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Plasma Kinins and Cortisol: A Possible Explanation of the Anti-Inflammatory Action of Cortisol

Abstract. *Kinins are naturally occurring vasoactive polypeptides thought to be mediators of acute inflammatory responses. Kinins are released from a plasma protein substrate by glass-activated plasma enzymes (kallikreins) or by isolated intact granulocytes. Cortisol in concentrations of* 2.5×10^{-6} *to* 2.5×10^{-5}M *prevented the release of active kinin from substrate by granulocytes or contact with glass. Deoxycorticosterone, progesterone, and etiocholanolone in comparable concentrations were significantly less effective in preventing kinin release. Plasma obtained from patients receiving prednisone released no kinin after activation by glass and less kinin than control plasma when exposed to granulocytes. Cortisol also partially inhibited the release of kinin by purified urinary kallikrein. Certain adrenocorticosteroids may exert their anti-inflammatory effect by inhibiting the release of plasma kinins. Steroids may act in part by preventing interaction between the activated kallikrein and its substrate.*

Kinins are small naturally occurring polypeptides of known structure. Their pharmacologic activity is extraordinarily high; in concentrations of 0.1 to 1 mμg/ml the kinins cause vasodilatation, produce edema by increasing capillary permeability, stimulate some smooth muscle, provoke pain, and are leukotactic (*1*). Since these pharmacologic effects resemble the manifestations of acute inflammation, kinins are believed to be a hormonal mediator of the response to tissue injury (*2*). This concept is supported by considerable experimental evidence of two general types: kinin concentrations are increased at the sites of inflammation, and when a synthetic kinin such as bradykinin is applied to certain tissues it mimics an inflammatory response (*3*).

Kinins are released from a plasma protein substrate by either plasma enzymes (kallikreins) or by granulocytes. The agent activating the plasma enzyme(s) that liberate kinins from biologically inactive substrate (kininogen) appears to be blood clotting factor XII (Hageman factor) in its activated configuration. Thus, release of kinin and initiation of the clotting mechanism are closely linked processes. Hageman factor can be activated in vitro by contact of plasma with a glass surface. The release of free kinin from plasma substrates or from purified kininogen by exposure to granulocytes of rabbits and man was recently described in preliminary reports (*4, 5*). This phenomenon is dependent on the concentration of mature granulocytes (neutrophils, metamyelocytes, and myelocytes) and is not produced by normal or neoplastic lymphocytes.

The present studies were designed to examine the interaction between the kinin system and the anti-inflammatory adrenocorticosteroids, cortisol, and prednisone.

Leukocytes and plasma were obtained from normal subjects and patients with hematologic diseases or arteriosclerosis (control subjects), and from a group of patients with nonbacterial inflammatory diseases or with comparable hematologic diseases who were receiving 60 to 80 mg of prednisone per day (experimental subjects). The isolation of leukocytes and the in vitro incubation conditions have been described in detail (*6*); the only modification was the use of Eagle's minimal essential medium (*7*) containing human albumin (25 mg/ml) as the suspending medium.

Plasma kinins were prepared for assay by the method of Diniz and Carvalho (*8*) and were measured by bioassay on the estrus rat uterus (*9*) with synthetic bradykinin (Sandoz, Inc.) as the standard. Assays were usually performed in duplicate, and the results differed from the mean by no more than 7 percent. These peptides were characterized by their insensitivity to trypsin, their inactivation by chymotrypsin, and their hypotensive effect in the rat. Substrate (kininogen) concentration was determined by the difference between the total kinin released from a sample by trypsin digestion and the kinin concentration of the undigested sample. Since the known individual kinins, which are nona-, deca-, and unadecapeptides, have an approximately equal effect in the bioassay system, no effort was made to differentiate them, and their combined activity was expressed as bradykinin concentration.

Plasma substrates (kininogen) were prepared by a minor modification of the method of Webster and Pierce (*10*) and shown to be free of kinin-destroying (kininase) and kinin-generating activity. Various preparations of substrate contained 0.05 to 0.2 μg of bradykinin per milliliter as contaminant; this amount was not sufficient to interfere with the interpretation of the results. Urinary kallikrein was prepared by the method of Moriya, Pierce, and Webster (*11*) and had a specific activity of 5.7 Frey units per milligram.

