Role of Kinin in Regulation of Rat Submandibular Gland Blood Flow

Torill Berg, Oscar A. Carretero, A. Guillermo Scicli, Barbara Tilley, and John M. Stewart

In tissues rich in kallikrein, vasodilator kinins, acting as paracrine hormones, may play a role in the local regulation of blood flow. We studied the role of kinins in the regulation of blood flow in the rat submandibular gland using a kinin analogue with antagonistic properties, [DArg°]Hyp3-Thi[4][Phe7]bradykinin. When infused into the carotid artery (20 µg/min/rat), this antagonist blocked the effect of bradykinin (25–250 ng/kg, intracarotid injection) on glandular blood flow. In nephrectomized rats, the antagonist also blocked the increase in glandular blood flow caused by enalaprilat, a kininase II converting enzyme inhibitor. At a dose of 20 µg/min/rat, the antagonist produced no detectable change in basal glandular blood flow; however, at a higher dose (100 µg/min/rat), it caused a significant decrease (p<0.001). In eight of 10 rats, blood flow decreased by 75% or more; this effect was not blocked by the α-adrenergic receptor antagonist phentolamine. After antagonist infusion was stopped, blood flow returned toward normal. Sympathetic nerve stimulation of the gland induced vasoconstriction followed by poststimulatory vasodilation. In rats displaying severe vasoconstriction after the antagonist, postsympathetic vasodilation was abolished even when stimulation was performed after the antagonist infusion had been stopped and blood flow returned toward normal. Although a direct vasoconstrictor effect of the kinin antagonist cannot be completely ruled out, these data suggest that, in the rat submandibular gland, kinins may play a role in regulation of basal blood flow and vasodilation after converting enzyme inhibitor or sympathetic stimulation. (Hypertension 1989;14:73–80)
min from Schwarz/Mann Biotech, Cleveland, Ohio; bradykinin from Bachem, Inc., Torrance, California; heparin sodium from Elkins-Sinn, Cherry Hill, New Jersey; norepinephrine from Winthrop-Breon Laboratories, New York; phenolamine (Regitine) from CIBA-GEIGY, Basel, Switzerland; and silicone (Dow Corning 200 fluid) from Dow Corning Corporation, Midland, Michigan. The kinin antagonist [dArg¹Hyp³-Thr⁵-phényl]bradykinin-TFA (Hyp, 4-hydroxyproline; Thr, β-(2-thienyl)-L-alanine; TFA, trifluoroacetic acid) was produced by John Stewart of the University of Colorado as previously described for similar peptides, and angiotensin I CEI or kininase II inhibitor (enalaprilat, MK422) was provided by Merck, Sharp & Dohme, West Point, Pennsylvania.

Male Wistar or Sprague-Dawley rats (400–500 g body wt) were anesthetized with ether and nephrectomized via a flank incision to avoid interference from the renin-angiotensin system, particularly for studies involving CEI. Twenty-four hours later, the rats were anesthetized with pentobarbital sodium (60 mg/kg body wt i.p.) and tracheotomized. The abdominal aorta was catheterized via the femoral artery, and blood pressure was recorded with a Brush recorder and Statham strain-gauge transducer. A stretched polyethylene catheter (PE 10) was inserted into the carotid artery facing the submandibular gland; to avoid leakage, glue was applied at the point of insertion. Vehicle (0.9% NaCl containing 0.01% bovine serum albumin to avoid adhesion of the peptide to tubes and catheters) or kinin antagonist was infused with a Harvard pump. A three-way stopcock placed on this catheter was used for bolus injections of bradykinin and CEI. Blood flow in the submandibular gland was measured as previously described. Briefly, the effluent was collected from a catheter in the right submandibular gland vein close to the hilus, passed over a photoelectric drop counter, and then returned to the animal through the femoral vein. The drop counter was partially filled with 0.9% saline containing 100 USP units/ml heparin covered by a layer of silicone. It was connected to a Brush recorder, and the time per drop was calculated from a calibrated ramp that was restarted every time a drop of blood passed the photoelectric cell. When the time per drop exceeded maximum ramp time (19 seconds), paper speed was used to calculate flow rate. Vascular resistance was calculated as blood pressure/blood flow. Rats were given heparin (0.20 ml, 1,000 USP units/ml i.p.) before fine dissection and cannulation of the submandibular gland vein but after major surgery was completed. If sympathetic nerve stimulation was to be performed, the cervical sympathetic nerve was first dissected free and covered with mineral oil; then, just before stimulation, it was carefully placed on a silver electrode. A Grass SD9 stimulator (7.5 V, 2 msec, 10 Hz, 1 minute) was used. Submandibular gland blood flow and blood pressure were measured continuously while the following protocols were performed.

