

Adrenergic effects on exocrine secretion of rat submandibular epidermal growth factor

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SUMMARY The present study was undertaken to investigate the effect of alpha- and beta-adrenergic agonists on secretion of epidermal growth factor (EGF) from the rat submandibular glands and to test the possibility of intestinal absorption of EGF. Alpha-adrenergic agonists increased the concentration of salivary EGF by approximately a hundred times, while the serum concentration of EGF was unchanged. The contents of EGF in the submandibular glands decreased upon administration of the alpha-adrenergic agonist noradrenaline, and this was confirmed on immunohistochemical investigation of the glands. Beta-adrenergic agonists had no effect on secretion of EGF from the submandibular glands. Intestinal absorption of EGF could not be confirmed, as stimulation by noradrenaline with free passage of saliva to the gastrointestinal tract and intrajejunal infusion of EGF had no influence on the concentration of EGF in serum. This study shows that alpha-adrenergic agonists stimulate exocrine secretion of submandibular EGF and that EGF in physiological amounts are not absorbed in the gastrointestinal tract.

Epidermal growth factor (EGF), a polypeptide comprising 53 amino acids, was originally isolated from mouse submandibular glands and from human urine.¹ The peptide stimulates cellular growth and differentiation, inhibits gastric acid secretion and prevents experimentally induced gastric and duodenal ulcers.²⁻⁴ The effect of EGF at the cellular level has been the subject of investigation for nearly 20 years,⁵ whereas the physiological role of EGF remains unknown. Investigation of the regulation and mode of secretion of submandibular EGF is important for further elucidation of the physiology of the peptide, especially in relation to the gastrointestinal tract.

In the submandibular glands, EGF is localised to the cells of the granulated convoluted tubules (GCT cells), which are surrounded by an intense network of adrenergic nerves.^{6,7} A single intraperitoneal injection of noradrenaline in mice increases the concentration of EGF in plasma and in saliva collected from the oral cavity, while the effect of beta-adrenergic agonists is controversial.⁸⁻¹⁰

The purpose of the present study has been to clarify the adrenergic regulation of the secretion of submandibular EGF and to investigate whether the peptide is secreted in an exocrine or an endocrine way. In addition, we investigated the possibility of intestinal absorption of the peptide as previously suggested.¹¹

Methods

ANIMALS

Experimental design

Studies were performed on 112 male Wistar rats in groups of eight weighing 200-220 g. The rats were fasted overnight before the experiment, but were allowed free access to water and kept in raised mesh bottom cages to prevent coprophagy. Under ether anaesthesia, a 0.8 mm polyethylene catheter was placed in a jugular vein for infusions. For collection of saliva, a laparotomy was performed and through an incision in the forestomach a catheter was placed with the tip in the distal part of the oesophagus and secured with a purse string suture in the forestomach (Fig. 1). Saliva was collected for three hours in glass syringes connected to the catheter filled with 1 ml of distilled water containing 500 kIU aprotinin

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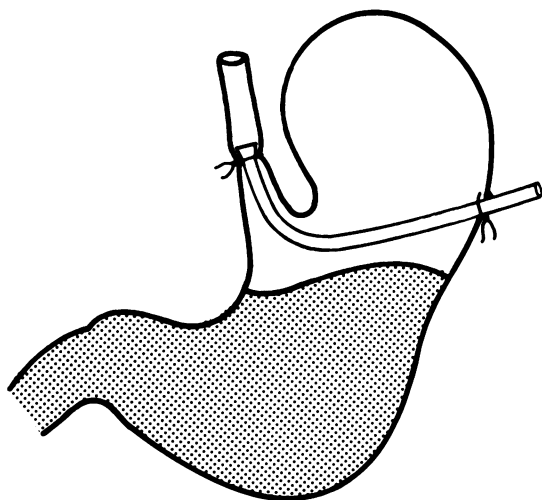


Fig. 1 Through a stab wound in the forestomach a polyethylene catheter was placed with the tip in the distal oesophagus for collection of saliva.

