FLUORIDE-INDUCED RESISTANCE TO INSULIN IN THE RAT

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SUMMARY: The insulinic response of rats challenged with glucose at different times after an oral dose of 40 µmol NaF/100 g bw revealed the concentration of the molecular species reacting with anti-insulin antibody was significantly lower after 3 hr, and the glucose levels were somewhat higher than in controls. At the 4th and 5th hr, plasma F levels were still higher than in the controls, at which times the response of β-cells toward glucose was assessed by a dose-response function. The concentration of glucose that produced 50% of the insulinic response was significantly higher in rats treated with NaF (6.3±0.2 g/L) compared with the control group (4.3±0.4 g/L), P<0.05. These results indicate that F-treated rats exhibit a significantly reduced sensitivity to glucose stimulus. Measurements of plasma insulin at the 5th hr after F load by gel filtration of plasma, followed by measurement of insulin in the fractions containing 5–8 kDa peptides, revealed that only one-fifth of the species reacting against the antiserum corresponds to this hormone. The secretion of C-peptide did not exhibit the expected relationship with immunoreactive insulin-like species. The clearance of intravenously injected ^{125}I-insulin was not affected by the presence of fluoride. The incorporation of ^{3}H-leucine into β-cell proteins in the range of 5 to 13 kDa was higher in F-treated than in control slices, supporting the hypothesis that F does not interfere with the synthesis of insulin. This work provides further data on the dysfunction induced on pancreatic β-cell physiology by increased F in the extracellular fluid, a dysfunction that most probably involves disturbance of the enzymatic processing of insulin precursors.

Keywords: β-Cell proteins; C-peptide secretion; Fluoride and insulin; Glucose tolerance; Insulin; Insulin resistance; Rat glycemia.

INTRODUCTION

It has been demonstrated that, after the intake of NaF by human volunteers and by rats, plasma fluoride (F) increased and plasma insulin decreased accompanied by an increase in serum glucose levels.[1] In standard glucose tolerance tests done on people in Argentina living in an area of endemic fluorosis, the area under the curve of plasma insulin levels as a function of time showed an inverse relationship with fluoremia.[2] In agreement with these findings, abnormal glucose tolerance tests on inhabitants of endemic fluorosis areas in India were interpreted as resistance to insulin, and glucose tolerance was normalized when the water supply was replaced by water with low F content.[3] In another study, higher plasma glucose levels and a delay in the peak of plasma insulin were observed after a glucose tolerance test on inhabitants with high F intake.[4]

When F was administered in the drinking water to rats (5 mM), bone mass and bone F content increased, without any noticeable effect on glucose metabolism. These results could be explained by the low rate of F intake. As bone mass increased by the stimulus of F, the latter was incorporated into the bone mineral, and plasma F levels were kept below 1 µM.[5]

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A direct effect of F on insulin secretion was demonstrated by *in vitro* experiments with isolated Langerhans' islets. These experiments showed that plasma ionic F above 5 μM causes a decrease in insulin secretion.[1] Perfusion of isolated rat pancreas showed that the presence of F in the buffer inhibits both the first and the second phases of insulin secretion stimulated by glucose. Removal of F from the incubation buffer produced an immediate increase in the secretion of insulin that had a direct relationship with the previous concentration of F in the incubation buffer, thereby indicating that F disturbs secretion but not the synthesis of insulin.[6]

This paper reproduces the above-mentioned effect *in vivo* in the rat after a glucose tolerance test, demonstrating that F decreases insulin secretion while the concentration of F is high, but when the concentration of F is decreased, the glucose tolerance test does not normalize, although high levels of immunoreactive insulin peptides remain higher than in controls. This effect is evidently the consequence of the production of proteins reactive to the antibody anti-insulin but without a normal hypoglycemic effect.

MATERIALS AND METHODS

The experiments reported in this paper were conducted according to directions detailed in the NIH Guide.[7] The project was approved by the Ethics Committee of the School of Medicine, Rosario National University, Rosario, Argentina.

