

## A Comparison of Ghrelin, Glucose, Alpha-amylase and Protein Levels in Saliva from Diabetics

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During the past decade, many salivary parameters have been used to characterize disease states. Ghrelin (GAH) is recently-discovered peptide hormone secreted mainly from the stomach but also produced in a number of other tissues including salivary glands. The aim of this work was to examine the relationship between active (aGAH) and inactive (dGAH) ghrelin in the saliva and other salivary parameters in type II diabetic patients and healthy controls. Salivary parameters were assessed in a single measurement of unstimulated whole saliva from 20 obese and 20 non-obese type II diabetes patients, and in 22 healthy controls. Total protein and alpha-amylase were determined by colorimetric methods, and glucose by the glucose-oxidase method. Saliva aGAH and dGAH levels were measured using a commercial radioimmunoassay (RIA) kit. Salivary concentrations of aGAH and dGAH ghrelin were more markedly decreased in obese diabetic subjects than in the two other groups. Glucose and alpha-amylase levels were higher in diabetic subjects than in controls. Furthermore, there were correlations between GAH levels and BMI, and between GAH and blood pressure. However, there was no marked variability in saliva flow rates among the groups. These results indicate that measurement of salivary GAH and its relationship to other salivary parameters might help to provide insight into the role of ghrelin in diabetes.

**Keywords:** Active/Inactive ghrelin, Alpha-amylase, Diabetes, Saliva

### Introduction

Ghrelin (Ghrelin Appetite Hormone, GAH; this abbreviation was proposed by Aydin, 2006a), an endogenous ligand for the growth hormone (GH) secretagogue receptor (GHSR), was originally discovered in extracts of rat and human stomach, where it is localized in the endocrine X/A-like cells of the gastric mucosa (Kojima *et al.*, 1999). This hormone is composed of 28 amino acid residues, of which the third, usually a serine but in some species a threonine, is acylated by a fatty acid; this modification is essential for GAH activity. Ghrelin is the first known example of a bioactive peptide modified by acylation (Kojima *et al.*, 2005). Two major forms of GAH are present in tissues and blood: des-acylated [dGAH (inactive); this abbreviation was proposed by Aydin, 2006a] and octanoylated [aGAH (active); this abbreviation was proposed by the same author in 2006a]. Both forms of ghrelin, especially aGAH, are now known to have important physiological roles (Kojima *et al.*, 2005; Aydin, 2006b), the only difference being that des-acylated ghrelin can cross the blood brain barrier (Banks *et al.*, 2002); but in terms of diabetes and insulin resistance the "inactive" form may be more physiologically important.

GAH is predominantly produced in the stomach (Kojima *et al.*, 1999; Kojima *et al.*, 2005), but it is expressed in many other organs including bowel, pancreas, myocardium, kidney, pituitary and hypothalamus (Kojima *et al.*, 2005). It promotes appetite. GAH mRNA has also been found in other tissues (Gnanapavan *et al.*, 2002) and GAH is present in human milk (Aydin *et al.*, 2006c). We have also reported a substance in plant parenchyma cells that is strongly immunoreactive against human ghrelin (Aydin *et al.*, 2006d). More recent work in our laboratory (Aydin *et al.*, 2005b), and others (Groschl *et al.*, 2005) has shown that GAH is produced and secreted by salivary glands and exhibits biological rhythm in humans (Aydin *et al.*, 2006e).

In humans, GAH affects cardiovascular activity by acting as a vasodilator, glucose metabolism by modulating insulin

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secretion, amino acid uptake, bone formation (Fukushima *et al.*, 2005) and appetite (increases food intake: Wren *et al.*, 2001; Kojima *et al.*, 2005). It contributes to loss of appetite in gastric cancers (Aydin *et al.*, 2005c). Plasma GAH levels are increased under negative energy balance conditions such as fasting, anorexia nervosa and cachexia (Otto *et al.*, 2005), and decreased in obesity (Tschop *et al.*, 2001; Bellone *et al.*, 2002; Hansen *et al.*, 2002; Haqq *et al.*, 2003; Rosicka *et al.*, 2003; Groschl *et al.*, 2005) and after food intake (Cumming *et al.*, 2002; Aydin *et al.*, 2006e). Marzullo and co-workers have reported that the circulating level of aGAH decreases in obese patients and that it is significantly correlated with BMI (Marzullo *et al.*, 2004). Circulating levels of aGAH are diminished in patients with type 2 diabetes mellitus and are inversely correlated with BMI in non-diabetic and type 2 diabetic subjects (Katsuki *et al.*, 2004; Celi *et al.*, 2005).

