to aspirin (100 M) or indomethacin (10 M), which did not modify the response. Pretreatment with pertussis toxin (15 M) also failed to prevent 8-epi PGF$_{2\alpha}$ induced shape change (data not shown). A structural isomer of 8-epi PGF$_{2\alpha}$, 8-epi PGF$_{5\alpha}$, did not evoke platelet shape change at concentrations up to 50 M (data not shown). The effects of 8-epi PGF$_{2\alpha}$ and that of the PGH$_2$ analog, U46619, on the shape change were additive (Fig. 2).

8-Epi PGF$_{2\alpha}$ alone failed to induce irreversible aggregation or a rise in platelet TxB$_2$ at concentrations up to 100 M. Weak, reversible aggregation was evoked by concentrations in excess of 20 M (Fig. 3A). Because 8-epi PGF$_{2\alpha}$ seemed to act as weak platelet agonist, we decided to investigate if it could modulate the platelet response to more common agonists. No significant effect on the maximal aggregation response to threshold concentrations of ADP (2 M), thrombin (1 unit/ml), U46619 (1 M), collagen (2 M), or arachidonic acid (100 M) was observed when 8-epi PGF$_{2\alpha}$ was coinubated with these ligands. However, the lag phase of the response to collagen was shortened (Fig. 3B). We noted that, 8-epi PGF$_{2\alpha}$ combined with arachidonic acid or collagen, there was a significant increase of TxB$_2$ production above that seen when these agonists were used alone (Table 1).

To address further the possibility that 8-epi PGF$_{2\alpha}$ might amplify the platelet response to conventional agonists, we explored its effects on the response to subthreshold concentrations of these compounds which are insufficient to induce full aggregation when used alone. 8-Epi PGF$_{2\alpha}$, dose-dependently increased the magnitude of the platelet aggregation response when combined with the subthreshold concentrations of the agonists. The EC$_{10}$ values for ADP, arachidonic acid, U46619, and collagen were, under these conditions, 0.8, 0.2, 0.6, and 0.5 M, respectively (Table II). Furthermore, platelet TxB$_2$ production was commensurately increased when 8-epi PGF$_{2\alpha}$ was combined with subthreshold concentrations of the agonists (data not shown). Both effects were prevented by aspirin or SQ29,548 (data not shown).

8-Epi PGF$_{2\alpha}$ and Thromboxane Receptor Activation—The thromboxane analog, I-BOP, dose-dependently displaced $^{3}$H-SQ29,548 from its binding sites. PGF$_{2\alpha}$ and collagen were, under these conditions, 0.8, 0.2, 0.6, and 0.5 M, respectively (Table II). Furthermore, platelet TxB$_2$ production was commensurately increased when 8-epi PGF$_{2\alpha}$ was combined with subthreshold concentrations of the agonists (data not shown).

Platelet Desensitization—To address further the possibility that 8-epi PGF$_{2\alpha}$ activates receptors distinct from these activated by PGH$_2$ or thromboxane A$_2$, we studied its ability to desensitize the platelet response to these ligands. Concentrations of 8-epi PGF$_{2\alpha}$ in the range (EC$_{50}$ 100 M to 1 M) which evokes platelet shape change, failed to desensitize the shape change response to either U46619 or I-BOP (Fig. 6A). Very high concentrations of 8-epi PGF$_{2\alpha}$ (50 M) caused a small decrease in the shape change response to U46619 (not shown). Preincubation of platelets with similar concentrations of 8-epi PGF$_{2\alpha}$ failed to modulate the aggregation responses to either U46619.
or I-BOP (Fig. 6B). Each of these two ligands induced homolo-
gous desensitization of their own shape change and aggrega-
tion responses.

