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EFFECT OF AN ALPHA-BLOCKING AGENT, NICERGOLINE, ON THE INTERACTION BETWEEN BLOOD PLATELETS, ELASTIN AND ENDOTHELIAL CELLS

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Abstract—The action on an α-blocking agent, Nicergoline, and of a β-blocking agent, Acebutolol, on platelet–elastin and platelet–endothelial cell interaction was investigated. Nicergoline (10^{-4}M), but not Acebutolol, added to PRP, decreased the adhesion of platelets to Sepharose–elastin columns by about 60 per cent. Nicergoline at 10^{-7}M completely inhibited the adhesion of platelets to endothelial cells 'activated' by epinephrine. The same inhibition was obtained when the endothelial cells or the platelets were preincubated with the α-blocking agent before mixing. Acebutolol was inefficient. These results confirm the efficiency of this α-blocking agent in preventing platelet adhesion to elastic fibers and to endothelial cells. They confirm the inefficiency of the β-blocking agent in both models used. They also suggest that platelet α-receptors may be involved in platelet–elastin interaction as well as in platelet–endothelial cell interaction.

The interaction between blood platelets and cellular and molecular elements of the vessel wall were shown to play an important role in the pathogenesis of arteriosclerosis as well as in the formation of thrombi [1–6]. Among the macromolecules which can interact with blood platelets, collagen plays a particularly important role [7, 8]. It was, however, recently demonstrated that platelets can also adhere to fibrous elastin [9]. It is also known that blood platelets can interact with endothelial cells when they are first treated with aggregation inducing agents such as epinephrine [10, 11]. Among the anti-aggregating agents which are actively used in therapeutics, alpha-blocking substances play an especially important role because they have several favorable effects on the circulation due to their vasodilating and alpha-blocking activity [12–15].

It was therefore decided to investigate the effect of such an alpha-blocking agent, Nicergoline, γ-β-(5 bromonicotinoyl hydroxymethyl)-1.6-dimethyl-10α-methoxy-ergoline, on the interaction between blood platelets and fibrous elastin on the one side and blood platelets and endothelial cells on the other side. This drug was shown to inhibit efficiently platelet aggregation induced by ADP or collagen [16]. Its metabolism in man and animals was also studied [17].

As a control drug a β-blocking agent, Acebutolol (Sectral), (hydroxy-2 isopropylamino-3 proposy)-2 butyramido-5 acetophenon, was used [18, 19].

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MATERIALS AND METHODS

Blood platelets. These were obtained freshly from normal human donors (for interaction with elastin) or from bovine blood (for interaction with endothelial cells). Acid citrate–dextrose (ACD, 0.8% citric acid, 2.2% Na-citrate, 2.45% dextrose) was used as an anticoagulant (1 ml of ACD per 8–9 ml blood) and were used immediately after centrifugation at 500 *g* for 5 min at 18° and after counting in a Coulter Counter.

Elastin. Elastin was purified from bovine ligamentum nuchae by NaOH treatment as described [20] and characterized by its amino acid composition and its desmosine content. Fibrous elastin was finely powdered in a hammer-mill to a granule size of about 100 μm.§

Table 1 shows the amino acid composition of the elastin used for these experiments. The relatively low Asp + Glu value (22 residues per 1000), the absence of methionine and the negligible histidine content can be taken as an indication for the absence of microfibrillar components [20]. The low hydroxyproline content (10 residues per 1000) is an indication of the absence of collagenous contaminants. It was shown previously that elastin purified by this procedure has a much lower polar amino acid content and lower microfibrillar contamination as judged by electron microscopy than elastin purified by other procedures [21]. This is true also for the hydroxyproline content and collagen contamination.

