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Presence of Insulin-Like Immunoreactivity and its Biosynthesis in Rat and Human Parotid Gland

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Summary. Extracts from homogenates of rat and human parotid glands contained insulin-like immunoreactivity. The values were 5.6 ± 2.8 ng/g wet tissue in six groups of rat parotid glands and 23.8 and 39.7 ng/g wet tissue in two human extracts. Upon gel filtration immunoreactive insulin of rat origin was eluted in a peak corresponding to the elution volume of isotopically labelled insulin. The material obtained from the two peak fractions showed an immunoassay dilution curve identical with that of rat insulin. Furthermore, biosynthesis of insulin-like immunoreactivity in rat and human parotid glands was confirmed in vitro by a specific separation method using anti-insulin antibody. These findings suggest that the parotid gland may be a further extrapancreatic source of insulin, and that insulin biosynthesis does occur in extrapancreatic tissues.

Key words: Parotid gland, extrapancreatic insulin, insulin extraction, gel filtration, insulin biosynthesis.

A relationship between the pancreas and the parotid gland has been suggested by diseases such as mumps, diabetes [1] and pancreatitis [2]. These organs have similarities in histological features, both serving exocrine gland roles, though it has not yet been confirmed that the salivary gland has an endocrine function. Lawrence et al. [3] have found glucagon-like immunoreactivity in salivary glands. Recently, the presence of insulin has also been reported in the gastrointestinal tract [4], brain [5] and other organs [6] in rats and man with concentrations higher than that found in plasma. There have been no reports, however, of the biosynthesis of insulin in these organs and no clear-cut demonstration of insulin-like immunoreactivity in the salivary gland. We have therefore looked for such immunoreactivity and for evidence of insulin biosynthesis in rat and human parotid glands.

Materials and Methods

Parotid Glands

Anaesthetized (50 mg/kg sodium pentobarbital) male Wistar rats weighing 250–280 g were perfused with a solution of 0.154 mol/l saline through an aortic catheter after an overnight fast, and the parotid glands were removed. The mean plasma insulin concentration of these rats was 0.9 ± 0.2 ng/ml (n = 10). Normal human parotid glands were obtained at surgery from two male patients aged 48 and 64 years. Both had cancer of the tongue and had not received radiotherapy. They had no history of mumps, pancreatitis or other major illness and had normal glucose tolerance. Informed consent

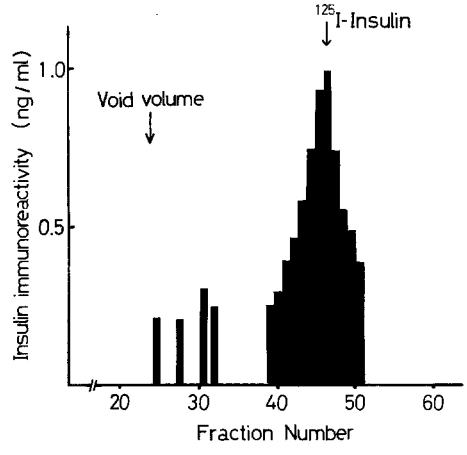

Fig. 1. Elution pattern of acid ethanol extracts of parotid glands of ten rats filtered on a Sephadex G-50 superfine column (0.9 × 60 cm). The column was equilibrated with 1 mol/l acetic acid; 0.4 ml of sample was applied. Fractions of 0.6 ml were collected. Marker points for void volume and ^{125}I-insulin were not applied to the extract depicted. This procedure was repeated four times – similar results were obtained

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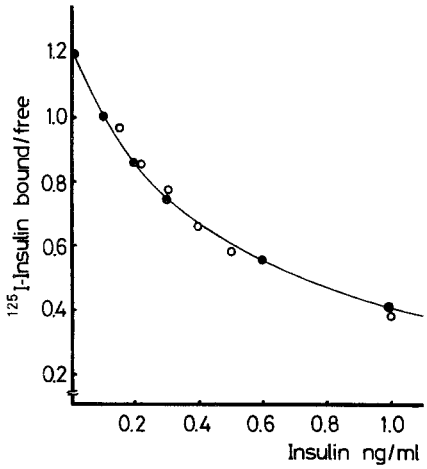

