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 μM induce a dose-dependent increase in platelet shape change, in calcium release from intracellular stores $[Ca^{2+}]_i$ 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 μM) 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 $PGF_{2\alpha}$. 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)[1] 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, effects also prevented by thromboxane receptor antagonists (10, 11). However, while 8-epi $PGF_{2\alpha}$ induces platelet shape change, it has variously been described to evoke weak, reversible 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 PGE_2 (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.

[1] The abbreviations used are: PG, prostaglandin; COX, cyclooxygenase enzyme; PRP, platelet-rich plasma; WP, washed platelets; TPα, placental thromboxane receptor; TPβ, endothelial thromboxane receptor; HEK cells, human embryonic kidney cells; Tx, thromboxane; HBSS, Hanks' balanced salt solution.

EXPERIMENTAL PROCEDURES

Materials

5-Heptenoic acid, 7-[3-[3-hydroxy-4-(4-idophenoxy)-1-butenyl]-7-oxabicyclo[2.2.1]hept-2-yl]-[1*S*-[1α,2α(*Z*0,3(1*E*,3*S*),4α]], I-BOP; 5-heptenoic acid, 7-[6-(3-hydroxyl-1-octenyl)-2-oxabicyclo[2.2.1]hept-5-yl]-, [1*R*-[1α,4α,5β(*Z*),6α(1*E*,3*S*)]], U46619; 8-epi PGF$_{2\alpha}$; PGF$_{2\alpha}$; and SQ29,548 were purchased from Cayman Chem. Co. (Ann Arbor, MI). 8-Epi PGF$_{3\alpha}$ was a generous gift from Dr. Kirk Maxey of Cayman Chem. Co. Indomethacin, aspirin, ADP, arachidonic acid, saponin, EGTA, EDTA, and bovine serum albumin were purchased from Sigma. Pertussis toxin and Fura-2/AM were obtained from Calbiochem. Dowex 1-X8 AG anion exchange resin (formate form) was from Bio-Rad. Collagen was purchased from Biodata Corp. (Horsham, PA). *myo*[2-^{3}H]Inositol was purchased from Amersham Corp. ^{3}H-SQ29,548 and ^{125}I-BOP were purchased from DuPont NEN. Fetal calf serum, Dulbecco's modified Eagle's medium, Dulbecco's phosphate-buffered saline, Hanks' balanced salt solution (HBSS), and HEPES were from Life Technologies, Inc. 7E3 was a generous gift from Dr B. Coller, Mt. Sinai Hospital, New York.

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).

Desensitization Assay—Blood anticoagulated with 5 mM EDTA and containing indomethacin (5 μg/ml) was centrifuged at 160 × *g* for 10 min. The PRP was removed. The platelets were desensitized by incubation for 30 min with U46619 (1 μM), I-BOP (100 nM), or 8-epi PGF$_{2\alpha}$ (1 μM). The presence of EDTA prevents activation during the incubation with the desensitizing agonist. The desensitized platelets were then washed twice by centrifugation and resuspended in HBSS buffer.

TxB$_2$ Measurement—Platelet TxA$_2$ production was assessed, 3 min after the stimulus, as its hydrolysis product TxB$_2$, using a gas chromatography/mass spectrometry assay, as described previously (23).

Platelet Shape Change—Platelet shape change was determined in 500-μl aliquots of PRP by light transmission. Platelet aggregation was prevented by prior addition of 7E3 10 μg/ml, a Fab(2′) fragment of an antibody to the platelet glycoprotein IIb/IIIa (24). Agonists and antagonists were added in volume of 5% or less to avoid dilution artifacts. The signal was amplified and recorded as the maximum reduction in light transmission within 2 min of adding the agonist, as described previously (10).

Thromboxane Receptors Studies

Cell Culture and Transfections—Human embryonic kidney (HEK) 293 cells, obtained from the American Type Culture Collection, were routinely grown in Dulbecco's modified Eagle's medium, containing 10% heat-inactivated fetal calf serum. Cells were plated in 100-mm culture dishes 24 h prior to transfection at a density of 1.5 × 10^6 cell/dish. The cDNAs encoding the placental (TPα) (25) or endothelial (TPβ) human thromboxane receptor isoforms were subcloned into the *Eco*RI-*Eco*RI or the *Eco*RI-*Xba*I site, respectively, of the G418-resistant mammalian expression vector pcDNAIII (Invitrogen, San Diego, CA). Cells were transfected by cationic liposome mediated transfer (DOTAP, Boehringer Mannheim) with 10 μg of DNA/dish, according to the manufacturer's instructions. Approximately 6 h after transfection, the medium was replaced with fresh Dulbecco's modified Eagle's medium containing G418 (1 mg/ml). Cell lines were grown from single colonies which were apparent after approximately 10 days in culture. Receptor expression was verified in several cells lines by Northern analysis using the re-spective cDNAs as probes. The TPβ cDNA was a generous gift of Dr. Anthony Ware (21).

