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C Patrono, ... , I A Blair, G A FitzGerald

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### Research Article

We have measured the excretion of a major urinary metabolite of thromboxane B<sub>2</sub> (TxB<sub>2</sub>), i.e., 2,3-dinor-TxB<sub>2</sub>, during the infusion of exogenous TxB<sub>2</sub> over a 50-fold dose range to enable estimation of the rate entry of endogenous TxB<sub>2</sub> into the bloodstream. Four healthy male volunteers received 6-h i.v. infusions of vehicle alone and TxB<sub>2</sub> at 0.1, 1.0, and 5.0 ng/kg X min in random order. They were pretreated with aspirin at a dose of 325 mg/d in order to suppress endogenous TxB<sub>2</sub> production. Urinary 2,3-dinor-TxB<sub>2</sub> was measured before, during, and up to 24 h after the infusions and in aspirin-free periods, by means of radioimmunoassay. The nature of the extracted immunoreactivity was characterized by thin-layer chromatography and confirmed by negative ion-chemical ionization gas chromatography/mass spectrometry. Aspirin treatment suppressed urinary 2,3-dinor-TxB<sub>2</sub> excretion by 80%. The fractional elimination of 2,3-dinor-TxB<sub>2</sub> was independent of the rate of TxB<sub>2</sub> infusion and averaged 5.3 +/- 0.8%. Interpolation of metabolite values obtained in aspirin-free periods onto the linear relationship between the quantities of infused TxB<sub>2</sub> and the amount of metabolite excreted in excess of control values ( $y = 0.0066x$ ,  $r = 0.975$ ,  $P$  less than 0.001) permitted calculation of the mean rate of entry of endogenous TxB<sub>2</sub> into the circulation as 0.11 ng/kg X min. The rate of disappearance of immunoreactive TxB<sub>2</sub> from the circulation was monoexponential [...]

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# Estimated Rate of Thromboxane Secretion into the Circulation of Normal Humans

Carlo Patrono and Giovanni Ciabattoni

Department of Pharmacology, Catholic University School of Medicine, 00168 Rome, Italy

Francesco Pugliese and Alessandro Pierucci

Division of Nephrology, Department of Medicine, University of Rome, La Sapienza, 00100 Rome, Italy

Ian A. Blair and Garret A. FitzGerald

Division of Clinical Pharmacology, Department of Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

## Abstract

We have measured the excretion of a major urinary metabolite of thromboxane B<sub>2</sub> (TxB<sub>2</sub>), i.e., 2,3-dinor-TxB<sub>2</sub>, during the infusion of exogenous TxB<sub>2</sub> over a 50-fold dose range to enable estimation of the rate entry of endogenous TxB<sub>2</sub> into the bloodstream. Four healthy male volunteers received 6-h i.v. infusions of vehicle alone and TxB<sub>2</sub> at 0.1, 1.0, and 5.0 ng/kg·min in random order. They were pretreated with aspirin at a dose of 325 mg/d in order to suppress endogenous TxB<sub>2</sub> production. Urinary 2,3-dinor-TxB<sub>2</sub> was measured before, during, and up to 24 h after the infusions and in aspirin-free periods, by means of radioimmunoassay. The nature of the extracted immunoreactivity was characterized by thin-layer chromatography and confirmed by negative ion-chemical ionization gas chromatography/mass spectrometry. Aspirin treatment suppressed urinary 2,3-dinor-TxB<sub>2</sub> excretion by 80%. The fractional elimination of 2,3-dinor-TxB<sub>2</sub> was independent of the rate of TxB<sub>2</sub> infusion and averaged 5.3±0.8%. Interpolation of metabolite values obtained in aspirin-free periods onto the linear relationship between the quantities of infused TxB<sub>2</sub> and the amount of metabolite excreted in excess of control values ( $y = 0.0066x$ ,  $r = 0.975$ ,  $P < 0.001$ ) permitted calculation of the mean rate of entry of endogenous TxB<sub>2</sub> into the circulation as 0.11 ng/kg·min. The rate of disappearance of immunoreactive TxB<sub>2</sub> from the circulation was monoexponential over the first 10 min with an apparent half-life of 7 min. This corresponded to a maximal estimate of the plasma concentration of endogenous TxB<sub>2</sub> of 2.0 pg/ml. These results suggest that *ex vivo* platelet activation and/or analytical problems confound estimates of endogenous thromboxane release based on plasma TxB<sub>2</sub> and provide a rationale for seeking longer-lived enzymatic metabolites of TxB<sub>2</sub> in plasma.