Sepharose-2B–elastin mixed affinity columns were prepared as described previously at a concentration of 1.0 mg fibrous elastin per ml of Sepharose [9]. The fibrous elastin suspension was thoroughly mixed

Table 1. Amino acid composition of the elastin preparation used for the experiments, after 24 hr hydrolysis in 6N HCl at 110°. Results in res. per 1000 residues

Hypro	10
Asp	7
Thr	7
Ser	6
Glu	15
Pro	110
Gly	330
Ala	224
Val	136
Cys	0
Met	0
I-Leu	38
Leu	91
Tyr	3
Phe-Ala	12
i-Des*	4
Des*	6
Arg	4
His	1
Lys	4

* Isodesmosine and desmosine given in lysine equivalents

with the Sepharose suspension in order to achieve a uniform distribution of the elastin fibers in the Sepharose gel. This was controlled by preparing cryostat sections through the Sepharose–elastin gels prepared in plastic tubings. The Sepharose–elastin suspension was then poured in siliconated columns of 30 × 1.2 cm and were percolated with sterile saline as previously described [9]. Control columns of Sepharose-2B were of identical size but did not contain elastin. The elution profile of the platelet rich plasma (PRP) was monitored on a recording Acta III Beckman Spectrophotometer. The platelets recovered were counted in a Coulter Counter, and these data were used for the calculation of retention.

The inhibiting effect of the alpha-blocking agent, Nicergoline, and of a beta-blocking agent, Acebutolol, was studied by adding them to the PRP before the percolations through the elastin columns at a concentration varying between 10^{-7} and 10^{-4}M, 15 min before their percolation on the column.

Endothelial cells. Endothelial cells were obtained from bovine aortae and cultured in Dulbecco's Modified Eagle's medium (DME) supplemented with 10% foetal calf serum in an atmosphere of 10% CO_2 in air as previously described [22]. Secondary cultures were cloned and subcultured three times prior to being grown on plastic coverslips (9 × 35 mm) for these experiments. Fifth passage cells were seeded at 5×10^5 cells per Leighton tube, reached confluency in 3 days and were kept at a post-confluency for 4 days on the coverslips (ca. 3.7×10^5 cells per coverslip) [23].

Activation of endothelial cells. Four days post-confluent cultures on coverslips were rinsed twice with Dulbecco's medium and the cells 'activated' (or treated) with epinephrine (2×10^5 M for 2 min), Nicergoline (1×10^{-4}, 1×10^{-6}, 1×10^{-7} M for 4 min) and Acebutolol (1×10^{-4}, 1×10^{-6}, 1×10^{-7} M for 4 min). After each activation (or treatment) step the coverslips were rinsed twice with Dulbecco's medium and subsequently floated on ^{125}I-platelets resuspended in Dulbecco's medium. Incubation times of 2–20 min with Nicergoline or Acebutolol gave identical results.

Platelet–endothelial interactions were terminated after 4 min by immersing the coverslips in 4 ml of 2% glutaraldehyde (37°) containing 0.1M cacodylate buffer, pH 6.9 for 30 min. Fixed cells were washed three times with the cacodylate buffer (pH 6.9) and counted directly in a counter.

Preparation and iodination of platelets. Platelets were isolated from freshly drawn bovine blood containing ACD as anticoagulant (1 ml of ACD per 8 ml blood). Platelet rich plasma (PRP) was prepared by