Radioligand Binding Studies of Stable Transfectants—HEK cells stably expressing the TPα or TPβ cDNAs were washed with Dulbecco's phosphate-buffered saline (without calcium and magnesium), harvested in HED buffer (20 mM HEPES, pH 7.6, 0.5 mM dithiothreitol, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μM indomethacin), and lysed by sonication. Membranes were prepared by centrifugation (130,000 × *g*) for 1 h at 4 °C. The pellets were resuspended in HEDG buffer (20 mM HEPES, pH 7.6, 0.5 mM dithiothreitol, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μM indomethacin, 10% glycerol, 100 mM NaCl) and were stored at −80 °C. Receptors (25–50 μg of membrane protein/reaction) were radiolabeled with 0.5 nM ^{125}I-BOP (~350 cpm/fmol; ~16,000 cpm/tube) for 30 min at 30 °C in the presence or absence of increasing concentrations of competing ligand (26). Reactions were stopped by the addition of 4 ml of ice-cold wash buffer (10 mM Tris, pH 7.4, containing 0.1% BSA) and immediate filtration through G/FC filters (Whatman International Ltd., Maidstone, UK) which had been soaked in ice-cold wash buffer and radioactivity associated with the filters was counted using a scintillation counter (Beckman Instruments).

Platelet Binding Studies—Blood was anticoagulated with EDTA 5 mM and indomethacin (5 μg/ml). PRP was prepared as described above. The platelets were resuspended in Tris buffer, pH 7.4 (50 mM Tris-HCl, 150 mM NaCl, 5 mM dextrose). ^{3}H-SQ29,548 (0.5 nM) (27) was incubated with platelets in the presence of varying concentrations of unlabeled I-BOP, U46619, 8-epi PGF$_{2\alpha}$, and PGF$_{2\alpha}$ to assess competitive displacement of the agonist ligand. Platelets were incubated with excess of unlabeled SQ29,548 (10 μM) to determine the nonspecific binding. Incubations were performed as described above. Washed platelets were resuspended in hypotonic buffer (Tris-HCl 20 mM, EDTA 5 mM, indomethacin 1 mg/ml, pH 7.4) and centrifuged at 1000 × *g* for 10 min at 4 °C to prepare platelet membranes. They were resuspended again in hypotonic buffer and allowed to stand for 20 min on ice. Following sonication, they were centrifuged at 130,000 × *g* for 30 min at 4 °C and resuspended in buffer (Tris-HCl 20 mM, MgCl$_2$ 10 mM, EDTA 5 mM) pH 7.4. The protein concentration was determined by using a Bio-Rad DC protein assay system (Bio-Rad). One hundred micrograms of the membrane protein were then incubated with various concentrations of I-BOP, U46619, 8-epi PGF$_{2\alpha}$, PGF$_{2\alpha}$, and SQ29,548 as described above.

Platelet Biochemistry Studies

Pertussis Toxin Studies—To address the possibility that 8-epi PGF$_{2\alpha}$ 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 in order to remove the extracellular Fura-2, and resuspended in HBSS, pH 7.4. Fluorescence measurement was carried out at 37 °C in a Perkin-Elmer LS 50-B spectrofluorimeter equipped with magnetic stirrer (Perkin-Elmer, Buckinghamshire, UK). The fluorescence signal was monitored at 510 nm with the excitation wavelength of 340 and 380 nm. The ratios of maximum and minimum fluorescence were determined by the addition of 250 μM digitonin in the presence of 1 mM CaCl$_2$ and in the presence of 10 mM EGTA (pH >8.5), respectively, as described previously (22).

Inositol Phosphate Formation—Platelets were labeled with *myo*-[^{3}H]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-^{3}H]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 Dowex 1-X8 AG anion exchange resin (formate form). Following elution of [^{3}H]inositol and [^{3}H]glycerophosphorylinositol with 60 mM ammonium formate containing 5 mM disodium tetraborate, total inositol phosphates were eluted with 12 ml of 1 M ammonium formate containing 100 mM formic acid. An aliquot (2 ml) of the elution was taken, 8 ml of scintillation liquid were added, and the radioactivity was determined by using a scintillation counter (Beckman Instruments).

Plasma 8-Epi PGF$_{2\alpha}$—Plasma levels of 8-epi PGF$_{2\alpha}$ were assayed as described previously (18) using a stable isotope dilution assay by gas chromatography/mass spectrometry. Blood anticoagulated with EDTA and immediately centrifuged to separate the plasma, was obtained from
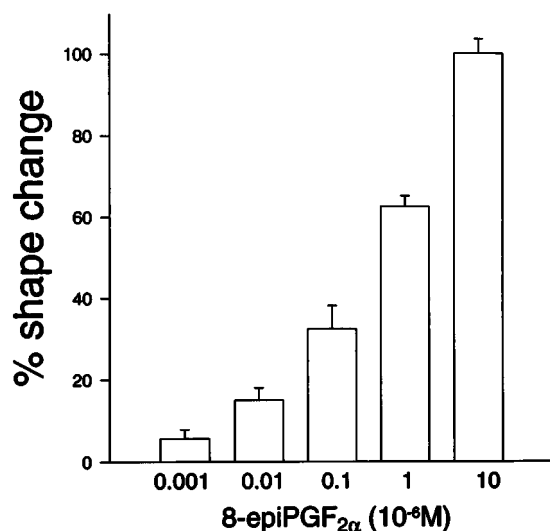
FIG. 1. **Dose-dependent increase in platelet shape change induced by 8-epi PGF$_{2\alpha}$.** The platelet shape change was studied in PRP in the presence of 7E3 (10 μg/ml) to prevent platelet aggregation. The signal was amplified and recorded as the maximum reduction in light transmission within 2 min of adding the agonist. Each point represents the mean $\pm$ S.E. of four different experiments.

