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Potentiation by adrenaline of human platelet activation and the inhibition by the alpha-adrenergic antagonist nicergoline of platelet adhesion, secretion and aggregation

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Abstract

Adrenaline (1 to 10μM) can induce the aggregation of human platelets suspended in citrated plasma but does not induce the aggregation of washed human platelets at doses as high as 1 mM, although these platelets respond normally to ADP, PAF-acether, collagen, arachidonic acid, thrombin, the endoperoxide analog U-46619 and the Ca^{2+} ionophore A23187. Adrenaline (0.5 μM) potentiates the aggregation and secretion induced by all the previous agonists in citrated platelet-rich plasma (cPRP) or in washed platelets. The activation by adrenaline of human platelets is mediated by alpha 2-adrenergic receptors, as demonstrated by inhibition with a series of adrenergic antagonists. The alpha-adrenergic antagonist nicergoline inhibits the activation of human platelets by adrenaline in the following situations: i) nicergoline inhibits the aggregation and secretion caused by adrenaline in cPRP (IC_{50} 0.22 μM and 0.28 μM respectively); ii) nicergoline inhibits the aggregation and secretion induced by the combination of adrenaline and each aggregating agent listed above in cPRP (IC_{50} ranging from 0.1 to 2.5 μM) or in washed platelets (IC_{50} ranging from 0.1 to 0.8 μM); iii) nicergoline inhibits the biding of ^{3}H-yohimbine to washed human platelets (IC_{50} 0.26μM); iv) the intravenous administration of nicergoline (0.5 mg/kg per day) to patients inhibits significantly the *ex vivo* response of their platelets to adrenaline in cPRP. High concentrations of nicergoline also inhibit the aggregation and secretion induced by the aggregating agents listed above in cPRP (IC_{50} range 108 to 670 μM) and in washed platelets (IC_{50} range 27 to 140 μM) and the adhesion of platelets to collagen-coated surfaces. This latter effect is not mediated through blockade of alpha-adrenoceptors. A possible role of adrenaline in platelet activation *in vivo* could justify the use of nicergoline (Sermion®), an alpha-adrenergic antagonist in combination therapy to prevent arterial thrombosis.

Introduction

As early as 1963 [1], it was shown that adrenaline can induce the aggregation of human platelets suspended in citrated plasma. Adrenaline-induced aggregation has specific characteristics: i) it is not preceded by shape change; ii) adrenaline fails to aggregate platelets from a number of human donors and from most mammalian species [2]; iii) platelets suspended in hirudin anticoagulated plasma and washed platelets do not aggregate in the presence of adrenaline [3, 4]. Under these conditions, adrenaline is able to activate human platelets by potentiating the aggregation induced by other agonists like ADP, PAF-acether, collagen, thrombin and arachidonate [2, 5].

The development of tritiated ligands has made possible the study of the binding of adrenergic agonists and antagonists to human platelets. These studies have shown that human platelet adrenergic receptors are essentially of the alpha 2-type [5–7].

Specific alpha 2-adrenergic antagonists or mixed alpha 1 + alpha 2-adrenergic antagonists can selectively inhibit the aggregation induced by adrenaline in cPRP, the proaggregatory effect of adrenaline and the binding of tritiated alpha-adrenergic ligands to human platelets[2,5]. There is less information on the effect of these alpha-adrenergic antagonists on the *in vivo* effect of adrenaline on human platelets. There is no direct evidence of the *in vivo* effect of adrenaline on blood platelets, but it could exert a proaggregatory effect in some pathological conditions where the level of adrenaline is increased, e.g. stress [8], pheochromocytoma [9], coronary artery disease [10, 11], essential hypertension [12] and myocardial infarction [13].

It therefore seems of interest to study in detail the effects on platelet function of an alpha-adrenergic antagonist, nicergoline (Sermion®), which is already used in clinical situations. We report here the effects of nicergoline on the activation of human platelets by several agonists in

the presence or absence of adrenaline, on the binding of ^{3}H-yohimbine to alpha 2-adrenergic receptors of intact platelets and on the adhesion of platelets to collagen. The *ex vivo* effects of nicergoline on platelet aggregation were also studied after intravenous administration of Sermion® to patients.

Materials and methods

Drugs and reagents

(0-methyl-^{3}H)-yohimbine (85 Ci/mmol), 5-hydroxy (G-^{3}H) tryptamine creatinine sulphate (13 Ci/mmol), sodium ^{51}Chromate, ^{111}Indium oxinate were purchased from the Radiochemical Center, Amersham, U.K. The purity of ^{3}H-yohimbine was checked by thin layer chromatography on silica gel in chloroform: methanol: 880 ammonia (96:4:1) and ranged from 92 to 98%.

Adenosine 5'-diphosphate (ADP) sodium salt n°A-6127, (–) adrenaline bitartrate, DL-propranolol hydrochloride, collagen from bovine tendon type I n° C-9879, arachidonic acid sodium salt and diisopropylfluorophosphate (DFP) were purchased from Sigma, St Louis, Mo, U.S.A.

The following chemicals or drugs were kindly given or purchased from the following companies: nicergoline bitartrate (Spécia, Paris, France), phentolamine hydrochloride (Ciba-Geigy, Basel, Switzerland), prazosin hydrochloride (Pfizer, New York, N.Y., U.S.A.), yohimbine hydrochloride (Coopération Pharmaceutique Française, Paris, France), paraformaldehyde (Prolabo, Paris, France), calcium ionophore A23187 (Boehringer, Mannheim, Germany), PAF-acether (Bachem, Bubendorf, Switzerland), endoperoxide analog U-46619 and prostacyclin, PGI_2 (Upjohn, Kalamazoo, Mi, U.S.A.), bovine thrombin (Hoffmann-la Roche, Basel, Switzerland), human fibrinogen (Kabi, Stockholm, Sweden).

Preparation of human platelets

The techniques used to separate and isolate human platelets from blood by centrifugation have been previously described in detail [14]. Briefly, blood was collected by venipuncture from healthy blood donors who denied having taken any drugs in the previous 8 to 10 days.

