

Identification of Ghrelin in Human Saliva: Production by the Salivary Glands and Potential Role in Proliferation of Oral Keratinocytes

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Background: We investigated whether ghrelin is present in human saliva, is produced by salivary glands, and physiologic consequences of these findings.

Methods: Expression of ghrelin and specific receptor mRNA was determined by PCR. Proteins were identified by immunoblotting and size-exclusion fast protein liquid chromatography (FPLC) with consecutive RIA. Specific RIAs were used for quantification of salivary total and bioactive ghrelin. Distribution of ghrelin was investigated by immunohistochemistry in cryosections of the salivary glands. The effect of ghrelin on incorporation of 5-bromo-2'-deoxyuridine as a measure of cell proliferation was investigated in primary oral keratinocytes.

Results: Ghrelin is produced by the salivary glands. The hormone was identified in saliva and glands by immunoblotting and by FPLC fractionation of saliva. Immunohistochemistry demonstrated ghrelin distribution in the salivary glands. The receptor was also produced by the glands and by oral keratinocytes and was shown to be functional. Comparison of total ghrelin values for healthy individuals (body mass index, 18–27 kg/m²) showed significantly lower concentrations in saliva than in serum ($P < 0.01$). The correlation between both matrices was $r^2 = 0.56$ ($P < 0.001$) with a negative correlation to body mass index ($r^2 = 0.314$; $P < 0.01$). Bioactive acylated ghrelin was also present in saliva. Incubation of keratinocytes with ghrelin led to signifi-

cantly increased cell proliferation ($P < 0.001$). This effect could be completely suppressed by co-incubation with NOX-B11 (50 nmol/L), a novel specific inhibitor of acylated ghrelin.

Conclusions: Ghrelin in saliva is produced and released by salivary glands. The effect of ghrelin on oral cell proliferation adds to the pro-proliferative action of other salivary growth factors.

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Ghrelin (growth hormone secretagogue), a 28-amino acid acylated peptide, is produced predominantly in the stomachs of humans and rodents (1). It has no relevant homology with most other biologically active peptides except motilin and has therefore equivalently been described as motilin-related peptide (2). Modification of the serine-3 residue of ghrelin by *n*-octanoic acid is mandatory for binding to the specific receptor for hormonal activity. This acylated peptide is described as the active form as opposed to the inactive des-acyl ghrelin (3).

One main function of ghrelin is its growth-hormone-releasing activity (4). Ghrelin is the natural ligand to a formerly "orphan" G-protein-coupled growth hormone secretagogue receptor (GHS-R),⁴ specific to a family of growth hormone secretagogues (5, 6). GHS-R, the main binding site for ghrelin, is produced throughout the brain (7) as well as in various peripheral tissues (8) in two described isoforms, GHS-R 1a and 1b (9). Research activities have focused on the stimulatory effect of ghrelin on food intake and its role in the modulation of energy expenditure (10). Ghrelin plays a major role in the gastrointestinal tract, stimulating gastric contractility and

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⁴ Nonstandard abbreviations: GHS-R, growth hormone secretagogue (ghrelin) receptor; BMI, body mass index; TBS, Tris-buffered saline; DAPI, 4',6'-diamidin-2'-phenylindoldihydrochloride; FPLC, fast protein liquid chromatography; and BrdUrd, 5-bromo-2'-deoxyuridine.

acid secretion (11), and it is responsible for the metabolic response to starvation by modulating insulin secretion (12), glucose metabolism (13), and amino acid uptake (14). Furthermore, it affects cardiovascular activity (15) by acting as a vasodilator.

Data on the influence of ghrelin on proliferation processes are contradictory. The peptide displays antiproliferative effects in lung carcinoma (16) and breast carcinoma cell lines (17) but increases proliferation in prostate cancer cell lines (18), pancreatic adenocarcinoma cells (19), and adrenal cells (20).

Although ghrelin was first isolated and purified from gastric mucosa, recent studies have demonstrated additional production sites for this peptide. In addition to the gastrointestinal tract, certain tissues of the amphibian central nervous system (21), the kidney (22), and the placenta (23) also produce ghrelin in notable amounts.

Given the hormone's importance in food intake and gastric motility, we focused our study on investigating the production and distribution of ghrelin and GHS-R in the major human salivary glands because they have been shown to be important production sites for various hormones and growth factors (24, 25). In case we found ghrelin in human saliva, an additional aim of this study was to examine potential ghrelin functions in the oral cavity by investigating its influence on the proliferation of oral keratinocytes *in vitro*.

Materials and Methods

SAMPLE MATERIALS

Human salivary glands (submandibular, parotid, and sublingual gland) were excised during surgery for otorhinolaryngologic disorders (neck dissection if the sublingual gland was involved; tumor surgery if the parotid or submandibular gland was involved). The glandular tissues used for investigations were not affected by the underlying disorders. Collection of these tissues for scientific purposes was approved by the University ethics board.

Primary oral buccal keratinocytes (26) were obtained from the Department of Oral and Maxillofacial Surgery, University of Freiburg, Germany.

Serum and saliva were collected simultaneously from healthy adults [$n = 25$; 11 male and 14 female; age range, 17–51 years; body mass index (BMI), 19.5–35 kg/m²], who gave informed consent for participation. None of the volunteers took medication or contraceptives, and all had fasted for at least 6 h.

Blood was collected (~10 mL) with Monovettes[®] to obtain 5 mL of serum; saliva was collected with Salivettes[®] (Sarstedt). Each saliva sample was divided into 2 aliquots (1 mL), and 100 μ L of 1 mol/L HCl and 10 μ L of phenylmethylsulfonyl fluoride were added to 1 aliquot to stabilize the *n*-octanoyl group on the Ser-3 residue, which is mandatory for the receptor binding of ghrelin (3). After centrifugation (4000g for 10 min), samples were stored at -80°C until measurement. Serial

dilutions of saliva were prepared to examine linearity and parallelism with the recombinant ghrelin calibration curves.

