Pinealectomy Alters Adipose Tissue Adaptability to Fasting in Rats

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This study investigated the effects of pinealectomy and fasting on rat adipose tissue metabolism, as well as on profiles of the hormones directly involved in its regulation (insulin, leptin, and corticosterone). Pinealectomized (PINX) and sham-operated (CONTROL) adult male Wistar rats were killed 6 weeks after surgery, in either fed or fasted (12 and 36 hours) states. Blood samples (for glucose and hormone determinations) and peri-epididymal adipocytes (for in vitro insulin-stimulated glucose uptake, oxidation, and incorporation into lipids) were collected. Pineal ablation decreased insulin-stimulated glucose uptake in adipocytes of both fed and fasted animals without affecting insulin-binding capacity. Pinealectomy attenuated the reduction in the ability to oxidize glucose in both basal and insulin-stimulated states during fasting. This alteration in the ability of adipocytes to oxidize glucose appeared together with a decrease in insulin-induced glucose incorporation into lipids in PINX animals. Additionally, pinealectomized rats showed higher corticosterone levels in both fed and fasted states, and a lower leptinemia with 36 hours of fasting, in comparison to CONTROLs. In conclusion, our data reinforce the hypothesis that the pineal gland has a role in the modulation of adipocyte metabolism, and its absence alters metabolic adaptation to fasting in rats.

THE PINEAL GLAND of vertebrates synthesizes and releases melatonin into the circulatory system almost entirely during the night,[1,2] and thus plays an important role in the interface between the cyclic environment and rhythmic physiological processes.[3] There is a growing body of evidence indicating melatonin involvement in the modulation of energy metabolism by direct action on peripheral tissues in some mammalian species.[4-8] In isolated adipocytes from rat epididymal fat, melatonin enhances cell sensitivity to insulin after a 4-hour preincubation period,[8] while pineal ablation lowers insulin responsiveness in these cells.[9] Additionally, it was been demonstrated that pinealectomy modifies several other physiological parameters of carbohydrate metabolism.[10-13]

In the fasting state, the organism adjusts its metabolism in order to avoid hypoglycemia. Short- and long-term mechanisms are triggered, involving the secretion of catecholamines and glucocorticoids that reflect the enzymatic activity related to carbohydrate and lipid metabolism. Despite the normoglycemia and hyperlipemia[14] observed in fasting, this state is associated with low insulin and leptin levels.[15-18] Leptin, an important hormone secreted by adipose cells, is regulated by insulin,[16,18,19] as well as cell size and the amount of stored fat.[20,21] Moreover, leptin gene expression and secretion are modulated by glucocorticoids both in vivo and in vitro.[15,22-25] However, it is unknown whether such physiological adjustments are functional in pinealectomized fasted rats.

Since melatonin has been shown to have an inhibitory effect on glucocorticoid secretion,[26] and pinealectomy is linked to hypercorticosteroidism,[27] the present study sought to evaluate the effects of pinealectomy on some adipose tissue metabolic parameters, and to analyze blood levels of insulin, leptin, and corticosterone from fed and fasted rats.

MATERIALS AND METHODS

Animals

Six-week-old male adult Wistar rats from the Animal Resource of the Institute of Biomedical Sciences of the University of Sao Paulo, weighing 100 to 135 g, were anesthetized with sodium pentobarbital (Hypnol, Cristalia, Itapira, Brazil) 4.0 mg/100 g body weight intraperitoneally and subjected to a pinealectomy or to a sham operation (PINX and CONTROL groups, respectively). After recovery from surgery (according to procedures described elsewhere[28]), the rats (3 or 4 animals per cage) were provided with food (Nuvilab balanced chow pellets, Nuvital SA, Columbo, Brazil) and water ad libitum, in a temperature-controlled room (25°C) under a 12/12-hour light/dark cycle (lights on at 7 AM). Body weight, as well as food and water consumption, was measured weekly. Eight weeks after surgery, the animals were decapitated at 8 AM in either a fed or fasted (12 and 36 hours) state. The success of the pinealectomy was verified at that time. Trunk blood was collected in heparinized tubes and used for the determination of plasma insulin, leptin, corticosterone, and glucose. The abdominal wall was opened and the peri-epididymal fat pads were excised, weighed, and processed for adipocyte isolation and metabolic studies: insulin-stimulated 2-deoxy-D-[^{3}H]glucose uptake (2DGU); incorporation of D-[U-^{14}C]-glucose into lipids; conversion of D-[U-^{14}C]-glucose into $^{14}CO_2$; and insulin binding. All procedures followed the institutionally approved protocol in accordance with the Ethical Principles in Animal Research adopted by the Institute of Biomedical Sciences Ethical Committee for Animal Research (CEEA) (no. 032/99).