Citrated platelet-rich plasma (cPRP) was prepared from blood collected in sodium citrate dihydrate (final concentration 0.38%). Blood was centrifuged at 175 g for 15 min, cPRP was decanted, platelets were counted with a Baker A 810 platelet automatic analyzer (Baker Instruments Co., Allentown, Pa, U.S.A.) and platelet count was adjusted to 300,000 per mm^3 by dilution with autologous citrated platelet-poor plasma.

Human platelets were also isolated from acid-citrate-dextrose (ACD) anticoagulated blood by differential centrifugation and washed at 37°C according to a modification [14] of the method described by Mustard [15]. The modifications are the use of 0.35% purified human albumin (Centre Régional de Transfusion Sanguine, Strasbourg, France) to replace bovine albumin and the inclusion of 1 μM PGI_2 in the washing buffer. The final suspension of washed human platelets was kept at 37°C, pH 7.3, in Tyrode's solution containing 0.35% human albumin, 5 mM HEPES and apyrase. Platelet count was adjusted to 300,000 per mm^3.

Binding of ^{3}H-yohimbine to intact washed human platelets

The binding of ^{3}H-yohimbine to platelets was measured as previously described [5]. Briefly, intact washed human platelets (300,000/mm^3) suspended in Tyrode's solution containing 0.35% human albumin and apyrase were incubated 10 min at 37°C with 7.5 nM ^{3}H-yohimbine with and without 10 μM phentolamine to estimate non specific binding. The incubation was terminated by rapidly diluting 250 μl of the platelet suspension in 5 ml of cold (4°C) Tyrode's solution containing 0.35% human albumin. Diluted platelets were then filtered on Whatman glass-fiber (GF/F). The filters were rinsed with another 5 ml of cold buffer. The filters were transfered into vials containing 5 ml of liquid scintillation fluid (ACS II, Amersham, U.K.). The radioactivity was counted in a beta-scintillation radioactivity counter (Minibeta 1211, LKB, Turku, Finland). Specific binding represented 40 to 60% of total binding. Each determination is the mean of 2 to 3 separate measurements.

Platelet aggregation studies *in vitro*

Aggregation of human platelets in citrated plasma or of washed platelets was measured at 37°C by a turbidimetric method in a dual-channel Payton aggregometer (Payton Associates, Scarborough, Ontario, Canada). Aggregation was studied exactly as previously described [14] in 0.5 ml platelet samples. The aggregation of washed human platelets was tested in the presence of human fibrinogen pretreated with DFP [14]. When thrombin was the aggregating agent, the addition of fibrinogen to the platelet suspension was omitted. In order to study the inhibition of the potentiating effect of adrenaline on aggregation, the drugs were added every 30 s in the following order: fibrinogen (only with washed platelets), adrenergic antagonist, adrenaline, aggregating agent.

Platelet aggregation studies *ex vivo*

The effects of intravenous administration of nicergoline were studied in 6 patients with peripheral arterial disease of the lower limbs who had not received any concurrent drug. This study was performed before surgery. Nicergoline (Sermion®, Spécia, Paris, France) was perfused intravenously, twice a day at 12 hr intervals for 3 days at a total dose of 0.5 mg/kg body weight per day. The duration of the perfusion was 3 hrs. During that time the blood pressure and the pulse rate were monitored. Blood (30 ml) was collected to prepare cPRP for platelet aggregation studies, before treatment and 3 days after the last intravenous administration of Sermion®.

Platelet secretion of ^{3}H-5HT

Platelets were incubated with ^{3}H-5 hydroxytryptamine (^{3}H-5HT) (0.2 μCi per ml of collected blood) either in cPRP, 15 min at 22°C, or during the first washing step, 15 min at 37°C. Secretion of serotonin was measured during aggregation. Three minutes after addition of the agonist, 100 μl of 10% paraformaldehyde was added to block instantaneously further secretion and reuptake of ^{3}H-5HT[16]. The content of the aggregation cuvette was transferred into a polypropylene conical Eppendorf tube and centrifuged 1 min at 8000 g. A 100 μl aliquot of the supernatant was removed, mixed with 2.5 ml of ACS II and counted in a Minibeta 1211 (LKB). The amount of secretion is expressed as a percentage of the total radioactivity present in labelled non stimulated platelets.

Platelet adhesion to a collagen-coated glass surface

The adherence of ^{51}Cr or ^{111}In-oxine-labeled platelets to a collagen-coated glass surface was measured as previously

described [17, 18]. Glass rods coated with collagen were rotated at 37°C at 200 rpm for 5 min in human citrated blood (hematocrit 47%, 140,000 platelets per mm^3) or in a suspension of washed human platelets (hematocrit 40%, 180,000 platelets per mm^3).

Analysis of data

The IC_{50} for inhibition of ^{3}H-yohimbine binding to intact platelets, i.e. the concentration required to inhibit binding by 50%, was calculated from experiments where the concentration of the radioligand used corresponded to its K_D. The IC_{50} for platelet aggregation or secretion was the concentration that inhibited by 50% the difference in maximum aggregation or secretion in response to an agonist plus adrenaline, minus the effect in response to the agonist alone. The IC_{50} were calculated graphically using at least 5 different concentrations of antagonists. Means were compared using Student's t test.

Results

Effects of nicergoline on human platelet aggregation and ^{3}H-5HT secretion in citrated plasma

The *in vitro* inhibitory effect of nicergoline on the aggregation of human platelets in cPRP is selective and markedly dependent on the nature of the aggregating agent used. Nicergoline inhibited the aggregation and the secretion of ^{3}H-5HT induced by PAF-acether, collagen, the calcium ionophore A23187, the endoperoxide analog U-46619, ADP and arachidonic acid at IC_{50} values ranging from 108 to 670 μM. These values are only an estimate, because nicergoline tended to form a hazy precipitate above 100 μM.

