Abnormal regulation of pancreatic glucagon secretion in obese *fa/fa* rats

F. Rohner-Jeanrenaud and B. Jeanrenaud

Laboratoires de Recherches Métaboliques, Faculty and Department of Medicine, University of Geneva, Switzerland

Summary. The results reported in the literature regarding glucagonaemia in genetically obese *fa/fa* rats are conflicting: normal, increased or decreased plasma glucagon levels have been reported. Due to the existence of several molecules endowed with glucagon-like immunoreactivity, it was thought that the conflicting data could be related to the degree of specificity of the different glucagon antibodies. Three antibodies that all qualified as being specific for pancreatic glucagon were used. It was found that, depending on the antibody, absolute values of basal glucagonaemia or arginine-induced glucagon output varied quantitatively and qualitatively in both lean and obese rats. When non-extracted basal or stimulated plasma samples were passed on a G-50 Sephadex column, glucagon-like immunoreactivity was present over a wide range of molecular weights, indicating the presence of non-pancreatic glucagon molecules. When an ethanol extraction was used, the fractions eluting from the G-50 Sephadex column contained only pancreatic glucagon immunoreactivity. It is concluded that ethanol extraction is necessary for the measurement of the 3500 daltons glucagon. Using this methodology it was found that: (1) basal glucagonaemia was low but identical in the two groups of rats; (2) arginine-induced glucagon secretion was greater in obese than in lean animals; (3) glucagonaemia was decreased by glucose administration in lean but not in obese rats. It is concluded that there are, in obese animals, dysfunctions of glucagon output that may play a role in their abnormal glucose tolerance.

Key words: *fa/fa* rats, glucagon output, pancreatic glucagon, ethanol extraction, specific antiglucagon antibodies.

When studied in the preobesity phase of the syndrome, the genetically obese *fa/fa* rat has been shown by our laboratories to hypersecrete glucagon as well as insulin in response to arginine, while basal glucagonaemia was identical to that of its lean littermate [1]. These arginine-induced glucagon and insulin hypersecretions were mediated by an activation of the parasympathetic nervous system, since they were normalised by an acute superimposed atropine administration [1]. With the duration of the syndrome, the early substrate-elicited insulin hypersecretion worsens and, from 3–4 weeks onwards, the obese animals do not have only an increased response of insulin to various stimuli, but they also have an increased basal insulinaemia [2]. As suggested by previous studies [3, 4], these alterations lead to obesity by overstimulating glucose metabolism and, more specifically, by channelling glucose carbon into lipids, a process that, contrary to other metabolic pathways, does not become insulin resistant [5]. In contrast with the observations concerning insulin, the reports about glucagon in adult *fa/fa* rats are conflicting. Thus, most studies reported normal or decreased basal

glucagon levels in these animals, together with a diminished response of the hormone to arginine [6–8]. When comparing the absolute values of plasma glucagon levels obtained by different research groups either in the basal or in the stimulated state, a great variability in the data becomes evident. To give an example, basal glucagonaemia has been reported to be either as low as 150 pg/ml [7] or as high as 600 pg/ml [6]. Since a great number of molecules are endowed with glucagon-like-immunoreactivity (GLI) [9], one could hypothesize, as has been clearly demonstrated in human obesity [10], that the variation of glucagonaemia thus far reported could be due to variable detection of GLI material having different molecular weights than that of the 3500 daltons glucagon, the so-called pancreatic glucagon.

The purpose of this study was therefore: (1) to attempt unravelling the origin of the prevailing controversy regarding the glucagonaemia values in adult obese *fa/fa* rats; (2) to provide an answer to the question of knowing whether the arginine-induced hypersecretion of glucagon observed in the preobesity phase

of the *fa/fa* syndrome [1] persists once the obese rats have become adult, and may be accompanied by another dysregulation of glucagon output.

Materials and methods

Animals

Ten to 15-week-old male or female lean (FA/FA) and genetically obese *(fa/fa)* rats bred in our laboratories were used throughout the experiments. They were kept in animal quarters at a constant temperature (23 °C) with a fixed (12 h) light cycle. The animals were fed a standard laboratory chow (Lacta 299, Provimi-Lacta S.A., Cossonay, Switzerland) and water ad libitum. Five to six hours-fasted animals were anaesthetized with Nembutal (60 mg/kg body weight) and an indwelling catheter was placed into the right atrium for blood sampling. After a resting period of 30 min, basal blood samples started to be collected and were followed or not by an intravenous bolus of arginine (600 mg/kg lean b.w.) or glucose (200 mg/kg lean b.w.). In the two instances, blood samples of 400 µl were withdrawn 3, 10 and 30 min after the acute arginine or glucose administration.

