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LOWERING OF KININOGEN IN RAT BLOOD BY ADRENALINE AND ITS INHIBITION BY SYMPATHOLYTIC AGENTS, HEPARIN AND ASPIRIN

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1 (−)-Adrenaline lowered the kininogen content and transitorily elevated the fibrinolytic activity of plasma following intravenous injection into the rat. Its effect on kininogen increased when administered by intravenous infusion.

2 Although less effective, (−)-noradrenaline had a similar action to adrenaline; (±)-isoprenaline was inactive and failed to inhibit the effect of adrenaline.

3 The effect of adrenaline on kininogen could be reproduced *in vitro* by incubation of whole blood, but not cell-free plasma, with the catecholamine for 5 min at 37° C.

4 Propranolol or phenoxybenzamine, as well as heparin or acetylsalicylic acid (aspirin), blocked the reduction of rat blood kininogen by adrenaline *in vivo* and *in vitro*.

Introduction

Rosa, Rothschild & Rothschild (1972) noted that adrenalectomized rats have increased levels of kininogen but not of total protein in plasma. Such animals fail to show the activation of the plasma fibrinolytic system which is normally evoked by an intravenous injection of cellulose sulphate (a powerful kinin-releasing, hypotensive agent; Rothschild, 1968), believed to activate fibrinolysin by the indirect release of catecholamines (Rosa *et al.*, 1972). Fibrinolysin (plasmin), may be a mediator of kinin release *in vivo* (Seidel, Stücker & Vogt, 1971). In order to substantiate further the assumption that the adrenalectomized rat has increased levels of kininogen in plasma because it is less able to release catecholamines, the effects of (−)-adrenaline and related compounds on plasma kinin precursor were examined. This report describes how the reduction in plasma kininogen induced by catecholamines in the rat is affected by adrenaline antagonists, heparin, aspirin and the absence of the formed elements of the blood.

Methods

Male Wistar rats (200-300 g) were used; drugs were injected through the venous sinus of the penis, or infused through this site after cannulation; a Palmer continuous slow injector was used. Samples of blood were withdrawn by cardiac puncture into 0.2% sodium oxalate, under pentobarbitone (30-40 mg/kg), anaesthesia; they were kept at 1-2° C until use. Plasma was separated by centrifugation at 2,000 x *g* for 20 minutes. Each animal served as its own control because a sample of blood was withdrawn 30 min before the administration of catecholamines or other drugs. Fibrinolytic activity was determined by the fibrin plate technique and plasma protein by the biuret reagent, as previously described (Rosa *et al.*, 1972). Total plasma kininogen was estimated by the trypsin digestion method according to Diniz & Carvalho (1963): catecholamines and antagonists which interfered with the bioassay of kinin in the last step of this procedure, were eliminated by submitting the plasma, after dilution with 0.2% acetic acid, to dialysis against 50-100 volumes of this diluent for 1 h, with stirring. Subsequent steps, i.e. heating and incubation with trypsin were as originally described. Dialysed samples of plasma yielded the same amounts of kinin as non-dialysed controls. The dialysis step was necessary only for plasma derived from blood which had been treated with catecholamine *in vitro*; plasma obtained from rats which had been injected or infused with catecholamines 5 min prior to blood withdrawal, had apparently been cleared of such amines *in vivo*: such plasma did not inhibit the response of the guinea-pig ileum to kinin. Kininogen levels are expressed in terms of the amount of kinin in μg yielded by 1 ml of plasma.