8-Epi PGF$_2\alpha$ and Platelet Calcium Movement—Human
platelets loaded with Fura-2/AM and stimulated with 8-epi
PGF$_2\alpha$ respond with a rapid, transient, increase in intracellular
calcium, which coincides with platelet shape change. The EC$_{50}$
for this response was $3.1 \pm 1.0 \mu M$ (Fig. 7). To investigate
whether the increase in calcium induced in platelets by 8-epi
PGF$_2\alpha$ was of extracellular or intracellular origin, Fura-loaded
human platelets were pretreated with 1 mM CaCl$_2$ to increase
a potential influx of extracellular calcium. Similarly, they were
pretreated with 2 mM EGTA to chelate extracellular calcium.
No change in the 8-epi PGF$_2\alpha$ evoked calcium signal were
observed upon treatment with either 1 mM CaCl$_2$ or EGTA in
comparison with untreated platelets. This result implies that
8-epi PGF$_2\alpha$ induces mobilization of calcium from intracellular
stores. This mobilization of intracellular calcium evoked by
8-epi PGF$_2\alpha$ was unaffected by pretreatment with the rela-
tively nonspecific (COX-1 versus COX-2) (31) COX inhibitors,
indomethacin (10 $\mu M$), or aspirin (100 $\mu M$). The thromboxane
receptor antagonist SQ29,548 (1 $\mu M$) was able to prevent it
completely (data not shown). However, consistent with the
functional responses, initial stimulation of platelets with 8-epi
PGF$_2\alpha$ did not desensitize the increment in intracellular cal-
cium evoked by U46619. Similarly, pretreatment with U46619
failed to desensitize the calcium response to 8-epi PGF$_2\alpha$ (Fig.
8, A and B). Similar results were observed when I-BOP was
used in place of U46619 (data not shown). 8-Epi PGF$_2\alpha$ was also
able to amplify the calcium responses to subthreshold concen-
trations of platelet agonists, such as arachidonic acid or
U46619, consistent with the data on platelet function (Table
III). Positional specificity of the response to 8-epi PGF$_2\alpha$ was
again evident in this system; 8-epi PGF$_3\alpha$, failed to evoke cal-
cium movement at concentrations up to 50 $\mu M$. It also failed to

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**Table I**

| Conditions | Light transmission | TxB$_2$ |
|------------|--------------------|---------|
| 8-Epi PGF$_2\alpha$ | $3 \pm 1$ | ND$^b$ |
| Arachidonic acid | $70 \pm 8$ | $280 \pm 30$ |
| Arachidonic acid + 8-epi PGF$_2\alpha$ | $78 \pm 10$ (NS$^c$) | $340 \pm 26$ |
| Collagen | $72 \pm 6$ | $200 \pm 20$ |
| Collagen + 8-epi PGF$_2\alpha$ | $80 \pm 5$ (NS) | $265 \pm 30$ |

$^a$ 8-Epi PGF$_2\alpha$ (1 $\mu M$) was always added 15 s after arachidonic acid (100 $\mu M$) or collagen (2 $\mu g/ml$). The aggregation trace was followed for 3 min and expressed as percentage of light transmission. Supernatants were collected to measure TxB$_2$ by gas chromatography/mass spectrometry as described in the text. Data with and without 8-epi PGF$_2\alpha$ are compared for each agonist and presented as the mean $\pm$ S.E. of four experiments.

$^b$ ND, not detectable.

$^c$ NS, not significant.

$^d$ p < 0.005.
8-Epi PGF2α desensitizes the intracellular calcium response to 8-epi PGF2α (data not shown). Similarly, PGF2α failed to evoke a calcium response when used at concentrations up to 50 μM. It also failed to desensitize the response to 8-epi PGF2α (data not shown).

8-Epi PGF2α and Platelet Inositol Phosphates—The addition of 8-epi PGF2α to platelet suspensions increased the level of total inositol phosphates in a concentration-dependent manner (Fig. 9). The production was rapid, with a maximum at 15 s after the stimulus was added. Stimulation of inositol phosphate production by 8-epi PGF2α at 1 μM was significantly greater than that evoked by either I-BOP or U46619 (Fig. 10). Furthermore, stimulation by 8-epi PGF2α appeared more resistant to pharmacological thromboxane receptor antagonism, than was the case for U46619 (Fig. 10) or I-BOP (data not shown). The 8-epi PGF2α-induced increase of platelet inositol phosphates was not inhibited by pretreatment with pertussis toxin (data not shown).

