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Salivary insulin concentrations in Type 2 (non-insulin-dependent) diabetic patients and obese non-diabetic subjects: relationship to changes in plasma insulin levels after an oral glucose load

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Summary. The presence of immunoreactive insulin in saliva and its relationship to plasma immunoreactive insulin was investigated in healthy subjects, newly diagnosed non-obese Type 2 (non-insulin-dependent) diabetic patients and obese non-diabetic subjects, basally and after an oral glucose tolerance test. The mean ± SEM fasting values of plasma and salivary immunoreactive insulin were significantly higher in diabetic patients and obese non-diabetic subjects than in normal volunteers ($p < 0.05$). During the glucose challenge, the increase of salivary insulin was related with that of plasma in the three groups of subjects, with a time lag in normal and obese subjects. In normal volunteers, plasma and salivary peak values were respectively 49.5 ± 13.4 μU/ml ($p < 0.05$ vs obese subjects) at 60 min and 12.0 ± 3.3 μU/min ($p < 0.05$ vs obese subjects) at 120 min; in diabetic patients, the values were 51.7 ± 5.6 μU/ml ($p < 0.05$ vs obese subjects) and 14.6 ± 4.1 μU/min at 120 min; in obese subjects, the peak value for plasma insulin was 111.5 ± 40.1 μU/ml at 90 min and for salivary insulin 15.6 ± 5.1 μU/min at 120 min. A positive linear relationship was shown between plasma and salivary insulin during the oral glucose tolerance test. The identity of salivary insulin was assessed by reversed-phase HPLC. We conclude that salivary immunoreactive insulin can be found in Type 2 diabetic patients and in obese non-diabetic subjects, as well as normal volunteers, that plasma and salivary insulin are related after a glucose load, and that differences exist in salivary insulin secretion patterns among the three groups of subjects.

Key words: Salivary insulin, obese subjects, Type 2 diabetic patients.

The presence of immunoreactive insulin in saliva and its relation to changes in serum insulin concentration was first noted in a small group of healthy subjects by Sweeney and Antoniades in 1967 [1]. From the few reports successively published about the significance of salivary immunoreactive insulin (S-IRI), it is possible to draw evidence that S-IRI is accumulated from the blood [2], and that, in normal subjects, its concentrations parallel those of plasma immunoreactive insulin (P-IRI) after an oral glucose tolerance test [3, 4]. However, no information is currently available regarding the presence of endogenous immunoreactive insulin in saliva from Type 2 (non-insulin-dependent) diabetic patients and obese non-diabetic subjects, or about the relationship between S-IRI concentrations and P-IRI levels in the fasting state and after physiological stimuli in such cases.

In this paper, we have evaluated whether immunoreactive insulin can be found in saliva from newly diagnosed Type 2 diabetic patients and obese non-diabetic subjects; moreover, we also investigated whether S-IRI changes in these patients reflect P-IRI variations after an oral glucose load and if the changes are similar to normal subjects. Finally, we tried to ascertain if basal and stimulated S-IRI concentrations could be used to distinguish healthy subjects from diabetic patients and obese non-diabetic subjects.

Subjects and methods

Subjects

Twenty subjects were included into the study: seven non-obese normal volunteers, aged 39 ± 4 years; eight newly diagnosed, untreated, non-obese Type 2 diabetic patients [5], aged 40 ± 3 years; and five obese non-diabetic subjects, with body mass index (BMI) > 30 kg/m^2, aged 41 ± 6 years.

Oral glucose tolerance test

All subjects maintained diets with at least 150 g carbohydrate per day for 3 days before an oral glucose tolerance test (OGTT). They were studied at 08.00 hours after an overnight fast. Each subject was given 150 ml 50% dextrose solution, so that 75 g glucose was ingested by each patient. Blood was drawn from an antecubital vein 10 min before

Table 1. Basal values and peak values of plasma-IRI and salivary-IRI from normal subjects, Type 2 diabetic patients and obese non-diabetic subjects

	Plasma-IRI (μU/ml)			Salivary-IRI (μU/min)		
	Normal subjects ($n=7$)	Diabetic patients ($n=8$)	Obese non-diabetic subjects ($n=5$)	Normal subjects ($n=7$)	Diabetic patients ($n=8$)	Obese non-diabetic subjects ($n=5$)
Basal value	8.4± 0.5	11.5±2.0[a]	14.1± 2.4[a]	2.2±0.4	2.8±0.3[a]	5.8±1.5[a]
Peak value	49.5±13.4	51.7±5.6	111.5±40.1[b]	12.0±3.3	14.6±4.1	15.6±5.1[a]

[a] $p<0.05$ vs normal subjects, [b] $p<0.05$ vs normal subjects and diabetic patients

the glucose load and 30, 60, 90, 120, 150 and 180 min afterwards. At each time patients were asked to collect saliva in a clean plastic tube for 2 min. No mechanical or chemical stimulus was necessary to facilitate the collection. The salivary flow ranged 150–650 μl/min in the basal state and remained fairly unchanged during the glucose load.