Animals and general experimental conditions: The experiments were carried out with 7-week old female rats, 180–220 g bw of the strain IIM/Fm subline "m", with normal glucose metabolism and standard glucose tolerance tests.[8] Rats were fed balanced food and water *ad libitum*. Food pellets contained 2.4 μmole fluorine/gram. Tap water had 15 μmole F/L. The load of F imposed by food and water intakes is small and does not affect plasma F levels and fasting glucose levels.[5]

Fluoride load: This was obtained by an oral dose of 0.5 mL/100 g bw of a 80 mM solution of NaF (40 μmole F/100 g bw).

Glucose challenge (glucose tolerance test): The animals received an intraperitoneal injection of 5 mL/100 g bw of a 10 g% (w/v) glucose solution, and they were sacrificed by heart puncture 1hr later, close to the moment where plasma glucose is at its maximum.[1,5]

Anesthesia: Urethane (120 mg urethane/100 g bw) was administered by intraperitoneal injection. All experiments were done with nonfasting rats beginning at 8.00–9.00 AM.

Blood sampling: Blood was obtained by heart puncture or from a catheter inserted into the femoral artery. Heparin was used as anticoagulant.

Drugs and chemicals: All drugs and chemicals were of analytical grade purchased from Sigma Chemical Co. (St Louis, MO, USA). ^{3}H-leucine was purchased from New England Nuclear (USA)

Analytical techniques: Plasma F was measured with an ion specific electrode (94-09, Orion Research Inc, Cambridge, MA, USA)[9] after its isolation by means

of the room temperature distillation method.[10] Plasma glucose was measured according to Washko and Price.[11] Insulin was measured in plasma, incubation medium or column eluates by radioimmuno-assay with rat insulin as a standard (Millipore Corporation, USA). Except when measuring chromatographic fractions (Sephadex G100 experiments), the β-cells secretion is probably (as discussed below) a mixture of insulin-like molecules. The data obtained using a polyclonal anti-insulin serum (Diagnostic Products Corporation) are reported as equivalent to μIU of insulin/mL. Plasma levels of C-peptide was measured by radioimmunoassay (Diagnostic Products Corporation, USA) and reported as pmoles/mL.

Plasma F, insulin, and C-peptide levels, one hour after glucose challenge, at several time periods following a F load: The aim of this experiment was to assess the relationships between plasma glucose, insulin and C-peptide at varying plasma F levels. A group of seven rats were sacrificed to obtain basal values of insulin, F, glucose, and C-peptide. Another group of 10 rats received a glucose load and blood was obtained 1 hr later (controls). Six groups of 5 rats each received the F load (treated) and, after 15, 60, 120, 180, 240, and 300 min, the animals received the glucose challenge. One hr after glucose load, rats were sacrificed by heart puncture and plasma samples were saved for glucose, insulin, C-peptide and F measurements.

Insulin content of plasma as determined by gel filtration and measurement with insulin antiserum in eluates: Two groups of five rats each received either the F load or the same volume of distilled water. Five hr later they were challenged with glucose. One hr after the latter, the animals were anesthetized and sacrificed by hearth puncture. Insulin was measured in plasma. Aliquots with known amounts of insulin were filtered through a Sephadex G-100 column.[12] The column (38-cm length × 1-cm diameter) was eluted with buffer (NaCl 0.16 M, sodium phosphate 50 mM, pH 7.4; flow rate 0.17-0.22 mL/min. Molecular weight markers were used to calibrate the column. Half-mL aliquots of fractions 18 to 30, known to receive molecular species of 6 kDa, were subjected to insulin analysis.

Plasma clearance of exogenous ^{125}I-insulin: Under urethane anesthesia, four rats received 3 nCi of ^{125}I-insulin in 0.25 mL of saline through a catheter inserted in the femoral artery 15 min after the F load. The same number of control animals received a similar volume of saline by i.v. injection and the same volume of distilled water by gavage. Blood samples were obtained after 7.5, 15, 30, 45, and 60 min through the same catheter and plasma (100-μL aliquots) were used for radioactivity measurements. The rate of clearance was calculated as the constant (K) of the one phase exponential decay function. Half-life of insulin was calculated as ln 2/K.

