Cigarette smokers, but not former smokers, excrete more thromboxane A₂ (TxA₂) metabolites in the urine than do lifelong nonsmokers, which suggests chronic activation of their platelets. To further characterize the effect elicited by smoking on platelet function, we followed the change in urinary excretion of the 2,3-dinor (Tx-M) and 11-dehydro (dTx) metabolites of TxA₂, analyzed by gas chromatography/mass spectrometry and radioimmunoassay, respectively, in eight healthy women who quit habitual smoking and compared it with the recovery of these metabolites after a single dose of acetylsalicylic acid (ASA). Tx-M and dTx before cessation of smoking were approximately 550 and 600 pg/mg creatinine, respectively. Within 3 days after quitting smoking, Tx-M and dTx had dropped to stable levels of approximately 300 and 350 pg/mg, respectively. The rates of change in excretion of Tx-M and dTx after smoking cessation were more rapid (p<0.02 and 0.02, respectively) than those observed during the recovery of platelet function after a single dose of ASA. The excretion of 2,3-dinor-6-keto-prostaglandin F₁α, a metabolite of prostacyclin, was not affected by smoking cessation. We conclude that cigarette smoking elicits an increase in platelet activity in the absence of vascular injury. This increase is reversible within the life span of the platelets.

**Key Words**  • acetylsalicylic acid  • cardiovascular risk factors  • cigarette smoking  • platelets  • prostacyclin  • thromboxanes  • urine

Cigarette smoking is a major risk factor for cardiovascular disease; the risk increases progressively as daily cigarette consumption increases.¹⁻³ After cessation of smoking the risk drops rather rapidly toward the level observed in nonsmokers.⁶⁻⁹ Platelets are thought to play an important role in cardiovascular disease,¹⁰⁻¹¹ but the effect of smoking on platelet function is poorly understood. Smokers have been reported to display biochemical evidence of increased activation of platelets in vivo.¹²⁻¹⁵ These data may indicate facilitation of platelet aggregability as a significant etiologic factor in smoking-associated cardiovascular disease.

Thromboxane A₂ (TxA₂) is formed in platelets from arachidonic acid.¹⁶ TxA₂ has platelet proaggregatory and proadhesive properties and is also a strong vasoconstrictor. The administration of acetylsalicylic acid (ASA) in doses that inhibit cyclooxygenase-dependent formation of TxA₂ in platelets has proven efficient in the primary¹⁷ and secondary¹⁸ prevention of acute myocardial infarction. These data indicate that TxA₂ formed in platelets is etiologically involved in certain acute cardio-vascular disorders as well as being a marker for platelet activity.

Cigarette smoking is linked to an increased incidence of cardiovascular disease in women.¹⁹⁻²² Although women seem to develop cardiovascular disease 10–15 years later than men,²³ it is a leading cause of death among women.²⁴ In several countries, women constitute a majority of the newly recruited cigarette smokers, and since the 1960s, fewer women than men have stopped smoking. Women start to smoke at younger ages than they used to; more young women than men smoke today, and each smoker uses more cigarettes.²⁵⁻²⁶ Despite these facts, most studies on platelet function in smokers have focused on men.

We recently reported that women who smoke cigarettes excrete larger amounts of the urinary 2,3-dinor metabolite of TxA₂ (Tx-M) than do nonsmokers.²⁷ In that study former smokers did not differ from lifelong nonsmokers in excretion of Tx-M. To further characterize the apparent reversibility in the effect of smoking, we followed urinary excretion of the TxA₂ metabolites Tx-M and 11-dehydro-TxB₂ (dTx)²⁸⁻²⁹ in women who were quitting habitual smoking. We also determined the urinary excretion of 2,3-dinor-6-keto-prostaglandin F₁α (PGI-M), a metabolite of prostacyclin,³⁰⁻³¹ since it has been proposed that an increased formation of prostacyclin in the vascular endothelium may reflect an increased interaction between the platelets and the vascular endothelium.³²