Protocol 1: Effect of Kinin Antagonist on Bradykinin-Induced Vasodilatation (Nine Wistar Rats)

Immediate changes after intracarotid injections of bradykinin (25, 100, and 250 ng/kg body wt, 0.5 ml, 1 minute infusion) were determined before and after administration of kinin antagonist (20 μg/min, 34 μl/min). Two rats that responded to the antagonist with prolonged vasoconstriction were excluded, as changes during the 1-minute bradykinin injections could not be monitored by the drop counter when flow was slower than 1 drop/min.

Protocol 2: Effect of Kinin Antagonist on Converting Enzyme Inhibitor–Induced Increase in Blood Flow

After a 5-minute control period, either the antagonist (20 μg/min) or vehicle (34 μl/min) was infused in six and eight Wistar rats, respectively. Ten minutes later, CEI (100 μg/ml enalaprilat at 30 μg/kg body wt) was injected into the carotid artery as a bolus. After 5 minutes, infusion of antagonist or vehicle was stopped, and blood flow and pressure were recorded for another 45 minutes.

Protocol 3: Effect of Kinin Antagonist on Basal and After Sympathetic Nerve Stimulation

After a 5-minute control period, continuous infusion of antagonist or vehicle was started. Sympathetic nerve stimulation was then performed in four groups of animals.

Group 1 rats (six Sprague-Dawley rats) received infused vehicle (55 μl/min) for 9 minutes with sympathetic nerve stimulation performed 5 minutes into the infusion. In five rats, stimulation was repeated 35 minutes after the infusion. In group 2 rats (six Sprague-Dawley rats), kinin antagonist (20 μg/min, 55 μl/min) was infused for 9 minutes. Sympathetic stimulation was performed only once, 5 minutes after the start of the infusion. In group 3 rats (10 Sprague-Dawley rats), kinin antagonist (100 μg/min, 55 μl/min) was infused for 9 minutes with stimulation performed 5 minutes into the infusion. In seven rats, stimulation was repeated approximately 35–50 minutes later (i.e., after blood flow had returned and stabilized near preantagonist values). Group 4 rats (six Sprague-Dawley rats) were identical to those in group 3, except that an intravenous bolus of 2 mg/kg body wt phenolamine, an α-adrenergic blocker, was injected 10 minutes before antagonist infusion. The blocking effect of phenolamine was tested in a separate group of five Sprague-Dawley rats by monitoring the blood pressure response to norepinephrine (50, 100, and 200 ng/rat, i.v. bolus injection) before and 10 minutes after phenolamine (2 mg/kg body wt, i.v. bolus injection; five rats). At this dose, phenolamine blocked 80–90% of the norepinephrine response (p<0.0001).
Statistical Analysis

All results are given as mean±SEM. In protocol 1, measured analysis of variance (ANOVA) was used and repeated to assess the response to bradykinin before and after antagonist infusion. In this analysis, the first step was to test for an interaction between bradykinin dose and the presence or absence of antagonist. No such interaction was detected, and the effect of different doses of bradykinin as well as antagonist versus vehicle were tested directly. Profile analysis was used in protocol 2 to compare groups (vehicle and antagonist) across time and to test for interaction. Because of interaction between group and time, paired t tests were used to compare times within groups and two-sample t tests were used to compare groups at a particular time. In protocol 3, the Kruskal-Wallis test was used to compare resistance among groups (vehicle, 20 and 100 µg/min antagonist) due to the presence of outliers in the resistance measurements. ANOVA was used for blood flow and pressure. In the event of any difference among groups, Wilcoxon’s test was used in pairwise comparisons for resistance and two-sample t tests were used for blood flow and pressure. Whenever it was necessary to adjust rejection levels for multiple comparisons, a conservative Bonferroni approach was used to maintain an overall p value of 0.05.

Results

Protocol 1: Effect of Kinin Antagonist on Bradykinin-Induced Vasodilatation

When bradykinin was injected into the carotid artery, submandibular gland blood flow increased (p<0.01 for all doses), while vascular resistance fell (p<0.004) (Figure 1). The change was marginally dose related for blood flow (p<0.06) but not vascular resistance (p>0.28), indicating that bradykinin was administered in a near-supramaximal dose. Injection of bradykinin was not associated with any detectable change in blood pressure (p>0.16). The antagonist blocked the effect of bradykinin on blood flow (p<0.006) and vascular resistance (p<0.004). During the antagonist infusion, the small vasodilatation induced by bradykinin was similar to that caused by saline alone.