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 $\pm$ 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-epi PGF$_{2\alpha}$ on platelet shape change at various concentrations ranging from 1 nM to 10 μM. 8-Epi PGF$_{2\alpha}$ induced a dose-dependent platelet shape change with an EC$_{50}$ of 0.6 $\pm$ 0.2 μM (Fig. 1). SQ29,548 (1 μM) completely inhibited the shape change induced by 8-epi PGF$_{2\alpha}$, in contrast to aspirin (100 μM) or indomethacin (10 μM), which did not modify the response. Pretreatment with pertussis toxin (15 μg/ml) 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$_{3\alpha}$, did not evoke platelet shape change at concentrations up to 50 μM (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 μM. Weak, reversible aggregation was evoked by concentrations in excess of 20 μM (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 μM), thrombin (1 unit/ml), U46619 (1 μM), collagen (2 μg/ml), or arachidonic acid (100 μM) was observed when 8-epi PGF$_{2\alpha}$ was coincubated with these ligands. However, the lag phase of the response to collagen was shortened (Fig. 3B). We noted that, when 8-epi PGF$_{2\alpha}$ was 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 I).
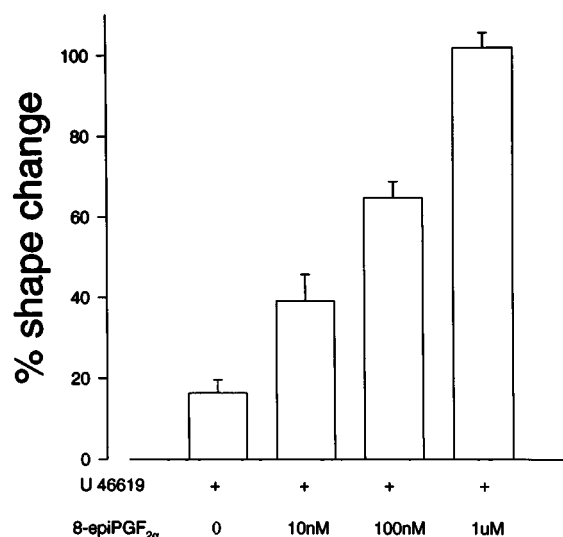
To address further the possibility that 8-epi PGF$_{2\alpha}$ might

FIG. 2. **Platelet shape change induced by U46619 and 8-epi PGF$_{2\alpha}$.** Human platelets pretreated with 7E3 (10 μg/ml) to prevent aggregation were stimulated with U46619 (0.4 μM) alone or in combination with increasing concentrations of 8-epi PGF$_{2\alpha}$. Each point represents the mean $\pm$ S.E. of four different experiments.

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$_{50}$ values for ADP, arachidonic acid, U46619, and collagen were, under these conditions, 0.8, 0.2, 0.6, and 0.5 μM, 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 ^{125}I-BOP from the TPα and TPβ isoforms which were stably expressed in HEK 293 cells. 8-Epi PGF$_{2\alpha}$, in contrast, failed to displace the agonist ligand from either isoform (Fig. 4).

I-BOP and U46619 both competed with SQ29,548 on WP membranes with an IC$_{50}$ values of 10 nM ($n = 4$) and 100 nM ($n = 4$), respectively. 8-Epi PGF$_{2\alpha}$, by contrast, was much (IC$_{50}$ = 100 μM) less effective than I-BOP or U46619 at displacing ^{3}H-SQ29,548 from its binding sites. PGF$_{2\alpha}$ was an even less effective competitor for the binding sites identified by I-BOP than was 8-epi PGF$_{2\alpha}$ (Fig. 5A). Similar results were obtained in platelet membranes. I-BOP and U46619 both exhibited high affinity for the binding site identified by SQ29,548 with IC$_{50}$ values of 10 nM ($n = 4$) and 100 nM ($n = 4$) respectively. Competition binding studies with 8-epi PGF$_{2\alpha}$ at concentrations up to 100 μM failed to displace ^{3}H-SQ29,548 completely (Fig. 5B).

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 nM to 1 μM) 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 μM) 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

