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Differential distribution and expression of leptin and the functional leptin receptor in major salivary glands of humans

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Abstract

Leptin plays a central role in the regulation of food intake and energy expenditure in rodents. However, it has become clear that this hormone has more than only a satiety-inducing function, and that there are other sources of leptin, such as the central nervous system, placenta and the gastrointestinal tract in addition to adipose tissue. Knowing about the important role of the salivary glands in food intake and digestion, it was the objective of the present study to investigate how leptin and its receptor are expressed and distributed in the major salivary glands of humans. We found leptin distributed throughout the major salivary glands with obvious intracellular concen-

trations in granula. In contrast, immunostaining for the leptin receptor was found exclusively in the membranes of the glandular cells. A high density of the leptin receptor was localised in the epithelia of the duct lumen. PCR analysis proved the autonomous expression of leptin by the salivary glands independently from adipocytes. Accordingly the long receptor isoform was expressed by any examined tissue. In the light of recent findings of leptin influencing the growth of rodent salivary glands, the presence and distribution of leptin and its receptor suggests an autocrine role of salivary leptin within the glands.

Introduction

Leptin is a mainly adipocyte-derived hormone which influences food intake and energy expenditure by interaction with receptors in the feeding centres of the hypothalamus (Hirschberg 1998). Especially for rodents (Air *et al.* 2002) and even birds (Lohmus *et al.* 2003) this influence of leptin on nutrition has been proved, whereas it remains still unclear whether this special role of the cytokine has similar importance for humans.

However, an increasing number of studies have shown that other tissues, such as the central nervous system (Morash *et al.* 1999), the placenta (Dötsch *et al.* 1999) and the gastrointestinal tract (Bado *et al.* 1998) are additional sources of leptin. Recently we demonstrated the expression of leptin by the human salivary glands (Gröschl *et al.* 2001). Other authors have shown a direct atrophic effect of leptin on the salivary glands of rats (Higa *et al.* 2002). This has led to the hypothesis that leptin released into saliva might have additional 'non-anorexic' effects in the upper gastrointestinal tract, as has been demonstrated *in vivo* for the stomach using recombinant leptin (Konturek *et al.* 1999a, 2001).

Certain effects appear obvious, since salivary leptin shows diverse physiological rhythmicities in humans,

such as diurnal ones (Randeve *et al.* 2003) and a clear dependence on the phase of the menstrual cycle (Gröschl *et al.* 2002).

Leptin signalling occurs after binding to various receptor isoforms (ob-R_a to ob-R_c) which are located ubiquitously (Halaas & Friedman 1997, Löllmann *et al.* 1997, Bjorbaek *et al.* 1998) and introduce different modes of signal transduction (Sweeney 2002). It is not yet clear whether salivary leptin plays a role as a ligand of gastrointestinal receptors, localised in the mucosa of the stomach (Bado *et al.* 1998, Breidert *et al.* 1999) and in other parts of the gastrointestinal tract (Morton *et al.* 1998). However, infusion of recombinant adenovirus containing the cDNA for rodent leptin induced hyperleptinaemia in rats accompanied by atrophic changes of the salivary glands (Higa *et al.* 2002). These findings suggest that the proliferation of salivary gland cells was reduced by a non-anorexic effect of leptin. However, our own observations showed that leptin enhances the proliferation of human oral keratinocytes *in vitro*, as has been described for human and murine keratinocytes in cutaneous wound repair (Goren *et al.* 2003). This is underlined by various other studies indicating that leptin acts in a pro-proliferative way in many cell types (Tsuchiya *et al.* 1999, Wolf *et al.* 1999, Martin Romero *et al.* 2000, Park *et al.* 2001).

Table 1 List of primers used for RT-PCR determination of human leptin, the long receptor isoform ob-R_b, and the adipocyte-specific protein apM-1

	Direction	Sequence
Gene product		
Leptin	Sense	5' CCA TCC AAA AAG TCC AAG ATG ACA CCA AAA 3'
	Antisense	5' CCA TGC AAT GCT CTT CAA TCC TGG AGA TAC C 3'
Leptin nested	Sense	5' ACC CCA TCC TGA CCT TAT CCA AGA TGG ACC 3'
	Antisense	5' GCA AGT GGC AGC TCT TAG AGA AGG CCA GC 3'
ob-R _b	Sense	5' CAA GGA CTT AAT TTT CAG AAG CCA ACG 3'
	Antisense	5' CCA TGA GCT ATT AGA GAA AGA ATC CTT CAA 3'
apM-1	Sense	5' TTT GAA CCA AAG CTC AAA ACA ATC AAG ACC 3'
	Antisense	5' CAA TTG TAC TTC AAA GCA TCA CAG GAC C 3'
apM-1 nested	Sense	5' GAC ACT GTT ATC AGA AAT AGG AGA GTG G 3'
	Antisense	5' CAG GAC CAT TAA TCC TGA AAT CTT GAC C 3'