Blood samples were collected at 0 °C in heparinised tubes containing trasylol (500 U/ml) and EDTA (1.2 mg/ml) as previously recommended, to remove proteolytic enzymes and non specific glucagon-like immunoreactivity of some plasma proteins that could potentially interfere with pancreatic glucagon measurements [11, 12]. After centrifugation, the supernatants were stored at −20 °C for subsequent determination of plasma glucagon levels.

Glucagon determinations

Once thawed, part of the samples was extracted with the ethanol procedure of Heding [11] before being assayed, the other part being directly radioimmunoassayed without prior extraction. This was done to detect the effect of the extraction procedure on each plasma sample. The percent recovery of the extraction procedure was measured using three different approaches: (1) A known amount of purified 125-I glucagon [13] mixed with cold glucagon in rat plasma was extracted, and the counts recovered after the procedure were compared to those obtained before the extraction; (2) Pools of plasma from rats stimulated with arginine or not were prepared and separated into several aliquots. In each immunoassay, one aliquot of these two pools was measured at 5 different dilutions, with or without prior ethanol extraction; (3) One aliquot of an internal standard containing a known amount of synthetic pancreatic glucagon (Novo Research Institute, Bagsvaerd, DK) that was extracted or not extracted was measured with each series of unknown samples at 5 different dilutions. The three approaches just summarised gave information on the effect of ethanol extraction, the effect of serial dilutions, and inter-assays variations. They gave analogous recoveries of the ethanol extraction procedure, i.e. 85–95%. The yield of each extraction procedure for all series of assay tubes was taken into account, to obtain final corrected values.

The first two antibodies used, referred to as antibody A and B in the text for the purpose of clarity, were obtained from the following sources (see acknowledgements). Antibody A was the rabbit anti-glucagon antiserum IBC 23/5. This antibody has been shown to cross-react < 1% with glycentin, gastric inhibitory polypeptide, gastrin, motilin, cholecystokinin-pancreozymin, secretin, vasoactive intestinal polypeptide, somatostatin, pancreatic polypeptide, and insulin. Antibody B (code number of the antibody: 51080) was obtained with the qualification of being specific for pancreatic glucagon.

With these two antibodies, 125-I-glucagon (500–600 µCi/mg, Centre National de Transfusion Sanguine, Orsay, France) and antisera were diluted in glycine buffer (0.2 mol/l, pH 8.6) containing human serum albumin (0.25%), bacitracin (0.5 g/l), trasylol (2000 KIE/ml) and normal sheep serum (1%). In both cases, the radioimmunoassay procedure was as follows: 300 µl 125-I-glucagon were purified for each assay according to Rosselin [13] and pipetted into plastic tubes. Two hundred µl of antibody as well as 100 µl of either known standards (Novo) or unknown samples were then added. After 4 to 5 days incubation at 4 °C, the separation of free and bound glucagon was performed with dextran coated charcoal (Norit SXII, Amersfoort, the Netherlands). When antibody A was used, the zero binding was around 70%, the 50% displacement was obtained at a mean glucagon concentration of 400 pg/ml and the lower limit of detection of the assay system was 18 pg/ml (glucagon concentration corresponding to the counts obtained by adding those at zero binding plus two standard deviations, as defined theoretically [14]). With antibody B, these parameters were 60% for the zero binding, 310 pg/ml for the 50% displacement and 25 pg/ml for the lower limit of detection of the assay. The standard curves were represented by plotting the percent free as a function of the glucagon concentration. Due to the fact that the standard points are close to each other (12.5, 25, 50, 100, 200, 300, 400, 500, 600, 1000, 1200 and 2000 pg/ml), each point was joined to the next one by a straight line. All the calculations were made using an Apple computer program.

Antibody C was that provided by a glucagon radioimmunoassay kit CM-GLU (Oris S.A., Gif-Sur-Yvette, France). According to the instruction procedure specifications, the cross-reactivity of this antibody with dog gut glucagon-like-immunoreactivity was < 3% whereas it was < 1% with the glucagon fragment 1–26, and with extracts of jejunum or ileum. Using the kit as indicated, the methodology adopted for the separation of bound and free fractions was based on the use of a double antibody. The calculations were made in this case by plotting the percent B/Bo values as a function of the glucagon concentration. The lower limit of detection of this kit glucagon system was 150 pg/ml.

