Effects of prolonged reduction in blood flow on submandibular secretory function in anesthetized sheep

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The present experiments were undertaken in an attempt to define the extent to which submandibular secretory responses depend on the blood flow to the gland, in the longer term. The results show that reducing the blood flow (by ~50%) is associated with a rapid reduction in the flow of saliva, together with the outputs of Na⁺, K⁺, and protein, with no sign of adaptation to the constraint over a period of 40 min.

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The physiological relation between the flow of blood to exocrine glands and secretory function is clearly of fundamental importance and yet has received scant attention. Over the last century, salivary glands have provided the most popular functional, experimental model with which to investigate exocrine secretion; they are readily accessible and, in some, both secretion and blood flow can be measured simultaneously, allowing precise quantification of the responses to autonomic stimulation.

The mechanism whereby the blood flow increases in response to parasympathetic stimulation has attracted enormous interest, ever since it was shown to be resistant to atropine (originally in the submandibular gland of the dog [10]). Numerous theories were put forward to account for this phenomenon (for example, see Ref. 5), and it is now established that it is due to the release of vasoactive intestinal peptide from the parasympathetic nerve terminals, which elicits a nitric oxide-dependent increase in intracellular cGMP (reviewed in Ref. 3). In most of these studies, the dependence of secretion on blood supply in vivo has generally been assumed rather than investigated. Langley (11) thought that a reduction in blood flow would result in a diminished salivary secretion. This was supported by Emmelin (6), who showed that a reduction in salivary secretion was proportional to the degree of sympathetic stimulation that was superimposed on a period of supramaximal parasympathetic stimulation. However, this ignores the complex interactions between transmitters released from the two populations of nerve terminals, which are revealed most obviously after the administration of small amounts of atropine (see Refs. 4, 12, 15).

Lung (13, 14) was the first to address the question of the blood flow dependence of salivary secretion directly, using an in vivo vascular perfused preparation in anesthetized dogs in which the arterial flow rate to the mandibular gland was controlled. She found that a 2-min cessation or reduction in arterial blood flow resulted in a secretory response that was completely independent of blood flow during parasympathetic stimulation at low to moderate frequencies (below 8 Hz). However, research from this laboratory has shown that, over longer periods (5 min), salivary secretion is highly dependent on blood flow. Various methods have been used to reduce blood flow through the submandibular gland, including the induction of generalized hypotension and intracarotid (ic) infusions of endothelin-1 (ET-1) (8, 9, 17). ET-1 is a potent vasoconstrictor peptide that acts directly on vascular smooth muscle cells, increasing their intracellular calcium concentration and hence causing their contraction (20). Each of these studies has shown that a reduction in blood flow substantially reduces the secretion of saliva in response to parasympathetic stimulation in both cats and sheep over periods of 5 min.
infusion of the barbiturate into the right femoral vein at a jugular vein, and subsequently maintained by a continuous injection of 3% pentobarbital sodium (Sagatal; Rhône Merieux, Harlow, UK), at a dose of 15–20 mg/kg, into the jugular vein, and subsequently maintained by a continuous infusion of the barbiturate into the right femoral vein at a rate of 0.1–0.3 mg·kg⁻¹·min⁻¹. The rate was adjusted to maintain a stable blood pressure and was always carefully monitored, because the anesthetic is initially absorbed by fat but then slowly released over time.

