# Bradykinin Inhibition by Serum from Animals Subjected to Thermal Injury[1] (36057)

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The concept of liberation or formation of toxic substances following burns has been postulated for several years (1–5). Some investigators have denied the existence of a burn toxin (6) and others have attributed "toxic" postburn products to microorganisms (7). The initial purpose of this investigation was to study the effect of pre- and postthermal burn serum or plasma on the biological activity of bradykinin, since kinins have been implicated in thermal trauma (8, 9).

Pathologically occurring "specific inhibitors of natural kinins" have not been reported in the literature, although two kininases have been demonstrated (10). However, analogs of bradykinin possessing "varying degrees of antibradykinin activity as well as bradykinin like potency" have been studied (11). None of these antibradykinin analogs exhibit reliable antibradykinin action.

Studies in this laboratory (12) indicate that the serum or plasma of thermal burned animals (rats, rabbits) inhibits the activity of the natural kinins, bradykinin, lysylbradykinin, and methionyllysylbradykinin on smooth muscle but without action on nonmammalian kinin, eledoisin. The inhibitory activity of 48 to 72 hr postburn serum appears to be different from the two kininases. The isolated rat uterus bioassay procedure for bradykinin was chosen because of the high degree of sensitivity of the uterus muscle to the action of kinin. The earlier thermal burn research was done on male rats, but the bulk of later work was performed on rabbits.

*Methods and Materials.* A male rabbit (6–7 lb) was strapped to an animal board; and hair was clipped from the hind quarters (dorsal and ventral areas) to the level of the lower abdomen. The genitalia and hind limbs were placed in plastic ice packs. The animal was deeply anesthetized with ether and the clipped area was immersed in 80° for 15 sec, after which the scalded area was placed in an ice-water bath for several seconds. Approximately 25 to 30% of the total body surface was burned.

Animals were exsanguinated via the ear vein 48 to 72 hr postburn. Rabbit bleeding apparatus[2] consisted of a glass cylinder closed at one end with two side arms, one for attachment to vacuum line and the other for collection of blood. A single lateral slit was made in the marginal ear vein and the open end of the cylinder was placed over the rabbit ear; vacuum was applied gradually until the blood flowed freely into the container. Blood was collected in polyethylene tubes, processed to serum, and stored at —20° or freeze dried. To obtain plasma the blood was heparinized. Edema fluid, when present in the hind quarters, was collected under sterile conditions.

The various aliquots of normal and postburn serum were tested in the presence of bradykinin (synthetic bradykinin, BRS 640 Sandoz, Inc.) in a modified smooth muscle bioassay apparatus. The uterine horn of a virgin albino female rat (150–175 g) was employed. Constant volume (15 ml), aeration rate, temperature (28°), as well as fresh Tyrode's solution were maintained in the muscle bath. A smoke drum kymograph

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[2] Bellco Biological Glassware and Equipment Company, Vineland, NJ.

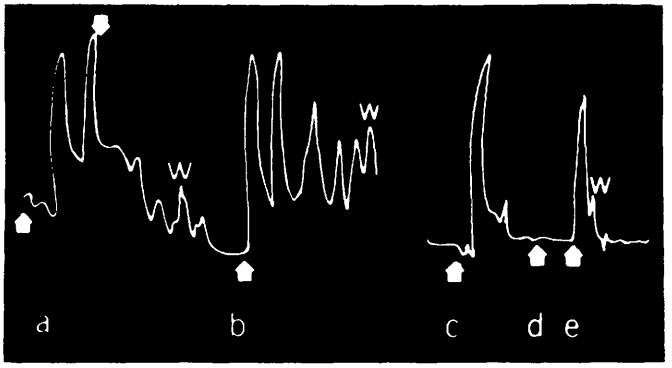


FIG. 1. Inhibition of BK by scald burn serum (rabbit): (a) Response of uterus muscle in Tyrode's solution (15 ml volume) at 28° to 0.125 ng/ml of BK. As indicated by the arrow, 0.5 ml of burn serum was added with subsequent fall in muscle contraction. (b) After washing muscle (W), 0.125 ng/ml of BK was added, resulting in continued contraction. (c) Response of uterus muscle to 0.125 ng/ml of BK + 1 ml of normal rabbit serum. (d) Response of uterus muscle to 0.125 ng/ml of BK + 0.25 ml of burn serum (incubated before adding), with complete inhibition of BK action. (e) On addition of 0.125 ng/ml of BK, the muscle responds with normal contraction.

