Br. J. Pharmac. (1984), **81**, 055–060

Effect of nicotine on the formation of prostacyclin-like activity and thromboxane in rabbit aorta and platelets

Pawel Alster & Åke Wennmalm

Department of Clinical Physiology, Huddinge University Hospital, S-141 86 Huddinge, Sweden

1 The effect of nicotine on the bioformation of prostacyclin (PGI_2) and of thromboxane (Tx)B_2 in rabbit aorta and platelets, respectively, was investigated.

2 Rabbit aortic rings were incubated with [^{14}C]-arachidonic acid ([^{14}C]-AA) and the incubation products were separated with thin layer chromatography (t.l.c.). Alternatively, the aortic rings were incubated without substrate and their spontaneous formation of platelet anti-aggregatory activity was measured. Rabbit platelet microsomes were incubated with [^{14}C]-AA and the products formed were separated with t.l.c.

3 Rings of aorta were found to be incapable of converting added [^{14}C]-AA to labelled 6-keto-$PGF_{1\alpha}$ (the stable hydrolysis product of PGI_2). Rings of aorta incubated in saline medium spontaneously formed PGI_2-like activity. This formation was dose-dependently inhibited by nicotine, with an I_{50} of about 10^{-4} M.

4 Platelet microsomes converted [^{14}C]-AA to labelled TxB_2. This formation was unaffected by nicotine.

5 It is concluded that a true difference in sensitivity to nicotine exists between cyclo-oxygenase in rabbit aorta and platelets. The data also demonstrate a tissue difference between rabbit aorta and platelets concerning their utilization of exogenous AA as substrate in the formation of platelet active compounds.

Introduction

The initial oxygenation of arachidonic acid (AA), catalyzed by fatty acid cyclo-oxygenase, produces prostaglandin endoperoxides (PGG_2/PGH_2), the immediate precursor of prostacyclin (PGI_2), thromboxane (Tx)A_2 and the primary prostaglandins. Prostacyclin inhibits platelet aggregation, whereas thromboxane A_2 induces such aggregation (Hamberg, Svensson & Samuelsson, 1975; Bunting, Gryglewski, Moncada & Vane, 1976). It has been suggested that the aggregatory state of the platelets is regulated by a delicate balance between the formation of proaggregatory TxA_2 and anti-aggregatory PGI_2 (Moncada, Korbut, Bunting & Vane, 1978).

Earlier investigations have demonstrated that the formation of PGI_2 by isolated specimens of vascular tissue is decreased in the presence of nicotine. Thus, in the pulsatingly perfused rat aorta the efflux of PGI_2-like activity was counteracted by nicotine and a similar effect of the alkaloid was observed in our laboratory after incubation of rabbit aorta or human veins in a saline medium (Ten Hoor & Quadt 1979; Sonnenfeld & Wennmalm, 1980). The action of nicotine has recently been shown to be based on competitive inhibition of cyclo-oxygenase (Alster & Wennmalm, 1983).

In contrast to the inhibitory action of nicotine in animal vascular tissue, the compound fails to affect the conversion of AA to TxB_2 in microsomal fractions of humans platelets, although such formation also requires the enzyme cyclo-oxygenase (Alster & Wennmalm, 1981a). In the present study we have simultaneously investigated the effect of nicotine on the formation of PGI_2 and TxB_2 in rabbit aorta and platelets, in order to find out whether the above-mentioned differences in sensitivity to nicotine between the PGI_2 and TxA_2 synthesis pathways was based on inter-species or inter-tissue differences.

Methods

Aortic ring experiments

Rabbits of either sex and mixed strains, weighing 2.0–3.0 kg, were used. They were killed by cervical dislocation and exsanguinated via the left carotid

artery. The thoracic aorta was rapidly dissected free and rinsed in chilled buffered saline. The cleaned aorta was then cut into rings, approximately 2 mm wide and weighing about 5 mg. Equal numbers of rings were used in two parallel experiments, namely for incubation with [^{14}C]-AA and for assessment of the spontaneous formation of PGI_2.

Incubation of aortic rings with [^{14}C]-arachidonic acid

Incubation of rabbit aortic rings, weighing 30–50 mg (6–10 rings), was carried out in 1.2 ml Tris buffer (0.05 M, pH 8.0) containing 1 mM EDTA and 0.15 mM NaCl. [^{14}C]-AA (New England Nuclear, sp.act. 56 mCi mmol^{-1}) was converted into its sodium soap by mixing with Na_2CO_3 solution (0.15 M) and diluting with a small volume of Tris buffer. About 0.8 μCi of ^{14}C-arachidonate (final substrate concentration about 2×10^{-6} M) was used in each incubation.

