Redirection of Prostaglandin Endoperoxide Metabolism at the Platelet-Vascular Interface in Man

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Abstract

Prostacyclin (PGI₂) is an inhibitor of platelet function *in vitro*. We tested the hypothesis that PGI₂ is formed in biologically active concentrations at the platelet-vascular interface in man and can be pharmacologically modulated to enhance its inhibitory properties. This became feasible when we developed a microquantitative technique that permits the measurement of eicosanoids in successive 40- μ l aliquots of whole blood emerging from a bleeding time wound.

In 13 healthy volunteers the rate of production of thromboxane B₂ (TXB₂) gradually increased, reaching a maximum of 421 ± 90 (mean $\pm$ SEM) fg/ μ l per s at 300 ± 20 s. The hydration product of PGI₂, 6-keto-PGF_{1 α} , rose earlier and to a lesser degree, reaching a peak (68 ± 34 fg/ μ l per s) at 168 ± 23 s. The generation of prostaglandins PGE₂ and D₂ resembled that of PGI₂. Whereas the threshold concentration of PGI₂ for an effect on platelets *in vitro* is ~ 30 fg/ μ l, only < 3 fg/ μ l circulates under physiological conditions. By contrast, peak concentrations of 6-keto-PGF_{1 α} obtained locally after vascular damage averaged 305 fg/ μ l.

Pharmacological regulation of PG endoperoxide metabolism at the platelet-vascular interface was demonstrated by administration of a TX synthase inhibitor. The rate of production of PGI₂, PGE₂, and PGD₂ increased coincident with inhibition of TXA, as reflected by three indices; the concentration of TXB₂ in bleeding time blood and serum, and excretion of the urinary metabolite, 2,3-dinor-TXB₂.

These studies indicate that PGI₂ is formed locally in biologically effective concentrations at the site of vessel injury and provide direct evidence in support of transcellular metabolism of PG endoperoxides in man.

Introduction

Prostacyclin (PGI₂)¹ is a potent platelet-inhibitory, vasodilator compound *in vitro* (1), but its circulating concentrations are

too low to exert an effect on platelet function or vascular tone under physiological conditions in man (2–5). PGI₂ is classified as an autacoid and thought to exert its action close to its site of generation, although quantitative evidence compatible with this concept has yet to be provided. Indeed, such low rates of metabolite formation may merely reflect a relative lack of agonism to its production rather than question the importance of this eicosanoid in the regulation of platelet-vessel wall interactions. In keeping with this hypothesis is the capacity of vascular endothelium to synthesize PGI₂, its stimulation by physical and chemical factors associated with vascular perturbation (6–8), and increased excretion of the PGI₂ metabolite, 2,3-dinor-6-keto-PGF_{1 α} , in human syndromes accompanied by laboratory evidence of platelet activation (9, 10).

TX synthase inhibitors represent a potential pharmacological approach to regulation of the formation of arachidonic acid metabolites at the site of platelet-vessel wall interactions in man (11–13). Such compounds dose-dependently inhibit the generation of TXA₂, a proaggregatory vasoconstrictor compound, by blocking its formation from the PG endoperoxide substrate, PGH₂. Furthermore, it has been demonstrated that endothelial cells can enzymatically use the accumulated platelet endoperoxide as a substrate for PGI₂ synthase in perturbed, mixed cell suspensions *in vitro* (14, 15) and that excretion of urinary 2,3-dinor-6-keto-PGF_{1 α} is enhanced after administration of TX synthase inhibitors in man (16). However, the *in vitro* experiments were performed in the absence of such potentially critical variables as albumin (17) and flow (18), and it is impossible to determine whether the alterations in metabolite excretion reflect events at the platelet-vascular interface or in a tissue with the capacity to synthesize both TXA₂ and PGI₂, such as the lung or the kidney.

To address the hypothesis that eicosanoid formation may be regulated at the platelet-vascular interface in man by administration of a TX synthase inhibitor, we developed methodology that permits their quantitative analysis in successive aliquots of whole blood obtained at the site of a bleeding time wound using a highly specific and sensitive technique, gas chromatography-negative ion, chemical ionization-mass spectrometry (GC-NICI-MS).

Methods

The blood emerging from a standard template skin incision (16) was collected in successive 40- μ l samples drawn with a pipetman (Oscar Fisher Co., Inc., Newburgh, NY) and added to 200 μ l saline containing heparin (0.5 USP unit), indomethacin solution (final concentration 5.5×10^{-5} M) and tetradeuterated standards of eicosanoids (500 pg). The samples were then mixed with 50 μ l 2% methoxyamine HCl in aqueous solution and after 30 min at room temperature the respective methoximes formed were extracted with 1 ml ethyl acetate. The ethyl acetate was subsequently evaporated and the dry samples were treated with acetonitrile, α -bromo-2,3,4,5,6-pentafluorotoluene and diisopropylethylamine for 30 min at room temperature. After evaporation the

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1. Abbreviations used in this paper: GC-NICI-MS, gas chromatography-negative ion, chemical ionization-mass spectrometry; PGI₂, prostacyclin.