was used to record the smooth muscle contractions at a speed of 0.5 cm/min.

Fractionation of normal and burn plasma with polyethylene glycol (PEG) 6000 was accomplished by the procedure of Polson (13). Four protein fractions were prepared as follows: gamma globulin precipitated with 0–10 g/100 ml (pH 5.8); beta globulin, 10–16 g/100 ml (pH 5.8); alpha globulin, 0–9 g/100 ml (pH 4.6); albumin, 14–20 g/100 ml (pH 7.0).

Ammonium sulfate fractionation of normal and burn plasma was as follows: gamma globulin, 0–33% sat.; alpha and beta globulins, 33–50% sat.; albumin, 50–75% sat. All precipitations were adjusted to pH 7.0.

Seromucoid protein separation was carried out in 0.6 *M* perchloric acid according to the procedure of Chandler and Neuhaus (14) and Neuhaus *et al.* (15).

Determination of the approximate molecular weight of the inhibitor was accomplished by ultrafiltration with Amicon Diaflo membranes, XM-50 and XM-100, using pressures from 10 to 25 psi.

*Results.* Figure 1 indicates the inhibitory effect of scald burn serum or plasma (rabbit, rat) on the bradykinin elicited contraction of uterus muscle of the virgin rat. On addition of the burn serum during contraction subsequent to the addition of bradykinin, an immediate fall in the amplitude of the contracting muscle occurs. It is not essential to preincubate the burn serum and bradykinin before addition to the muscle bath in order to elicit inhibition. Direct addition of burn serum to the muscle preparation following the introduction of bradykinin usually results in no response of the muscle, hence, complete inhibition.

The burn serum was assayed shortly after the animal was exsanguinated, and the remaining serum was stored in 50 ml plastic

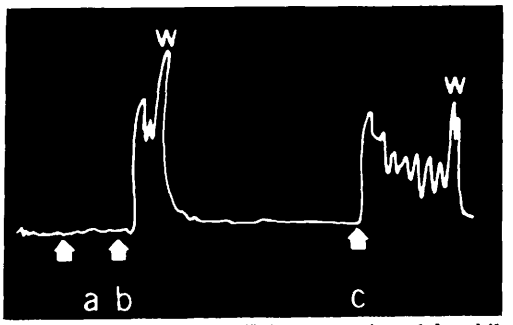


FIG. 2. Inhibition of BK by reconstituted lyophilized burn serum: (a) Response of uterus muscle in Tyrode's solution at 28°, to 0.5 ng/ml of BK + 0.25 ml of reconstituted lyophilized burn serum to original volume. (b) Addition of 0.5 ng/ml of BK results in contraction of muscle. (c) Same as A except that reconstituted lyophilized normal rabbit serum (3× concentrated) was used.

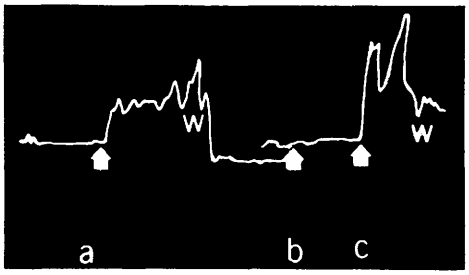


FIG. 3. Inhibition of bradykinin by ultrafiltrates from burn serum (rabbits): (a) Response of uterus muscle in Tyrode's solution, at 28° to 0.5 ng/ml of BK + 1 ml of ultrafiltrate of burn serum <50,000 mol wt. (b) Same as A except 0.5 ml of ultrafiltrate >50,000 mol wt. (c) Normal response of uterus muscle to 0.5 ng/ml of BK.

bottles at —20°. Storage of the serum at 6° (refrigerator temperature) resulted in complete loss of inhibitory action after 10 days. Frozen serum samples remained active for a longer interval of time, but gradually lost their activity. Presumably, proteases released during the burn trauma are responsible for degradation of the inhibitor.