We investigated the influence of salivary flow rate on ghrelin concentrations in 5 healthy individuals. After collecting a baseline sample, we placed 100 μ L of lemon juice on each person's tongue. We then collected a second sample 10 min after this stimulus. The participants then rinsed their mouths with water, and a third sample was collected after a 10-min wait. The amount of saliva was quantified volumetrically after centrifugation before ghrelin was assayed by RIA.

GHRELIN STABILITY STUDIES

It was mandatory to investigate the stability of salivary ghrelin with regard to shipment of sample material as has been shown for serum and plasma (27). We aliquoted fresh saliva from healthy persons into 5 identical portions, which were stored at room temperature or at 4°C for up to 5 days. We studied ghrelin stability by freezing 1 specimen of each series every day at -80°C until the end of the experiment. Additionally, aliquots of these saliva samples were repeatedly frozen and thawed to investigate the stability of salivary ghrelin under laboratory conditions.

RNA extraction and reverse transcription-PCR. We extracted total RNA from the tissues with guanidine–thiocyanate acid–phenol (TRIzol[®]; WAK Chemie, Medical GmbH). Contrary to the manufacturer's protocol, we used 1 mL of TRIzol/100 mg of glandular tissue and added 400 μ L of CHCl₃. To determine the RNA concentration, we measured the absorbance at 260 and 280 nm on a spectrophotometer (Bio-Photometer; Eppendorf). For PCR, 1 μ g of RNA was reverse-transcribed in a volume of 20 μ L containing 4 μ L of 5 \times first-strand buffer, 2 μ L of deoxynucleotide triphosphate mixture (10 mM), 1 μ L (0.4 μ g) of Random-Primer p(dN6), 1 μ L of dithiothreitol, 1 μ L of RNase inhibitor, 1 μ L of Moloney murine leukemia virus (MMLV) polymerase, and distilled water (HPLC grade; to bring the volume up to 20 μ L). All chemicals were obtained from Promega. Reverse transcription was performed at 39°C for 60 min. Oral keratinocytes from the incubation experiments were harvested with 0.5 mL of TRIzol/well, and RNA was extracted and transcribed as described above.

Human gastric cell lines (AGS/HGTA) and human placenta served as positive controls, and water as a negative control. The primers were 5'-GGCCAAAGAG-GCCCA-3' (forward) and 5'-AGCAAGCGAAAAGC-CAGATG-3' (reverse) for ghrelin (amplicon size, 90 bp) and 5'-GTGAAGCTGGTCATCTTCGTCA-3' (forward) and 5'-CACCCTACAGCCAGCATTTTC-3' (reverse) for GHS-R 1a (348 bp). For GHS-R 1b, the same forward primer was used, but the reverse primer was 5'-GCT-GAGACCCACCCAGCA-3' (amplicon size, 349 bp). Primers were obtained from MWG Biotech AG.

After initial denaturation at 94 °C (10 min), samples were PCR-amplified (40 cycles). Each cycle consisted of denaturation at 94 °C, annealing at 67 °C, and a final extension at 72 °C. After PCR amplification, cDNA was analyzed by electrophoresis on 2% agarose in Tris-acetate-EDTA buffer and visualized by ethidium bromide staining.

Western blotting. For 40-fold concentrated saliva, homogenized salivary glands, and oral keratinocytes, the protein was denatured and the charge equalized in sodium dodecyl sulfate-mercaptoethanol lysis buffer. After heating (90 °C for 5 min) and recooling on ice, proteins were separated on Tris-tricine gradient gels (10–20%; Bio-Rad), transferred to nitrocellulose membranes (Roth), blocked with 50 g/L nonfat powdered milk in Tris-buffered saline (TBS) overnight (4 °C), and incubated for 2 h at room temperature with specific rabbit antibodies against human ghrelin (Phoenix) and GHS-R (Alpha Diagnostic) diluted 1:500 or 1:200, respectively, in blocking buffer. After washing with TBS, the immune complexes were visualized with horseradish peroxidase-labeled goat anti-rabbit conjugates (New England Biolabs) at a dilution of 1:2000 (1 h at room temperature). Biotin-labeled protein markers (New England Biolabs) or color-labeled protein markers (Bio-Rad) were used for molecular weight determinations. Human gut and stomach cells served as positive controls for ghrelin and the receptor (9).

Immunohistochemistry. Submandibular, parotid, and sublingual glands were frozen in liquid nitrogen and cut on a cryostat (Microm). Sections (7 µm) were thawed and fixed with acetone on microscopic slides (Superfrost Plus; Labor Schubert). Sections were preincubated with TBS buffer containing 10 mL/L bovine serum albumin, 5 mL/L Triton X-100, and 100 mL/L goat serum for 30 min at room temperature. After the sections were rinsed with buffer, anti-human ghrelin antiserum (Phoenix) was added (1:50 in TBS buffer) and incubated for 2 h at room temperature in a humid box. After a buffer rinse, rhodamine-tagged goat anti-rabbit IgG (Biotrend) was applied at a final dilution of 1:30 in buffer, including 4',6'-diamidin-2'-phenylindoldihydrochloride (DAPI) for nuclear staining, for 1 h at room temperature. After a final washing step, the sections were coverslipped in VectaShield.

Negative controls were prepared by replacing the first antibody solution with TBS buffer or by preincubation of the antibody with ghrelin (Phoenix Pharmaceuticals), respectively. Microscopy was performed with a confocal microscope (Bio-Rad).