Adrenaline at a concentration of 4.5 μM induced, without shape change, a first wave of aggregation, followed by a second wave of aggregation leading to a 20 to 60% increase in light transmission. During the second wave of aggregation, platelets secreted 40 to 70% of their contents of ^{3}H-5HT (Fig. 1). Nicergoline inhibited the aggregation (IC_{50} 0.22 μM) and ^{3}H-5HT secretion (IC_{50} 0.28 μM) induced by 4.5 μM adrenaline (Table 1).

When adrenaline was used at a low concentration (0.4 μM), it induced a first wave of aggregation of small amplitude (2 to 7% increase in light transmission) and secretion of 15 to 30% of the total platelet ^{3}H-5HT. At this concentration, adrenaline (0.4 μM) greatly potentiated (2 to 10-fold) the aggregation and ^{3}H-5HT secretion induced by low concentrations of several agonists such as PAF-acether, ADP, collagen, A23187, arachidonic acid and U-46619 (Fig. 2). Nicergoline inhibited the potentiating effect of adrenaline on all the aggregating agents tested, with IC_{50} values ranging from 0.1 to 2.5 μM (Table 2).

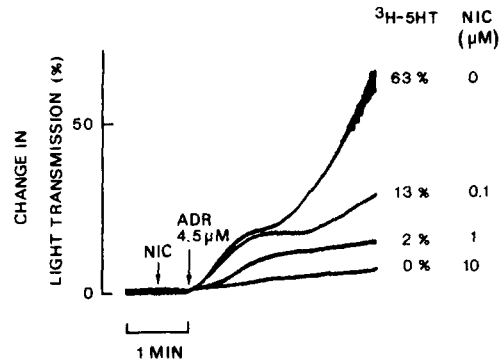

Figure 1
Effect of nicergoline (NIC) on the aggregation and ^{3}H-serotonin (^{3}H-5HT) secretion of prelabelled human citrated platelet rich plasma induced by adrenaline (ADR). The addition of each reagent is indicated by an arrow. One of four similar experiments.

When a high dose of nicergoline was used, the potentiating effect of adrenaline was suppressed and the aggregation was similar to that induced by the other aggregating agent alone. As an example, the effect of nicergoline on the synergistic effect of adrenaline and U-46619 is shown in Figure 2.

Yohimbine, a selective alpha2-adrenergic antagonist, and prazosin a selective alpha1-adre-

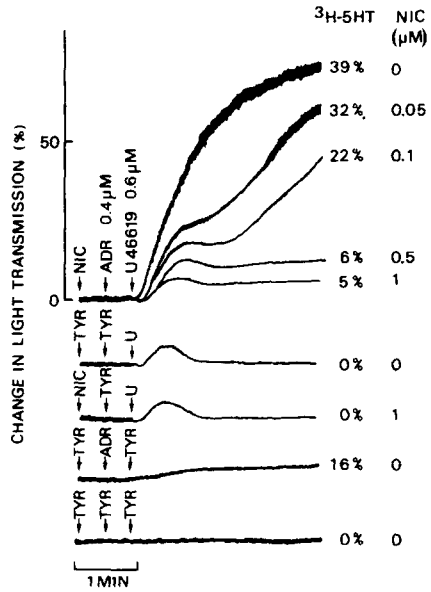

Figure 2
Effect of nicergoline (NIC) on the potentiation by adrenaline (ADR) of the endoperoxide analog U-46619-induced aggregation and ^{3}H-serotonin (^{3}H-5HT) secretion of prelabelled citrated platelet rich plasma. The addition of each reagent is indicated by an arrow. TYR = Tyrode's solution. One of two similar experiments.

Table 1
Inhibition by nicergoline of the aggregation and ^{3}H-5HT secretion of human platelets suspended in citrated plasma induced by various agonists.

Agonist	(f.c.)	Inhibition of aggregation IC_{50} (μM)	Inhibition of ^{3}H-5HT secretion IC_{50} (μM)
Adrenaline	4.5 μM	0.22 ± 0.07	0.28 ± 0.15
PAF-acether	0.1 μM	108 ± 54	400
Collagen	25 ng/ml	215 ± 56	407 ± 112
A23187	5 μM	253 ± 35	290 ± 55
ADP	5 μM	287 ± 89	140 ± 110
U-46619	5 μM	330 ± 83	397 ± 135
Arachidonic acid	500 μM	567 ± 84	670 ± 57

Platelets (300,000/mm^3) suspended in citrated plasma were incubated with the agonists and varying concentrations (4 to 6) of nicergoline. The results are expressed as mean ± S.E.M. from 3 to 4 separate experiments from different donors. f.c. = final concentration.

Table 2
Inhibition by nicergoline of the potentiating effect of adrenaline on the aggregation and ^{3}H-5HT secretion of human platelets suspended in citrated plasma induced by low concentrations of various agonists.

Adrenaline μM	Agonists (f.c.)		Inhibition of the potentiation	
			Aggregation IC_{50} (μM)	^{3}H-HT secretion IC_{50} (μM)
0.4	PAF-acether	0.008 μM	0.6	0.6
0.4	Collagen	1.5 ng/ml	2	2.5
0.4	A23187	0.4 μM	1.3	—
0.4	ADP	0.5 μM	0.2	—
0.4	U-46619	0.6 μM	0.1	0.1
0.4	Arachidonic acid	210 μM	0.9	—

Platelets (300,000/mm^3) suspended in citrated plasma were incubated with several concentrations (5 to 8) of nicergoline, adrenaline and the various agonists at low concentrations. Mean values from 1 to 3 separate experiments. f.c. = final concentration.

nergic antagonist, were compared to nicergoline in their capacity to inhibit completely the potentiating effect of adrenaline on aggregation and secretion induced by the various aggregating agents listed above. To inhibit completely the potentiating effect of adrenaline, 0.5 to 1 μM yohimbine, 1 to 5 μM nicergoline and 100 μM prazosin were necessary.

Effects of nicergoline on the aggregation and ^{3}H-5HT secretion of washed human platelets

While nicergoline is an alpha-adrenergic antagonist at submicromolar concentrations, it was also an inhibitor of the aggregation and ^{3}H-5HT secretion of washed human platelets induced by all aggregating agents tested, with IC_{50} values ranging from 27 to 140 μM (Table 3). Using washed human platelets suspended in media containing physiological Ca^{2+} levels, ADP or PAF-acether will only cause aggregation in the presence of added fibrinogen without inducing the platelet release reaction [14].