Fig. 2. Dilution profile of rat parotid gland extract. Serial dilution of the peaked 47th and 48th fractions from column-chromatographed rat parotid glands extract. (○——○, was compared with standard curve of rat insulin (●——●) in the radioimmunoassay

to the study was obtained before operation. The tissues were washed in 0.154 mol/l saline and were used for insulin extraction and biosynthesis within one hour of surgery.

Insulin Extraction

After weighing, tissues were homogenised in a Potter-Elvehjem homogeniser with 20 ml/g wet tissue of ice-cold 0.2 mol/l HCl / ethanol (25 : 75 v. v.). The suspension was left at 4 °C for 24 h and insulin was extracted according to Melani's modification [7] of the method of Davoren. The extracts were evaporated before use. Recovery varied between 33 and 44%, and the insulin concentrations reported here have been corrected for the recovery. Insulin immunoreactivity of these extracts was determined by radioimmunoassay. Pancreatic insulin concentrations in the rats were measured by the same method. Extracts from the parotid glands of ten rats were chromatographed at 4 °C using a Sephadex column (G-50 superfine, 0.9 × 60 cm) eluted with 1 mol/l acetic acid. The column was calibrated with ^{125}I-insulin (Dainabot Nuclear Company, Tokyo, Japan). Each fraction of 0.6 ml was lyophilised and dissolved with borate buffer 0.05 mol/l (pH 8.6) containing bovine serum albumin 2.5 g/l (Armour Pharmaceuticals, USA) for insulin assay.

These procedures were repeated four times and parotid glands from 40 rats were used. Serial dilution of the mixture of the peaked eluant from the 47 and 48th fractions was performed and compared with the dilution curve of rat insulin (a mixture of rat insulin I and II, Lot 615-1112B-295C, Lilly Research Laboratories, USA).

Insulin Biosynthesis

In order to investigate the biosynthesis of insulin in rat and human parotid glands, 50 mg of tissues obtained from rat and human glands respectively were pre-incubated in 0.5 ml of Krebs-Henseleit bicarbonate buffer, pH 7.4, containing bovine serum albumin 5 g/l, glucose 3.3 mmol/l under a gas phase of 95% O_2–5% CO_2 at 37 °C for 20 min. The tissues were then transferred to an incubation medium containing glucose 16.7 mmol/l and L-(4,5-^{3}H) leucine 10 μCi (specific activity 393 mCi/mg), The Radiochemical Centre, Amersham, Bucks, UK) for 3 h, otherwise under the same conditions as for the pre-incubation. Immunoreactive insulin was extracted from the tissues using the method described above.

The biosynthesis was performed by three different procedures from one extract divided into three aliquots. The first procedure was gel filtration of the extracts at 4 °C on a Bio-gel P30 (0.9 × 60 cm) column with acetic acid 3 mol/l.

The second method was an immunological technique: 3 ml of borate buffer 0.05 mol/l (pH 8.6) containing bovine serum albumin 2.5 g/l, and anti-insulin antiserum 0.1 ml diluted 600-fold, which bound 32 ng of insulin (donated by Dr. Kohga, Shimizu Pharmaceuticals, Shimizu, Japan) were added to the acid ethanol extracts. The crossreactivity of this anti-insulin antiserum with glucagon, somatostatin, bovine pancreatic polypeptide, vasoactive intestinal polypeptide, gastric inhibitory polypeptide, secretin, gastrin, thyrotropin releasing hormone and neurotensin was less than 0.001%. After 48 h incubation at 4 °C carrier normal guinea pig serum and anti-guinea pig globulin antiserum (rabbit) 0.5 ml were added, and a further 24 h incubation at 4 °C was performed. Following centrifugation (2000 g at 4 °C for 30 min) the supernatant was decanted and the precipitate was rinsed twice with borate buffer 0.05 mol/l (pH 8.6). For the separation of newly synthesised insulin from insulin-antibody complex, HCl (0.5 ml of 0.2 mol/l) was added to the precipitate and incubated for 24 h at 4 °C. This was then gel-filtrated at 4 °C on a column of Bio-gel P 30 (0.9 × 60 cm) with acetic acid 3 mol/l.