Chromatographic separation of salivary ghrelin. Fast protein liquid chromatography (FPLC) separation was performed on an ÄctaPrime apparatus (Amersham Pharmacia Biotech). Salivary proteins were separated on a Superose 12 HR 10/30 (10-mm diameter, 30-cm length) column from the same manufacturer. In brief, 500 µL of either

saliva or human ghrelin calibrator (500 µg/L) was injected and eluted with elution buffer (pH 7.4) containing 0.01 mol/L KH₂PO₄, 0.15 mol/L NaCl, and 1g/L NaN₃ at room temperature at a flow rate of 0.5 mL/min. Fractions (0.5 mL) were collected and assayed by a commercial RIA for total ghrelin (Phoenix Pharmaceuticals).

Measurement of salivary ghrelin. All measurements related to the identification and characterization of salivary ghrelin were performed with a commercial RIA for the measurement of total ghrelin (Phoenix Pharmaceuticals) (27).

In a later investigation we wanted to determine whether acylated (physiologically active, octanoylated) ghrelin and des-acyl ghrelin are present in saliva. For this purpose, we used two commercial RIA systems designed to differentiate between active and total (acylated plus des-acylated) ghrelin (Linco Research). The procedure and specifications for the total ghrelin measurement and the differences in the values obtained by both methods have been reported previously (28).

The lower limit of detection of the octanoyl-ghrelin RIA was 7.8 ng/L. The intra- and interassay CVs are 9.5% (intraassay) and 13.7% (interassay) at 237 ng/L and 6.7% (intraassay) and 9.6% (interassay) at 139 ng/L, respectively (data provided by the manufacturer). Matched samples were measured within the same assay run to rule out any preanalytical influences.

Measurement of ghrelin in keratinocyte culture supernatants. Human oral keratinocytes were cultured in a 5% CO₂, humidified atmosphere at 37 °C in Keratinocyte FBS medium (Gibco), containing 100 kIU/L penicillin and 100 g/L streptomycin (Sigma). The cells were grown in 6-well plates to an initial confluence of ~50%. Three plates were prepared with wells containing growth medium with 1.2, 2.5, 5, 10, or 20 µg/L leptin added as stimulus for ghrelin production (29); 1 well (control well) in each plate contained no leptin. Supernatants were harvested consecutively after 24, 48, and 72 h and measured by Linco RIAs for total and acylated ghrelin.

Functionality of GHS-R in oral keratinocytes. Human oral keratinocytes were cultured in 6-well plates to ~70% confluence. Cells were stimulated with 50, 10, or 0 µg/L acylated ghrelin, with incubation for 15, 30, 60, or 90 min (37 °C). Each concentration at each time point was run in duplicate. At each time point, cell supernatants were collected, and cells were lysed with 0.1 mol/L HCl. Cell lysates and culture supernatants (diluted 1:5 in 0.1 mol/L HCl) were measured with the low pH cAMP EIA (R&D Systems) as recommended by the manufacturer.

Cell proliferation assay. In the first experiment, keratinocytes were transferred to sterile 96-well microtiter plates (Falcon) at a density of ~10³ cells/well. After adhesion of the cells (24 h), the culture medium was changed to a

medium containing acylated ghrelin at 0, 1.8, 3.7, 7.5, 15, or 30 $\mu\text{g}/\text{L}$. Acylated human ghrelin was purchased from Phoenix.

In the second experiment, geometrically diluted saliva (in sterile phosphate-buffered saline) was sterile-filtered (Minisart 0.45 μm ; Sartorius). These dilutions were mixed 1:1 (by volume) with culture medium and applied to the microtiter plates in consecutive order. In each experiment, we used 10 samples of each concentration.

The cell proliferation assay was purchased from Roche (Mannheim, Germany). 5-Bromo-2'-deoxyuridine (BrdUrd) was added to the wells as a pyrimidine analog (except for the background control) after 48 h of preculture. After an additional 24 h of BrdUrd incorporation, cells were fixed, and tetramethylbenzidine turnover was measured at 370 nm.

We designed a control experiment to verify the specificity of a proliferative effect. For this purpose, we performed a proliferation assay under the same conditions and with the same cell density as described above. The cells were preincubated with a highly specific ghrelin-inhibitor, the RNA-Spiegelmer, NOX-B11 (NOXXON Pharma AG) at concentrations of 50, 25, 12, or 6 nmol/L for 10 min ($n = 10$ for each concentration). After this preincubation, acylated ghrelin was added at a concentration of 25 $\mu\text{g}/\text{L}$ to each well. NOX-B11 binds to the octanoylated NH_2 terminus of ghrelin and prevents binding to the GHS-R (30). One lane containing only ghrelin without the inhibitor and one lane containing 50 nmol/L NOX-B11 lacking ghrelin served as controls. The BrdUrd assay then was performed as described above.

STATISTICAL ANALYSIS

The correlations between matched salivary and serum ghrelin values and between calculated and measured ghrelin values after serial dilution were calculated by linear regression analysis. Differences between salivary and serum ghrelin values were calculated by Wilcoxon signed-rank test. Preanalytical influences on salivary ghrelin values were calculated by ANOVA (Kruskal-Wallis), as were the differences in BrdUrd incorporation between the ghrelin-stimulated cells. A P value <0.05 was considered significant.

Results

RNA ANALYSIS

Ghrelin and GHS-R mRNA expression was detected in the 3 major salivary glands as well as in oral keratinocytes. PCR yielded cDNA products with the expected length of 90 bp for ghrelin (Fig. 1A) and 348 and 349 bp for the two receptors GHS-R 1a (data not shown) and GHS-R 1b (Fig. 1B) in the tissues and cells examined, with human gastric cells and placenta serving as positive controls.