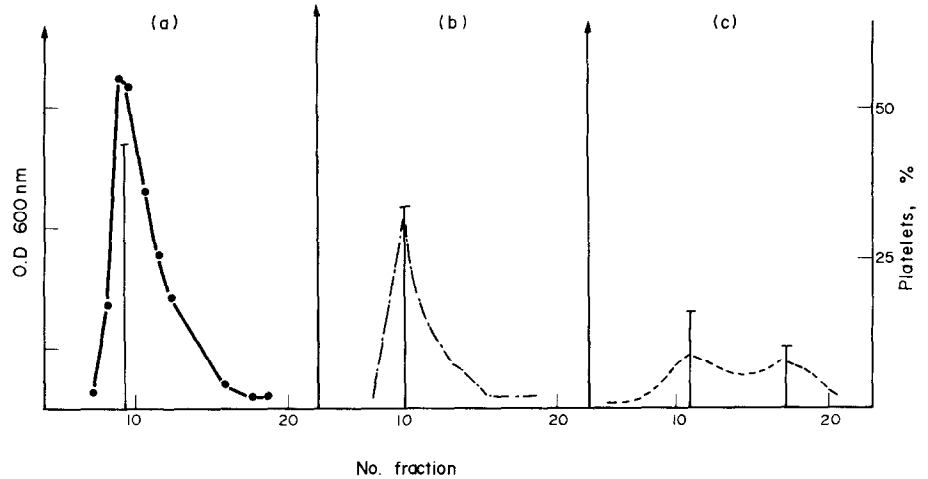

Fig. 1. Elution profile of human blood platelets (PRP) on a Sepharose-2B column (a), on a Sepharose-2B elastin (1 mg/ml) mixed affinity column (c) and on the same Sepharose-2B elastin column in the presence of Nicergoline (1×10^{-4} M) (b). Nicergoline was added to the PRP 15 min before the percolation through the column. Elution with 0.15 M saline and monitoring at 600 nm on a recording spectrophotometer (curves and left hand ordinates) and Coulter-counting of the platelets (vertical lines and right hand ordinates). Abscissa: fraction number.

Table 2. Retention of platelets on a Sepharose-2B elastin column (1mg/ml fibrous elastin) in the absence and in the presence of Nicergoline*

Nicergoline concentration	Platelets retained (%)	Inhibition by Nicergoline (%)‡
0	71 ± 0.13†	
10^{-6} M	62 ± 1	12
10^{-4} M	20 ± 6	72

* For details see Materials and Methods. Results represent mean ± S.E.M. of three determinations.

† In some experiments lower recoveries were obtained, 48 + 8% for instance. Compared to this value, the inhibition obtained with 10^{-4} M Nicergoline would be 58% ($P < 0.05$).

‡ $P < 0.02$.

centrifugation of whole blood for 3 min at 450 *g*. The PRP was centrifuged for 10 min at 1500 *g* and the platelet pellet resuspended and washed three times in 0.10 M NaCl containing 1 ml of ACD per 25 ml of 0.15 M NaCl. The washed platelet pellet was resuspended in Dulbecco's medium (phosphate-buffered saline containing Ca^{2+} and Mg^{2+}) to provide 6×10^5 platelets per μl. The platelets were labeled with carrier-free iodine-125 (specific activity: 6 Ci/nmole) using the lactoperoxidase method. Iodinated platelets were washed two times with Dulbecco's medium and resuspended in Dulbecco's medium to contain 6×10^5 platelets per μl. Labeled platelets were used within 30 min after preparation and also met the criteria of being aggregated by thrombin (0.25 U/ml) and ADP (1×10^{-5}M). In addition, no obvious changes in morphology were observed. In these experiments 1×10^6 ^{125}I-labeled platelets = 3.0×10^3 counts per minute.

Platelet-endothelial cell interaction. Activated (or treated) washed endothelial cells on coverslips were floated (cells down) on 1 ml of ^{125}I-labeled platelets suspended in Dulbecco's medium (6×10^5 platelets/μl). After 4 min rotation on a rotary shaker (120 r.p.m. at 35°), the coverslips were carefully removed with tweezers and processed as described above. All experiments were performed on triplicate coverslips and appropriate non activated controls (Dulbecco's medium only) were simultaneously run in triplicate in each experiment.

RESULTS

Interaction between blood platelets and elastin. Figure 1a shows the elution profile of a platelet rich plasma on a Sepharose-2B column in the absence of elastin. It can be seen that the platelets appear in the breakthrough volume and the recovery of the platelets is of the order of 50–80 per cent. When the Sepharose-2B column contains 1 mg/ml fibrous elastin the profile is considerably changed (Fig. 1c) and only about 20–30 per cent of the platelets are recovered, partially in the breakthrough peak and partially in a second smaller peak appearing in a retarded position.

In the presence of 1×10^{-4} M of Nicergoline, the recovery of platelets in the first breakthrough peak is significantly higher than in the absence of Nicergoline, about 40–75 per cent of the platelets are recovered in the breakthrough peak and no second retarded peak appeared (see Table 2 and Fig. 1b) The average retention of platelets on Sepharose-2B elastin column was decreased by 10^{-4} M Nicergoline by about 58–72 per cent. At a concentration of 1×10^{-6} M of Nicergoline, about 12 per cent inhibition could still be observed.

