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SALIVARY GLAND GLUCAGON IS A FICTITIOUS SUBSTANCE DUE TO TRACER-DEGRADING ACTIVITY RESISTANT TO PROTEASE INHIBITORS

Y. Tahara, K. Shima, M. Hirota, H. Ikegami, A. Tanaka, and Y. Kumahara

Department of Medicine and Geriatrics, Osaka University Medical School
Fukushima-ku, Osaka, 553, Japan

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SUMMARY A high level of glucagon immunoreactivity was apparently detected in acid-saline extract from rat submandibular glands, but tracer glucagon added to the assay mixture was mostly damaged in spite of the presence of protease inhibitors commonly used in radioimmunoassay. Gel-filtration of the extract on a Bio-Gel P-10 column revealed strong tracer-degrading activity at the void fraction where the apparent immunoreactivity was eluted. Serial changes in apparent immunoreactivity of the extract fit well on the theoretical curve of an exponential tracer degradation. These findings indicate that the salivary gland glucagon is a fictitious substance due to tracer degradation during radioimmunoassay. Further study revealed that the glucagon molecule was hydrolyzed at the arginyl bonds and split into two fragments during incubation with the acid-saline extract from rat submandibular glands.

Extrapancreatic sources of glucagon other than the gastrointestinal tract have been recognized since Penhos et al.(1) demonstrated that the total evisceration of rats resulted in an increase in circulating glucagon levels. Salivary glands were reported as the extrapancreatic sources of glucagon by several authors (2-6). They reported that the salivary gland glucagon was a peptide with biological and immunological properties close to those of the pancreatic glucagon but with much greater molecular weight. However, the data for salivary gland glucagon contradict the data for pancreatic glucagon at many points. The microgram order of glucagon was extracted from the rat submandibular gland by an acid-saline (AS) procedure (2-4), while only nanogram order was extracted by an acid-ethanol (AE) procedure (5,6). Its molecular weight seems to be too large to show biological activities, since gastrointestinal glucagon-like materials with molecular weight greater than the pancreatic glucagon were almost biologically inactive (7). In immunohistochemical studies, no specific staining with glucagon-specific antisera has been observed in salivary glands (3,8).

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The present study was undertaken to make these conflicting points clear. During this investigation we have discovered that there exist fundamental problems in the traditional assay system of salivary gland glucagon.

MATERIALS AND METHODS

ACID-SALINE AND ACID-ETHANOL EXTRACTS Submandibular glands from male Wistar rats were extracted by AS procedure (4) or by AE procedure (9). The extracted materials were redissolved in assay buffer and used for investigation. Part of the redissolved samples was boiled for 10 min and centrifuged at 3000 rpm for 30 min. The supernatants were used as boiled samples.

RADIOIMMUNOASSAY (RIA) Glucagon immunoreactivity was measured by RIA using C-terminal antiserum OAL-123 (10). Assay buffer was 0.2 M glycine buffer, pH 8.8, containing 5 mM EDTA, 10 mM benzamidine and 1000 KIU/ml Trasylol. B/F separation was carried out by the double antibody method. Total incubation time was 72 hr at 4°C.

TRACER DEGRADATION STUDY Tracer degradation was examined by incubating an assay mixture identical with RIA but in the absence of antisera for 48 hr at 4°C, followed by addition of an equal volume of 10% TCA. The radioactivity of the precipitate was considered that of the intact tracer glucagon.

GEL-FILTRATION The AS extract was submitted to gel-filtration on a Bio-Gel P-10 column (1.7 x 90 cm) equilibrated in 50 mM NH_4HCO_3 buffer, pH 8.6. Each 3 ml eluate was collected and used in RIA and tracer degradation studies. Tracer glucagon 0.2 μCi and unlabeled porcine glucagon 25 ng, incubated with the AS extract for 48 hr at 4°C, were applied to a Bio-Gel P-6 column (0.8 x 60 cm) equilibrated in 50 mM NH_4HCO_3 buffer. Glucagon immunoreactivity and glucagon-like immunoreactivity of each fraction (1 ml) were measured by RIA using C-terminal antiserum OAL-123 and N-terminal antiserum OAL-196, respectively (10,11).

