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11-Dehydrothromboxane B_2: A quantitative index of thromboxane A_2 formation in the human circulation

(platelets/atherosclerosis/gas chromatography–mass spectrometry)

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ABSTRACT **In human plasma, 11-dehydrothromboxane (TX) B_2 is a major long lived metabolite ($t_{1/2}$ 45 min) formed from infused TXB_2, the hydration product of biologically active TXA_2. Plasma concentrations of TXB_2 itself are readily confounded by *ex vivo* platelet activation and, theoretically, an enzymatic derivative of this compound, not subject to formation in whole blood, would more accurately reflect TXA_2 formation *in vivo*. To address this hypothesis, we developed a sensitive assay for both 11-dehydro-TXB_2 and TXB_2, using capillary gas chromatography/negative-ion chemical ionization mass spectrometry. We established that whole blood possesses a minor capacity to form 11-dehydro-TXB_2, attributable to nonenzymatic formation in erythrocytes. However, the nonenzymatic formation of 11-dehydro-TXB_2 was not a practical limitation to its use as an index of TX biosynthesis. Blood was drawn from healthy volunteers (*i*) via an indwelling catheter at the time of insertion and at 30, 60, 90, 180, and 240 min thereafter and (*ii*) via separate venipunctures at 0 time and at 90 and 240 min thereafter. Plasma TXB_2 drawn via the catheter at baseline (66 ± 63 pg/ml) was substantially greater than the maximal estimate of endogenous TXB_2 (1–2 pg/ml) in plasma [Patrono, C., Ciabattoni, G., Pugliese, F., Perruci, A., Blair, I. A. & FitzGerald, G. A. (1986) *J. Clin. Invest.* 77, 590–594] and increased in magnitude and variance over time (339 ± 247 pg/ml at 240 min). By contrast, 11-dehydro-TXB_2 did not change significantly in the sequential catheter samples or in the samples drawn by separate venipuncture. Basal plasma concentrations in volunteers were depressed by pretreatment with 325 mg of aspirin. Furthermore, the range of concentrations in patients with severe atherosclerosis in whom urinary 2,3-dinor-TXB_2 was increased was significantly higher (5–50 pg/ml, $P < 0.01$) than in healthy subjects (0.9–1.8 pg/ml). Concentrations of 11-dehydro-TXB_2 were increased in patients who had recently suffered a pulmonary embolism to a greater extent than either the 11-dehydro-13,14-dihydro-15-keto-TXB_2 or the 2,3-dinor-TXB_2 metabolites in plasma. These results indicate that plasma TXB_2 is readily confounded by platelet activation *ex vivo*. Measurement of enzymatic metabolites of TXB_2 minimizes this problem. The 11-dehydro metabolite is the most appropriate analytic target to detect phasic release of TXA_2 in the human circulation, such as might occur in human syndromes of platelet activation.**

Thromboxane (TX) A_2 is a cyclooxygenase product of arachidonic acid that causes irreversible platelet aggregation and causes bronchial and vascular smooth muscle to contract (1–3). Pharmacological probes have been used to clarify the biological importance of this compound. For example, progression of kidney disease following subtotal renal ablation in the rat has been delayed by pretreatment with a TX synthase inhibitor (4) and an antagonist of the prostaglandin endoperoxide/TXA_2 receptor(s) markedly reduces the incidence of reperfusion arrythmias in a canine model of coronary vascular occlusion (5). However, the pharmacological action of these compounds is not restricted to their inhibition of the functional effects of TXA_2. Additional properties include altered biosynthesis of other eicosanoids (6, 7) and modulation of their biological activity (8, 9).

Biochemical assessment of TXA_2 biosynthesis would complement the use of pharmacological agents to elucidate the role of this compound in the mediation of human disease. TXA_2 is extremely evanescent in biological fluids and cannot be measured directly (10). Attention has consequently focused on the measurement of more stable, but biologically inactive, metabolites. Urinary excretion of the TXA_2 hydration product, TXB_2 and its 2,3-dinor metabolite have been utilized as indices of predominantly renal (11) and extrarenal (10) TXA_2 biosynthesis. Urinary TXB_2 is increased in animal (12) and human models (13) of glomerular disease and enhanced excretion of the dinor metabolite has been demonstrated in a variety of human syndromes of platelet activation (14–16). Although such an approach may be usefully combined with pharmacological probes, the measurement of urinary metabolites provides a time-integrated, albeit noninvasive, index of TX biosynthesis. To address this limitation, the analysis of TXB_2 in plasma has been widely employed to assess the formation of TXA_2 by platelets in the circulation more discretely in time.