Gel chromatography

For these studies, three different pools of plasma obtained from fed female rats either in the basal state (referred to as "low plasma") or just after an arginine (600 mg/kg) bolus (referred to as "high plasma") were prepared and divided into different aliquots.
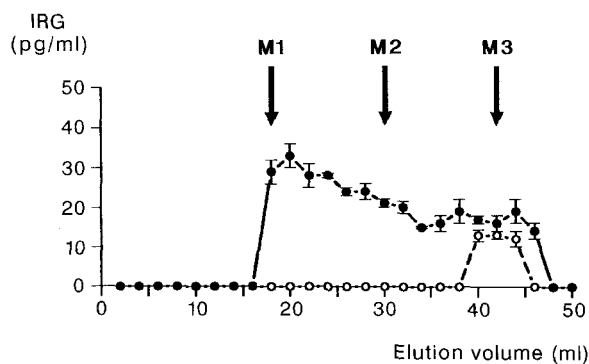
A 1.5 × 60 cm column of Sephadex G-50 superfine (Pharmacia Fine Chemicals, Uppsala, Sweden) was used at 4 °C and calibrated with Dextran Blue (M1), 125-I-insulin (M2) and 125-I-glucagon (M3). Aliquots of 2 ml plasma were lyophilised and resuspended in 1 ml 40 mmol/l NH_4HCO_3, pH 8.8 containing 1 g/l human serum albumin (Red Cross, Berne, Switzerland), as suggested before [15]. The two-fold concentrated plasma samples thus obtained or aliquots of the same pools of plasma that were previously extracted as described above and also concentrated twice were applied on the column and eluted at a flow rate of 8 ml/h with the buffer just mentioned. Fractions of 2 ml were collected and, after lyophilisation, they were resuspended in 1 ml assay buffer (0.2 mol/l glycine, pH 8.6), in order to achieve another two-fold concentration of the fractions. At that time, they were frozen at −20 °C until the determination of the glucagon immunoreactivity, using antibody A. The results had to be divided by four due to the two, two-fold concentration steps mentioned above to increase the actual glucagon concentration of each individual fraction. There were 28 fractions per run of the G-50 Sephadex column, making a total of 336 samples to be measured in duplicate since there were three basal and three stimulated pools of plasma that were both either ethanol-extracted or not. The sum of the glucagon immunoreactivity of the different eluted fractions was calculated and compared to the glucagon levels found by directly assaying one aliquot of each individual plasma pool. The yields of the chromatography thus calculated were: "low plasma" without ethanol extraction 76±3%; "low plasma" after extraction 61±4%; "high plasma" without ethanol extraction 77±3%; "high plasma" after extraction 65±4%.

Statistical analysis

All values are means ± SEM. The statistical analysis was carried out with the two-tailed Student's t-test for unpaired data.
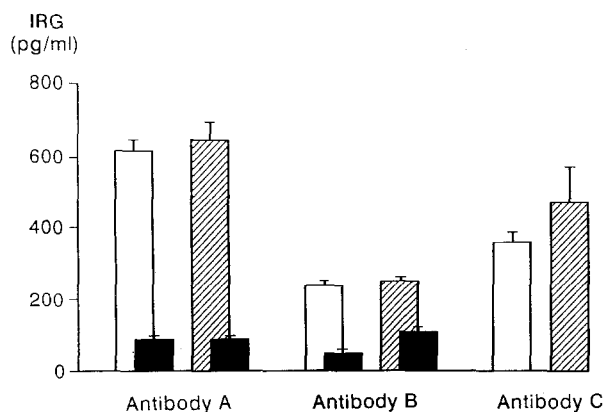
Fig. 1. Basal plasma glucagon (IRG) levels in lean (open bars) and genetically obese *fa/fa* rats (hatched bars) as measured with three different so-called "pancreatic-specific" glucagon antibodies (antibody A, B, and C) with and without an ethanol extraction of the plasma samples (ethanol extraction: black bars). The origin and specifications of these three antibodies are given in "Materials and methods". The values are means ± SEM of 5–50 measurements. For the ethanol-extracted samples, the values obtained with antibody A were: lean = 87 ± 6 pg/ml, obese = 89 ± 8 pg/ml; and with antibody B: lean = 49 ± 5 pg/ml, obese = 62 ± 7 pg/ml