Therefore, with regard to a possible autocrine function of leptin in the glandular tissues and its role in the oral cavity, it was the objective of the present study to investigate the expression and distribution of leptin and its receptor in the major salivary glands in humans.

Material and Methods

Sample material

Submandibular, sublingual and parotid gland had been excised because of otorhinolaryngological disorders in the Department of Otorhinolaryngology of the University of Erlangen. The glandular tissues were not affected by the underlying disorders. Patients gave informed consent for the use of the tissue samples for research use. The specimens were stored at -80°C until use. Histological evaluation of the specimens was done on sections stained with haematoxylin–eosin.

Immunohistochemistry

Salivary glands were cut on a cryostat (Microm, Walldorf, Germany). Seven-micron thick frozen sections were fixed for 5 min in absolute alcohol, exposed to 10% normal bovine serum and incubated in a 1:100 dilution of antisera against recombinant human leptin (Gröschl *et al.* 2000) or human soluble leptin receptor (Lammert *et al.* 2001) for 45 min at room temperature. Negative controls were prepared by replacing the first antibody solution with PBS. The sections were then washed in PBS and exposed to a 1:100 dilution of the secondary rhodamine-tagged goat anti-rabbit antibody (Dianova, Hamburg, Germany) combined with a 4',6-diamidino-2-phenylindole (DAPI) 1:1000 solution, labelling for DNA.

The sections were examined under a Zeiss Axioplan fluorescence microscope. Photographs were taken under identical conditions with the same exposure times.

RNA extraction and reverse transcription

Total RNA was extracted from the tissues using guanidine–thiocyanate acid phenol (TRIzol; WAK Chemie, Medical GmbH, Bad Homburg, Germany). Different from the manufacturer's protocol, we used 1 ml TRIzol/100 mg glandular tissue and added 400 μl CHCl_3 . RNA concentration was determined spectrophotometrically. One microgram of RNA was reverse transcribed in a volume of 20 μl at 39°C for 60 min (all chemicals were obtained from Boehringer, Mannheim, Germany).

Nested RT-PCR yielded a 158 bp fragment of leptin cDNA. Following an initial denaturation at 94°C (2 min), samples were PCR amplified (25 cycles). Each cycle consisted of denaturation at 94°C , annealing at 67°C and extension at 72°C . To examine whether the leptin-specific signal was caused by contamination of the gland tissues with adipocytes, a nested RT-PCR for adipocyte-specific apM-1 mRNA (Schaffler *et al.* 1998) was carried out. Nested RT-PCR yielded a 292 bp fragment of apM-1 cDNA. In contrast to the amplification of leptin cDNA, the annealing temperature for apM-1 cDNA was 62°C . For the receptor no additional nested primers were used. The size of the fragment of ob-R_b was 435 bp. The initial denaturation occurred at 94°C for 2 min. Each amplification cycle consisted of denaturation at 94°C , annealing at 67°C and extension at 72°C .

After PCR amplification, cDNA was analysed by electrophoresis on 1% agarose in Tris–borate buffer and visualised by ethidium bromide staining. Human placenta and fat tissue served as positive controls, water as negative control. Primers for RT-PCR are listed in Table 1.

Results

Leptin was localised ubiquitously in the parotid, sublingual and submandibular glands. The leptin-specific rhodamine

