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Obestatin is present in saliva: alterations in obestatin and ghrelin levels of saliva and serum in ischemic heart disease

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Ghrelin and obestatin are a single gene products and are a multiple functional peptides that regulates energy homeostasis, and food intake. In the present work, we studied the secretion of ghrelin and its co-secreted peptide obestatin in 44 patients with ischemic heart disease with that of 27 healthy matched controls. Here we first conducted using an immunohistochemistry assay to screen whether human salivary glands have any obestatin immunoreactivity. Then, serum and saliva obestatin and acylated ghrelin levels were determined by using Radioimmunoassay. Our immunohistochemical analysis demonstrated that obestatin was localized in the striated and excretory duct of human salivary gland. We also report for the first time that obestatin, like ghrelin, is present in human salivary gland and saliva. No evidence of the role of obestatin or ghrelin saliva levels in the context of ischemic heart disease was found. Salivary ghrelin and obestatin levels are correlated in controls with the blood levels. Determination of salivary values could represent a non-invasive alternative to serum ones that can be useful in clinical practice. [BMB reports 2008; 41(1): 55-61]

INTRODUCTION

Ischaemic heart disease (IHD) is the most common cause of death in most western countries as well as world-wide (1). Genarally IHD is a result of atherosclerosis. Atherosclerosis is characterized by endothelial dysfunction, lipoprotein oxidation, leukocyte infiltration, relase of various chemotactic and growth factors and the build up of cholesterol, lipids and calcium. The earliest pathologic lesion of atherosclerosis is the fatty streak. The fatty streak may progress to form a atherosclerotic plaque, lipid accumulation, acute and chronic luminal obstruction, abnormalities of blood flow and restricted the supply of blood to the target organs (2) and thus oxygen. When arteries are narrowed, less blood and oxygen reaches to the target organs, this leads to ischemia within these organs and might cause a necrosis.

In IHD, several hormones and other regulatory mediators are recruited that either worsen or ameliorate cardiovascular alterations following restricted the supply of blood to the heart. It has been indicated that myocardial ischemia could cause acute alterations in circulating natriuretic peptide levels (3). Experimental studies have also addressed the alterations in intracellular calcium during ischemia and reperfusion (4). That is, cellular metabolism was manipulated by regulatory mediators and several hormones, including recently discovered an acylated peptide hormone ghrelin (5). Studies in humans (6) and animal models (7) have exhibited beneficial effects of ghrelin in the cardiovascular system, such as a gain in left ventricular mass, an increase in left ventricular ejection fraction and the administration of an acylated peptide hormone ghrelin causes a significant decrease in mean arterial blood pressure (MABP) and has a strong inotropic action, which suggests its important regulatory role in cardiovascular homeostasis (8). Ghrelin (lipopeptide hormone) in rats and in human are recognized for every organ of the body (9-11). In human (12) had found type 1a growth hormone secretagogue receptor (GHSR 1a) in the normal pituitary and at a much lower level in the thyroid gland, pancreas, spleen, myocardium and adrenal gland. They also demonstrate that the distribution of GHSR 1b a truncated form of the type 1a receptor with pharmacological inactivity, is widespread, indeed they detected this form in all tissues studied (12). Papotti and his co-workers using [^{125}I]Tyr-Ala-hexarelin described GHS receptors binding site in the myocardium, in adrenal, gonads, arteries, lung, liver, skeletal muscle, kidney, pituitary, thyroid, adipose tissue, veins, uterus, skin, and lymphnode. They suggested that it may exist a still unknown receptor subtype, different from GHsR1a and 1b, in the heart and in other tissues (13).

Ghrelin structure in the mammalian, the avian and the fish, the third residue that is octanoylated is a serine, whereas in the amphibian this third residue is a threonine, which is essential

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for binding to the GHSRA 1a and n-octanoyl bearing ghrelin known as active ghrelin (acylated-ghrelin). (9, 11). Data in the animal and in the human have described activity of octanoylated ghrelin and des-octanoylated ghrelin in the cardiovascular system (14-17), but in term of ischaemic heart disease the "active" form may be more physiologically crucial.