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respective methoxime-pentafluorobenzyl esters obtained were separated by TLC. Recovery from the TLC plates was assessed with radiolabeled standards and exceeded 95%. The derivatization was completed by trimethylsilylation. The quantitative analyses were accomplished using the Nermag R 10-10 mass spectrometer meter coupled to Varian Vista 6,000 gas chromatograph. Chromatography was performed on 10-m fused silica nonpolar capillary column SPB 1 (Supelco, Inc., Bellefonte, PA) with the oven temperature of 190°C and increasing 20°C/min for 6 min. The mass spectrometer was operated in the negative ion mode with methane ionization at a pressure of 7×10^{-2} torr.

Eicosanoid production rates during bleeding time were calculated by dividing the amount of prostanoid recovered in 40- μ l aliquots of blood by this volume and the individual collection time.

Serum TXB₂ was measured using a specific RIA as previously described (19). Urinary excretion of 2,3-dinor-TXB₂ was by a previously described method using sample purification by immunoaffinity chromatography and analysis by GC-NICI-MS (20).

Results

Initial experiments were performed to address the pattern and reproducibility of TXA₂ and PGI₂ formation in bleeding time blood by measurement of their respective hydration products, TXB₂ and 6-keto-PGF_{1 α} . Similar results were obtained when eicosanoids were measured after a bleeding time incision in different arms of the same individual (Fig. 1). While the results were similar in the same individual on different occasions, more variability in the maximal concentrations achieved was evident between volunteers (Figs. 1 and 2). The pattern of formation of both eicosanoids differed substantially. In the group data generation of TXB₂ gradually increased, reaching a maximum (mean $\pm$ SEM) of 421 $\pm$ 90 fg/ μ l per s ($n = 13$) at 300 $\pm$ 20 s. By contrast, the production rate of 6-keto-PGF_{1 α} was initially low and then rose to a lower and more variable peak production rate than TXB₂ (68 $\pm$ 34 fg/ μ l per s). On average this reached a maximum earlier in the bleeding time (168 $\pm$ 23 s), although in some cases later, transient increases occurred during the course of bleeding. The generation of

Figure 1. TXB₂ (○) and 6-keto-PGF_{1 α} (■) formation (fg/ μ l per s) in blood emerging from the bleeding time wound on different occasions (A and B) from the same individual. TXB₂ formation increases gradually in the successive 40- μ l aliquots of blood while the increase in 6-keto-PGF_{1 α} formation is early and transient.

Figure 2. Formation of TXB₂ (○) and platelet count (■) in blood emerging from bleeding time wound site on a separate occasion in a healthy volunteer. This is a different individual than is depicted in Fig. 1. Note the similar pattern of increase in TXB₂ but the difference in maximal concentrations attained between individuals in contrast to the within individual variability depicted in Fig. 1.

PGE₂ and PGD₂ resembled that of 6-keto-PGF_{1 α} in that there was a phasic rather than a continuous increase in their production rates during the bleeding time. While a contribution to the TXB₂ concentrations by TXA₂ formation by platelets *ex vivo* is theoretically possible, this is unlikely. 40- μ l samples were collected continuously into indomethacin from the incision site so visible accumulation of blood on the skin surface was not evident. Furthermore, there was no relationship between the platelet count in bleeding time blood (which remained constant) and the steadily increasing concentrations of TXB₂ (Fig. 2). Compared with circulating blood the absolute platelet counts were low in the blood emerging from the wound site. This may have reflected consumption in the hemostatic plug.

To investigate the effects of a TX synthase inhibitor on eicosanoid formation at the platelet-vascular interface, we administered 100 mg of imidazo (1,5-2) pyridine-5-hexanoic acid (CGS 13080; Ciba Geigy Corporation, Summit, NJ) to six healthy male volunteers. This compound selectively inhibits TX synthase and has been previously shown to cause increased excretion of urinary 2,3-dinor-6-keto-PGF_{1 α} , coincident with inhibition of TXB₂ generation in both healthy volunteers and patients with severe atherosclerosis and evidence of platelet activation (21). Bleeding time eicosanoids were measured before administration of this compound, at a time corresponding to peak drug action (1 h after dosing), and when partial recovery from drug effect would be anticipated (6 h after dosing) (21). Two additional indices of TX generation were measured in addition to TXB₂ in bleeding time blood: TXB₂ generation in serum and excretion of the urinary TX metabolite, 2,3-dinor-TXB₂.