FIG. 3. **Platelet aggregation induced by 8-epi PGF$_{2\alpha}$ alone or in combination with platelet agonists.** *A*, representative traces of the effect of 8-epi PGF$_{2\alpha}$ alone (10 μM) or in combination with a threshold concentration of ADP (2 μM) on platelet aggregation. *B*, when a threshold concentration of collagen (2 μg/ml) was combined with 8-epi PGF$_{2\alpha}$, a reduction in the lag phase was seen, along with an increase in TxB$_2$ (see Table I). 8-Epi PGF$_{2\alpha}$ was always added 15 s after ADP or collagen. The aggregation trace was followed for 3 min and expressed as percentage of the change of light transmission as described in the text. The data shown are representative of four experiments.
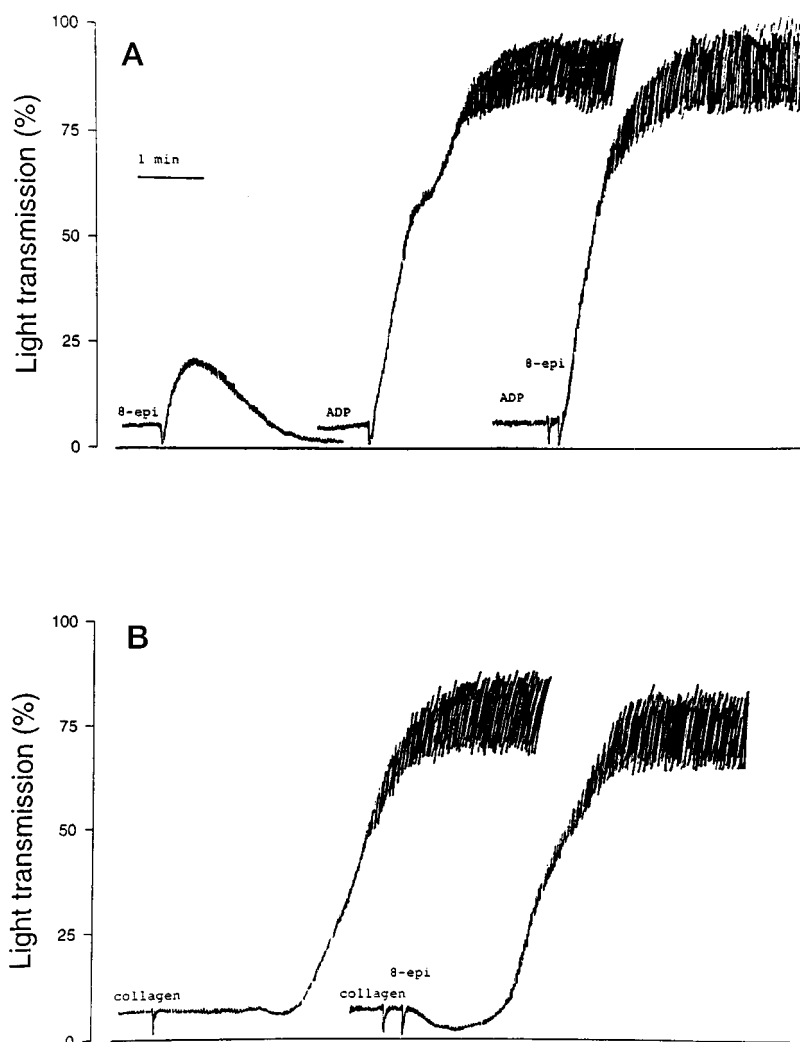
TABLE I
Platelet aggregation and TxB$_2$ production by human platelets stimulated with threshold concentrations of agonists with without 8-epi PGF$_{2\alpha}$

Conditions[a]	Light transmission	TxB$_2$
	%	ng/ml
8-Epi PGF$_{2\alpha}$	3 ± 1	ND[b]
Arachidonic acid	70 ± 8	280 ± 30
Arachidonic acid + 8-epi PGF$_{2\alpha}$	78 ± 10 (NS)[c]	340 ± 25[d]
Collagen	72 ± 6	200 ± 20
Collagen + 8-epi PGF$_{2\alpha}$	80 ± 5 (NS)	265 ± 30[d]

[a] 8-Epi PGF$_{2\alpha}$ (1 μM) was always added 15 s after arachidonic acid (100 μM) or collagen (2 μg/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 ± S.E. of four experiments.
[b] ND, not detectable.
[c] NS, not significant.
[d] $p < 0.005$.

or I-BOP (Fig. 6*B*). Each of these two ligands induced homologous desensitization of their own shape change and aggregation 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 ± 1.0 μM (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 relatively nonspecific (COX-1 *versus* COX-2) (31) COX inhibitors, indomethacin (10 μM), or aspirin (100 μM). The thromboxane receptor antagonist SQ29,548 (1 μM) 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 calcium 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 concentrations 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 calcium movement at concentrations up to 50 μM. It also failed to

Amplification of Platelet Function by 8-Epi PGF$_{2\alpha}$

TABLE II

Effect of increasing concentrations of 8-epi PGF$_{2\alpha}$ on platelet aggregation induced by subthreshold concentration of platelet agonists

Conditions[a]	Light transmission				
	Control	0.01 μM 8-Epi PGF$_{2\alpha}$,	0.1 μM 8-Epi PGF$_{2\alpha}$,	1 μM 8-Epi PGF$_{2\alpha}$,	10 μM 8-Epi PGF$_{2\alpha}$,
			%		
Arachidonic acid (10 μM)	7 ± 3	25 ± 6	45 ± 8	68 ± 5	75 ± 5
Collagen (0.4 μg/ml)	3 ± 2	15 ± 8	27 ± 5	70 ± 6	75 ± 4
ADP (0.5 μM)	6 ± 2	21 ± 6	38 ± 8	65 ± 7	70 ± 5
U46619 (0.4 μM)	4 ± 2	21 ± 6	35 ± 5	60 ± 7	70 ± 5
I-BOP (50 nM)	4 ± 3	18 ± 6	30 ± 6	65 ± 5	72 ± 7