Obestatin is another product of proghrelin that was recently discovered by (18). That is, ghrelin and obestatin have been shown to be functions of single genes. The latter bears a 23-residues that is yielded in the cells lining the stomach and small intestine of humans and including other mammals. Ghrelin is orexigenic whereas obestatin is anorexigenic. Ghrelin regulates body fluid homeostasis, food intake and energy metabolism, whereas obestatin seems to induce the opposite effects (reviewed by (9)).

The goals of the present study was to determine the serum and saliva concentrations of ghrelin and obestatin in subjects with and without complication of ischemic heart disease, whether obestatin is synthesized locally in salivary glands, whether blood ghrelin and obestatin changes are reflected in saliva.

RESULTS

The demographic characteristics of the subjects are shown in Table 1. Immunohistochemical analysis showed that obestatin was localized in the striated and extcretory ducts of salivary glands (Fig. 1A, negative control; Fig. 1B, obestatin immunoreactive cells of parotid gland; Fig. 1C, negative control; Fig. 1D, obestatin immunoreactive cells of submandibular gland). Our RIA analysis indicated that mean acylated-ghrelin and obestatin levels in salivary gland was 13.4 ± 4.2 pg/mg of tissue, 82.6 ± 28.4 pg/mg of tissue, respectively. Saliva obestatin

Table 1. Demographic characteristics and biochemical data of groups

	Group 1 (n = 27)	Group 2 (n = 44)	P value
Age (Years)	54.8 ± 5.6	56.7 ± 5.8	>0.05
Gender (Female/Male)	13/14	17/27	>0.05
Body mass index (kg/m^2)	28.1 ± 4.5	27.1 ± 3.8	>0.05
Smoking (Present/Absent)	8/19	17/27	>0.05
Heredity (Present/Absent)	4/23	7/37	>0.05
Systolic blood pressure (mmHg)	118.1 ± 13.4	118.9 ± 10.5	>0.05
Diastolic blood pressure (mmHg)	75 ± 13.3	75.4 ± 7.6	>0.05
Total Cholesterol (mg/dL)	191 ± 47.8	193 ± 55	>0.05
Triglyceride (mg/dL)	161 ± 50.3	203 ± 62.2	<0.05
LDL-Cholesterol (mg/dL)	124 ± 45.3	149 ± 29.4	<0.05
HDL-Cholesterol (mg/dL)	36.8 ± 5.9	35.9 ± 2	>0.05
White blood cell	**7,866 ± 2.49**	**7,851 ± 2.32**	**>0.05**