Adrenaline, even at concentrations as high as 1 mM, did not induce aggregation and ^{3}H-5HT secretion of washed human platelets [5]. Nevertheless, a low concentration (0.5 μM) of adrenaline was able to potentiate the effects of low concentrations of aggregating agents such as PAF-acether, ADP, A23187, U-46619, thrombin and arachidonic acid. The concentration of the second aggregating agent to be potentiated by adrenaline was chosen in order to give shape change alone and no or minimal (less than 5%) aggregation. This is illustrated for arachidonic acid in Fig. 3. Inhibition of the potentiation by adrenaline of aggregation and secretion by all the

Table 3
Inhibition by nicergoline of the aggregation and ^{3}H-5HT secretion of washed human platelets induced by various agonists.

Agonist	(f.c.)	Inhibition of aggregation IC_{50} (μM)	Inhibition of ^{3}H-5HT secretion IC_{50} (μM)
PAF-acether	0.1 μM	32.3 ± 5.6	N.D.
Collagen	12.5 ng/ml	38 ± 14	37 ± 21
A23187	t μM	47 ± 14	64 ± 10
ADP	5 μM	27 ± 7.5	N.D.
U-46619	5 μM	52 ± 18	65 ± 11
Arachidonic acid	450 μM	86 ± 31	70 ± 7
Thrombin	0.05 U/ml	46.6 ± 15	140 ± 41

Platelets (300,000/mm^3) suspended in Tyrode's albumin buffer were incubated with purified human fibrinogen (0.08%) (with the exception of thrombin), various concentrations (5 to 6) of nicergoline and the different agonists. The results are expressed as mean ± SEM from 3 to 4 separate experiments from different donors. N.D. = no detectable secretion.

Table 4
Inhibition by nicergoline of the potentiating effect of adrenaline on aggregation and ^{3}H-5HT secretion of washed human platelets induced by different agonists.

Adrenaline (μM)	Agonists (f.c.)		Inhibition of the potentiation	
			Aggregation IC_{50} (μM)	^{3}H-5HT secretion IC_{50} (μM)
0.5	PAF-acether	0.08 μM	0.1	N.D.
0.5	A23187	2.6 μM	0.1	N.D.
0.5	ADP	0.4 μM	0.1	N.D.
0.5	U-46619	0.5 μM	0.7	0.2
0.5	Arachidonic acid	9 μM	0.2	0.2
0.5	Thrombin	0.03 U/ml	0.8	0.1

Platelets (300,000/mm^3) suspended in Tyrode's albumin buffer were incubated in the presence of purified human fibrinogen (0.08%), varying concentrations (5 to 8) of nicergoline, adrenaline and the different agonists listed. Mean values from 1 to 3 separate experiments. f.c. = final concentration, N.D. = no detectable secretion.

agonists tested was achieved with low concentrations of nicergoline (Table 4). To inhibit completely the potentiating effect of adrenaline on the various aggregating agents listed in Table 4, 0.1 μM yohimbine, 0.1 to 1 μM nicergoline and 100 μM prazosin were required. In addition, the effects of yohimbine, nicergoline and prazosin on the potentiating effect of adrenaline on ADP-induced aggregation were studied in more detail (Table 5). Nicergoline was 5.5 times less potent than yohimbine and 103 times more potent than prazosin in inhibiting the potentiating effect of adrenaline on ADP-induced aggregation.

Effect of intravenous administration of nicergoline in man on the *ex vivo* aggregation of cPRP by adrenaline

The intravenous administration of nicergoline (0.5 mg/kg b.w./d in two injections) to 6 patients suffering from chronic lower limb arteritis resulted in a significant inhibition ($p < 0.05$) of the velocity of the first wave of aggregation in cPRP induced by 1 and 5 μM adrenaline (Fig. 4). The aggregation induced by 0.25 μM ADP was inhibited in only 2 patients (20% and 100% inhibition respectively). The administration of nicergoline did not inhibit the aggregation induced by 1mM arachidonic acid (data not shown).

Effect of nicergoline on the binding of ^{3}H-yohimbine to intact washed human platelets

We have previously [5] characterized alpha 2-adrenergic receptors on intact washed platelets by measuring the binding of ^{3}H-yohimbine. The binding of ^{3}H-yohimbine is rapid, saturable and reversible with a K_D of 8.1 nM and a Bmax of 395 sites per platelet. Nicergoline, yohimbine, phentolamine (a mixed alpha 1 + alpha 2-antag-

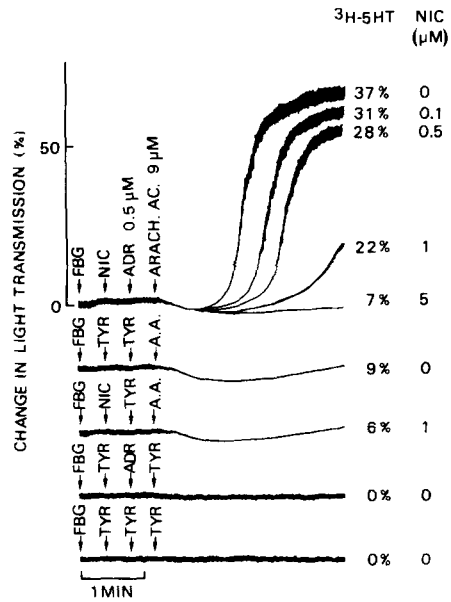

Figure 3
Effect of nicergoline (NIC) on the potentiation by adrenaline (ADR) of arachidonic acid (ARACH.AC.) -induced aggregation and ^{3}H-serotonin (^{3}H-5HT) secretion of prelabelled intact washed human platelets suspended in Tyrode's albumin solution (TYR). The addition of each reagent is indicated by an arrow. FBG = Fibrinogen. One of two similar experiments.