The same experiments were repeated with the beta-blocking agent, Acebutolol, which showed no inhibition of the interaction of elastin and blood platelets up to a concentration of 10^{-4}M using this same technique. Even in the presence of 10^{-4}M of this drug, the retention of platelets on the Sepharose-elastin column was unchanged.

Interaction between blood platelets and endothelial cells. Table 3 shows the results of the experiment concerning the interaction between activated bovine endothelial cells and bovine blood platelets. If the endothelial cells are not pretreated, only a very low radioactivity sticks to them, corresponding to about 2×10^5 platelets per coverslip. If the endothelial cells are incubated in the presence of 2×10^{-5} M epinephrine for 2 min, there is more than a 100 fold increase in the radioactivity showing a very strong interaction between platelets and activated endothelial cells.

When Nicergoline was added at concentrations of 10^{-4}, 10^{-6} and 10^{-7} M, a total inhibition of platelet–endothelial cell interaction could be seen as shown on Table 3 (experiment V). Nicergoline alone had no detectable effect on the interaction between blood platelets and endothelial cells (experiment III).

An inhibition of about 90 per cent of platelet–endothelial cell interaction was obtained when the blood platelets were preincubated with Nicergoline before adding the endothelial cells (experiment VII). This shows that the same inhibition can be obtained by incubation of either endothelial cells or of blood platelets with Nicergoline.

The same experiments were carried out with Acebutolol, the beta-blocking agent, instead of Nicergoline. It can be seen that no inhibition of platelet endothelial cell interaction was observed with this drug, whether it was added to the endothelial cells or to the blood platelets prior to their mixing (experiments V and VIII).

This experiment confirms that the inhibition of the interaction between blood platelets and elastin and blood platelets and endothelial cells is obtained with the alpha-blocking Nicergoline and not with the beta-blocking Acebutolol.

DISCUSSION

Although the precise mechanism of the interaction between blood platelets and vessel wall macromolecules such as collagen, elastin or microfibrils on the one hand and endothelial cells on the other is not known, it can be assumed that some of the glycoproteins of the blood platelet surface play an important role in this phenomenon. It was proposed recently that fibronectine present in the blood platelets may be responsible for its interaction with collagen [23]. Some other membrane glycoproteins are

Table 3. Interaction between bovine blood platelets and bovine aorta endothelial cells in culture, as described in Materials and Methods*

Expt No.	Treatment	Platelets per coverslip, mean ± S.E.M.	Per cent inhibition of platelet–endothelial cell interaction
I	Control (washed, untreated endothelial cells)	$1.96 \times 10^5 \pm 0.13$	
II	Epinephrine treated (2×10^{-5}M, 2 min) endothelial cells	$2.95 \times 10^7 \pm 0.16$	
III	Endothelial cells (non activated) preincubated with Nicergoline, 4 min		
	1×10^{-4}M	$1.81 \times 10^5 \pm 0.05$	
	1×10^{-6}M	$1.91 \times 10^5 \pm 0.28$	
	1×10^{-7}M	$1.87 \times 10^5 \pm 0.10$	
IV	Endothelial cells (non activated) preincubated with Acebutolol, 4 min		
	1×10^{-4}M	$1.83 \times 10^5 \pm 0.15$	
	1×10^{-6}M	$1.89 \times 10^5 \pm 0.13$	
	1×10^{-7}M	$1.82 \times 10^5 \pm 0.19$	
V	Epinephrine 'activated' endothelial cells (as in expt. II) preincubated with Nicergoline for 4 min†		
	1×10^{-4}M	$2.03 \times 10^5 \pm 0.23$	~ 100
	1×10^{-6}M	$2.03 \times 10^5 \pm 0.19$	~ 100
	1×10^{-7}M	$2.24 \times 10^5 \pm 0.22$	~ 100
VI	Epinephrine 'activated' endothelial cells (as in expt. II) preincubated with Acebutolol for 4 min†		
	1×10^{-4}M	$2.45 \times 10^7 \pm 0.065$	~ 0
	1×10^{-6}M	$2.59 \times 10^7 \pm 0.16$	~ 0
	1×10^{-7}M	$2.67 \times 10^7 \pm 0.30$	~ 0
VII	Epinephrine 'activated' endothelial cells (as in expt. II) added to platelets preincubated 4 min with Nicergoline‡		
	1×10^{-4}M	$3.26 \times 10^5 \pm 0.93$	~ 90
	1×10^{-6}M	$2.71 \times 10^5 \pm 0.13$	~ 90
	1×10^{-7}M	$2.40 \times 10^5 \pm 0.28$	~ 90
VIII	Endothelial cells 'activated' (as in expt. II) and added to platelets preincubated 4 min with Acebutolol‡		
	1×10^{-4}M	$1.91 \times 10^7 \pm 0.34$	0
	1×10^{-6}M	$3.21 \times 10^7 \pm 0.41$	0
	1×10^{-7}M	$3.54 \times 10^7 \pm 0.39$	0