Incorporation of ^{3}H-Leucine into pancreatic peptides: Four rats were anesthetized, and pancreatic tissue was rapidly excised, submerged in buffer at 4°C and cut with iris scissors into small pieces of approximately 1 mm^3. Fifty mg aliquots of slices from pancreatic tissue were incubated for 10 min in the buffer reported by Patzelt[13] and by Tager.[14] Six experiments were carried out with and

without 20 μM NaF in the incubation buffer. The media were gassed with carbogen (95% oxygen, 5% carbon dioxide). At the end of this period, the vials received the addition of 50 μCi of ^{3}H-Leucine and were stimulated with glucose (added to attain a concentration of 3 g/L) for an additional 10 min of incubation. At this time, pancreatic tissue was separated from the buffer, washed twice with buffer without F and homogenized in 1 mL saline and centrifuged. Aliquots of the supernatant containing 10 μg of protein were mixed with the same volume of buffer (120 mM Tris buffer, pH 6,8, 2% sodium dodecyl sulphate, 20% glycerol, 10% 2-mercaptoethanol), heated in a boiling water bath for 5 min and subjected to polyacrylamide gel electrophoresis. The procedure employed a discontinuous system of 4% stacking and 15% separating gels.[15] Aliquots of a mixture of proteins of molecular mass markers were run simultaneously (Triose-phosphate isomerase (26.6 kDa), myoglobin (17 kDa),α-lactalbumin (14.2 kDa), aprotinin (6.5 kDa), insulin B chain (3.49 kDa) and bradykynin (1.06 kDa)). After electrophoresis, three sections of the lanes were cut, corresponding to molecular weights 5-8, 8-11 and 11-13 kDa. The pieces of gel were disrupted with liquid scintillation solution with aid of a glass rod into scintillation vials. Radioactivity was measured to a constant 2% error.

Statistical techniques: When two independent samples were compared, the analysis was performed using a two-tailed Student's t test for continuous variables. When more than two media were compared, one-way analysis of variance (ANOVA) and the multiple comparison test of Newman-Keuls were employed. Nonlinear regression was used to evaluate the decay of radioactivity after the injection of I^{125} insulin and the dose-response of insulin as a function of plasma glucose levels. Differences were considered significant when $p<0.05$. Data are expressed as the mean±standard error (SEM).

RESULTS

Plasma F and peptide levels after glucose challenge following a F load: Figure 1 and Table 1 display plasma levels of F, insulin, and C-peptide, 1 hr after the glucose tolerance test done at variable times after the F load. Basal values and values after a glucose load without F (controls) are also shown.

Table 1. Plasma levels of insulin (μUI/mL), C-peptide (pmole/mL), and F (μM) one hr after glucose challenge of controls (without F) and at varying time from F dose

	Insulin	C-peptide	F
Basal values (n=7)	17±3	13±8	1.1±0.2
Controls (n=10)	370±26	135±8	1.5±0.8
Hours elapsed between F dose and glucose load			
0.25 (n=5)	49±5*	91±12	40.0±15.0*
1 (n=5)	48±6*	135±20	26.3±12.6*
2 (n=5)	33±10*	137±25	19.3±6.0*
3 (n=5)	148±50	161±41	13.8±5.5
4 (n=5)	379±66	77±13	9.3±5.2
5 (n=5)	700±80*	99±4	3.4±3.1

Results are expressed as mean±SEM. *Represents statistical differences compared to controls $p<0.05$.