However, the capacity of platelets to generate TXB_2 greatly exceeds the calculated production rate *in vivo* under physiological conditions (17) and such measurements are consequently liable to substantial artifact *ex vivo*.

Another strategy would be to measure enzymatic products of TXB_2 in plasma. Such metabolic transformation is more likely to occur predominantly in the liver rather than in the bloodstream (18) and would theoretically bypass the problem of *ex vivo* platelet activation. We have identified 11-dehydro-TXB_2 as a major enzymatic metabolite of infused TXB_2 in the human circulation and developed a sensitive assay for this compound employing capillary gas chromatography/negative-ion chemical ionization mass spectrometry (GC-NICI-MS) (19). The purpose of the present study was to explore the utility of this approach to the assessment of TX biosynthesis in the human circulation.

MATERIALS AND METHODS

Analyses of TXB_2 and 11-Dehydro-TXB_2. TXB_2 and 11-dehydro-TXB_2 (Fig. 1) were measured by a stable isotope dilution assay utilizing GC-NICI-MS as described (19). Internal standards were biosynthesized from tetradeuterated TXB_2 (19). Briefly, 10 ml of acidified plasma was extracted on a C_{18} SEP-PAK column (Waters Associates) and eluted

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Abbreviations: GC, capillary gas chromatography; NICI, negative-ion chemical ionization; MS, mass spectrometry; TX, thromboxane.
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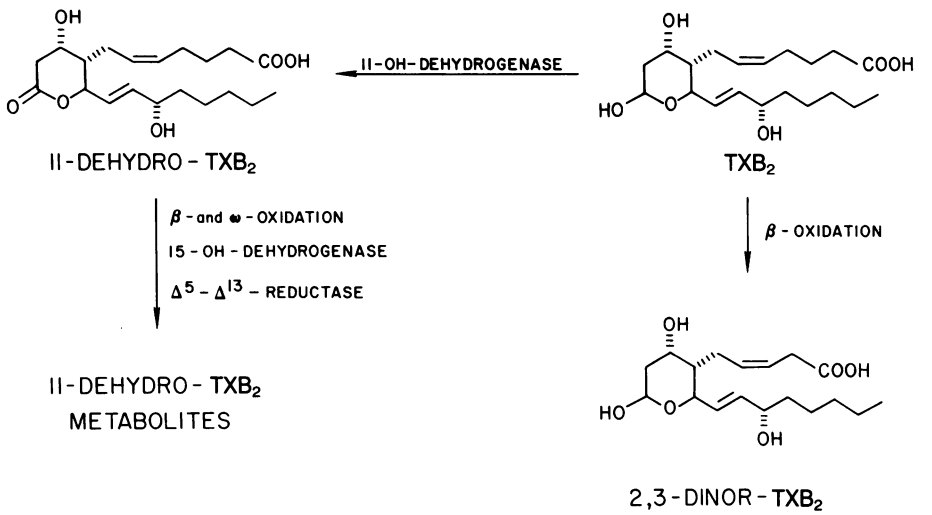

FIG. 1. The major routes of metabolism of TXB_2, the hydration product of TXA_2. While 2,3-dinor-TXB_2 is the most abundant product of systemically administered TXB_2 in human urine (21), 11-dehydro-TXB_2 is the predominant metabolite detected in plasma.

with ethyl acetate. TXB_2 was separated from 11-dehydro-TXB_2 on silica gel TLC plates (Whatman). Following formation of the pentafluorobenzyl ester and further purification on TLC, derivatization of 11-dehydro-TXB_2 was completed by trimethylsilylation. Quantitative analysis was accomplished on a NERMAG 10-10C GC/MS monitoring m/z 511 for endogenous 11-dehydro-TXB_2 and m/z 515 for the deuterium-labeled internal standard. TXB_2 was derivatized as the methoxime, pentafluorobenzyl ester and, after further purification by TLC, derivatization was completed by formation of the trimethylsilylether derivative. Quantitation was accomplished by GC/MS in NICI mode monitoring m/z 614 and m/z 618 for endogenous TXB_2 and tetradeuterated internal standard, respectively.