TX biosynthesis was reversibly depressed by administration of CGS 13080. The average TXB₂ production rate fell from 88 $\pm$ 16 fg/ μ l per s to 6 $\pm$ 2 fg/ μ l per s ($P < 0.05$) 1 h after dosing. There was a significant, although incomplete, recovery 6 h after dosing (Fig. 3). Qualitatively similar results were obtained for this and other eicosanoids whether the data were expressed as production rates or actual concentrations in bleeding time blood (Figs. 3–5). Reversible inhibition of TX formation was also documented by measurement of serum TXB₂ and excretion of 2,3-dinor-TXB₂ (Table I).

Figure 3. Formation of 6-keto-PGF_{1α} (A) and TXB₂ (B) in bleeding time blood before and after administration of a TX synthase inhibitor (CGS 13080). Data are expressed both as an absolute concentration and a production rate. Differences from measurements performed before drug administration are indicated as ** = $P < 0.01$.

Coincident with depression of TX generation, formation of the other eicosanoids, PGI₂ (measured as 6-keto-PGF_{1α}; Fig. 3), PGE₂, and PGD₂ (Fig. 4), was increased in bleeding time blood. PGF_{2α} was measured before (both 1 fg/μl per s) and 1 h after (6 and 12 fg/μl per s) administration of the TX synthesis inhibitor in two individuals. Additional evidence that these compounds were formed from platelet-derived endoperoxides was provided by the temporal pattern of formation of these eicosanoids in bleeding time blood (Fig. 6). Thus, after CGS 13080 the pattern of formation of 6-keto-PGF_{1α}, PGE₂, and PGD₂ had converted to resemble the progressive increase with time characteristic of TXB₂ under control conditions.

Discussion

The results of this study provide the first evidence for transcellular metabolism of arachidonate at the platelet-vascular interface in man. They are consistent with the "endoperoxide shunt" hypothesis (22, 23). Additional evidence for this phenomenon has been obtained in mixed cell suspensions in vitro (14, 15) and includes the increase in urinary 2,3-dinor-6-keto-PGF_{1α} excretion after TX synthase inhibition in man (16, 21). An alternative, or perhaps additional possibility, is that free arachidonate released by platelets might have been used by vascular tissues to augment eicosanoid production (24). The use of GC-NICI-MS rather than RIA (25, 26) was critical in this regard as we have previously shown that only minor de-

Figure 4. Formation of PGD₂ (A) and PGE₂ (B) in bleeding time blood before and after administration of a TX synthase inhibitor (CGS 13080). Data are expressed both as an absolute concentration and a production rate. Differences from measurements performed before drug administration are indicated as ** = $P < 0.01$.

grees of antibody cross-reactivity can result in substantial errors of quantitation when endoperoxide rediversion is assessed after TX synthase inhibition in whole blood in vitro (27). In addition, this approach provided the sensitivity sufficient to assay several eicosanoids in multiple aliquots over the course of the bleeding time. Thus, the demonstration of com-

Figure 5. Mean ($n = 6$) changes in eicosanoid formation in bleeding time blood from pretreatment levels, 1 h after administration of the TX synthase inhibitor, CGS 13080. Data are expressed both as absolute concentrations and production rates.

Table 1. TX Formation and Bleeding Time after Administration of the TX Synthase Inhibitor, CGS 13080, to Six Healthy Volunteers

	Predrug	Postdosing	
		1 h	6 h
Serum TXB ₂ (ng/ml)	375±102	3±1*	126±29†
Bleeding time (s)	404±63	445±55	398±41
Bleeding time blood volume (μl)	231±66	254±49	312±101
Urinary 2,3-dinor-TXB ₂	108±29	43±12‡	

* $P < 0.01$; † $P < 0.05$. Differences expressed reflect two-tailed comparisons with predosing values (Wilcoxon signed rank test). Urine was collected for measurement of 2,3-dinor-TXB₂ in 2-h aliquots before and after dosing.

parable time courses of TXB₂ formation before, and that of other eicosanoids after TX synthase inhibition was dependent upon this methodology.

Figure 6. The temporal patterns of formation of TXB₂ and 6-keto-PGF_{1α} in the same individual before (○) and 1 h after (●) administration of the TX synthase inhibitor CGS 13080. This is a different individual from those depicted in Figs. 1 and 2. Data are expressed as actual concentrations rather than rate (which gives qualitatively similar results) for convenience of display. Cumulative time during which blood emerged from the skin incision is expressed on the abscissa. The increases in 6-keto-PGF_{1α} and PGE₂ formation early in the bleeding time, before administration of the inhibitor, are obscured by the compression of the scales necessary to demonstrate the drug-related rise in eicosanoid concentrations. Note how the temporal pattern of augmented 6-keto-PGF_{1α} formation after CGS 13080 resembles that of TXB₂ before drug administration.

