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Identification of Leptin in Human Saliva

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Leptin is produced predominantly in adipose tissue but has recently also been found in gastric mucosa. It has been shown that the oral application of leptin induces neuronal activity in the brain stem of rodents. The objective of the present study was to identify this hormone in human saliva and to examine the production and stability of salivary leptin. We have demonstrated production of leptin in salivary glands and oral mucosa by RT-PCR, its storage by immunocytochemistry, and the release of the peptide by RIA. Chromatographic analysis and immunoblotting confirmed the identity of leptin. There is a strong linear correlation ($r^2 = 0.78$) between leptin concentrations from simultaneously collected saliva and plasma samples (n = 61). Stimulation of saliva flow increases total leptin secretion up to 3-fold ($P < 0.001$). As to the stability of leptin in gastric fluid, we found the peptide was not degraded above pH 3.5. Additionally, salivary leptin remains stable up to 5 d at 4 C. With regard to the presence of leptin receptors in gastric mucosa, we suggest salivary leptin as being a possible ligand for gastric leptin receptors. Furthermore, the determination of leptin in saliva allows for noninvasive sample collection.

SINCE THE DISCOVERY of leptin in 1994, research activities have been directed at understanding the regulation and actions of this peptide hormone. Leptin is the product of the obese gene with a single-chain structure and a molecular mass of 16 kDa. It is produced by differentiated adipocytes (1, 2) as well as in the placenta (3, 4). More recently, storage and secretion of leptin has also been demonstrated in the stomach (5–7). The signal function of leptin on the central nervous system has been the major area of research (8–12). Leptin influences food intake (*e.g.* by suppressing neuropeptide Y in the hypothalamus [11], and stimulates energy expenditure and thermogenesis (*e.g.*, by interaction with the adrenal cortex [13, 14]). However, specific receptors for leptin have been found ubiquitously in the body (*e.g.*, thyroid gland, adrenal glands, lung, placenta, kidney, liver, and endothelial cells [15–17]). This suggests a peripheral role for leptin. Interestingly, a specific leptin receptor was recently identified in the gastric mucosa (6) and in other parts of the gastrointestinal tract (18). To date, the sources of leptin as a gastrointestinal receptor ligand have been only partially investigated.

It was therefore an objective of the present study to identify and characterize the presence of leptin in saliva. A second aim was to investigate the regulation of leptin in the salivary glands and its stability under gastric conditions.

Materials and Methods

Sample material

Plasma and saliva were collected simultaneously from healthy adult males (n = 23; age 28–80 yr; body mass index [BMI] 20–50 kg/m^2) and females (n = 25; age 22–85 yr; BMI 19.5–54 kg/m^2) and healthy adolescents (male: n = 13; female: n = 5; age 10–18 yr; BMI: 15–39.9 kg/m^2) recruited in our hospital, who gave informed consent of participation. None of the volunteers took medication or contraceptives and all participants had fasted for at least 2 h. The study was approved by the Ethics Review Board of the University of Erlangen.

Plasma was collected with S-Monovettes, saliva was collected with the Salivette device (both Sarstedt, Nürnbrecht, Germany). Collection time was between 0900 and 1100 h. After centrifugation (4,000× *g;* 10 min), samples were stored at −25 C until measurement. Serial dilutions of saliva were prepared to examine linearity and parallelism with recombinant leptin.

In 10 healthy subjects (26–30 yr; BMI 20–22 kg/m^2), the influence of stimulating salivary flow rate with citric acid was investigated. After collecting a baseline sample, 100 μl lemon juice were applied onto the tongue. One minute after this stimulus, a further sample was collected. After rinsing the mouth with water and a latency of 1 min, a third sample was collected. The volume of saliva was quantified.

To investigate the stability of leptin under pH conditions comparable to those in the stomach, recombinant leptin standard material (~1 μg/liter) was incubated at pH 7.4, 5.3, 4.1, 3.5, 2.4, and <1, respectively. Samples were neutralized with $CaCO_3$ before RIA measurement.

Furthermore, leptin in saliva was kept under various storage conditions to investigate the stability of the parameter as already described for other salivary hormones (19): Fresh saliva from healthy subjects was pooled and divided into three series of seven identical portions. Each series was duplicated. One series consisted of blank saliva, the second was centrifuged before storage. NaN_3 (0.5%) was added to the third series to avoid bacterial contamination. One of the duplicate series was stored at reverse transcription (RT) (18 C); the other was stored in the refrigerator (4 C). The temporal pattern of leptin stability was studied by freezing one specimen of each series every day at −25 C until the end of the experiment and measurement after 1 wk.