(Trasyolol[®], Bayer, Leverkusen, FRG). In the first series of experiments, the following drugs were infused in a volume of 2 ml/h for three hours: adrenaline 1.36 $\mu\text{mol/kg/h}$ (250 $\mu\text{g/kg/h}$) (DAK, Copenhagen, Denmark) alone or in combination with phenoxybenzamine 16.8 $\mu\text{mol/kg/h}$ (5 mg/kg/h) (Alfred Benzon, Copenhagen, Denmark) or propranolol 19.3 $\mu\text{mol/kg/h}$ (5 mg/kg/h) (Frekven[®], Ferrosan, Copenhagen, Denmark). In a second series of experiments noradrenaline 1.48 $\mu\text{mol/kg/h}$ (250 $\mu\text{g/kg/h}$) (DAK, Copenhagen, Denmark) was infused alone or in combination with phenoxybenzamine 16.8 $\mu\text{mol/kg/h}$. Also isoproterenol 1.18 $\mu\text{mol/kg/h}$ (250 $\mu\text{g/kg/h}$) (DAK, Copenhagen, Denmark) was given alone or in combination with propranolol 19.3 $\mu\text{mol/kg/h}$. Eight untreated rats and eight rats who had the submandibular glands removed 10 days previously served as controls and received NaCl 0.154 mol/l in a volume of 2 ml/h for three hours. At the end of each infusion, blood was drawn from the inferior vena cava, the volume of saliva collected was determined, the submandibular glands were removed and together with saliva and serum stored at -20°C for later determination of the concentration of epidermal growth factor.

Immunohistochemical investigation of EGF in the submandibular glands was performed in two groups of five rats receiving either noradrenaline 1.48 $\mu\text{mol/kg/h}$ or isoproterenol 1.18 $\mu\text{mol/kg/h}$. After five hours, the submandibular glands were removed and fixed for 24 hours in Bouin's fixative (without

acetic acid). The tissues were then dehydrated and embedded in paraffin. Sections were stained for EGF by the peroxidase-antiperoxidase method (PAP). The EGF antiserum (8136) was used at dilutions of 1/1600, 1/3200 and 1/6400.

In the third series of experiments a possible endocrine secretion of submandibular EGF was investigated in eight rats who underwent ligation of the submandibular ducts and received an infusion of noradrenaline 1.48 $\mu\text{mol/kg/h}$ for three hours. For control, another eight rats were sham-operated and received saline intravenously for three hours. Both groups of rats had a 0.8 mm polyethylene catheter placed in the bladder for collection of urine during the infusion. After three hours, 1 ml of blood was drawn from the inferior vena cava and the volume of urine collected was measured.

Absorption of EGF from the gastrointestinal tract was investigated in another three groups of rats by infusion of noradrenaline 1.48 $\mu\text{mol/kg/h}$ for three hours without collection of saliva, and by intra-jejunal infusion at 1 ml/h of a submandibular extract prepared as previously described¹² containing 1000 nmol/l or 10 000 nmol/l of EGF for three hours. All rats had urine collected for three hours and blood taken from the inferior vena cava at the end of the study.

Laboratory analysis

Epidermal growth factor was measured by a homologous radioimmunoassay using antibody 8136 as previously described.¹² Detection limit of the assay is 0.06 nmol/l and coefficient of variation 0.10 for values between 0.06 nmol/l and 4 nmol/l. Purified submandibular EGF was used for calibration and production of tracer. Serum was tested undiluted, saliva was diluted 1+5 or 1+449 and urine 1+49 in assay buffer (0.1 mol/l phosphate buffer, human albumin 0.1%, pH 8.0). The submandibular glands were homogenised in 5 ml water with 500 kIU Trasyolol/ml and centrifuged for 20 min at 1500 g. The supernatant was lyophilised, reconstituted in 2 ml distilled water and diluted 1+999 with assay buffer before analysis. The substance concentration of creatinine in urine was measured with an automatic analyser (Greiner Electronics AG, Langenthal, Switzerland).

Isoelectric focusing of saliva from rats given noradrenaline was performed with a LKB equipment (LKB, Bromma, Sweden) on a 5% polyacrylamide gel with a pH gradient from 4.0 to 6.5. The gel was cut in 5 mm slices and eluted in 1 ml assay buffer overnight, before EGF analysis.

For statistical evaluation, the Mann-Whitney test for unpaired differences was used. P values of less than 0.05 were considered significant.

Results

In intact controls the median output of EGF in saliva was 8.1 pmol/3 h (5.5–19.4) and after removal of the submandibular glands it was reduced to 0.40 pmol/3 h (0.10–0.70). The two groups of rats had identical concentrations of EGF in serum (Table 1).