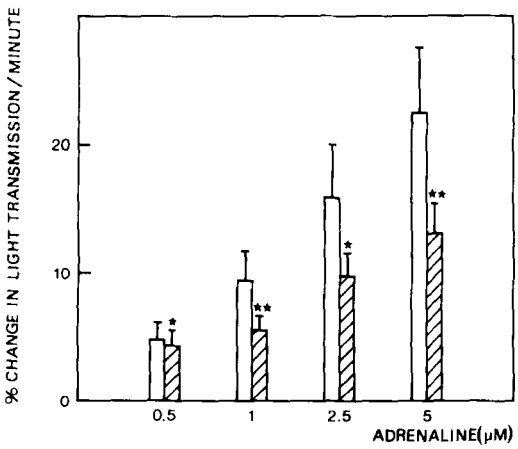

Figure 4
Effect of intravenous administration of nicergoline (SERMION®) on the velocity of the first wave of aggregation induced by different concentrations of adrenaline. The results before (empty columns) and after (shaded columns) treatment are expressed as mean ± SEM from six experiments. *0.05 < P < 0.1; **P < 0.05 using a one-tailed Student's t test.

onist) and prazosin were tested for their ability to displace ^{3}H-yohimbine bound to intact washed human platelets (Table 5). Nicergoline was equi-

Table 5
Comparison of the inhibition of ^{3}H-yohimbine binding and the inhibition of the potentiating effect of adrenaline on ADP-induced aggregation of intact washed human platelets by alpha-adrenergic antagonists.

Antagonist	Inhibition of ^{3}H-yohimbine binding IC_{50} (μM)	Inhibition of the potentiation of aggregation IC_{50} (μM)
Yohimbine	0.013 ± 0.009	0.019 ± 0.003
Nicergoline	0.265 ± 0.178	0.105 ± 0.043
Phentolamine	0.317 ± 0.130	0.024 ± 0.016
Prazosin	13.767 ± 7.250	10.733 ± 2.150

For binding experiments, platelets (300,000/mm^3) were incubated with ^{3}H-yohimbine (7.5 nM) and varying concentrations (6 to 10) of the competing compound. The values of 3 separate experiments are expressed as mean ± SEM. For aggregation studies, platelets (300,000/mm^3) were incubated in the presence of purified human fibrinogen (0.08%), adrenaline (0.5 μM), ADP (1 μM) and varying concentrations (5 to 8) of the competing compound. The values of 3 to 6 separate experiments are expressed as mean ± SEM.

potent to phentolamine in displacing ^{3}H-yohimbine. The IC_{50} for the displacement of ^{3}H-yohimbine by nicergoline was 52-fold lower than that of prazosin and 20-fold higher than that of yohimbine.

Effect of nicergoline on human platelet adhesion to collagen-coated surfaces

The adhesion of radiolabelled human platelets suspended in citrated blood to a collagen-coated glass surface was not significantly inhibited by nicergoline at concentrations as high as 1 mM (Table 6). In experiments conducted with ^{111}In-labelled washed human platelets suspended in

Table 6
Effect of nicergoline on the adherence of ^{51}Cr-labelled human platelets to a collagen-coated surface in citrated blood.

Nicergoline (μM)	Adhesion (platelets/mm^2)
0	24,600 ± 2,200
10	26,700 ± 7,000
100	25,800 ± 5,500
1000	24,300 ± 3,200

Nicergoline was incubated for 10 min with citrated blood (3.8%) containing ^{51}-chromium labelled platelets. The hematocrit was adjusted to 47% and the platelet count to 140,000/mm^3. The collagen-coated glass rods were rotated for 5 min at 37°C and 200 rpm. The results are expressed as mean ± SEM from 8 experiments.

Table 7
Effect of nicergoline on the adherence of ^{111}In-labelled washed human platelets to a collagen-coated surface.

Nicergoline (μM)	(n)	Adhesion (platelets/mm^2)	P
0	32	33,200	
10	8	36,900 ± 6950	< 0.6
100	16	20,100 ± 2486	< 0.005
500	16	13,700 ± 1749	< 0.001
1000	16	6,500 ± 1260	< 0.001

Nicergoline was incubated for 10 min with washed human platelets (180,000/mm^3) suspended in Tyrode's solution containing 0.35% albumin and 40% hematocrit. The red cells were added 1 min before the beginning of the experiment. The collagen-coated glass rods were rotated in this suspension for 5 min at 200 rpm and 37°C. The results are expressed as mean ± SEM from 8 to 32 separate measurements. P values calculated with respect to control suspension without drug using a two-tailed Student's t test.

Tyrode's solution containing 0.35% human albumin and 40% red blood cells, nicergoline (100 μM) inhibited platelet adhesion (Table 7).

Discussion

One characteristic of the action of adrenaline on human platelets is its ability to strongly potentiate the action of aggregating agents such as ADP [5, 19, 20], thrombin [21], collagen [21], PAF-acether [22, 23] and arachidonate [24]. The potentiation by adrenaline of ADP-induced aggregation is directly explained by its binding to the platelet alpha 2-adrenergic receptor [5].

In this study, we report that low doses of adrenaline potentiate the aggregation and 3H-5HT secretion in cPRP or in washed platelets induced by a wide variety of agonists acting through different mechanisms. The potentiating effect of adrenaline on all the agonists tested is mediated by its interaction with alpha 2-adrenergic receptors because: i) this effect of adrenaline is obtained at a dose (0.5 μM) which is similar to its IC_{50} for ^{3}H-yohimbine binding (0.33 μM ± 0.15) [5], when both these parameters are determined using the same intact washed platelet preparation; ii) the potentiating effect of adrenaline is selectively inhibited by alpha 2-(yohimbine) or mixed alpha 1 + alpha 2-(phentolamine) adrenergic antagonists and poorly inhibited by alpha 1-(prazosin) or beta-(propranolol) adrenergic antagonists [5].