Blood and saliva samples were centrifuged at 10,000 *g* for 15 min respectively to separate plasma and to remove food fragments and cellular detritus. After the measurement of glucose concentration, plasma and saliva samples were stored at −20 °C until insulin analysis.

Analytical methods

Plasma and saliva glucose levels were determined by the glucose oxidase method on a Beckman glucose analyser; the inter- and between-assay coefficients of variation resulted in less than 5%. P-IRI was measured using HPLC purified A_{14}-^{125}I-insulin as labelled antigen [6] and dextran-coated charcoal to separate free from bound hormone; details regarding this procedure are reported elsewhere [7]. The intra- and between-assay coefficients of variation for the method were 4% and 7% respectively. Reference saliva samples with known amounts of immunoreactive insulin were prepared as follows. Thirty millilitres of saliva were collected from one healthy volunteer after an overnight fast during 1 h and centrifuged at 10,000 *g* for 15 min at 4 °C. Ten grams of dextran-coated charcoal were added and the suspension was stirred at 4 °C for 12 h. After centrifugation at 20,000 *g* for 60 min at 4 °C, the supernatant was measured for immunoreactive insulin by the method described below. Immunoreactive insulin levels lower than 0.5 μU/ml were present. Aliquots of a stock solution containing 200 μU/ml of porcine insulin, as measured by optical density [7], were added to saliva samples to obtain standard S-IRI values ranging 1.25–200 μU/ml. These values were used to fit a standard curve on which the bound/total radioactivity of saliva samples from subjects was read. The within-assay and between-assay coefficient of variation was respectively 9% and 13%. The appropriate measure of saliva insulin was made in microunits per time interval, $\frac{\mu U/ml}{ml/min}$, which reduces to μU/min.

To further characterize S-IRI, saliva samples were chromatographed isocratically by reversed-phase high-performance liquid chromatography. A model 510 pump and a C_{18} μBondapak column (Waters Associates, Milford, Mass, USA) were used. The eluent was as follows: 0.01 mol/l phosphate buffer:acetonitrile:isopropanol = 68:21:11 by vol, 0.15 mol/l ammonium acetate, adjusted to pH 2.5 with HCl [8]. One ml fractions were collected and radioimmunoassay performed. The results were compared with those obtained by chromatographing saliva samples in which insulin had been added.

Statistical analysis

The results are expressed as mean ± SEM; differences between means were compared by Student's t-test. Univariate regression analysis and Spearman's rank correlation coefficient were used to calculate correlations between parameters.

Results

Table 1 shows basal and maximum values for P-IRI and S-IRI in normal subjects, Type 2 diabetic patients and obese non-diabetic subjects during the OGTT. Both P-IRI and S-IRI were significantly higher in Type 2 diabetic patients and obese subjects than normal subjects in the basal state ($p<0.05$). After the glucose load, P-IRI reached a peak of 49.5 ± 13.4 μU/ml at 60 min in normal subjects; in diabetic patients the P-IRI maximum value was found after 120 min and was similar to the peak value of normal subjects; the P-IRI peak in obese subjects was observed at 90 min and was significantly higher than in normal volunteers and Type 2 diabetic patients ($p<0.05$). The S-IRI maximum value was observed at 120 min in the three groups, and the value found in obese subjects was significantly higher ($p<0.05$) than in healthy subjects (Table 1).

The area under the curve of mean stimulated P-IRI was significantly higher ($p<0.02$) in obese subjects (11,750 ± 2,937 μU/ml) than in normal subjects (5,521 ± 1,320 μU/ml) and diabetic patients (6,882 ± 2,294 μU/ml). The amount of immunoreactive insulin which accumulated into saliva during the glucose load was 1,255 ± 313 μU in normal subjects, 1,444 ± 380 μU in Type 2 diabetic patients and 1,879 ± 447 μU in obese subjects ($p<0.05$ vs normal subjects and diabetic patients). Thus, in obese subjects P-IRI increase was approximately 100% higher than that of normal subjects and diabetic patients; in the same group, the S-IRI increase was 50% higher than in normal volunteers and 30% higher than in diabetic patients.

The time course of P-IRI and S-IRI changes during the glucose load is shown in Figure 1. A delay in reaching peak accumulation by S-IRI was observed in normal and obese subjects, but not in Type 2 diabetic patients.