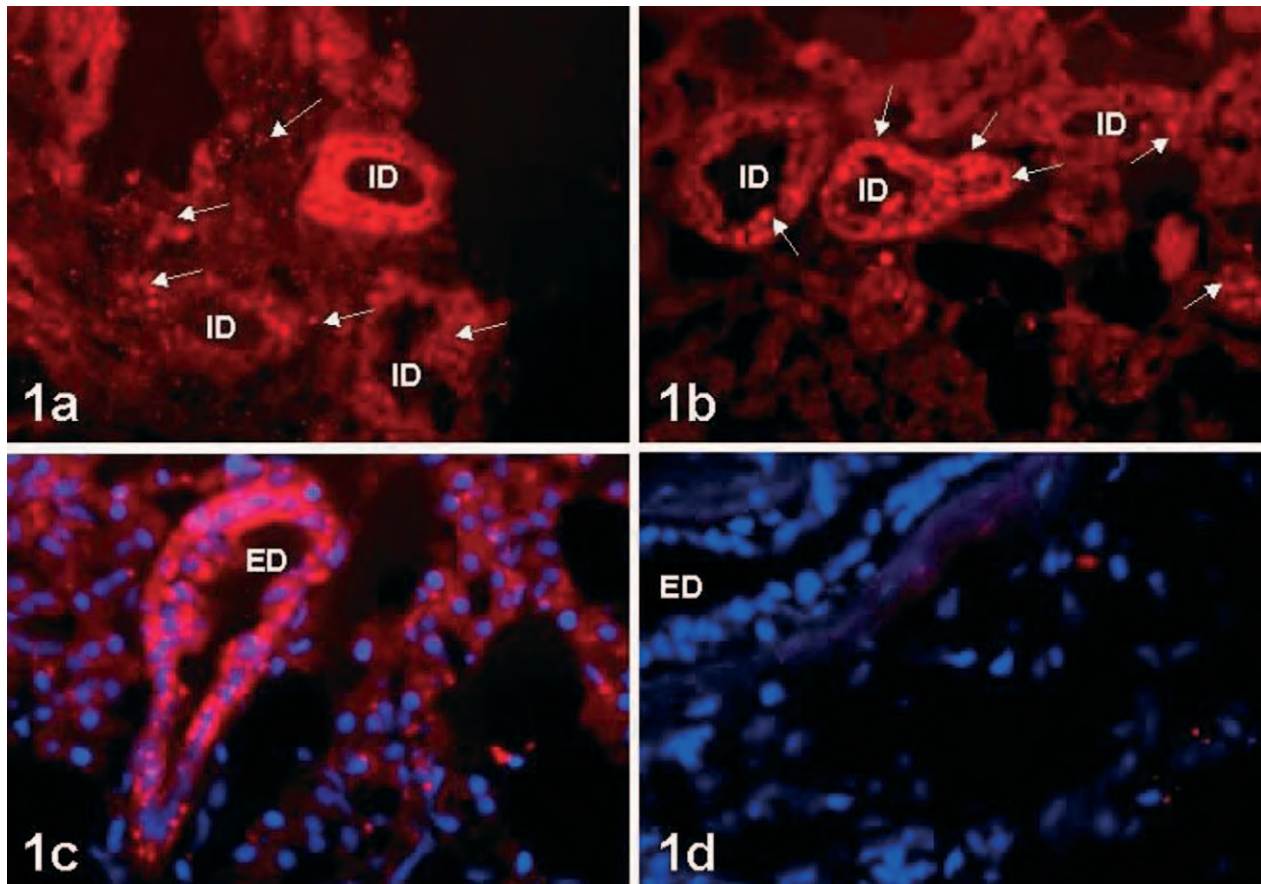


Figure 1 Rhodamine staining of leptin in 7 μm sections of human salivary glands ($\times 200$) with DAPI staining (c and d) of the DNA. (a) Submandibular gland: intercalated ducts (ID); (b) sublingual gland: intercalated ducts (ID); (c) sublingual gland: excretory duct (ED); (d) negative control: excretory duct. Granular staining for leptin was found all over the glandular tissues (arrows) with further concentration in the epithelia of the ducts. The cellular structure of the salivary ducts is clarified by the distribution of the DAPI staining in (c and d).

staining was found ubiquitously in acinar endpieces and the salivary ducts of the glands (Fig. 1). A distinct concentration occurred in granula, found in both acini and ducts (indicated by arrows in Fig. 1a and b). Further intense rhodamine staining was present in the epithelia of the salivary ducts (Fig. 1c). The blue DAPI staining localises the DNA, exemplified in Fig. 1c and d, to clarify the cellular structure. Immunoreactivity was absent in the buffer controls (Fig. 1d).

In contrast to the widespread granular distribution of leptin, rhodamine staining for ob-R was observed exclusively in the cell membranes. The staining was thereby more intense in those membranes directed towards the lumina of the salivary ducts and in those membranes which demarcate the acinar endpieces.