Formation of 11-Dehydro-TX in Whole Blood. Two studies were performed. In the first, 80 ml of blood was obtained by direct venipuncture from a healthy volunteer who had abstained from aspirin-like drugs for the preceding 2 weeks. The blood was aliquoted into four 20-ml samples and either centrifuged immediately or incubated at 37°C for 15, 30, or 60 min. All supernatants were then centrifuged at 1000 × g for 15 min. The serum was then separated and maintained at −20°C until subsequent analysis for TXB_2 by radioimmunoassay (RIA) and 11-dehydro-TXB_2 by GC-NICI-MS as described (6, 19).

The second study was designed to clarify the origin of 11-dehydro-TXB_2 formed in whole blood. From a healthy volunteer in whom endogenous TXA_2 synthesis was inhibited by aspirin (325 mg), 25 ml of whole blood were drawn into a syringe containing heparin at 5 units/ml and mixed with 5 ml of 6% (wt/vol) dextran 75 (Gentran 75; Travenol Laboratories, Deerfield, IL) and allowed to sediment at room temperature for 90 min. The supernatant was removed, and the erythrocytes were resuspended in Dulbecco's phosphate-buffered saline (PBS). The supernatant was then centrifuged at 280 × g for 10 min, and the leukocyte pellet was resuspended in 2 ml of PBS. Hypotonic lysis was induced with distilled water, 9 ml for 30 sec, and terminated by adding 1 ml of concentrated (10×) PBS. The cells were then washed three times in PBS by repeated centrifugation (280 × g, 10 min) and decantation. Finally, the leukocyte pellet was resuspended in PBS containing glucose, calcium, and magnesium. TXB_2 at 0.2 μg/ml was then added to the leukocyte and erythrocyte fractions prior to incubation at 37°C for 60 min and subsequent quantitative analysis for 11-dehydro-TXB_2.

From another volunteer, platelet-rich plasma was prepared by centrifugation of citrate-treated blood (280 × g, 10 min). Again, TXB_2 at 0.2 μg/ml was added prior to incubation at 37°C for 60 min and subsequent analysis for 11-dehydro-TXB_2.

Circulating Concentrations of 11-Dehydro-TXB_2. The clinical studies were approved by the Committee for the Protection of Human Subjects of Vanderbilt University. Blood samples (20 ml) were drawn by fresh venipuncture into heparinized syringes from five additional healthy volunteers, before and 8 hr after oral administration of 325 mg of aspirin. The blood was immediately centrifuged at 1000 × g for 15 min, the plasma was separated, 14 ng of tetradeuterated 11-dehydro-TXB_2 was added, and then the plasma was frozen and kept at −20°C until assayed.

Analogous procedures were used to collect blood from six consenting male patients (aged 58–71 years) with angiographically confirmed obstructive arterial disease in whom urinary 2,3-dinor-TXB_2 excretion was increased (15). Two were insulin-dependent diabetics and five were smokers. Additional samples were obtained from three patients (2 males) in whom the diagnosis of pulmonary embolism was supported by a recent (<1 hr) ventilation/perfusion scan. All had recently undergone orthopedic operations. All patients had abstained from nonsteroidal antiinflammatory drugs for at least 2 weeks prior to the study. The atherosclerotic patients had not smoked for at least 6 hr prior to blood withdrawal.

Indwelling Catheter Study. Five healthy volunteers, aged 26–39 years, were studied. An indwelling catheter (Flashcath; Travenol Laboratories) was inserted into the left arm and blood drawn into ice-cold heparinized syringes containing indomethacin (5 μg/ml) at time zero and after 30, 60, 90, 180, and 240 min for the determinations of TXB_2 and 11-dehydro-TXB_2 by GC-NICI-MS. In this catheter system the proximal portion of the lumen of the catheter is occluded by a teflon plunger when not in use. Patency of the catheter was maintained by saline irrigation after each blood withdrawal. No heparin was employed. A further sample for both compounds was drawn at 240 min from the cannula into a syringe without indomethacin. In addition, samples were drawn by fresh venipuncture from the opposite arm at the time of insertion of the indwelling catheter and 90 and 240 min after.