A preliminary account of this study was presented at the 1985 Winter Prostaglandin Conference, 12 January 1985, Keystone, CO, and at the annual meeting of the American Federation for Clinical Research, 6 May 1985, Washington, DC, and has been published as an abstract (1985. *Clin Res.* 33:287A).

Address reprint requests to Dr. FitzGerald, Division of Clinical Pharmacology, Vanderbilt University School of Medicine, Nashville, TN 37232.

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## Introduction

Thromboxane (Tx)<sup>1</sup> A<sub>2</sub> is a labile derivative of prostaglandin (PG) endoperoxide metabolism (1) with potent contractile activity on bronchial and vascular smooth muscle (2), glomerular mesangium (3), and platelets (1). The last effect is currently held to account for arachidonate-induced irreversible platelet aggregation (4). Because of chemical instability of the oxane ring, TxA<sub>2</sub> is rapidly converted to the chemically stable and relatively biologically inactive hydration product TxB<sub>2</sub> (1).

Many reports have appeared during the last 6 years, describing peripheral plasma levels of TxB<sub>2</sub> in health and disease (see Reference 5 for a review). Such measurements largely rely on two assumptions: (a) that TxA<sub>2</sub> is continuously produced by platelets and other cell types endowed with Tx synthase and (b) that TxB<sub>2</sub>—because of its chemical stability—represents a long-lived metabolite in the circulation. Inasmuch as neither of these assumptions was proven to be valid in the case of primary PGs (6), including prostacyclin (PGI<sub>2</sub>) (7), it seemed to us that they should be subjected to a critical appraisal in the case of TxB<sub>2</sub>. Thus, the aims of the present investigation were: (a) to estimate the rate of entry of endogenous TxB<sub>2</sub> into the circulation in humans, (b) to calculate the clearance rate of TxB<sub>2</sub>, and (c) to obtain a maximal estimate of the circulating concentration of TxB<sub>2</sub>.

## Methods

**Subjects and study design.** Four healthy male volunteers aged 32-40 yr were investigated. Their weight ranged between 65 and 80 kg, and they abstained from all medication for 2 wk before the study. Each subject received 6-h intravenous infusions of vehicle alone (normal saline) and TxB<sub>2</sub> at 0.1, 1.0, and 5.0 ng/kg·min in random order. The safety of intravenous TxB<sub>2</sub> infusion of similar duration was previously reported by Roberts et al. (8) at 65 ng/kg·min. Infusions were separated by at least 6-d intervals. The subjects were treated with aspirin (325 mg/d for 3 d) prior to each infusion, in order to minimize the contribution of endogenous TxB<sub>2</sub> production to the measurement of plasma and urinary metabolites. For infusion, the sodium salt of TxB<sub>2</sub> (a generous gift of Dr. J. Pike, The Upjohn Co., Kalamazoo, MI) was prepared by dissolving the TxB<sub>2</sub> in an equimolar amount of 50 mM Na<sub>2</sub>CO<sub>3</sub> and was diluted to a final volume of 60 ml with normal saline, as described by Roberts et al. (8). Peripheral plasma samples were obtained before, during, and

1. **Abbreviations used in this paper:** GC/MS, gas chromatography/mass spectrometry; NICI, negative ion-chemical ionization; PG, prostaglandin; PGI<sub>2</sub>, prostacyclin; RIA, radioimmunoassay; TLC, thin-layer chromatography; Tx, thromboxane.