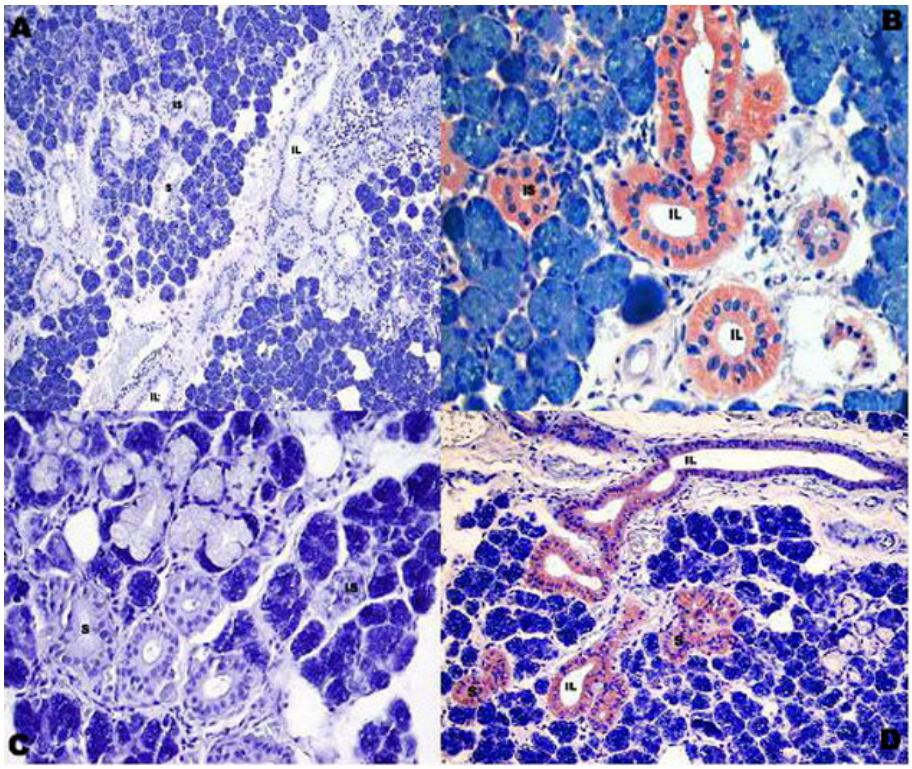

Fig. 1. Obestatin İmmunohistochemistry of human salivary gland. A and B) Interlobular duct (IL) and intercalated (IS) in the parotid gland; C and D) striated duct (S) and interlobular duct (IL) in the submandibular gland. Magnification, A, × 100, B × 400, C, and D, × 200).

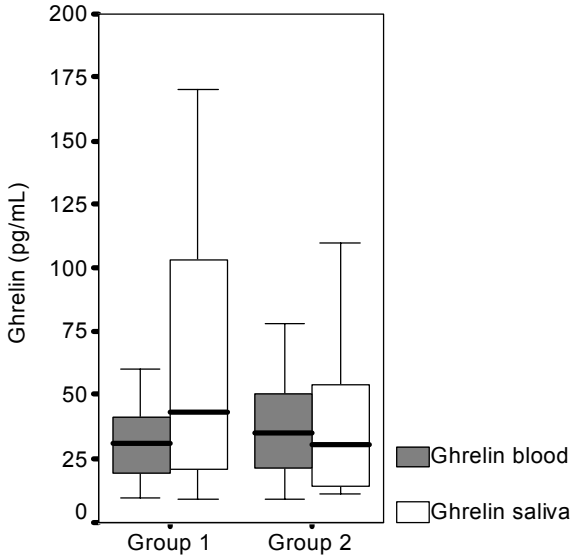

Fig. 2. Serum and saliva ghrelin levels of groups; Group 1: Control; Group 2: Patient with ischemic heart disease.

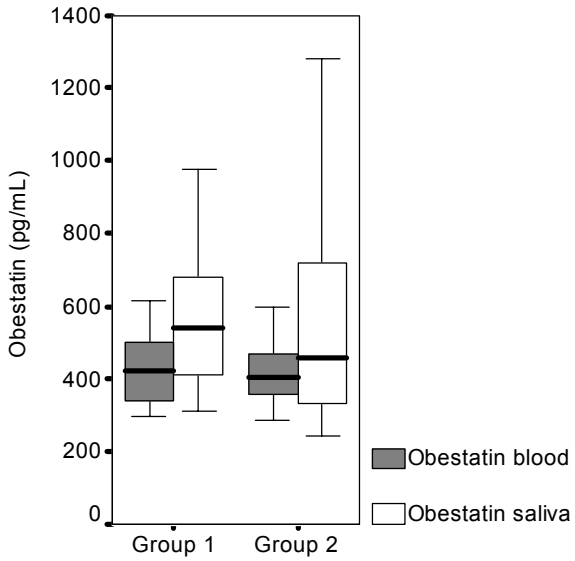

Fig. 3. Serum and saliva obestatin levels of groups. Group 1: Control; Group 2: Patient with ischemic heart disease.