PROTEIN IDENTIFICATION

Western blot analysis of saliva and glandular tissues confirmed the presence of ghrelin in the samples exam-

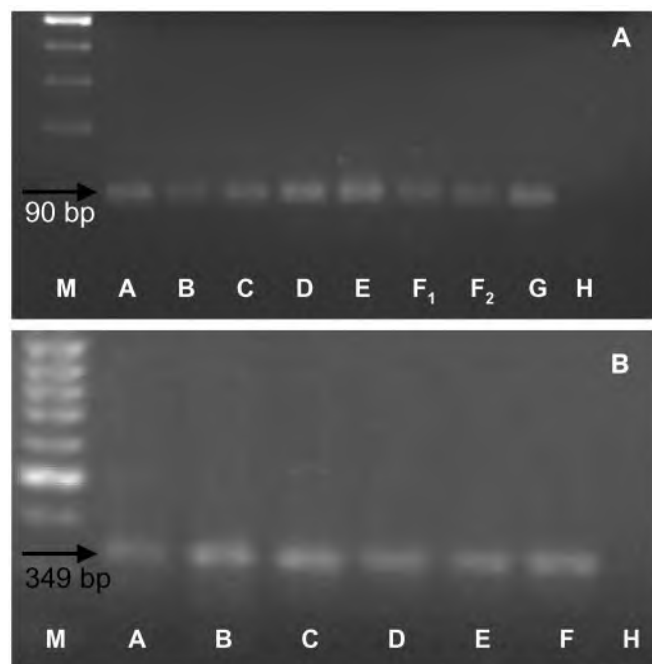


Fig. 1. Expression of ghrelin (A) and GHS-R 1b (B) mRNA in the 3 major salivary glands.

Lanes: M, molecular markers; A, gastric cells (HGTA; positive control); B, gastric cells (AGS; positive control); C, sublingual gland; D, submandibular gland; E, parotid; F, keratinocytes; G, placenta (positive control); H, water (negative control). F₁ and F₂ indicate different keratinocyte samples.

ined. The immunoreactive band in saliva (molecular mass ~ 3 kDa) migrated at the same position as the ghrelin calibrator (Fig. 2A). Ghrelin was also present in all 3 major salivary glands and oral keratinocytes (Fig. 2B). Additionally, the ghrelin receptor was detected in the glandular tissues and oral keratinocytes, as 2 specific bands with molecular masses of ~ 30 and ~ 40 kDa (Fig. 2C). These bands were also present in the positive controls from gut and stomach cells.

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Specific rhodamine staining was observed in sections of all 3 major salivary glands after incubation with an antibody raised against human ghrelin. The staining was absent in negative controls (Fig. 3C). The results shown in Fig. 3 are for the submandibular gland. Cells throughout the entire duct system showed staining concentrated in granulae (Fig. 3A, examples indicated by arrows), which were also present in the acinar endpieces. To facilitate orientation, the same section after DAPI staining of the cell nuclei is shown in Fig. 3B.

FPLC SEPARATION

Using size-exclusion chromatography and subsequent RIA measurements, we confirmed that ghrelin is the positive immunoreactive analyte in human saliva. The recombinant human ghrelin calibrator yielded 1 broad peak around fraction 40. In saliva samples, immunoreactive ghrelin eluted in the same fractions (Fig. 4).

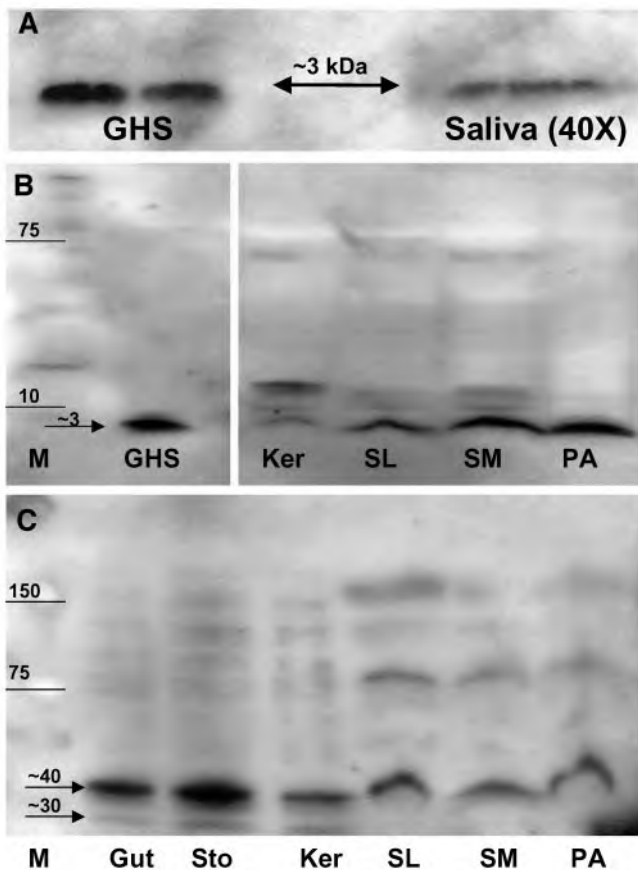


Fig. 2. Western blot analysis of salivary ghrelin.

(A), recombinant human ghrelin (*GHS*; 3.3 kDa) and 40-fold concentrated saliva samples. (B), Western blot analysis of ghrelin in the major salivary glands [*SL*, sublingual gland; *SM*, submandibular gland; *PA*, parotid gland] and oral keratinocytes (*Ker*). Lane *M*, molecular markers. (C), Western blot analysis of the ghrelin receptor in the same tissues and in the gut and stomach (*Sto*).

