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Short communication

Age-dependent changes in insulin-like immunoreactivity in rat submandibular salivary glands

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In recent years, a growing interest had arisen in hormonal factors in salivary glands. We have investigated the changes in the content of an insulin-like immunoreactive (ILI) compound in the submandibular salivary glands of Sprague Dawley rats during physiological aging, in the range 15 days -27 months. The amount of ILI in the submandibular glands of young adult rats was found to be doubled in the post-natal period until the age of puberty and was maintained in senescence. No significant correlation was found between age-dependent variations in ILI levels of submandibular salivary glands and circulating insulin concentrations, further supporting previous indications that ILI is being synthesized in situ. It is possible that ILI could exert paracrine effects within the glands, as regards the development of other glandular structures during the first months of life, as well as the preservation of glandular function in senescent animals as well.

Key words: insulin-like immunoreactivity; submandibular salivary glands; aging; rats

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The major salivary glands of various animal species, including man, appear to produce an array of biologically active peptides and growth factors, which are usually secreted into saliva (1) and may also be released into the blood (2, 3). For some of them, such as epidermal growth factor (EGF) and nerve growth factor (NGF), submandibular salivary glands in rodents represent the major site of synthesis in the body (4). Among these factors, insulin or more generally an insulin-like immunoreactive compound (ILI) should be included, as identified by immunohistochemistry and localized in the cells of the intercalated ducts (5–7). ILI is likely to be synthesized in situ and has recently been confirmed to possess at least some biological properties of insulin (8).

We have previously shown that streptozotocin-induced diabetes is associated with an increase in ILI concentration in the rat submandibular salivary glands (SSG) (9), and that this increase is prevented by long-term correction of diabetic metabolic alterations (10). These results have prompted us to investigate whether the ILI production could be influenced by the metabolic changes occurring during physiological aging. We have therefore studied the ILI content in submandibular salivary glands of rats of different ages (15 days–27 months) and its correlation with blood glucose and insulin levels.

Materials and methods

Animals

Male Sprague Dawley rats of various ages (15 days, 2–3, 8, 18 and 24–27 months-old) were used. They were housed in a climatized vivarium under artificial lighting (12 h light-dark cycle) and received standard laboratory pellet food, except suckling 15-day-old animals. Just before the experimental

procedure, blood samples were collected from the tail vein of conscious animals into EDTA pre-treated tubes and centrifuged to separate plasma for successive determinations of non-fasting glucose and insulin levels.

Submandibular salivary peptide extraction

After anaesthesia with sodium pentobarbital (50 mg/kg b.w.), submandibular salivary glands were separated from the sublingual glands and immediately frozen. Each gland of animals aged between 2 and 27 months was homogenized separately in 2.5 ml of cold acidified ethanol (0.7 M HCl-ethanol, 1:3 v/v), and the insulin-like peptide was extracted for 48 h at 4°C.

For younger rats, 4 glands removed from 2 15-day-old animals or both glands of each 1-month-old animal were pooled before homogenization. After centrifugation (5000 *g* for 30 min at 4°C), the supernatants were stored until assay, whereas the pellets were dissolved in 1 M NaOH for total protein determination.

Assays

Plasma glucose was assayed by the glucose oxidase method using a commercially available kit (Sclavo Diagnostics, Siena, Italy). In 15-day-old rats, glycaemia was determined on whole blood by a Boehringer glucometer. Total protein in the acid ethanol was measured by the method of BRADFORD (11), using bovine serum albumin as standard. Immunoreactive plasma insulin and insulin-like material in salivary gland extracts were measured by a radioimmunological method using ^{125}I-insulin kindly provided by Professor R. NAVALESI, Cattedra di Malattie del Ricambio, University of Pisa. Anti-insulin antibody and standard rat insulin were supplied by Linco Research (St. Louis, MO, USA). The detection limit of our assay was 0.1 ng/ml. The results were expressed as ng/g of glandular tissue wet weight.

Statistical analysis

Statistical significance of age-dependent changes was evaluated by analysis of variance. When significant differences were found ($p<0.05$), then the unpaired Student *t*-test was performed to make two-by-two comparisons.

Results and discussion

Fig. 1 shows the effect of ageing on insulin-like immunoreactivity in submandibular salivary glands

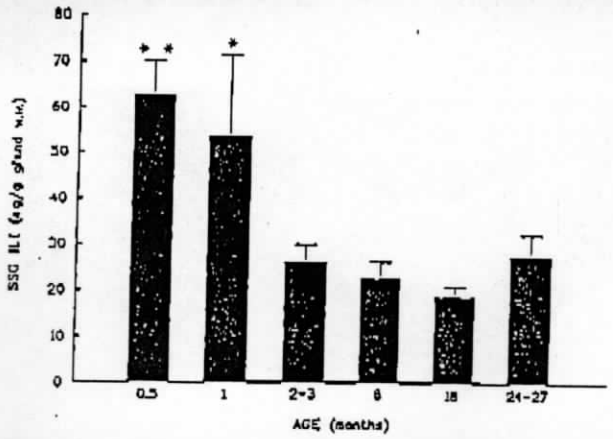

Fig. 1. Insulin-like immunoreactivity (ILI) in submandibular salivary glands (SSG) of Sprague-Dawley rats of various ages. Results are expressed as mean±SEM of 8–34 observations. Statistical significance of age-dependent changes was firstly evaluated by analysis of variance and secondly by the Student *t*-test. $*p<0.05$; $**p<0.01$ versus 2–3-month-old rats (unpaired Student *t*-test).

of Sprague-Dawley rats. Analysis of variance showed that age-dependent changes were highly significant ($F=8.98$, $p<0.01$). Compared to young adult rats (2–3-month-old), usually used as reference in the literature, gland ILI content was more than doubled in both suckling (15-day-old) and pubescent (1-month-old) animals. In mature (8-month-old) and elderly (18-month-old) animals, there was a trend to reduction of ILI levels compared to young adults. Gland ILI content of senescent rats (24–27-month old) increased somewhat to values closer to those of young adults.

