# Pinealectomy causes glucose intolerance and decreases adipose cell responsiveness to insulin in rats

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**Lima, Fabio B., Ubiratan F. Machado, Ione Bartol, Patricia M. Seraphim, Doris H. Sumida, Solange M. F. Moraes, Naomi S. Hell, Maristela M. Okamoto, Mario J. A. Saad, Carla R. O. Carvalho, and José Cipolla-Neto.** Pinealectomy causes glucose intolerance and decreases adipose cell responsiveness to insulin in rats. *Am. J. Physiol.* 275 (*Endocrinol. Metab.* 38): E934–E941, 1998.—Although the pineal gland influences several physiological systems, only a few studies have investigated its role in the intermediary metabolism. In the present study, male Wistar rats, pinealectomized or sham-operated 6 wk before the experiment, were submitted to both intravenous glucose tolerance tests (IVGTT) and insulin binding as well as glucose transport assays in isolated adipocytes. The insulin receptor tyrosine kinase activity was assessed in liver and muscle. The insulin secretory response during the IVGTT was impaired, particularly in the afternoon, and the glucose transport responsiveness was 33% lower in pinealectomized rats. However, no difference was observed in the insulin receptor number of adipocytes between groups as well as in insulin-stimulated tyrosine kinase activity, indicating that the initial steps in the insulin signaling were well conserved. Conversely, a 40% reduction in adipose tissue GLUT-4 content was detected. In conclusion, pinealectomy is responsible for both impaired insulin secretion and action, emphasizing the influence of the pineal gland on glucose metabolism.

pineal gland; glucose transport; GLUT-4; insulin receptor tyrosine kinase; adipocytes

IT IS WELL ESTABLISHED that the response to a variety of (metabolic, hormonal, and neural) stimuli and the pattern of insulin secretion by the pancreatic β-cell vary according to the time of the day (6). In humans, the pancreatic β-cell response to a glucose challenge is more intense in the morning hours and declines as evening approaches, provided the same amount of glucose is administered (40). In rodents, on the other hand, the overall situation is basically similar, except that the daily fluctuation in response to a glucose load oscillates inversely in relation to humans (5).

The pineal gland, by synthesizing and releasing melatonin, plays an important role in the interface between the cyclic environment and the rhythmic vertebrate's physiological phenomena. The circadian release of norepinephrine by the sympathetic terminal of the superior cervical ganglia, acting on $\beta_1$- and $\alpha_1$-adrenoceptors, leads to the activation of the rate-limiting enzyme arylalkylamine *N*-acetyltransferase (or serotonin acetyltransferase), resulting in the circadian production of melatonin (35a).

The functional characteristics of the neuroendocrine system, which controls the pineal gland, make the circadian production and secretion of melatonin tightly related to the dark phase of the diurnal environmental lighting cycle. Thus the daily plasma melatonin variation (evaluated by one or several of its parameters: height, area, and the time to peak), following the profile of the environmental darkness, is able to transmit daily and seasonal timing cues to the internal milieu. This internal synchronizing role has already been demonstrated for several functions (3, 7, 9), indicating a very wide range of actions of melatonin, regulating and modulating several physiological processes.

The relationship among pineal gland, melatonin, and the regulation of carbohydrate metabolism has for a long time been suggested in both humans (1) and rodents (25). It has been demonstrated in rats that pinealectomy induces a decrease in hepatic and muscular glycogenesis and an increase in blood pyruvate concentration (25). Moreover, pinealectomy is followed by an increase in blood sugar levels, and after alloxan treatment, pinealectomized rats show a far higher increase than intact control rats (11). In addition, it has been demonstrated that pinealectomy modifies several other physiological parameters involved in carbohydrate metabolism (12). Conversely, it was reported that the infusion of pineal extracts led to hypoglycemia and increased both glucose tolerance and hepatic and muscular glycogenesis after glucose loading (25). We have shown, in isolated adipocytes from rat epididymal fat (23), that melatonin elicited an enhancement in cell sensitivity to insulin (a leftward shift in the insulin-stimulated dose-response curve) after a 4-h preincubation period.

Therefore, this study in rats investigates the effects of pinealectomy on *1*) the ability to cope with an in vivo glucose load, *2*) insulin binding and glucose transport in isolated adipocytes, and *3*) insulin receptor tyrosine kinase in liver and muscle.

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## MATERIALS AND METHODS

*Animals.* Twenty-two male Wistar rats, aged 6 wk and weighing 100–135 g, were initially anesthetized with sodium pentobarbital (Hypnol, 4.0 mg/100 g body wt ip) and subjected to pinealectomy (20) (PINX rats) or sham operation (SHAM rats). After recovery from surgery, they were placed in individual cages with food (Purina chow pellets) and water ad libitum under a 12:12-h light-dark cycle (lights on at 0600). Food consumption was measured each day and body weight every 2 days. By the 5th wk after surgery, rats were submitted to an intravenous glucose tolerance test (IVGTT). The tests were done at 8:00 AM or 4:00 PM (2 h after lights on and 2 h before lights off, respectively) in fasted animals, under anesthesia with Hypnol, as described in *IVGTT*. Blood samples were collected for glucose and insulin determinations. A week later, animals were killed under anesthesia at 8:00 AM or 4:00 PM. Blood samples were again collected for glucose and insulin determinations, and epididymal fat pads were taken off, weighed, and processed for adipocyte isolation. The cells were submitted to an insulin-stimulated 2-deoxy-D-[$^3$H]glucose uptake dose-response test and to insulin binding studies.