Nicotine, obtained from the chemical analysis laboratory at the Swedish Tobacco Company, was dissolved in saline (0.15 M) before each experiment and added to the Tris buffer mixture to produce final concentrations of 2×10^{-7}–2×10^{-4} M.

The rings were pre-incubated with nicotine in the medium for 1 min before addition of [^{14}C]-AA. After substrate had been added, the reaction mixture was incubated at 37°C for 15 min. Controls, in which the same amounts of tissue were incubated without nicotine, were also performed. Aliquots (20 μl) of the reaction mixture were spotted on thin layer chromatography (t.l.c.) plates (0.02 mmDC Plastikfolien, Kieselgel 60, Merck) under a stream of nitrogen. Standards of AA (Sigma), PDG_2, PGE_2, $PGF_{2\alpha}$, and 6-keto-$PGF_{1\alpha}$ (Upjohn Co) were also spotted, in a different lane. The plates were developed 18 cm in the organic phase of the solvent mixture: ethyl acetate, acetic acid, 2:2:4 trimethylpentane, water (110:20:50:100 v/v, Flower, Cheung & Cushman, 1973). After development the t.l.c. plates were cut into 36 0.5 cm horizontal strips. The radioactivity of these strips was measured individually in an LKB Wallac liquid scintillation spectrometer.

Studies on aortic ring spontaneous formation of prostacyclin-like activity

Rabbit aortic rings, 30–50 mg (6–10 rings), were incubated for 45 min at room temperature in a modified Tyrode solution (0.1 ml mg^{-1} tissue) of the following composition (mM): NaCl 137, KCl 2.7, $MgCl_2$ 1.0, $NaHCO_3$ 12, NaH_2PO_4 0.4 and glucose 5.6. Incubation was stopped by removing the rings from the medium. The medium was subsequently put on ice.

In one series of experiments nicotine (2×10^{-7}–2×10^{-4} M) or indomethacin (Merck, Sharp & Dohme, 2×10^{-8} and 2×10^{-6} M) was added to the medium before incubation; control incubations without drug were also performed. A small volume (20–50 μl) of the medium was added to an aggregometer tube, containing 0.5 ml of a human platelet-rich plasma (HPRP)-Tris buffer mixture, and 90 s later platelet aggregation was induced by addition of 2 μg of adenosine-5′-diphosphate (ADP, Sigma Chemicals).

In another series, the effect of addition of adenosine deaminase (ADA, Sigma Chemicals) on the anti-aggregatory effect of the incubate was studied. The anti-aggregatory effect of PGI_2 was analysed as above, but the incubate/HPRP mixture was added with 1 iu of ADA 2 min before the induction of aggregation with ADP. An external standard of adenosine was used to check the efficiency of ADA.

The aggregation of platelets was recorded in a Payton Dual Channel aggregometer. HPRP was prepared from peripheral venous blood obtained from volunteers who had not taken aspirin-like drugs for at least 10 days. Before use, the HPRP was diluted with an equal volume of 0.1 M Tris buffer pH 7.4.

Platelet experiments

Rabbits of either sex (2.0–3.0 kg) were anaesthetized with an injection of pentobarbitone sodium (Mebumal, 60 mg kg^{-1}). Blood was withdrawn from a cannulated carotid artery. It was collected in plastic tubes containing 1 ml of 0.15 M disodium edetate (Na-EDTA) and immediately centrifuged for 15 min at 200 *g*. The platelet-rich plasma thus obtained was recentrifuged for 15 min at 650 *g* to give a platelet pellet. Platelet pellets from 200 ml blood were resuspended in 0.1 M phosphate buffer, pH 7.4, and sonicated at 0°C, using an Ultrasonic A180G: six 5 s treatments separated by 1 min intervals for cooling. The microsomal fraction sedimenting between 9000 *g* × 12 min and 100,000 *g* × 90 min was resuspended in 10 ml of 0.1 M phosphate buffer, pH 7.4. Each such microsomal suspension was divided into four incubation portions. The portions were pre-incubated with nicotine (2×10^{-7}–2×10^{-4} M) or indomethacin (2×10^{-10}–2×10^{-7} M) for 1 min at 37°C. Subsequently 0.25 μCi of [^{14}C]-Na-arachidonate was added (final substrate concentration about 2×10^{-7} M) and the incubation was continued for another 2 min at 37°C before being terminated by addition of 25 ml of methanol. The methanol-containing incubation mixture was further diluted with 25 ml of water and after acidification with acetic acid to pH 4.3, the mixture was extracted twice with an equal volume of ether. After evapora-