Plasma 8-Epi PGF2α—The levels of 8-epi PGF2α in plasma from healthy volunteers ranged from 31 to 65 pmol/liter, with a median of 48 pmol/liter. The plasma levels in patients with cirrhosis were elevated, ranging from 57 to 204 pmol/liter with a median value of 147 pmol/liter (p < 0.001).

**DISCUSSION**

8-Epi PGF2α activates human platelets. It induces platelet shape change coincident with an increase in inositol phos-
phosphates and calcium release from intracellular stores. These effects are prevented by pretreatment with thromboxane receptor antagonists, but not by cyclooxygenase inhibitors. Unlike the stable carbacyclin derivative of thromboxane A$_2$ (32), or structural analogs of either thromboxane A$_2$ or its prostaglandin endoperoxide precursor, PGH$_2$ (11), we have found that there is a clear distinction between the concentrations of 8-epi PGF$_{2\alpha}$ which induce shape change and those which affect either platelet thromboxane formation or irreversible aggregation. The concentrations of 8-epi PGF$_{2\alpha}$ which induce shape change do not inhibit irreversible aggregation induced by a range of platelet agonists, including the PGH$_2$ analog, U46619. Much higher concentrations (10$\mu$M versus 10 nM) induce weak, reversible aggregation. These findings are in accord of those of Morrow et al. (12) and Yin et al. (13). Despite these observations, 8-epi PGF$_{2\alpha}$ does facilitate the induction of aggregation by subthreshold concentrations of platelet agonists. Thus, concentrations of 8-epi PGF$_{2\alpha}$ ranging from 10 nM to 10$\mu$M cause dose-dependent, irreversible platelet aggregation in the presence of concentrations of collagen, ADP, arachidonic acid, and...
The ability of 8-epi PGF2α liver injury (33), may be associated with platelet activation. Stress, such as vascular reperfusion (17) and alcohol induced acid. Many syndromes, putatively associated with oxidant radical formation coincide. Relevance to settings where platelet activation and enhanced free radical formation coincide.

The effects of pharmacological thromboxane antagonists on the platelet responses to 8-epi PGF2α imply that it exerts these effects via thromboxane receptors. However, this seems highly unlikely. A single gene encoding thromboxane receptors has been identified (34). Like other eicosanoid receptors, it is a member of the heptahelical G protein coupled receptor family (35). We (36) and others (8) have reported that 8-epi PGF2α competes weakly for binding cells transiently transfected with a form of the receptor cloned from human placenta. However, a feature of at least the PGE subfamily of eicosanoid receptors, feature of at least the PGE subfamily of eicosanoid receptors, a form of the receptor cloned from human placenta. However, a feature of at least the PGE subfamily of eicosanoid receptors, a form of the receptor cloned from human placenta. However, a feature of at least the PGE subfamily of eicosanoid receptors, a form of the receptor cloned from human placenta. However, a feature of at least the PGE subfamily of eicosanoid receptors, a form of the receptor cloned from human placenta. However, a feature of at least the PGE subfamily of eicosanoid receptors, a form of the receptor cloned from human placenta. However, a feature of at least the PGE subfamily of eicosanoid receptors, a form of the receptor cloned from human placenta. However, a feature of at least the PGE subfamily of eicosanoid receptors.

Amplification of Platelet Function by 8-Epi PGF2α

**Table I**

| Conditions            | Control | 0.01 μM 8-Epi PGF2α | 0.1 μM 8-Epi PGF2α | 1 μM 8-Epi PGF2α | 10 μM 8-Epi PGF2α |
|-----------------------|---------|----------------------|---------------------|-----------------|------------------|
| U46619 (0.4 μM)       | 260 ± 40| 310 ± 30             | 380 ± 25            | 460 ± 30        | 500 ± 40         |
| Arachidonic acid (20 μM) | 180 ± 30| 220 ± 30             | 265 ± 20            | 350 ± 20        | 380 ± 30         |

* Human platelets were loaded with Fura-2/AM (2 μM) as described in the text. Subthreshold concentrations were defined as the highest concentration that evokes less than a 10% increase in light transmission. Ca2+ release was measured as relative fluorescence intensity over the basal at excitation wavelength of 340 nm and 380 nm, followed for 3 min. Data represent the mean ± S.E.M. of three different experiments.