In any given experiment the release of kinin from plasma or purified substrate was accomplished in one of three ways: (i) Leukocytes in a final concentration of 3×10^6 cells per milliliter and consisting of at least 70 percent mature neutrophils and metamyelocytes were added to undiluted plasma or to plasma diluted 1 : 3 with minimal essential medium. After incubation at 37°C for 10 to 30 minutes, the cells were removed by centrifugation and the suspending medium was assayed for kinin activity. Plasma and leukocytes were obtained from the same person or from subjects of compatible blood groups who had not received transfusions. In certain experiments, purified plasma substrate (kininogen) containing in each milliliter 0.1 or 1.0 μg of material equivalent to bradykinin was substituted for whole plasma. (ii) Seven glass beads, 4 mm in diameter, were added to 1.5 ml of cell-free plasma in a 25-ml siliconized erlenmeyer flask. The bead suspension was shaken mechanically at room temperature for 15 minutes at 4 cy/sec. Samples of plasma were then removed and assayed for kinin. (iii) Purified urinary kallikrein, 1 Frey unit per milliliter, was added to whole plasma or purified substrates and allowed to react for 5 minutes at 20°C. Samples were then assayed for kinin concentration.

When cell-free plasma from the control subjects was added to granulocytes or placed in contact with a glass surface, the kallikrein system was activated as indicated by a rise in the concentration of free kinin and a decrease in the concentration of kininogen (Table 1). Because kinins were simultaneously released from substrate and destroyed by plasma and granulocyte-kininase activity, their concentration at any time was a function of these opposing two processes. Consequently, the depletion of substrate concentration was used as an index of the total generation of kinin from substrate.

The release of active kinin from substrate by granulocytes or by glass contact (*12*) was prevented by the addition of cortisol to the plasma in the form of the free alcohol or as the hemisuccinate derivative in concentrations from 2.7×10^{-6} to $2.5 \times 10^{-5} M$. Deoxycorticosterone, progesterone, and etiocholanolone in comparable concentrations were less effective or totally ineffective (Table 1). Higher concentrations of these steroids were not tested. Cortisol, in the concentrations used in the test system, had no effect on the action of kinin in the bioassay system. In the absence of glass contact, the three other steroids did not liberate kinin from plasma and had no effect on the bioassay system. Cortisol in the concentrations employed had no effect on the kininase activity of intact leukocytes or leukocyte fragments (*4*).

To simplify the test system, purified kininogen was substituted for whole

Table 1. Effect of steroids on release of kinin from plasma or purified kininogen by leukocytes or contact with glass. Results are expressed as means ± S.E.

Mode of activation	Steroid added	Concentration (mmole/ml)	No. of substrates tested	Kinin concentration after activation (% of control)	Residual kininogen after activation (%)
	Substrate: Plasma (3000 to 6000 mμg kininogen per milliliter)				
Leukocytes	None		10	259 ± 55	50 ± 10
	Cortisol	2.5×10^{-5}	4	114 ± 8	96 ± 3
	Deoxycorticosterone	2.5×10^{-5}	4	154 ± 9	24 ± 5
	Progesterone	2.5×10^{-5}	3	139 ± 18	41 ± 1
	Etiocholanolone	2.5×10^{-5}	3	123 ± 46	46 ± 1
Glass	None		8	209 ± 29	35 ± 5
	Cortisol	2.7×10^{-7}	2	69	38
		2.7×10^{-6}	5	88 ± 12	100 ± 4
	Deoxycorticosterone	2.7×10^{-6}	5	292 ± 67	47 ± 10
	Progesterone	2.7×10^{-6}	3	223 ± 62	45 ± 12
	Etiocholanolone	2.7×10^{-6}	3	267 ± 42	40 ± 5
	Substrate: Purified kininogen (0.1 to 1.0 mμg per milliliter)				
Leukocytes	None		4	138 ± 18	29 ± 10
	Cortisol	5.0×10^{-6}	4	138 ± 20	80 ± 6
	Deoxycorticosterone	5.0×10^{-6}	4	190 ± 55	19 ± 8

Table 2. Disappearance of kininogen from plasma of four control subjects and of four patients receiving prednisone.* Plasma was activated by contact with glass, with autologous leukocytes, or with isologous leukocytes from prednisone-treated patients.