Adipocyte Isolation

Epididymal fat pads were minced with fine scissors, and added to a flask containing 4.0 mL of EHB buffer (Earle's salts, 25 mmol/L HEPES, 4% bovine serum albumin [BSA]), 5 mmol/L glucose and 1.25 mg/mL collagenase type II, pH 7.4, at 37°C.[29] The mixture was incubated for 30 minutes 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 3 times with 25.0 mL EHB (Earle's

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salts, 20 mmol/L HEPES, 1% BSA, no glucose, 2 mmol/L Na pyruvate, and 4.8 mmol/L $NaHCO_3$), pH 7.4, at 37°C, and resuspended to a final cell concentration of 20% (vol/vol, corresponding to 7 to 9 $\times 10^5$ cells/mL in fed rats and 8 to 11 $\times$ 10^5 cells/mL in fasted rats). These cell suspensions were maintained in a water bath for 30 minutes before initiating the biological tests described below. Adipocyte viability was tested with trypan blue, and cell number was determined as previously described.[30]

Insulin-Stimulated 2DGU

Isolated adipocytes (20% cell suspension in EHB buffer) were incubated in the presence or absence of a maximally stimulating insulin concentration (10 nmol/L). At the end of the incubation period (30 minutes), the basal and maximal rates of 2DGU were evaluated according to protocols described elsewhere.[31]

Insulin Binding to Receptors

From the same 20% adipocyte suspension, 450 μL aliquots in EHB (pH 7.8) were transferred to 12 $\times$ 75 mm polypropylene test tubes prepared with a 10-μL mixture of A_{14}-monoiodo-^{125}I-labeled insulin (10,000 counts $\cdot$ min^{-1} $\cdot$ $tube^{-1}$; Amersham Biosciences, Sao Paolo, Brazil) in the presence or absence of "cold" insulin (0 and 1 μmol/L), in a 500-μL final reaction volume. This mixture was incubated for 180 minutes in a water bath at 16°C. The assay was interrupted by the centrifugation of 200-μL aliquots through silicone oil. The radioactivity trapped in the cell pellets was measured as described elsewhere.[31] The results measure specific insulin-binding, and are expressed as the percent of total insulin bound per 10^6 cells.

Incorporation of D-[U-^{14}C]-Glucose Into Lipids, and Conversion of D-[U-^{14}C]-Glucose Into $^{14}CO_2$

From a 20% adipocyte suspension in Krebs/Ringer/phosphate buffer pH 7.4, with 1% BSA and 2 mmol/L glucose (at 37°C) and saturated with a gas mixture of CO_2 5%/O_2 95%, 450-μL aliquots were transferred to polypropylene test tubes (17 $\times$ 100 mm), containing D-[U-^{14}C]-glucose (0.1 μCi/tube), in the presence or absence of insulin (10 nmol/L). These samples were then incubated (500 μL final volume) for 1 hour at 37°C in an orbital shaker water bath (150 rpm). The tubes had an upper-isolated well containing a loosely folded piece of filter paper moistened with 0.2 mL of ethanolamine. After incubation, the medium was acidified with 0.2 mL of H_2SO_4 (8N) and incubated for an additional 30 minutes. At the end of incubation, the filter paper was removed and placed into scintillation vials for measurement of adsorbed radioactivity. The remaining reaction mixture was treated with 2.5 mL of Dole's reagent (isopropanol, n-heptane, and H_2SO_4, 4:1:0.25 vol/vol/vol) for lipid extraction.[29] The results were expressed as nanomoles of glucose incorporated into lipids per (10^6 cells $\cdot$ h).