The age-dependent variations in ILI content were also apparent if expressed as a function of gland protein. Indeed, protein levels in the SSG homogenates varied slightly during aging; means±SEM were 39.7±1.6; 49.2±1.8; 46.1±1.6; 37.1±0.2; 46.7±2.1; 37.6±1.3 mg/g w.w. tissue in 15-day, 1-month, 2–3-month, 8-month, 18-month and 24–27-month-old animals, respectively.

In Table 1, some metabolic characteristics of the animals are shown. Body weight increased progressively with age until 18 months. Analysis of variance indicated that plasma glucose varied with age, although to a limited extent (in the range 101–144 mg/dl). Plasma insulin levels also showed moderate age-dependent variations, with the lowest value at puberty, and the highest in 24–27-month-old animals, presumably due to the development of peripheral insulin resistance. However, neither circulating glucose, nor insulin concentrations were found to be significantly correlated with submandibular gland ILI content (correlation coefficients 0.17 and 0.22, respectively).

Table 1

*Metabolic characteristics of Sprague-Dawley rats of various ages; results are expressed as mean ± SEM of the number of observations indicated in parentheses; *p<0.05; **p<0.01 versus 2–3-month-old rats (unpaired Student t-test)*

Age (months)	Body weight (g)	Plasma glucose (mg/dl)	Plasma Insulin (ng/ml)
0.5	26±0.4** (12)	101±4.7 (8)	2.56±0.47 (15)
1	64±1.6** (9)	122±7.3 (9)	0.89±0.20** (9)
2–3	271±11.7 (23)	116±6.5 (10)	3.76±0.90 (8)
8	606±12.3** (10)	144±4.8** (8)	5.14±0.80 (10)
18	706±13.4** (14)	128±5.9 (14)	4.20±0.53 (14)
24–27	595±43.4** (8)	103±6.2 (6)	6.61±0.92* (7)

These results are substantially in agreement with the recent data of CARTER et al. (7) who, however, did not study animals older than 12 months. ILI content in salivary glands is the highest during the early post-natal period, which is characterized by the histological and morphological organization of the intercalated ducts of submandibular salivary glands (12).

Cells located in the first part of intercalated ducts as they exit from the acini are considered the most likely site of production and/or accumulation of ILI, on the basis of immunohistochemical studies (13). The striated and convoluted tubular compartments in rat salivary glands are poorly differentiated until sexual maturity. Typical striated cells and granular convoluted tubular (GCT) cells are not observed until puberty (12) and, consequently, growth factors produced by these cells, such as NGF, EGF (12) and insulin-like growth factor I (14) are found only in trace amounts during this period. Thus, we would suggest that the early local synthesis of insulin-like material, in the absence of other growth factors, could play an important rôle in local glandular development and possibly permit the organization of granular convoluted tubules, whose cells differentiate definitively during the 6th–7th week in rats (12). This hypothesis is also supported by a recent report of CHEN et al. (16), who, on the basis of morphological observations, concluded that intercalated duct cells were involved with the generation of the other cell types in mouse submandibular glands.

The stabilisation of SSG ILI content after 2–3 months of age is in line with the fact that by 3 months of age, the adult pattern of distribution of the various cellular elements is established in the gland (12).

On the other hand, senescence in rodents is accompanied by a functional decline of GCT cells, as judged by the reduction of immunocytochemical staining for EGF and NGF (15, 17), as well as by a reduction in the volume of acini with a concomitant increase in the ductal volume in comparison with younger ages (18). Our data show a 20% reduction in protein concentration but no decrease in ILI content of SSG in 24–27-month-old rats compared to young adults. This could actually help to maintain, at a suitable level, factors which may exert compensatory paracrine effects within the glands, thus limiting the hypotrophic alterations and the loss of functional efficiency in senescent animals. For instance, it has recently been observed that insulin can enhance salivary gland amylase gene expression in 24-month-old rats (19).

SSG ILI might also contribute to the maintenance of glucose homeostasis during senescence, since this period of life is usually associated with insulin resistance and glucose intolerance (20). From our data, this possibility appears most unlikely, since SSG ILI levels do not increase significantly in senescent rats, and the hyperinsulinaemia in these animals indicates an adequately efficient compensatory effect of the endocrine pancreas.

Since specific insulin-binding kinetics have been reported for rat submandibular salivary gland (21), it cannot be excluded that the SSG ILI content, at least in part, could be accounted for by trapped insulin through a ligand-receptor endocytotic process. However, the lack of correlation between age-dependent changes in plasma insulin levels and SSG ILI content adds further evidence to the assumption that most insulin-like material is synthesized in situ. Other evidence includes the previously reported increase in ILI content of submandibular glands in streptozotocin-diabetic rats with negligible circulating insulin levels (9) and the direct demonstration of the presence of insulin mRNA in the mouse salivary glands (22).

In conclusion, it may be suggested that the salivary glands may act as an extrapancreatic source of insulin, probably not rich enough to play an important rôle in the maintenance of glucose homeostasis in the course of physiological aging, but at least capable of exerting paracrine effects within the glands, throughout the life-span of the animals.

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