Parotid gland (Fig. 2a: acinar endpieces), submandibular gland (Fig. 2b: acini) and sublingual gland (Fig. 2c: excretory duct) presented similar staining for ob-R in the membranes surrounding the DAPI-stained nuclei. More-

over, intense rhodamine staining was found in the membrane epithelia of the ducts, as demonstrated in Fig. 2c showing details of an excretory duct in the sublingual gland. A negative control (absence of specific antiserum) is shown in Fig. 2d.

Since PCR amplification for leptin using the outer primer set yielded bands in the parotid and the sublingual gland only, but not in the submandibular gland and the oral mucosa, a further amplification of this PCR product with a nested primer set was added. The nested RT-PCR then yielded the expected 158 bp fragment of leptin cDNA in all large salivary glands as well as in oral mucosa (Fig. 3A).

Although the adipocyte-specific marker apM-1 was present in the parotid and sublingual glands, yielding a 292 bp fragment, it was not detected in the submandibular gland and oral mucosa even after nested PCR (Fig. 3B).

Furthermore, expression of ob-R_b mRNA was present in all examined tissues (Fig. 3C). For the receptor no

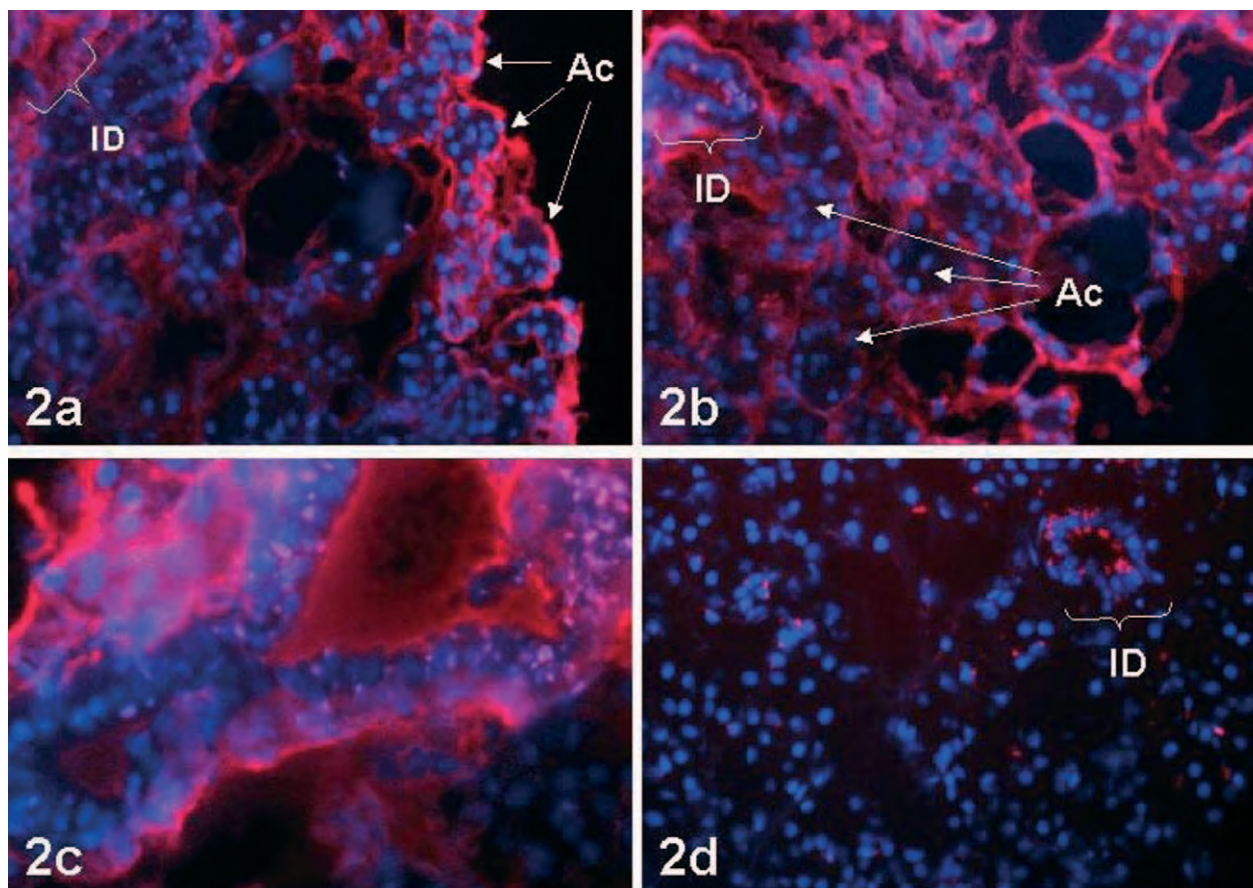


Figure 2 Rhodamine staining of ob-R in 7 μm sections of human salivary glands ($\times 200$) with DAPI staining of the cell nuclei. (a) Parotid gland: excretory duct (ED); (b) submandibular gland: acinar endpieces (Ac) and intercalated duct (ID); (c) sublingual gland: acinar endpieces (Ac) and intercalated duct (ID); (d) negative control. Rhodamine staining was localised exclusively in the membranes of the cells of both the acini and the salivary ducts. The cellular structure of the acinar endpieces and the salivary ducts is clarified by the distribution of the DAPI staining.