Plasma Hormones and Glucose Measurements

Plasma glucose determination was performed using the enzymatic glucose-oxidase/peroxidase method[32] available as a commercial kit (Glicose SL-e, CELM, Sao Paulo, Brazil). Plasma leptin and insulin levels were quantified using rat leptin and insulin radioimmunoassay (RIA) kits (Linco Research, St Charles, MO). Total corticosterone was also quantified using a RIA commercial kit (Biotrak Rat Corticosterone, Amersham Biosciences, Buckinghamshire, England).

Statistical Analysis

Statistical procedures were performed using 2-way analysis of variance (ANOVA), followed by Bonferroni post-tests for multiple comparisons among groups. *P* values less than .05 were considered statistically significant. Data are presented as means $\pm$ SEM. We used the statistical software package GraphPad Prism version 3.0 for Windows (GraphPad Software, San Diego, CA).

Table 1. Mean Values of Body Weight, Food Intake, and Adipocyte Size in CONTROL and PINX Fed and Fasted Rats

	PINX	CONTROL
Body weight (g)		
Fed	274.4 $\pm$ 5.3	270.3 $\pm$ 4.1
Fasted (36 h)	252.2 $\pm$ 6.5	252.7 $\pm$ 5.7
Fed - fasted (36 h)	22.2 $\pm$ 3.9	17.6 $\pm$ 4.3
Food intake (g/24 h)	21.7 $\pm$ 0.6	21.9 $\pm$ 0.5
Adipocyte volume (pL)		
Fed	239.6 $\pm$ 21.2	223.7 $\pm$ 21.1
Fasted (36 h)	187.8 $\pm$ 21.1	206.4 $\pm$ 20.5

NOTE. Values are mean $\pm$ SEM (n = 12 fed rats; n = 12 fasted [36 h] rats). None of these comparisons were significant using a 2-way ANOVA.

RESULTS

General Features of PINX and CONTROL Groups

Body weight, food intake, and adipocyte volume were not significantly different ($P > .05$) between PINX and CONTROL fed animals. Fasting (36 hours) promoted a similar ($P > .05$) reduction in body weight for both PINX and CONTROL ($\pm$8%). Adipocyte volume after 36 hours of fasting was also not statistically different ($P > .05$) among PINX and CONTROL animals (Table 1).

Insulin-Stimulated 2DGU and Insulin-Binding Studies

Figure 1 demonstrates the effects of pinealectomy and fasting on 2DGU. The fasting state minimized the basal ($P < .0001$) and the maximal rates ($P < .0001$) of glucose transport in control animals. Pinealectomy provoked an additional decrease in maximal insulin-stimulated uptake in fed and fasted (12 and 36 hours) animals ($P = .0003$). Additionally, the difference between maximal and basal 2DGU, which represents cell responsiveness to insulin, was estimated. The ability of adipocytes from control rats to respond to insulin was higher than that seen in adipocytes from PINX in both the fed and fasted states. These reductions indicate that the pinealectomy reduced the range of the cell responsiveness to insulin. The insulin-binding studies demonstrated that pineal ablation had no effect on the maximal capability of adipocytes to bind insulin under both fasting and fed conditions (4.7 $\pm$ 0.7 *v* 4.6 $\pm$ 0.4 percent bound ^{125}I/10^6 cells, n = 7, respectively, for PINX and CONTROL fed animals, and 4.6 $\pm$ 0.3 *v* 4.7 $\pm$ 0.5 percent bound ^{125}I/10^6 cells, n = 6, respectively, for 36-hour fasted PINX and CONTROL animals).