GHRELIN MEASUREMENT

Ghrelin measured in the serial dilutions of saliva gave linear results ($r^2 = 0.99$; $P < 0.0001$; Fig. 5), indicating the reliability of salivary ghrelin measurements. There was a significant linear correlation between salivary and plasma total ghrelin when measured with the Phoenix assay ($r^2 = 0.56$; $P < 0.001$; Fig. 6A) and the Linco assay ($r^2 = 0.48$; $P < 0.01$), with salivary ghrelin values being significantly lower than serum values ($P < 0.0001$). The ratios of salivary and serum ghrelin ranged from 1:1.5 to 1:8. Additionally, salivary ghrelin concentrations were higher in lean persons than in obese persons (Fig. 6B). Concentrations of salivary total ghrelin ranged, depending on the RIA, from 10 to 198 ng/L (Phoenix) and from 550 to 2470 ng/L (Linco), according to the described assay discrepancies in ghrelin quantification (28).

The biologically active acylated form of ghrelin was also present in all saliva samples measured with the Linco active ghrelin assay (range, 55–451 ng/L; median, 126 ng/L). Interestingly, there was no correlation between the total salivary ghrelin concentrations and the values for

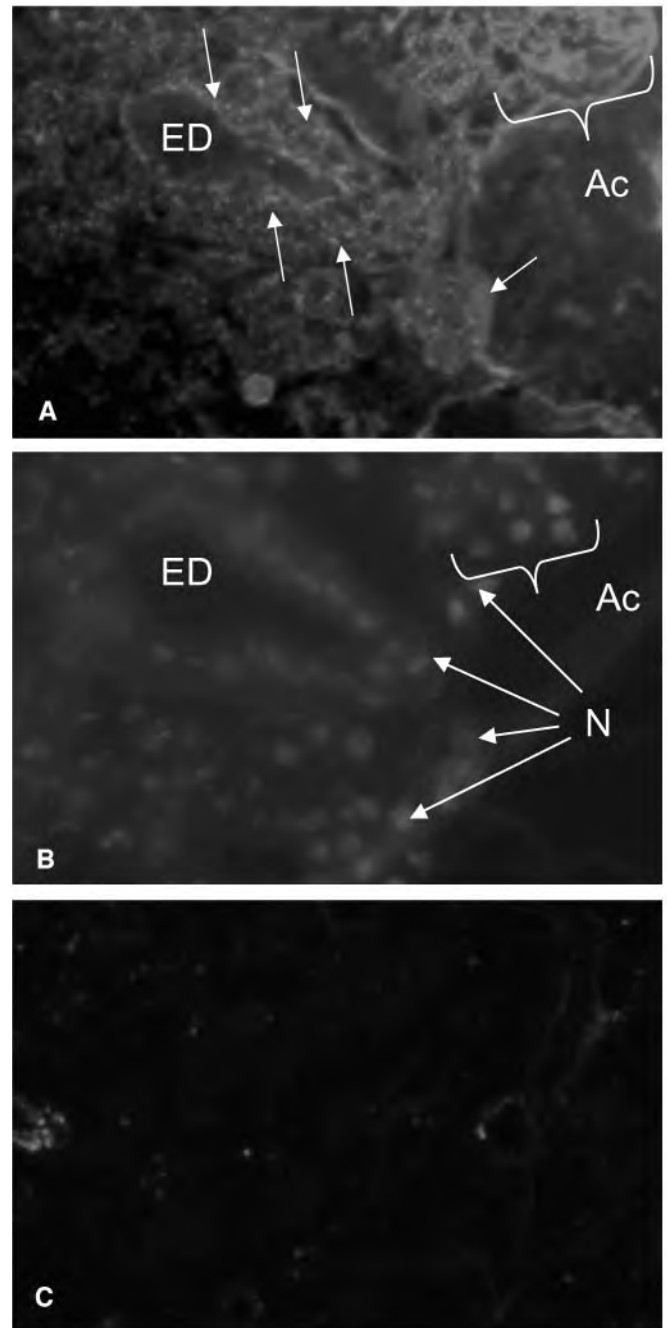


Fig. 3. Visualization of ghrelin by immunofluorescence (rhodamine staining) in sections of human sublingual gland ($\times 40$).

(A), rhodamine staining for ghrelin. Staining was localized in granulae, which were concentrated near the cell membranes (arrows). (B), DAPI staining of nuclei (arrows) from the same section. (C), negative control without ghrelin antiserum.

acylated ghrelin from the same samples ($P = 0.197$). The ratios of acylated vs total salivary ghrelin ranged from 1:2 to 1:17.

STIMULATION OF SALIVA FLOW RATE

Stimulation of salivary flow by the application of citric acid to the tongue produced a slight but statistically

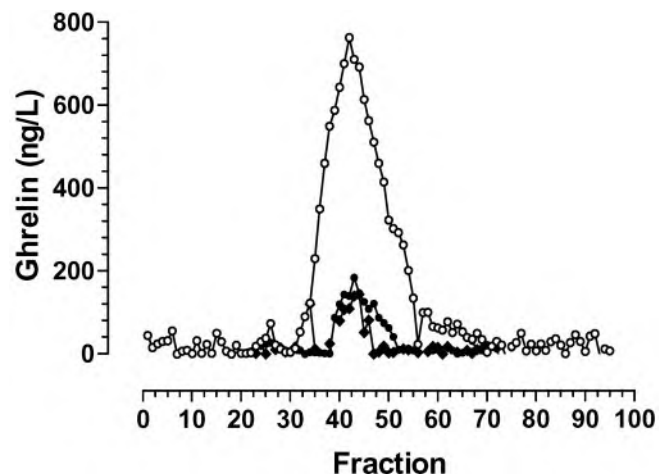


Fig. 4. Ghrelin immunoreactivity in fractions obtained from FPLC separation of concentrated human saliva samples (● and ◆) and recombinant ghrelin calibrator (○).

significant decrease of salivary ghrelin concentration ($P < 0.05$); the value after citric acid application was ~70% of the prestimulation value. At the same time, saliva volume increased up to 4-fold ($P < 0.001$). We therefore conclude that the total secretion of ghrelin increased 2- to 3-fold ($P < 0.001$). After the participants rinsed their mouths with water, ghrelin and saliva production returned to prestimulation values.