Diabetes mellitus (DM) is a group of disorders of carbohydrate metabolism characterized by hyperglycemia, with an estimated 170 million patients worldwide in the year 2000. Its prevalence is rapidly increasing (Wild *et al.*, 2004). It is clinically complex and associated with many serious complications including kidney failure, blindness and cardiovascular disease (Babligna *et al.*, 2006). The chronic nature of DM entails a substantial decrease in quality of life and life expectancy and accounts for billions of dollars in health care spending (Coffey *et al.*, 2002; Stephens *et al.*, 2006).

Many recent studies have assessed the insight that might be gained into the pathophysiology of this disorder of carbohydrate metabolism by investigating saliva composition in diabetic patients (Harrison *et al.*, 1981; Tenovuo *et al.*, 1986; Ben-Aryeh *et al.*, 1988; Streckfus *et al.*, 1994; Belazi *et al.*, 1998; Data *et al.*, 2004) and experimental animals (Reuterving, 1986). However, there has been no consensus about saliva composition in diabetic patients. For example, total salivary protein concentration has been found to be similar in diabetic and control groups in some studies (Harrison *et al.*, 1981; Tenovuo *et al.*, 1986), while others have found salivary protein concentrations from diabetics to be either lower (Streckfus *et al.*, 1994) or higher (Ben-Aryeh *et al.*, 1988). Dembinski *et al.* (2003) found a dramatic impairment of amylase activity in diabetic animals as compared to controls. Zhang *et al.* (2005) showed that ghrelin inhibits potassium-stimulated amylase secretion in incubated pancreatic lobules, and there is a correlation between ghrelin and glucose (Hirsh *et al.*, 2005). It has been shown that pretreatment with ghrelin reduces pancreatic damage in caerulein-induced pancreatitis and inhibits the development of gastric lesions induced by ethanol (Dembinski *et al.*, 2003).

To the best of my knowledge, although several studies have examined serum ghrelin levels in patients with diabetes (Katsuki *et al.*, 2004; Celi *et al.*, 2005), measurements of saliva ghrelin levels, alpha-amylase, total protein and glucose in type 2 diabetes have not been undertaken to date. The present study was therefore undertaken to investigate: (i) whether the salivary levels of dGAH and aGAH are decreased

or increased in diabetic subjects; (ii) the relationship between ghrelin and alpha-amylase, total protein and glucose in diabetic patients. The results may provide important information concerning the activity of saliva aGAH/dGAH in diabetic patients and its association with alpha-amylase, total protein level and glucose.

## Materials and Methods

**Subjects.** The group of patients included in this study consisted of 20 obese (BMI >30 kg/m<sup>2</sup>) and 20 non-obese (BMI <25 kg/m<sup>2</sup>) patients with type 2 diabetes mellitus, aged from 41 to 66 years, who were referred by the Endocrinology Service of the Firat Medical Center, Elazig. The diagnosis of type 2 diabetes mellitus was based on the criteria of the American Diabetes Association (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1997). Type 1 diabetic subjects were excluded. The control group consisted of 22 clinically healthy humans (11 female and 11 male) between 38 and 59 years of age. Exclusion criteria for the control group were: pregnancy, alcohol consumption, tobacco products (former and current), BMI >25 kg/m<sup>2</sup>, chronic medical illness, history of drug treatment or therapy within the previous months, and history of diabetes. Subjects were asked not to eat, smoke or drink (except water) for an overnight fast prior to collection of saliva samples. Their diets were similar with respect to protein content and uptake of fat and carbohydrates. Socio-economical status was similar for both groups (survey data).