Plasma donor	Concentration of kininogen (mμg/ml)	Mode of activation	Residual kininogen after activation (%)
	Experiment 1		
Control	2930	None	100
		Glass	46
Prednisone-treated	2880	None	100
		Glass	104
	Experiment 2		
Control	3130	None	100
		Glass	46
		Control leukocytes	48
		P leukocytes †	77
Prednisone-treated	2780	None	100
		Glass	103
		Control leukocytes	34
		P leukocytes †	41
	Experiment 3		
Control	5900	None	100
		Glass	25
		Control leukocytes	31
		P leukocytes †	55
Prednisone-treated	3925	None	100
		Glass	100
		Control leukocytes	33
		P leukocytes †	100
	Experiment 4		
Control	3400	None	100
		Glass	29
Prednisone-treated	3050	None	100
		Glass	97

* In experiment 4, plasma was obtained from the same patient before (control) and during prednisone therapy. † P = from prednisone-treated patient.

Table 3. Effect of steroids on release of kinin from plasma or purified kininogen by urinary kallikrein. Urinary kallikrein was added to plasma or purified kininogen in a concentration of 1 Frey unit per milliliter. The reaction was stopped after 5 minutes at 20°C by the addition of 10 volumes of 0.2-percent acetic acid. Samples were then prepared and assayed for kinin activity. Results are expressed as means ± S.E.

Steroid added	Concentration (mM/ml)	No. of substrates tested	Kinin concentration after activation (% of control)	Residual kininogen after activation (%)
Substrate: Plasma (2810 to 3900 mμg kininogen per milliliter)				
None		5	386 ± 123	33 ± 6
Cortisol	4×10^{-6}	3	383 ± 67	46 ± 3
Cortisol	4×10^{-5}	3	242 ± 80	74 ± 13
Deoxycorticosterone	4×10^{-5}	3	350 ± 104	37 ± 9
Substrate: Purified kininogen (1000 mμg per milliliter)				
None		5	372 ± 34	
Cortisol	4×10^{-5}	4	147 ± 7	
Deoxycorticosterone	4×10^{-5}	5	312 ± 27	

or diluted plasma as substrate. When well-washed granulocytes were added to the purified kininogen (*p*H 7.4), biologically active peptide was released and substrate concentration decreased. When cortisol, $5 \times 10^{-6}M$, was added to the system, formation of kinin was partially inhibited (Table 1). Deoxycorticosterone in comparable concentrations did not prevent generation of kinin by leukocytes. Progesterone and etiocholanolone were not examined in this system. These observations suggest that cortisol in concentrations that are four to eight times greater than physiological levels is capable of specifically interfering with granulocyte kallikrein activity in vitro.

The possibility that the cortisol effect was an artifact of the in vitro addition of the steroid to the test system was examined by experiments on plasma obtained from patients receiving large doses of prednisone. Such plasma was exposed to glass surfaces or to granulocytes obtained either from the plasma donor or from control subjects with similar diseases who were not being treated with prednisone. The results (Table 2) indicate that plasma obtained from patients receiving prednisone was no longer susceptible to glass activation under the described experimental conditions. Such plasma could still be partially activated by granulocytes, but compatible cells from control subjects (not treated) were more effective in activation than autologous granulocytes from the prednisone-treated patients. The available data, although limited, suggest that leukocytes from patients receiving prednisone may be less effective than normal white blood cells in releasing kinin from normal plasma. Thus, "glucocorticoids" administered to patients appear to inhibit the formation of kinins from their plasma.

