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Glucose, Insulin and Renin Activity after Sodium Loading and Depletion in *Vipera aspis*

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ABSTRACT. Sodium, potassium, chloride, glucose, insulin and renin activity were investigated in fasted *Vipera aspis* subjected for 3 days to administration of 3% NaCl 5 ml, or injection of a diuretic and water loading to produce sodium depletion. After sodium loading, plasma sodium and glucose were significantly elevated if compared with those of controls, while plasma renin-like activity and plasma insulin were depressed. The insulin and somatostatin producing cells (B- and D-cells) showed only a weak immunoreactivity, while in the glucagon producing cells (A-cells) the immunoreactivity was stronger if compared with the handled controls. After sodium depletion, plasma sodium and glucose were significantly depressed and plasma renin-like activity and plasma insulin were significantly elevated. A strong immunoreactivity was present in B- and D-cells and only a weak immunoreactivity was detectable in the A-cells. These data suggest that the secretory activity of the endocrine pancreas and kidney may be affected, in vipers, by sodium and/or volume status. COMP BIOCHEM PHYSIOL 113C, 375–380, 1966.

KEY WORDS. Endocrine pancreas, renin activity, insulin, glucose, immunohistochemistry, sodium loading, sodium depletion, *Vipera aspis*

INTRODUCTION

Changes in extracellular sodium concentration seem to influence, in mammals, the secretion of pancreatic somatostatin (10). In a preliminary study some of us (15) observed that changes in plasma sodium concentration affect the secretion of pancreatic islets and plasma insulin levels in the rat. Insulin, glucose and somatostatin are thought to be involved in the control of transepithelial transport of ions and water: Insulin affects sodium and water excretion by the isolated dog kidney (19) and has a sodium retaining effect in normal man (7), glucagon stimulates chloride secretion in the chloride cells of teleosts (9) while somatostatin stimulates transepithelial intestinal transport of sodium (13).

Recently, components of the renin-angiotensin system (RAS) have been demonstrated in the canine and rat pancreas and high affinity binding sites for Angiotensin II have been localized by autoradiography in the pancreatic tissue in both dog and rat (5,28).

The presence of an RAS in mammalians' pancreatic tissue supports the hypothesis of Epple and Brinn (8) that the original role of the gland was osmoregulation and only later it was involved in metabolic activities.

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An RAS has been demonstrated in several reptiles: in crocodilians and turtles (20,24), in terrestrial chelonians (30), and in the snakes *Elaphe climocophora* (16) and *Vipera aspis* (29).

In this study we investigated by immunohistochemistry the activity of the endocrine pancreas in the snake *Vipera aspis* subjected to sodium loading and administration of a diuretic to produce sodium depletion. The aim of the study was of demonstrating the possible correlation between sodium status, plasma renin activity and insulin secretion in fasting vipers.

MATERIALS AND METHODS

Animals and Treatments

Adult male *Vipera aspis* ranging in length approx. 50 cm were captured in September in North Italy. The animals were housed in cages in the laboratory for 2 weeks at 20°C and kept unfed. Water was supplied once a day. After acclimation, groups of animals were subjected to the following experiments that were performed on conscious vipers:

1. Sodium loading: three animals were administered saline solution (3% NaCl, 5 ml) by cannulation of the gut; the intubation procedure was repeated once a day for 3 days.
2. Three animals were injected daily for 3 days with furosemide (Lasix, Hoechst 20 mg/kg bwt/day i.p.) and subsequently water loaded (1 ml/100 g i.p.).
3. Three animals were used as handled controls: vipers were extracted from their cages once a day for 3 days, sham

intubated, and injected with 0.8% NaCl (1 ml/100 g bwt, i.p.).

Water and food were not supplied to the controls or the treated animals during the 3 days of experiments.

A fourth group of animals was fed with a little mouse once a day.