The *in vitro* effects of adrenaline on platelet activation could suggest a role for this catecholamine in platelet activation *in vivo* [2]. In such a case, the use of an alpha-adrenergic antagonist in preventive antithrombotic therapy could be considered. We have explored this possibility by studying the effects of nicergoline on human platelets. Nicergoline has been described as a more selective alpha 1-adrenergic antagonist, when studied on the cardiovascular system of rats and dogs [25] and on central adrenergic receptors in rats [26]. Nicergoline inhibits the potentiating effect of adrenaline on the aggregation and ^{3}H-5HT secretion induced by low concentrations of all aggregating agents tested, with IC_{50} ranging from 0.1 to 2.5 μM in human cPRP and between 0.1 to 0.8 μM in intact washed human platelets. Higher concentrations are necessary to inhibit the effect of adrenaline in cPRP than in washed platelets; this can be explained by a possible binding of nicergoline to plasma proteins. Nicergoline is 5.5 times less potent than yohimbine and 103 times more potent than prazosin in inhibiting the potentiating effect of adrenaline on platelet aggregation and secretion in washed platelets. Nicergoline also inhibits the aggregation and secretion induced by adrenaline alone in cPRP, with IC_{50} 0.22 and 0.28 μM respectively. These data are in agreement with previous reports on the inhibition of platelet aggregation in human cPRP by nicergoline [27, 28]

Nicergoline inhibits the binding of ^{3}H-yohimbine to intact washed human platelets with an IC_{50} (0.26 μM) 20 times higher than that of yohimbine and 52 times lower than that of prazosin. Nicergoline inhbits ^{3}H-yohimbine binding to human platelets, adrenaline induced aggregation and secretion in cPRP and the potentiating effect of adrenaline on aggregation and secretion in cPRP or washed platelets in the same dose range (0.1–1 μM). These results show that nicergoline inhibits the effect of adrenaline on platelet activation by interacting with alpha 2-adrenergic receptors. Nicergoline inhibits the binding of alpha 1-adrenergic antagonists to rat cerebral cortex, with IC_{50} between 0.012 and 0.2 μM, and is 100 to 232 times less effective on alpha 2-adrenergic receptors as determined with ^{3}H-clonidine [26] and ^{3}H-guanfacine binding [29]. Thus these authors suggested that nicergoline is essentially an alpha 1-adrenergic antagonist. Human platelets possess essentially alpha 2-adrenergic receptors [2]. Our results suggest that nicergoline possesses alpha 2-adrenergic antagonist properties at least with respect to human platelets and can be better considered as a mixed alpha 1 + alpha 2-adrenergic antagonist. These discre-

pancies could be explained by the use of different adrenergic ligands. A recent study has shown that the affinities of various adrenergic ligands are similar in rat brain and human platelet adrenergic receptors, when ^{3}H-yohimbine is used in both tissues. In contrast, there was no correlation between ^{3}H-yohimbine binding in human platelets and ^{3}H-clonidine binding in rat brain [30].

Nicergoline inhibits the aggregation and ^{3}H- 5HT secretion indued by all agents tested in cPRP or washed platelets at concentrations 50 to 1000 times higher than the concentrations that inhibit the potentiating effect of adrenaline. These results are in agreement with previous experiments using cPRP describing an inhibition by nicergoline of ADP-, collagen-[27, 28, 31], thrombin-, arachidonate- and A23187- [31] induced aggregation at concentrations from 100 to 700 μM. Nicergoline is able to inhibit the adhesion of platelets to elastin [32] at 100 μM and we report here that, at the same concentration, nicergoline inhibits the adhesion of washed platelets to a collagen-coated surface.

The non specific inhibition by nicergoline of platelet aggregation, secretion and adhesion is not mediated through an inhibition of the cyclooxygenase pathway because: i) nicergoline inhibits the aggregation of washed platelets induced by ADP and by PAF-acether, which is not accompanied by thromboxane A_2 generation [14]; ii) nicergoline (100 μM) inhibits in cPRP or washed platelets the aggregation and secretion induced by the endoperoxide analog U-46619; iii) nicergoline (100 μM) does not inhibit the formation of MDA induced by 0.1 mM arachidonate, but inhibits that caused by thrombin (10 U/ml) (data not shown). Largarde et al. [31] have shown that 200 μM nicergoline does not inhibit MDA formation induced by arachidonate (0.01 mM), but inhibits that caused by collagen (2.5 μg/ml) or thrombin (0.1 U/ml). These authors have also demonstrated that nicergoline inhibits platelet prostaglandin biosynthesis induced by thrombin or collagen, but not when exogenous arachidonate is used. They suggested that nicergoline may inhibit phospholipase activity. It is however possible that the effect of nicergoline is mediated through cyclic AMP [31]. We have found that nicergoline inhibits a crude cyclic AMP phosphodiesterase preparation from human platelets, with an IC_{50} of 201 μM (Stierlé A., personal communication), although nicergoline does not significantly raise platelet cyclic AMP levels [31]. Other cyclic AMP phosphodiesterase inhibitors are able to inhibit platelet functions without a detectable rise in platelet cyclic AMP, probably by acting on a small pool of platelet cyclic AMP [33]. The fact that nicergoline inhibits the action on platelets of agents acting through at least three different mechanisms also supports a possible involvement of cyclic AMP.

Other explanations for this general inhibitory effect of nicergoline include Ca^{2+} chelation. Nicergoline (1 mM) does not modify a global coagulation test (activated partial thromboplastin time, data not shown), a Ca^{2+}-dependent process. Thus, it is not likely that the action of nicergoline is due to Ca^{2+} removal. Another possible effect of nicergoline could be a generalized perturbation of the platelet plasma membrane, as has been demonstrated for high concentrations of propranolol [34]. The inhibition by nicergoline of platelet adhesion could be explained by interaction with the binding of von Willebrand factor to the platelet membrane, but we were not able to demonstrate an inhibition of ristocetin-induced platelet agglutination in cPRP by up to 200 μM nicergoline (data not shown).