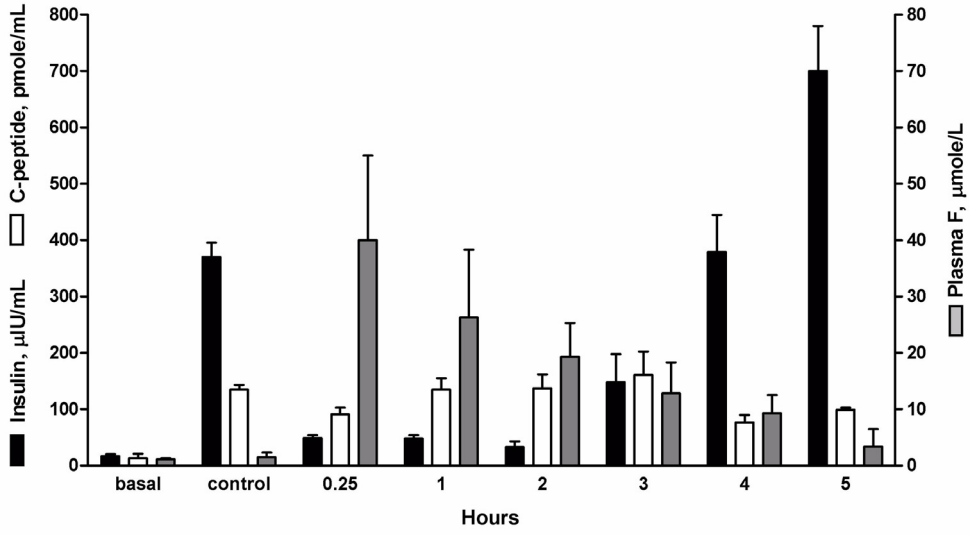

Figure 1. Plasma levels of F, C-peptide, and insulin 1 hr after glucose challenge. Bars and vertical lines represent mean±SEM. The horizontal axis indicates the time elapsed between the oral dose of NaF and the glucose challenge.

Between 15 min and 2 hr after the F load, the plasma F level was higher and the insulin level lower than in the control rats administered the glucose load without the F dose. From the 3rd to the 5th hr after the F load, plasma F decreased and insulin increased significantly. C-peptide plasma levels decreased and then increased and then again decreased. Overall, they did not change significantly during the experiment and did not show any clear relationship with insulin levels.

As seen in Figure 2, insulinemia of treated rats and controls as a function of serum glucose levels showed a sigmoidal dose-response function. The ED_{50}, corresponding to the value of serum glucose levels when the insulin level is the mean of the bottom and the top of the function, was calculated to evaluate insulin sensitivity to glucose levels. Treated rats had higher value of ED_{50} (6.3±0.2 g/L) than controls (4.3±0.4 g/L), $P<0.05$. These values indicate that F-treated rats exhibit a significantly reduced sensitivity to glucose stimulus. Points of treated rats at the bottom of the dose-response line had lower levels of insulin than controls and come from rats that received F 3 hr before glucose challenge. On the other hand, points of treated rats above the bottom come from rats that received F 4 hr before glucose load.

Figure 3 displays the plasma insulin recovered after chromatography on Sephadex G-100 of known amounts of plasma from F-treated rats, obtained 5 hr after the F load (Figure 1) and from controls, in both cases 1 hr after the glucose challenge. In control and treated rats, species eluting with a peak in fraction 23, corresponding to a molecular mass of 6 kDa, reacted against insulin antibody. The recovery of plasma insulin from control animals was complete: amount of insulin chromatographed = 51±6.5 µIU, amount recovered: 54±3 µIU; Recovery: 106±19%. The same experiment done with plasma of animals treated with F 5 hr

before and receiving the standard glucose challenge 1 hr before sacrifice, gave a significantly lower recovery: amount of insulin chromatographed=83.5±9.3 µIU, amount recovered: 15.0±7.0 µIU; Recovery: 18±19%.

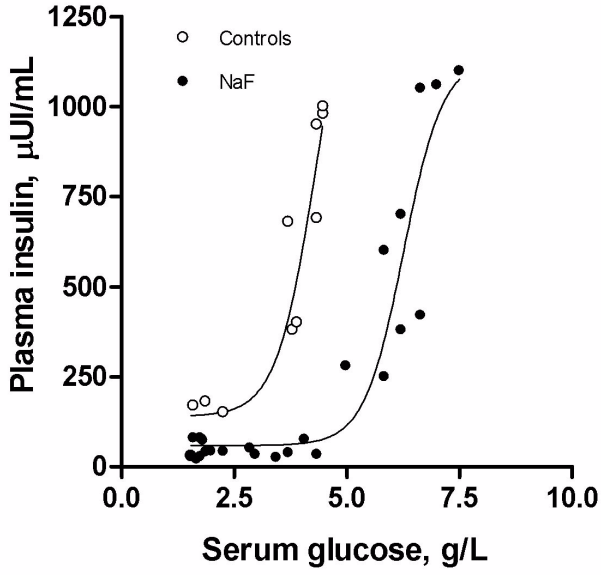

Figure 2. Plasma levels of insulin as a function of plasma glucose levels in control and F-treated rats 1 hr after a glucose challenge. Treated rats received a F dose at varying times before glucose challenge. (See materials and Methods.)