STABILITY OF SALIVARY GHRELIN

Total salivary ghrelin concentrations remained stable in centrifuged saliva after storage in the refrigerator (4 °C) for up to 3 days ($P = 0.34$). However, storage (or shipment) at room temperature is not to recommend because of a dramatic decrease in values ($P < 0.001$). Repeated freezing and thawing also led to decreased measured values for salivary total ghrelin. This investigation was

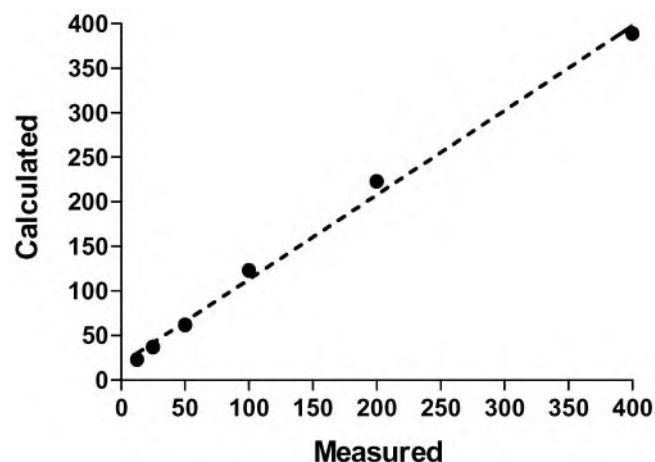


Fig. 5. Correlation between calculated and measured ghrelin content of a serially diluted saliva sample ($r^2 = 0.99$; $P < 0.001$).

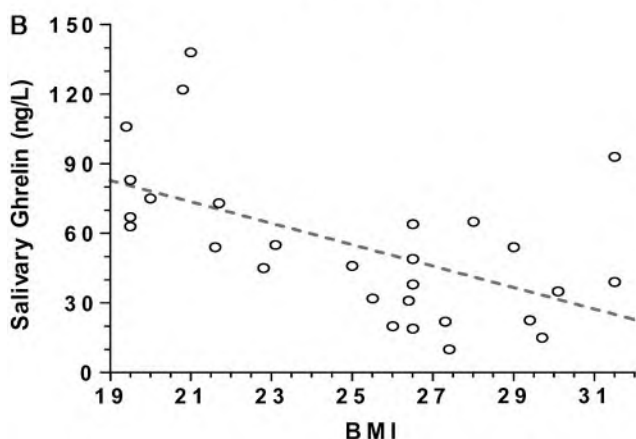
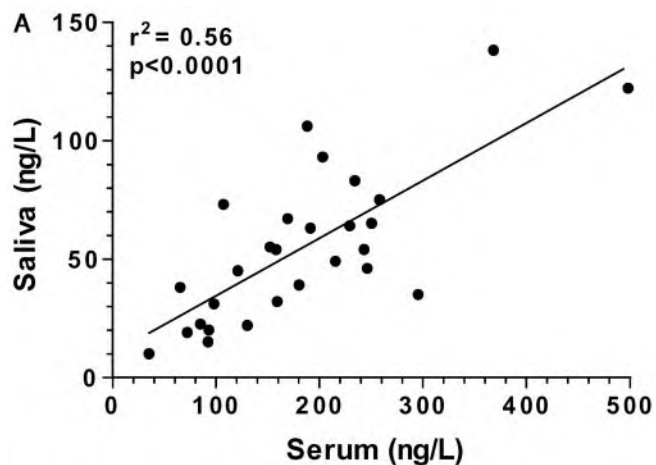


Fig. 6. Relationship between plasma and salivary concentrations of ghrelin from matched sample collections from healthy volunteers (A) and between salivary ghrelin concentrations and BMI (B).

Results of linear regression analysis were as follows: (A), $r^2 = 0.56$; $P < 0.001$; (B), $r^2 = 0.314$; $P = 0.019$.

performed only for total ghrelin with the Phoenix RIA before introduction of the other assays to the study.

GHRELIN RELEASE FROM ORAL KERATINOCYTES AFTER LEPTIN INCUBATION

Results obtained with both assays for total and acylated ghrelin indicated that ghrelin was present in keratinocyte culture supernatants. Total ghrelin concentrations ranged between 1067–1499 ng/L (0 $\mu\text{g/L}$ leptin) and 1246–1427 ng/L (20 $\mu\text{g/L}$ leptin), whereas acylated ghrelin ranged between 125–128 ng/L (0 $\mu\text{g/L}$ leptin) and 145–297 ng/L (20 $\mu\text{g/L}$ leptin). There was no obvious correlation between leptin concentration or time and the measured ghrelin concentration.

FUNCTIONALITY OF GHS-R IN ORAL KERATINOCYTES

cAMP was significantly increased in ghrelin-stimulated cells and supernatants ($P < 0.001$) after 15, 30, and 60 min, respectively. The highest values were observed after 30 min. After 90 min, there was no difference between stimulated and nonstimulated cells (Fig. 7).