(626.4 ± 133 pg/ml) and acylated-ghrelin (45.5 ± 13 pg/ml) level were higher than blood obestatin (436 ± 114 pg/ml) and ghrelin level (35.1 ± 13 pg/ml) in controls ($P > 0.05$) (Fig. 2, 3). In IHD, we also observed the patterns. That is, saliva obestatin (635.6 ± 196 pg/ml) and acylated-ghrelin (59.9 ± 18 pg/ml) level were higher than blood obestatin (434.7 ± 127 pg/ml) and ghrelin level (38.8 ± 16 pg/ml) ($P > 0.05$). Saliva ghrelin and obestatin levels and blood ghrelin were slightly higher in ischaemic heart disease subjects in comparison with control subjects, but blood obestatin level were the same in both groups (Fig. 2, 3). There was a correlation between blood obestatin and white blood cell in control subjects ($r = 0.389$, $P = 0.04$). Similarly, correlation was also observed between saliva obestatin and and total cholesterol levels in control subjects ($r = 0.503$, $P = 0.008$). Furthermore, there was a correlation between saliva obestatin and LDL and HDL levels in control subjects ($r = 0.490$ $P = 0.01$; $r = 0.406$ $P = 0.03$, respectively), but there was no correlation in IHD. A correlation was also found between blood levels of ghrelin and Diastolic Blood Pressure ($r = 0.568$, $P = 0.002$) and between saliva ghrelin and BMI ($r = -0.590$, $P = 0.001$) in control subjects, but there was no correlation in IHD. A correlation was found between saliva levels of obestatin and saliva ghrelin. ($r = 0.413$, $P = 0.005$) and between blood ghrelin and saliva ghrelin ($r = 0.424$, $P = 0.006$) in ischaemic heart disease. A weak negative correlation was also found between blood levels of obestatin and saliva obestatin. ($r = -0.245$, $P = 0.011$) in ischaemic heart disease, but not in control groups. Phoenix kit detected obestatin quantitatively in saliva. The lowest sensitivity of salivary obestatin was reported to be 9.7 pg/ml. The intra (within-day) and inter-assay (between-days) percentage coefficients of variation were found to be 7.8 and 11.4, respectively. Saliva obestatin assay showed linearity and its recovery was 93.75%. Linearity and recovery results were indicated below.

Linearity

Two saliva (S) samples were diluted with distilled water and assayed. (Obestatin concentrations in pg/ml.)

	Undiluted	1/2	1/4	1/8
S 1	438	464	410	452
	(100%)	(106%)	(94%)	(104%)
S 2	510	492	548	566
	(100%)	(104%)	(108%)	(111%)

Recovery

Two saliva (S) samples were enriched with increasing amounts of obestatin. The percentage recovery was calculated as follows: observed value-baseline value/amount added × 100. The concentrations are given in pg/ml.

	Initial concentration	Amount added	Amount recovered	Amount expected	Recovery (%)
S	532	100	626	632	94.0
	644	200	831	844	93.5

DISCUSSION

The present study examined the concentrations of ghrelin and obestatin in blood and saliva in 44 persons with ischaemic heart disease and 27 healthy controls. Our first experiment in this study concerned the expressions of obestatin in human salivary glands. The present work demonstrated for the first time that human saliva and the duct in human salivary gland contained obestatin as in ghrelin. It also can be expected that obestatin in human salivary gland might be yielded and secreted by the acinar cells like the most salivary peptides (19). In IHC reaction for obestatin of salivary gland in present study, however, ıt was exhibited that the duct in a human salivary gland bears obestatin. These observation are supported by the fact that some other salivary proteins (including ghrelin) have been demonstrated to be yielded in the striated ducts of human salivary gland (20, 21). Another supporting evidence that portions of some of these striated duct protein relased basally into circulation (20). Here we can not rule out the possibility of basal secretion of obestatin, since we do not have evidence of mRNA for obestatin in human salivary glands, but Gröschl and his co-workers have demonstrated ghrelin mRNA expression in the 3 majors' salivary glands (sublingual and submandibular gland, parotid) as well as in oral keratinocytes (22). Ghrelin and obestatin are derived from the same precursor, thus there is a possibility of basal secretion of obestatin.

Determined high ghrelin and obestatin level in saliva is very likely that the consequence of the salivary glands function since obestatin is produced by salivary gland. We (23) and others (22) previously independly have reported that the salivary gland have an own-production of ghrelin. This reported saliva high ghrelin level is agrement with our previous report (23), but is in contrast to a previous study demonstrating a blood concentrations ghrelin higher than saliva concentrations (22). Also here, we can not rule out the possibility that in our condition of blood and saliva collection, it subsist a part of degradation of ghrelin and obestatin due to the temperature of collection and centrifugation. Therefore, reported the high values of ghrelin and obestatin in saliva could be due to a much greater degradation in blood than in saliva. For the future, we are confident that strict standardisation of the pre-analytic procedure, such as when collecting chilling samples, and therefore might improve reliability of saliva and blood for peptide hormone measurements. In the present study we have also showed that there is correlation between saliva obestatin and saliva ghrelin levels and between blood and saliva ghrelin. The latter findings are in agreement with a previous studies (for ghrelin concentrations) demonstrating a correlation between blood and saliva ghrelin concentrations (23, 24).