Protocol 2: Effect of Kinin Antagonist on Converting Enzyme Inhibitor-Induced Increase in Blood Flow

In the vehicle group, average blood flow doubled within 30 seconds after administration of CEI but subsequently decreased and returned to pre-CEI values after 33±4 minutes (Figure 2, Table 1). The antagonist blocked the immediate effect of CEI on blood flow. When the infusion was stopped 5 minutes after CEI, blood flow steadily increased, reaching a maximum of 89±27 µl/min (p<0.03 compared with pre-CEI flow) before returning to pre-CEI values. Peak flow occurred at 26±5 minutes in the antagonist group compared with 1 minute in the vehicle group. This relation between vehicle and antagonist groups across time represents a statistical interaction that was confirmed by profile analysis.


Infusion with vehicle alone produced no detectable change in blood flow, vascular resistance, or blood pressure (p>0.13) (Figure 3, Tables 2 and 3). After 5 minutes of antagonist infusion (20 µg/min), no change in blood pressure, blood flow, or vascular resistance could be detected, except for two rats that exhibited markedly decreased blood flow (38% and 26%, respectively). Infusion of 100 µg/min significantly decreased basal blood flow (by 75% or more in eight of 10 rats) (p<0.001) and increased vascular resistance (p<0.003). Pretreatment with phentolamine was associated with a significant
DECREASE IN BLOOD PRESSURE (103±5 mm Hg before and 64±6 mm Hg after, \( p<0.0005 \)) and blood flow (65±5 \( \mu l/min \) before and 41±4 \( \mu l/min \) after, \( p<0.002 \)). The antagonist further increased vascular resistance and decreased blood flow though not as much as in rats not pretreated with phentolamine; however, actual blood flow in both groups during antagonist infusion was similar (\( p>0.05 \)).

Sympathetic nerve stimulation in the vehicle group induced vasoconstriction (data not shown) followed immediately by marked vasodilatation. A similar response was seen with the second (postinfusion)

### TABLE 1. Mean Blood Pressure, Submandibular Gland Blood Flow, and Vascular Resistance Before and 1 and 35 Minutes After Converting Enzyme Inhibitor Administration in Rats Infused With Vehicle or Kinin Antagonist

<table>
<thead>
<tr>
<th>Measure*</th>
<th>Group</th>
<th>Before CEI</th>
<th>1 minute after CEI</th>
<th>35 minutes after CEI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood pressure</td>
<td>Vehicle</td>
<td>94±7</td>
<td>86±10</td>
<td>99±9</td>
</tr>
<tr>
<td>(mm Hg)†</td>
<td>Antagonist</td>
<td>99±4</td>
<td>102±3</td>
<td>91±10</td>
</tr>
<tr>
<td>Blood flow</td>
<td>Vehicle</td>
<td>52±4</td>
<td>103±11§</td>
<td>48±3 NS</td>
</tr>
<tr>
<td>(( \mu l/min ))</td>
<td>NS</td>
<td>58±7</td>
<td>65±10 NS</td>
<td>NS</td>
</tr>
<tr>
<td>Resistance†</td>
<td>Vehicle</td>
<td>1.86±0.15</td>
<td>0.86±0.08§</td>
<td>2.12±0.27 NS</td>
</tr>
<tr>
<td>(BP/BF)</td>
<td>NS</td>
<td>§</td>
<td>§</td>
<td>§</td>
</tr>
<tr>
<td></td>
<td>Antagonist</td>
<td>1.80±0.16</td>
<td>1.71±0.21 NS</td>
<td>1.43±0.63 NS</td>
</tr>
</tbody>
</table>

*Profile analysis was used to compare all rats monitored as long as 35 minutes after converting enzyme inhibitor (CEI) administration (vehicle, 5 of 8; antagonist, 6 of 6).

†No group- or time-related effects (\( p>0.8 \)) or interaction between group and time (\( p>0.08 \)) were detected.

‡Statistically significant interaction between group and time was detected for both blood flow (BF) and vascular resistance (\( p<0.02 \)). Increased blood flow and decreased vascular resistance in the vehicle group within 1 minute after CEI were significantly blunted in the antagonist group. However, there was no significant difference in the change in blood flow (\( p>0.26 \)) or vascular resistance (\( p>0.28 \)) between the two groups from before to 35 minutes after CEI, BP, blood pressure.