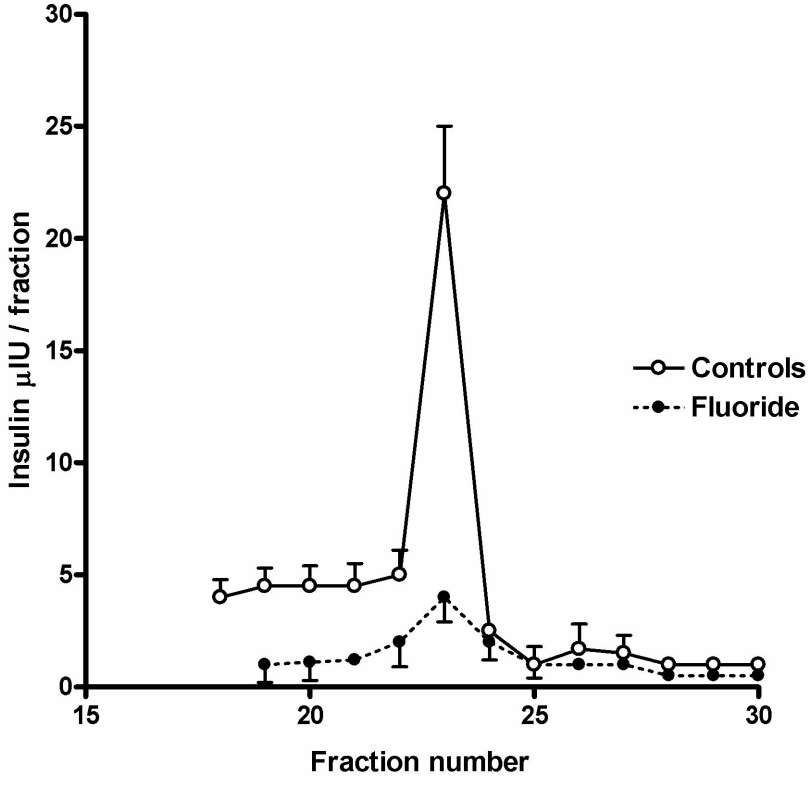

Figure 3. Chromatography of serum from control and F-treated rats. Only fractions 18 to 30 are displayed. Molecular species of 6 kDa are eluted in these fractions.

Clearance of exogenous ^{125}I-insulin: After an intravenous injection of a tracer amount of ^{125}I-insulin, the clearance of plasma radioactivity was assumed to be a direct function of insulin clearance. Radioactivity fitted a one-phase-exponential-decay function ($I=I_o\ e^{-kt}$). No significant differences were observed in half-life times: Controls = 4.43±2.69 min^{-1}, n=4; F-treated: 2.06±1.05 min^{-1}, n=4.

Radioactivity incorporation into proteins from pancreatic tissue after glucose stimulus: Table 2 gives the results of radioactivity present in sections of polyacrylamide gel at different molecular mass levels. The treatment of pancreatic slices with F produced an increase of radioactivity into proteins in the molecular weight range of insulin, proinsulin, and preproinsulin.

Table 2. Radioactivity measured in sections of the polyacrylamide gel after electrophoresis of homogenates of pancreatic tissue incubated with ^{3}H-leucine for 10 min

Relative molecular mass	Radioactivity, cpm		
	NaF 0 µM	NaF 20 µM	% of increase
13-11 kDa	396±13	464±11*	17.2
11-8 kDa	136±10	159±9*	16.9
8-5 kDa	69±5	85±4*	23.0
TOTAL	601±28	708±24*	17.8

Results are expressed as mean±SEM of 6 experiments. *Represents significant differences compared with NaF 0 µM, $p<0.05$.

DISCUSSION

In these experiments, although plasma F levels above 5 µM inhibited the secretion of insulin, insulin and serum glucose levels gradually returned to normal levels.[1] When the F intake by rats occurred at low rates in the drinking water, the animals showed disturbances of glucose homeostasis evident only after a glucose load.[5] The evidence indicates that plasma ionic F is the active species involved in this phenomenon.[16] The phenomenon was also demonstrated with isolated Langerhans' islets[1] and *in situ* perfusion of the pancreas.[6] The effect would not be caused by inhibition on glycolysis, which occurs with F concentrations of 1.5 mM.[17]

The present results confirm previous experiments from this laboratory. In the first 3 hr after the F load the concentration of the molecular species reacting with anti-insulin antibody was lower than in the controls. After the 4th and 5th hr plasma F levels were still higher than in the controls and much higher than the threshold level of 5 µM.