**Methods**

**Study Population**

Eight women smokers (age, 38±3 years [mean±SEM]; range, 23–45 years) were recruited from the hospital.
staff. Basic data on the study population are presented in Table 1. All participants were healthy and took no regular drugs (including oral contraceptives). Six of them were mothers. All had regular menstrual cycles with a mean age of 35±3 years, a mean weight of 53±3 kg and body length of 167±2 cm. Their daily tobacco use ranged from 2 to 20 cigarettes (mean, 13±2 cigarettes/day). Their lifetime tobacco use ranged from 20,000 to 124,000 cigarettes (mean, 76,100±13,700 cigarettes). No participant smoked pipes or cigars or used smokeless tobacco. The subjects studied were carefully informed about the nature and purpose of the investigations before giving their voluntary consent to participate. The study protocol was approved by the local human investigations committee.

**Protocol**

All subjects were interviewed about their previous and present health states, gynecological data, and smoking habits. They were carefully instructed not to take any form of nonsteroidal anti-inflammatory or otherwise platelet-active drugs, except that included in the study, from 1 week before the start until the end of the study. After cessation of smoking no other supply of nicotine (e.g., nicotine gum or nicotine plaster) was allowed. Compliance was checked verbally in association with each urine collection, and cotinine, the major urinary metabolite of nicotine, was analyzed in each subject as well.

On two occasions during the week before the cessation of smoking, a 24-hour urine sample was collected without additives. The subjects were instructed to maintain their ordinary smoking habits during these collections. On day 4 (midnight) of their individual menstrual cycle, counting the first day of the menstrual cycle as day 1, the participants stopped smoking. Thereafter, a 24-hour urine specimen was collected without additives every second day until day 23 of the menstrual cycle, i.e., until 19 days after the cessation of smoking. During the next menstrual cycle an analogous procedure was repeated; however, instead of smoking cessation the participants took a single 1-g oral dose of ASA at 9 PM on day 4 in the menstrual cycle. Urine was collected as above.

The urine was immediately frozen at −20°C and transferred to −80°C within 1 week. Analysis of the urine was performed within 4 weeks after collection.

**Analyses**

All urine samples were analyzed for Tx-M, PGI-M, and creatinine. In addition, urine samples from two occasions before cessation of smoking/intake of ASA and from four or five occasions after cessation of smoking/intake of ASA were analyzed for dTx. Urinary cotinine was analyzed once before cessation of smoking and on days 7 and 17 after cessation of smoking.

Tx-M was analyzed by a stable isotope dilution assay using gas chromatography/negative ion–chemical ionization mass spectrometry as previously described. Briefly, 2 ng of a deuterated internal standard was added to 5-mL aliquots of urine. The samples were then treated with methoxyamine hydrochloride, and the resulting dinor-TxB2 methoxime was adsorbed onto a phenylboronic acid column. After elution, the dinor-TxB2 methoxime was further purified on a reversed-phase Sep-Pac and eluted into ethyl acetate. The dried residue was applied to a straight-phase thin-layer chromatography (TLC) plate and developed in the organic layer of ethyl acetate:acetic acid:hexane and shaken with water (54:12:25:100, v/v/v/v). The area corresponding to 2,3-dinor-TxB2 methoxime was scraped and eluted in ethyl acetate. The organic layer was transferred to another tube, dried, and converted to its pentafluorobenzyl ester. This material was further purified by another straight-phase TLC and developed in the organic layer of iso-octane:ethyl acetate and shaken with water (65:85:100, v/v/v), followed by scraping and elution of the appropriate area on the TLC plate. The derivatization was completed by formation of the trimethylsilyl ether.

Quantitative analysis was accomplished by using a Finnigan Incos 50 mass spectrometer coupled to a Varian 3400 gas chromatograph. The instrument was operated in the negative ion–chemical ionization mode, using methane as the reactant gas and monitoring the mass/charge ratio = 586 for endogenous Tx-M and mass/charge ratio = 590 for the tetra(deuterated) internal standard. Before gas chromatography/mass spectrometry, the trimethylsilyl derivatization mixture was dried under a stream of dry nitrogen and the sample was dissolved in 10 μL hexane, of which 2–5 μL was injected into a splitless injector operated at 250°C. The column oven was kept at 280°C.