*IVGTT.* To submit the two groups (8 AM and 4 PM) to the same fasting condition, temporal aspects of their eating behavior were taken into account, as previously described (10) for the same condition in our animal facility room. In summary, under the lighting conditions just described, the rats concentrate their eating behavior during the dark phase, showing a bimodal pattern, with the first bout occurring immediately after lights off (6:00 PM) and the last bout occurring 2 h before lights on (4:00 AM). Therefore, we chose to withdraw the food at 8:00 PM from the animals selected to be tested at 8:00 AM and at 7:00 AM from those selected to be tested at 4:00 PM. As the latter rats were, probably, starving for about 3 h before food withdrawal, we assume that the tests accomplished in the morning and in the afternoon were preceded by an equivalent period of fasting, i.e., ~12 h.

At least five PINX or SHAM rats were tested at each scheduled time. The animals were anesthetized (Hypnol, 4.0 mg/100 g body wt ip), and an indwelling Silastic catheter was implanted in the right jugular vein for glucose injection and blood sampling. The test was performed under anesthesia and started 45 min after catheterization to reduce the stress response due to surgical procedures.

A glucose pulse (50% glucose, 50 mg/100 g body wt) was injected as a bolus, and 0.4-ml blood samples were collected at 0 (immediately before injection) and 5, 10, 15, 20, 30, 45, and 60 min after injection. The blood was heparinized and kept at 4°C before centrifugation. The obtained plasma was frozen (−20°C) until glucose and insulin determinations were performed.

*Adipocyte isolation.* After the collection of a 2.0-ml heparinized blood sample from the inferior vena cava (for determination of glucose and insulin concentration), the epididymal fat pads were excised, weighed, minced with fine scissors, and poured into a flask containing 4.0 ml of EHB buffer (Earle's salts-25 mM HEPES-4% BSA), 5 mM glucose, and 1.25 mg of type II collagenase, pH 7.40, at 37°C (32). The mixture was incubated for 30 min at 37°C in an orbital shaker (New Brunswick Scientific, Edison, NJ) at 150 rpm. The isolated adipocytes were filtered through a fine plastic mesh, washed three times with 25.0 ml EHB (20 mM HEPES, 1% BSA, no glucose, 2 mM Na pyruvate, and 4.8 mM $NaHCO_3$), pH 7.40, at 37°C, and resuspended up to a final cell concentration of 20% (vol/vol). This cell suspension was maintained in a water bath for ≥30 min before the assays for insulin-stimulated 2-deoxy-D-[$^3$H]glucose (2-DG) uptake and insulin binding. Adipocyte viability was tested with trypan blue, and cell counting was performed as described elsewhere (13).

*Insulin-stimulated 2-DG uptake.* From the 20% adipocyte suspension, 100-ml aliquots were transferred to plastic test tubes (17 × 100 mm) previously prepared with insulin diluted in EHB (pH 7.4) at 37°C in increasing amounts to achieve a final concentration of 0, 25, 50, 75, 100, 150, 250, 1,000, 2,500, or 10,000 pM (15). The final reaction volume was 400 ml, and the cells were incubated for 30 min at 37°C in a reciprocating water bath (100 strokes/min). At the end of the incubation period, a 10-ml aliquot of 2-deoxy-D-[2,6-$^3$H]glucose (Amersham International, UK; 0.1 mM final concentration and 0.12 mCi/tube) was pipetted, and the uptake reaction was allowed for exactly 3 min. After ~2 min, two 150-ml duplicates were transferred to the top of formerly set 400-ml microfuge tubes layered with 100 ml of silicone oil, and at the completion of the 3-min period the samples were centrifuged (Microfuge E, Beckman Instruments, Palo Alto, CA) for 6 s. The cell pellet on the top of the oil layer was removed to scintillation vials containing 3.0 ml of scintillation cocktail (Universol, ICN Pharmaceuticals), and the trapped radioactivity was measured. In a parallel tube, a similar amount of cells from the same source was pulsed with L-[1-$^{14}$C]glucose (0.1 mCi/tube, Amersham International) instead of 2-DG to evaluate the unspecific radiolabel trapping, because L-[$^{14}$C]glucose is not a substrate for the glucose transporters and therefore is not taken up by the cells. This value was discounted from the total trapping, and the resultant specific uptake was recalculated to be expressed as picomoles per squared centimeter per cell surface area, which is a good index of the glucose transporter population present in adipocyte plasma membrane.