**FIG. 9.** Inositol phosphate formation by human platelets. Dose-dependent stimulation of [3H]inositol phosphate formation by 8-epi PGF2α. Platelets were prelabeled with myo-[3H]inositol (50 μCi/ml), and experiments were carried out in the presence of LiCl (10 mM). The level of total inositol phosphate formation was measured by Dowex-1 anion exchange chromatography as described in the text. Each point represents the mean ± S.E. of three separate experiments.

**FIG. 10.** Inositol phosphate formation by platelets stimulated with 8-epi PGF2α and U46619. Human platelets were prelabeled with myo-[3H]inositol (50 μCi/ml) and stimulated with 8-epi PGF2α (1 μM) or U46619 (1 μM) in the absence (open bars) or in the presence (closed bars) of the thromboxane receptor antagonist, SQ29,548 (1 μM). Each point represents the mean ± S.E. of three separate experiments.

Analogues of both thromboxane A2 (I-BOP) and PGH2 (U46619) which alone fail to aggregate platelets. This phenomenon is accompanied by a dose-dependent increment in platelet thromboxane formation and in the intracellular calcium response to these agonists. It is prevented by pretreatment with either cyclooxygenase inhibitors or thromboxane antagonists, indicating the dependence of aggregation on the secondary formation of thromboxane A2. These findings are in accord with those of Yin et al. (13), but contrast with Morrow et al. (12) who found that 8-epi PGF2α inhibited aggregation induced by arachidonic acid. Many syndromes, putatively associated with oxidant stress, such as vascular reperfusion (17) and alcohol induced liver injury (33), may be associated with platelet activation. The ability of 8-epi PGF2α, to amplify the aggregation response to subthreshold concentrations of platelet agonists may be relevant to settings where platelet activation and enhanced free radical formation coincide.

The effects of pharmacological thromboxane antagonists on the platelet responses to 8-epi PGF2α imply that it exerts these effects via thromboxane receptors. However, this seems highly unlikely. A single gene encoding thromboxane receptors has been identified (34). Like other eicosanoid receptors, it is a member of the heptahelical G protein coupled receptor family (35). We (36) and others (8) have reported that 8-epi PGF2α competes weakly for binding cells transiently transfected with a form of the receptor cloned from human placenta. However, a feature of at least the PGE subfamily of eicosanoid receptors, a form of the receptor cloned from human placenta. However, a feature of at least the PGE subfamily of eicosanoid receptors, a form of the receptor cloned from human placenta. However, a feature of at least the PGE subfamily of eicosanoid receptors, a form of the receptor cloned from human placenta. However, a feature of at least the PGE subfamily of eicosanoid receptors,
tion induced by U46619 or 1-BOP is not desensitized by preincubation of platelets with concentrations of 8-epi PGF$_{2\alpha}$ which induce platelet shape change or support aggregation induced by subthreshold concentrations of conventional platelet agonists.

Similarly, such concentrations of 8-epi PGF$_{2\alpha}$ failed to desensitize the calcium or inositol phospholipid response to platelet stimulation by these agonists. Finally, the effects elicited by 8-epi PGF$_{2\alpha}$ on platelets exhibit considerable structural specificity. Thus, 8-epi PGF$_{2\alpha}$ failed to cause platelet shape change or aggregation or a rise in intracellular calcium or inositol phosphates. It did not cross-desensitize such responses elicited by 8-epi PGF$_{2\alpha}$. All of these findings are consistent with the hypothesis that 8-epi PGF$_{2\alpha}$ may activate a closely related, but distinct receptor from that for thromboxane A$_2$. Our observations also confirm and extend the data of Badr and colleagues (8), who found discrepancies between the relative potencies of the isoprostane and thromboxane/PGH$_2$ analogs in stimulating inositol phosphates and DNA synthesis in rat vascular smooth muscle cells. We have found a similar discrepancy in inositol phosphate stimulation by the two classes of agonist in platelets. 8-Epi PGF$_{2\alpha}$ was the more potent stimulus and was less susceptible to blockade by thromboxane antagonists.