**Saliva collection and conservation.** Unstimulated saliva (5 ml) from the diabetic and control groups was collected using the previously published method (Aydin *et al.*, 2005b; Aydin *et al.*, 2006e) and the results obtained were confirmed by Groschl *et al.* (2006). Briefly: in the morning, at 8 o'clock (before breakfast), the subjects were asked to rinse their mouths thoroughly with water, then to bend their heads forward and allow saliva to flow into an ice-chilled sterile container bearing the appropriate preservatives. The containers were brought immediately to the laboratory from the Endocrinology Clinic. Collection took 5 min and the salivary flow rate was defined as the volume of saliva secreted per min. Once the saliva was collected, it was centrifuged at 4000 rpm for 15 min to remove any particulate material. Each supernatant was divided into three aliquots and stored at 70°C until analysis. To exclude the possibility of effects of the menstrual cycle on the ghrelin rhythm, the data for each subject were obtained during the luteal phase.

Ghrelin was measured in unstimulated total saliva using the same human-ghrelin-RIA kit, which is designed to test any biological fluid with sufficient levels of the peptide to be determined. Validation of the GAH radioimmunoassay in whole human saliva has been reported elsewhere (Aydin *et al.*, 2005b; Groschl *et al.*, 2005; Aydin *et al.*, 2006e). All samples were read with a gamma counter. Salivary dGAH was calculated as follows: inactive GAH = total GAH concentration - aGAH concentration. Active salivary GAH was measured using the human-ghrelin-RIA kit. Active saliva GAH measurements were first validated as follows;

**Sensitivity:** The lowest concentration that could be distinguished from the zero standard was 15 pg/ml.

**Precision:** The intra-assay (within-day) variation was determined for two different saliva (S) and two different plasma (P) samples using the means of 2 replicates of each. The coefficient of variation (CV) is calculated as:  $CV = \text{Standard Deviation (SD)}/\text{Mean}$ .

Sample	Number	Mean (pg/ml)	SD (pg/ml)	CV (%)
S1	3	34	3.6	10.5
S2	2	31	4.2	13.5
P1	2	44	8.1	2.3
P2	2	68	6.6	9.7

The inter-assay (between-days) variation was also determined for two different saliva (S) and two different plasma (P) samples using the means of several 2 replicates of each.

Sample	Number	Mean (pg/ml)	SD (pg/ml)	CV (%)
S1	3	28	4.5	16
S2	3	42	6.6	15.7
P1	2	74	8.4	11.4
P2	2	66	7.4	11.2

**Linearity:** Two saliva (S) and plasma (P) samples were diluted with distilled water and assayed. (Concentrations in pg/ml.)

	Undiluted	1/2	1/4	1/8
S1	34 (100%)	40 (118%)	28 (83%)	38 (112%)
S2	42 (100%)	36 (86%)	50 (119%)	52 (124%)
P1	66 (100%)	72 (100%)	84 (127%)	78 (118%)
P2	74 (100%)	86 (116%)	80 (108%)	92 (124%)

**Recovery:** Two saliva (S) and plasma (P) samples were enriched with increasing amounts of active ghrelin. The percentage recovery was calculated as follows:  $\text{observed value} - \text{baseline value} / \text{amount added} \times 100$ . The concentrations are given in pg/ml.

	Initial concentration	Amount added	Amount recovered	Amount expected	Recovery (%)
S	34	64	86	98	88
	42	128	190	170	112
P	66	64	136	130	104
	78	128	210	206	102

These results verified that the kit detected ghrelin quantitatively in saliva. The lowest sensitivity of salivary active ghrelin was found to be 15 pg/ml. The intra- and inter-assay percentage coefficients of variation were found to be 2.3 and 16.0, respectively.

Total protein and alpha-amylase were determined immediately after collection in order to avoid daily variations caused by endogenous proteolytic activity. Total protein concentration was measured by the Bradford method (Bradford *et al.*, 1976) and amylase activity was determined by the colorimetric method of Winn-Deen and co-workers (Winn-Deen *et al.*, 1988). Glucose was determined in 200  $\mu\text{l}$  of saliva by the glucose-oxidase method (Reljic *et al.*, 1992). Salivary total proteins (g/min), amylase activity (U/ml) and glucose level (mg/ml/min) were expressed in the units shown for ease of comparison with previous studies on diabetic subjects (Belazi *et al.*, 1998).