*Insulin binding studies.* From the same 20% adipocyte suspension, 200-ml aliquots were transferred to 12 × 75-mm polystyrene test tubes prepared with a mixture of $A_{14}$-monoiodo-$^{125}$I-labeled insulin (10,000 counts $\cdot min^{-1} \cdot tube^{-1}$, Amersham International) and "cold" insulin in increasing concentrations (25, 50, 100, 250, 1,000, 2,500, 5,000, 10,000, and $10^6$ pmol, final concentrations) diluted in 200 ml of EHB (pH 7.80) at room temperature. The final mixture was incubated for 60 min in a water bath at room temperature (22°C), and the assay was interrupted by ice-cooling the tubes, followed by centrifugation of 150-ml aliquots through silicone oil, as described in *Insulin-stimulated 2-DG uptake*. The radioactivity trapped in the cell pellets was measured in a gamma counter (Abbott AutoLogic, Abbott Laboratories). The amount of radioactivity measured in the cell pellets incubated in the presence of 1.0 $M^{-6}$ insulin was assumed to be unspecific, and this value was discounted from the remaining tubes. Competitive binding curves and Scatchard analysis (34) were performed to determine insulin receptor number and affinity for the ligand. Nonlinear regression curves were obtained using GraphPad Prism2 software.

*Adipose tissue membrane preparation, extraction, and Western blot determination of GLUT-4.* In a parallel study designed to obtain adipose cell membranes (24), epididymal fat pads from similarly prepared PINX and SHAM animals were excised, weighed, dipped into 40 ml of TES buffer (10 mM Tris·HCl, 1 mM EDTA, and 255 mM sucrose, pH 7.40, at 4°C), and homogenized with a Polytron (PT-MR3000, Kinematica, Littau, Switzerland) set at 20,000 rpm for 30 s at 4°C. This crude homogenate was centrifuged at 3,000 *g* for 15 min at 4°C. The fat layer on top and *pellet I* (consisting of nuclei, mitochondria, and cell debris) were discarded, and the supernatant of fat-free extract was collected and recentrifuged at 12,000 *g* for 15 min at 4°C. *Pellet II*, or the plasma membrane

fraction (PM), was resuspended in the same buffer and kept frozen (−70°C) until its handling for Western blot procedures. *Supernatant II* was respun at 22,000 *g* for 15 min, *Pellet III* was discarded, and *supernatant III* was finally centrifuged at 146,000 *g* for 75 min. *Pellet IV*, a low-density microsomal fraction (M), was resuspended and frozen (−70°C). For Western blotting, PM and M were simultaneously processed.

An anti-GLUT-4 antiserum, raised against the carboxy-terminal dodecapeptide of rat GLUT-4 (developed in the Laboratory of Dr. Masayuki Saito, Department of Biochemistry, Faculty of Veterinary Medicine, Hokkaido University, Japan), was used for immunoblotting at 1:200 dilution. Equal amounts of membrane protein were solubilized in Laemmli sample buffer, subjected to SDS-PAGE (10%), and electrophoretically transferred to nitrocellulose paper. After blocking with BSA, the sheets were incubated with the anti-GLUT-4 antibody, followed by washing and incubation with $^{125}$I-labeled protein A (Amersham International). After autoradiographic exposure, the radioactive spots were excised, and their radioactivity was counted in a gamma counter.

*Insulin receptor tyrosine kinase activity determination*. In a separate experiment, 6 wk after surgery, PINX and SHAM rats were anesthetized with sodium pentobarbital (4 mg/100 g body wt), and small samples of gastrocnemius muscle and liver were saved for further procedures to extract and prepare tissue proteins for Western blot analysis of insulin receptor tyrosine kinase activity. The activity found in these tissues was considered basal activity. Soon afterward, the portal vein was exposed and injected with 1.5 U of regular porcine insulin. About 30 s after insulin injection, another sample of the liver was taken off, and ~60 s afterward, a sample of the contralateral gastrocnemius muscle was excised. Both tissues were processed to extract and measure insulin-stimulated tyrosine kinase activity.

The tissue samples were homogenized in an extraction buffer (100 mM Tris, 10% SDS, 10 mM EDTA, 100 mM NaF, 100 mM Na pyrophosphate, and 10 mM $Na_3VO_4$, pH 7.5) at 100°C with a Polytron PT-MR3000 (Kinematica) in a boiling water bath. Ten minutes later, the extracted material was transferred to 1.5-ml microfuge tubes and kept in an iced water bath; then it was centrifuged at 12,000 rpm for 40 min at 4°C. The supernatant was collected and diluted in Laemmli buffer (5:1, vol/vol) containing 200 mM dithiothreitol.

Two hundred micrograms of tissue-extracted protein were submitted to 10% SDS-PAGE minigels (25 V for 2 h followed by 120 V for 2 h), and the resolved proteins were electrotransferred to nitrocellulose membranes (120 V for 2 h), as previously described (36). The washed membranes were incubated for 4 h at room temperature with antiphosphotyrosine antibodies and then with $^{125}$I-protein A (Amersham International). The autoradiographs were analyzed by optic densitometry (Sharp JX-330 densitometer) by use of the software Image Master 1D (Pharmacia) to analyze the blots.

*Statistical analysis*. Results are expressed as means ± SE of five or six experiments for each group of animals. Comparisons of the data were made using Student's *t*-test for unpaired samples, and the differences between groups were considered significant when $P < 0.05$.