Although the molecular characterization of the receptor activated by 8-epi PGF$_{2\alpha}$ remains to be identified, it bears a marked similarity to a receptor which we have previously characterized pharmacologically on human platelets. Thus, the thromboxane receptor antagonist [1R-(1aZ),2a,3,5ta]-7-(5-[[1,1-biphenyl]-4-yl][methoxy]-3-hydroxy-2-(1-piperidinyl)cyclopentyl]-4-heptenoic acid hydrochloride (GR 32191) (10) identifies two binding sites activated by thromboxane A$_2$/PGH$_2$. One of these, to which it binds irreversibly, mediates a small increase in [Ca$^{2+}$], an increase in inositol phosphates and protein kinase C activation and supports irreversible platelet aggregation, when stimulated by thromboxane A$_2$/PGH$_2$ analogs. The other, to which GR 32191 binds reversibly, mediates the preponderance of the stimulated calcium release from intracellular stores and platelet shape change. It supports irreversible aggregation only in the presence of subthreshold concentrations of other platelet agonists (10). Consistent with the possibility that the latter is a distinct, but related receptor to that which mediates thromboxane A$_2$ dependent platelet aggregation, it is not desensitized by stimulation of the aggregation receptor (41). The biological profile of this receptor is consistent with the receptor activated by 8-epi PGF$_{2\alpha}$ in human platelets.

Given that 8-epi PGF$_{2\alpha}$ may exert biological actions on platelets, the relationship between the concentrations necessary for these effects and those which circulate in vivo is of relevance to its potential function. We measured 8-epi PGF$_{2\alpha}$ in healthy volunteers and in patients with hepatic cirrhosis, who have elevated levels of the compound in plasma and in urine (30). We found a marked discrepancy between the median concentrations in volunteers (48 pmol/L) and the patients (147 pmol/liter) and the EC$_{50}$ for shape change (0.6 ± 0.2 μM), calcium release (3.1 ± 1 μM) and the rise in inositol phosphates (100 nM) induced by 8-epi PGF$_{2\alpha}$. Similarly, relatively high concentrations (100 nM) are required to support aggregation by other agonists and even higher concentrations (50 μM) fail to displace ligand from the thromboxane receptor variants. Given that the highest plasma levels which we recorded in the patients were outside the range of concentration necessary to evoke biological responses in platelets, or indeed, in other cells (8), it is most unlikely that 8-epi PGF$_{2\alpha}$ functions as a conventional, circulating hormone in vivo. This is an important observation, because unlike primary prostaglandins, which undergo rapid metabolism to inactive products, a significant fraction of 8-epi PGF$_{2\alpha}$ is likely to circulate unmetabolized prior to its excretion by the kidney. Thus, increments in 8-epi PGF$_{2\alpha}$ are observed in both plasma and urine in syndromes of exaggerated oxidant stress (16, 17, 30, 33). Unlike primary prostaglandins, which act as autacoids in the immediate microenvironment of their formation, 8-epi PGF$_{2\alpha}$ may theoretically exert biological actions by incidentally activating receptors distal from its site of formation, particularly under conditions of oxidant stress. However, our data indicate that this is highly unlikely. Indeed, even autacoidal function of 8-epi PGF$_{2\alpha}$ may require highly concentrated forms of delivery to local receptors, such as may occur when membrane microvesicles are shed from activated platelets (42).