After the induction of anesthesia, an endotracheal tube was introduced into the trachea. Oxygen (British Oxygen, Manchester, UK; 2–3 l/min) was generally provided via this tube to stabilize respiration. An arterial catheter was introduced into the abdominal aorta via the right femoral artery and connected to an Elekma pressure transducer to monitor aortic blood pressure and heart rate with a polygraph recorder. Samples of arterial blood were also withdrawn periodically from this catheter for hematocrit measurements and arterial plasma Na⁺ and K⁺ concentrations. The right femoral vein was then cannulated for continuous infusion of the anesthetic. After a midline cervical incision, the ipsilateral ascending cervical sympathetic nerve was identified and sectioned to prevent re-occurrence of sympathetic activation of the gland. The submandibular duct was identified running beneath the chorda-lingual (C-L) nerve and cannulated by using nylon tubing with the widest diameter possible. The free end of this tube was placed over a photoelectric drop counter to record the flow of saliva. The branches of the external jugular vein were isolated and ligated, except for the small vein draining the submandibular gland itself. At this point, the animal was heparinized (Multiparin; CP Pharmaceuticals, Wrexham, UK) with an initial dose of 1,000 U/kg iv, and subsequent doses of 500 U/kg were given at intervals of 2 h. The external jugular vein was then cannulated with a short length of polythene tubing (1.4 mm ID). This was accomplished in such a way as to prevent any buildup of pressure in the venous drainage from the gland, as would occur if the outflow was occluded. The distal end of this tube was then placed over another photoelectric drop counter to record the blood flow from the gland. All tubing was cut to the shortest possible length to minimize the dead spaces. The submandibular venous effluent blood was returned to the animal via the left femoral vein by an electronically controlled pump, the rate of which was adjusted as necessary to match input to output. The ipsilateral carotid artery was cannulated with a small-diameter needle, with the use of Superglue (Bostik, Leicester, UK) to secure it in position. This needle was connected to a length of narrow-bore tubing (0.50 mm ID) through which ET-1 (Peninsula Laboratories Europe) could be infused (2.5 pmol·kg⁻¹·min⁻¹). A shielded bipolar electrode was then placed on the C-L nerve as close to the hilum of the submandibular gland as possible. Its position was adjusted, and then it was clamped securely in position once a satisfactory secretory and vascular response had been achieved during C-L stimulation. At the end of each experiment, the animal was given a lethal dose of pentobarbital sodium (Pentoject; Animalcare, York, UK). At postmortem examination, both the experimental and nonexperimental glands were then excised and weighed, and a representative sample from each gland was removed and fixed in formaldehyde. They were then processed to paraffin wax, sectioned (7-μm sections), and stained with hematoxylin and eosin for inspection by light microscopy.

Experimental protocol. After surgery, the animals were left undisturbed until a stable blood flow had been established. The parasympathetic innervation to the gland (i.e., the C-L nerve) was then stimulated continuously at 4 or 8 Hz (10- to 30-V square wave; 5-ms pulse width) for 50 min. Samples of saliva and submandibular venous effluent blood were collected at 5-min intervals before and after stimulation and at 2-min intervals throughout the period of stimulation itself. After 10 min of stimulation, ET-1 was infused (2.5 pmol·kg⁻¹·min⁻¹ ic) for 10 min (Fig. 1). Collection of the subman-

Fig. 1. Diagrammatic representation of the protocol. ET-1, endothelin-1. Arrows, times at which collection of blood and saliva samples were started.

METHODS

Animals. The experiments were carried out on adult Welsh ewes (28–46 kg body wt), which had been fed on sheep nuts (Emerald 18 Cake, Avn, Edmunds, UK), hay, and fresh water. Food, but not water, was withheld for at least 24 h before each experiment.

Preparatory procedures. Anesthesia was induced by an injection of 3% pentobarbital sodium (Sagatal; Rhône Merieux, Harlow, UK), at a dose of 15–20 mg/kg, into the jugular vein, and subsequently maintained by a continuous infusion of the barbiturate into the right femoral vein at a rate of 0.1–0.3 mg·kg⁻¹·min⁻¹. The rate was adjusted to maintain a stable blood pressure and was always carefully monitored, because the anesthetic is initially absorbed by fat but then slowly released over time.

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Fig. 2. Changes in mean heart rate (A), arterial blood pressure (B), and submandibular vascular resistance (C) in response to parasympathetic stimulation at 4 Hz before, during, and after an intracarotid infusion of ET-1 (2.5 pmol·min⁻¹·kg⁻¹) in anesthetized sheep. C-L, chorda-lingual. Error bars represent SE of each mean value; n = 4 sheep. ***P < 0.01 with respect to the mean average value before ET-1 infusion.

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A dibular blood and saliva samples was delayed for 2 min after the onset of stimulation and the initiation of the ic infusion of ET-1 to ensure complete evacuation of the dead spaces and equilibration of the preparation. Samples of blood and saliva were measured photometrically and gravimetrically, and the blood that had been collected was returned to the animal as soon as possible. The samples of saliva were stored at $-40^\circ C$ for analysis later.

Estimations. Salivary Na$^+$ and K$^+$ concentrations were measured with a Corning 435 flame photometer, which was calibrated by using a standard commercial solution (Ciba Corning Diagnostics; Halstead, Essex) (Na$^+$ = 140 mmol; K$^+$ = 5 mmol). The protein in the samples was measured with the Bio-Rad protein assay, dependent on the Coomassie brilliant blue G-250 reaction in acidic solution (Bio-Rad Laboratories, München, Germany). Blood flow, salivary flow, and outputs of cations and protein in the saliva are expressed per unit weight of the contralateral, nonexperimental gland, as is customary to eliminate any error arising from edema in the experimental gland. Submandibular vascular resistance (SVR) was estimated by dividing the perfusion pressure (aortic blood pressure) by the submandibular blood flow (SBF). Submandibular plasma flow was estimated by correcting the blood flow for packed cell volume; this was found to vary between animals but remained constant throughout each individual experiment. The global mean value was 32 ± 1%. All results are expressed as means ± SE. Statistical significance was determined by using the Student’s paired t-test, with $n$ representing the number of animals.