tion of the organic phase, the residue was reconstituted in 0.2 ml of ethanol and submitted to t.l.c. Chromatographic separation was performed, using Fertigplatten Kieselgel F254 (Merck) in solvent benzene : dioxane : acetic acid (60 : 30 : 3, v/v), against unlabelled standards of AA, TxB_2, PGD_2, PGE_2 and $PGF_{2\alpha}$. Radioscans of the chromatograms were developed with a Berthold Dünnschickt Scanner. The areas in the radiochromatograms corresponding to the authentic standards of AA and TxB_2 were scraped off and eluted in 2 ml of ethanol. The radioactivity in the eluted fractions was monitored in a LKB Wallac liquid scintillation spectrometer.

Calculations

The effect of nicotine on the spontaneous formation of platelet anti-aggregatory activity in the aortic ring incubations was calculated as follows: a dose-response curve for the anti-aggregatory effect of authentic PGI_2 on ADP-induced aggregation was established for each batch of HPRP. This curve was used to estimate the amount of platelet anti-aggregatory activity (expressed as PGI_2 equivalents) formed during the incubation of aortic rings in the absence or presence of nicotine.

The effect of nicotine on the formation of [^{14}C]-TxB_2 by the platelet microsomal fractions was calculated as follows: the ratio between the amount of radioactivity in the eluted fractions corresponding to TxB_2 and the total radioactivity was calculated within each chromatogram. The ratio thus obtained in chromatograms from incubations without nicotine was used as control and the ratio obtained in chromatograms from incubations with nicotine was expressed as a percentage of this control.

Results

Aortic ring experiments

Incubation of aortic rings with [^{14}C]-arachidonate When rabbit aortic rings were incubated with [^{14}C]-arachidonate, no apparent conversion of substrate took place, as evidenced by the lack of radiopeaks, apart from authentic [^{14}C]-AA, in the eluted fractions of the t.l. chromatograms.

Studies on aortic ring spontaneous formation of prostacyclin-like activity Incubation of rabbit aortic rings in Tyrode solution at room temperature resulted in formation of PGI_2-like (anti-aggregatory) activity in the medium (Figure 1), at an estimated rate of about 80 pg g^{-1} tissue min^{-1} (Table 1). In the presence of nicotine, this formation was dose-dependently inhibited (Figures 1 and 2). The I_{50} of nicotine was about 10^{-4} M. Indomethacin was a more potent inhibitor of the formation of anti-aggregatory activity than nicotine, with an I_{50} of about 10^{-6} M (Figure 2).

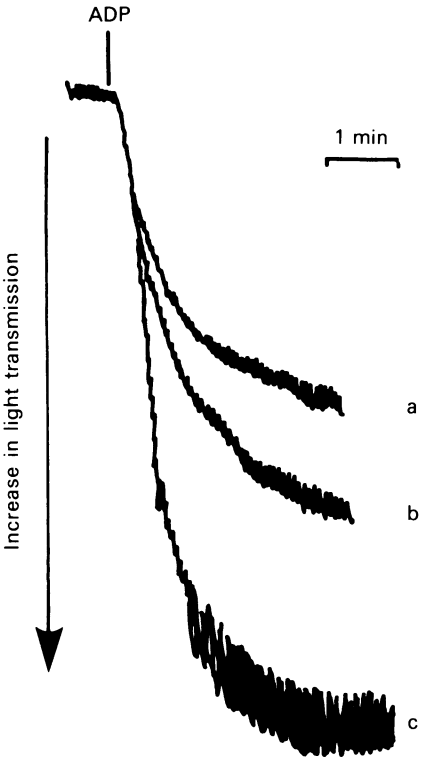

Figure 1 Typical aggregation recordings, displaying the inhibitory effect of aortic ring incubates on ADP-induced platelet aggregation in a mixture of human platelet-rich plasma and Tris buffer (HPRP/Tris), and the attenuating effect of nicotine on this inhibition. In (a) 30 µl of the medium resulting from incubation for 45 min of rings of rabbit aorta in Ca-free Tyrode solution (0.1 ml mg^{-1} tissue) were added to 0.5 ml of HPRP/Tris and 90 s later ADP was added. In (b) the procedure was identical except that nicotine (2×10^{-4} M) was present in the medium during the incubation. Recording (c) is a control without incubate added.