PGI-M was also analyzed by a stable isotope dilution assay. In summary, 1 ng of a deuterated internal stan-

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<th>Subject</th>
<th>Age (years)</th>
<th>Height (cm)</th>
<th>Weight (kg)</th>
<th>Estimated lifetime cigarette consumption (×1000)</th>
<th>Present cigarette consumption (daily)</th>
<th>Basal Tx-M (pg/mg)</th>
<th>Basal Day 3 Tx-M (pg/mg)</th>
<th>Basal dTx (pg/mg)</th>
<th>Basal Day 3 dTx (pg/mg)</th>
<th>Plasma cotinine (ng/mL)</th>
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<th>Basal Day 17 Plasma cotinine (ng/mL)</th>
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Tx-M, 2,3-dinor-thromboxane B2; dTx, 11-dehydro-thromboxane B2. Tx-M and dTx are metabolites of thromboxane A2.
Calculations

The rate of change of the urinary excretion of Tx-M after cessation of smoking was calculated as follows. The excretion of Tx-M at time $t$ was assumed to be determined by the formula

$$\text{Tx-M} = c + e^{a(t+b)}$$

where $a$, $b$, and $c$ are constants. The constant $c$ is the subject's basal level of Tx-M, i.e., the excretion in the absence of smoking, and $e$ is the change in Tx-M excretion elicited by smoking. Being negative, $b$ represents the rate of return toward $c$ after cessation of smoking. According to this formula, Tx-M at time zero (i.e., the stable level of Tx-M before cessation of smoking) is $[c+e^0]$, and Tx-M at time $t$ (i.e., the stable level of Tx-M after cessation of smoking) is $[c]$. Applying the same assumptions to the rate of change of the urinary excretion of Tx-M during recovery after intake of ASA yields

$$\text{Tx-M} = c - e^{a(t-b)}$$

where $a$, $b$, and $c$ are constants, in accordance with the above. The constant $c$ is the subject's basal level of Tx-M, i.e., the excretion in the absence of ASA, and $e$ is the change in Tx-M excretion elicited by the intake of a single dose of ASA. The rate of return toward $c$ after the intake of a single dose of ASA is represented by $b$. Tx-M at time zero (i.e., after the intake of a single dose of ASA) is $[c-e^0]$, and Tx-M at time $* \ (i.e., \ the \ stable \ level \ of \ Tx-M \ when \ the \ effect \ of \ ASA \ has \ disappeared)$ is $[c]$. For each subject the values of the constants $a$ and $c$ were determined on the basis of the obtained individual Tx-M excretion values. The constant $b$ was then calculated for each subject to fit the observed recovery phase from $[\text{Tx-M}=c+e^0]$ to $[\text{Tx-M}=c]$ as closely as possible.

Comparison of the absolute values of the constant $b$ for the rate of change after cessation of smoking and during recovery after ASA, respectively, was performed using the Wilcoxon signed rank test. Comparisons of the relative levels of urinary Tx-M and dTx before and after cessation of smoking were also performed using Student's $t$ test for paired means. All tests were two tailed. A value of $p<0.05$ was considered significant. All values are given as mean±SEM.

Results

The mean urinary excretion of Tx-M before cessation of smoking was $548±86 \, \text{pg/mg \ creatinine}$. After cessation of smoking urinary Tx-M dropped to approximately $300 \, \text{pg/mg}$. The fall in excretion was completed in $3$ days; on the third day after cessation, urinary Tx-M was $296±35 \, \text{pg/mg}$ (Figure 1).

Urinary dTx displayed a pattern that closely resembled that of Tx-M. Thus, the basal mean excretion of dTx was $586±41 \, \text{pg/mg}$. It fell to about $350 \, \text{pg/mg}$ after cessation of smoking. The fall in excretion was completed in $3$ days; on the third day after cessation, urinary Tx-M was $355±59 \, \text{pg/mg}$ (Figure 1).

Values of Tx-M and dTx before and on day $3$ after cessation of smoking for each subject are given in Table $1$. Values of cotinine before and on days $7$ and $17$ after cessation of smoking are also given in Table $1$.