Figure 2 shows complete inhibition of bradykinin by reconstituted lyophilized burn serum after several months storage at —20°.

Ultrafiltration of burn serum (Amicon Diaflo-membranes XM-50; XM-100) (Fig. 3) shows that no inhibitory activity exists below a molecular weight of approximately 50, 000, with all inhibitory activity above this magnitude, between 50,000 and 100,000.

Since it appears that the inhibitor is protein in nature, of large molecular size, and is heat labile, a comparative study of some bradykininolytic enzymes with the inhibitor from burn plasma and chemical inhibitors is shown in Table I. The data for the inhibition of the inactivation of bradykinin by carboxypeptidase B (pancreas), kininase I and II was compiled from the literature (16). This comparative study indicates a similarity of inhibition of plasma kininase II and the burn plasma inhibitor with regard to 1, 10-phenanthroline and EDTA. Likewise, $CdSO_4$ and arginine are not effective in producing inhibition of kininase II and the burn plasma inhibitor.

Fractionation of burn plasma by gel filtration on sephadex G 200 was not successful in separating one major fraction that was active as a bradykinin inhibitor. On the contrary, a number of fractions were eluted from the column that had some activity. DEAE-cellulose fractionation of burn plasma presented similar patterns of activity of the inhibitor.

Preliminary study on the use of protein precipitants without denaturation of the protein was more successful in separating the active inhibitor from the burn plasma. Polyethylene glycol (mol wt 6000) was employed in the fractionation process. The major fraction that appeared to be the most active inhibitor was albumin, followed by alpha$_2$ globulin—gamma globulin had no activity. Ammonium sulfate precipitation of the burn plasma, 50–75% saturation, likewise, showed concentration of the active inhibitor in the albumin fraction.

*Discussion.* Increased kininogen and bradykinin itself has been implicated as one of the postburn toxic products and Jacobsen and Waaler (17), have shown that various components of the kallikrein–kininogen system in lymph including the kininase are increased during the scalding of the hind leg of the dog. Our experiments, however, which indicate a bradykinin inhibitor as a postburn product, are not necessarily inconsistent with the above. Since nearly all of our thermal injured animals survived at least 72 hr, one could postulate that the bradykinin inhibitor served as a protective mechanism to counteract toxic products, [*i.e.*, bradykinin and kallidin (kinins)] and thus insure survival.

In contrast to acetylcholine and histamine, no physiological occurring low molecular weight organic compounds, have been found which would specifically block the action of kinins. Kinins are rapidly inactivated in blood plasma; the liberation and inactivation occur as independent processes. Erdös (18) has characterized carboxypeptidase N (kininase I), an enzyme in human plasma fraction IV-1 which inactivates bradykinin by cleaving the C-terminal arginine of the peptide. This enzyme was found to be distinct from pancreatic carboxypeptidase B which is also a kinin inactivator. Both kallidin and bradykinin are inactivated equally fast and only a fraction of kallidin is convert-