Thirdly, in order to confirm that the biosynthesised peak is indeed insulin, excess bovine insulin (Sigma, St. Louis, USA) was added to the acid ethanol extracts: the same immunological separation procedure was then carried out.

In the biosynthesis study, these procedures were repeated three times; parotid glands from three rats and two patients were used.

Radioactivity in each fraction was measured with a liquid scintillation counter (Model 3330, Packard Tricarb Liquid Scintillation Spectrometer, USA) at pH 5.5. Insulin immunoreactivity in extracts of rat and human parotid glands was determined according to the immunoassay of Morgan and Lazarow [8] using rat insulin (Lot 615-1112B-295C, Lilly Research Laboratories, USA) and porcine insulin (Shimizu Pharmaceuticals, Shimizu, Japan) as standards, respectively. The sensitivity of this assay was 0.1 ng/ml.

Results

Insulin-like immunoreactivity was found in extracts from both rat and human parotid glands. The insulin concentration of extracts from six groups of rat parotid glands was 5.6 ± 2.8 ng/g wet tissue (range: 0.6–19.0 ng/g wet tissue), and the values from two human extracts were 23.8 and 39.7 ng/g wet tissue. The mean pancreatic insulin concentration for the rats was 38.6 ± 7.3 μg/g wet tissue ($n = 7$).

Figure 1 demonstrates that insulin-like immunoreactivity occurred in a single peak corresponding to the isotopically labelled insulin fraction. The same pattern was obtained on chromatography in the three other studies. Furthermore, serial dilution of the peak 47th and 48th fractions was parallel to that of rat insulin (Fig. 2). In the biosynthesis study, the acid ethanol extracts showed several peaks including one corresponding to ^{125}I-insulin (Fig. 3, upper panel). By the specific immunological separation technique, the biosynthesis of insulin-like immunoreactivity was demonstrated as a peak corresponding to the isotopically labelled portion, and its radioactivity showed a si-