RESULTS

TRACER DEGRADATION BY SUBMANDIBULAR ACID-SALINE EXTRACT Apparent content of glucagon immunoreactivity in rat submandibular AS extract was 3.6 ± 1.5 μg/g-tissue (mean ± SEM, n=5) in good agreement with that reported previously (2-4). However, the tracer glucagon added to the assay mixture of the AS extract was almost completely damaged in spite of the presence of 5 mM EDTA, 10 mM benzamidine and 1000 KIU/ml Trasylol (Table 1). When the AS extract was boiled, the tracer glucagon was not significantly damaged during incubation, but glucagon immunoreactivity could not be detected. Since glucagon and

Table 1. Apparent glucagon immunoreactivity and tracer degradation

Extraction procedure	(dilution)	Apparent glucagon immunoreactivity (ng/ml)		Intact tracer glucagon* (%)	
		extract	boiled	extract	boiled
Acid-saline	(x 1000)	4.1	n.d.**	7.6	74.6
Acid-ethanol	(x 40)	n.d.	n.d.	81.5	76.9

* 78.1 ± 1.1 (mean ± SD, n=10) when incubated with buffer only.
** not detected.

glucagon-like materials are heat-stable (9), the immunoreactivity detected in the AS extract is likely to be a false one due to tracer degradation. No glucagon immunoreactivity was detected in the AE extract.

Figure 1 shows a fractionation profile of the AS extract on a Bio-Gel P-10 column. Glucagon immunoreactivity was apparently eluted at the void fraction. Tracer glucagon was mostly damaged when incubated with the eluate at the void fraction. These findings indicate that the salivary gland glucagon is a fictitious substance due to tracer degradation.

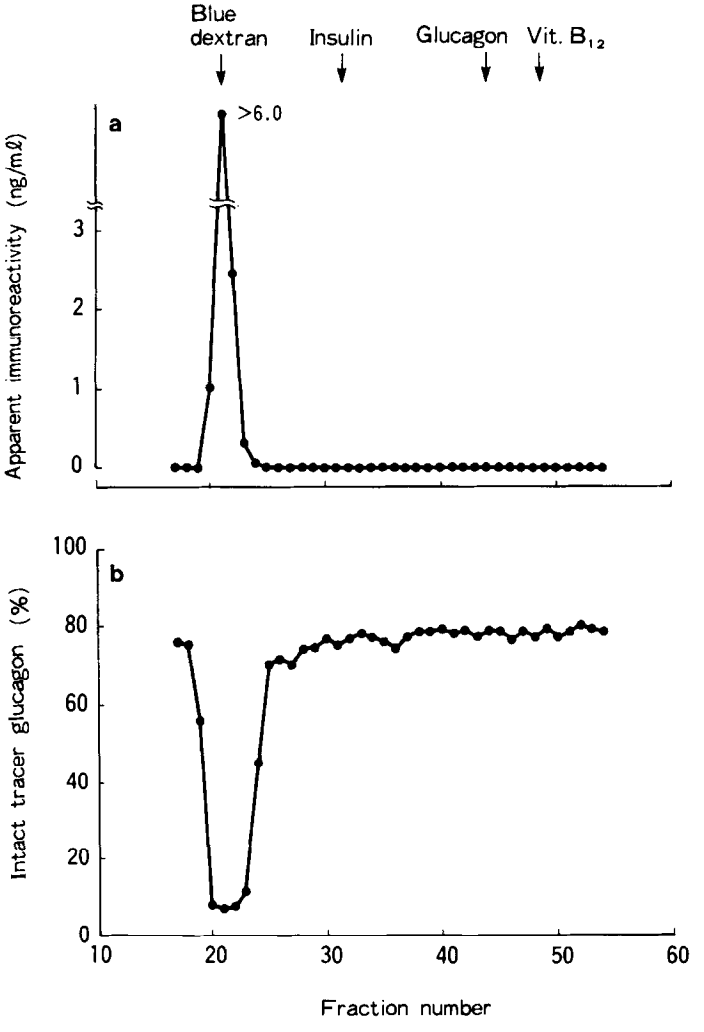

Fig. 1. Gel-filtration profile of AS extract on a Bio-Gel P-10 column; (a) apparent glucagon immunoreactivity in the eluates, (b) remaining intact tracer glucagon after incubation with the eluates.

SERIAL DILUTION CURVE Serially diluted AS extract was used in RIA and tracer degradation studies. The apparent displacement curve of the AS extract was not parallel to the standard curve of the porcine glucagon, but was parallel to the remaining intact tracer glucagon over the range of dilutions (upper panel of Fig. 2).