**Chemicals.** Total ghrelin (Phoenix-Germany) and active ghrelin were obtained from Linco Research, INC. USA. Other chemicals were purchased from Sigma-Aldrich.

**Statistical analysis.** Statistical analysis was performed using SPSS 10.0 for Windows software. All values are reported as mean  $\pm$  SD. The statistical significance of differences in BMI, aGAH, dGAH, amylase, glucose, total proteins, and systolic and diastolic blood pressure between the study groups and the controls was estimated by one-factor analysis of variance (ANOVA) with or without Tukey's post-hoc test.  $p$  values smaller than 0.05 were accepted as significant.

## Results

The demographic characteristics of the subjects are shown in Table 1. There were no significant inter-group differences in age or duration of diabetes (Table 1). Total protein did not differ among the groups (Table 2). Salivary glucose (200%) and alpha-amylase (27%) levels were significantly higher in obese diabetic subjects than in controls ( $p < 0.05$ ); and salivary glucose (192%) and alpha-amylase (24%) levels in non-obese diabetic subjects were also significantly higher than those of control. Salivary glucose and alpha-amylase levels in the obese diabetic subjects were almost the same (Table 2).

Salivary aGAH (52%) and dGAH (24%) levels were lower than control in obese subjects with type 2 diabetes (Table 2). Saliva aGAH (39%) and dGAH (19%) level were also lower than control in non-obese type 2 diabetes subjects (Table 2). Saliva aGAH (22%) and dGAH (6.5%) level were lower in the obese than in the non-obese patients (Table 2). This means that type 2 diabetes is associated with a decrease in both aGAH and dGAH. The aGAH and dGAH saliva levels were also found to be correlated with body mass index (BMI) in both obese ( $r = 0.48$ ,  $p < 0.05$ ) and non-obese ( $r = 0.34$ ,  $p < 0.06$ ) type 2 diabetic patients and control groups. When saliva aGAH levels were compared with salivary dGAH concentrations on an individual basis, a large variance was observed, with values ranging from 16 to 59 pg/ml for aGAH and from 74 to 289 pg/ml for dGAH.

The diastolic and systolic blood pressures were higher in type 2 diabetes patients than normal subjects (Table 1;  $P < 0.05$ ) but there was no marked difference between obese and non-obese

**Table 1.** Clinical characteristics of type 2 diabetic patients and controls. Data are expressed as arithmetic means  $\pm$  standard deviations (SD)

Parameters	Obese (n = 20)	Nonobese (n = 20)	Control n = 22
Age (years) <sup>a</sup>	47 $\pm$ 7	48 $\pm$ 8.5	49 $\pm$ 8.8
Sex (M/F) <sup>a</sup>	9/11	10/10	10/12
DBP (mm Hg) <sup>b</sup>	97 $\pm$ 4.2	92 $\pm$ 7.6	83 $\pm$ 6.8
SBP (mm Hg) <sup>c</sup>	137 $\pm$ 6.4	136 $\pm$ 8.2	126 $\pm$ 6.6
Duration of diabetes (years) <sup>d</sup>	9.4 $\pm$ 0.31	9.8 $\pm$ 0.5	none

DBP; Diastolic blood pressure, SBP; systolic blood pressure. <sup>a</sup>Age and sex were not different among the study groups. <sup>b,c</sup> $p < 0.05$  obese vs control, <sup>b,c</sup> $p < 0.06$  obese vs non-obese. <sup>d</sup>Duration of diabetes did not differ between obese and non-obese subjects.