Salivary glands (Gld. submandibularis, Gld. parotis, Gld. sublingualis) for immunocytochemical and genetic investigations were obtained during surgery in the Department of Otorhinolaryngology of the University of Erlangen. These glands had been excised because of otorhinolaryngological disorders (*e.g.*, neck-dissection in the case of Gld. submandibularis). The glandular tissues were not affected by these

Abbreviations: BMI, Body mass index; FPLC, fast-protein liquid chromatography; RT, reverse transcription.

TABLE 1. List of primers used for nested RT-PCR determination of human leptin and the adipocyte-specific protein ApM-1

Gen-product	Direction	Sequence
Leptin	Sense	CCATCCAAAAAGTCCAAGATGACACCAAACC
Leptin	Antisense	GGTATCTCCAGGATTGAAGAGCATTGCATGG
Leptin nested	Sense	ACCCCATCCTGACCTTATCCAAGATGGACC
Leptin nested	Antisense	GCTGGCCTTCTCTAAGAGCTGCCACTTGC
ApM-1	Sense	TTTGAACCAAAGCTCAAAACAATCAAGACC
ApM-1	Antisense	CAATTGTACTTCAAAGCATCACAGGACC
ApM-1 nested	Sense	GACACTGTTATCAGAAATAGGAGAGTGG
ApM-1 nested	Antisense	CAGGACCATTAATCCTGAAATCTTGACC

disorders. Cells from the oral mucosa of four healthy subjects were obtained by scratch biopsy with wooden spatulas.

Measurement

Leptin in plasma was determined by a specific RIA as described before (20). Salivary leptin was measured with a highly sensitive non-equilibrium version of the RIA. The standards ranged from 620 to 3 ng/liter. Total tracer activity was 9000 cpm/25 μl. The antibody dilution was 1:9000. Preincubation was performed overnight (RT) before adding the tracer. The sensitivity of this modification was 2 ng/liter. Inter- and intraassay coefficient of variation was 15.2% and 9.1%, respectively. Matched samples were measured within the same assay run.

Chromatographic separation of salivary leptin

Leptin was separated by gel permeation chromatography as described recently (21). The fast-protein liquid chromatography (FPLC) consisted of an Ismatec Reglo pump (Glattbrugg, Zürich, Switzerland), an LKB-Uvicord S 2138 detector (wavelength 278 nm; Stockholm, Sweden) and a fraction collector (LKB-2111). The separation of salivary proteins was conducted with a Superose 12 HR 10/30 column (Amersham Pharmacia Biotech, Freiburg, Germany). Elution buffer (pH 7.4) consisted of 0.01 M KH_2PO_4, 0.15 M NaCl, and 1 g/liter NaN_3. Elutions were performed at RT at a flow rate of 0.6 ml/min and collected for radioimmunological measurement. Fractions were measured directly without freezing.

Immunoblots

The proteins of saliva, plasma, and recombinant human leptin standard were separated by tricine-sodium-dodecyl-sulfate PAGE (22), transferred to PVDF-membranes (Roti-PVDF, Roth, Karlsruhe, Germany), and blotted with the same antibody used for RIA and immunocytochemistry (1:2000). The immune complexes were visualized using alkaline phosphatase conjugated goat antirabbit IgG (Bio-Rad Laboratories, Inc., Munich, Germany). Plasma and standard material were used directly, whereas saliva samples were concentrated 120-fold by centrifugation (Vivaspin, Sartorius, Göttingen, Germany). Color-labeled markers (Kaleidoscope Polypeptide Standards) with molecular mass of 36.5, 27.9, 17.9, 8.6, and 4.1 kDa were purchased from Bio-Rad Laboratories, Inc.