TABLE I. Comparative Inhibition of Some Bradykininolytic Enzymes with the Inhibitor from Burn Plasma.[a]

| Chemical | Conc (moles/liter) | Carboxypeptidase B–pancreas[b] | Carboxypeptidase N–plasma (kininase I) (human)[c] | Plasma kininase II (human)[c] | Burn plasma inhibitor (rabbit) |
|---|---|---|---|---|---|
| $CdSO_4$ | $3 \times 10^{-4}$ | Activation | ++ | 0 | 0 |
| $ZnCl_2$ | $3 \times 10^{-4}$ | Activation | 0 | 0 | 0 |
| $CoCl_2$ | $1 \times 10^{-4}$ | ++ | Activation | Activation | nt |
| 1,10-Phenanthroline | $1 \times 10^{-3}$ | ++ | ++ | ++ | ++ |
| EDTA $Na_2$ | $3 \times 10^{-3}$ | 0 | ++ | ++ | ++ |
| BAL | $2 \times 10^{-3}$ | ++ | ++ | ++ | 0 |
| Cys | $1 \times 10^{-4}$ | ++ | + | nt | 0 |
| Arg | $3 \times 10^{-3}$ | ++ | ++ | 0 | 0 |
| *E*-Amino-*n*-caproic acid | $3 \times 10^{-3}$ | ++ | + | 0 | 0 |
| Urea | 6M | 0 | + | nt | + |

[a] ++, complete inhibition; +, partial inhibition; 0, no inhibition; nt, not tested.
[b] Data from literature.
[c] Handb. Exp. Pharmakol. 35 (1970) (16).

ed to bradykinin by the converting enzyme aminopeptidase before being inactivated by kininase. More recently Yang and Erdös (10) have characterized a second bradykininase (kininase II) from human plasma. Kininase II cleaves the $Pro^7$–$Phe^8$ bond in bradykinin. Rabbit plasma does not contain this particular kininase (16). Both kininases of human plasma are inhibited *in vitro* by chelating agents such as EDTA, 1,10-phenanthroline, and 2,3-dimercaptopropanol (BAL) suggesting the presence of a metal cofactor for enzymatic activity.

Table I indicates a comparative study of the inhibition of some bradykininolytic enzymes with the thermal injury inhibitor from burn plasma. The data show that, with the exception of two chemical inhibitors 1, 10-phenanthroline and EDTA, no other chemical affects the activity of the burn inhibitor. Apparently BAL has no influence on the inhibitor, except only in higher concentrations.

An induction of plasma protein synthesis in response to trauma (laparotomy) has been reported by Neuhaus *et al.* (15). This hepatic synthesis involves the formation of seromucoid proteins over a period of 24 hr. This group of proteins is soluble in 0.6 *N* perchloric acid and may be readily separated from the other plasma proteins. The seromucoid isolated from thermal injury plasma had no inhibitory activity on bradykinin.

So far all efforts to prove that the inhibitor may be a kininase or peptidase induced by thermal injury have failed. Whereas kininases react slowly with bradykinin, requiring several minutes of incubation time with the substrate; the inhibitor, on the contrary, acts almost instantaneously with the kinin, either directly or at the receptive site on the smooth muscle. The inhibitor is heat labile, nondialyzable, of large molecular weight, and appears to be associated with $alpha_2$-globulin and albumin fractions. Earlier observations (12) on the immunoelectrophoretic patterns of normal and thermal burn serum of rabbits indicated that an $alpha_2$ macroglobulin was present in 48 hr postburn serum that could not be detected in normal serum. However, this protein has not yet been identified with bradykinin inhibitor.

All experiments were repeated a sufficient number of times to avoid any artifacts.

Further study is underway to elucidate the chemical nature of the inhibitory substance and its role in other biological reactions in the animal body, *i.e.*, capillary permeability, and vasodilation.

*Summary.* A substance appears in postburn serum and edema fluid of animals (rats, rabbits) subjected to thermal burn that inhib-

its the action of bradykinin on smooth muscle. It has not been detected in normal serum. Evidence suggests that it is not enzymatic in nature, and not a kininase. Ultrafiltration studies indicate that the inhibitor has a molecular weight of over 50,000. The substance appears to be confined to two fractions of the serum, alpha$_2$-globulin and albumin. Presumably the inhibitor acts by combining with bradykinin, therefore, antagonizing its action on the receptor site of the muscle.

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