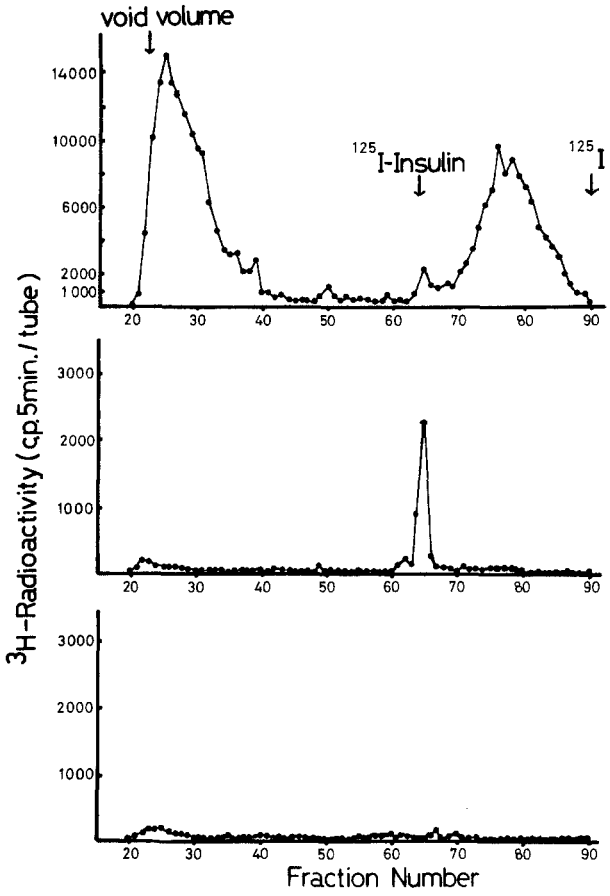

Fig. 3. Biosynthesis of insulin-like immunoreactivity in rat parotid glands. After 3 h incubation with ^{3}H-leucine, insulin in the tissues was extracted with acid ethanol. The extract was gel-filtrated (upper panel), or precipitated with anti-insulin antiserum followed by separation from the antibody with 0.2 mol/l HCl (middle panel) and subsequently displaced with excess insulin (100 µg) added in the specific immunological separation procedure (lower panel). For gel filtration, the Bio-gel P 30 column (0.9 × 60 cm) was equilibrated with 3 mol/l acetic acid and 0.4 ml of sample was applied. Fractions of 0.4 ml were collected

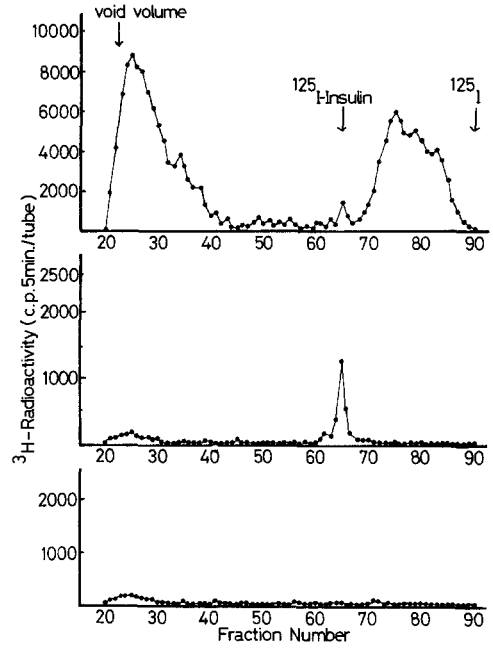

Fig. 4. Biosynthesis of insulin-like immunoreactivity in human parotid glands. The same biosynthetic procedures used for rat parotid glands were employed

milar peak area to that detected by the gel filtration immediately after the acid ethanol extraction (Fig. 3, middle panel). After the addition of excess insulin in the immunological separation procedure, the peak of insulin was completely abolished (Fig. 3, lower panel). These observations of biosynthesis were confirmed in the other experiments. The same results were obtained with human parotid glands (Fig. 4).

Discussion

The present investigation showed the presence of insulin, or a closely related peptide, in rat and human parotid glands. It must be emphasised, however, that the concentration of this substance is not as high as in the pancreas, but was six times higher than that present in the plasma. With column chromatography, peaks corresponding to proinsulin could not be detected as reported for other extrapancreatic tissues [5, 6]. This may be due to a low concentration of this substance in parotid glands or to the relatively low affinity for proinsulin of the anti-insulin antiserum used in this assay. More sensitive antisera might therefore disclose proinsulin. In addition to the detection of insulin in parotid glands, biosynthesis of insulin was confirmed in rat and human parotid glands. Despite the discovery of insulin in extrapancreatic tissues, the source of such insulins has not been identified. There have previously been no reports of the biosynthesis of insulin-like immunoreactivity in extrapancreatic tissues, probably because of its low concentration in these tissues. In this study, therefore, it was necessary to use an affinity technique in order to demonstrate biosynthesis. To ensure that genuine biosynthesis was being observed, three different procedures were employed on a single extract after incubation with ^{3}H-leucine. From these studies, it was confirmed that the peak observed corresponding to ^{125}I-insulin fractions was indeed synthesised insulin.

B cells have not been demonstrated in the parotid gland histologically, but the present observations suggest the presence of cells which contain insulin and have the ability to produce insulin in the parotid

glands. Recently insulin-like immunoreactivity has been found in human saliva and, furthermore, its concentration was increased by oral glucose loading [9].

Further investigations are necessary to demonstrate the physiological significance, if any, of this insulin-like immunoreactivity in the parotid gland.

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