

Does Bombesin-like Peptide Mediate Radiation-induced Anorexia and Satiety?

Yan Aalto, Sture Forsgren, Uno Kjöll, Ulrika Funegård, Lars Franzén and Roger Henriksson

From the Departments of Oncology (Y. Aalto, L. Franzén, R. Henriksson), Anatomy (S. Forsgren, U. Kjöll), and Cariology (U. Funegård), Umeå, Sweden

Correspondence to: Dr Roger Henriksson, Department of Oncology, Umeå University, S-901 85 Umeå, Sweden. Tel.: + 46 90 785 00 00. Fax: + 46 90 785 20 31

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Bombesin (BN) and its mammalian counterpart gastrin-releasing peptide (GRP) act as neuroregulatory hormones and peripheral and central satiety-inducing agents. Previously, we demonstrated that irradiation induces an increase in the expression of BN/GRP in the innervation of the salivary glands in rats. We therefore carried out a study using radioimmunoassay (RIA) analysis and immunohistochemistry to examine whether saliva contains BN and whether irradiation affects the BN release to saliva in rats. Immunoreactivity for BN was detected not only in the innervation of the parenchyma but also in the duct cells and in the lumina of the ducts, suggesting entrance of BN into saliva. The RIA analysis confirmed that rat saliva contains a BN-like peptide. The observation shows that saliva contains this peptide but that there is no significant increase following the radiation schedule used. Nevertheless, the occurrence of an enhanced expression of BN in different peripheral tissues such as the salivary and laryngeal glands should be taken into consideration when discussing the clinically important problem of reduced food intake and anorexia in cancer patients.

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Salivary glands are supplied with nerve fibres containing many biologically active neuropeptides, such as substance P, bombesin (BN), and vasoactive intestinal peptide (VIP) (1–5). It is suggested that these peptides modulate the secretory process evoked by ‘classical’ neurotransmitters and cause changes in blood flow in salivary glands. BN and its mammalian counterpart gastrin-releasing peptide (GRP) act as neuroregulatory hormones and tissue-specific growth factors, and have also been implicated as peripheral and central satiety inducing agents (6). GRP seems to be a physiological regulator of food intake (7). Central or peripheral administration of BN inhibits food intake in rats (8, 9) and peripherally induced satiety by BN is associated with changes in taste perception (6). Recently, we have demonstrated that irradiation induces an increase in the expression of specific neuropeptides such as BN and substance P in the innervation in various tissues such as salivary glands (4, 5, 10–12). These observations could thus be of importance, since BN has been implicated as a potential integrative peptide for feeding and satiety (6). We therefore found it of interest to further investigate whether parotid saliva contains BN.

MATERIAL AND METHODS

Animals

In our study nine-week-old female Sprague Dawley rats were used. Some of these animals were used for studies of saliva content. The saliva was collected before irradiation (5 animals), as well as 2 (4 animals) and 15 min (5 animals) after completed irradiation. Specimens from 13 irradiated rats and 5 controls were used for immunohistochemical studies.

Irradiation procedure

Irradiation was carried out on a 6MV medical linear accelerator and was given on five consecutive days with daily doses of 5–8 Gy (total dose 25–40 Gy). The irradiated volume included all the salivary glands. The rats were anaesthetized with Brietal (Methohexital) in the tail vein, fastened in a plastic mould and observed through a television camera in order to check that the animals were firmly positioned during irradiation. The absolute dosimetry was checked with an ionization chamber in a rat-like phantom and all scattering materials in the field were kept constant (4).

Collection of saliva

After a 10-h fast, saliva was collected according to a standardized method (13). The animals were anaesthetized with 0.1 ml/kg body weight of Hypnorm® (Janssen Pharmaceutical, Beerse, Belgium) containing the active substances fluanisone (10 mg/ml) and fentanyl (0.2 mg/ml), and 0.1 ml/kg body weight of Stesolid® (Dumex A/S, Copenhagen, Denmark) containing 5 mg/ml of the active component diazepam. The Hypnorm® and Stesolid® preparations were given as two separate intramuscular injections in the thigh. The rats were put on an inclining (10°) table with the mouth placed over a plastic cup in a position that prevented direct contamination from nasal secretion and tears. Saliva secretion was stimulated by a subcutaneous injection of a combination of 2.5 mg/kg body weight of pilocarpine (Sigma Chemical Co., St. Louis, MO) and 2.5 mg/kg body weight of isoproterenol (Sigma Chemical Co., St. Louis, MO). Saliva was collected for 15 min in pre-weighed plastic cups. After weighing of the saliva samples, the cups were kept ice-chilled. Volumes for the analyses were immediately pipetted into separate tubes and kept at -20°C until analysed. An aliquot of 1 ml saliva was collected between 9 a.m. and 12 a.m. and the collection time never exceeded 20 min; 25 µl of each saliva sample was frozen at -70°C and later lyophilized and used for peptide determinations.

Radioimmunoassay (RIA)

Saliva samples were lyophilized and then reconstituted and diluted in BSA-borate buffer. After centrifugation, the supernatants were assayed for the peptides. The concentrations of BN-like peptide in the extracts were determined using an RIA kit (¹²⁵I) purchased from Incstar Corp. (Stillwater, Minnesota, USA). The RIA was performed according to the instructions from the manufacturer. The BN antibody was directed against BN isolated from frog skin and was tested by Incstar to display 50% cross-reactivity with porcine GRP, but only <0.002% cross-reactivity with other peptides tested (including substance P).