Kinin formation following injection or incubation of cellulose sulphate with plasma is the result of the breakdown of the plasma-kallikrein-sensitive portion of kininogen. It is considered (Rothschild & Castania, 1970), that it reflects the kininogen I

(Jacobsen, 1966) content of plasma. The effect of catecholamines on this precursor was determined by incubating control or experimental fresh plasma with 40 μg/ml cellulose sulphate for 3 min at 37°C in the presence of 1 mg/ml 8-hydroxyquinoline and assaying released kinin on the atropine-treated guinea-pig ileum (Rothschild & Gascon, 1966). Reduced amounts of kinin released by trypsin from heat-denatured substrate, obtained from experimentally-treated plasma, can only reflect true loss of kininogen. Decreased kinin production by cellulose sulphate could be due to experimentally-induced loss of kininogen, but also to impairment of the enzymic process involving Hageman factor activation and increased esterase activity, which accompanies kinin generation by cellulose sulphate in plasma (Rothschild, 1968). However, the latter alternative is less likely because the same degree of plasma kininogen loss following adrenaline could be demonstrated by either the Diniz & Carvalho (1963), or the Rothschild & Gascon (1966) techniques.

The following materials were used: trypsin (twice crystallized, Worthington, Freehold, N.J.); bovine fibrinogen (fraction I, containing at least 60% coagulable protein), (−)-adrenaline, (−)-noradrenaline and (±)-isopropylnoradrenaline ((±)-isoprenaline) (Sigma, St. Louis, Mo.); bradykinin triacetate (Schwartz, Orangeburg, N.Y.); phenoxybenzamine (dibenzyline) (Smith, Kline & French); propranolol (inderal) (Imperial Chemical Industries Ltd); heparin (Roche Laboratories); acetylsalicylic acid (Aspirin) (U.S. Pharmacopoea). Solutions of all basic compounds were prepared in dilute hydrochloric acid at pH 3-4 just prior to use. Cellulose sulphate was prepared as previously described (Rosa *et al.*, 1972).

Results

The intravenous injection of 40 μg/kg (−)-adrenaline into the rat led to the appearance of fibrinolytic activity in plasma collected 1 min, but not 5 min after injection. Table 1 shows this effect, the decrease in both total and type I plasma kininogen, and the unchanged levels of total plasma protein in the treated animals. Loss of total kininogen, estimated by the trypsin digestion technique of Diniz & Carvalho (1963) was 0.55 bradykinin equivalents/ml; in the same group of animals, kinin release by cellulose sulphate fell from 1.13-0.55 μg/ml after adrenaline. These data illustrate the good agreement between these two methods of estimating kininogen loss in plasma. Figure 1 shows a comparison between the effects of (−)-adrenaline, (−)-noradrenaline and (±)-isoprenaline on kininogen levels in rat plasma, examined 5 min after the intravenous injection of various amounts of catecholamines into the animal. Adrenaline (40 μg/kg), caused a drop of 50% in kinin precursor levels; 20 and 10 μg/kg led to smaller, but still statistically significant effects. (−)-Noradrenaline was ineffective at 20 μg/kg; 40 μg/kg produced results comparable to those of 40 μg/kg adrenaline. (±)-Isoprenaline failed to affect kininogen even when injected at a dose of 80 μg/kg, which corresponds, in terms of the (−)-isomer, to the highest dose of adrenaline employed. The ineffectiveness of (±)-isoprenaline was not due to an inhibitory effect of the (+)-isomer (Ariens, 1967); additional experiments showed that the injection of a mixture of 40 μg/kg (−)-adrenaline plus 80 μg/kg (±)-isoprenaline failed to affect the kininogen-lowering action of the former.

The administration of catecholamines by slow infusion, raised their effectiveness. Table 2 shows that a total of 10 μg/kg adrenaline, infused at a rate of 2 μg kg^{-1} min^{-1}, reduced kininogen to the same extent as did a single injection of 40 μg/kg catecholamine. A total of 5 μg/kg adrenaline infused at the rate of 1 μg kg^{-1} min^{-1}, evoked no effect on kininogen, even though it raised arterial blood pressure during infusion, by 15 ± 2.5%. A

Table 1 Changes in fibrinolytic activity, kininogen content and total protein in the plasma of rats receiving an intravenous injection of (−)-adrenaline.