## RESULTS

*Growth profile of PINX and SHAM rats*. The growth pattern of PINX and SHAM animals was very similar; although PINX rats tended to be a little heavier, the differences were never significant along the entire period of observation. The body weights, at the moment animals were killed, were 302.3 ± 5.0 and 290.2 ± 9.3 g, and the average daily growing rate was 3.9 ± 0.1 and 3.8 ± 0.2 g, respectively, for PINX and SHAM animals. Also, no significant differences were detected between PINX and SHAM groups regarding the epididymal adipose tissue weight (2.85 ± 0.22 and 2.97 ± 0.22 g, respectively, for PINX and SHAM, $P > 0.05$) and adipocyte size (17,220 ± 490 and 18,050 ± 1,200 mm$^2$ of cell surface area, respectively, for PINX and SHAM, $P > 0.05$).

*IVGTT*. Figure 1 depicts the glycemic profiles in the tests performed at 8:00 AM and 4:00 PM. As can be seen, the blood glucose and insulin profiles of PINX and SHAM rats at 8:00 AM are very similar. On the other hand, in the 4:00 PM tests, although the basal glycemia and insulinemia were of same magnitude, blood glucose levels increased more intensely and significantly in PINX animals. During the initial 20 min, the pancreatic β-cell response was clearly enhanced in SHAM compared with PINX rats. However, the ability of the endocrine pancreas to face a glucose challenge is disturbed, even if we examine the morning tests. This phenomenon is better seen in Fig. 2. Here, the difference between the insulin concentrations at 5 and 0 min ($\Delta I_{5-0}$) was divided by the difference between the glycemia concentrations at the same times ($\Delta G_{5-0}$). When this $\Delta I_{5-0}$-to-$\Delta G_{5-0}$ ratio ($\Delta I_{5-0}/\Delta G_{5-0}$) is used as an index of the β-cell's ability to respond to a glucose load, it is clear that SHAM rats secreted more insulin in response to a comparable blood glucose variation. This maneuver demonstrated that the β-cell's ability to respond to insulin was in fact decreased in PINX rats at both 4:00 PM and 8:00 AM ($P < 0.05$).

*Insulin binding studies*. The Scatchard analysis of the insulin receptor binding experiments (Fig. 3) shows that the number of receptors increased from 8:00 AM to 4:00 PM. Both groups showed the same pattern of variation, and no differences were detected between them in consequence of pinealectomy (2.0 ± 0.4 and 4.1 ± 0.2 receptors/mm$^2$ cell surface area, respectively, at 8:00 AM and 4:00 PM for PINX rats, and 2.7 ± 0.6 and 4.2 ± 0.6 receptors/mm$^2$ cell surface area for SHAM rats). In neither case was the receptor's affinity for the ligand significantly affected.

*Insulin-stimulated 2-DG uptake studies*. Figure 4 shows the characteristic sigmoid-shaped curves of 2-DG uptake tests. In the inset, both the basal and maximal responses are depicted. As can be seen, a significant 33% decrease in maximal responsiveness occurred in the PINX group at both times of the day. On the other hand, there were no differences for the basal 2-DG uptake rates and for the $EC_{50}$ values in all studied groups.

*GLUT-4 glucose transporter status in PINX rats*. In Fig. 5, a characteristic blot obtained from a typical experiment (*top panels*) is shown, as well as results obtained from 6 different experiments pooled and expressed in counts per minute (cpm) per milligram of protein (prot) loaded per well in the electrophoresis plate. As can be seen, a persistent and generalized reduction in the GLUT-4 content was found in PINX adipose tissues (46.16 ± 13.70 vs. 10.68 ± 2.25 cpm/mg