Incorporation of D-[U-^{14}C]-Glucose Into Lipids and the Conversion of D-[U-^{14}C]-Glucose Into $^{14}CO_2$

During fasting, a progressive fall in oxidative activity occurred in both groups of animals (Fig 2A). However, there was a notable increase in the basal glucose oxidation rate in adipocytes from PINX animals; a similar pattern of response to insulin stimulation was also observed ($P < .05$). In addition, in the fed state, pinealectomy increased the amplitude of response to insulin (the difference between the maximal and the basal

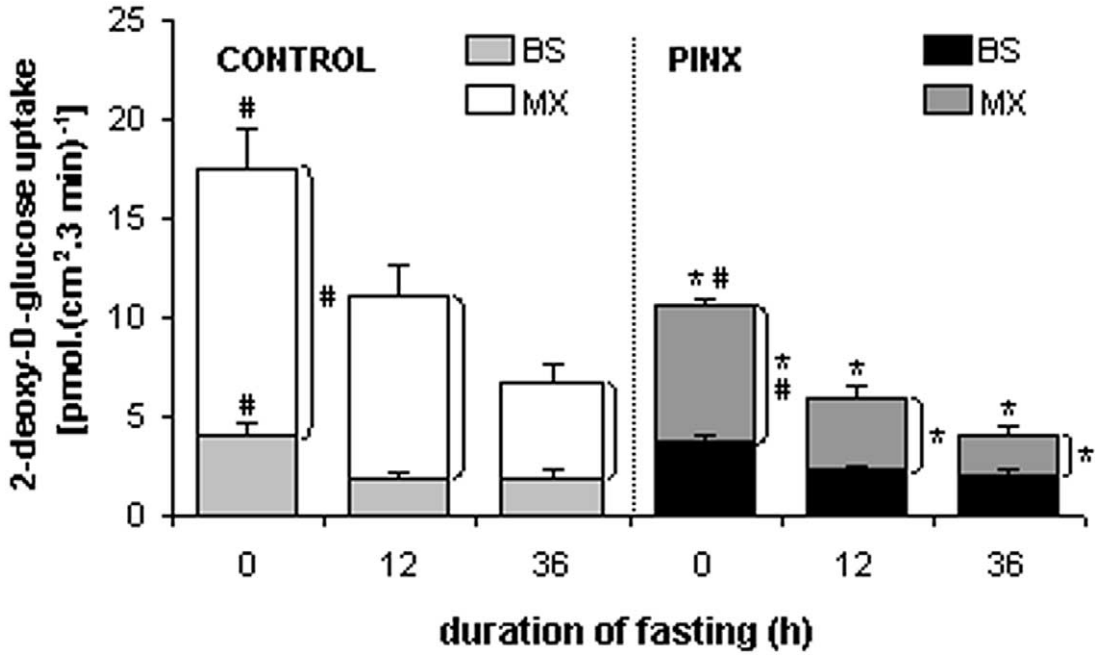

Fig 1. Influence of pinealectomy and fasting on [^{3}H]-2-deoxy-D-glucose uptake in isolated adipocytes. The differences between maximal (MX) and basal (BS) rates (corresponding to the area limited by the maximal and basal bars) illustrate the responsiveness to insulin, and are represented by the brackets to the right side of the bars. Values are mean ± SEM, n = 7. *$P < .05$, PINX *v* CONTROL under the same fasting duration; #$P < .05$, fed *v* 12- and 36-hour fasted rats within the same group.

rates) in terms of the ability of the adipocytes to oxidize glucose ($P = .0003$).

The results of glucose incorporation into lipids (Fig 2B) revealed that the basal rates were similar in fed state between PINX and CONTROL and were reduced by approximately 40% during fasting, with no significant differences between the groups. Also, among fed (PINX and CONTROL) animals, there were no differences in adipocyte responsiveness to insulin. On the other hand, the ability of adipocytes to respond to insulin increased during fasting in CONTROL but not in PINX animals ($P = .002$). In these animals, in fact, a visible reduction in the maximal insulin-stimulated rate of glucose incorporation into lipids was detected when compared to CONTROL. By means of 2-way ANOVA statistical analysis, comparison of the groups (PINX and CONTROL) against fasting time revealed that both factors interacted significantly to produce the observed differences ($P = .03$).