Quantitative analysis of the decrease in TXB₂ production and the increment in 6-keto-PGF_{1α}, PGE₂, and PGD₂ formation suggest that reusing PG endoperoxides is a relatively, albeit not completely, efficient process (Fig. 5). Although we cannot definitively address the cellular origin of the eicosanoids measured in bleeding time blood, several observations seem appropriate. First, PGE₂ is a much more abundant product of endoperoxide redirection than 6-keto-PGF_{1α}. Although both platelets and monocytes possess a minor capacity to generate this eicosanoid, PGE₂ rather than PGI₂ is the major eicosanoid formed by microvascular endothelial cells of rabbit coronary and human foreskin origin in culture (28, 29). Secondly, although bovine coronary microvessels possess PGD₂ isomerase, platelets also have a considerable capacity to generate this eicosanoid (30). Thus, the quantitatively impressive formation of PGD₂ after TX synthase inhibition may reflect reorientation of endoperoxide metabolism within a single cell type, the platelet, although vascular production cannot be discounted.

Evidence of the likely functional importance of platelet-inhibitory PG in the antithrombotic action of TX synthase inhibitors has recently been provided. TX synthase inhibitors have been shown to have synergistic effects with antagonists of the shared PG endoperoxide/TXA₂ receptor both in the prevention of electrically induced coronary artery thrombosis (31) and in the abolition of platelet-dependent cyclical flow phenomena in an experimental model of partial coronary occlusion (32). In both instances synergy was abolished by pretreatment with a cyclooxygenase inhibitor, suggesting its dependence on the generation of platelet-inhibitory eicosanoids, such as PGI₂ and PGD₂.

Study of the formation of eicosanoids in bleeding time blood also provides insight into their potential role at sites of vascular injury under physiological conditions, the temporal increase of TXB₂ in bleeding time blood coincides with the phase of platelet-platelet interaction after vascular injury in the rat mesentery (33). This would be consistent with other platelet agonists, such as ADP, being of primary importance during the initial phase of platelet recruitment at the site of vascular damage. Thus, TXA₂ might serve predominantly as an amplifying mechanism, analogous to its role in the "secondary" wave of ADP-initiated platelet aggregation in vitro. After the disruption of the integrity of the endothelium in the rat mesentery there is a rapid deposition of platelets that is localized to the site of the vascular injury. This is followed by the further deposition of platelets to overlay the initial monolayer (33). The earlier increase in PGI₂ formation corresponds in time to the phase of platelet-vessel wall interaction, suggesting that this eicosanoid may play a homeostatic role in limiting the extent of platelet accumulation. In keeping with this hypothesis, the quantity of PGI₂ detected in bleeding time blood is sufficient to inhibit platelet function. For example, studies performed in vitro indicate that the threshold concentration for an effect of PGI₂ on platelets is ~ 30 fg/μl, in excess of the concentrations (< 3 fg/μl) estimated to circulate under physiological circumstances in man (2-5). By contrast, peak concentrations averaged 305 fg/μl during the bleeding time. Thus, biologically effective concentrations of this eicosanoid are formed at the site of vascular injury in man.

Despite this augmentation in PGI₂ formation it is noteworthy that the bleeding time was only modestly prolonged after administration of the TX synthase inhibitor (Table I). Al-

though interpretation of these data must be limited by the possibility of artifact due to blood sampling, this is not an unexpected finding (16, 21, 34, 35). Indeed, sample collection might have been expected to prolong rather than shorten the bleeding time due to disturbance of hemostatic plug formation. This may imply that TX generation is of little importance in the control of hemorrhage. However, many factors influence the bleeding time and the explanation for this phenomenon was not addressed in the present study. However, in addition to their availability for further metabolism, PG endoperoxides may substitute for the action of TXA₂ at their shared receptor(s) after administration of TX synthase inhibitors (13, 16). Such an effect would be expected to limit the ability of such compounds to prolong the bleeding time.

In conclusion, we report the development of a microquantitative analytical technique that permits the precise and sensitive characterization of eicosanoid formation at the platelet-vascular interface. PGI₂ is generated locally in biologically effective concentrations after vascular injury. Furthermore, administration of a TX synthase inhibitor results in reorientation of PG endoperoxide metabolism, further augmenting PGI₂ generation at the platelet-vascular interface in man. However, the biological effects of these compounds are likely to reflect the augmented production of other eicosanoids such as PGE₂ and PGD₂ in addition to PGI₂. This approach can be readily applied to further study of the transcellular metabolism of eicosanoids in man.

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