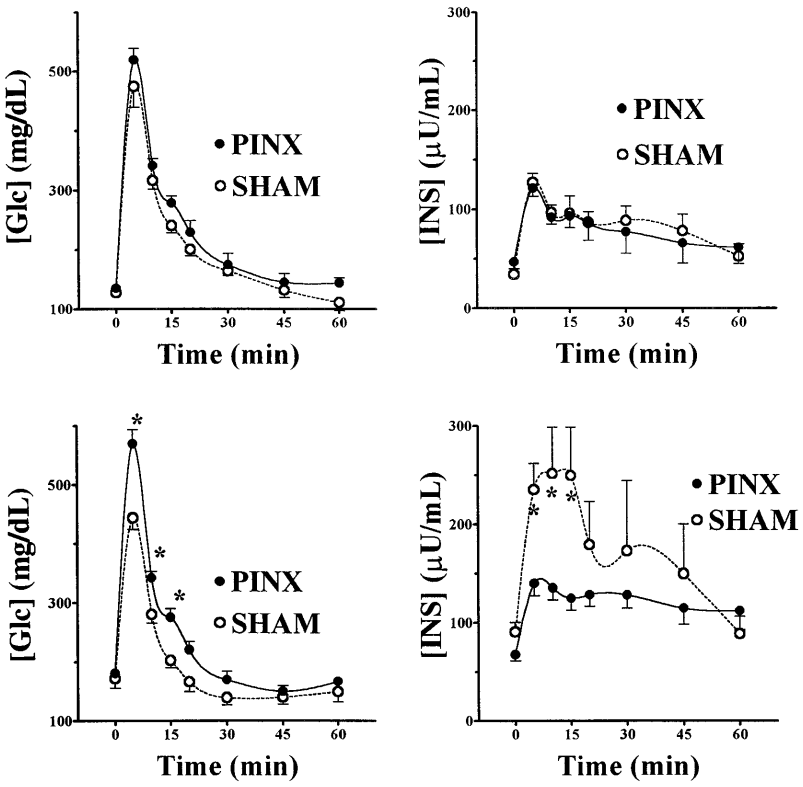


Fig. 1. Intravenous glucose tolerance test (IVGTT) results (means ± SE) in rats subjected to pinealectomy (PINX) or a sham operation (SHAM). Tests were performed with animals under pentobarbital anesthesia at *week 6* after surgery, as described in MATERIALS AND METHODS. Panels at *left* display the blood glucose profiles (Glc concn) at 8:00 AM (*top*) and 4:00 PM (*bottom*), respectively. Panels at *right* show blood insulin response (INS concn) at 8:00 AM (*top*) and 4:00 PM (*bottom*), respectively. * Significant difference between PINX ($n$ = 6) and SHAM ($n$ = 5), $P < 0.05$.

protein in PM and 110.35 ± 25.6 vs. 37.6 ± 3.88 cpm/mg protein in M fractions, for SHAM vs. PINX, $P < 0.05$).

*Insulin receptor tyrosine kinase activity.* Figure 6 depicts the results of the insulin receptor β-subunit and the IRS1 bands of liver and skeletal muscle. As can be seen, no differences in the degree of basal and insulin-stimulated phosphorylation status of both insulin receptor and IRS1 were evidenced as a consequence of pinealectomy.

## DISCUSSION

In the present study, some metabolic aspects that rats develop in consequence of pineal ablation have been characterized. The main features can be summarized as follows: *1*) loss of a daily rhythm in the pancreatic ability to respond to a glucose challenge; *2*) decrease in the in vitro responsiveness of the glucose transport system in adipocytes; *3*) a global reduction in cellular content of GLUT-4 [described here and elsewhere (35)]; *4*) insulin resistance. On the other hand, the variation in insulin receptor number, characterized by downregulation in the morning and upregulation in

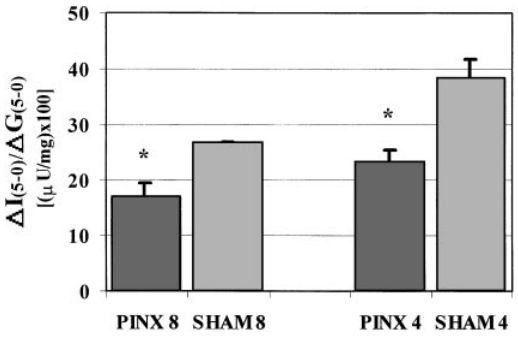


Fig. 2. Promptness of endocrine pancreas response to a glucose stimulus. Ability of endocrine pancreases to respond to a given glucose stimulation was estimated by use of the ratio of increments in insulin and glucose concentrations of blood samples collected immediately before (0) and 5 min after [$\Delta I_{(5-0)}/\Delta G_{(5-0)}$] glucose injection in each individual GTT. There was a tendency of pancreases to respond more intensely when tests were performed in the afternoon. However, this immediate response was defective even in morning tests in PINX compared with SHAM group (*$P < 0.05$). 8, 8:00 AM; 4, 4:00 PM.

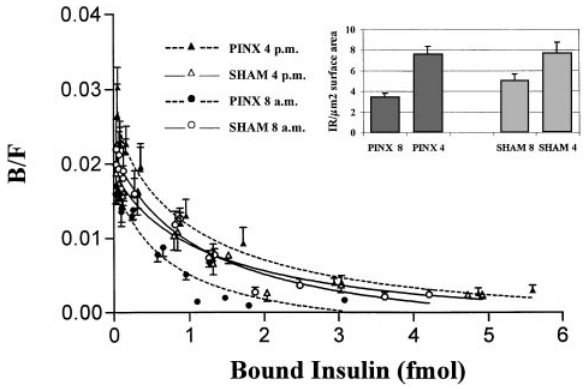


Fig. 3. Scatchard plots from insulin receptor binding tests in isolated adipocytes. Isolated adipocytes from PINX and SHAM rats in 6th wk postsurgery were submitted to insulin receptor (IR) binding tests as described in MATERIALS AND METHODS. B/F, ratio of bound to free insulin. Mean curvilinear plots were obtained from 6 PINX and 5 SHAM rats. Curves are roughly parallel, indicating that receptor's affinity for ligand did not vary in consequence of animal treatment or time of day. *Inset*: IR no. expressed per μm$^2$ of cell surface area. Although an increase in IR no. was observed in adipocytes at 4 PM compared with 8 AM regardless of the group of animals, no differences were detected between groups in consequence of pineal ablation.