[a] 8-Epi PGF$_{2\alpha}$ was always added 15 s after the agonists. The aggregation curves were followed for 3 min afterward. The data represent the mean ± S.E. of four different experiments and are expressed as percentage of change of light transmission as described in the text. Subthreshold concentration of agonist was defined as the highest concentration causing less than a 10% increase in light transmission.
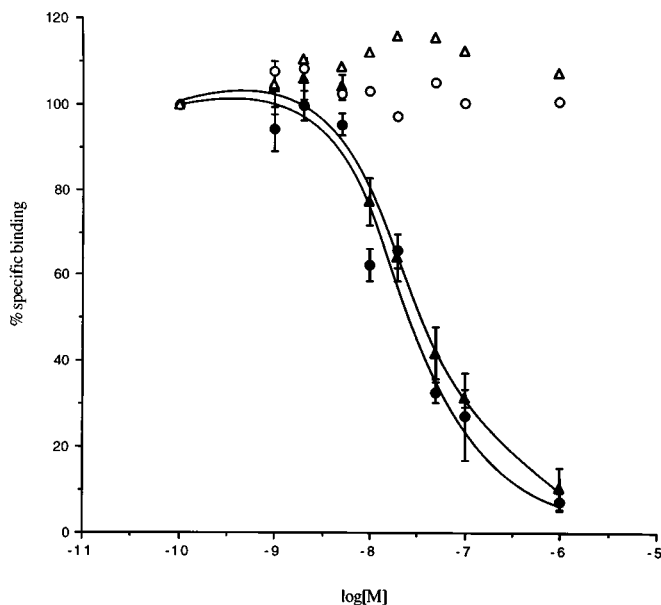

FIG. 4. **Displacement analysis of binding to thromboxane receptor membrane isoforms.** Membranes were prepared from HEK 293 cells stably expressing the endothelial (TPβ) (*triangles*) or the placental (TPα) (*circles*) isoforms of the human thromboxane receptor. Receptors were radiolabeled with ^{125}I-BOP in the presence of increasing concentrations of I-BOP (*closed symbols*) or 8-epi PGF$_{2\alpha}$ (*open symbols*), as indicated. Each point is the mean ± S.E. of four separate experiments.

FIG. 5. **Displacement analysis of SQ29,548 binding to human platelets.** Competition binding curves demonstrating the relative abilities of U46619 (▲) (*n* = 4), I-BOP (●) (*n* = 4), 8-epi PGF$_{2\alpha}$ (○) (*n* = 4), PGF$_{2\alpha}$ (△) (*n* = 4) to displace ^{3}H-SQ25,948 in washed platelets (*A*) and platelet membranes (*B*). The results are expressed as percentage of ^{3}H-SQ25,948 binding per mg of cell protein after the indicated concentrations of the various ligands. Each point is the mean ± S.E.

desensitize the intracellular calcium response to 8-epi PGF$_{2\alpha}$ (data not shown). Similarly, PGF$_{2\alpha}$ failed to evoke a calcium response when used at concentrations up to 50 μM. It also failed to desensitize the response to 8-epi PGF$_{2\alpha}$ (data not shown).

8-Epi PGF$_{2\alpha}$ and Platelet Inositol Phosphates—The addition of 8-epi PGF$_{2\alpha}$ 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 PGF$_{2\alpha}$ at 1 μM was significantly greater than that evoked by either I-BOP or U46619 (Fig. 10). Furthermore, stimulation by 8-epi PGF$_{2\alpha}$ 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 PGF$_{2\alpha}$ induced increase of platelet inositol phosphates was not inhibited by pretreatment with pertussis toxin (data not shown).

Plasma 8-Epi PGF$_{2\alpha}$—The levels of 8-epi PGF$_{2\alpha}$ 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 ($p < 0.001$).
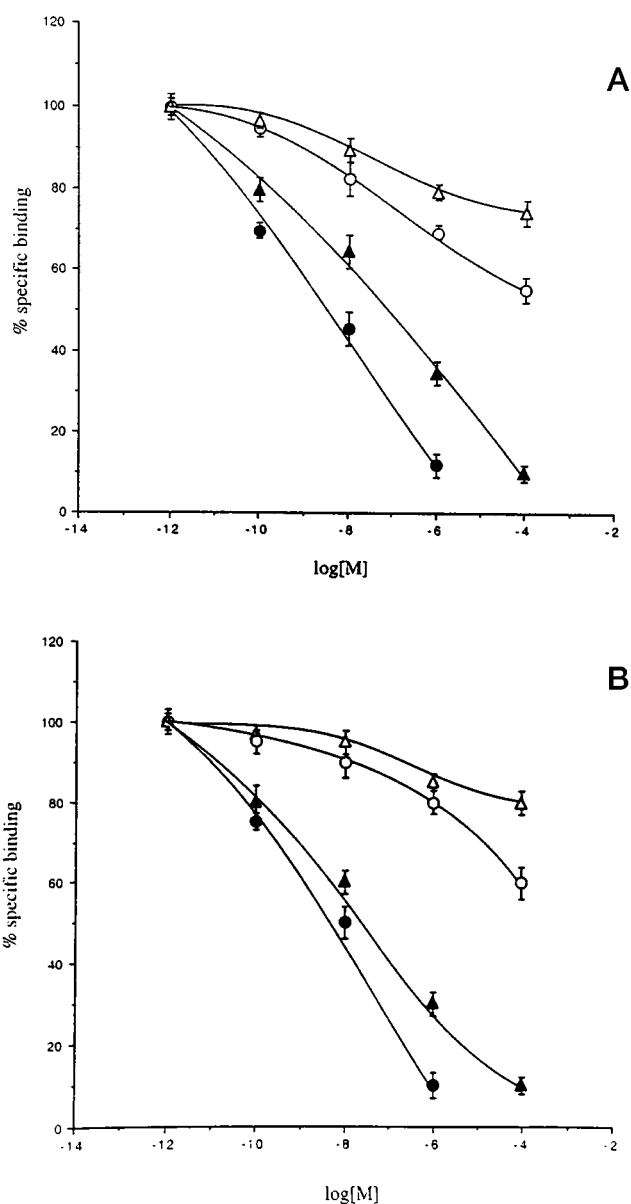
DISCUSSION