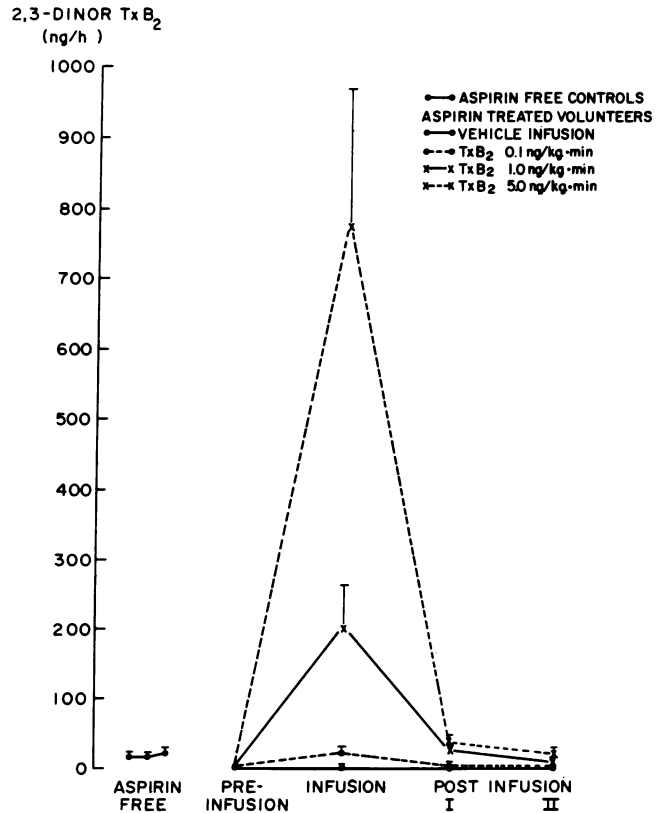
after the infusions for measurement of TxB<sub>2</sub>. Blood was collected by direct venipuncture via a butterfly needle with a cyclooxygenase inhibitor (fenoprofen sodium salt, 10 µg/ml, Eli Lilly & Co., Indianapolis, IN) in the syringe to suppress fully platelet TxB<sub>2</sub> production after sampling. Urinary samples were obtained before (midnight to 8 a.m.), during (8 a.m. to 2 p.m.) and after (2 p.m. to midnight; midnight to 8 a.m.) the infusions for measurement of 2,3-dinor-TxB<sub>2</sub>, the major urinary metabolite of infused TxB<sub>2</sub> in humans (8). A supine urine collection (midnight to 8 a.m.) was also obtained on three separate occasions in aspirin-free periods. Under the same circumstances, peripheral venous blood samples were obtained and triplicate 1-ml aliquots of whole blood allowed to clot at 37°C for 1 h, in order to measure the maximal capacity of platelets to produce TxB<sub>2</sub> when exposed to endogenous thrombin (9). Plasma, serum, and urine samples were immediately frozen and kept at -20°C until extracted or assayed. Blood pressure and heart rate were measured at 30-min intervals throughout the infusions.

**Analytical methods.** The rate of entry of endogenous TxB<sub>2</sub> into the circulation was estimated by quantification of the 2,3-dinor-TxB<sub>2</sub> metabolite in urine, similarly to our previous study of endogenous PGI<sub>2</sub> secretion (7). For measurement of urinary 2,3-dinor-TxB<sub>2</sub>, both radioimmunoassay (RIA) and a stable isotope dilution assay were employed. Immunoreactive 2,3-dinor-TxB<sub>2</sub> was extracted from 20-ml urine samples, run on SEP-PAK C18 cartridges (Waters Associates, Milford, MA) and eluted with ethyl acetate. Eluted 2,3-dinor-TxB<sub>2</sub> was separated from TxB<sub>2</sub> on silica gel thin-layer chromatography (TLC) plates (60 F254 E. Merck, Darmstadt, Federal Republic Germany). The details of the RIA procedure have been described elsewhere (10). A 5-ml aliquot of the same urine was spiked with 2.5 ng/ml tetradeuterated 2,3-dinor-TxB<sub>2</sub> (a generous gift of Dr. J. Pike, The Upjohn Company), purified on reverse-phase SEP-PAK, derivatized as the methoxime, and purified on TLC. After formation of the pentafluorobenzyl ester and further purification on TLC, derivatization was completed by trimethylsilylation. Quantitative analysis was accomplished on a NERMAG 10-10C gas chromatograph-mass spectrometer (GC/MS) in the negative ion-chemical ionization (NICI) mode, monitoring *m/z* 586.5 for endogenous 2,3-dinor-TxB<sub>2</sub> and *m/z* 590.5 for the tetradeuterated internal standard. Serum and plasma concentrations of TxB<sub>2</sub> were measured by RIA (9, 10). Unextracted samples were diluted in the standard diluent of the assay (0.02 M PO<sub>4</sub> buffer, pH 7.4) and assayed in a volume of 1.5 ml at a final dilution of 1:50 (plasma) or 1:15,000 (serum). The least detectable concentration which could be measured with 95% confidence was 1 pg of TxB<sub>2</sub>/ml of incubation mixture. Therefore, the detection limit of the assay was 50 or 15,000 pg/ml of plasma or serum, respectively.

**Statistical analysis.** Significance of the data was evaluated by Student's *t* test. Owing to the inequality of variance in the metabolite excreted at different rates of TxB<sub>2</sub> infusion, regression analysis was performed by a weighted least squares approach (11). All calculations were based on RIA data, as there was no statistically significant difference vs. GC/MS measurements.