	Controls	*Treated: 40 μg/kg (−)-adrenaline* 1 min	5 min
Fibrinolytic activity: Mean diameter of lysis area (mm ± s.e.)	0	72 ± 14*	0
Total kininogen content (μg kinin/ml ± s.e.)	1.95 ± 0.05	–	1.40 ± 0.11*
Protein content (g % ± s.e.)	4.7 ± 0.2	–	4.7 ± 0.2
Kinin formed after incubation with cellulose sulphate (μg/ml ± s.e.)	1.13 ± 0.12	–	0.55 ± 0.10*

Each result is the mean of five experiments in which each animal served as its own control. *Effect of treatment statistically significant at $P \leqslant 0.05$, as determined by Student's *t* test on paired samples.

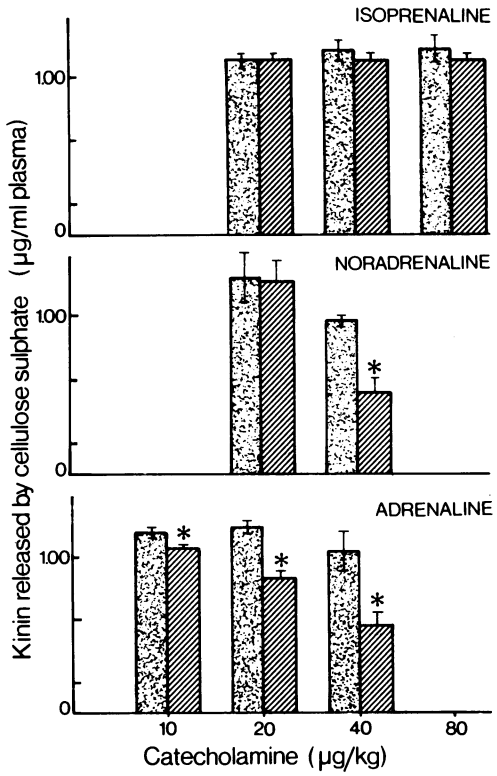

Fig. 1 Reduction of kininogen in plasma of rats injected with catecholamines, demonstrated by decreased kinin formation after incubation with cellulose sulphate. Stippled columns, control samples withdrawn 30 min before treatment; diagonally hatched columns, samples withdrawn 5 min after i.v. injection of catecholamine; results are means obtained from five animals, and are presented with their standard errors. * Effect of treatment significant at $P \leq 0.05$.

total of 10 µg/kg (−)-noradrenaline, infused at the rate of 2 µg kg^{-1} min^{-1}, produced the same effect as an identical dose of adrenaline. The infusion of a total of 10 or 20 µg/kg (±)-isoprenaline did not lead to a lowering of plasma kininogen.

A study of the effects of adrenoceptor blocking agents on the action of catecholamines revealed that both the α-adrenoceptor blocking agent phenoxybenzamine as well as the β-adrenoceptor blocking agent propranolol, markedly decreased the effect of infused (−)-adrenaline or (−)-noradrenaline on kininogen. This result is illustrated in Fig. 2, which also shows that, by itself, neither of these inhibitors affected plasma kininogen.

Heparin (1 mg/kg) or sodium acetylsalicylate (20 mg/kg) administered intravenously to groups of three rats each, prevented the decrease of kininogen caused by 40 µg/kg (−)-adrenaline given by the intravenous route, 15 min after either treatment.

Table 3 shows that oxalated rat whole blood, but not rat plasma separated from its formed elements, responded to incubation with catecholamines with a lowering of kininogen. (−)-Adrenaline was more effective than (−)-noradrenaline; (±)-isoprenaline was inactive. Figure 3 shows the effect of concentration on the reduction of kininogen induced by adrenaline: extrapolation of the results suggested that 35-50 ng/ml adrenaline would be required to produce threshold effects; a maximal response was obtained with approximately 500 ng/ml blood. As in the intact animal, the effects of adrenaline could be inhibited by either α- or β-adrenoceptor blocking agents. Table 4 shows the results of two experiments, each performed on blood pooled from two rats, indicating that phenoxybenzamine as well as propranolol was able to inhibit the action of the catecholamine. The α-adrenoceptor blocking agent appeared to be more effective on a molar basis.