Blood and Tissue Collection

All animals were killed by a blow to the head and blood was collected from the neck directly into lithium-heparinized tubes. After centrifugation at 4°C (700 **g**), separated plasma was analyzed for electrolytes (Na, K, Cl), plasma renin activity (PRA), plasma glucose and plasma insulin. Pancreas with the spleen was rapidly removed and fixed in Bouin's fluid. Bouin-fixed sections (5 μm) were placed on gelatin-coated glass slides and subjected to immunohistochemistry.

Plasma Glucose and Plasma Electrolytes

Plasma osmolalities were determined by freezing point depression (Automatic Roebling osmometer) and sodium, potassium, chloride and glucose were measured by ion-selective electrodes (ASTRA Beckman). Hematocrits were measured.

Plasma Insulin and Plasma Renin Activity

Plasma insulin was determined by radioimmunoassy using a commercial kit (INSIK, Cea-Sorin).

Plasma renin activity was determined by the radioimmunoassay of angiotensin I generated *in vitro*. The main steps of the assay were as follows: (a) Angiotensin I was generated in aliquots of plasma samples in presence of porcine angiotensinogen (Sigma, 7 mg/100 μl). Incubation was performed at 20°C and at 37°C for 1.5 hr in presence of 4 mM 2.3-dimercaptopropanol, 3.2 mM 8-hydroxyquinoline sulfate, and 5.9 mM EDTA to inhibit angiotensinases and angiotensin I converting enzyme. The samples were buffered at pH 6.5. Incubation was stopped by ice bath. (b) Blanks were prepared with aliquots of plasma samples placed directly on the ice bath. Controls were prepared by incubation of porcine renin substrate without addition of plasma samples. (c) Renin activity was assayed using a commercial kit (Amersham, Bucks, U.K.). The generated angiotensin I was expressed as nmol/ml plasma/hr. The antiserum used was raised in rabbit against angiotensin I. Serial dilutions of unlabelled angiotensin I were treated in the same way to obtain a standard curve whose sensitivity was $0.18 \pm 0.04 \times 10^6$ g/l. Intra assay and inter-assay coefficients of variation were respectively 5.5% and 16.8% ($n = 10$).

Bioassay

Aliquots of plasma samples incubated with porcine angiotensinogen, under the conditions described above, were injected into anaesthetised rats as a qualitative assay in order to test vasopressor response elicited by angiotensin II generated in viper's plasma.

Immunohistochemistry

The Bouin fixed sections, from control vipers ($n = 3$) and vipers subjected to sodium loading ($n = 3$) and sodium depletion ($n = 3$), were submitted to the indirect immunofluorescence technique (6) and to the peroxidase-antiperoxidase (PAP) method (26). The antisera used were: porcine Ab-Insulin (Milab-Sweden, dil. 1:100), porcine Ab-Glucagon middle-*N* terminal (CRB, dil. 1:400), porcine Ab-Somatostatin (Milab-Sweden, dil. 1:1600).

The specificity of the immunohistochemical localizations was tested by replacing each specific antiserum with: a) normal rabbit serum (or guinea pig serum in the case of insulin), b) buffer and c) specific antisera absorbed with the antigens (liquid phase).

Statistics

Data are given as means ± SD. Significance of the results was determined by Bonferroni's *t*-test.

RESULTS

Plasma Electrolyte Concentrations, Hematocrits, and Osmolarity

Table 1 summarizes the effects of sodium loading and administration of a diuretic on plasma osmolarity and electrolyte concentrations. Sodium loading produced a significant increase in plasma sodium, potassium and chloride concentrations and on plasma osmolarity, while furosemide administration produced significant decrease in plasma sodium, potassium and chloride concentrations and depressed osmolarity. Hematocrits were not statistically different.

TABLE 1. Effects of sodium loading and sodium depletion on plasma electrolytes, hematocrits and osmolarity in *Vipera aspis*.