* ^{125}I-labeled platelets were in contact with normal or epinephrine-activated endothelial cells for 4 min. Radioactivity attached to the endothelial cells was determined after washing away non-adherent platelets. Platelet adhesion is expressed as platelets per coverslip, every coverslip contained about 3.7×10^5 endothelial cells. The alpha-blocking agent, Nicergoline, or the beta-blocking agent, Acebutolol, were preincubated at the indicated concentrations (1×10^{-4}, 1×10^{-6}, 1×10^{-7}M) either with endothelial cells (normal or 'activated') or with blood platelets for 4 min prior to mixing platelets and endothelial cells. The results given are the mean average of three independent experiments ± S.E.M.

† Order of incubation: endothelial cells + Nicergoline or Acebutolol, followed after 4 min (without washing) by epinephrine.

‡ Platelet suspension preincubated with Nicergoline or Acebutolol and mixed without washing, after 4 min with the endothelial cells.

considered also as important for the interaction between platelet and vessel wall components, one of them being the Von Willebrand factor present in endothelial cell membranes [24, 25].

It is not yet clear whether the alpha-adrenergic receptors of blood platelets which play an important role in platelet aggregation [26] appear on the same or distinct membrane glycoproteins. As epinephrine does produce platelet aggregation and potentiation of collagen and ADP-induced aggregation, and isoproterenol does not [26], platelets are considered to possess only alpha-type receptors.

It is interesting to notice in this respect that an alpha-blocking agent which is a derivative of dihydroergotamine is able to prevent selectively the interaction of blood platelets with fibrous elastin on the one side and with activated endothelial cells on the other side. A beta-blocking agent, Acebutolol, used at the same concentration, proved to be inefficient in both reactions.

Our results suggest the involvement of the alpha-receptors of blood platelets in their interaction with fibrous elastin. This same receptors appear to be involved in the reaction between blood platelets and endothelial cells. Although the interaction between elastin and blood platelets is a weaker one than the one between collagen and blood platelets, it may play a role in the adhesion of platelets to the sub-endothelium. It is possible that in the denuded, atherosclerotic intima, blood platelets do come in contact with elastic fibrils or with the internal elastic lamina. Therefore the above described inhibition of the interaction between elastin and blood platelets may be of therapeutic significance. This is certainly the case as far as the inhibition of the interaction of endothelial cells activated with epinephrine and blood platelets is concerned. It is interesting to notice that this interaction can be inhibited down to 10^{-7}M concentration of the alpha-blocking agent, a much lower concentration than the one which was needed to obtain about 50 per cent inhibition of elastin blood platelet interaction. As the interaction of blood platelets with 'abnormal' endothelial cells may play an important role in thrombogenesis and atherogenesis, it is interesting to notice that concentrations of this alpha-blocking agent which are therapeutically useful can efficiently and completely inhibit this interaction.

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