Immunocytochemical determination of leptin in salivary glands

Salivary glands (Gld. submandibularis, Gld. parotis, Gld. sublingualis) were frozen in liquid nitrogen and cut on a cryostat (Microm, Walldorf, Germany). Sections (8–12 μm) were thawed and fixed with acetone on microscopic slides (Superfrost Plus, Diagonal, Münster, Germany). Sections were preincubated with TBS buffer containing 1% BSA, 0.5% Triton X-100, 10% porcine serum for 30 min at RT. After rinsing with buffer, rabbit antihuman leptin antiserum was added (1:5.000 in TBS-buffer) and incubated overnight at RT. After a buffer rinse, fluorescein isothiocyanate-tagged porcine antirabbit IgG (Biotrend, Cologne, Germany) was applied at a final dilution of 1:30 in buffer for 1 h at RT. After a final washing step, the sections were cover slipped in TBS-glycerol (pH 8.6). Negative controls were prepared by preabsorption of the first antibody by incubation with recombinant human leptin overnight (RT) or by replacing the first antibody solution with TBS buffer. Human placenta, known to produce leptin, was used as a positive control.

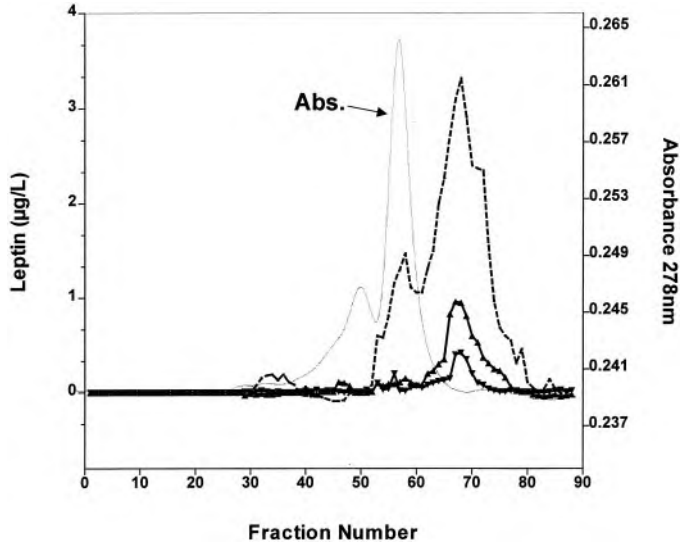

FIG. 1. Leptin immunoreactivity in fractions obtained from Fast Performance Liquid Chromatography of two different saliva samples (▲/▼) and recombinant leptin (—). Absorbance at 278 nm is also shown (Abs.).

Microscopy was performed with the Arisoplan-Microscope (Leitz, Stuttgart, Germany) and a confocal microscope (Bio-Rad Laboratories, Inc.).

RT-PCR

Total RNA was isolated from salivary gland tissues and mucosa cells using standard techniques (Trizol reagent, Life Technologies, Inc., Karlsruhe, Germany). For the preparation of cDNA, 1 μg total RNA in 50 μl final volume was used. 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 (23) was carried out. Nested RT-PCR yielded a 292-bp fragment of ApM-1 cDNA. In contrast to the amplification of leptin cDNA, annealing temperature for ApM-1 cDNA was 62 C. After PCR amplification, cDNA was analyzed by electrophoresis on 1% agarose in Tris-borate buffer and visualized by ethidium bromide staining. Human placenta and fat tissue served as positive controls, water as negative control. Primers used are listed in Table 1.

Statistics

The correlation between salivary and plasma leptin values was calculated by linear regression (Spearman). For comparison of the calculated and measured leptin values in serial dilution studies, Passing/Bablock regression (24) was used. Alterations of salivary leptin values owing to the storage conditions were calculated by ANOVA and post hoc Bonferroni's multiple comparison test. Changes in leptin concen-

tration, saliva flow, and leptin secretion during stimulation were compared using ANOVA for repeated measurements.

Results

FPLC separation

Leptin was confirmed as the immunopositive analyte in saliva by size exclusion chromatography and subsequent radioimmunological measurements. With recombinant human leptin standard, two peaks were detected. The first peak coeluted with albumin, an ingredient of the NaH_2PO_4 buffer (Fig. 1). Salivary leptin analyzed under the same conditions coeluted with the second peak of recombinant human leptin. Plasma samples showed similar chromatographic profiles as the standard material in NaH_2PO_4 buffer containing albumin.

Immunoblotting

Western blotting analysis of saliva and recombinant standard material confirmed the identity of the salivary leptin. The immunreactive bands in both recombinant human leptin standard material and in concentrated saliva were localized just below the 17.9-kDa marker (Fig. 2).