Immunohistochemistry

Tissue sampling and sectioning. Ten days after the last irradiation, the rats were anaesthetized with Mebumal (sodium pentobarbital 40 mg/kg i.p.). The submandibular, sublingual and parotid glands were dissected out and fixed by formaldehyde in 0.1 M phosphate buffer, pH 7.0. Thereafter, the specimens were thoroughly washed in Tyrode's solution containing 10% sucrose at 4°C overnight, and mounted on thin cardboard in OCT embedding medium (Miles Laboratories, Naperville, IL). Submandibular, sublingual and parotid glands of controls were usually mounted on the cardboard along with corresponding glands of irradiated animals. Series of 8–10 µm thick sections were cut using a cryostat. The sections were

mounted on slides precoated with chrome-alun gelatin, dried and processed for immunofluorescence or stained for demonstration of activity of NADH-tetrazolium reductase (NADH-TR) (14). The NADH-TR staining served as a reference for tissue morphology.

Staining procedures. Sections were incubated for 30 min in a 1% solution of detergent Triton X-100 in 0.01 M phosphate-buffered saline (PBS), pH 7.2, containing 0.1% sodium azide as preservative, rinsed three times for 5 min each in PBS, and incubated in 5% normal swine serum in PBS supplemented with 0.1% bovine serum albumin (BSA) for 15 min. The sections were then incubated with the primary antibody, diluted in PBS with BSA, in a humid environment. Incubation lasted for 60 min at 37°C. After incubation with specific antiserum and after three 10 min washes in PBS, the sections were immersed in fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit IgG, diluted 1.40, for 30 min at 37°C in a moist chamber, washed in three changes of PBS, mounted in glycerol : PBS (1 : 1) and examined under a photomicroscope equipped with epifluorescence optics. For details, see also Aalto et al. (12).

Antibodies and preabsorption tests. The BN/GRP antibody used was raised in rabbits using synthetic bombesin conjugated to bovine thyroglobulin with glutaraldehyde (Incstar Corp., Stillwater, MN; code: 368, working dilution 1 : 100). The specificity of the antiserum was tested by incubation of sections with antiserum preabsorbed with 10–20 µg synthetic BN (Sigma, USA), or 10–20 µg synthetic substance P (Sigma), in 1 ml antiserum.

RESULTS

The RIA analysis demonstrated immunoreactivity for BN-like peptide in the saliva, but there were no statistical differences between non-irradiated controls and irradiated animals (control: 1261 ± 192 (n = 5), 2 weeks: 1176 ± 89 (n = 4), 15 weeks: 1155 ± 26 (n = 5), mean $\pm$ S.E.M and expressed as pg/ml saliva). Immunohistochemical analysis revealed the occurrence of BN immunoreactivity in nerve fibres localized in the parenchyma, in duct cells and occasionally in duct lumina (Fig. 1). These reactions were the same after preabsorption with substance P. On the other hand, these reactions did not occur after preabsorption with BN.

DISCUSSION

Our study lends further support to the previous observations of BN in duct cells and in the lumina of the ducts in rat salivary glands (12). This suggests entry of BN from ducts into the saliva, a suggestion that is confirmed by our novel observation of BN-like peptide in rat saliva. As far as we know, the occurrence of BN in the saliva has not previously been described. However, somewhat in contrast to our previous observation of an increased BN im-

munoreactivity in the parenchyma in response to irradiation (5), irradiation did not cause detectable changes in the amount of BN-like peptide in the saliva.

BN is an amphibian tetradecapeptide originally purified from the skin of the frog *Bombina bombina* (15). The mammalian counterpart is gastrin-releasing peptide (GRP).

Immunoreactivity for BN/GRP has been detected in the central and peripheral nervous systems (16) as well as in the gastrointestinal tract (17) and in milk (18, 19). BN has been found to stimulate gastrin release and gastric acid secretion in humans (20). Moreover, peripheral administration of BN inhibits food intake in food-deprived rats (8) by causing generalized aversion or disruption of behaviour, e.g. chewing or swallowing. Central administration of BN also inhibits food intake in rats (9). Thus, it has been suggested that BN is a physiologically active peptide for digestion and a psychologically active peptide for satiety (6). Therefore, our previous findings of an increased BN content in the parenchyma of salivary (5) and laryngeal glands (21) following irradiation and the present observations that saliva indeed contains BN are important because of the known effects of BN on satiety. Previously, BN-like immunoreactants have also been found in maternal milk, and it has been proposed that BN may have a trophic response in the pancreas of suckling rats (18, 19). The existence of BN in saliva may contribute significant effects on the gastrointestinal tract, in which there is a wide distribution of BN-binding sites, and BN may produce a trophic response by a complex interplay between the periphery and the CNS. It is plausible to speculate that BN/GRP originating from duct cells by receptor interaction might directly affect target tissue in the gut, there

being an interesting linkage from salivary glands via saliva on different parts of the gastrointestinal tract. Furthermore, BN/GRP can act locally in the affected organ and via the blood stream.

Anorexia, primary caused by the tumour disease itself or being secondary to cancer treatment, is a major obstacle in the management of cancer patients. Nausea associated with direct or indirect activation of 5-HT₃ receptors in the gastrointestinal tract and the emetogenic centre by chemotherapy or radiotherapy is more or less accepted as one of the causes of anorexia. Different kinds of cytokines such as TNF are also known to induce a state of catabolism and are also linked with cachexia. Thus, since BN/GRP is associated with the physiological regulation of satiety and since BN-like immunoreactivity was detected in rat saliva and parenchyma, we propose that the observations of an enhanced content of BN/GRP in tissues in response to irradiation might be of clinical interest in the context of treatment-induced anorexia that develops in cancer patients. Further studies using methods to antagonize the effects of BN might also be considered of interest.

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Fig. 1. Sections of the sublingual (a) and submandibular (b) glands after processing for BN. After irradiation (a), and in a control animal (b). Immunoreactive granular structures can be seen in the duct cells (filled arrows) as well as in the duct lumen (open arrow) (b) $\times 500$.

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