Addition of 1 iu of adenosine deaminase (ADA) to the aortic ring incubate did not cause any change in its content of anti-aggregatory activity; the minor variations in the amounts of anti-aggregatory activity found in incubations with compared to without ADA were not statistically significant (Table 2). External standards of adenosine, upon incubation with ADA, completely lost their anti-aggregatory activity.

Table 1 Effect of nicotine and of indomethacin on the spontaneous formation of prostacyclin (PGI_2)-like (anti-aggregatory) activity formed by rabbit aortic rings incubated in a saline medium (pg g^{-1} tissue min^{-1})

Control	*Nicotine*				*Indomethacin*		
	2×10^{-7}	2×10^{-6}	2×10^{-5}	2×10^{-4}	2×10^{-8}	2×10^{-7}	2×10^{-6}
82±3	74±1	68±1	56±1	40±2	64±3	52±2	40±5
$n=9$	$n=5$	$n=5$	$n=6$	$n=7$	$n=4$	$n=2$	$n=5$

Values are mean ± s.e.mean.

Platelet experiments

Incubation of [^{14}C]-arachidonate with rabbit platelet microsomes resulted in the appearance of a radiopeak co-chromatographing with authentic TxB_2 in the chromatograms. The presence of nicotine (2×10^{-7}–2×10^{-4} M) in the medium during the incubations did not change the pattern of the chromatograms in comparison to controls, indicating that the drug did not affect the metabolism of [^{14}C]-arachidonate in the rabbit platelet microsomes (Figure 2). The lack of effect of nicotine on the formation of [^{14}C]-TxB_2 from labelled arachidonate is in contrast to the effect elicited by indomethacin in similar incubations. At a concentration of 2×10^{-9} M, this drug inhibited the formation of [^{14}C]-TxB_2 by about 32%, and at 2×10^{-7} M more than 80% inhibition was observed (Figure 2).

Discussion

In the present study the amounts of PGI_2-like activity spontaneously formed upon incubation of the aortic rings were assessed in terms of capacity to inhibit platelet aggregation. In addition to PGI_2, vascular tissue can form another compound with platelet anti-aggregatory activity, i.e. adenosine (Born & Cross, 1963). To exclude the possibility that the biological activity of the current incubates to any extent was due to such formation of adenosine, some incubates were treated with ADA, an enzyme that converts adenosine to is biologically inactive metabolite, inosine. No loss of anti-aggregatory activity was found following such treatment, indicating that adenosine did not contribute to the anti-aggregatory activity of the incubates. We therefore feel entitled to conclude that the platelet inhibitory effect of the incubates was due to PGI_2 formed by the aortic rings.

Table 2 Effect of treatment with adenosine deaminase on the content of prostacyclin (PGI_2)-like (anti-aggregatory) activity in incubates of rabbit aortic rings

	Content of PGI_2-like activity in incubate (ng ml^{-1})	(% of control)
Control	0.42±0.02 $n=4$	—
Adenosine deaminase	0.40±0.03 $n=4$	95±7 $n=4$

Adenosine deaminase did not affect the content of anti-aggregatory activity in the incubates. Data are presented as mean ± s.e.

The formation of anti-aggregatory activity was found to be dose-dependently decreased by nicotine. This observation is in accordance with earlier data on the effect of nicotine on PGI_2 synthesis in vascular tissue (TenHoor & Quadt, 1979; Sonnenfeld & Wennmalm, 1980; Alster & Wennmalm, 1983). The I_{50} of nicotine was about 10^{-4} M. In similar incubations, the I_{50} of indomethacin, an established inhibitor of cyclo-oxygenase, was about 4×10^{-7} M. This relation between the inhibitory efficiencies of nicotine and indomethacin is of the same order as that observed earlier (Alster & Wennmalm, 1981b).

The current data do not allow any definite conclusions concerning the mechanism behind the inhibitory effect of nicotine. However, on the basis of earlier studies in our laboratory, which indicate that cyclo-oxygenase is the only target for nicotine's inhibitory effect (Alster & Wennmalm, 1983), it seems likely that this was also the case in the present experiments.