Plasma Hormones and Glucose Measurements

Figure 3A shows that plasma glucose levels were not significantly altered by pinealectomy. In 36-hour fasted animals, plasma glucose levels were reduced by approximately 20% in relation to fed animals independently of their pineal status. A similar reduction was observed in plasma insulin levels, with a more pronounced fall (~46%) after 36 hours of fasting (Fig. 3B). In the fed state, leptin levels did not differ between the groups. After 36 h of fasting; however, CONTROL rats presented a reduction of 78% in leptinemia, while pinealectomy reduced leptin levels (98%) over and beyond the effects of fasting ($P = .006$, Fig 3C). A 2-fold increase in the circulating levels of corticosterone was observed in PINX fed rats. Additionally, corticosterone was 56% higher in PINX compared to CONTROL fasted animals ($P < .05$, Fig 3D).

DISCUSSION

In this study, we investigated whether pinealectomy could influence the metabolic adaptations usually observed under fasting conditions. Previously, we had demonstrated a fall in insulin-induced glucose uptake in fed PINX rats.[9] It is well established that fasting reduces insulin responses by 50% in intact rats, as evaluated by glucose transport in adipocytes.[33] Our results show a more striking reduction in insulin-stimulated glucose uptake rates during fasting in PINX rats, and indicate that the glucose transport system was affected by the pineal ablation, and that this effect occurred without any corresponding interference with insulin binding capacity.

As corticosterone (a classical insulin counter-regulatory hormone) markedly increases in a fasting state,[34,35] we examined corticosterone levels in PINX rats in both fed and fasting situations. Our studies showed that fed animals subjected to a pinealectomy demonstrated an increase in plasma corticosterone levels and, when submitted to a fasting period, presented an even greater increase in corticosterone levels as compared to the CONTROL group (Fig 3D). These results suggest that melatonin has an important effect on the hypothalamic-pituitary-adrenal (HPA) axis, and is a negative corticosterone modulator in normal as well as stress situations. This view is strongly supported by a recent in vitro study describing melatonin functional MT1 receptors in primate adrenal gland cortex