When the concentration of tracer glucagon is c and that of unlabeled glucagon is x, the bound tracer concentration, B, can be given by

$$B = B_0 \cdot c / (x + c). \qquad (1)$$

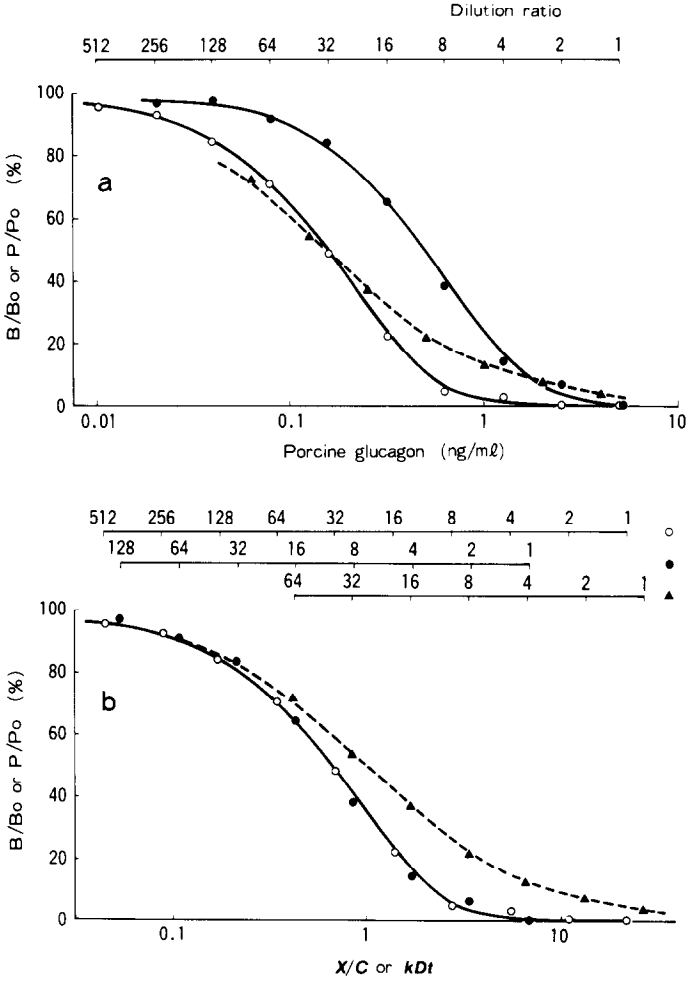

Fig. 2. Standard curve of porcine glucagon (▲), apparent displacement curve of AS extract (●), and remaining intact tracer glucagon after incubation with serially diluted AS extract (○). The broken and solid lines in the lower panel represent the theoretical curves of B and P calculated by the equations (1) and (2), respectively.

On the other hand, during an exponential tracer degradation, the remaining intact tracer glucagon, P, precipitable by TCA can be given by

$$P = P_0 \cdot \exp (-kDt), \qquad (2)$$

where D is the tracer-degrading activity, t the incubation time, and k the proportional constant. Theoretical curves of B and P for the change of x and D are drawn in the lower panel of Fig. 2. Serial changes in apparent immunoreactivity of the AS extract as well as the remaining intact tracer glucagon fit well on the theoretical curve of an exponential tracer degradation. These facts further support that the salivary gland glucagon is a fictitious substance due to tracer degradation.

GEL-FILTRATION OF THE DAMAGED GLUCAGON In order to investigate the degradation mechanism, 0.2 μCi tracer glucagon was incubated with the AS extract and applied to a Bio-Gel P-6 column. As shown in Fig. 3a, the damaged tracer glucagon gave a single peak several fractions later than the intact. Its molecular weight was roughly estimated to be 2000. No other radioactive component was observed.

Unlabeled glucagon 25 ng, treated with the AS extract, was applied to the same column (Fig. 3b). The component reacting with OAL-196 was eluted out at the same fraction as the damaged tracer glucagon. The total amount of glucagon - like immunoreactivity eluted out was 11.3 ng, which was 45% of the initial glucagon treated. The component reacting with OAL-123 was further delayed. Its molecular weight was estimated to be about 1300 since it was eluted at the same fraction as Vitamine B_{12}. The total amount of glucagon immunoreactivity eluted out was 1.9 ng, which was 8% of the initial glucagon.