RESULTS

Effects of C-L stimulation and ET-1 on cardiovascular responses. Mean heart rate remained relatively stable during C-L stimulation at 4 Hz with no significant change during or after ET-1 infusion compared with the baseline value before infusion (Fig. 2A). Mean aortic blood pressure also remained stable during stimulation, before the infusion of ET-1. However, there was a small but highly significant rise during the infusion of the peptide, providing immediate evidence of its biological potency. Thus the mean of the average values during the infusion (87.9 ± 1.2 mmHg) was significantly higher than the corresponding mean average value before ET-1 infusion.

<table>
<thead>
<tr>
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<th>4-Hz Stimulation</th>
<th>8-Hz Stimulation</th>
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<tbody>
<tr>
<td>Blood flow during ET-1 infusion</td>
<td>48 ± 4</td>
<td>21 ± 4</td>
</tr>
<tr>
<td>Saliva flow during ET-1 infusion</td>
<td>50 ± 1</td>
<td>28 ± 1</td>
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Values are averages of the mean values ± SE expressed as a percentage of the values before the infusion of endothelin-1 (ET-1).
average value during C-L stimulation before ET-1 was infused (78.5 ± 0.5 mmHg; P < 0.001; Fig. 2B). C-L stimulation produced a substantial fall in mean SVR from ~25 to ~10 ml·min·mmHg⁻¹. When ET-1 was infused, SVR rose steadily to a peak value of 31 ± 9 and then subsided steadily to a final value of 19 ± 3 ml·min·mmHg⁻¹ at 50 min when stimulation was discontinued (Fig. 2C). These changes in SVR were reflected in the mean SBP through the gland, which rose from ~300 to ~600 μl·min⁻¹·g gland⁻¹ in response to C-L stimulation and then fell back during the infusion of ET-1 to a final nadir of 283 ± 80 μl·min⁻¹·g gland⁻¹. Thereafter, it rose slowly and steadily to a final value of 387 ± 89 μl·min⁻¹·g gland⁻¹ at 50 min (Fig. 3A).

The same protocol was employed in two additional sheep except that the C-L nerve was stimulated at 8 Hz. As with the group in which the nerve was stimulated at 4 Hz, there was no significant effect on either heart rate or aortic blood pressure (data not shown). The pattern of changes in SBF and SVR was similar to that in the group in which the nerve was stimulated at 4 Hz, but the vasoconstrictor effect of ET-1 was substantially reduced (Table 1).

The effects of ET-1 persisted long after the ic infusion had been discontinued. Thus the mean average values for arterial blood pressure, SVR, and SBF, over the last 10 min of stimulation (40–50 min in Fig. 1), were all still significantly different from the corresponding values at 0–10 min before the infusion; arterial blood pressure was 89.0 ± 0.2 compared with 78.5 ± 0.2 mmHg (P < 0.001), SVR was 18.8 ± 0.1 compared with 9.8 ± 0.1 ml·min·mmHg⁻¹ (P < 0.001), and SBF was 385 ± 1 compared with 618 ± 5 μl·min⁻¹·g gland⁻¹ (P < 0.001).

**Effects of C-L stimulation and ET-1 on submandibular secretory responses.** C-L stimulation at 4 Hz produced an abrupt increase in the flow of submandibular saliva (Fig. 3B) to an average mean value of 67 ± 2 μl·min⁻¹·g gland⁻¹ (P < 0.001) over the first 10 min. The corresponding value in two animals in which the nerve was stimulated at 8 Hz was significantly higher at 103 ± 4 μl·min⁻¹·g gland⁻¹ (P < 0.001), showing that the response was frequency dependent over this range. The ic infusion of ET-1, during ongoing C-L stimulation at 4 Hz, produced a substantial and highly significant fall in SBF to an average mean value of 34 ± 3 μl·min⁻¹·g gland⁻¹ over the period of the infusion (10–20 min; P < 0.001; Fig. 3A). Like the effect of ET-1 on SBF, the reduction in the flow of saliva persisted for the duration of the period of stimulation, and the mean average value during the final 10 min of C-L stimulation (38 ± 1 μl·min⁻¹·g gland⁻¹; **Table 3. Changes in submandibular Na⁺, K⁺, and protein output in response to an intracarotid infusion of ET-1 (2.5 pmol·min⁻¹·kg⁻¹) during stimulation of the chorda-lingual nerve at either 4 or 8 Hz in anesthetized sheep**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Sodium</th>
<th>Potassium</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before ET-1 infusion</td>
<td>62 ± 5</td>
<td>11.9 ± 0.2</td>
<td>1,677 ± 36</td>
</tr>
<tr>
<td>During ET-1 infusion</td>
<td>49 ± 2</td>
<td>9.0 ± 0.4‡</td>
<td>1,443 ± 18‡</td>
</tr>
<tr>
<td>After ET-1 infusion</td>
<td>52 ± 2</td>
<td>11.2 ± 0.1†</td>
<td>1,323 ± 31‡</td>
</tr>
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</table>