additional nested PCR was necessary. The size of the fragment of ob-R_b was 435 bp.

Discussion

A proliferative effect of leptin has been described for various organs and tissues (Konturek *et al.* 1999a, Wolf *et al.* 1999, Frank *et al.* 2000). The recent discovery of both leptin and leptin receptors in the stomach (Bado *et al.* 1998, Cinti *et al.* 2000) and the presence of leptin in saliva (Gröschl *et al.* 2001) has led to further questions about peripheral leptin actions, which may influence food uptake, digestion or mucosal proliferation in a still unknown manner.

In the present study, we could show that leptin and its receptor ob-R have dissimilar distribution within the salivary glands. The concentration of the cytokine in

distinct granula is in line with the immunohistochemical localisation of leptin in other tissues (Tsuruo *et al.* 1996, Cinti *et al.* 1997, Sobhani *et al.* 2000). This leads to our hypothesis that the peptide is stored within the glandular cells before its release into saliva. This might also explain why a nested PCR was necessary to detect leptin expression in any examined tissue. Our finding of leptin mRNA expression in the oral mucosa and the submandibular gland without parallel apM-1 mRNA expression underlines the idea of an adipocyte-independent leptin production by the salivary glands and the oral mucosa.

In contrast, ob-R was localised exclusively in the membranes of the glandular cells with further intense staining in the luminal epithelia of the salivary ducts. As in neuronal tissues (Funahashi *et al.* 2000, Iqbal *et al.* 2001), the presence of the receptor protein indicates that leptin derived from salivary glands acts directly via an autocrine