Treatments	*n*	Na mmol/l	K mmol/l	Cl mmol/l	Hemat. (%)	Osm mOsm/l
Controls	3	157.0 ± 3.2	3.5 ± 0.2	122.0 ± 1.0	22.8 ± 2.2	298.6 ± 1.1
Na loading	3	263.3 ± 23.9*†	4.6 ± 0.4*†	236.6 ± 8.9*†	19.5 ± 0.5	509.3 ± 39.0*
Na depletion	3	98.2 ± 2.3*	1.9 ± 0.1*	97.0 ± 8.8*	21.3 ± 0.5	196.1 ± 8.9*

Values are mean ± SD; *$p < 0.05$ versus controls; †$p < 0.05$ sodium loading versus sodium depletion; Bonferroni *t* test.

TABLE 2. Effects of sodium loading and sodium depletion on plasma renin activity (PRA), Plasma Insulin (PI), and Glucose in fasted *Vipera aspis*

Treatments	n	PRA ng ANG I/ml/h	PI μU/ml	Glucose mg/100 ml
Controls	3	18.2 ± 3.5	10.5 ± 1.2	39.3 ± 0.5
Sodium loading	3	6.0 ± 0.4*†	2.3 ± 0.2*†	79.3 ± 0.2*†
Sodium depletion	3	38.6 ± 5.1*	24.8 ± 3.2*	8.6 ± 0.3*

Values are mean ± SD; *$p < 0.05$ versus controls; †$p < 0.05$ sodium loading versus sodium depletion; Bonferroni t test.

Plasma Renin Activity, Plasma Insulin and Glucose Concentration

In nonfasted vipers, used as controls, values of plasma glucose and insulin varied greatly. We chose therefore to submit to treatments only fasted vipers in which stable values of plasma glucose and insulin were available.

Data on plasma renin activity, insulin and plasma glucose concentration are given in Table 2.

Injection of samples containing Angiotensin I generated in vipers' plasma gave in rats a vasopressor response similar to that obtained injecting angiotensin I.

After furosemide administration, that caused a significant sodium depletion, plasma renin activity was higher and an elevation in plasma insulin concentration was observed. Plasma glucose diminished significantly.

In sodium loading plasma renin activity was very low and plasma insulin was depressed, while plasma glucose was significantly higher than in the controls.

Immunohistochemical Results

Incubation with antiserum to insulin resulted in immunostaining of insulin producing cells, B-cells, columnar or slightly wedge-shaped, mainly localised at the centre of the islets (Fig. 1a). Insulin-immunoreactive cells were however found randomly distributed also in the exocrine tissue.

Immunoreactivity for glucagon was found in a large number of elongated cells, A-cells, in the central portion of the islets (Fig. 2a).

Somatostatin immunoreactive cells, D-cells, round or oval in shape, are peripherally localised in the islet or gathered in small groups in the acinar tissue (Fig. 3a).

In vipers submitted to sodium loading, B- and D-cells showed a very weak immunoreactivity (Figs. 1b, 3b), while in A-cells the immunoreactivity was stronger if compared with the controls (Fig. 2b).

In vipers submitted to a diuretic administration and consequent sodium depletion, a strong immunoreactivity was present in the B- and D-cells (Figs 1c, 3c) and only a weak immunoreactivity was detectable in the A-cells (Fig. 2c).

In the controls, performed to test the specificity of the immunoreactions, immunostaining was negative.