Statistical Analysis. Pairwise comparisons were performed using an adaptation of the t test for small sample sizes (20) to avoid assumptions as to the distributions of the variables involved.

Table 1. Formation of TXs in whole blood

Time, min	TX, ng/ml	
	Serum TXB_2	Serum 11-dehydro-TXB_2
0	3.0	0.003
15	196	0.06
30	363	2.9
60	374	5.0

RESULTS

Formation of TXs in Whole Blood. Analysis of TXB_2 and 11-dehydro-TXB_2 indicated minor, but detectable formation of the latter compound in whole blood *in vitro* (Table 1). Addition of tetradeuterated TXB_2 (1 μg/ml) did not depress formation of the endogenous 11-dehydro-TXB_2, suggesting that the system was not saturable. Formation of 11-dehydro-TXB_2 was not depressed by heating to 60°C, further suggesting that its formation in whole blood is nonenzymatic. Finally, following whole blood incubation and fractionation as described, endogenous 11-dehydro-TXB_2 formation was largely confined to the erythrocyte fraction (9.8 ng/ml) rather than the leukocyte (0.1 ng/ml) or platelet rich (0.01 ng/ml) fractions. Thus, these results suggest that a minor capacity to form 11-dehydro-TXB_2 from TXB_2 exists in whole blood. This is apparently nonenzymatic and occurs predominantly in erythrocytes.

Circulating Concentrations of 11-Dehydro-TXB_2. The plasma concentrations of 11-dehydro-TXB_2 ranged from 0.9 to 1.8 pg/ml (n = 5) in healthy individuals. Because of the extreme sensitivity of the assay and the low blank in the internal standard (<0.2 parts per thousand), depression of these concentrations following cyclooxygenase inhibition was readily quantifiable, ranging from <0.1 to 1.1 pg/ml (P < 0.001) 8 hr after administration of 325 mg of aspirin orally. In patients with severe atherosclerotic peripheral vascular disease (Fig. 2) and in the three patients with pulmonary embolism, plasma 11-dehydro-TXB_2 was markedly higher than in healthy individuals (14, 43, and 512 pg/ml). Plasma concentrations of the 11-dehydro-13,14-dihydro-15-keto-TXB_2 and 2,3-dinor-TXB_2 metabolites (19) were below these respective detection limits (1.5 and 2.0 pg/ml), save in the patient with pulmonary embolism who had the highest level of 11-dehydro-TXB_2. However, even in this individual, the 11-dehydro-13,14-dihydro-15-keto (23 pg/ml) and dinor (24 pg/ml) metabolites were considerably less abundant than 11-dehydro-TXB_2 in plasma.

Plasma Thromboxanes and *ex Vivo* Platelet Activation. In the samples obtained via an indwelling catheter, mean TXB_2 concentrations rose progressively as did the variance of these measurements (Fig. 3). By contrast, plasma concentrations of 11-dehydro-TXB_2 did not alter significantly during the study, although there was a tendency to rise toward the end of the observation period. Plasma concentrations of 11-dehydro-TXB_2 remained in the low pg/ml range following repeated venipunctures in the contralateral arm (Fig. 4). Samples obtained from the indwelling catheter at the end of the study period (240 min) were withdrawn into syringes with and without indomethacin. Omission of the cyclooxygenase inhibitor resulted in a significant, additional increase in plasma TXB_2 but did not influence the concentration of 11-dehydro-TXB_2.