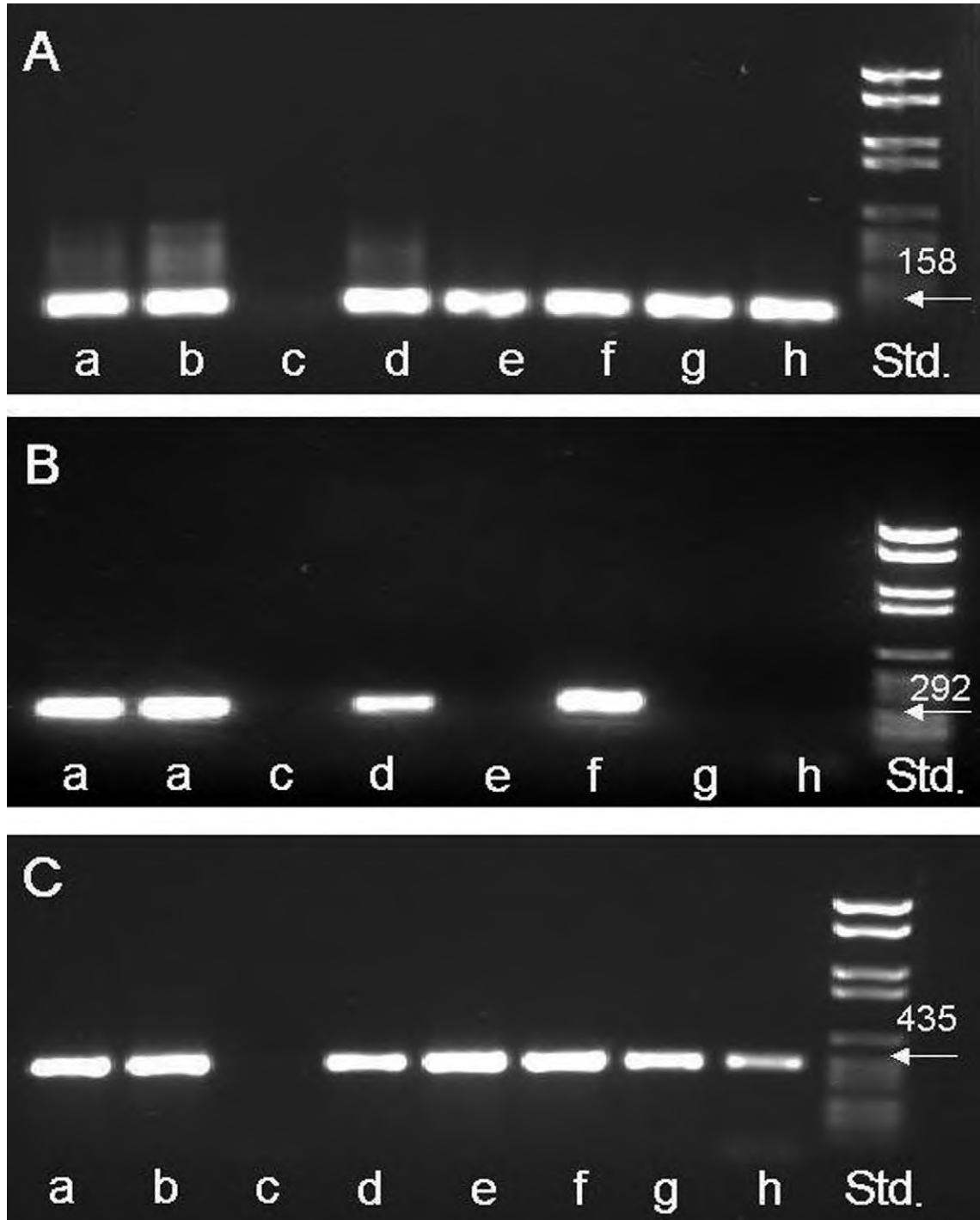


Figure 3 mRNA expression of leptin (A), the adipocyte-specific apM-1 (B), and the functional leptin receptor ob-R_b (C), in human oral mucosa and salivary glands: a, adipose tissue (positive control); b, placenta (positive control); c, water (negative control); d, sublingual gland; e, submandibular gland; f, parotid gland; g and h, oral mucosa; Std, DNA marker. For the determination of apM-1, adipose tissue was used twice as positive control (two 'a' lanes). Arrows indicate the expected size of the PCR products.

activation. In addition to a postulated autocrine action of salivary leptin, which may be in the context of growth regulation of the glands (Higa *et al.* 2002), other gastrointestinal actions of this hormone appear possible. It has been shown by microelectrode recording that direct application of leptin into the stomach leads to neuronal activities in the brainstem of rodents (Yuan *et al.* 1999), possibly through vagal fibres projecting from the stomach to the caudal part of the brainstem. Furthermore, gastric application of leptin has been shown to reduce pepsin secretion and gastric acid production in rats (Brzozowski *et al.* 1999). However, interaction with gastric peptides as described for cholecystokinin and ghrelin seems not to be the only target of gastrointestinal leptin action. Moreover leptin seems to have a strong gastroprotective function (Brzozowski *et al.* 1999, Konturek *et al.* 1999a,b), either by inducing proliferation of the gastric mucosa itself or via up-regulation of further growth factors.

It must be mentioned that our protein analysis of the leptin receptor in glandular tissues is not able to differentiate between the long receptor isoform ob-R_b and other truncated forms such as ob-R_a. We used an antibody raised against the soluble leptin receptor sob-R (Lammert *et al.* 2001), which is identical to the extracellular domain of the membrane-bound isoforms. Therefore we can state that there are extracellular binding sites for salivary leptin within the glands, located in the cell membranes with a clear concentration in membranes demarcating the acinar endpieces and salivary duct lumina. PCR analysis, however, proved the expression of the ob-R_b isoform. This indicates that this so-called functional receptor isoform was responsible for the rhodamine staining.

A recently published work also focused on immunohistochemical determination of leptin and its receptor in submandibular and parotid gland (De-Matteis *et al.* 2002). Interestingly this study could determine immunoreactivity for leptin within salivary microglobules, whereas the seromucous material was negative. This finding underlines our hypothesis of an active release of the hormone into saliva.

In summary, this study suggests a functional role of salivary leptin in the salivary glands and the oral cavity. Certainly it remains to be investigated why hyperleptinaemia (in a transfection experiment) leads to weight loss of the salivary glands themselves (Higa *et al.* 2002), whereas salivary leptin increases proliferation of oral keratinocytes *in vitro* (Gröschl *et al.* 2003). Maybe negative feedback loops can explain the controversial action of leptin in the glands and the oral cavity.

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