The action of nicergoline on platelet function is also demonstrated *ex vivo*, after intravenous administration to patients. Nicergoline inhibits selectively adrenaline-induced aggregation but is ineffective on ADP- or collagen-induced aggregation. A previous report has shown an inhibition of ADP-, adrenaline-, thrombin- and collagen-induced aggregation after oral administration of a single dose of 20 mg of nicergoline [35]. Another study, using 30 mg/day orally for two days, showed an inhibition of ADP-induced aggregation, but no inhibition of adrenaline-induced aggregation [36]. The discrepancies between these results could be explained in part by the mode of administration (oral or intravenous), the duration of the treatment, the total concentration administered and by the lag time after cessation of the treatment. Our results obtained *ex vivo* are in agreement with the action of nicergoline *in vitro*, where it inhibits the action of adrenaline at doses 50 to 500 times lower than those necessary to inhibit other types of platelet activators.

It has been shown that the association of adrenaline, ADP and vasopressin, at concentrations that can be attained *in vivo*, can induce the aggregation of human platelets [10]. *In vivo*, platelet activation measured by the secretion of platelet-specific proteins (PF4, βTG) or by the

formation of thromboxane B_2, may occur during exercise [37] and after emotional stress [8, 38], which are known to increase the level of catecholamines in plasma. The specific inhibition by nicergoline of the activation of platelets by adrenaline *in vitro* and *ex vivo* and the reports that nicergoline inhibits platelet-mediated thrombosis in stenosed coronary arteries [11] and cortical arteries [39] in experimental animals *in vivo* could increase the potential therapeutic applications of nicergoline. The administration of an alpha-adrenergic antagonist like nicergoline, with limited side effects on blood pressure, could be a useful adjunct in the prophylactic treatment of platelet mediated arterial thrombosis, alone or in combination with other inhibitors of platelet functions, such as aspirin or dipyridamole, which are acting through different mechanisms [40].

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References

[1] J. R. O'Brien, *Some effects of adrenaline and adrenaline compounds on platelets* in vitro *and* in vivo, Nature *200*, 763–765 (1963).

[2] R. Kerry and M. C. Scrutton, *Platelet adrenoceptors*. In The Platelets: Physiology and Pharmacology pp. 113–159 (Ed. G. L. Longenecker). Academic Press, Inc., Orlando 1985.

[3] E. Glusa and F. Markwardt, *Adrenaline-induced reactions of human platelets in hirudin plasma*, Haemostasis *9*, 188–192 (1980).

[4] J.-P. Cazenave, A. Sutter, S. Hemmendinger, M. L. Wiesel, F. Lanza and J. Daver. *Adrenaline activates human platelets but does not cause primary aggregation if thrombin generation is inhibited by hirudin*, Thromb. Haemostas. *46*, 95 (1981).

[5] F. Lanza and J. P. Cazenave, *Studies of alpha 2-adrenergic receptors of intact and functional washed human platelets by binding of 3H-dihydroergocryptine and 3H-yohimbine. Correlation of 3H-yohimbine binding with the potentiation by adrenaline of ADP-induced aggregation*, Thromb. Haemostas. *54*, 402–408 (1985).

[6] K. D. Newman, L. T. Williams, N. N. Bishopric and R. J. Lefkowitz, *Identification of alpha-adrenergic receptors in human platelets by 3H-dihydroergocryptine binding*, J. Clin. Invest. *61*, 395–402 (1978).

[7] H. J. Motulsky, S. J. Shattil and P. A. Insel, *Characterization of alpha 2-adrenergic receptors of human platelets using 3H-yohimbine*, Biochem. Biophys. Res. Commun. *97*, 1562–1570 (1980).

[8] S. P. Levine, B. L. Towell, A. M. Suarez, L. K. Knieriem, M. M. Harris and J. N. George, *Platelet activation and secretion associated with emotional stress*, Circulation *71*, 1129–1134 (1985).

[9] E. L. Bravo, R. C. Tarazi, R. W. Gifford and B. H. Stewart, *Circulating and urinary catecholamines in pheochromocytoma*, N. Engl. J. Med. *301*, 682–686 (1979).

[10] N. G. Ardlie, H. A. Cameron and J. Garrett, *Platelet activation by circulating levels of hormones: a possible link in coronary heart disease*, Thromb. Res. *36*, 315–322 (1984).

[11] R. Bolli, J. A. Ware, T. A. Brandon, D. G. Weilbaecher and M. L. Mace, *Platelet-mediated thrombosis in stenosed canine coronary arteries: inhibition by nicergoline, a platelet-active alpha-adrenergic antagonist*, J. Amer. Coll. Cardiol. *3*, 1417–1426 (1984).

[12] N. J. Christensen, *Catecholamines and essential hypertension*, Scand. J. Clin. Lab. Invest. *42*, 211–215 (1982).

[13] R. A. Nadeau and J. Dechamplain, *Plasma catecholamines in acute myocardial infarction*, Am. Heart. J. *98*, 548–554 (1979).

[14] J.-P. Cazenave, S. Hemmendinger, A. Beretz, A. Sutter-Bay and J. Launay, *L'agrégation plaquettaire: outil d'investigation clinique et d'étude pharmacologique, Méthodologie*. Ann. Biol. Clin. *41*, 167–179 (1983).

[15] R. L. Kinlough-Rathbone, J. F. Mustard, M. A. Packham, P. W. Perry, H. J. Reimers and J.-P. Cazenave, *Properties of washed human platelets*, Thromb. Haemostas. *37*, 291–308 (1977).

[16] J. L. Costa and D. L. Murphy, *Platelet 5-HT uptake and release stopped rapidly by formaldehyde*, Nature *255*, 407–408 (1975).

[17] J.-P. Cazenave, D. Blondowska, M. Richardson, R. L. Kinlough-Rathbone, M. A. Packham and J. F. Mustard, *Quantitative radioisotopic measurement and scanning electron microscope study of platelet adherence to collagen-coated surface and to subendothelium with a rotating probe device*, J. Lab. Clin. Med. *93*, 60–70 (1979).

