Estimated Rate of Thromboxane Secretion into the Circulation of Normal Humans

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Abstract

We have measured the excretion of a major urinary metabolite of thromboxane B2 (TxB2), i.e., 2,3-dinor-TxB2, during the infusion of exogenous TxB2 over a 50-fold dose range to enable estimation of the rate entry of endogenous TxB2 into the bloodstream. Four healthy male volunteers received 6-h i.v. infusions of vehicle alone and TxB2 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 TxB2 production. Urinary 2,3-dinor-TxB2 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-TxB2 excretion by 80%. The fractional elimination of 2,3-dinor-TxB2 was independent of the rate of TxB2 infusion and averaged 5.3±0.8%. Interpolation of metabolite values obtained in aspirin-free periods onto the linear between the quantities of infused TxB2 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 TxB2 into the circulation as 0.11 ng/kg·min. The rate of disappearance of immunoreactive TxB2 from the circulation was monoexponential over the first 10 min with an apparent half-life of 7 min. This corresponded to a maximum estimate of the plasma concentration of endogenous TxB2 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 TxB2 and provide a rationale for seeking longer-lived enzymatic metabolites of TxB2 in plasma.

Introduction

Thromboxane (TX) 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, TX A is rapidly converted to the chemically stable and relatively biologically inactive hydration product TX B (1).

Many reports have appeared during the last 6 years, describing peripheral plasma levels of TX in health and disease (see Reference 5 for a review). Such measurements largely rely on two assumptions: (a) that TX A is continuously produced by platelets and other cell types endowed with TX synthase and (b) that TX B—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 (PG I) (7), it seemed to us that they should be subjected to a critical appraisal in the case of TX B. Thus, the aims of the present investigation were: (a) to estimate the rate of entry of endogenous TX B into the circulation in humans, (b) to calculate the clearance rate of TX B, and (c) to obtain a maximal estimate of the circulating concentration of TX B.

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 TX B at 0.1, 1.0, and 5.0 ng/kg·min in random order. The safety of intravenous TX B 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 TX B production to the measurement of plasma and urinary metabolites. For infusion, the sodium salt of TX B (a generous gift of Dr. J. Pike, The Upjohn Co., Kalamazoo, MI) was prepared by dissolving the TX B in an equimolar amount of 50 mM Na2 CO3 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

Abbreviations used in this paper: GC/MS, gas chromatography/mass spectroscopy; NICI, negative ion-chemical ionization; PG, prostaglandin; PG I, prostacyclin; RIA, radioimmunoassay; TLC, thin-layer chromatography; TX, thromboxane.
after the infusions for measurement of TxB2. 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 TxB2 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-TxB2, the major urinary metabolite of infused TxB2 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 TxB2 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 TxB2 into the circulation was estimated by quantification of the 2,3-dinor-TxB2 metabolite in urine, similarly to our previous study of endogenous PGI2 secretion (7). For measurement of urinary 2,3-dinor-TxB2, both radioimmunoassay (RIA) and a stable isotope dilution assay were employed. Immunoreactive 2,3-dinor-TxB2 was extracted from 20-ml urine samples, run on SEPAK C18 cartridges (Waters Associates, Milford, MA) and eluted with ethyl acetate. Eluted 2,3-dinor-TxB2 was separated from TxB2 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 tetraduterated 2,3-dinor-TxB2 (a generous gift of Dr. J. Pike, The Upjohn Company), purified on reverse-phase SEPAK, 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-TxB2 and m/z 590.5 for the tetraduterated internal standard. Serum and plasma concentrations of TxB2 were measured by RIA (9, 10). Unextracted samples were diluted in the standard diluent of the assay (0.02 M PO4 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 TxB2/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 TxB2 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-TxB2 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 TxB2 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) 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 TxB2 infusion. A mean of 5.3% of the infused TxB2 appeared as 2,3-dinor-TxB2 (Table I). Interpolation of metabolite values obtained in aspirin-free periods onto the linear relationship between the quantities of infused TxB2 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 TxB2 into the circulation (Fig. 3). For this purpose, it was assumed that the linear relationship between rate of TxB2 infused and the quantity of metabolite excreted also existed for TxB2 <0.1 ng/kg·min. Although it would be logically expected that when the y-variable (infused TxB2) was 0 the x-variable (quantity of 2,3-dinor-TxB2 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 TxB2 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-TxB2 excretory rates measured prior to and during TxB2 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 TxB2 during 1-h whole blood clotting averaged 274±52 ng/ml of serum. In addition, immunoreactive TxB2 was measured in peripheral venous plasma during the last 60 min of TxB2 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 TxB2 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 TxB2 may be obtained by dividing the rate of entry of TxB2 into the bloodstream by this rate of plasma clearance, resulting in 2 pg/ml.