The basal urinary excretion of Tx-M before intake of ASA was $338±27 \, \text{pg/mg}$. This value did not differ significantly from the corresponding value on day $19$ after cessation of smoking. On the day after intake, it had dropped to $65±10 \, \text{pg/mg}$. It subsequently increased progressively to reach a stable level of approximately $270 \, \text{pg/mg}$ on day $13$ after intake of ASA (Figure 1).

Urinary dTx was $396±62 \, \text{pg/mg}$ before intake of ASA. This value did not differ significantly from the corresponding value on day $17$ after cessation of smoking. It fell to $79±12 \, \text{pg/mg}$ on the day after intake, to increase subsequently to approximately $390 \, \text{pg/mg}$ on day $19$ after intake of ASA (Figure 1).

While the facilitating effect of smoking on urinary Tx-M or dTx seemed to be completely abolished within $3$–$5$ days after cessation, the inhibitory effect of a single dose of ASA appeared to influence metabolite excretion during a substantially longer period, i.e., up to $13$ days. The difference between the time constants (see “Methods”) for recovery after quitting and recovery after intake of ASA was significant ($p<0.02$ for both Tx-M and dTx), implying that smoking affected platelet function during a shorter period than did ASA.

The relative contribution of Tx-M and dTx to the excretion of thromboxane metabolites in the urine was calculated. Before cessation of smoking, Tx-M accounted for $54±3\%$ of the $\Sigma[\text{Tx-M}+\text{dTx}]$ excretion; dTx accounted for $47±3\%$. Before intake of ASA, i.e., about $25$ days later, Tx-M accounted for $49±4\%$ of the metabolite excretion, and the dTx fraction was $51±4\%$.

The urinary excretion of PGI-M before cessation of smoking was $221±28 \, \text{pg/mg \ creatinine}$. After cessation it displayed no clear-cut changes (Figure 2). Before intake of ASA the urinary excretion of PGI-M was $169±18 \, \text{pg/mg}$. During the following days there was no significant change in urinary PGI-M (Figure 2).

Discussion

The urinary excretion of thromboxane metabolites decreased rapidly after cessation of cigarette smoking in...
FIGURE 1. Line graphs showing urinary excretion of thromboxane A2 metabolites. Upper panel: Urinary excretion of 2,3-dinor-thromboxane B2 (Tx-M, solid circles) in eight healthy female habitual cigarette smokers who quit smoking on day 0. Open circles represent urinary excretion of 11-dehydro-thromboxane B2 (dTx). Lower panel: Urinary excretion of Tx-M and dTx (symbols as in upper panel) after intake of a single 1-g oral dose of acetylsalicylic acid (ASA). The time constants for the decay in excretion of Tx-M and dTx after cessation of smoking (days 0-3) differ (p<0.02 and 0.02, respectively) from the time constants for the increase in excretion of Tx-M and dTx after intake of ASA (days 1-13).

healthy women in the present study, whereas the excretion of the prostacyclin metabolite was not affected. The effect of smoking on thromboxane metabolite excretion was more short-lasting than that elicited by ASA.

Previous studies in our laboratory have suggested that the increase in excretion of Tx-M elicited by cigarette smoking disappears upon cessation. Thus, in a population study of healthy 18-19-year-old men, former cigarette smokers did not excrete more Tx-M than did lifelong nonsmokers.13 Similar signs of a reversible effect of smoking on Tx-M excretion were recently obtained in a study on smoking and nonsmoking women aged 18-59 years.27 Controlled studies on the change in Tx-M excretion immediately after cessation of smoking have, however, not been reported. Tx-M and dTx are metabolites of TxA2.28 Their excretion in the urine mainly reflects the formation of TxA2 in the platelets.29 The excessive excretion of Tx-M in smokers is mainly platelet derived: a 20-mg dose of ASA, which selectively inhibits platelet cyclooxygenase, abolished the difference in Tx-M excretion between smokers and nonsmokers.13 In addition, after intake of ASA the recovery of Tx-M excretion to pretreatment levels paralleled that of platelet cyclooxygenase func-
tion. It therefore seems reasonable to assume, by analogy, that the increased excretion of $\text{TxA}_2$ metabolites in the present healthy women was also of platelet origin.