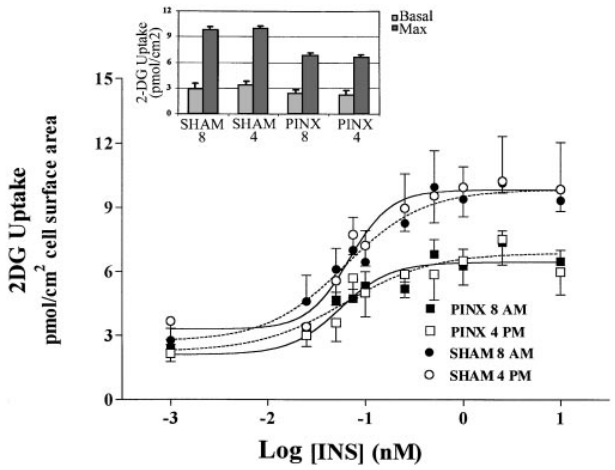


Fig. 4. Effect of pinealectomy on insulin-stimulated 2-deoxy-D-[$^3$H]glucose (2-DG) uptake rates. Isolated adipocytes from control SHAM and PINX rats were submitted to an insulin-stimulated 2-DG uptake test, as described in MATERIALS AND METHODS. Cells were obtained at 2 different times of day (8:00 AM and 4:00 PM). Each curve represents means ± SE of 6 PINX and 5 SHAM animals. *Inset*: basal and maximal values calculated from curves. Note that maximal insulin-stimulated rates of glucose transport were clearly diminished in PINX rats ($P < 0.05$). On the other hand, no statistical difference was detected in sensitivity ($EC_{50}$) to the hormone.

the afternoon, and the initial steps in the spreading of intracellular hormone signal were not disturbed by pinealectomy.

Although the development of obesity is not a common feature (33) in pinealectomized animals, it was important to check out the weight profile of our rats after surgery, because the eventual occurrence of obesity could argue against the hypothesis of insulin resistance due to the absence of pineal gland. In our case, the growth pattern, the weight of the epididymal adipose tissue, and the adipocyte size of PINX rats were very similar to that of the control animals, and no tendency to obesity was detected, at least during the 6-wk period of observation. Therefore, it seems that the differences seen in the responses to glucose load in vivo and in the cell responsiveness to insulin in vitro can be explained by the lack of the pineal gland rather than by the degree of adiposity of the animals.

It is known that several metabolic parameters, such as blood glucose levels, show a very remarkable diurnal rhythm (29). In fasted rats kept under a 12:12-h light-dark cycle, the highest blood glucose concentrations were obtained between 2.5 and 4.5 h after lights off, and the lowest values were obtained at 2.5 h after lights on (29). These data and free-running experiments point to the possible role of endogenous factors, in addition to environmental synchronizers such as food availability and the light-dark cycle, on the regulation of 24-h rhythmic fluctuations of carbohydrate metabolism. Moreover, in fasted rats kept under a 14:10-h light-dark cycle, a clear diurnal fluctuation on the plasma insulin level was shown, with values rising as the end of the light period approaches in anticipation of the main daily feeding onset (4). It should be stressed that, in the above experiment, the rise in insulinemia was preceded by a fall and not a rise in the blood glucose level, and that this circadian oscillation of insulin is, in part, independent of the feeding status (30). Furthermore, it is well known that both humans (22, 37) and rats (5) show a diurnal fluctuation on the oral and intravenous glucose tolerance tests and a clear diurnal variation in the peripheral sensitivity to insulin (16, 17).

In this study, SHAM rats exhibited a diurnal variation in the standard response of the pancreas to a glucose load. In the tests brought about at 4:00 PM, there was an intense insulin secretion during the first 20 min after the bolus injection. A response of such magnitude was not seen at 8:00 AM. On the other hand, as a consequence of pinealectomy, rats showed, in morning and afternoon tests, an insulin secretory response of the same intensity. Their profiles reproduced those of the tests performed at 8:00 AM in control