Immunocytochemistry

Intense immunofluorescent staining was observed in sections from all three salivary glands after incubation with a highly specific antibody raised against human leptin. This positive immunoreactivity was absent in preabsorbation and buffer controls. Only preparations of the submandibular gland are shown (Fig. 3), but the other glands showed the same results. Intercalated ducts (A), striated ducts (B), and excretory ducts (C) showed cytoplasmatic staining. Additionally, granular staining was observed in cells of the outer layer of the double-layer epithelium (a/b/c). In the acini (D), basal peripheral staining could be shown. Intensive epithelial staining was observed in capillary blood vessels of the salivary glands. Negative and positive controls are not shown here.

RT-PCR

Leptin mRNA expression could be detected in all large salivary glands as well as in oral mucosa (Fig. 4A). Human adipose tissue and placenta served as positive controls. The adipocyte-specific protein ApM-1 was detected in the parotid gland and sublingual gland but was not present in the submandibular gland and oral mucosa (Fig. 4B). For ApM-1, human adipose tissue served as positive control.

Leptin measurement

There was a strong linear correlation ($r^2 = 0.78$) between salivary and plasma leptin (Fig. 5). The correlation between

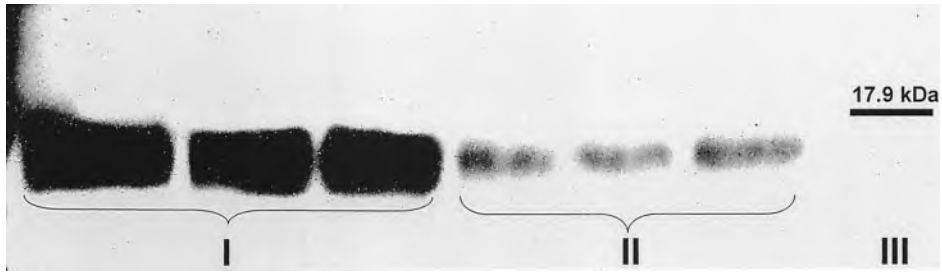

FIG. 2. Immunoblotting of salivary leptin. I, Concentrated (120-fold) saliva samples; II, recombinant human leptin (16 kDa); III, localization of kaleidoscope marker (17.9 kDa).

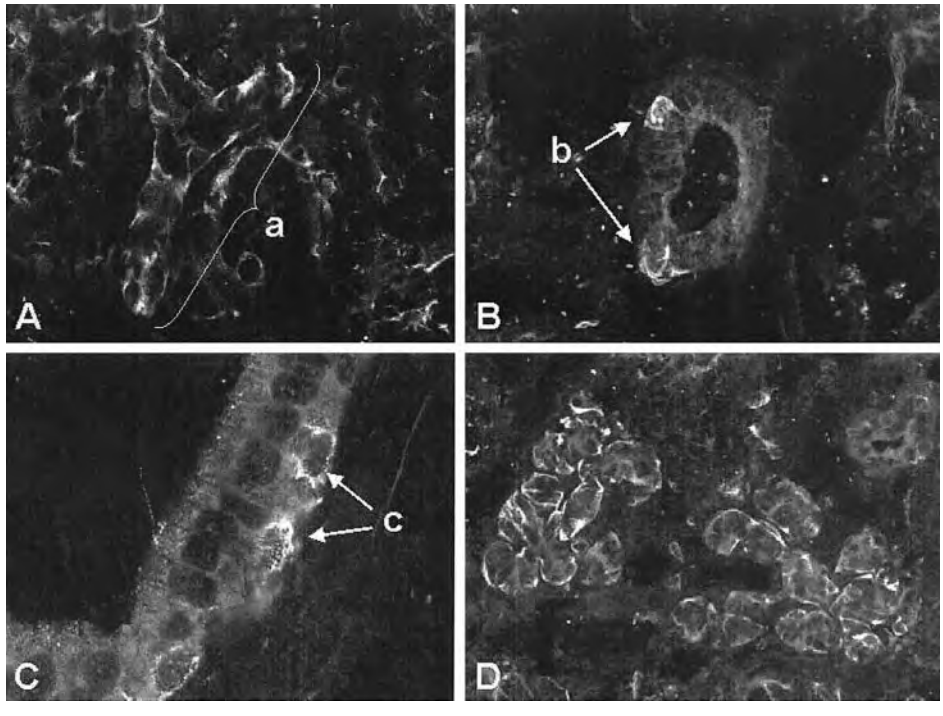

FIG. 3. Immunostaining of leptin in acetone sections of human Gld. submandibularis. A, Striated duct (a) in overview (×40). B, Single immunoreactive cells (b) in epithelium of an intercalated duct (×64). C, Single immunreactive cells (c) in the basement layer of an excretory duct epithelium (×64). D, Positive immunostaining of leptin in seromucose acini (×40).