Table 2 Kinin formed by cellulose sulphate *in vitro* in the plasma of rats that had first received an i.v. infusion of (−)-adrenaline, (−)-noradrenaline or (±)-isoprenaline.

		Kinin yield (µg/ml ± s.e.)		
Drug	*Dose (µg kg^{-1} min^{-1})*	*Controls*	*After treatment*	
(−)-Adrenaline	1.0	1.17 ± 0.03	1.12 ± 0.03	(5)
,,	2.0	1.07 ± 0.04	0.60 ± 0.07*	(5)
(−)-Noradrenaline	2.0	1.03 ± 0.08	0.53 ± 0.06*	(5)
(±)-Isoprenaline	2.0	1.00 ± 0.04	1.00 ± 0.04	(3)
,,	4.0	1.04 ± 0.05	1.04 ± 0.05	(3)

Catecholamine solutions were infused at constant rate of not over 0.2 ml/min for a 5 min period; blood was withdrawn 30 min before (controls) and 5 min after the infusion. Figures in parentheses refer to the number of animals employed. *Results significantly different from controls.

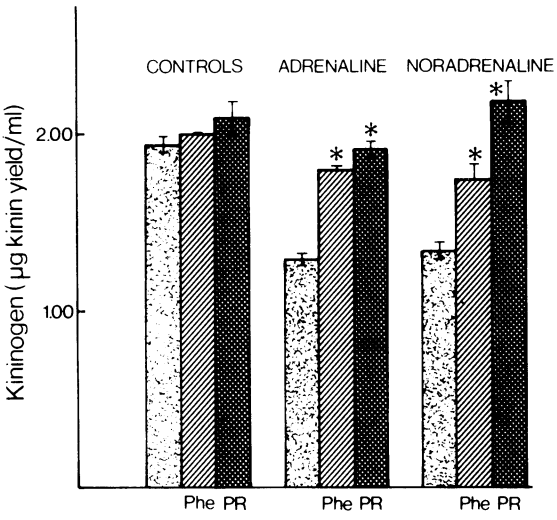

Fig. 2 Inhibition by phenoxybenzamine (Phe), or propranolol (PR), of the decrease of kininogen evoked in the rat plasma by an intravenous infusion of (−)-adrenaline or (−)-noradrenaline. Stippled columns, animals receiving no pre-treatment; diagonally hatched columns, animals receiving 10 mg/kg of phenoxybenzamine by slow i.v. infusion over a 60 min period terminating 1 h before catecholamine treatment; cross-hatched columns, animals receiving 10 mg/kg of propranolol i.p., 1 h before catecholamine treatment. Adrenaline or noradrenaline were infused at a rate of 2 µg min^{-1} kg^{-1} over a 5 min period, terminating 5 min before withdrawal of the blood sample. Results from controls or adrenaline-treated animals represent means and standard errors obtained from five animals; those from noradrenaline-treated rats, from three. * Effect of inhibitors significant at $P \leq 0.05$.

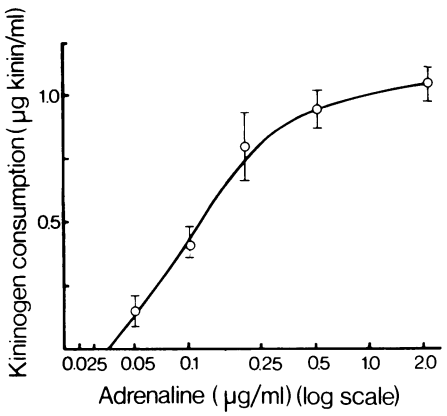

Fig. 3 Lowering of plasma kininogen following incubation of oxalated, whole rat blood with various concentrations of adrenaline for 5 min at 37°C. Each point represents the mean and standard error of four experiments.