Fig. 2. Sephadex G-50 gel chromatogram of basal plasma glucagon (IRG) samples (referred to as "low plasma") before (solid line) or after ethanol extraction (broken line). A 1.5 × 60 cm column of Sephadex G-50 s.f. was used after calibration with Dextran Blue (M1), ^{125}I-insulin (M2) and ^{125}I-glucagon (M3). (Yield of the procedure, see "Material and methods".) Mean ± SEM of 3 pools of basal plasma from normal rats. IRG was measured with antibody A

Results

Figure 1 shows the results of basal plasma glucagon levels in lean and genetically obese *fa/fa* rats as measured with three different antibodies, antibody A, B and C whose origin and specifications are given in "Materials and methods". As can be seen, the results obtained varied considerably according to the antibody used, as the basal glucagonaemia of lean or obese rats could either be of about 600 pg/ml (antibody A) or of about 250 pg/ml (antibody B). When, on the same plasma samples, the extraction procedure of Heding [11] was used prior to the assay, the basal glucagon levels were reduced to about 80 pg/ml and became comparable with two antibodies, namely anti-
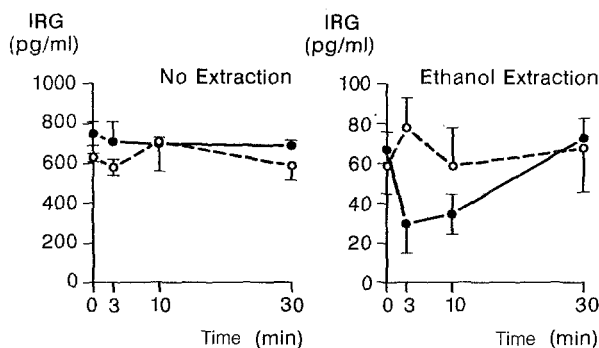
body A and B. The reason why the ethanol extracted values with antibody C are not shown by the figure is, as mentioned in "Materials and methods", that this antibody was not able to detect values smaller than 150 pg/ml making the measurement of the extracted samples impossible. Of note is the fact that the values of the extracted plasma samples of lean and obese rats (as measured with antibody A or B) were similar. To ensure that the ethanol extraction was able to selectively measure pancreatic glucagon, aliquots of three different pools of basal plasma ("low plasma") that were either extracted or not were placed on a G-50 Sephadex column. The results, illustrated by Figure 2, showed that when the non-extracted "low plasma" samples were passed through the column, glucagon-immunoreactive material was measured in a whole range of fractions (from 18 ml up to 48 ml of the eluent), thereby indicating that they contained products with high molecular weights that cross-reacted with pancreatic glucagon (M3). However, following the ethanol extraction of the same samples, glucagon-immunoreactivity was only present in three fractions that co-eluted with 125-I 3500 daltons glucagon (M3). When, after the collection of a basal blood sample, a bolus of arginine was administered, the values of non-extracted plasma glucagon levels differed considerably according to the antibody used, as did the comparison between lean and obese rats. Indeed, when using antibody A, there was no stimulation of glucagon output in either lean or obese rats (left pannel of Fig. 4). On the contrary, when antibody B was used, the arginine-induced glucagon output was higher in obese (peak value of 582 ± 76 pg/ml, with a basal value of 257 ± 13 pg/ml) than in lean rats (peak value of 326 ± 13 pg/ml, with a basal value of 243 ± 11 pg/ml). Finally, the results obtained with antibody C gave a third version of the same data since glucagon stimulation following arginine was similar in lean and obese rats (lean, peak value = 781 ± 135 pg/ml and basal value = 410 ± 29 pg/ml; obese, peak value = 850 ± 153 pg/ml and basal value = 475 ± 96 pg/ml).

Figure 3 shows the results obtained by passing arginine-stimulated pools of plasma ("high plasma") with or without prior ethanol extraction on a G-50 Sephadex column. Similarly to what was observed with the "low plasma glucagon" (see Fig. 2), it can be seen from Figure 3 that immunoreactive glucagon material was present in a whole range of fractions when no prior extraction was performed. Once the samples were ethanol-extracted, the immunoreactivity ran only in the range of molecular weights corresponding to the 3500 daltons glucagon (M3).