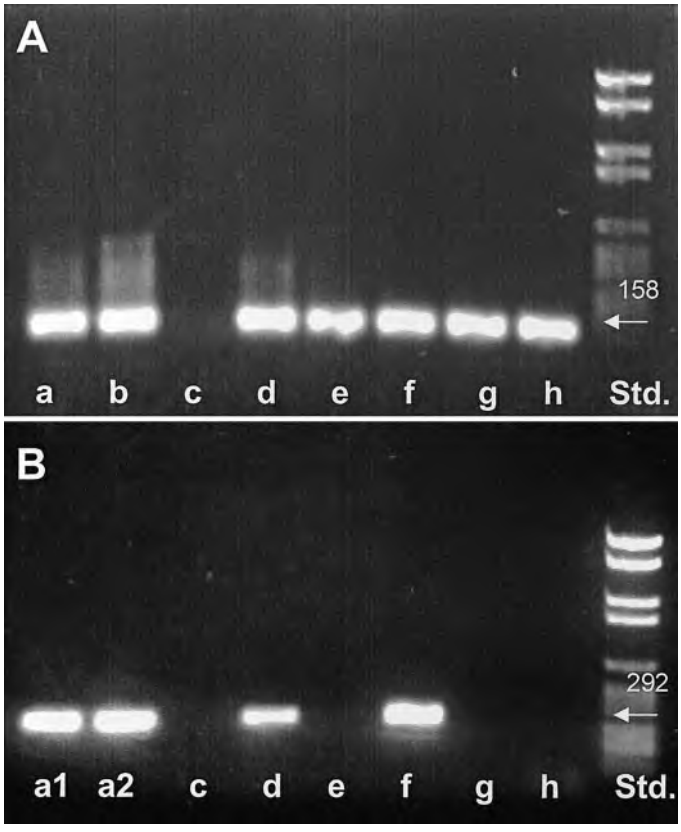

FIG. 4. Messenger RNA expression of leptin (A) and the adipocyte-specific ApM-1 (B) in human oral mucosa and salivary glands: a, positive control 1 (adipose tissue); b, positive control 2 (placenta); c, water (negative control); d, sublingual gland; e, submandibular gland; f, parotid gland; g, oral mucosa 1; h, oral mucosa 2; Std, DNA marker. For the determination of ApM-1, adipose tissue was used twice as positive control (a1 and a2). *Arrows* indicate the expected size of the PCR products.

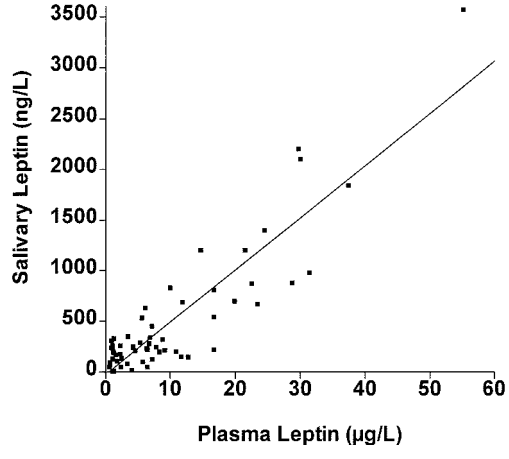

FIG. 5. Relationship between the concentrations of plasma and salivary leptin from corresponding sample collections from healthy volunteers with BMIs from 15 to 54. The line indicates linear regression ($r^2 = 0.78$).

salivary leptin and the BMI was $r^2 = 0.52$, whereby a correlation of $r^2 = 0.53$ was calculated for plasma levels and the BMI. The ratios of salivary and plasma leptin values were significantly lower ($P < 0.05$) in obese (BMI > 25) subjects in comparison with lean subjects (BMI < 20) (Fig. 6). Serial dilution of saliva showed linearity of leptin concentrations ($r = 0.997$, $P < 0.001$) and paralleled the standard curve (Fig. 7).