More than 95% of the $\text{TxA}_2$ formed is metabolized to degradation products before excretion in the urine. Among these products, $\text{Tx-M}$ and d$\text{Tx}$ are the most abundant. $\text{Uedelhoven et al}^{39}$ recently demonstrated that smoking increased the fraction of $\text{TxA}_2$ undergoing $\beta$-oxidation to $\text{Tx-M}$ threefold and decreased the fraction subjected to dehydrogenation to d$\text{Tx}$ by about 50%. Their observation was not supported by our data, in which the fractions being $\beta$-oxidized and dehydrogenated, respectively, were the same before and after quitting. The differences between their data and ours may be explained by the apparent intersubject differences in cigarette use; their smokers used an average of 28 cigarettes/day, whereas ours smoked less than half that number.

The method applied in the present study to characterize the effect of smoking on platelet formation of $\text{TxA}_2$ is to compare it with the effect elicited by ASA. ASA inhibits platelet formation of $\text{TxA}_2$ by irreversible acetylation of cyclooxygenase. Hence, the recovery of platelet $\text{TxA}_2$ formation after a single dose of ASA reflects the appearance of newly formed platelets. Any change in platelet $\text{TxA}_2$ formation having another time constant than that appearing after intake of a single dose of ASA must consequently be based on another mechanism than irreversible influence on cyclooxygenase. Certainly, smoking increases platelet $\text{TxA}_2$ formation, whereas ASA inhibits this formation. Despite these opposite effects, their respective time constants provide information concerning their respective mechanisms of action. Hence, a time constant for return from a stimulated level to a basal level (such as after cessation of smoking) that differs from a time constant for return from an inhibited level to a basal level (such as after intake of a single dose of ASA) indicates that the observed effects differ not only qualitatively, i.e., concerning stimulation versus inhibition, but also quantitatively, i.e., concerning time to complete reversal. Quitting smoking resulted in a drop in $\text{TxA}_2$ formation that was completed in about 3 days in the present women. In contrast, the effect of ASA had not disappeared completely until after about 13 days. Hence, smoking does not irreversibly affect platelet formation of $\text{TxA}_2$. The present data do not allow further conclusions with respect to the mechanism behind the effect of smoking on platelet $\text{TxA}_2$ formation. We have previously demonstrated that platelet lifetime is not shortened in smokers compared with nonsmokers and hence, that the platelets appear instead to be chronically activated by some factor related to the smoking habit. Since the rate-limiting factor in eicosanoid synthesis is the availability of its precursor, arachidonic acid, present and previous data are compatible with an effect of smoking on the platelet membrane. Such an effect of smoking might be to reversibly enhance acyl hydrolase-induced mobilization of arachidonic acid from the membrane phospholipids. Other possibilities must, however, be considered as well.

The excretion of $\text{Tx-M}$ and d$\text{Tx}$ decreased after cessation of smoking without any parallel decay in the excretion of PGI-M. The latter is a metabolite of prostacyclin, and the urinary excretion of PGI-M accurately reflects the formation of prostacyclin in the vascular endothelium. Several states of acute and chronic cardiovascular diseases are characterized by increased urinary excretion of both $\text{Tx-M}$ and PGI-M, probably reflecting increased interaction between platelets and the injured vessel walls. The current women smokers had no clinically evident signs of cardiovascular disease. Hence, it may be assumed that vascular injury, if at all, was present only to a very limited, subclinical degree. In harmony with this assumption, the excretion of PGI-M in these subjects did not decrease after cessation of smoking. Hence, it seems that platelet activation is present in the absence of an increased platelet--vessel wall interaction.

In summary, our data indicate that cigarette smoking elicits a facilitating effect on platelet $\text{TxA}_2$ formation in the absence of vascular injury. This effect is reversible within the lifetime of the platelets.

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References

38. Curvall M, Enzell CR: Monitoring absorption by means of determina-
Excretion of thromboxane metabolites in healthy women after cessation of smoking.
C Rångemark, G Ciabattoni and A Wennmalm

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