The concentration of glucose needed to produce a 50% of insulin secretion (ED_{50}) was higher in rats that received F before the glucose load, as compared with controls. This finding suggests that F produced either a strong insensitivity of the β-cell to glucose stimulus or induced the secretion of molecular species with lower biological activity than insulin. With respect to the insensitivity of the β-cell hypothesis, assessment of the data (Figure 2) indicates that secretion of molecular species reacting against the insulin immunoserum requires higher glucose levels than in controls. However, the fact that the clearance of intravenously injected ^{125}I-insulin was not affected by F treatment also suggests that the half-life of

plasma insulin is not involved in the abnormal glucose metabolism induced by F. The second hypothesis (lower biological activity) was partially supported by the Sephadex experiments. Measurement of plasma 6 kDa-insulin in the eluate of gel filtration of plasma 5 hr after the F load (Figure 3) supports the hypothesis that β-cells secrete a mixture of peptides, and that only about one-fifth of the species reported in Figures 1 and 2 correspond to insulin.

A second argument in favor of a dysfunction of β-cell secretion is the lack of an expected relationship between levels of insulin and C-peptide. Further investigations including a more specific assay for rat insulin and analysis of the molar ratio insulin:C-peptide could improve the understanding of the effect of F on insulin secretion.

The incorporation of ^{3}H-leucine into β-cell proteins, investigated in the range of 5 to 13 kDa, was higher in the F-treated than in control slices. This finding can be interpreted to support the hypothesis that F does not interfere with the synthesis of insulin. The overall information afforded by these experiments support the previous conclusion that F hinders the secretion of insulin and strongly suggests that it affects the enzymatic processing of its precursors. Within the β-cell granules, proinsulin in converted by a process of enzymatic cleavage into insulin and C-peptide.[18] In addition, C-peptide and the partially processed forms of proinsulin may have biological activity.[18,19] Chromatographic studies have shown heterogeneity of C-peptide in serum, which may arise either through *in vivo* metabolism and/or through degradation on storage.[20-23] The present data are not sufficient to conclude that the increased plasma levels of peptides reacting against the polyclonal anti-insulin serum are due to proinsulin and/or partially processed forms of proinsulin. Cross reactivity insulin-proinsulin is reported to vary from 38 to 100%.[23,24]

We believe we have a clear case of β-cell dysfunction caused by F as indicated by the decreased sensitivity of pancreatic tissue toward glucose stimulus. In a first stage, F reduces the secretion of insulin. In a second stage, under glucose stimulus, high levels of F appear to induce an abnormal secretion of a mixture of insulin, its precursors, and various metabolites. Literature reports indicate that β-cell dysfunction is associated with the secretion of intact and partially processed proinsulin.[24-26]

There is also solid evidence that thyroid hormones influence insulin secretion. In hyperthyroidism an increase in plasma insulin, C-peptide, and proinsulin levels have been observed as well as an increase in the area under the curve of serum glucose levels after a glucose tolerance test.[27] These variables return to normal values after antithyroid treatment. There is also evidence of a deficiency in insulin processing in hyperthyroidism.[28] On the other hand, in hypothyroidism a decrease in the insulin peak after an oral glucose tolerance test was observed.[29] An increase in counter regulatory hormones and a negative relationship between secretion and sensitivity to insulin has also been observed in related work.[30] It should also be noted that F affects thyroid function, and the effect depends on the level of iodine in the diet.[31] However, the inhibitory action of F on insulin secretion is a

consequence of a direct action on β-cells, as it was demonstrated by incubation of Langerhans' islets[1,6] and *in situ* perfusion of pancreas.[6] The effect of F on insulin sensitivity described in this paper could be the consequence of an interaction among F, pancreas, and thyroid glands, but this possibility needs more evidence to be established.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Consejo Nacional de Investigaciones Científicas y Técnicas, CONICET (PIP 5018).

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