Values are averages of the mean values ± SE given in mmol/l; n = 2 sheep. *P < 0.02, †P < 0.01, ‡P < 0.001 with respect to the values before ET-1.

Fig. 6. Changes in mean submandibular salivary Na⁺ and K⁺ output in response to parasympathetic stimulation at 4 Hz before, during, and after an intracarotid infusion of ET-1 (2.5 pmol·min⁻¹·kg⁻¹) in anesthetized sheep. Error bars represent SE of each mean value; n = 4 sheep. **P < 0.02, ***P < 0.01, ****P < 0.001 with respect to the mean average value before ET-1 infusion.
30–40 min) was not significantly different from that during the infusion \( (P > 0.2) \).

The effect of ic ET-1 on SBF and the secretion of saliva is best illustrated by an individual record from an experiment in which the recorder was accelerated at the point in time at which the infusion was initiated (Fig. 4). Both responses appear to diminish concurrently, and there was in fact a strictly linear relation between the flow of plasma through the gland and secretion of saliva throughout these experiments (Fig. 5).

**Effects of C-L stimulation and ET-1 on submandibular electrolyte and protein output.** The concentrations of \( \text{Na}^+ \), \( \text{K}^+ \), and protein in the submandibular saliva produced in response to parasympathetic (C-L) stimulation at 4 Hz all fell in response to the ic infusion of ET-1 (Table 2). In the case of \( \text{Na}^+ \), the effect just failed to achieve statistical significance in this small group of animals, but the reductions in both \( \text{K}^+ \) and protein concentration were statistically significant (both \( P < 0.001 \)). None of these values had recovered to the pre-infusion level 30 min later, and a very similar pattern of responses was observed during C-L stimulation at 8 Hz (data not shown).

The concentration of \( \text{Na}^+ \) in the plasma varied between 136 and 157 mmol/l, and that of \( \text{K}^+ \) between 4.4 and 6.1 mmol/l, in different animals but remained quite constant during individual experiments.

The fall in the rate of flow of submandibular saliva during C-L stimulation at 4 Hz, in response to the ic infusion of ET-1, was reflected in a highly significant fall in both salivary \( \text{Na}^+ \) and \( \text{K}^+ \) output, which persisted until stimulation was discontinued (Fig. 6, Table 3). The outputs of both ions were also linearly related to the rate of flow of both saliva and plasma (Figs. 7 and 8). Similar results were obtained when the C-L nerve was stimulated at 8 Hz (data not shown).

The output of protein in the submandibular saliva was also substantially reduced by ET-1 and linearly related to the flow of saliva throughout these experiments both during C-L stimulation at 4 Hz (Fig. 9) and at 8 Hz (data not shown).

Postmortem histological examination failed to reveal any gross differences between glands that had been tested and those on the contralateral side, with both appearing normal.

Fig. 7. Relations between mean submandibular plasma flow and the outputs of \( \text{Na}^+ \) (A) and \( \text{K}^+ \) (B) in response to parasympathetic stimulation at 4 Hz before, during, and after an intracarotid infusion of ET-1 (2.5 pmol·min\(^{-1}\)·kg\(^{-1}\)) in anesthetized sheep \((n = 4)\).

Fig. 8. Relations between mean submandibular salivary flow and output of \( \text{Na}^+ \) (A), \( \text{K}^+ \) (B), and protein (C) in response to parasympathetic stimulation at 4 Hz before, during, and after an intracarotid infusion of ET-1 (2.5 pmol·min\(^{-1}\)·kg\(^{-1}\)) in anesthetized sheep \((n = 4)\).
Fig. 9. A: changes in mean submandibular salivary protein output in response to parasympathetic stimulation at 4 Hz before, during, and after an intracarotid infusion of ET-1 (2.5 pmol·min⁻¹·kg⁻¹) in anesthetized sheep. Error bars represent SE of each mean value; n = 4 sheep. ***P < 0.02, ****P < 0.001 with respect to the mean average value before ET-1 infusion. B: relation between mean submandibular plasma flow and the output of protein in response to parasympathetic stimulation at 4 Hz before, during, and after an intracarotid infusion of ET-1 (2.5 pmol·min⁻¹·kg⁻¹) in anesthetized sheep (n = 4).