Stimulation of saliva flow rate

The stimulation of salivary flow by the application of citric acid to the tongue resulted in a slight but statistical decrease of salivary leptin concentration ($P < 0.05$). About 70% of the prestimulation values were reached (Fig. 8A). At the same time, saliva volume (Fig. 8B) increased up to 4-fold ($P < 0.001$). Therefore, it must be concluded that the total secretion of leptin (Fig. 8C) increased 2- to 3-fold ($P < 0.001$). After rinsing the mouth with water, leptin and saliva production returned to prestimulation values.

Stability of salivary leptin

Recombinant leptin standard material was incubated at different pH values and was measured after buffering with carbonate. Leptin remained stable between pH 7.2 and 3.5. Furthermore, leptin remained stable in centrifuged saliva after storage in the refrigerator (4 C) for up to 5 d ($P = 0.34$) and at RT after the addition of sodium azide to prevent microbial growth.

Discussion

The role of leptin in energy turnover has been intensively studied, emphasizing the importance of this peptide as a feedback signal from the adipose tissue to the central nervous system (10, 11, 25), especially with respect to the regulation of appetite (1, 12). The recent discovery of leptin in the stomach (5, 7) raises the question of whether gastric leptin directly influences digestion and energy uptake. It is still controversial whether leptin is produced (5, 7) or only stored and secreted (6) in the stomach. Specific leptin receptors are located in the gastric mucosa of rats (5) and humans (6) and in intestinal cells of mice (18). It has been shown by microelectrode recording that direct application of leptin into the stomach leads to neuronal activities in the brain stem of

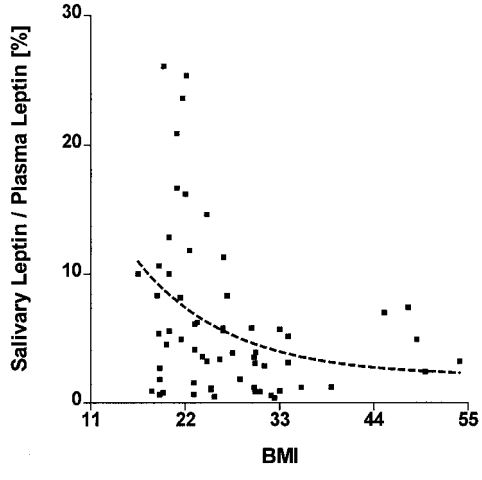

FIG. 6. Ratios between salivary and plasma leptin values in lean and obese subjects in percent. —, Nonlinear regression fitted to the data.

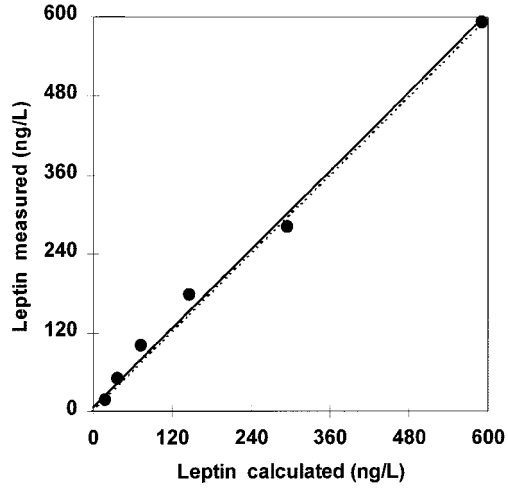

FIG. 7. Serial dilution of saliva in relation to the calculated amount. —, Ideal correlation y = x; the *connected line* shows the calculated Passing/Bablock regression.