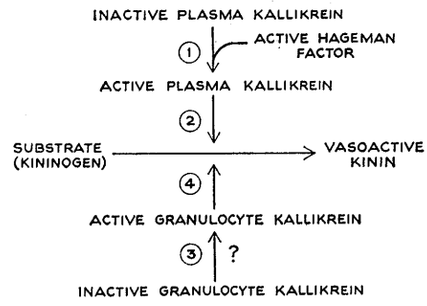

Fig. 1. The formation of kinins from kininogen.

The two likely mechanisms of cortisol action in preventing kinin formation are (i) inhibition of the activation of leukocyte and plasma kallikrein (sites 1 and 3, Fig. 1), and (ii) interference with the interaction of activated kallikrein with substrate (sites 2 and 4, Fig. 1). These alternatives were indirectly tested by the addition of activated urinary kallikrein to purified kininogen or plasma. Such a test system circumvented the first step of kallikrein activation. In experiments in which both purified substrate and purified kallikrein were used, neither leukocyte nor plasma kininase was present and the kinin concentration reflected kinin generation. As shown in Table 3, the addition of cortisol partially prevented the release of kinin from plasma and purified substrate by activated urinary kallikrein. Deoxycorticosterone was less effective. Thus, at least one site of cortisol action appears to be at the interaction between activated kallikrein and its substrate. The data do not preclude the possibility that cortisol also affects kallikrein activation, substrate configuration, or both.

Despite the fact that glucocorticoids have been used as anti-inflammatory agents for approximately 15 years, their mode of action is poorly understood. The most attractive hypothesis thus far offered to explain their anti-inflammatory action is the stabilization of lysosomal membranes, with consequent inhibition of the release of "irritating" hydrolytic enzymes (*13*). To produce this effect, however, the cortisol-like steroids must be present in much greater concentrations (10^{-3} to $10^{-4}M$) than can be achieved by drug administration. Furthermore, anti-inflammatory steroids inhibit the early phenomena of the inflammatory process: capillary dilatation, edema formation, fibrin deposition, and migration of phagocytic granulocytes into the inflamed area. The activities cannot be explained readily by the effect of steroids on lysosomal membranes. On the basis of the investigations reported here, we propose that cortisol and similar steroids may exert their anti-inflammatory effect in part by preventing the formation of vasoactive kinins, which are responsible for many of the manifestations of acute inflammation. This effect of cortisol is demonstrable at concentrations that can be achieved by drug administration. The observation that cortisol partially inhibits release of kinin from substrate by urinary kallikrein suggests that one site of steroid action may be at the interaction of activated kallikrein and substrate.

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Odor Discrimination in Pigeons

Abstract. *An operant procedure was employed to investigate odor discrimination in the pigeon. Amyl acetate concentrations of 6-percent saturation in air, and lower, when paired with electric shock markedly reduced key-pecking during the odor stimulus period. Sectioning the olfactory nerves eliminated this selective suppression behavior. After the operation, the suppression was again conditioned when the concentration of amyl acetate was increased to 15-percent saturation in air. This is theoretically possible through mediation by the trigeminal system.*

Although it has long been known that all birds possess an olfactory epithelium and an olfactory bulb (*1*), it is only recently that Tucker (*2*) has studied the functional status of the receptors. Electrical activity was recorded from the primary olfactory nerves in pigeons, quail, turkey vultures, and eleven other species of birds during olfactory stimulation with amyl acetate and other odorants. Tucker concluded that the olfactory receptors of birds are functional.

Behavioral studies have produced controversial results. By such methods as studying change in heart rate and respiration, some evidence has been obtained that birds can perceive olfactory stimulations (*3*). However, most experiments designed to study the learning of olfactory discriminations have yielded negative results (*4*). Other investigators (*5*) have shown some behavioral change in learning to discriminate olfactory stimuli. Michelsen, using an operant conditioning technique, reported successful odor discrimination in the pigeon. Fink (*6*), using the gray goose as a subject, was unable to repeat the work of Michelsen.