In summary, 8-epi PGF$_{2\alpha}$ may be formed as an isoprostane or as a minor product of either COX-1 or COX-2. It modulates platelet function in a manner distinct from that caused by analogs of thromboxane A$_2$ or PGH$_2$, despite prevention of its effects by pharmacological thromboxane antagonists. It may amplify the effects of platelet activation coincident with oxidant stress. Enzymatic formation of 8-epi PGF$_{2\alpha}$, but not other F$_2$ isoprostanes, would be consistent with it having a distinct membrane receptor. Failure of 8-epi PGF$_{2\alpha}$ to compete for binding to either recombinant thromboxane receptor isoform or for thromboxane binding sites on platelets would support this notion, as does the absence of cross desensitization of thromboxane receptors at biologically effective concentrations of 8-epi PGF$_{2\alpha}$ and the specificity of its responses relative to its trienoic analog or to PGF$_{2\alpha}$. Despite the circulation of unmetabolized 8-epi PGF$_{2\alpha}$, activation of such a receptor, even incidentally by 8-epi PGF$_{2\alpha}$, formed as an isoprostane, requires concentrations considerably in excess of those which circulate under conditions of oxidant stress.

Acknowledgment—We are grateful for the technical assistance of Dr. Yu Min Shen.

REFERENCES

1. O’Connor, D. E., Mihelic, E. D., and Coleman, M. C. (1984) J. Am. Chem. Soc. 106, 3577–3584
2. Morrow, J. D., Hill, K. E., Burk, R. F., Namr, M. T., Badr, K. F., and Roberts, L. J. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 9383–9387
3. Morrow, J. D., Minton, T., Mukundan, C. R., Campbell, M. D., Zackert, W. E., Daniel, V. C., Badr, K. F., Blair I. A., and Roberts, L. J. (1994) J. Biol. Chem. 269, 4317–4326
4. Pratico, D., Lawson, J. A., and FitzGerald, G. A. (1995) Blood 86, Suppl. 1, 270a
5. Harrison, K. A., and Murphy, R. C. (1995) J. Biol. Chem. 270, 17273–17278
6. Banerjee, M., Kang, K. H., Morrow, J. D., Roberts, L. J., and Newman, J. H. (1992) Am. J. Physiol. 263, H660–H663
7. Takahashi, K., Namr, M. T., Fukunaga, M., Ebert, J., Morrow, J. D., Roberts, L. J., Hoover, R. L., and Badr, K. F. (1992) J. Clin. Invest. 90, 136–141
8. Fukunaga, M., Makita, N., Roberts, L. J., Morrow, J. D., Takahashi, K., and Badr, K. F. (1993) Am. J. Physiol. 264, C1639–C1624
9. Mais, D. E., Saussy, D. L., Chakroun, A., Kode, P. J., Knapp, D. R., Hamaanika, N., and Haluska, P. V. (1985) J. Pharmacol. Exp. Ther. 233, 418–424
10. Takahara, K., Murray, R., FitzGerald, G. A., and FitzGerald, D. J. (1990) J. Biol. Chem. 265, 6836–6844
11. Mayeux, P. R., Morton, H. E., Lord, A., Morinelli, T. A., Boehm, A., Mais, D. E., and Haluska, P. V. (1988) Biochem. Biophys. Res. Commun. 157, 733–739
12. Morrow, J. D., Minton, T., and Roberts, L. J. (1992) Prostaglandins 44, 155–163
13. Yin, K., Haluska, P. V., Yan, Y. T., and Wong, P. Y. K. (1994) J. Pharmacol. Exp. Ther. 270, 1192–1196
14. Longmore, A. W., Roberts, L. J., and Morrow, J. D. (1994) Prostaglandins 48, 247–256
15. Delanty, N., Reilly, M., Pratico, D., Lawson, J. A., and FitzGerald, G. A. (1996) J. Clin. Pharmacol. in press