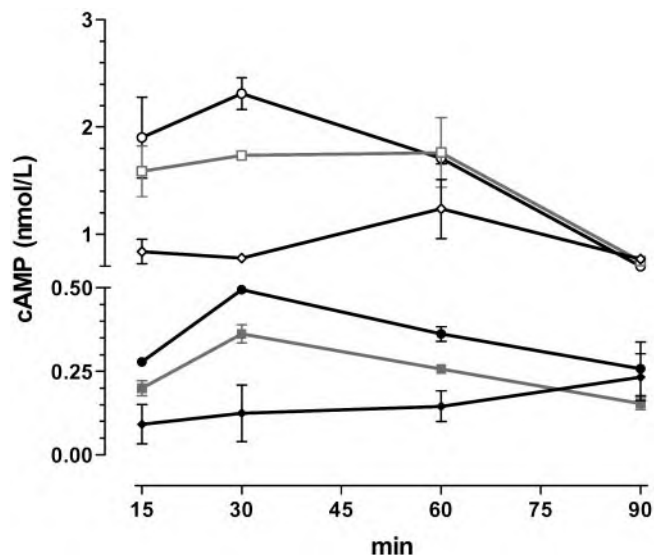


Fig. 7. Measurement of cAMP in keratinocytes (cell lysates; *open symbols*) and supernatants (*filled symbols*) after stimulation with 10 (\square and \square for cell lysates and supernatants, respectively) or 50 $\mu\text{g/L}$ ghrelin (\bullet and \circ for cell lysates and supernatants, respectively) for 15, 30, 60, and 90 min in relation to nonstimulated cells. \blacklozenge and \blacklozenge , control supernatant and cell lysate (no ghrelin), respectively. Differences were significant at any time point other than 90 min ($P < 0.001$). Highest values were observed after 30 min. At the end of the experiment (90 min), there was no difference between stimulated and nonstimulated cells. Error bars, SE.

CELL PROLIFERATION ASSAY

One possible physiologic role of salivary ghrelin in the oral cavity could be clarified by performing cell proliferation experiments. We observed a dose-dependent increase in BrdUrd incorporation (3.7 $\mu\text{g/L}$ ghrelin, $P < 0.05$; 7.5 $\mu\text{g/L}$, $P < 0.01$; 30 $\mu\text{g/L}$, $P < 0.001$; Fig. 8A). Moreover, results obtained with sterile dilutions of whole saliva with its natural ghrelin content showed a dose-dependent increase in BrdUrd ($P < 0.001$; Fig. 8B), indicating that ghrelin, in addition to many other salivary factors, also promotes oral keratinocyte proliferation. It is important to mention that this finding was observed only when freshly prepared ghrelin additive was used. The effect disappeared when the experiments were performed with material subjected to repeated freezing-thawing or with ghrelin solutions stored several days in the refrigerator. These findings are consistent with the use of the ghrelin inhibitor NOX-B11, which specifically binds to the active form of ghrelin and neutralizes its binding to the GHS-R. A sevenfold molar excess of NOX-B11 (50 nmol/L) completely blocked ghrelin-induced (25 $\mu\text{g/L}$) cell proliferation ($P < 0.001$), whereas the inhibitor itself had no statistically significant effect on cell growth.

Discussion

In the present study, we could show that ghrelin and the two receptor isoforms, GHS-R 1a and GHS-R 1b, are produced by the human salivary glands, with subsequent

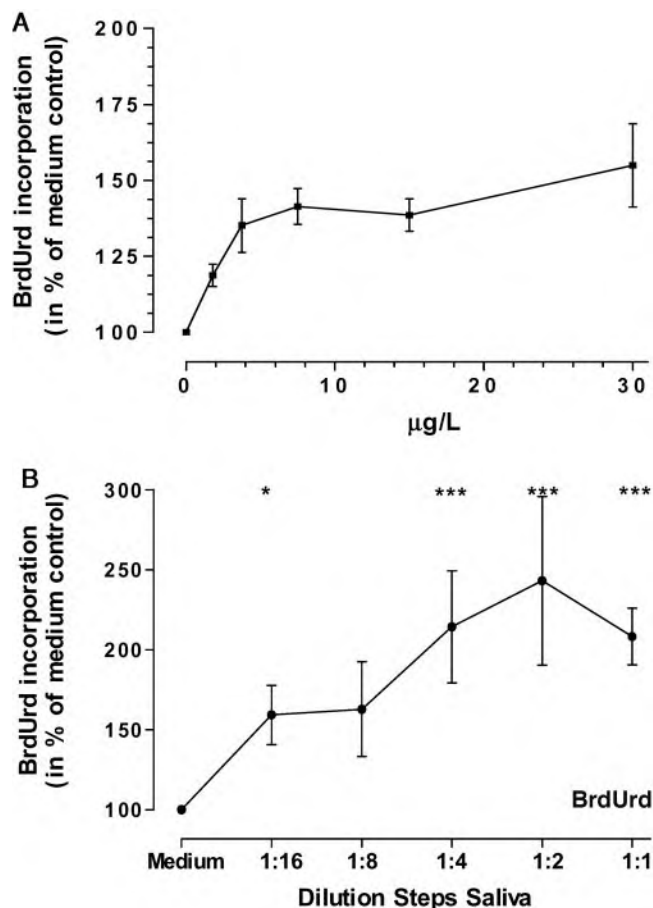


Fig. 8. Proliferation of human oral keratinocytes after incubation with ghrelin compared with medium controls (A), and BrdUrd incorporation in cells incubated with sterile filtered human saliva, serially diluted in phosphate-buffered saline and mixed with an equal volume of medium compared with medium controls (B).