NS, \( p>0.05 \); \( \rho<0.005 \); \( \rho<0.05 \) values after data indicate intragroup comparison (before compared with 1 or 35 minutes after CEI), while values between lines indicate intergroup comparison (vehicle compared with antagonist group).
stimulation in the rats infused with antagonist (20 
\mu g/ml), there was no detectable difference in
the postsympathetic vasodilatory response compared
with the vehicle group. In the rats given 100 
\mu g/ml, blood flow was almost totally shut off before
stimulation in eight out of 10 animals; these animals
exhibited no subsequent vasodilatation when stim-
ulated during antagonist infusion. In the two remain-
ing rats that did not respond to the antagonist with
decreased basal blood flow, postsympathetic vaso-

**FIGURE 3.** Typical recordings of submandibular gland blood flow in response to sympathetic nerve stimulation (●) performed during and after infusion of vehicle (top), 100 
\mu g/ml antagonist (middle), or 100 
\mu g/ml antagonist after pretreatment with phentolamine (P) (bottom). A high dose of antagonist results in decreased blood flow (middle), which was not prevented by phentolamine (bottom). The vasodilatory response after both first and second stimulations in the vehicle group (top) was blocked by the antagonist (middle). The duration of infusion of vehicle (V) or antagonist (A) is indicated by the horizontal bars (■) above the tracings.

**TABLE 2. Effect of Kinin Antagonist on Basal Mean Blood Pressure, Blood Flow, and Vascular Resistance**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Blood pressure (mm Hg)</th>
<th>Blood flow (\mu l/min)</th>
<th>Vascular resistance (BP/BF)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before infusion</td>
<td>5 minutes after infusion</td>
<td>Before infusion</td>
</tr>
<tr>
<td>Vehicle</td>
<td>6</td>
<td>88±9</td>
<td>86±9</td>
</tr>
<tr>
<td>20 \mu g antagonist</td>
<td>6</td>
<td>80±5</td>
<td>82±3</td>
</tr>
<tr>
<td>100 \mu g antagonist</td>
<td>10</td>
<td>92±6</td>
<td>84±7</td>
</tr>
<tr>
<td>Phentolamine</td>
<td>6</td>
<td>64±6</td>
<td>55±4</td>
</tr>
<tr>
<td>+ 100 \mu g antagonist</td>
<td>6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Analysis of variance (Kruskal-Wallis test for resistance) showed significant differences in the change in blood pressure, basal blood flow, and vascular resistance among groups that were administered the vehicle and 20 and 100 
\mu g antagonist from before to 5 minutes after the start of infusion (p<0.03, <0.003, and <0.005, respectively). However, pairwise comparisons between groups show significant differences only in blood flow and vascular resistance in the 100 
\mu g antagonist group (*,†,‖ when compared with the vehicle group, †,‖ when comparing values before and after infusion). There was also a significant difference in blood flow between the 20 and the 100 
\mu g antagonist groups (*,‖ between lines). No other significant differences were observed.

NS, p>0.017; †p<0.006; *,†p<0.001; †,‖ p<0.01.
vasoactive intestinal peptide or substance P may have been involved. The objective of the present study was not to study this atropine-resistant parasympathetic vasodilatation but to investigate the role of kinin in basal blood flow regulation in the submandibular gland of the rat because this gland contains more kallikrein than any other organ known. Furthermore, we wanted to study the role of kinin in the vasodilatation observed after CEI where a kinin dependency was strongly suggested by previous results and in the vasodilatation after sympathetic nerve stimulation, which is the only stimulus that induces massive release of kallikrein and kinin into the vascular compartment.

To further study the role of the kallikrein-kinin system in the regulation of glandular blood flow, we used a recently developed analogue of bradykinin that has antagonistic properties. Previous studies showed that this analogue is particularly effective in blocking exogenous bradykinin-induced vasodilatation in the dog hind limb and hypotension in the rat. The specificity of the antagonist has also been tested by its lack of inhibitory effect on the vasodilatory response to acetylcholine and nitroprusside. Furthermore, we know from unpublished studies (Paolo Madeddu and Oscar Carretero) that this antagonist does not block the vasodilatory or hypotensive effect of dopamine or prostaglandin E2.