Further, we tried to show the changes in ghrelin and obestatin levels of saliva and serum in ischaemic heart disease. Concentrations of obestatin in blood were similar in the two groups while we have demonstrated slightly higher concentrations of saliva ghrelin and obestatin in ischaemic heart disease persons compared with control groups. Gnanapavan and his co-workers indicated that ghrelin mRNA expression occurs in the myocardium and vein (12). In their work, they only detected GHSR-1a in the myocardium and not in the vein, and they detected the splice variant GHSR-1b in myocardium and vein, which confirms that the cardiovascular system is a target for ghrelin eventhough the cardioprotective role of ghrelin still remains to be determined, but here we tentatively suggest that acutely and locally produced ghrelin in infarct tissue may accelerate tissue repair in an autocrine- or paracrine-dependent manner, as well as participating directly in the regulation of enery metabolism in ischemic heart tissue (25). Furthemore, eventhough Iglesias and his co-workers have recently described that in their conditions obestatin have no effect on cardiomyocyte viability and metabolism (26). However, pathophysiological role of obestatin ischaemic heart disease still remains an important research topic. A strong correlation was also found between blood ghrelin and diastolic blood pressure. Our diastolic blood pressure result lends credence to that administration of ghrelin causes a significant decrease in mean arterial blood pressure (8).

In this work, It is perhaps necessary to point out that both patients and controls have a mean BMI which is higher than that considered normal all over the world, and even though subjects with this BMI are not to be considered obese. Main reason why our all subjects was overweighted was that mean BMI for all patients at study was 27.1 kg/m^2 (SD, $\pm$ 3.8 kg/m^2). So, BMI of controls (28.1 kg/m^2 (SD, $\pm$ 4.5 kg/m^2) were also matched to patients. If BMI of both control subject and patients were not matched. This might influence obestatin and ghrelin secretion, independently of the heart disease. Because the increase in ghrelin was correlated with the decrement in body weight (27). A negative correlation was also found between saliva ghrelin and BMI ($r = -0.590$, $P = 0.001$) in control subjects, but there was no correlation in IHD. Our results lend credence to these reports (22, 23, 27, 28).

In conclusion, obestatin in saliva is yielded and secreted by salivary glands. Circulated ghrelin and obestatin levels were slightly higher in ischaemic heart disease compared with healty controls, but this difference was neither statistically nor clinically significant. Determination of salivary ghrelin and obestatin values could represent a non-invasive alternative to serum ones that can be useful in metabolism studies.

MATERIALS AND METHODS

Chemicals

Ghrelin (active; Cat. #: GHRA-88HK) was obtained from Linco Research, INC. USA. Obestatin (Human, Monkey; Cat. #: RK-031-92) was purchased from Phoenix, Pharmaceuticals, Inc. USA. Other chemicals were from Sigma-Aldrich.

Subject and sample collection

We studied 44 people with ischemic heart disease (IHD) and Body Mass Index and age-matched 27 healthy controls. This study was carried out on the volunteers whose written consent was taken prior to the study together with the institutional Ethical Committee approval of the study protocol (issue no: 2007, 47). All healthy controls had no family history of obesity or history of abdominal surgery or gastrointestinal diseases or cardiopathies and took no medication for at least 7 days before or during the period of sample collection. The mean age of healthy controls was 54.8 ± 5.6 while the mean age of people with IHD was 56.7 ± 5.8. Body Mass Index (BMI) of IHD patients and controls was 27.1 ± 3.8, 28.1 ± 4.5, respectively. All subjects (including controls) underwent coronary angiography. IHD was defined as ≥ 70% diameter stenosis dioagnosed by coronary angiography. None of subjects had hypertension. Patient and control subjects were advised not to eat, smoke or drink (except water) for an overnight fast prior to the saliva and blood samples collection. From each patients and controls, 5 ml blood sample was drawn into plain biochemistry tubes. Serum tubes were kept at room temperature for 30-45 minutes, allowing clotting, before centrifugation. To protect ghrelin and obestatin from proteolytic action (if any) 20 µl (200 kallikrein inactivator unit, KIU) aprotinin was added per of ml samples. Samples were also acidified to stabilize the labile side chain of active ghrelin and prevent a rapid desacylation of ghrelin, as suggested (29). Blood and saliva sampling were taken simultaneously and also measured in the same experimental set. All blood samples were centrifuged at 4,000 rpm at room temperature for 5 minutes and serum aliquots were stored at −20°C pending analysis. On analysis all samples were measured in duplicates. For saliva collection, the particiipants were instructed to rinse their mouth thoroughly in tap water, and then to lean forward and spit out their saliva into an ice-chilled sterile container bearing the appropriate preservatives. Saliva was centrifuged at 4,000 rpm and at room temperature for 10 minutes (twice) and all sera taken into eppendorf, then placed in zip-closure plastic bags prior to storage at −20°C pending analysis.