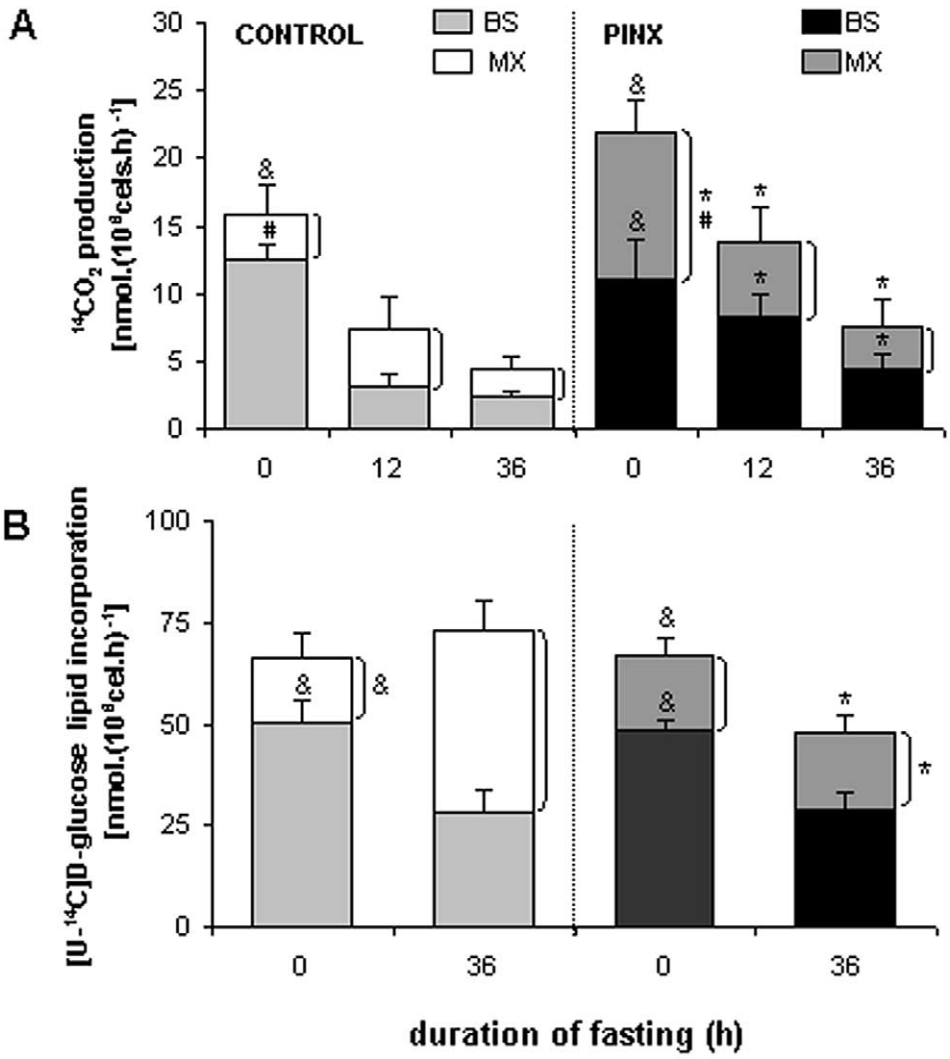

Fig 2. Influence of pinealectomy and fasting on (A) CO_2 production from [U-^{14}C]-glucose and (B) [U-^{14}C]-glucose incorporation into lipids in isolated adipocytes. The differences between maximal (MX) and basal (BS) rates (corresponding to the area limited by the maximal and basal bars) illustrate the responsiveness to insulin, and are represented by the brackets to the right side of the bars. Values are mean ± SEM, n = 7. *$P < .05$, PINX *v* CONTROL under the same fasting duration; $^{\#}P < .05$, fed *v* 12- and 36-hour fasted rats within the same group; $^{\&}P < .05$, fed *v* 36-hour fasted rats within the same group.

and their inhibitory effect on corticotropin-stimulated cortisol production.[26]

Glucocorticoids stimulate leptin release (as mentioned above), while agents that elevate cyclic adenosine monophosphate (cAMP) inhibit it.[25,36-40] In addition, other studies have shown a relationship between the pineal gland and serum leptin levels.[41,42] In the present study, the higher increase in corticosterone levels detected in 36-hour fasted PINX rats did not prevent a more abrupt fall in serum leptin levels than that detected in CONTROL rats. Indeed, the pinealectomy interacted synergistically with fasting to provoke a more accentuated fall in leptinemia levels.

These results suggest that the pineal gland plays a crucial role in corticosterone-induced leptin secretion during fasting. As melatonin (the major secretion product of the pineal gland) acts directly on adipocytes (through the specific receptors MT1 and MT2) by inhibiting adenylyl cyclase (and, consequently, by decreasing cAMP generation[5-7]), we hypothesize that pineal ablation may attenuate the melatonin inhibitory effect of intracellular cAMP generation, resulting in suppression of the stimulatory effect of glucocorticoids on leptin synthesis. Nonetheless, there is also evidence indicating a correlation between melatonin action and leptin plasma levels,[43-46] although these findings are controversial. However, none of these studies investigated the relationship between these 2 hormones in fasting animals.

Our studies of glucose oxidation revealed that pinealectomy increased adipocyte responsiveness to insulin in fed animals. During fasting, a reduction of glucose oxidation in CONTROL rats was observed, similar to the results of another study.[33] However, this reduction was attenuated by pinealectomy. Measurements of the rate of glucose incorporation into lipids demonstrated that fasted CONTROL rats were able to sustain the same maximal insulin-stimulated rates of glucose incorporation as fed animals, although their basal rates of triacylglycerol synthesis decreased. Thus, this particular response was not affected by fasting, indicating that the phenomenon of insulin resistance does not necessarily encompasses all aspects of the pleiotropic action of this hormone. On the other hand, the 36-hour fasted PINX rats did not achieve the same maximal