**Table 2.** Mean salivary values of glucose, total protein, alpha-amylase, and aGAH and dGAH levels in obese/non-obese diabetic and control groups

Parameters	Obese (n=20)	Non-obese patients (n = 20)	Control n = 22
Glucose (mg/ml/min) <sup>a</sup>	3.9 $\pm$ 0.8	3.8 $\pm$ 0.6	1.3 $\pm$ 0.3
Total protein (g/min) <sup>b</sup>	1.74 $\pm$ 0.3	1.68 $\pm$ 0.2	1.84 $\pm$ 0.4
Amylase (U/ml) <sup>c</sup>	628 $\pm$ 62	612 $\pm$ 57	494 $\pm$ 44
aGAH (pg/ml) <sup>d</sup>	21 $\pm$ 2.9	27 $\pm$ 4.6	44 $\pm$ 7.1
dGAH (pg/ml) <sup>e</sup>	146 $\pm$ 34	156 $\pm$ 47	192 $\pm$ 38
Flow rate (ml/min) <sup>f</sup>	0.97 $\pm$ 0.2	1.09 $\pm$ 0.1	1.2 $\pm$ 0.3

<sup>a</sup> $p < 0.001$  obese vs control, <sup>a</sup> $p < 0.05$  obese vs non-obese and non-obese vs control, <sup>b</sup> $p < 0.05$  obese vs control, non-obese vs control, obese vs non-obese, <sup>c</sup> $p < 0.001$  obese vs control, <sup>c</sup> $p < 0.05$  obese vs non-obese and non-obese vs control, <sup>d</sup> $p < 0.001$  obese vs control and non-obese, <sup>d</sup> $p < 0.05$  obese vs non-obese; <sup>e</sup> $p < 0.001$  obese vs control and non-obese, <sup>e</sup> $p < 0.05$  obese vs non-obese. <sup>f</sup>The flow rate did not differ among study groups.

diabetic subjects ( $P < 0.06$ ). Active ghrelin levels correlated weakly with the systolic and diastolic blood pressure in type 2 diabetes patients ( $r = 0.329$ ;  $p = 0.064$  and  $r = 0.356$ ;  $p = 0.058$ , respectively). Inactive ghrelin levels also correlated weakly with the systolic and diastolic blood pressure in these patients ( $r = 0.344$ ;  $p = 0.057$  and  $r = 0.356$ ;  $p = 0.069$ , respectively). There was no variability in saliva flow rate among the groups, suggesting that there are no differences in secretion rate from salivary gland. When salivary glucose was related to flow rate, no linear correlation was found.

In the present study, the lowest sensitivity of saliva active ghrelin was found to be 15 pg/ml. The intra- and inter-assay percentage coefficients of variation for salivary ghrelin were 10.5 and 16, respectively. Determinations in serial dilutions of saliva indicated that the salivary ghrelin measurements were reliable. Mean recovery was 114% and sensitivity was 89%. Thus, it was verified that the human plasma-ghrelin-RIA kit could detect saliva ghrelin quantitatively.

## Discussion

The use of saliva rather than blood for diagnosis has recently been promoted. Obtaining saliva is advantageous for patients, especially children and diabetic subjects, since the procedure is non-invasive, stress-free and allows multiple samplings. Salivary composition in diabetic subjects has been reported in

a number of previous studies (Dodds *et al.*, 1997; Belazi *et al.*, 1998; Mata *et al.*, 2004). Two major forms of ghrelin are present in saliva; des-acylated (dGAH) and active (aGAH). Both forms, especially the active one (Kojima *et al.*, 2005; Aydin, 2006b), are now known to have important physiological roles, the only difference being that des-acylated ghrelin can cross the blood brain barrier (Banks *et al.*, 2002), but in terms of diabetes and insulin resistance the "inactive" form may be more physiologically significant. Therefore, it is important to differentiate between active and inactive GAH. The present study has shown for the first time that aGAH and dGAH levels in saliva are lower in obese patients than in non-obese patients and controls. In agreement with the results recently reported by Katsuki *et al.* (2004), the obese type 2 diabetic patients in this study exhibited significantly lower serum aGAH levels than the non-obese subjects, who shared a similar pattern with the healthy subjects.