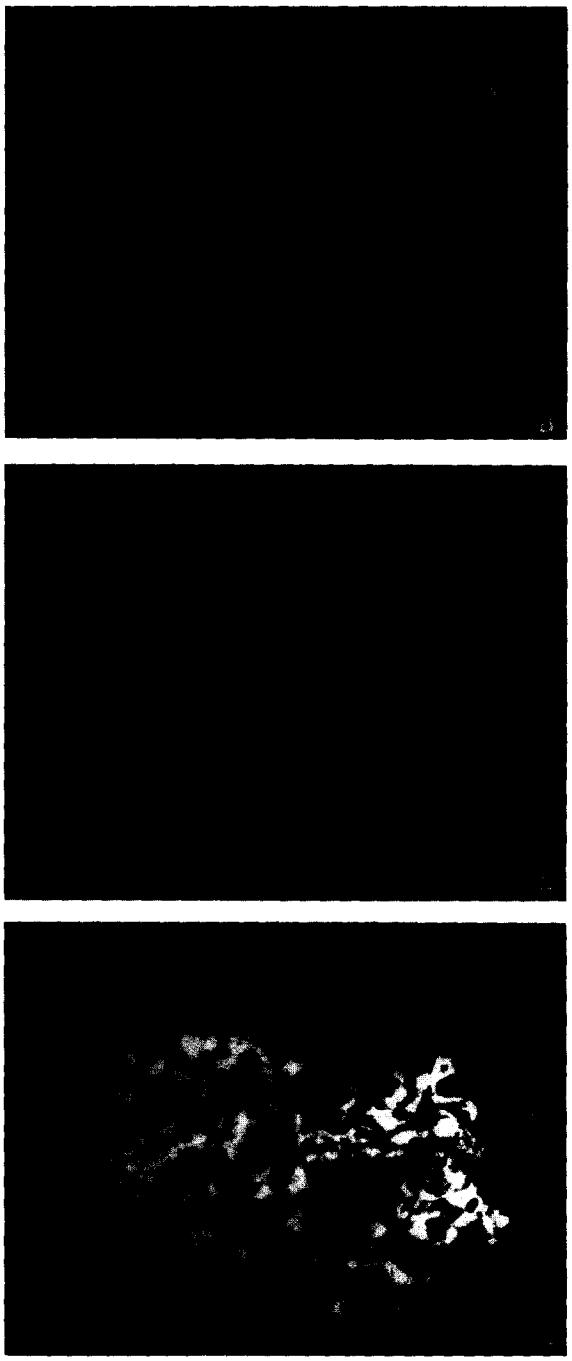

FIG. 1. Insulin-immunoreactive cells, localised at the center of the islets, show a weak immunoreactivity after sodium loading (1b, ×340) and a stronger reaction after sodium depletion (1c, ×340) if compared with the control (1a, ×340).

DISCUSSION

Administration of NaCl resulted in a rise in plasma sodium concentration, and, injections of the diuretic furosemide de-