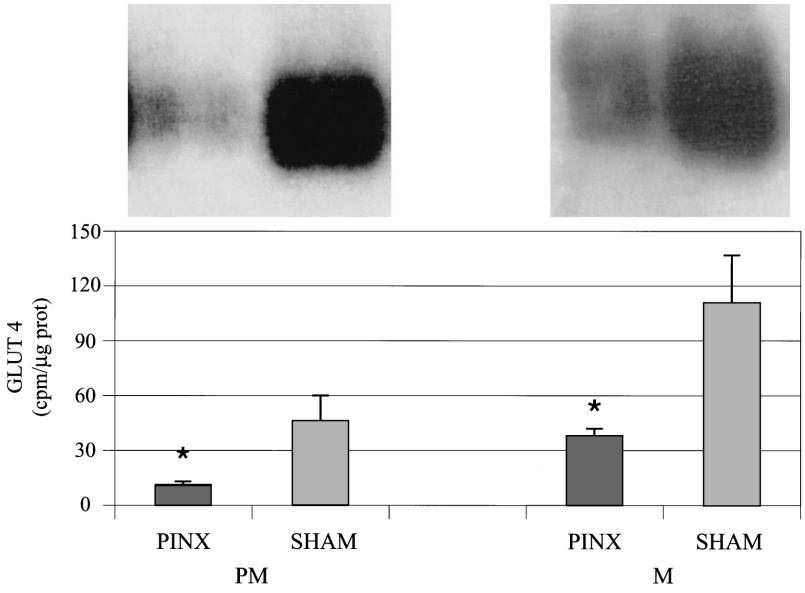


Fig. 5. Western blot analysis of GLUT-4 in plasma membrane (PM) and low-density microsomal (M) fractions of adipose tissue from PINX and SHAM rats. Two hundred micrograms of protein from PM and M of adipose tissue from PINX and SHAM rats were loaded in wells for PAGE. Gels were electrotransferred to nitrocellulose membranes, and GLUT-4 transporters were immunodetected as described in MATERIALS AND METHODS. Representative blots are depicted. *Left*: PM; *right*: M. In each blot, 1st lane is from PINX, and 2nd is from SHAM animals. Bars beneath blots represent means ± SE of 4 different experiments. *Significantly different from SHAM ($P < 0.05$).

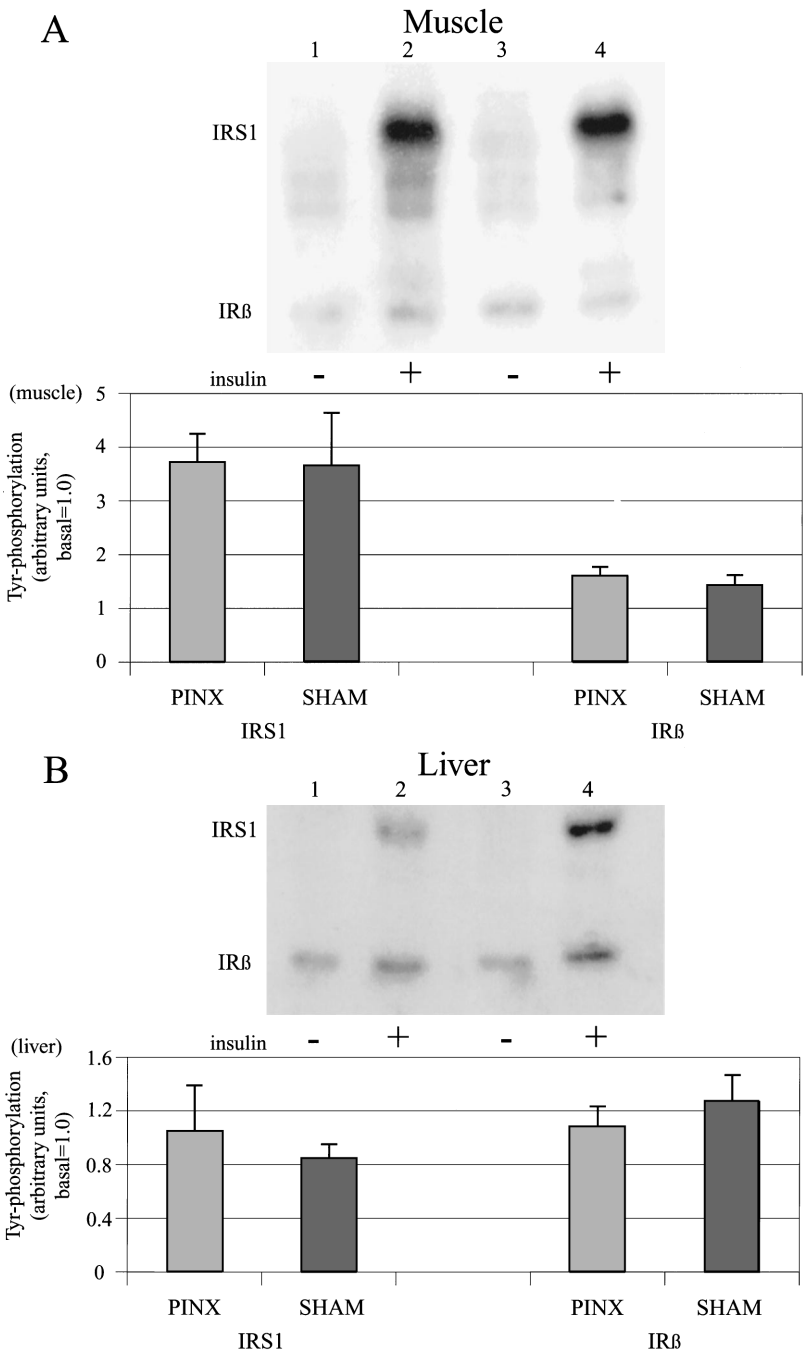


Fig. 6. Insulin receptor-stimulated tyrosine kinase activity (IRS1) in SHAM and PINX rats. *A*: typical blot of phosphorylation status of IRS1 and insulin receptor β-subunit (IRβ) in gastrocnemius muscle before (−) and after (+) intraportal insulin injection. *Lanes 1* and *2*, samples taken from SHAM rats; *lanes 3* and *4*, samples from PINX rats. *B*: same aspects of IRS1 in samples from liver of SHAM and PINX rats, with lanes corresponding to those in *A*. No differences were detected at these early steps of intracellular signal propagation between the 2 groups of animals. Graphs below blot show intensity of signal produced after insulin stimulation. In *A* and *B*, each bar shows ratio of insulin-stimulated to basal phosphorylation. There was no statistically significant difference in basal phosphorylation status between groups. Results are means ± SE of 4 different experiments.

animals. Therefore, the pancreatic response to a glucose load in PINX rats was invariant throughout the day and was also reduced compared with normal rats in the afternoon, characterizing a worsening of the β-cell responsiveness to glucose at that time of the day. Because melatonin seems to be the main putative timing molecule in several physiological systems (3, 31), and because it plays an important role in the regulation of peripheral action of insulin (23), it is reasonable to postulate that its secretion is critical for the daily regulation of the β-cell's ability to respond to glucose.