Both heparin and acetylsalicylic acid (aspirin) proved to be efficient inhibitors of the action of adrenaline on blood kininogen (see Table 5).

Discussion

Adrenaline and noradrenaline were able to lower rat plasma kininogen normally available for kinin release by trypsin or cellulose sulphate. This action did not involve non-specific loss of plasma protein since this was not observed in the adrenaline-treated animals.

The infusion of catecholamines proved a more efficient means of lowering kininogen than the

Table 3 Plasma kininogen in oxalated rat whole blood or plasma after incubation with catecholamines for 5 min at 37°C.

Catecholamine (µg/ml)	*Incubation with*	*Kininogen* (µg kinin yield/ml ± s.e.)*	
–	Plasma	2.0 ± 0.0[c]	(4)
(−)-Adrenaline, 1.0	Plasma	2.0 ± 0.0	
–	Blood	2.2 ± 0.05[c]	(6)
(−)-Adrenaline, 1.0	Blood	1.0 ± 0.05	
–	Blood	1.9 ± 0.0[c]	(4)
(−)-Noradrenaline, 1.0	Blood	1.4 ± 0.05	
–	Blood	1.75[c]	(2)
(±)-Isoprenaline, 2.0	Blood	1.75	

Figures in parentheses refer to the number of experiments performed. [c]Controls incubated with 0.9% w/v NaCl solution (saline). *Determined according to Diniz & Carvalho (1963).

administration of equivalent or even higher doses of the amines by single intravenous injection. The effective rate of infusion (2 $\mu g\ kg^{-1}\ min^{-1}$) probably just sufficed to compensate for the rapid clearing of catecholamines from the circulation (Whitby, Axelrod & Weil-Malherbe, 1961), thus allowing for the build-up of enough hormone to trigger the kininogen-degrading process.

It was difficult to classify the effect of catecholamines as an action on either α- or β-adrenoceptors. Although lack of sensitivity to isoprenaline suggested a predominant involvement of the former, the achievement of blockade by the use of equivalent doses of either phentolamine or propranolol, did not warrant this conclusion. While none too frequent, sensitivity to both α- and β-adrenoceptor inhibitors of responses to catecholamine has been described. Ahlquist & Levy (1959) were the first to report this phenomenon in intestinal smooth muscle. Bydgeman & Johnsen (1969) have shown that platelet aggregation induced in human platelet-rich plasma by adrenaline or noradrenaline, could be inhibited by either phenoxybenzamine or propranolol. Mills & Roberts (1967) have suggested that in this system, propranolol owes its action to an imipramine-type of inhibition of catecholamine uptake, rather than to β-receptor blockade. Sensitivity to both α- and β-receptor inhibitors has been described for the lipolysis-stimulating effect of adrenaline in rat liver: a mixed type of receptor, having both α- and β-sensitive sites was proposed. However, later results (Robison, Butcher & Sutherland, 1971) showed that at least two steps were involved, each having distinct sensitivities to adrenoceptor blocking agents. It is possible that a similar situation prevails in the kininogen-lowering process triggered by catecholamines in the rat.

Table 4 Inhibition by phenoxybenzamine (Phe) or propranolol (Pro) of the decrease in kininogen in oxalated rat blood incubated with (−)-adrenaline.

	Kininogen		
Additions to blood (M)	*Sample 1*	*Sample 2*	*Average reduction*
	(μg kinin yield/ml plasma)		*(%)*
–	1.8	2.0	–
(−)-Adrenaline (Ad), 10^{-5}	0.8	1.0	53
Phe, 10^{-5} + Ad	1.2	1.2	37
Phe, 10^{-4} + Ad	1.5	1.8	13
Pro, 10^{-5} + Ad	0.8	1.0	53
Pro, 10^{-4} + Ad	1.5	1.6	19

Inhibitors were incubated with blood for 15 min at 37° C before the addition of adrenaline; they did not affect plasma kininogen levels. Samples 1 and 2 each represent the pooled blood obtained from two rats. Samples were incubated with adrenaline for 5 min at 37° C.