Adrenaline increased median concentration and total output of salivary EGF about a 100 times, but had no effect on the concentration of EGF in serum. The stimulatory effect of adrenaline could be completely prevented by simultaneous infusion of phenoxybenzamine while propranolol had no effect (Table 1).

In the second series of experiments the effect of alpha-adrenergic stimulation with adrenaline was confirmed by infusion of noradrenaline. When phenoxybenzamine was given together with noradrenaline, the increase in the concentration of EGF in saliva was abolished. Isoproterenol had no effect on the concentration of EGF in saliva. Isoelectric focusing of salivary EGF from rats given noradrenaline showed two isopeptides with pI 4.8 and 5.4. Neither noradrenaline nor isoproterenol increased the serum concentration of EGF above the detection limit of the assay (Table 2).

In the control group the median content of EGF in the submandibular glands was 3200 pmol/gland (1900–5400). After stimulation with noradrenaline the contents of EGF in the submandibular gland decreased significantly to 2450 pmol/gland (1500–2840). When both noradrenaline and phenoxybenzamine were infused, the contents of EGF was unchanged as compared with controls (median 3625 pmol/gland (1500–6150)). Isoproterenol had no effect on the contents of EGF in the submandibular glands as compared with controls (median 3050 pmol/gland) (1625–5440). Immunohistochemical

investigation of the submandibular glands from rats given noradrenaline showed a marked decrease of EGF immunoreactivity in the GCT cells, whereas controls and rats receiving isoproterenol exhibited identical reactions (Fig. 2).

In the third series of experiments (Table 3), a possible endocrine secretion of EGF from the submandibular glands could not be confirmed, as ligation of the submandibular ducts and subsequent infusion of noradrenaline had no effect on the concentration of EGF in serum and EGF output in urine. Moreover, we found no support for the assumption that EGF can be absorbed from the gastrointestinal tract. The concentration of EGF in serum and the total output of EGF in urine were unchanged after infusion of noradrenaline in rats with free passage of saliva to the gastrointestinal tract and also after infusion of exogenous EGF into the small intestine.

Discussion

The submandibular glands produce a number of biologically active polypeptides such as renin and nerve growth factor.¹³ Nearly all these peptides are androgen dependent and are released in large amounts into the saliva and to the circulation. The mode of secretion is therefore assumed to be endocrine as well as exocrine.^{11, 13} Exocrine secretion of biologically active peptides from the submandibular glands into the lumen of the upper gastrointestinal tract has increased interest in the physiology of these peptides in connection with the gastrointestinal tract.

In the present study it is shown that salivary EGF mainly originates from the submandibular glands, though small amounts from other sources can still be measured after removal of the submandibular

Table 1 *Effect of submandibulectomy and infusion of adrenaline alone or in combination with adrenergic antagonists on EGF in saliva and serum*

Treatment	No	EGF in saliva nmol/l	EGF output in saliva pmol/3 h	EGF in serum nmol/l
Control	8	6.8 (4.1–13.5)	8.1 (5.5–19.4)	<0.06 (<0.06–0.10)
Control + submandibulectomy	8	0.90* (0.20–1.20)	0.40* (0.10–0.70)	<0.06 (<0.06–0.10)
Adrenaline	8	413.5* (206.5–531.0)	567.0* (355.0–816.0)	<0.06 (<0.06–0.10)
Adrenaline + phenoxybenzamine	8	4.4 (2.5–10.5)	6.2 (4.8–13.8)	<0.06 (<0.06–0.08)
Adrenaline + propranolol	8	500.0* (270.0–616.5)	588.0* (324.0–712.5)	<0.06 (<0.06–0.10)

Values are given as median and total range.

Significance is indicated by *: $p < 0.01$ as compared to untreated controls.