There have been some contradictory reports on the influence of pineal products on the physiology of the pancreatic islets (14, 19). The presumed action of melatonin as a regulator of β-cell responsiveness to glucose may be exerted by both direct and indirect mechanisms. It has been reported that melatonin receptors are present in the pancreas (38). Moreover, it is well known that melatonin can act on its target cells by binding to membrane-bound receptors. One of these, the $Mel_{1a}$ receptor, was shown to inhibit adenylate cyclase and potentiate phospholipase C (PLC) activity (18), leading to amplification of protein kinase C (PKC) activity. Because it has been emphasized that the PLC/PKC system is of fundamental importance for the regulation of insulin secretion (39), it is possible that the harmful consequence of pinealectomy to the regula-

tion of insulin secretion could be direct damage in the cellular processes that trigger the PLC/PKC system in β-cells. At any rate, the damage caused by pinealectomy to β-cells was profound, because, even in the morning period, we detected a significant loss of responsiveness of the endocrine pancreas to glucose (see Fig. 2).

Indirect influences of pineal activity over insulin target tissues cannot be discarded. The pineal gland can modify the functioning of some hypothalamic nuclei [the suprachiasmatic nuclei, in particular (29)], which could lead to modifications in the secretory patterns of certain related endocrine systems, such as the hypothalamus-hypophysis-adrenal axis (27), which is well established as a modulator of carbohydrate metabolism. Moreover, it has been reported that melatonin inhibited the corticosteroidogenesis stimulated by adrenocorticotropic hormone directly in the adrenal cortex; an instance of this may be that PINX rats exhibited an increased blood corticosterone concentration throughout the day compared with control animals (27). Furthermore, a modulatory influence of melatonin on cell response to glucocorticoids has already been reported (2, 26). These studies strongly suggest that the lack of the pineal gland can cause hyperfunctioning of the adrenocortical physiology, which can lead to insulin resistance.

Other indirect roles of the pineal gland can be recalled. For example, it was demonstrated that functional pinealectomy modifies both the tissue responsiveness to autonomic neurotransmitters and the level of activity of the autonomic nervous system (7, 8). Therefore, a possible inhibiting effect on both the β-cell's ability to secrete insulin and insulin target tissues to respond to the hormone in animals under pinealectomy because of modifications in the autonomic nervous activity cannot be ruled out.

The glucose transport studies showed that adipocytes from PINX rats lost their ability to maximally take up 2-DG under insulin stimulation. Because the data are expressed in picomoles per squared centimeters of cell surface area, the results are suggestive that the lost ability to transport glucose is a consequence of a diminished presence of glucose transporters in the cell surface.

Adipose cells in PINX rats seemed to be unable to maximally respond to insulin with the same effectiveness as the adipocytes from the SHAM group did, irrespective of the time of the day. The reduced ability of PINX adipocytes to respond to insulin could be due either to a defect in the translocation apparatus caused by pinealectomy or to a downregulation of the insulin-dependent GLUT-4 glucose transporter gene expression, or to both.

The present data showed that this protein species was diminished in the adipocytes of PINX rats. In a former study (35), we found a reduction of the GLUT-4 content also in some other insulin target tissues, such as heart, gastrocnemius muscle, and brown adipose fat, suggesting that the influence of the pineal over GLUT-4 seems to be a more widespread phenomenon. Therefore, an important implication of this study is that GLUT-4 production is somehow under pineal control and that the observed 40% reduction in the amount of GLUT-4 justifies, almost thoroughly, the decrease in the adipocyte responsiveness to insulin. Moreover, neither gastrocnemius muscle nor liver from PINX and SHAM exhibited any differences in the insulin-triggered tyrosine kinase activity and IRS1 phosphorylation. These data, if transposed to adipocytes, indicate that the first steps in the pathway leading to the translocation of GLUT-4 to plasma membrane would be intact. This hypothesis is reasonably plausible, because the insulin receptor number present at the cell surface in adipocytes collected in the same period of the day did not differ between PINX and SHAM rats. Therefore, the initial steps of the intracellular signal triggered by insulin seem to be running normally.

In conclusion, PINX rats developed a disturbance in the carbohydrate homeostasis that was characterized by insulin resistance and a defective insulin secretion, particularly a disruption of daily rhythm. The insulin resistance was portrayed as a loss of the ability of adipocytes to maximally transport glucose under insulin stimulation because of a global drop in GLUT-4 content. Therefore, the pineal gland, probably through the action of melatonin (23, 35), somehow interferes with GLUT-4 synthesis regulation.

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