Using antibody A, glucagon immunoreactivity was measured before and after an arginine bolus in lean and obese rats, with or without prior ethanol extraction. The results, illustrated by Figure 4, showed that, when no extraction was performed, the glucagon-immunoreactive material behaved identically in lean and
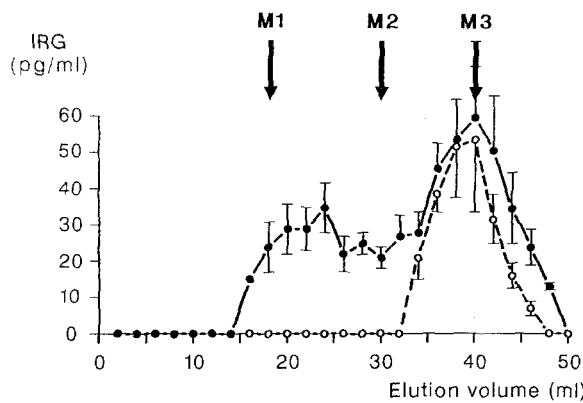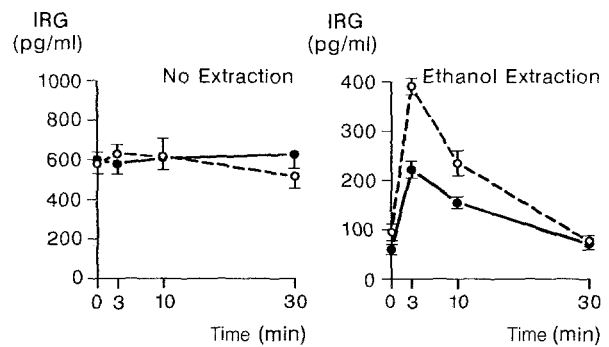
Fig. 3. Sephadex G-50 gel chromatogram of stimulated plasma glucagon (IRG) samples (referred to as "high plasma") before (solid line) or after ethanol extraction (broken line). A 1.5 × 60 cm column of Sephadex G-50 s.f. was used after calibration with Dextran Blue (M1), ^{125}I-insulin (M2) and ^{125}I-glucagon (M3). (Yield of the procedure, see "Materials and methods".) The stimulated pools of plasma were obtained from blood of anaesthetized rats, 3 min after an arginine bolus (600 mg/kg lean b.w.). Mean ± SEM of 3 pools of plasma from normal rats. IRG measured with antibody A

Fig. 4. Arginine-induced plasma glucagon (IRG) levels in lean (solid lines) and genetically obese *fa/fa* rats (broken lines) with (right panel) or without (left panel) ethanol extraction of the plasma samples. Arginine given as a bolus (600 mg/kg lean b.w.) just after time 0. IRG measured with antibody A. Mean ± SEM of 10 lean and 10 obese animals. Statistical analysis: obese statistically different from lean rats at 3 min ($p < 0.001$) and 10 min ($p < 0.02$) only after ethanol extraction

Fig. 5. Plagma glucagon (IRG) levels before and after an intravenous glucose load in lean (solid lines) and genetically obese *fa/fa* rats (broken lines) with (right panel) or without (left panel) ethanol extraction of the plasma samples. Glucose given as a bolus (200 mg/kg lean b.w.) just after time 0. IRG measured with antibody A. Mean ± SEM of 7 lean and 8 obese animals. Statistical analysis: obese statistically different from lean rats at 3 min ($p < 0.05$) only after ethanol extraction

obese rats with no stimulation following the arginine stimulus, as mentioned above. In clear contrast, after extraction, the same samples obtained from the same animals yielded different results. Basal glucagon values were lower in both lean and obese rats. In addition, following arginine administration, the plasma glucagon levels increased in both the lean and the obese group, as expected from previous work carried out on the perfused pancreas of normal rats [16], but the stimulatory effect of the amino acid was much more pronounced in obese than in lean animals. The same approach was adopted to study the effect of a glucose bolus on glucagonaemia in the two groups of animals, as shown by Figure 5. It can be seen that, when plasma samples were not extracted, glucose had no effect whatsoever whether tested in lean or obese rats. On the contrary, ethanol extracted samples of lean animals

showed a clear, transient inhibition of plasma glucagon levels, whereas such was not the case in obese animals in which glucagonaemia was not inhibited following the glucose bolus.