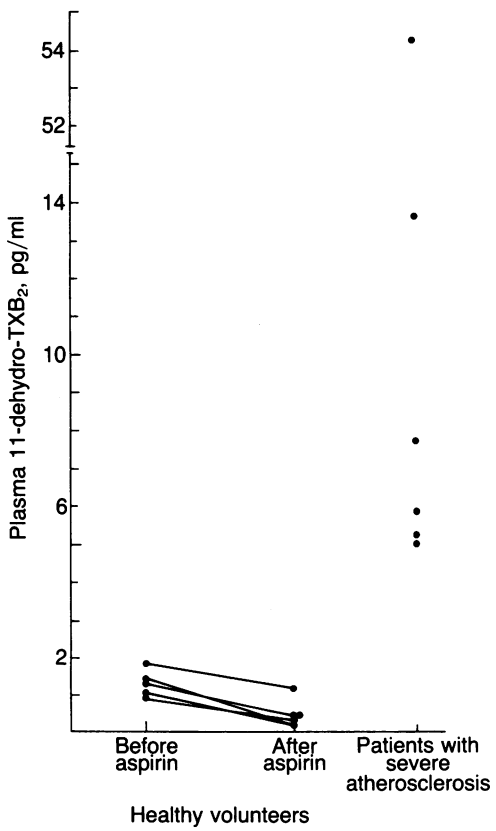

FIG. 2. Plasma concentrations of 11-dehydro-TXB_2 in healthy volunteers before (n = 5) and 8 hr after (n = 6) administration of 325 mg of aspirin orally. In two individuals the concentrations after aspirin administration were at the limit of analytical detection, i.e., <0.1 pg/ml. Elevated concentrations were observed in six patients with severe atherosclerotic peripheral vascular disease.

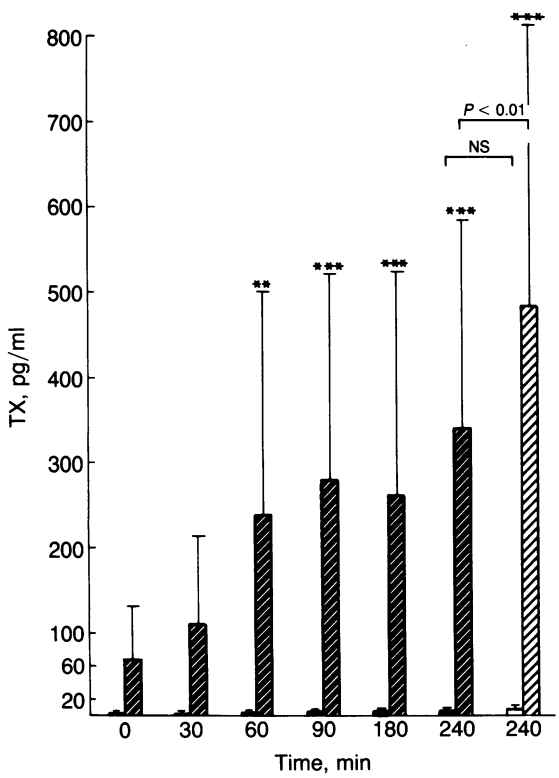

FIG. 3. Plasma concentrations of TXB_2 (solid and open-hatched bars) increased in magnitude and variance when drawn sequentially through an indwelling catheter while plasma 11-dehydro-TXB_2 (solid and open bars) did not change significantly. Omission of the cyclooxygenase inhibitor indomethacin (open bar and open-hatched bar) from the collecting syringe resulted in a further significant increment in TXB_2 levels. Presence of indomethacin (solid and solid-hatched bars). **, P < 0.01. ***, P < 0.001. NS, not significant.

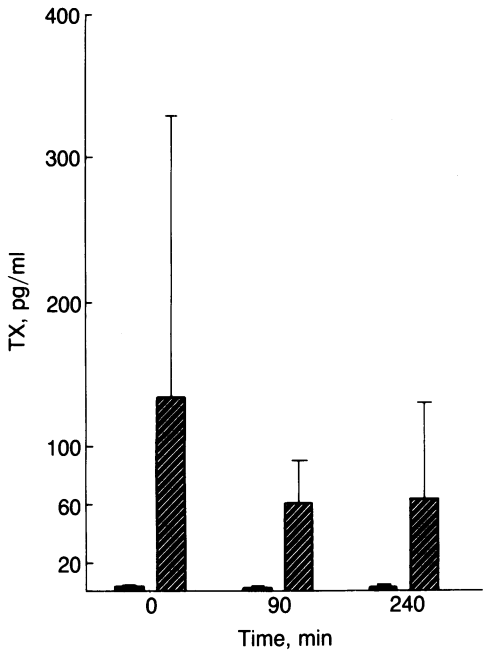

FIG. 4. Plasma TXB_2 (hatched bars) concentrations were highly variable even when samples were obtained by direct venipuncture. Plasma 11-dehydro-TXB_2 (solid bars) remained in the low pg/ml range.