[18] M. Eber, J.-P. Cazenave, J. C. Grob, J. Abecassis and G. Methlin, *111Indium labelling of human washed platelets: kinetics and* in vivo *sequestration sites*. In Blood Cells in Nuclear Medicine, part I. Cell Kinetics and Biodistribution pp. 29–43 (Eds. M. R. Hardeman and Y. Najean). Martinus Nijhoff Publishers, Boston 1984.

[19] D. C. B. Mills and G. C. K. Roberts, *Effects of adrenaline on human blood platelets*, J. Physiol. *193*, 443–453 (1967).

[20] N. G. Ardlie, G. Glew and C. J. Schwartz, *Influence of catecholamines on nucleotide-induced platelet aggregation*, Nature *212*, 415–417 (1966).

[21] D. P. Thomas, *The role of platelet catecholamines in the aggregation of platelets by collagen and thrombin*, Exp. Biol. Med. *3*, 129–134 (1968).

[22] B. B. Vargaftig, F. Fouque, J. Benveniste and J. Odiot, *Adrenaline and PAF-acether synergize to trigger cyclooxygenase-independent activation of plasma-free human platelets*, Thromb. Res. *28*, 557–573 (1982).

[23] A. Beretz, F. Lanza and J.-P. Cazenave, *Adrenaline potentiates platelet aggregation induced by PAF-acether and endoperoxide analog U-46619 through stimulation of alpha2 receptors independently of the cyclooxygenase pathway*, Thromb. Haemost. *50*, 41 (1983).

[24] G. H. R. Rao and J. G. White, *Epinephrine potentiation of arachidonate-induced aggregation of cyclooxygenase-deficient platelets*, Amer. J. Hematol. *11*, 355–366 (1981).

[25] A. M. HUCHET, P. MOUILLE, J. CHELLY, B. LUCET, M.-F. DOURSOUT, P. LECHAT and H. SCHMITT, *Effects of nicergoline on the cardiovascular system of dogs and rats*, J. Cardiovasc. Pharmacol. *3*, 677–691 (1981).

[26] F. HUGET, K. BIZIERE, M. BRETEAU and G. NARCISSE, *Effects of nicergoline on rat cerebral neurotransmitter receptors; neurochemical profile*, J. Pharmacol. *2*, 257–267 (1980).

[27] J. MIGNE, *Activité anti-agrégante plaquettaire de la nicergoline. Etude* in vitro, *ultrastructurale et* in vivo. In Actualités de Chimie Thérapeutique (5ème série) pp. 31–38 (Ed. LAVOISIER). Paris 1977.

[28] C. PRAGA, V. TANTALO and R. MARANGONI, *Nicergoline and platelet aggregation. A review of experimental and clinical studies*, Arzneim. Forsch. *29*, 1270–1277 (1979).

[29] A. CARDOT, J.-P. DAUSSE and P. MEYER, *Affinités respectives de la nicergoline et de l'ergoline pour les récepteurs adrénergiques alpha 1 et alpha 2 du cerveau de rat.* Compte Rendu Symposium Alpha-bloquants et Nicergoline, Dijon, pp. 26–35 (1982).

[30] K. SOGABE, H. HASHIMOTO and M. NAKASHIMA, *Affinities of adrenergic drugs for alpha 2-adrenergic receptors in dog saphenous vein in comparison with those in rat brain or human platelets*, Blood Vessels *22*, 127–138 (1985).

[31] M. LAGARDE, M. GUICHARDANT, I. GHAZI and M. DECHAVANNE, *Nicergoline, an anti-aggregating agent which inhibits release of arachidonic acid from human platelets phospholipids*, Prostaglandins *19*, 551–557 (1980).

[32] M. E. RAFELSON, J. MIGNE, R. SANTONJA, J. E. DEROUETTE and L. ROBERT, *Effect of an alpha-blocking agent, nicergoline, on the interaction between blood platelets, elastin and endothelial cells*, Biochem. Pharmacol. *29*, 943–947 (1980).

[33] A. BERETZ, F. BRIANCON-SCHEID, A. STIERLE, G. CORRE, R. ANTON and J.-P. CAZENAVE, *Inhibition of human platelet cyclic AMP phosphodiesterase and of platelet aggregation by a hemisynthetic flavonoid, amentoflavone hexaacetate*, Biochem. Pharmacol. (in press) (1985).

[34] R. KERRY, M. C. SCRUTTON and R. B. WALLIS, *Beta-adrenoceptor antagonists and human platelets: relationship of effects to lipid solubility*, Biochem. Pharmacol. *33*, 2615–2622 (1984).

[35] E. POGLIANI, A. DELLA VOLPE, R. FERRARI, P. RECALCATI and C. PRAGA, *Inhibition of human platelet aggregation by oral administration of nicergoline. A double blind study*, Il Farmaco *30*, 630–635 (1975).

[36] J. MIGNE, J.-P. SAINT-MAURICE, R. SANTONJA and S. KUNZ, *Activité anti-agrégante plaquettaire; Effet d'un alpha-bloqueur: la nicergoline*, Sem. Hôp. Paris (Thér.) *50*, 649–655 (1974).

[37] J. R. STRATTON, T. W. MALPASS, J. L. RITCHIE, M. A. PFEIFER and L. A. HARKER, *Studies of platelet factor 4 and beta-thromboglobulin release during exercise: lack of relationship to myocardial ischemia*, Circulation *66*, 33–43 (1982).

[38] H.-J. MEST, U. ZEHL, W. SZIGOLEIT, CH. TAUBE and W. FORSTER, *Influence of mental stress on plasma level of prostaglandins, thromboxane B2 and on circulating platelet aggregates in man*, Prostagland. Leuk. Med. *8*, 553–563 (1982).

[39] P. DER AGOPIAN, A. ROSA, J. C. GAUTIER and F. LHERMITTE, *Thromboses artérielles expérimentales. Effet d'un alpha-bloquant: la nicergoline*, Nouv. Presse Méd. *38*, 251–254 (1973).

[40] I. A. GREER, J. J. WALKER, A. A. CALDER and C. D. FORBES, *Aspirin with an adrenergic or a calcium-channel-blocking agent as new combination therapy for arterial thrombosis*, Lancet, *1*, 351–352 (1985).