In contrast to its inhibitory effect on the appearance of PGI_2-like activity in the aortic ring incubates, nicotine was completely unable to inhibit the formation of TxB_2 in platelet microsomes. This lack of effect on platelet formation of TxB_2 is analoguous to that observed in human platelets (Alster & Wennmalm, 1981a). One possible basis for the difference in effect of nicotine on aortic rings and platelet microsomes could be that nicotine, when inhibiting the formation of aortic PGI_2, interferes with an enzyme not present in platelets, i.e. PGI_2 synthetase. For reasons discussed above, this possibility seems rather unlikely. Another basis for the difference in effect could be that nicotine failed to reach the enzymatic sites in the platelets, and so was unable to inhibit the formation of TxB_2. This explanation is also unlikely, since in the platelet experiments micro-

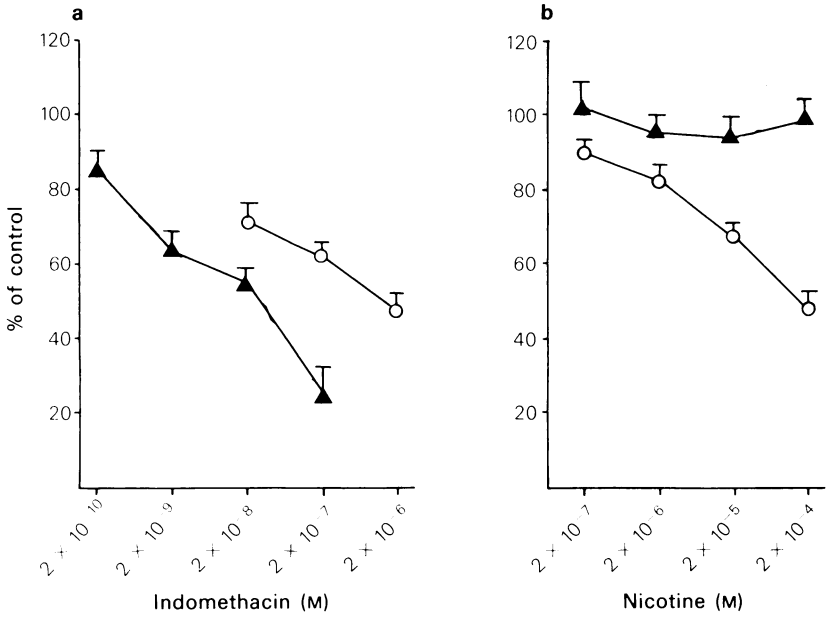

Figure 2 Dose-response curves demonstrating the effect of indomethacin (a) and nicotine (b) on the spontaneous formation of prostacyclin (PGI_2)-like (anti-aggregatory) activity by incubated rings of rabbit aorta (○) and on the formation of [^{14}C]-thromboxane (Tx)B_2 from [^{14}C]-arachidonic acid by platelet microsomes (▲). As is evident from the curves, indomethacin inhibited both the formation of PGI_2-like activity and TxB_2, while nicotine impaired only the formation of PGI_2-like activity.

somes were used, implying that the enzymatic sites were, if anything, more accessible to nicotine than those in the aortic experiments, in which specimens of intact tissue were used. It rather seems that a true tissue difference in sensitivity to the inhibitory action of nicotine exists between the cyclo-oxygenase in rabbit aorta and platelets. In this context it is worth noting that a reversed difference in cyclo-oxygenase sensitivity between TxA_2- and PGI_2-forming cells, compared to the present experiments, has been reported in humans ingesting low doses of aspirin: their platelets are more sensitive to the drug than their vascular endothelium (FitzGerald, Maas, Lawson, Oates, Roberts & Brash, 1982; Patrignani, Filabozzi & Patrono, 1982). Both those studies, however, concerned human tissues investigated *in vivo*; a closer comparison with the present data is therefore not possible.

An incidental observation of significance also resulted from the present experiments. For reasons of comparison, we incubated both aortic tissue and platelets with [^{14}C]-AA, in order to obtain conversion of this substrate to labelled 6-keto-$PGF_{1\alpha}$ and TxB_2, respectively. Using the same type of substrate for incubation, we found that the platelet microsomes readily converted [^{14}C]-AA to labelled TxB_2, while the aortic rings did not convert [^{14}C]-AA to chromatographically distinguishable amounts of any other compound. This could certainly be due to the fact that the substrate penetrated the platelet microsomes more easily than the aortic cell membranes (cf. above). In this connection, however, it should be noted that guinea-pig aortic tissue in similar incubations converted [^{14}C]-AA to labelled 6-keto-$PGF_{1\alpha}$ (Alster & Wennmalm, unpublished observation).

In conclusion, the present investigation strongly suggests that a true tissue difference in sensitivity to nicotine exists between cyclo-oxygenase in rabbit aorta and platelets. Whether this difference is of importance in relation to nicotine absorbed in smoking humans deserves further investigation.

This study was supported by the Council for Tobacco Research-USA, Inc., by the Swedish Tobacco Company, and by Stiftelsen Clas Groschinskys Minnesfond.

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(*Received April 27, 1983.*
Revised August 8, 1983.)