Table 5 Inhibition by aspirin or heparin of the effect of (−)-adrenaline on kininogen in rat oxalated blood *in vitro*.

	Kininogen		
Additions to blood (μg/ml)	*Sample 1*	*Sample 2*	*Average reduction*
	(μg kinin yield/ml plasma)		*(%)*
–	1.9	2.0	–
Adrenaline (Ad), 2.0	1.1	1.2	46
Heparin, 10 + Ad	1.8	2.0	4
Aspirin, 10 + Ad	1.5	1.9	13
Aspirin, 100 + Ad	1.8	1.9	5

Conditions were as for Table 4.

Lowering of kininogen level occurred readily in whole rat blood incubated with catecholamines, but not in cell-free plasma submitted to the same treatment. This contrasts with the action of anionic activators of kinin release, such as glass (Margolis, 1958), cellulose sulphate (Rothschild & Gascon, 1966) or ellagic acid (Gautvik & Rugstad, 1967), which function well in clear plasma. It is possible that blood platelets are the initial target of the action of catecholamines. Adrenaline and noradrenaline cause platelet aggregation (O'Brien, 1963) and release of nucleotides and enzymes (Mills, Robb & Roberts, 1968). In producing these effects, as with reduction of kininogen level, adrenaline is the most active, noradrenaline the intermediate, and isoprenaline the inactive member of the series. The infusion of adrenaline into rabbits leads to the activation of Hageman factor and to a marked, transient reduction (aggregation) of circulating platelets (McKay, Latour & Parrish, 1970). In view of the recognized role of Hageman factor in the kinin releasing process (Margolis, 1960), this observation also suggests an involvement of platelets in the lowering of blood kininogen by catecholamines.

Maximum disappearance of kininogen following adrenaline treatment was 2.0 units/ml rat blood; the same value was obtained in adult rat plasma treated with a maximally effective amount of cellulose sulphate or glass powder (Rothschild & Castania, 1970). This probably represents the yield of kinin which can be obtained from rat plasma following maximal activation of its kininogenase regardless of whether it is initiated by the direct action of acidic macromolecules like cellulose sulphate, glass or ellagic acid on soluble components of plasma or, as suggested by the present results, by an initial action of catecholamines on the formed elements of the blood. It is worth noting that the initial 'cellular' phase of this process is the probable target of the inhibitory action of heparin and aspirin: neither of these compounds prevented kininogen breakdown by cellulose sulphate, when added to rat plasma at the same concentrations which effectively blocked kininogen decrease in rat blood by adrenaline.

The anti-inflammatory action of aspirin is well known but its mechanism has not been firmly established. The observation that aspirin can block kininogen breakdown induced by such ubiquitous mediators as adrenaline or noradrenaline, represents an interesting subject for further studies. Aspirin inhibits platelet aggregation by adrenaline *in vivo* and *in vitro* (O'Brien, 1968), and, like other non-steroid anti-inflammatory compounds, effectively decreases the intensity of bacterial endotoxin shock (Hinshaw, Solomon, Erdös, Reins & Gunter, 1967), a condition shown to involve both catecholamine (Palmerio, Zetterstrom, Shamash, Euchbaum, Frank & Fine, 1963) and kinin release (Rothschild & Castania, 1968; Melmon & Cline, 1967). Exposure of rats to an abnormally hot environment leads to kininogen breakdown (Greeff, Luhr & Strobach, 1966). The etiology of this phenomenon has not been explained; it represents a condition of stress in which an initial sympathetic discharge could cause catecholamine-mediated kininogen breakdown and kinin release.

The observation (Rosa *et al.*, 1972) that adrenalectomized rats have higher levels of plasma kininogen, considered together with the present results, lends weight to the assumption that, apart from its pathological implications, the release of the potent vasodilator kinin by adrenaline might be yet another safety measure by which the organism counteracts excessive local sympathetic vasoconstriction.

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