## Results

At no time during the infusion was there any significant change in blood pressure or heart rate, and no clinically apparent adverse effects were observed in any of the subjects. Prior to the infusions, the urinary excretion of 2,3-dinor-TxB<sub>2</sub> averaged 3.3±1.8 ng/h (mean±SD, *n* = 10). This represented an 80% reduction from a mean value of 16.1±6.8 ng/h measured on the day prior to aspirin administration. A dose-dependent increase of metabolite excretion was detected during TxB<sub>2</sub> infusions, which returned to baseline levels 10–18 h after discontinuing the infusion (Fig. 1). No statistically significant change in metabolite excretion was found during vehicle infusion. No significant difference in metabolite excretion was noted on the days of vehicle infusion between those samples collected when the volunteers were supine (pre- and intrainfusion samples, midnight to 8 a.m. postinfusion)



**Figure 1.** Changes in the rate of excretion of 2,3-dinor-TxB<sub>2</sub> (as measured by NICI-GC/MS) during and up to 24 h after infusions of TxB<sub>2</sub> and vehicle alone. The excretory rate measured on three separate occasions in aspirin-free periods is also shown.

and those collected when they were ambulant (2 p.m. to midnight postinfusion). A highly significant correlation ( $r = 0.933$ ,  $P < 0.001$ ,  $n = 61$ ) was found between measurement of the dinor metabolite by TLC-RIA and measurement by NICI-GC/MS for each of the four different infusions (Fig. 2). The fractional elimination of the metabolite was independent of the rate of TxB<sub>2</sub> infusion. A mean of 5.3% of the infused TxB<sub>2</sub> appeared as 2,3-dinor-TxB<sub>2</sub> (Table I). Interpolation of metabolite values obtained in aspirin-free periods onto the linear relationship between the quantities of infused TxB<sub>2</sub> and the amount of metabolite excreted in excess of control values (i.e., during the infusion of vehicle alone) permitted calculation of the rate of entry of endogenous TxB<sub>2</sub> into the circulation (Fig. 3). For this purpose, it was assumed that the linear relationship between rate of TxB<sub>2</sub> infused and the quantity of metabolite excreted also existed for TxB<sub>2</sub> <0.1 ng/kg·min. Although it would be logically expected that when the *y*-variable (infused TxB<sub>2</sub>) was 0 the *x*-variable (quantity of 2,3-dinor-TxB<sub>2</sub> excreted in excess of control values) would also be 0, the regression line was tested against the hypothesis that the *y*-intercept was 0 prior to forcing the line through the origin (12). Having established that the *y*-intercept did not differ significantly from 0, the linear regression was constrained to pass through the origin. Insertion of individual metabolite values obtained in aspirin-free periods into the appropriate linear equation permitted estimation of the rate of entry of endogenous TxB<sub>2</sub> into the circulation in three of the four volunteers. In the fourth, sample collection was incomplete during vehicle infusion (Table II). The estimated rate ranged between 0.05 and 0.18 ng/

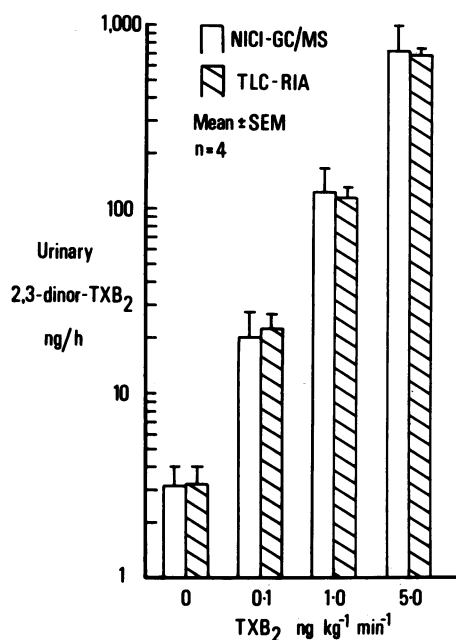


Figure 2. A comparison of urinary 2,3-dinor-TxB<sub>2</sub> excretory rates measured prior to and during TxB<sub>2</sub> infusions by NICI-GC/MS and TLC-RIA.