By using the unilinear regression analysis, stimulated P-IRI and S-IRI concentrations were positively correlated in Type 2 diabetic patients ($r=0.50$, $p<0.01$, Fig. 2). Because of the lag in the increase of S-IRI in normal and obese subjects, a significant correlation between P-IRI and S-IRI levels during the glucose load emerged when each P-IRI value was compared with the S-IRI value obtained in the saliva sample collected 30 min later ($r=0.52$, $p<0.01$ in normal subjects;

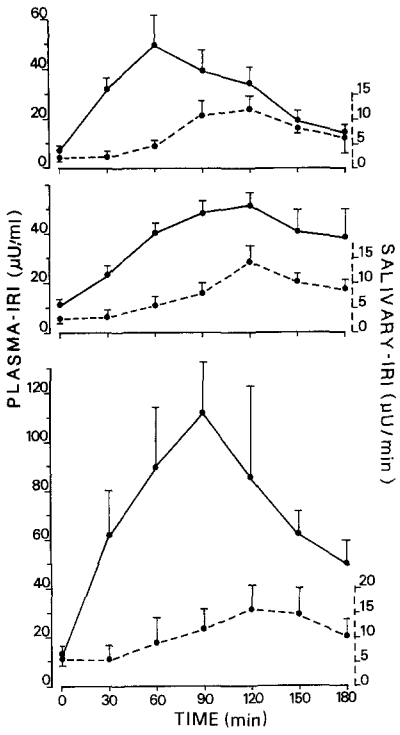

Fig. 1. Time course of plasma-IRI (●—●) and salivary-IRI (●-----●) after an oral glucose tolerance test in normal subjects (top panel), Type 2 diabetic patients (middle panel) and obese non-diabetic subjects (bottom panel)

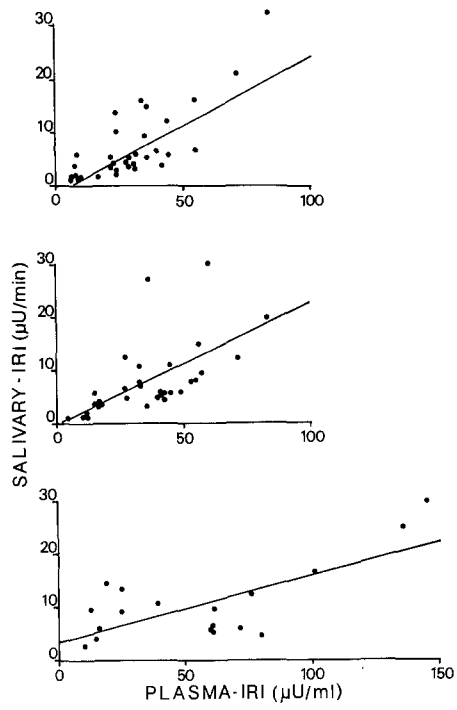

Fig. 2. Correlation coefficients between plasma IRI (P-IRI) and salivary IRI (S-IRI) in normal subjects ($r=0.52$, $p<0.01$, top panel), Type 2 diabetic patients ($r=0.50$, $p<0.01$, middle panel) and obese non-diabetic subjects ($r=0.69$, $p<0.01$, bottom panel)

$r=0.69$, $p<0.01$ in obese subjects, Fig. 2). Since it is not known whether S-IRI is normally distributed, we also compared P-IRI and S-IRI by a non-parametric comparison. Again, the correlation between P-IRI and S-IRI was statistically significant in normal subjects (Spearman's correlation coefficient, $R_s=0.84$, $p<0.001$), in Type 2 diabetic patients ($R_s=0.69$, $p<0.001$) and in obese subjects ($R_s=0.52$, $p<0.01$).

Figure 3 shows the chromatogram obtained by eluting saliva from patients by a reversed-phase HPLC system. The position of salivary immunoreactive insulin, as detected by radioimmunoassay, was the same as that of a standard saliva sample eluted at the same conditions.

Both in the fasting state and at each time of the glucose tolerance test, plasma glucose levels were significantly higher ($p<0.02$) in Type 2 diabetic patients than in normal and obese subjects. Salivary glucose values were superimposable among the three groups basally and throughout the challenge. No delay was observed in the changes of salivary glucose in comparison with plasma glucose during the OGTT. A positive correlation emerged between plasma and salivary glucose levels in normal and obese subjects ($r=0.56$, $p<0.05$ and $r=0.61$, $p<0.02$ respectively), but not in Type 2 diabetic patients ($r=0.14$).