Immunohistochemistry (IHC)

Immunohistochemistry was performed on 4 µm thick sections using the avidin-biotin-peroxidase complex (ABC) technique (30) with minor modifications (20), with reagents supplied by Lab Vision Corporation, USA). Following de-waxeing in xylene and dehydrated with alcohol, endogenous peroxidase activity was blocked with 0.3% H_2O_2/methanol for 10 min. ABC was prepared according to manufacturer's protocol (Lab Vision Corporation). The tissue sections were then placed in citrate buffer (pH 6.0), incubated in a 650 W microwave oven for 5 min, and washed with 0.01 M phosphate buffer, pH 7.4 (PBS). Blocking reagent was applied at this point in the assay for 10 min to eliminate nonspecific antibody binding. Next, the sections were incubated at 38°C for 30 min in rabbit anti-obestatin, diluted 1:500. (Phoenix Inc. sugesgested dilution of 1 in 70, was found to produce unacceptable levels background staining), then washed in PBS, incubated with biotinylated goat anti-polyvalent (Lab Vision Corporation) at 38°C for 2 × 15 min, and washed again in PBS. Streptavidin-biotin-peroxidase complex was applied to each tissue section for 10 min and then, Between each step, sections were washed twice times in phosphate-buffered saline (PBS) for 5 min. Amino ethyl carbazole (AEC) was applied as a chromogen for 10 min. Finally, after counterstaining with Mayer's haematoxylin for 1-2 min, slides were dehydrated, cover slipped and examined under a light microscope. Controls were incubated with PBS in place of the primary antibody and no positive staining was observed. The internal positive controls were performed using normal human somach tissue.

Serum and saliva ghrelin assay

The ghrelin concentration in whole saliva and serum was determined by a commercially available Radioimmunoassay (ghrelin active; Cat.#: GHRA-88HK) kit utilizes an antibody, which is specific for ghrelin bearing the octanoyl group on serine 3, called active form of ghrelin. Validation of the acylated ghrelin radioimmunoassay in whole human saliva has been reported elsewhere (31).

Preparation of tissue homogenate

The used salivary gland samples were from Pathology Tissue Archives of Firat Medical Center. The homogenate was prepared by carefully removing 10 mg of salivary gland tissue and crushing with an iron mold. The crushed samples were then homogenized in PBS (5%, w/v) using a stainless-steel mortar and the homogenates were centrifuged at 4,000 rpm for 10 min at room temparuture. The supernatant was separated, supplemented with 20 µl (200 kallikrein inactivator unit, KIU) /ml aprotinin and 1/10 volume 1 N HCl, and stored frozen (9, 11).

Serum, saliva and tissue obestatin assay

The concentration of obestatin in whole saliva and plasma was determined by a commercially available immunological assay [Obestatin (Human, Monkey, RIA Kit; Cat.#: RK-031-92)] based on competitive technique using a 125-I-labeled obestatin tracer. Saliva obestatin measurements were first validated as previously explained (31). We read all samples with using LKB-Wallac, MultiGamma 1261, Turku 10, Finland.

Statistical analysis

Statistical analyses were done using an SPSS 12 statistical package. Pearson correlation test are applied for group comparisons. P values smaller than 0.05 were accepted as significant.

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