kg·min in nine different determinations and averaged 0.1 ng/kg·min. In the same subjects, the maximal capacity of platelets to synthesize TxB<sub>2</sub> during 1-h whole blood clotting averaged 274±52 ng/ml of serum. In addition, immunoreactive TxB<sub>2</sub> was measured in peripheral venous plasma during the last 60 min of TxB<sub>2</sub> infusion at 5.0 ng/kg·min and during the first 60 min after discontinuing the infusion (Fig. 4). Its steady-state concentration averaged 88±12 pg/ml. Upon discontinuing the infusion, the rate of disappearance of TxB<sub>2</sub> from the circulation was linear over the first 10 min with an apparent half-life of 7 min. Based on these measurements, a total plasma clearance of 52.3 ml/kg·min was obtained. A maximal estimate of the circulating concentration of TxB<sub>2</sub> may be obtained by dividing the rate of entry of TxB<sub>2</sub> into the bloodstream by this rate of plasma clearance, resulting in 2 pg/ml.

## Discussion

Because platelet-derived TxA<sub>2</sub> might play a role in coronary thrombosis and vasospasm, several investigators have tried to

Table I. Fractional Conversion of TxB<sub>2</sub> to Urinary 2,3-dinor-TxB<sub>2</sub>

Rate of TxB <sub>2</sub> infusion ng/kg·min	2,3-dinor-TxB <sub>2</sub> %
0.1	6.2±1.6
1.0	4.7±1.7
5.0	4.9±1.2
Mean of three infusions	5.3±0.8

The fractional conversion rate of infused TxB<sub>2</sub> into 2,3-dinor-TxB<sub>2</sub> is expressed for the 24-h period commencing with each infusion.

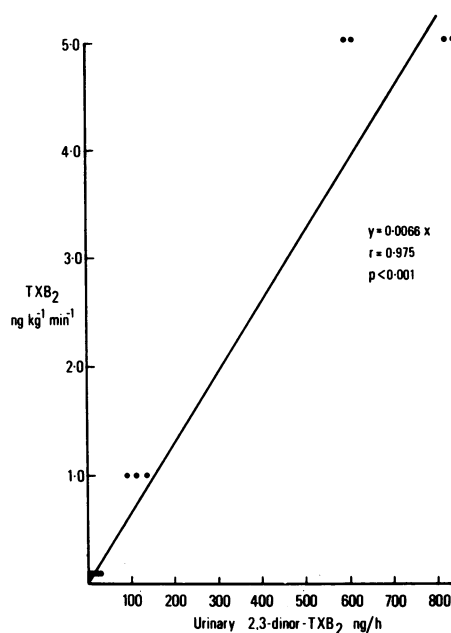


Figure 3. Infused TxB<sub>2</sub> is regressed upon the amount of 2,3-dinor-TxB<sub>2</sub> excreted in excess of control values (i.e., during the infusion of vehicle alone). After having established that the  $y$ -intercept did not differ significantly from 0, we found that the linear regression was constrained to pass through the origin.

obtain measurements of its stable breakdown product, TxB<sub>2</sub>, in peripheral or coronary sinus blood, and have reported levels in the range of 7–300 pg/ml (reviewed in References 5 and 13). Sampling-related activation of platelet arachidonate deacylation and subsequent oxygenation to PG endoperoxides was presumed to be prevented, in some studies, by drawing the blood into a

Table II. Estimated Rate of Entry of Endogenous TxB<sub>2</sub> into the Circulation

Subject	Control excretion of 2,3-dinor-TxB <sub>2</sub> ng/h	Entry rate of TxB <sub>2</sub> ng/kg·min
C.P.		
1	11.4	0.075
2	7.3	0.048
3	17.1	0.112
G.C.		
1	8.9	0.058
2	19.8	0.130
3	16.6	0.109
F.P.		
1	12.2	0.080
2	27.4	0.180
3	24.0	0.157
Mean±SEM	16.1±2.3	0.105±0.015

Insertion of individual excretory rates of 2,3-dinor-TxB<sub>2</sub> measured prior to aspirin administration into the appropriate linear equation (Fig. 3) permits estimation of the rate of entry of endogenous TxB<sub>2</sub> into the circulation.