DISCUSSION

TXA_2 is a labile metabolite of prostaglandin G_2 with potent biological activity. Roberts *et al.* (21) have identified 2,3-dinor-TXB_2 as the major urinary metabolite of systemically administered $[^3H]_8TXB_2$ in man—analysis of the excretion of this compound is of proven value in the noninvasive assessment of TX biosynthesis (9). A second major pathway for biotransformation of TXB_2 was also identified involving dehydrogenation of the hemiacetal alcohol group at C-11, resulting in the formation of a series of metabolites with a δ-lactone ring structure (21). A product of this pathway, 11-dehydro-TXB_2, was the second most abundant urinary metabolite of TXB_2 identified in these studies. Because of its lesser polarity, we reasoned that this compound and another product of this pathway, 11-dehydro-13,14-dihydro-15-keto-TXB_2 would be more likely than the dinor compound, or TXB_2 itself, to accumulate in plasma following an increase in TXA_2 biosynthesis. We have developed sensitive GC-MS assays for these compounds and confirmed that 11-dehydro-TXB_2 is a major metabolite of infused TXB_2 in man (19). This is consistent with the report of Granstrom *et al.* (22) that identified 11-dehydro-TXB_2 as the major metabolite formed in plasma following a bolus injection of $[^3H_8]TXB_2$.

The results of the present study indicate that measurement of 11-dehydro-TXB_2 represents an approach to the assessment of TXA_2 biosynthesis in the human circulation. Analysis of this compound has particular appeal because its post-infection half-life is prolonged (45 min), a potential advantage in the detection of phasic increments in TXA_2 generation, such as might characterize platelet activation *in vivo* (19). We have demonstrated that there is a minor capacity to form 11-dehydro-TXB_2 in whole blood, probably attributable to nonenzymatic hydrogen abstraction from TXB_2, predominantly in erythrocytes. However, formation of the 11-dehydro metabolite was markedly less (1%) than that of TXB_2 itself following incubation at 37°C for 60 min *in vitro*. More importantly, we have established that the utility of this index is not constrained by this observation under standardized conditions *in vivo*. While plasma concentrations of 11-dehydro-TXB_2 did not change significantly during the entire study period, a tendency to increase from basal levels (2.0 ± 0.4 pg/ml) was apparent when the catheter had been *in situ* for 240 min (5.1 ± 3.5 pg/ml). By this time, in the absence of local anticoagulation, blood samples were obtained with considerable difficulty, probably reflecting partial obstruction of the catheter tip by platelet thrombus. No such suggestion of *ex vivo* formation was apparent when blood samples were drawn via the catheter when it had been *in situ* for less than 90 min or when blood was drawn by direct venipuncture from the contralateral arm.

We have reported that patients with severe atherosclerotic disease of the lower limbs have increased excretion of the dinor metabolite of TXB_2 in urine (15). The observations that plasma concentrations of 11-dehydro-TXB_2 are also increased in these patients and in patients with pulmonary embolism and that they are depressed by aspirin administration to healthy volunteers also support the hypothesis that this metabolite may be utilized as an index of TXA_2 generation in the human circulation. Furthermore, combined analysis of 11-dehydro-TXB_2 with the diketo-dihydro and dinor metabolites under conditions of increased endogenous TXB_2 formation are in accord with the results that we obtained from infusion of exogenous TXB_2 (19), suggesting that 11-dehydro-TXB_2 is the most appropriate metabolite to monitor TX synthesis in the human circulation. The development of biochemical methods that permit the assessment of relatively short-term alterations in TX biosynthesis combined with the evaluation of selective antagonists of TXA_2 is likely to elucidate the role of this potent autocoid in human disease.

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