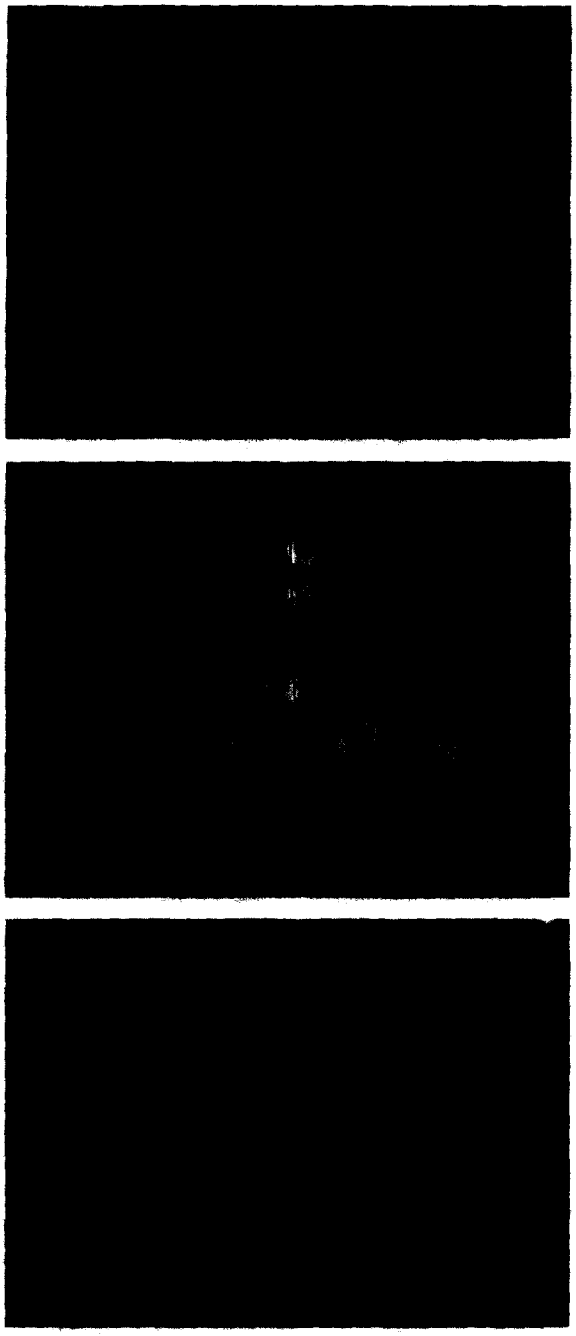

FIG. 2. **Glucagon-immunoreactive cells, localised in the central portion of the islets, show a strong immunoreaction after sodium loading (2b, ×340) and a very weak immunoreactivity after sodium depletion (2c, ×340) if compared with the control (2a, ×340).**

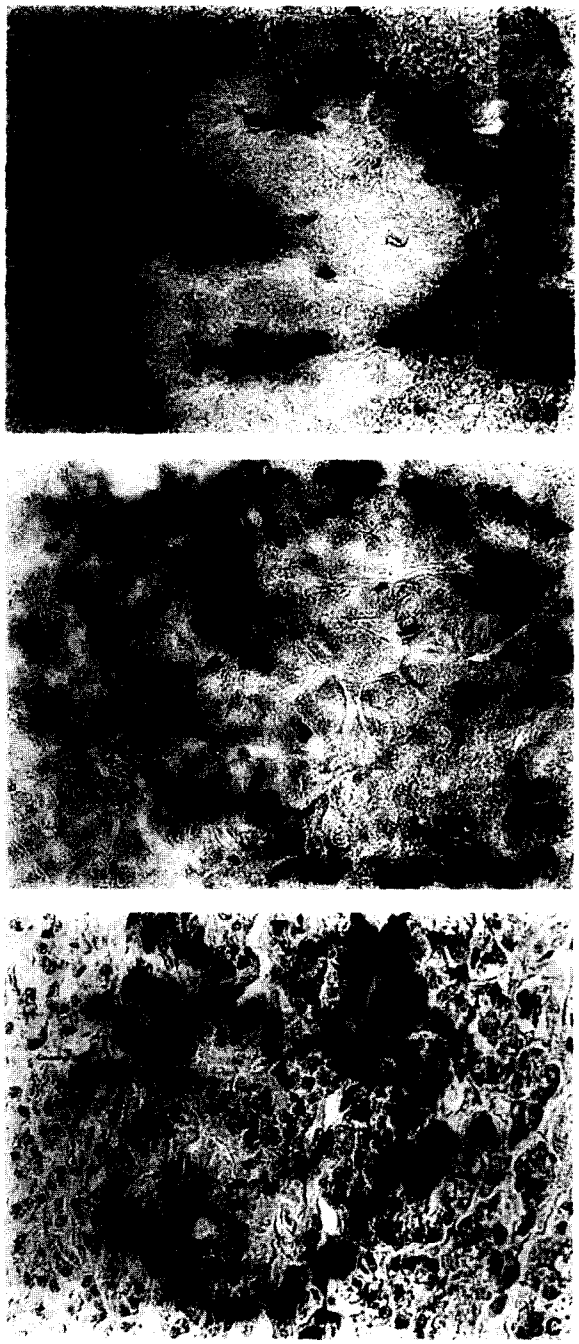

FIG. 3. **Somatostatin-immunoreactive cells, D cells, are localised in a peripheral position. They display a weak immunoreactivity after sodium loading (3b, ×340) and a strong immunoreactivity after sodium depletion (3c, ×340) if compared with the control (3a, ×340).**

pressed plasma sodium. This is in agreement with the data offered by Le Brie and Boelcskevy (12) in the water snake *Natrix*. The author stated that in snakes furosemide has a proximal and distal tubular site of action and is therefore able to act also in animals without a loop of Henle.