8-Epi PGF$_{2\alpha}$ activates human platelets. It induces platelet shape change coincident with an increase in inositol phos-
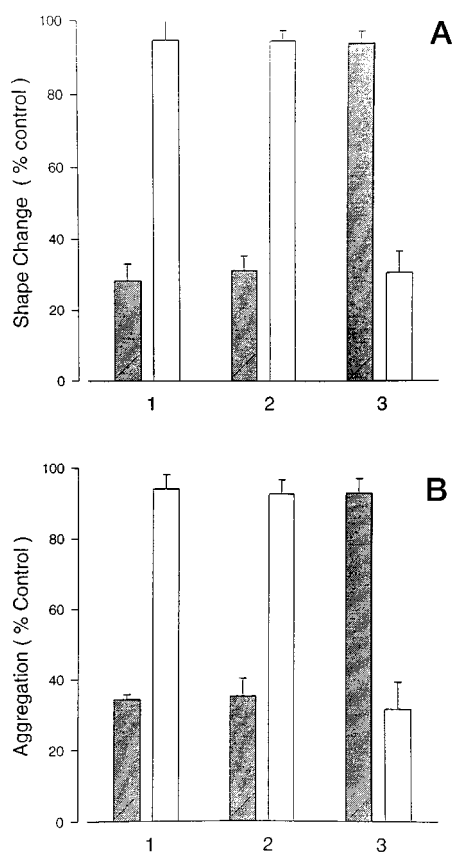
FIG. 6. **Desensitization of the platelet shape change (*A*) and aggregation (*B*) responses.** Platelets were desensitized with U46619 (*solid bars*) or 8-epi PGF$_{2\alpha}$ (*open bars*) and subsequently stimulated with (*1*) U46619 (1 μM), (*2*) I-BOP (100 nM), (*3*) 8-epi PGF$_{2\alpha}$ (1 μM). Results are expressed as percentage stimulation in control platelets and represent the mean $\pm$ S.E. of 5 (*A*) and 6 (*B*) experiments.
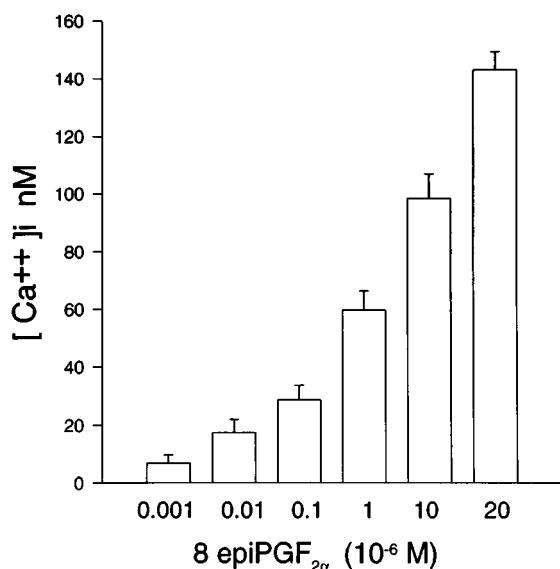

FIG. 7. **The effects of 8-epi PGF$_{2\alpha}$ on movement of Ca^{2+} in human platelets.** Fura-2 loaded human platelets were stimulated with increasing concentrations of 8-epi PGF$_{2\alpha}$. Ca^{2+} release was measured as relative fluorescence intensity over the basal at excitation wavelengths of 340 and 380 nm as described in the text. The data shown are the mean $\pm$ S.E. of five experiments.

FIG. 8. **Platelet response to 8-epi PGF$_{2\alpha}$ and U466619.** Human platelets were loaded with Fura-2/AM and the change in cytosolic free calcium concentration was measured. Platelets were stimulated with 8-epi PGF$_{2\alpha}$ (1 μM) (*A*). Human platelets were also prestimulated with U46619 (0.5 μM) and, after 120 s, with 8-epi PGF$_{2\alpha}$ (1 μM) (*B*). The data shown are representative of three experiments.