A very similar antagonist (Thi-[DPhe7]bradykinin) that has antagonistic properties has been shown to not block the effect of substance P on guinea pig ileum. The present study shows that this antagonist blocks the vasodilator effect in the submandibular gland induced by injection of exogenous bradykinin into the gland circulation. In addition, we have shown that the antagonist prevents vasodilatation induced by CEI. We have previously shown this particular response to be blocked by kinin antibodies, and it is therefore not likely to be mediated by substance P, even though CEI has been shown to inhibit substance P degradation. These data support the conclusion that the antagonist is specific for kinin and works through inhibition of kinin receptors.
It was not clear why the vasodilatory effect of CEI would wear off within 30 minutes as CEI is known to have a long-lasting effect. However, it is possible that the gland became desensitized to the effect of kinin or that other compensatory mechanisms were activated to return flow to baseline values. Furthermore, after infusion of antagonist, it took 26 minutes for CEI to reach a maximum effect, whereas in the control group the effect was immediate. The most likely explanation for this was that some time was needed for degradation and washout of the kinin antagonist, which also may have a prolonged half-life due to CEI.

In eight of 10 rats receiving antagonist at a rate of 100 μg/min, basal blood flow decreased by 75% or more. While this strong vasoconstriction was unexpected, it did not appear to be an artifact because blood flow returned after the infusion was discontinued; moreover, no such changes were ever observed in rats infused with vehicle. The magnitude of the response may be explained by the fact that kinin blockade could leave the gland unprotected against vasoconstrictive agents such as tonin or endothelin. It is also possible that vasoconstriction was due to local release of catecholamines from sympathetic ganglia and nerves through residual agonistic effects of the antagonist as bradykinin can stimulate catecholamine release. However, this seems unlikely because the vasoconstrictive effect was not affected by pretreatment with phentolamine, an α-adrenergic blocker. Thus, these results suggest that in the rat submandibular gland, locally generated kinins may play a role in the regulation of basal blood flow. To be sure, we cannot rule out the possibility that the kinin antagonist itself possesses a vasoconstrictive effect not mediated by kinin receptor blockade; however, we know of no evidence to support this theory.

The disparity between the dose necessary to alter basal blood flow and that sufficient to block kinin-induced vasodilation after exogenous bradykinin or CEI may be due to a difference in cellular compartments for peptide formation and action in these two situations. Converting enzymes are localized to the surface of endothelial cells, thus for both CEI and exogenous bradykinin, kinins most likely act on receptors within the vascular compartment, probably in the endothelium. On the other hand, endogenous kinin formation may take place within the interstitial space and act on receptors near this compartment, either directly or through the release of prostaglandins, thereby requiring a higher dose of antagonist.

We observed a clear difference in postsympathetic vasodilation between rats that responded to the antagonist with markedly reduced basal blood flow and those that did not. In the latter animals, vasodilatation was observed identical to controls. Among those that did respond, vasodilatation was abolished both during antagonist infusion and when stimulation was performed after antagonist-induced vasoconstriction had subsided and blood flow returned toward normal. This could be explained by the fact that return of blood flow was induced by vasodilators other than kinins (e.g., CO₂, adenosine) accumulating during the lengthy period of vasoconstriction. The ability to respond to kinins released during the second stimulation could still be blocked by the kinin antagonist, which is probably only gradually washed out of the interstitial space during vasoconstriction. However, we see no obvious explanation as to why one dose of antagonist should vary so markedly from animal to animal in its ability to block basal blood flow or why this same dose would still be sufficient to block postsympathetic vasodilatation with a 700-fold increase in kinin release. It cannot be excluded that the prolonged lack of flow after the high dose of antagonist damaged the tissue and made it unable to vasodilate. However, the tissue was at this point able to vasoconstrict on sympathetic stimulation. Furthermore, even though no proof exists to indicate that the kinin antagonist used in the present study should have pharmacological effects other than to block kinin, other biological effects cannot be excluded.

The results of the present study indicate that the rat submandibular gland responds to intra-arterial kinins with vasodilation that can be blocked by low doses of kinin antagonist. Though basal blood flow is not affected by such a low dose, the CEI-induced increase in glandular blood flow is abolished; this suggests that kinins mediate CEI-induced glandular vasodilatation, as also indicated by previous studies with kinin antibodies. Higher doses markedly decreased basal glandular blood flow in eight of 10 rats. Furthermore, vasodilatation subsequent to stimulation of the cervical sympathetic chain was also blocked by a high dose of antagonist. These data are consistent with the hypothesis that kallikrein released from the submandibular gland, either in basal conditions or during sympathetic stimulation, generates kinins that in turn play a role in the regulation of blood flow to the gland. Unless this antagonist has other pharmacological effects, the results from the present study are consistent with the hypothesis that the glandular kallikrein-kinin system acts as a regulator of local vasodilatation.

References

**KEY WORDS** • bradykinin • kinins • kallikrein • vasodilation • converting enzyme inhibitors • blood flow • salivary glands
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Hypertension. 1989;14:73-80
doi: 10.1161/01.HYP.14.1.73
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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