In this study, the decrease in GAH was more pronounced in the obese diabetic patients than the non-obese ones. A fall in serum aGAH in diabetic subjects has been noted previously (Katsuki *et al.*, 2004). It is not known whether the decrease in both forms of ghrelin in the saliva of obese type 2 diabetic patients is due to obesity or diabetes; it is well established that plasma GAH concentration (which correlates with salivary GAH concentration, Groschl *et al.*, 2005) is low in obese people and high in lean people (Tschop *et al.*, 2001; Bellone *et al.*, 2002; Hansen *et al.*, 2002; Haqq *et al.*, 2003; Rosicka *et al.*, 2003).

However, there is a reasonable presumption that the decrease is due to diabetes, because non-obese patients had even lower GAH levels than the controls in this study (Table 2).

Moderate correlations were found between salivary ghrelin value and BMI in non-obese type 2 diabetic patients ( $r = 0.329$ ,  $P = 0.061$ ) and controls ( $r = 0.440$ ,  $P = 0.058$ ), and a stronger correlation was found in the obese patients ( $r = 0.540$ ,  $P = 0.001$ ). This finding is in agreement with our previous data (Aydin *et al.*, 2005) and other reports (Whatmore *et al.*, 2003; Groschl *et al.*, 2005). A slight hypertension is well known to be associated with diabetes. However, the origin of diabetes-induced hypertension has not been precisely explained. On the basis of the present results, it was assumed that circulating GAH (originating from serum or saliva) should be at an acceptable physiological concentration in order to control blood pressure. Since intracerebroventricular administration of GAH suppresses the sympathetic nervous system resulting in a decrease in arterial pressure in rats (Lin *et al.*, 2004) and humans (Nagaya *et al.*, 2001), it is tempting to speculate that high or low GAH may not play a role in physiological adaptation to blood pressure in diabetic patients.

Alpha-amylase hydrolyzes internal 1-4 glycosidic bonds to produce maltose, alpha-dextrin and maltotriose (Shuler and Kargi, 1992; Aydin, 2005a). A dramatic impairment of salivary amylase activity has been found in diabetic animals compared to controls. The present study has shown that the alpha-amylase levels in unstimulated saliva from type 2 diabetic patients are higher than in control groups. There is considerable disagreement in the literature about salivary amylase activity in diabetic patients; different researchers have reported that salivary amylase concentrations from diabetics are higher (Varletzidis *et al.*, 1978; Dodds and Dodds, 1997), lower (Lopez *et al.*, 2003) or the same (Newrick *et al.*, 1991). Alpha-amylase also showed large SD values, as reported previously by Jenkins (1981).

No difference in salivary total protein was found among the three groups examined. Some studies have reported similar total salivary protein concentrations in diabetic and control groups (Harrison *et al.*, 1987; Belazi *et al.*, 1998) although others have found salivary protein levels from diabetics to be either higher (Makkonen *et al.*, 1986; Lopez *et al.*, 2003; Mata *et al.*, 2004) or lower (Streckfus *et al.*, 1994; Yavuzylmaz *et al.*, 1996). These conflicts could be explained by the fact that the different studies examined NDDM patients in different disease stages.

There are remarkable discrepancies in the literature about the effect of NIDDM on salivary flow rates (Lamey *et al.*, 1986; Dodds *et al.*, 1997; Belazi *et al.*, 1998; Meurman *et al.*, 1998; Chavez *et al.*, 2001; Lopez *et al.*, 2003; Dodds *et al.*, 2005). In this study, unstimulated salivary flow rate did not differ among the three groups examined. This finding is in agreement with the one previous study of salivary function under different levels of glycemic control, which showed no alterations in stimulated parotid output as blood glucose levels were reduced (Dodds *et al.*, 1997; Meurman *et al.*, 1998;

Dodds *et al.*, 2005;). Decreased salivary flow rates in diabetic subjects have also been noted in a number of reports (Belazi *et al.*, 1998; Chavez *et al.*, 2001; Lopez *et al.*, 2003), but not in others (Lamey *et al.*, 1986).

In summary, the present study has shown for the first time that the saliva ghrelin level is decreased in obese type 2 diabetic patients. Glucose and amylase were higher in patients than in controls, whereas total proteins did not differ among the groups. It is concluded that changes in ghrelin may have adverse effects on diabetes. These alterations may have a causal role in the developments and severity of disease.

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