phates and calcium release from intacellular stores. These effects are prevented by pretreatment with thromboxane receptor antagonists, but not by cyclooxygenase inhibitors. Unlike
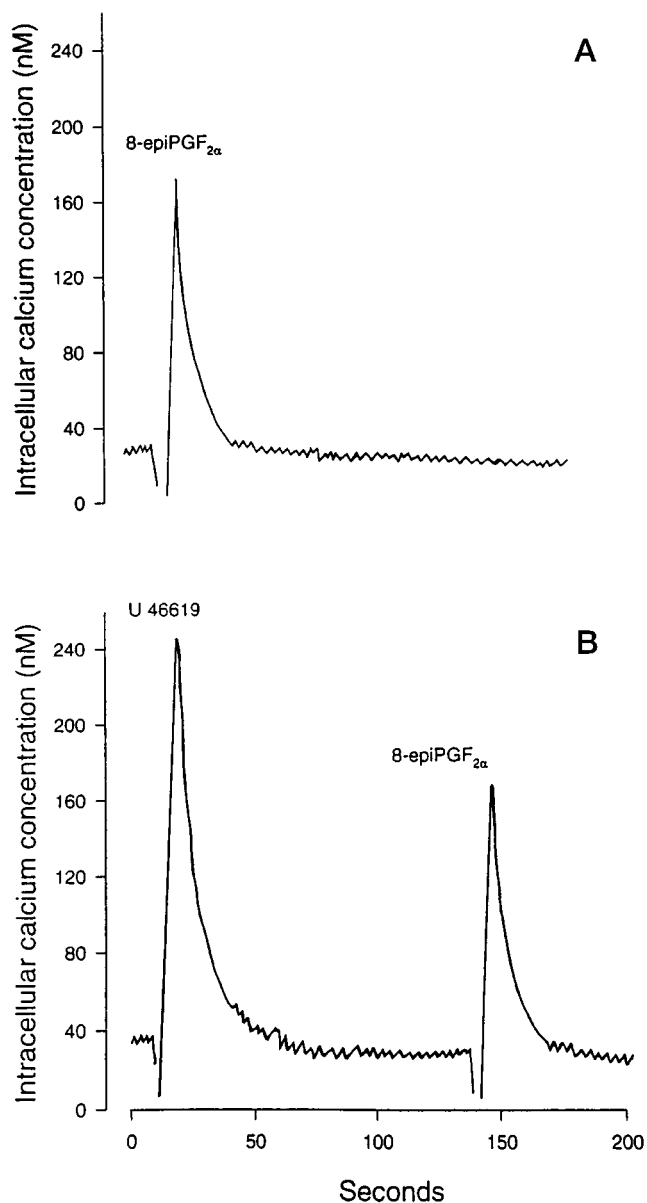
the stable carbacyclin derivative of thromboxane A$_2$ (32), or structural analogs of either thromboxane A$_2$ or its prostaglandin endoperoxide precursor, PGH$_2$ (11), we have found that there is a clear distinction between the concentrations of 8-epi PGF$_{2\alpha}$ alone which induce shape change 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 μM *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 μM cause dose-dependent, irreversible platelet aggregation in the presence of concentrations of collagen, ADP, arachidonic acid, and

TABLE III

Effects of increasing concentrations of 8-epi PGF$_{2\alpha}$ on platelet intracellular calcium movement induced by subthreshold concentration of agonists

Conditions[a]	Intracellular calcium movement				
	Control	0.01 μM 8-Epi PGF$_{2\alpha}$	0.1 μM 8-Epi PGF$_{2\alpha}$	1 μM 8-Epi PGF$_{2\alpha}$	10 μM 8-Epi PGF$_{2\alpha}$
			nM		
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

[a] Human platelets were loaded with Fura-2/AM (2 μM) as described in the text. 8-Epi PGF$_{2\alpha}$ was always added 15 s after the agonists. Subthreshold concentrations are defined as the highest concentration that evokes less than a 10% increase in light transmission. Ca^{++} 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.
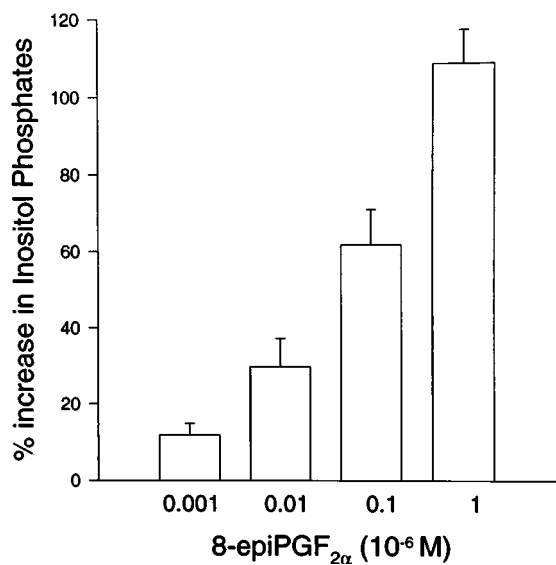

FIG. 9. **Inositol phosphate formation by human platelets.** Dose-dependent stimulation of [^{3}H]inositol phosphate formation by 8-epi PGF$_{2\alpha}$. Platelets were prelabeled with *myo*-[^{3}H]inositol (50 μCi/ml), and experiments were carried out in the presence of Li^{2+} (10 mM). The level of 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 PGF$_{2\alpha}$ and U46619.** Human platelets were prelabeled with *myo*-[^{3}H]inositol (50 μCi/ml) and stimulated with 8-epi PGF$_{2\alpha}$ (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.