Sodium loading and depletion caused variations in the plasma renin activity. Radioimmunoassay of renin activity in reptiles has been discussed by Stephens and Creekmore (25) and by Vallarino (31) who tested in *Testudo hermanni* the renin-like activity at different pH conditions and temperature. In some poikilothermic animal the optimal temperature of incubation for renin activity is 20°C (18), however in reptiles, renin activity is higher at 37°C than at 20°C, as observed by Vallarino (31) in turtles and in snakes by Seyama *et al.* (23).

In our experiments we observed in vipers the maximum of renin activity at 37°C. This discrepancy may reflect a species-specificity of the enzyme activity. Stephens and Creekmore stated that in turtles the angiotensin-like substance is bound by human angiotensin I antibody, enabling the determination of plasma renin activity. Seyama *et al.* (23) also found a renin-like activity in plasma and kidney of the snake *Elaphe quadrivirgata* and Uva *et al.* (29) demonstrated the presence of a renin-like activity in the plasma of *Vipera aspis*. Angiotensin I has been sequenced in several reptiles among which turtles and alligators (27). The amino acid sequence of angiotensin I liberated from the snake homologous substrate, differs in positions 5 and 9 from the mammalian angiotensin I (17). We therefore chose to perform our radioimmunoassay using porcine renin substrate, our data show the presence of a renin-like activity but cannot offer evidence that the rates of cleavage of homologous and heterologous substrates of viper's renin-like enzyme are the same.

In our experiments a close relationship was observed between sodium status and renin-like activity: plasma sodium loading resulted in reduction and sodium depletion in a significant rise, of plasma renin-like activity. Plasma insulin levels and glucose also changed following sodium status. Sodium loading caused a lowering in insulin plasma level and hyperglycemia, while sodium depletion resulted in the opposite conditions: hyperinsulinemia and hypoglycemia. The immunohistochemical data conformed the blood patterns showing a weak immunoreactivity in the B and D cells and a strong immunoreaction in the A cells after sodium loading, while the opposite patterns could be observed after sodium depletion.

The immunohistochemistry was performed with mammalian antibodies that cross reacted with the viper's antigens, as was stated for several snakes by Buchan and Polak (3) and Buchan (2). Insulin amino acid composition differs from the mammalian insulin by 5 amino acid in the A chain and 5 amino acid in the B chain (11), in pancreatic glucagon the primary structure is mostly identical in all mammals and differs for very few amino acids in birds and fish. The lack of specificity between the mammalian and the other pancreatic peptides revealed by these studies suggest that the structure of the hormones among vertebrates has been strongly conserved. Our data on the immunodetection of the three cell types agree with other results obtained by different authors in snakes (22,2,14) as respect the localisation of the A, B and D cell inside the pancreatic islets, and the detection of immunoreactive cells within the exocrine pancreas. Our data are also in agreement with the observation of Masini (14) on the absence of a significant difference in percentage of the A and B cells in the vipers' pancreas, but are in disagreement with the general trend which considers that in almost all squamata the A cells should prevail as reported by Epple and Brinn (8). Therefore in vipers the concept that glucagon is the main pancreatic hormone involved in regulation of plasma glucose may not be correct. The level of plasma glucose in vipers is fairly lower than that measured by other authors (21) in lizards, this discrepancy may be due to the fasting conditions of our animals or to a specie-specific difference.

Regulation of insulin secretion as well as the physiology of the pancreatic hormone in nonmammalian vertebrates is still under investigation. The presence of specific binding sites for angiotensin II in the mammalian endocrine pancreas (4,28) as well as the modifications of insulinemia in vipers after changed plasma sodium level (closely correlated to modification of plasma renin activity) support the hypothesis offered by Epple and Brinn (8) that the pancreatic tissue was originally involved in ion-water balance. The effect on blood glucose has been a secondary functional activity that reached a predominant role in mammals.

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