DISCUSSION

Many authors have reported the presence of immunoreactive glucagon in the salivary glands (2-6). The properties of the salivary gland glucagon reported previously were similar to those of the pancreatic glucagon, but were contradictory at many points. We have found that the tracer glucagon was mostly damaged during incubation with AS extract from rat submandibular glands even in the presence of the protease inhibitors commonly used in RIA. The salivary

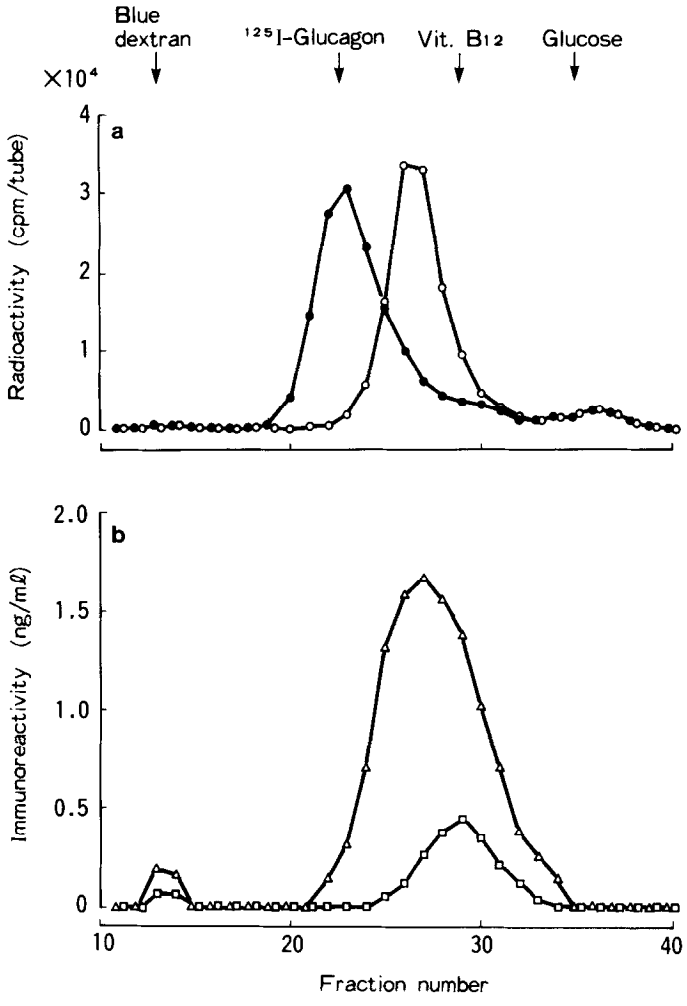

Fig. 3. (a) Gel-filtration profiles (Bio-Gel P-6 column) of tracer glucagon before (●) and after (○) incubation with AS extract. (b) Gel-filtration profiles of immunoreactivity of porcine glucagon incubated with AS extract; (△) measured with N-terminal antiserum OAL-196, (□) measured with C-terminal antiserum OAL-123.

gland glucagon proved to be a fictitious substance due to tracer degradation. This result is also supported by the fact that no specific staining with glucagon-specific antisera has been observed in salivary glands (3,8). Competitive binding to the glucagon receptors in rat liver membrane (4) is considered a result of tracer degradation. The circulating immunoreactive glucagon in the totally eviscerated rat (1) and the big plasma glucagon in human plasma (12) could be the same phenomena caused by tracer degradation. The minimal extraction efficiency of the AE procedure may be attributed to

denaturation of tracer-degrading activity during the extraction procedure. It is yet to be investigated whether the hyperglycemic effect of intravenous injection of AS extract in rats (2-4) and the hypoglycemic effect of salivary duct ligation in diabetic mice (13) are related to the tracer-degrading activity described here. We have not yet identified the tracer-degrading activity included in the AS extract. One possible candidate is the protease A isolated from mouse submandibular glands (14).

Gel-filtration of the damaged glucagon revealed that the glucagon molecule was split into two or more fragments by the submandibular AS extract. One of the fragments is considered N-terminal fragment with MW 2000 since it reacts with OAL-196. Another one is considered C-terminal fragment with MW 1300 since it reacts with OAL-123. Possible sites where the glucagon molecule is hydrolyzed into these fragments are the 17th and 18th arginyl bonds, which are susceptible to trypsine-like proteases. The fragment(1-17) thus produced has a MW of about 2000, crossreacts with OAL-196 and includes both tyrosine residues labeled with radioactive iodine. Since it does not crossreact with C-terminal antisera (10), the hydrolysis of the arginyl bonds results in a decrease in the percent bound in RIA. The fragment(19-29) reacts only with OAL-123, but has 10 times smaller affinity to the antiserum than the pancreatic glucagon (10). If 25 ng glucagon was completely hydrolyzed at the arginyl bonds, 2 or 3 ng glucagon immunoreactivity will be detected in the gel-filtrated samples. The observed value 1.9 ng is in good agreement with this expected value. Therefore we conclude that the glucagon molecule was hydrolyzed at the arginyl bonds and split into these two fragments during incubation with the submandibular AS extract.

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