16. Reilly, M., Delanty, N., Lawson, J. A., and FitzGerald, G. A. (1996) Circulation, in press
17. Delanty, N., Reilly, M., Lawson, J. A., McCarthy, J., FitzGerald, D. J., and FitzGerald, G. A. (1996) Circulation, in press
18. Pratico, D., Lawson, J. A., and FitzGerald, G. A. (1995) J. Biol. Chem. 270, 9800–9808
19. Pratico, D., and FitzGerald, G. A. (1996) J. Biol. Chem. 271, 8919–8924
20. Wang, Z., Ciabattoni, G., Creminon, C., Lawson, J. A., FitzGerald, G. A., Patrone, C., and Madourd, J. (1995) J. Pharmacol. Exp. Ther. 275, 94–100
21. Raykowadzai, M. K., Yukawa, M., Collins, L. J., McGail, S. H., Kent, K. C., and Ware, J. A. (1994) J. Biol. Chem. 269, 19256–19261
22. Praticò, D., Iuliano, L., Pulcinelli, F. M., Gazzaniga, P. P., Bonavita, M. S., and Violi, F. (1992) J. Lab. Clin. Med. 119, 364–370
23. Reilly, I., and FitzGerald, G. A. (1987) Blood 69, 180–186
24. Coller, B. S. (1985) J. Clin. Invest. 76, 101–108
25. Hirata, M., Hayashi, Y., Ushikubi, F., Yokota, Y., Kageyama, R., Nakanishi, S., and Narumiya, S. (1991) Nature 349, 617–620
26. Dorn, G. W., II (1990) J. Biol. Chem. 265, 4240–4246
27. Ogletree, M. L., Harris, D. V., Greenberg, R., Hasiander, M. F., and Nakane M. (1985) J. Pharmacol. Exp. Ther. 234, 435–441
28. Watson, S. P., Ruggiero, M., Abrahams, S. L., and Lapetina, E. G. (1986) J. Biol. Chem. 261, 5368–5372
29. Lapetina, E. G., and Siess, W. (1987) Methods Enzymol. 141, 176–192
30. Praticò, D., Basili, S., Cordova, C., Iuliano, L., Ferro, D., Camasta, C., FitzGerald, G. A., and Violi, F. (1996) J. Invest. Med. 44, 301A
31. Laneuville, O., Breuer, D. K., DeWitt, D. L., Hla, T., Funk, C. D., and Smith, W. L. (1994) J. Pharmacol. Exp. Ther. 271, 927–934
32. Bhagwat, S. S., Hamann, P. R., Still, W. C., Bunting, S., and Fitzpatrick, F. A. (1985) Nature 315, 511–513
33. Meagher, E. A., Lucey, M., Bensinger, S., and FitzGerald, G. A. (1996) J. Invest. Med. 44, 300A
34. Nusing K. M., Hirata, M., Kakizuka, A., Eki, T., Ozawa, K., and Narumiya S. (1993) J. Biol. Chem. 268, 25253–25259
35. Lefkowitz R. J. (1991) Nature 351, 253–254
36. Kinsella, T. B., O’Mahony, D. J., Lawson, J. A., Praticò, D., and FitzGerald, G. A. (1994) Ann. N. Y. Acad. Sci. 714, 270–278
37. Negishi, M., Sugimoto, Y., Irie, A., Narumiya, S., and Ichikawa A. (1993) J. Biol. Chem. 268, 9517–9521
38. Namba, T., Sugimoto, Y., Nagishi, M., Irie, A., Ushikubi, F., Kakizuka, A., Ito, S., Khikawa, A., and Narumiya S. (1993) Nature 365, 166–170
39. Hirata, T., Kakizuka, A., Ushikubi, F., Narumiya, S., and Okuma, M. (1995) Thromb. Haemostasis 73, 60A
40. Habib, A., Shen, Y. M., Creminon, C., Madouf, J., and FitzGerald, G. A. (1996) J. Invest. Med. 44, 263A
41. Foroud, L., Fitzgerald, D. J., and FitzGerald, G. A. (1991) J. Pharmacol. Exp. Ther. 250, 74–81
42. Fourcade, O., Simon, M. F., Vióè, C., Rugani, N., Leballe, F., Ragab, A., Fournié, B., Sarda, A., and Chap, H. (1995) Cell 80, 919–927
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J. Biol. Chem. 1996, 271:14916-14924.
doi: 10.1074/jbc.271.25.14916

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