Table 2 Effect of noradrenaline and isoproterenol alone or in combination with adrenergic antagonists on EGF in saliva and serum

Treatment	No	EGF in saliva nmoll/l	EGF output in saliva pmoll/3 h	EGF in serum nmoll/l
Control	8	6.8 (4.1-13.5)	8.1 (5.5-19.4)	<0.06 (<0.06-0.10)
Noradrenaline	8	651.0* (246.0-810.5)	769.0* (394.0-1134.5)	<0.06 (<0.06-0.13)
Noradrenaline + phenoxybenzamine	8	8.1 (5.2-12.0)	9.2 (4.1-14.6)	<0.06 (<0.06-0.10)
Isoproterenol	8	8.3 (2.0-19.1)	8.9 (3.2-15.3)	<0.06 (<0.06-0.08)
Isoproterenol + propranolol	8	10.0 (5.2-21.0)	8.5 (4.5-16.4)	<0.06 (<0.06-0.10)

Values are given as median and total range. Significance is indicated by *: p<0.01 as compared with controls.

glands as has previously been reported for renin.¹⁴ We found that alpha-adrenergic agonists stimulate secretion of EGF from the submandibular glands and decrease the contents of EGF in the glands as confirmed immunohistochemically. Beta-adrenergic agonists had no influence on the concentration of EGF in saliva and on the contents of EGF in the gland. The observed stimulatory effect of alpha-adrenergic agonists is in accordance with previous *in vivo* and *in vitro* studies as well as the morphological observation of a degranulation of the granulated convoluted tubular cells after alpha-adrenergic stimulation.^{8 15} A lack of effect of beta-adrenergic agonists as shown in the present study has also been reported in other studies.^{8 16 17} Others, however, found the concentration of EGF in saliva to increase although they could not confirm this by morphological studies.^{9 17}

We believe the present design to be as close to the physiological conditions as possible. The animals are awake during the experiment and manipulation in

connection with collection of saliva is minimal. The present study gives data on total output of EGF after stimulation for three hours, and errors caused by sampling of saliva from the oral cavity are avoided.

The mouse submandibular glands contain large amounts of EGF and nerve growth factor, and it is now evident that the submandibular glands are exocrine organs for nerve growth factor which is released by alpha-adrenergic stimulation.^{18 19} Similar experiments with EGF have suggested an exocrine as well as endocrine mode of secretion, although recent morphological investigations give support only to the exocrine path of secretion.

In the rat, barely detectable amounts of EGF were found in serum from control animals before and after removal of the submandibular glands. The serum concentrations of EGF were unchanged after alpha- and beta-adrenergic stimulation with collection of saliva. This suggests that the submandibular glands are exocrine rather than

Table 3 Absorption of EGF from the gastrointestinal tract

Treatment	No	EGF in serum nmoll/l	EGF in urine nmoll/l	EGF output in urine pmoll	EGF-creatinine ratio nmoll/mmol
Control	8	<0.06 (<0.06-0.10)	35.5 (17.0-82.5)	40.5 (13.5-99.0)	5.3 (3.4-9.1)
Noradrenaline + ligation of the submandibular ducts	8	<0.06 (<0.06-0.13)	38.0 (21.5-52.5)	40.9 (23.0-56.4)	5.7 (4.7-8.3)
Noradrenaline without collection of saliva	8	<0.06 (<0.06-0.10)	33.2 (18.8-66.5)	47.1 (11.3-89.8)	6.2 (3.4-10.9)
Intrajejunal EGF infusion 5 nmol/kg x h	8	<0.06 (<0.06-0.10)	35.8 (21.2-71.0)	40.2 (18.6-66.4)	5.4 (4.2-7.8)
Intrajejunal EGF infusion	8	<0.06 (<0.06-0.10)	40.1 (22.8-48.1)	44.5 (26.8-73.7)	5.7 (4.7-8.3)

Values given are median and total range. No statistical difference was observed between the individual groups.