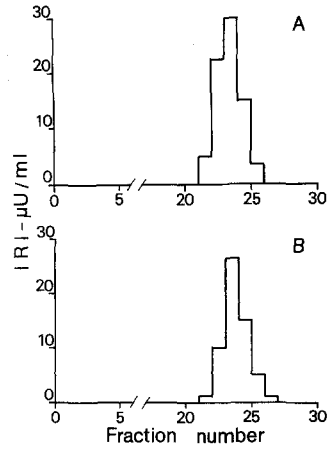

Fig. 3. Reversed-phase HPLC analysis of salivary sample (bottom panel) demonstrating that salivary IRI had the same retention time as insulin standard (top panel) eluted at similar chromatographic conditions. The position of IRI was detected by radioimmunoassay

Discussion

The present work demonstrates the presence of immunoreactive insulin in saliva from Type 2 diabetic patients and obese non-diabetic subjects, and describes the relation between P-IRI and S-IRI after an oral glucose tolerance test in the two groups.

These findings supplement previous studies regarding the presence of immunoreactive insulin in saliva and the changes of its concentrations in comparison to plasma insulin after oral glucose in normal subjects [1-4].

Our results also demonstrate that the same differences in basal P-IRI values observed among normal

subjects, Type 2 diabetic patients and obese non-diabetic subjects are found when S-IRI levels are considered. Moreover, the relationship between changes in P-IRI and S-IRI during the OGTT is shown.

Finally, by analysing samples of salivary IRI by reversed-phase HPLC, our study chromatographically identifies salivary insulin and insulin standards, confirming previous reports which show that salivary IRI is not a cross-reacting compound [2, 9].

In the present paper, the previously reported delay of S-IRI changes in comparison to P-IRI in healthy subjects [1–4] is confirmed; a similar behaviour is also demonstrated in obese non-diabetic subjects but not in Type 2 diabetic patients. As the delay in S-IRI variations is probably due to the time that it takes the insulin molecules to travel the distance from the blood vessels across salivary glands into the exocrine duct system [3], diabetic patients and subjects with normal glucose tolerance appear to handle insulin differently in the salivary glands.

In obese non-diabetic subjects, the amount of S-IRI secreted into saliva after a glucose load was higher than in normal subjects and Type 2 diabetic patients, but the difference was not as striking as for P-IRI. This could suggest that in obese non-diabetic subjects salivary glands process P-IRI in a different way than in normal subjects. Alternatively, cross-reacting substances in P-IRI could be included which do not travel into the salivary glands; proinsulin plasma levels, for example, are reported to be higher in obese non-diabetic subjects [10].

In conclusion, our results demonstrate the presence of S-IRI in Type 2 diabetic patients and in obese non-diabetic subjects, illustrate the relation between changes in S-IRI and P-IRI after a physiological stimulus in these patients, argue that in Type 2 diabetes and obesity an altered handling of blood substances by salivary glands may be present, and suggest the possibility of using S-IRI measurement in clinical practice.

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References

1. Sweeney EA, Antoniades HN (1967) The presence of immunologically reactive insulin in parotid saliva and its relation to changes in serum insulin concentration. Vox Sang 13: 54–56
2. Vallejo G, Mead PM, Gaynor DH, Devlin JT, Robbins DC (1984) Characterization of immunoreactive insulin in human saliva: evidence against production in situ. Diabetologia 27: 437–440
3. Sweeney EA, Juan CS, Avruskin TW (1979) Turner's syndrome and carbohydrate metabolism. Parotid salivary insulin concentration in normal subjects and in patients with gonadal dysgenesis. Am J Med Sci 277: 153–162
4. Doi K, Kawara A, Fujii S, Mastsuura M, Yoshida Y, Kisara Y, Yoshida M, Kaneko S, Baba S (1978) Insulin like substance in saliva. Igakunoayumi 106: 477–478
5. National Diabetes Data Group (1979) Classification and diagnosis of diabetes mellitus and other categories of glucose tolerance. Diabetes 28: 1039–1057
6. Marchetti P, Benzi L, Cecchetti P, Navalesi R (1984) A rapid separation of A14-125I-insulin from heterogeneous iodination mixture by high-performance liquid chromatography (HPLC). J Nucl Med Allied Sci 28: 1–4
7. Navalesi R, Pilo A, Ferrannini E (1978) Kinetic analysis of plasma insulin disappearance in non ketotic diabetic patients and in normal subjects. J Clin Invest 61: 197–206
8. Benzi L, Pezzino V, Marchetti P, Gullo D, Cecchetti P, Masoni A, Vigneri R, Navalesi R (1986) A14-125I-monoiodoinsulin purified by different high-performance liquid chromatography procedures and by polyacrylamide gel electrophoresis: preparation, immunochemical properties and receptor binding affinity. J Chromatogr Biomed Appl 377: 339–344
9. Rosenzwerg JL, Havronkova J, Lesniak MA, Brownstein M, Roth J (1980) Insulin is ubiquitous in extrapancreatic tissues of rats and humans. Proc Natl Acad Sci USA 77: 572–576
10. Melani F, Rubenstein AM, Steiner DF (1970) Human serum proinsulin. J Clin Invest 49: 497–507

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