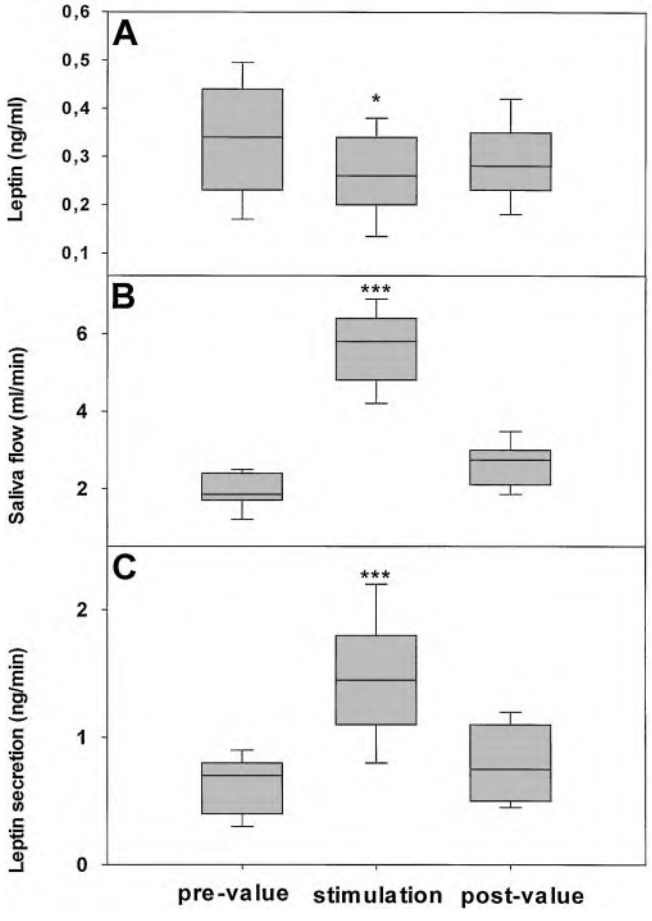

FIG. 8. Alterations in salivary leptin concentrations (A) and salivary flow rate (B) during stimulation with citric acid. C, Total secretion of salivary leptin before, during, and after stimulation (calculated as $Conc._{leptin} \times Vol._{saliva}$). All three corresponding samples were taken in a time interval <5 min. *, $P < 0.05$; ***, $P < 0.001$.

rodents (26), possibly through vagal fibers projecting from the stomach to the caudal part of the brain stem. Furthermore, leptin has been shown to reduce pepsin secretion and gastric acid production significantly after gastric application in rats (27). These findings suggest a functional role of leptin in the digestive tract.

In the present study, we could show that leptin is produced, stored, and secreted by the salivary glands and is expressed in oral mucosa. Using FPLC analysis, free immunoreactive leptin could clearly be identified in human saliva. The identity of leptin was confirmed by Western blotting presenting the 16-kDa band of leptin. Cross-reactions of the leptin antibody with other salivary components appear to be unlikely because of the strict linearity of leptin values measured in serial dilutions.

Owing to the strong correlation between salivary and plasma leptin values, a transport from blood vessels into the salivary glands appears plausible. Because diffusion is unlikely, owing to the protein's molecular weight, an active transport may be postulated. A similar mechanism has been shown for the plasma-cerebrospinal transport of leptin (28). However, the transport of circulating leptin into the salivary glands and its subsequent secretion does not appear to be the sole mechanism accounting for the presence of leptin in saliva. We could demonstrate leptin mRNA synthesis in the salivary glands and oral mucosa. Because the adipocyte-specific ApM-1 mRNA (23) was not detectable in the submandibular gland and oral mucosal tissue, which contains a high density of small salivary glands, contamination with fat cells can be excluded for these tissues. Owing to this autonomous leptin production in the glands, the strong correlation between plasma and salivary leptin values may alternatively be explained by similar regulatory mechanisms. This will be of interest for further investigations. Interestingly, we found significantly lower ratios of salivary and plasma leptin values in obese subjects in comparison with lean ones, indicating that higher circulating leptin values are not completely reflected by rising salivary leptin levels. The reason for this finding remains unclear. Studies are ongoing to investigate whether there is a saturable leptin transport through the endothelia in the salivary glands, similar to the one identified in the blood-brain barrier (28) or whether the production of leptin in the salivary glands is autonomous.

Stimulation of salivary flow rate with citric acid led to slightly decreased leptin concentrations; however, the volume of saliva rose 4-fold. Consequently, higher total amounts of salivary leptin are available. Because leptin is stable above pH 3.5, and pH values in the stomach increase with food intake up to 5 (29), the peptide is possibly not inactivated in the stomach during digestion. It is still not clear to what extent leptin is produced in the stomach. A recent study (6) was unable to detect leptin mRNA synthesis in the gastric mucosa. Therefore, leptin measured in the stomach might, at least partially, be a product of the salivary glands.

In conclusion, the findings of this study suggest an interaction of salivary leptin with the leptin receptors in the gastric mucosa, possibly for the purpose of regulating digestion.

Until now, for routine purposes, leptin had been measured exclusively from serum and plasma. Because salivary leptin levels changed during stimulation of saliva flow rate, the determination of salivary leptin appears suitable only for noninvasive sample collection, if a basic saliva flow can be guaranteed.

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