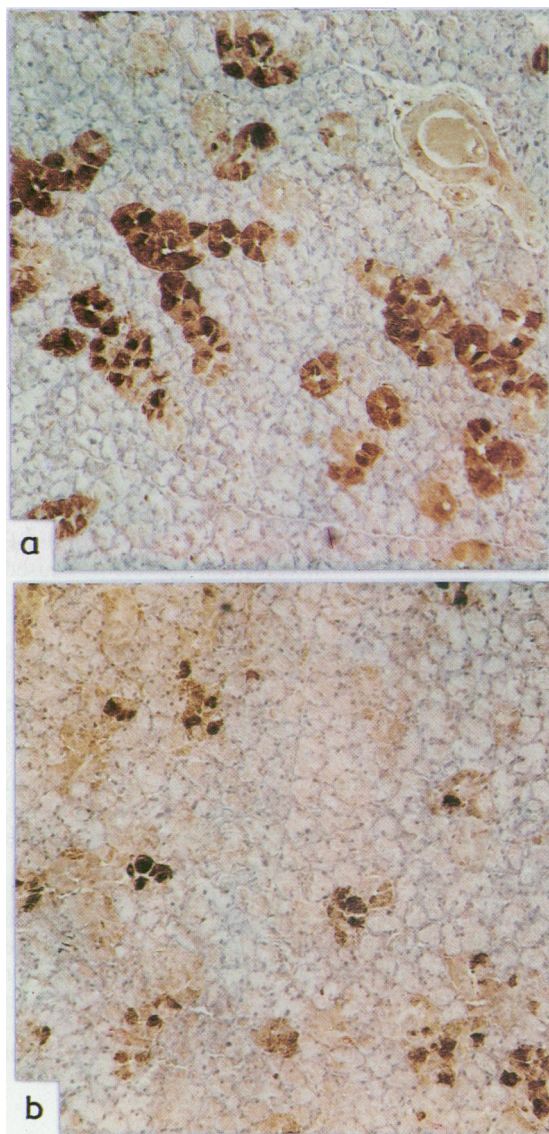


Fig. 2 Immunoreaction for EGF in male rat submandibular gland (antisera diluted 1:1600). (a) Untreated control showing strong positive immunoreaction in the granular convoluted tubular cells (GCT cells). (b) Rat given norepinephrine 250 µg/kg/h for five hours. Only a few GCT cells with positive reaction are seen ($\times 140$ original magnification).

endocrine organs for EGF in the rat, and this is supported by the observation that ligation of the submandibular ducts and subsequent administration of noradrenaline has no effect on the concentration of EGF in serum or in the urinary output of EGF.

In male mice treated with alpha-adrenergic agonists the concentration of EGF in plasma is less than 0.5% of the concentration in saliva, and removal of the submandibular glands has no effect on the basal concentration of EGF in plasma.^{8,9} One explanation is that during stimulation large amounts of EGF enter the gastrointestinal tract in swallowed saliva, and it can then be absorbed and reach the circulation. This assumption is based on the observation that oral administration of EGF to newborn mice induces precocious eyelid opening and incisor eruption to the same extent as a subcutaneous injection of the peptide.²⁰ In the rat we found neither endogenous salivary EGF nor that instillation of large amounts of exogenous EGF in the small intestine influenced the concentration of EGF in serum or urinary output of EGF. This can be explained by the observation that absorption of substantial amounts of intact protein occur only in newborn animals.²¹ The mature gut does not have the same ability, although recent investigations have shown that a small fraction of ingested protein can be absorbed.²² This could explain the small rise in plasma concentration of EGF in mice during stimulation, where saliva with a concentration of up to 100 µmol/l of EGF enters the gastrointestinal tract.¹³

Salivary EGF was found to consist of two isopeptides with the same pI as previously shown for submandibular EGF, but different from the pI of duodenal EGF, indicating that EGF is a heterogeneous peptide, which is in accordance with a recent description of the EGF gene.^{12,23}

The observation that large amounts of submandibular EGF enters the gastrointestinal tract with saliva and that EGF seems not to be absorbed from the intestine, strongly suggests a physiological function of EGF in the gastrointestinal tract. Epidermal growth factor initiates its action by binding to specific cellular receptors, which have been found in the stomach and small intestine.^{5,24,25} Oral administration of EGF has a trophic effect on the gastroduodenal mucosa and prevents the development of experimental gastric and duodenal ulcers.^{3,4,26,27} It is therefore likely that EGF plays a role in growth and protection of the gastrointestinal mucosa. The latter is probably caused by the rapid effects of EGF on epithelial cells such as changes of the cytoskeleton or stimulation of the synthesis of glucosaminoglycans, a component of mucus.^{28,29,30}

In conclusion, this study shows that alpha-

adrenergic but not beta-adrenergic agonists stimulate release of EGF from the rat submandibular glands by exocrine secretion and that physiological amounts of EGF in saliva do not appear to be absorbed from the gastrointestinal tract. Although exogenous EGF can inhibit gastric acid secretion and has a trophic effect on the gastrointestinal mucosa,^{31 32} the significance of EGF-isopeptides in saliva for the function of the gastrointestinal tract is still largely unknown.

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