(A), at 3.7 $\mu\text{g/L}$ ghrelin, $P < 0.05$; at 7.5 $\mu\text{g/L}$, $P < 0.01$; and at 30 $\mu\text{g/L}$, $P < 0.001$. Each data point represents the mean (SE; error bars) of 10 observations. (B), *, $P < 0.05$; ***, $P < 0.001$. Index for proliferation of the cells is the incorporation of BrdUrd into the DNA. Data are the mean (SD; error bars) for 10 measurements.

secretion of the hormone into saliva. Results of FPLC analysis indicated that ghrelin is present in human saliva, with further confirmation by Western blotting. Cross-reactions of the commercial antibody with other salivary components are unlikely: the ghrelin values measured in serial dilutions were strictly linear. Concentrations of salivary ghrelin were lower than those in serum with a significant correlation between both body fluids. In addition, we found a linear correlation between salivary ghrelin values and BMI of our participants. This finding is in agreement with previous data on lower ghrelin concentrations in obese compared with lean persons (31, 32).

Stimulation of the salivary flow rate with citric acid led to significantly decreased ghrelin concentrations, an effect described for other salivary peptides (25), indicating that the use of saliva for ghrelin determinations can be recommended only when constant flow conditions can be

guaranteed. With strict regard to preanalytical standardization, saliva might then be considered as a noninvasive alternative to serum. However, it was not the principal goal of the current study to describe an alternative to plasma ghrelin measurements.

Our results with salivary ghrelin to date may be considered with regard to two possible explanations:

- The correlation between salivary and serum ghrelin indicates transport from the blood vessels into the glandular cells. This is possible because other small peptide hormones, such as melatonin (33) and insulin (34), pass through the capillary endothelia into the salivary glands, with a strong correlation between concentrations in both body fluids. However, in our study, the statistical correlation was too weak ($r^2 = 0.48-0.56$) and the sample number too low to predict serum values by salivary ghrelin values.
- There is autonomous production of ghrelin by the salivary glands, as has been shown for other hormones and growth factors (35, 36). We could demonstrate production of ghrelin mRNA in all major salivary glands and in oral keratinocytes. Thus, the relatively high correlation between serum and salivary ghrelin values may be explained by general regulatory mechanisms, such as the body composition and the BMI, and with special respect to the gastrointestinal tract being the predominant origin of ghrelin.

In any case, whether salivary ghrelin could be useful in the diagnosis of metabolic or gastrointestinal disorders remains to be determined.

The immunohistologic staining of ghrelin with granular concentration near the cell membranes of the ducts indicates that ghrelin is stored within the glands before release into the ductal lumen. Because the salivary glands and oral mucosal keratinocytes produce both receptor isoforms, salivary ghrelin seems to act in a paracrine manner in these tissues, in agreement with similar postulated autoregulatory feedback loops for another peptide hormone, leptin, within the salivary glands (37). Moreover, the increase in cAMP supports the functionality of the GHS-R because cAMP has been shown to be involved in ghrelin signaling (38, 39).

Consequently, it was our aim to investigate possible functions of salivary ghrelin in the oral cavity. Beyond the role of ghrelin in the digestive tract and central nervous system feeding centers, it was not clear whether ghrelin acts for (18) or against (16, 17) proliferation in studies using pathologic cell lines.

We investigated *in vitro* whether ghrelin has any influence on the proliferation of primary human oral keratinocytes obtained from the buccal mucosa. We observed significantly increased incorporation of BrdUrd into the primary cells compared with untreated control cells. Because this effect could be completely blocked by a ghrelin inhibitor, we believe that the effect of ghrelin on the cell growth of keratinocytes is specific.

The concentrations used in the proliferation experiment were higher than those measured in saliva, based on the results obtained with the Phoenix RIA. However, saliva production is a continuous and very difficult process. Our findings of the influence of saliva flow on the ghrelin content as well as data of others on the restricted stability of the octanoyl group of ghrelin (40) led us to use superphysiologic concentrations. After des-acylation of the extremely unstable octanoyl branch, a hormone receptor interaction seems to no longer be possible (3). A recent publication demonstrated a half-life of acylated ghrelin of <20 min and a very limited stability of the active form to repeated freeze-thaw cycles (41). The authors of another topical publication assumed that des-acylated ghrelin may even counteract certain functions of the acylated portion (42), indicating that the story is not as simple as distinguishing ghrelin in active and nonactive forms in relation to the intactness of the octanoyl branch. Therefore, further investigations are planned to assess the possible roles of the different ghrelin forms in modulation of cell proliferation in the oral cavity and the gastrointestinal tract.

Nevertheless, we found both acylated and des-acylated ghrelin in saliva. On the basis of our findings, we believe that ghrelin acts pro-proliferatively on mucosal cells of the oral cavity. *In vivo* this effect might be supported by the presence of a variety of pro-proliferative peptides in saliva, such as epidermal growth factor or leptin. Interestingly, a recent study provided data on the pro-proliferative effects of ghrelin in the stomach (43). A link between the actions of ghrelin in the oral cavity and other parts of the gastrointestinal tract is therefore conceivable. As shown in our study, sterile filtered native saliva containing ghrelin and many other salivary components accelerates keratinocyte proliferation, demonstrating the importance of saliva for oral health. The pro-proliferative, and therefore wound-healing, effect of whole saliva in animals is well known (44, 45), and salivary gland-derived peptides such as ghrelin may be responsible for this in addition to the cooling and antiseptic or antibacterial effects of saliva (46-48).

In conclusion, the data provided here show for the first time the presence of ghrelin in saliva. It remains to be clarified whether salivary ghrelin might be a useful tool in clinical chemistry for diagnosis of metabolic or gastrointestinal disorders as has been postulated for serum ghrelin (49, 50).

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