Discussion

To solve the controversy regarding glucagon levels in genetically obese *fa/fa* rats, three different antibodies that are said to measure specifically pancreatic glucagon have been used. The results showed that, depending on the antibody (when no prior ethanol extraction was performed), the absolute values of basal or arginine-stimulated glucagonaemia varied to a large extent in both lean and obese rats, but were always high and without consistent intergroup differences. Moreover, in these conditions and in contrast to what is known to occur in several other species [17], no inhibitory effect of glucose administration on glucagonaemia of lean rats could be detected. Previous studies have reported decreased basal plasma glucagon levels in obese rats when compared to those of their lean littermates (668 pg/ml vs 815 pg/ml) [6]. In addition, the kinetics of an arginine test performed in genetically obese rats have revealed a lack of glucagon secretion in response to the stimulus [18]. This was in keeping with the observation that non-pancreatic glucagon also responded minimally to arginine [19]. On the other hand, arginine has been shown by others to stimulate glucagon both in lean and in obese rats, the stimulation being more effective in the obese than in the lean group [20].

In the present study, it was observed that pools of non-extracted plasma passed on a G-50 Sephadex column contained glucagon immunoreactive material in a wide range of fractions with higher molecular weights than that of the 3500 daltons pancreatic glucagon. It was therefore suggested that the above-mentioned conflicting results may be related to the degree of specifici-

ty of the antibody used for pancreatic glucagon. This viewpoint was strengthened by the previous observation that the elevated pancreatic glucagon levels found in human obesity have been shown to be masked by mere measurements (i.e. without extraction procedure) of total plasma glucagon immunoreactivity [10]. Using the same Sephadex G-50 column, it was further shown that the ethanol extraction of plasma according to Heding [11] prior to the glucagon radioimmunoassays, allowed to measure the 3500 daltons pancreatic glucagon only, excluding all glucagon-like immunoreactive material of higher molecular weight. The use of this or other procedures that are able to extract the 3500 daltons molecule (i.e. acetone, polyethylene glycol extraction) is therefore necessary to measure pancreatic glucagon, even when using specific glucagon antibodies.

When using extracted plasma of lean and obese rats, the underlying pathology of the glucagon regulation in the obese group becomes evident. Thus, when ethanol-extracted plasma were used, arginine administration produced increases in glucagon output in both groups, the output being clearly greater in obese than in control animals (Fig. 4). Furthermore, the administration of a glucose bolus decreased plasma pancreatic glucagon levels in lean animals, while no such suppression of glucagonaemia was observed in the obese *fa/fa* rats (Fig. 5).

These findings are of interest when other types of obesity in animals or in humans are considered. Indeed, it has been shown that plasma glucagon levels are elevated in ob/ob mice [21, 22] and in ventromedial hypothalamic (VMH)-lesioned rats [23, 24]. Perfused pancreases from VMH-lesioned animals also oversecrete glucagon in response to an amino acid mixture used at "physiological" concentrations [25]. In this case, an ethanol extraction was also necessary to measure exclusively pancreatic glucagon as the perfusate of this preparation (that comprises a duodenal loop) was shown to also secrete glucagon-like-immunoreactive material [25]. Pancreatic glucagon has been shown to be elevated in human obesity, particularly in Type 2 (non-insulin-dependent) diabetic patients with glucose intolerance [10, 26-29]. This is in keeping with the concept of the existence of an A cell dysfunction in Type 2 diabetes that would consist in increased pancreatic glucagon levels, over-secretion of the hormone in response to arginine and lack of suppression following glucose [30]. Along this line, it is well known that obese *fa/fa* rats are glucose intolerant [31]. Recently, using the measurement of non-steady state glucose turnover, the mechanism of this intolerance has been partly unravelled by showing the existence of a transient stimulation of hepatic glucose production following the ingestion of a glucose load [32]. One may thus propose that the stimulation of the hepatic glucose production just mentioned could be related to a lack of suppression or to a transient stimulation of pancreatic glucagon. Such a hypothesis will be proven or disproven

when portal glucagonaemia is measured, a task that is hampered by the difficulty of chronically and successfully catheterising the portal vein of the rat.

To sum up, the present study shows the importance of extracting plasma samples for measuring pancreatic glucagon levels, even when using specific antibodies. It also helps to reveal the existence of dysfunctions in the regulation of glucagon secretion in the obese *fa/fa* rat, that may play a role in their abnormal glucose tolerance.

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Dr. F. Rohner-Jeanrenaud
Laboratoires de Recherches
Métaboliques
64 Avenue de la Roseraie
CH-1211 Geneva 4
Switzerland