analogs of both thromboxane A$_2$ (I-BOP) and PGH$_2$ (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 A$_2$. These findings are in accord with those of Yin *et al.* (13), but contrast with Morrow *et al.* (12) who found that 8-epi PGF$_{2\alpha}$ 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 PGF$_{2\alpha}$ 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 PGF$_{2\alpha}$ 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 hepatahelical G protein coupled receptor family (35). We (36) and others (8) have reported that 8-epi PGF$_{2\alpha}$ 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,
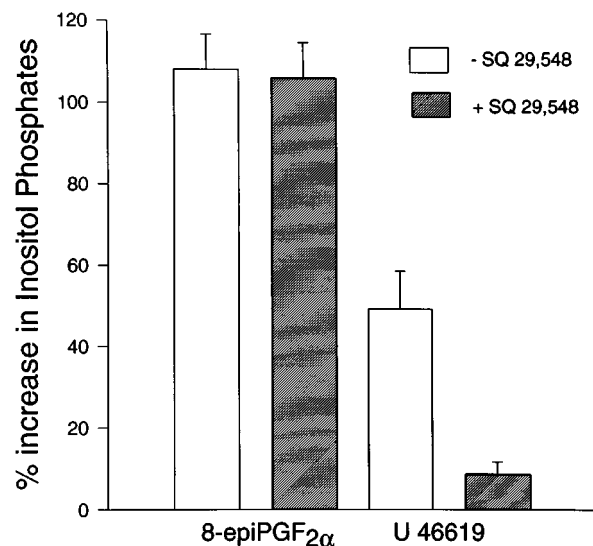
has been the existence of splice variation involving the carboxyl-terminal tail (37). Such E prostaglandin receptor variants have been shown to couple differentially to downstream signals in expression systems and to differ in their rates of desensitization (38). Recently, a splice variant of the thromboxane receptor has been cloned from human endothelial cells (TPβ), which has a longer carboxyl-terminal extension than the placental variant (TPα). mRNA for both variants has been detected in human platelets (39). We have stably expressed both variants in HEK cells and confirmed high levels of expression of mRNA and of binding sites, detected by the specific antagonist SQ29,548 (27). We have also confirmed receptor expression, using antibodies which discriminate between the two isoforms (40). Whereas I-BOP displaced the antagonist from both isoforms in a potent (IC$_{50}$ 10 nM) and dose dependent manner, concentrations of 8-epi PGF$_{2\alpha}$ up to 1 μM caused no displacement. Three additional observations were consistent with this finding. Thus, 8-epi PGF$_{2\alpha}$ was similarly unimpressive in displacing the antagonist from washed human platelets or platelet membranes, requiring concentrations (IC$_{50}$ 50–100 μM) similar to that for PGF$_{2\alpha}$. Parenthetically, 8-epi PGF$_{2\alpha}$ does not activate the recombinant PGF$_{2\alpha}$ receptor in a *Xenopus* expression system[2] and we have found that PGF$_{2\alpha}$, at concentrations up to 50 μM, fails to stimulate platelet [Ca^{2+}]$_i$ release or cross desensitize the 8-epi PGF$_{2\alpha}$ induced platelet [Ca^{2+}]$_i$ response in human platelets.

Second, thromboxane receptor dependent platelet aggrega-

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[2] A. Ford-Hutchinson, personal communication.

tion induced by U46619 or I-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 phosphate 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$_{3\alpha}$ 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-[1α(Z),2β,3β,5α]]-(+)-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 rise in [Ca^{2+}]$_i$, 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$_{50}$s 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 extrarenal 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.

REFERENCES

1. O'Connor, D. E., Mihelich, E. D., and Coleman, M. C. (1984) *J. Am. Chem. Soc.* **106,** 3577–3584
2. Morrow, J. D., Hill, K. E., Burk, R. F., Nammour, T. M., 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., II (1994) *J. Biol. Chem.* **269,** 4317–4326
4. Praticò, D., Lawson, J. A., and FitzGerald, G. A. (1995) *Blood* **86,** Suppl. 1, 509a
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., Nammour, T. M., 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,** C1619–C1624
9. Mais, D. E., Saussy, D. L., Chaikhoun, A., Kochel, P. J., Knapp, D. R., Hamanaka, N., and Halushka, 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 Halushka, P. V. (1988) *Biochem. Biophys. Res. Commun.* **157,** 733–739
12. Morrow, J. D., Minton, T. A., and Roberts, L. J. (1992) *Prostaglandins* **44,** 155–163
13. Yin, K., Halushka, P. V., Yan, Y. T., and Wong, P. Y. K. (1994) *J. Pharmacol. Exp. Ther.* **270,** 1192–1196
14. Longmire, A. W., Roberts, L. J., and Morrow, J. D. (1994) *Prostaglandins* **48,** 247–256
15. Delanty, N., Reilly, M., Praticò, D., Lawson, J. A., and FitzGerald, G. A. (1996) *Br. 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. Praticò, D., Lawson, J. A., and FitzGerald, G. A. (1995) *J. Biol. Chem.* **270,** 9800–9808
19. Praticò, 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., Patrono, C., and Maclouf, J. (1995) *J. Pharmacol. Exp. Ther.* **275,** 94–100
21. Raychowdhury, M. K., Yukawa, M., Collins, L. J., McGrail, 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., Haslanger, 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., Camastra, 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. In-*
vest. 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,** 353–354
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., Maclouf, J., and FitzGerald, G. A. (1996) *J. Invest. Med.* **44,** 263A
41. Furci, L., Fitzgerald, D. J., and FitzGerald, G. A. (1991) *J. Pharmacol. Exp. Ther.* **258,** 74–81
42. Fourcade, O., Simon, M. F., Viodé, C., Rugani, N., Leballe, F., Ragab, A., Fournié, B., Sarda, A., and Chap, H. (1995) *Cell* **80,** 919–927

Local Amplification of Platelet Function by 8-Epi Prostaglandin F$_{2\alpha}$ Is Not Mediated by Thromboxane Receptor Isoforms

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