Calvin (*4*), using a classical conditioning technique developed by Orgel and Smith (*7*), was unable to show discrimination of olfactory stimuli in pigeons. Later work by Smith and others in our laboratory has shown that the classical conditioning methods with birds are quite insensitive, and has led to the development of a more nearly adequate technique for the study of sensory phenomena in these animals (*8*). This technique is a variety of the conditioned suppression method and employs dual contingencies of positive reinforcement and aversive stimulation. Base lines for steady, on-going behavior are generated, and can be discretely disrupted by repeatedly pairing the warning stimulus with electric shock. The elimination of responding during the warning stimulus, when compared with base-line responding, gives high signal-to-noise ratios.

The purpose of the present study was to use olfactory stimulation as the warning signal in utilization of the conditioned suppression technique to study olfactory discriminations in the pigeon. The apparatus used for delivery of the olfactory stimulus, and the bird test chamber, are illustrated in Fig. 1. The air was cleaned with silica gel and activated charcoal and was saturated with either distilled water or amyl acetate. The breathing chamber was equipped with a pigeon key and an opening allowing access to a grain hopper. Perforated discs at the intake and exhaust ports of the breathing chamber allowed for more nearly uniform flow of air. A "white" masking noise was present throughout the sessions.

The subjects for the experiment were two Silver King pigeons, 6 and 7 years old, and one 2-year-old white Carneaux. The three subjects were run at approximately 80 percent of normal body weight. Stainless-steel wires were implanted around the pubis bones and terminated in a small plug which was attached to the back of a light canvas jacket worn by the bird (*9*). In the presence of air flow in the chamber (94 cm^3/sec) each of the birds was trained to peck the key for grain reinforcement. Training on a variable interval (V.I.) schedule of reinforcement (average inter-reinforcement period equaled 1 minute) ensued for 1 hour a day for approximately 14 days, at which time the rate of responding was stable. After stabilization of the response rate, suppression training was initiated. During the 1-hour session while the bird was pecking the key (average, approximately 100 pecks per minute), ten suppression trials, three control trials, and two base-line control trials were given. A suppression trial consisted of a 30-second period of stimulation with amyl acetate (6 cm^3/sec) added to the air flow, followed by an 85-msec electric shock administered to the pubis bones. Recordings of the key pecks during the olfactory stimulation and during the 30-second period prior to stimulation were made on two electrical impulse counters. To insure that the animal was responding to the presentation of amyl acetate rather than to an increase in air flow, control trials were run in exactly the same manner except that a volume of air equivalent to that of the amyl acetate

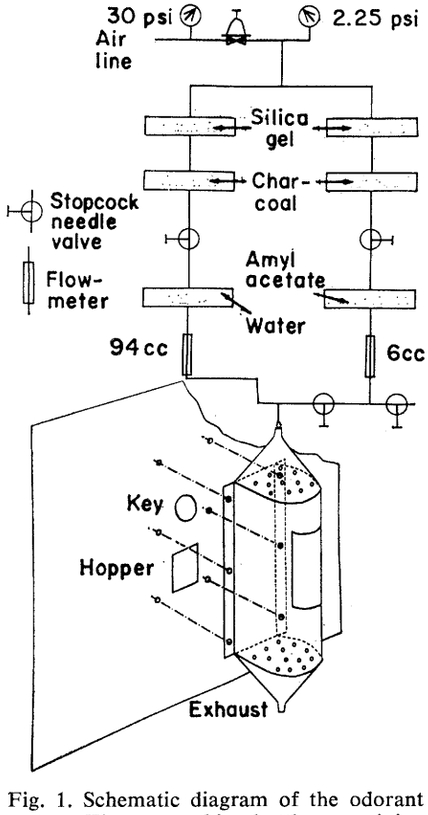

Fig. 1. Schematic diagram of the odorant system. The gas-washing bottles containing the charcoal, distilled water, and amyl acetate were continuously bathed in water at 20°C. Stopcocks and needle valves permitted control and mixture of the air in the two channels, as measured by the flow meters. The resultant concentration (6 percent shown in the diagram) was passed through the pigeon's breathing chamber and exhausted with a vacuum pump.