DISCUSSION

The results of this study show that a reduction in blood flow through the gland during C-L stimulation causes a significant reduction in salivary flow (P < 0.01), confirming previous findings in both the cat and the sheep (9, 18). Salivary flow was found to be directly proportional to blood or, more accurately, plasma flow through the gland, and the relation showed a strong linear correlation (r² = 0.98) throughout the experimental protocol, before, during, and after the infusion of ET-1. In cats, the gradient increases when ET-1 is infused, such that the flow of saliva amounts to ~6% of the SBF in the absence of ET-1 and to 9% while the peptide is infused (18). Thus there is an increase in the efficiency of salivation, when the blood flow through the gland is reduced in this species. This has been attributed to compensatory mechanisms that enable the gland to incur an oxygen (1) or water and electrolyte (7) debt, which can be replenished later when blood flow is no longer restricted. No evidence for any such mechanism was obtained in the present study in which the flow of saliva consistently amounted to ~10% of the blood flow before, during, and after the infusion of the peptide. Ruminants produce much greater volumes of saliva than monogastric species, and constancy of the pH of the rumen contents is largely dependent on the buffering action of this component of the rumen liquor. The fact that stimulation of the parasympathetic, secretomotor innervation provokes a flow amounting to such a high proportion of the blood flow may therefore represent a genuine species difference and leave no room for any further increase in efficiency, or the development of any fluid or electrolyte debt, when the blood flow is restricted, as here, for more than a few minutes.

The outputs of the salivary constituents that were monitored all fell when the salivary blood flow was reduced, and the effect on Na⁺, K⁺, and protein output was linearly related to both plasma flow and salivary secretion. Salivary secretion is an active two stage process. Initially, the acini secrete a primary secretion containing protein in a solution whose ionic concentration is similar to plasma (16). This is then modified as it flows through the salivary ducts, where K⁺ and HCO₃⁻ are secreted in exchange for Na⁺ and Cl⁻. The present results accord with the expectation that reduction in salivary blood flow compromises the primary secretion from the acinar cells, which is reflected in the highly significant reduction in salivary flow and electrolyte and protein output during ET-1 infusion (P < 0.01). The concentrations of both Na⁺ and K⁺ fell in response to ic ET-1 and the effect on Na⁺ concentration could be accounted for by increased reabsorption in the ducts when the time taken to traverse them increases as the volume of saliva produced diminishes. During the plateau phase of salivary secretion, K⁺ is derived from the plasma through the intracellular pool (2). It seems most likely that availability of K⁺ from this pool becomes limited when the blood flow through the gland is restricted, thus reducing the concentration in the saliva during prolonged stimulation, as employed here.

Reduction in blood flow through the gland during and after the infusion of ET-1, must inevitably reduce substrate availability for the de novo synthesis of protein, secreted by the vesicular route (17). This is reflected in the substantial reduction in protein output that occurred at both frequencies of stimulation during and after the infusion of ET-1. The reduction in output will be further augmented by the depletion of protein granules from the prolonged stimulation of the gland. Resynthesis of these granules is very slow (~12 h), as shown by Sreenby et al. (19) in the parotid gland of the rat, which may account for the fact that there was no sign of recovery in protein output after the infusion of ET-1 was discontinued. It is noteworthy that, in the present study, ET-1 at a dose of 2.5 pmol·min⁻¹·kg⁻¹ ic produced a greater reduction in protein output (P < 0.01) than has been reported previously, when the peptide was infused at a higher dose (10–20 pmol·min⁻¹·kg⁻¹ ic) during C-L stimulation at the same frequency (9). Accordingly, the present result provides the more convincing evidence that the protein
secretion by the ovine submandibular gland is compromised when the blood flow is reduced.

It is concluded that electrical stimulation of the parasympathetic secretomotor innervation of submandibular gland of the sheep elicits secretion of saliva that approximates 10% of the blood (and 15% of the plasma) flow through the gland. This proportion is higher than that found in monogastric species, such as the cat, and cannot be exceeded when the blood supply is reduced over a period of minutes.

We gratefully acknowledge the skilled technical assistance provided by Dr. P. M. M. Bischam.

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