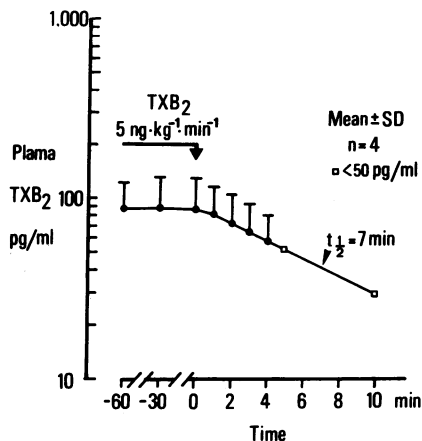


Figure 4. Plasma-immunoreactive TxB<sub>2</sub> concentrations measured during the last 60 min of TxB<sub>2</sub> infusion at 5.0 ng/kg · min and during the first 10 min after discontinuing the infusion. The concentrations measured at 5 and 10 min were interpolated on the standard curve above the 1 pg/ml point and are therefore indicated as <50 pg/ml.

cyclooxygenase inhibitor. However, based on the very high capacity of blood platelets to synthesize TxB<sub>2</sub> ex vivo, it could be argued—on theoretical grounds—that as little as 0.1% platelet activation during and after sampling would lead to apparent plasma concentrations of 200–400 pg/ml.

In the present study, we have estimated the rate of entry of endogenous TxB<sub>2</sub> into the bloodstream by quantification of a major urinary metabolite of infused TxB<sub>2</sub> in humans, i.e., 2,3-dinor-TxB<sub>2</sub> (8), by both TLC-RIA and NCI-GC/MS. A similar approach was used previously for estimating the entry rate of endogenous PGI<sub>2</sub> into the circulation in humans, through measurement of the urinary excretion of 2,3-dinor-6-keto-PGF<sub>1α</sub> (7). It is noteworthy that the fractional conversion of TxB<sub>2</sub> to 2,3-dinor-TxB<sub>2</sub> herein reported (5.3%) is comparable to the previously described rate of conversion of PGI<sub>2</sub> to its dinor metabolite (6.8%), when administered at similar low rates of infusion (7). Our present findings are somewhat at variance with previous observations by Roberts et al. (8, 14) indicating that 2,3-dinor-TxB<sub>2</sub> represented 16.8% of the total radioactivity in the urine (23% of total recovered radioactivity) after infusion of (<sup>3</sup>H)<sub>8</sub> TxB<sub>2</sub> into a healthy male volunteer (8, 14). A possible explanation for this discrepancy is that the conversion of TxB<sub>2</sub> to 11-dehydro-TxB<sub>2</sub> and subsequent metabolites was saturated at the much higher infusion rate (65 ng/kg · min) used by Roberts et al. (14).

The low rate of endogenous TxB<sub>2</sub> secretion (0.1 ng/kg · min) in healthy subjects may reflect the low frequency and/or intensity of stimuli to its secretion. Taken together with its rapid plasma clearance, this would argue for a local nature of TxA<sub>2</sub> synthesis and action, as previously suggested for PGI<sub>2</sub> (7). Similar to the vascular synthesis of PGI<sub>2</sub>, the biosynthetic capacity of platelets to produce TxB<sub>2</sub> greatly exceeds its actual production in vivo. Thus, 1 ml of whole blood clotting for 1 h in vitro can produce approximately the same amount of TxB<sub>2</sub> entering the circulation in vivo during the same period of time.

Peripheral plasma measurements of TxB<sub>2</sub> during infusion at 5 ng/kg · min, i.e., 50-fold higher than the estimated endogenous entry rate, gave levels ≤ 100 pg/ml consistently with the basal circulating concentration of TxB<sub>2</sub> being ≤ 2 pg/ml, as es-

timated. Previously Zipser and Martin (15) had suggested that the maximum arterial blood concentration of TxB<sub>2</sub> is <2 pg/ml, on the basis of fractional excretion of unmetabolized [<sup>3</sup>H]TxB<sub>2</sub> into human urine. Thus, these results suggest that previous measurements of plasma TxB<sub>2</sub> have largely overestimated its true circulating levels due to sampling and/or analytical artifacts.

The demonstration of significant protection against myocardial infarction and death afforded by single daily dosing with aspirin at 324 mg (16) does suggest a potential role for platelet-derived TxA<sub>2</sub> in mediating coronary thrombosis in patients with unstable angina. The central role of platelet activation in this condition is further supported by autopsy evidence of recurrent mural thrombosis in a major coronary artery of such patients (17). Clarification of the pathophysiologic and possibly predictive role of acute changes in platelet TxA<sub>2</sub> production now requires the development of adequate analytical tools bypassing the unreliability of plasma TxB<sub>2</sub> measurements. The identification of 11-dehydro-TxB<sub>2</sub> as a major enzymatic metabolite of TxB<sub>2</sub> with an extended plasma half-life (5, 18) and development of related analytical techniques may provide a new insight into TxA<sub>2</sub> pathophysiology in humans.

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