Discussion

Because platelet-derived TxA2 might play a role in coronary thrombosis and vasospasm, several investigators have tried to obtain measurements of its stable breakdown product, TxB2, 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 I. Fractional Conversion of TxB2 to Urinary 2,3-dinor-TxB2

<table>
<thead>
<tr>
<th>Rate of TxB2 infusion (ng/kg·min)</th>
<th>2,3-dinor-TxB2 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>6.2±1.6</td>
</tr>
<tr>
<td>1.0</td>
<td>4.7±1.7</td>
</tr>
<tr>
<td>5.0</td>
<td>4.9±1.2</td>
</tr>
<tr>
<td>Mean of three infusions</td>
<td>5.3±0.8</td>
</tr>
</tbody>
</table>

The fractional conversion rate of infused TxB2 into 2,3-dinor-TxB2 is expressed for the 24-h period commencing with each infusion.

Table II. Estimated Rate of Entry of Endogenous TxB2 into the Circulation

<table>
<thead>
<tr>
<th>Subject</th>
<th>Control excretion of 2,3-dinor-TxB2 ng/h</th>
<th>Entry rate of TxB2 ng/kg·min</th>
</tr>
</thead>
<tbody>
<tr>
<td>C.P.</td>
<td>11.4</td>
<td>0.075</td>
</tr>
<tr>
<td></td>
<td>7.3</td>
<td>0.048</td>
</tr>
<tr>
<td></td>
<td>17.1</td>
<td>0.112</td>
</tr>
<tr>
<td>G.C.</td>
<td>8.9</td>
<td>0.058</td>
</tr>
<tr>
<td></td>
<td>19.8</td>
<td>0.130</td>
</tr>
<tr>
<td></td>
<td>16.6</td>
<td>0.109</td>
</tr>
<tr>
<td>F.P.</td>
<td>12.2</td>
<td>0.080</td>
</tr>
<tr>
<td></td>
<td>27.4</td>
<td>0.180</td>
</tr>
<tr>
<td></td>
<td>24.0</td>
<td>0.157</td>
</tr>
<tr>
<td>Mean±SEM</td>
<td>16.1±2.3</td>
<td>0.105±0.015</td>
</tr>
</tbody>
</table>

Insertion of individual excretory rates of 2,3-dinor-TxB2 measured prior to aspirin administration into the appropriate linear equation (Fig. 3) permits estimation of the rate of entry of endogenous TxB2 into the circulation.
cyclooxygenase inhibitor. However, based on the very high capacity of blood platelets to synthesize TxB2 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 TxB2 into the bloodstream by quantification of a major urinary metabolite of infused TxB2 in humans, i.e., 2,3-dinor-TxB2 (8), by both TLC-RIA and NCI-GC/MS. A similar approach was used previously for estimating the entry rate of endogenous PGI2 into the circulation in humans, through measurement of the urinary excretion of 2,3-dinor-6-keto-PGF1α (7). It is noteworthy that the fractional conversion of TxB2 to 2,3-dinor-TxB2 herein reported (5.3%) is comparable to the previously described rate of conversion of PGI2 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-TxB2 represented 16.8% of the total radioactivity in the urine (23% of total recovered radioactivity) after infusion of [3H] TxB2 into a healthy male volunteer (8, 14). A possible explanation is that the conversion of TxB2 to 11-dehydro-TxB2 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 TxB2 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 TxA2 synthesis and action, as previously suggested for PGI2 (7). Similar to the vascular synthesis of PGI2, the biosynthetic capacity of platelets to produce TxB2 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 TxB2 entering the circulation in vivo during the same period of time.

Peripheral plasma measurements of TxB2 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 TxB2 being ≤2 pg/ml, as estimated. Previously Zipser and Martin (15) had suggested that the maximum arterial blood concentration of TxB2 is <2 pg/ml, on the basis of fractional excretion of unmetabolized [3H] TxB2 into human urine. Thus, these results suggest that previous measurements of plasma TxB2 have largely overestimated its true circulating levels due to sampling and/or analytical artifacts.

The demonstration of significant protective against myocardial infarction and death afforded by single daily dosing with aspirin at 324 mg (16) does suggest a potential role for platelet-derived TxA2 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 TxA2 production now requires the development of adequate analytical tools bypassing the unreliability of plasma TxB2 measurements. The identification of 11-dehydro-TxB2 as a major enzymatic metabolite of TxB2 with an extended plasma half-life (5, 18) and development of related analytical techniques may provide a new insight into TxA2 pathophysiology in humans.

Acknowledgments

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References