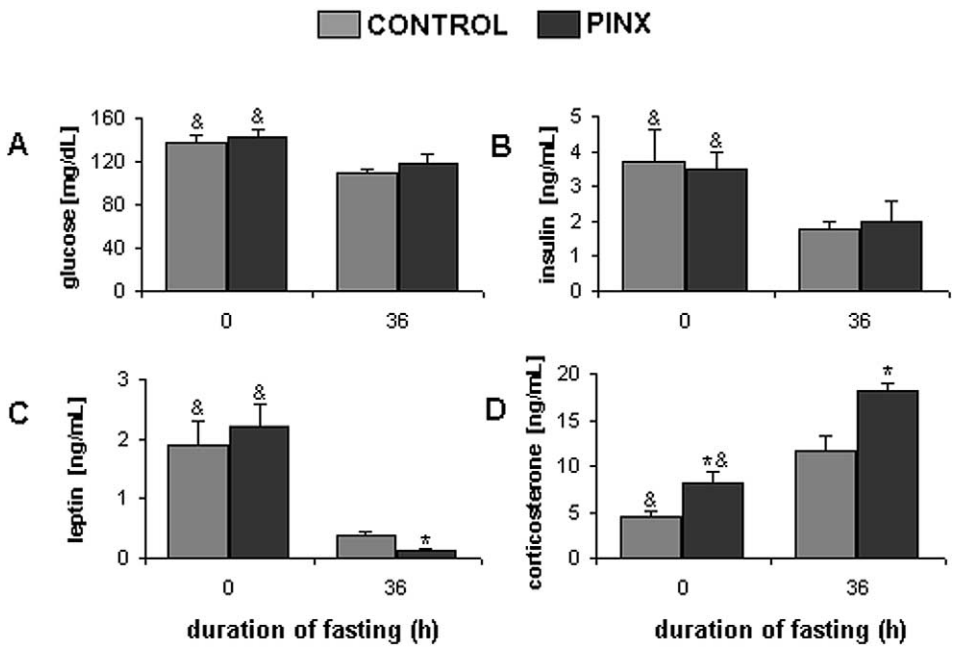

Fig 3. Plasma (A) glucose, (B) insulin, (C) leptin, and (D) corticosterone levels in fed and 36-hour fasted CONTROL and PINX rats. Two groups of animals were decapitated and blood was collected for the determination of plasma glucose and hormone levels. Bars represent the mean ± SEM, n = 7. *$P < .05$, PINX *v* CONTROL under the same fasting duration; &$P < .05$, fed *v* 36-hour fasted rats within the same group.

rates of glucose incorporation into lipids. Despite these differences in the metabolic fate of glucose, insulin receptor-binding capacity was unaffected by pinealectomy in both fed and fasted rats.

It is possible that the decreased triacylglycerol synthesis observed in fasted PINX rats may be the result of a defective fatty acid synthetic pathway, with decreased rates of re-esterification, as well as more intense lipolysis due to the elevated plasma corticosterone levels. Possibly, both aspects—a higher corticosterone level and the absence of melatonin—play a role in these effects. More detailed study need be performed to fully understand the mechanisms of the triacylglycerol biosynthesis in adipocytes, and processes underlying this anabolic pathway may help to elucidate the apparent reduction of adipocyte size observed in PINX rats.

Our results of the effects of pinealectomy and fasting on glucose incorporation into lipids, insulin-induced glucose uptake, and insulin-induced glucose oxidation suggest that pinealectomy readjusts anabolic responses to a lower level in adipocytes, and that this response is even greater in fasting rats. This effect was not due to a decrease in insulin levels during fasting, since it was similar in both groups. Likewise, we did not observe any differences in glycemic levels between PINX and CONTROL animals in fed and fasted states.

The striking elevation of the plasma glucocorticoid levels in PINX may be acting as a limiting factor to insulin's ability to stimulate triacylglycerol synthesis in adipocytes during fasting. Nevertheless, as MT1 and MT2 functional melatonin receptors are present in human and rat adipocytes, and in vitro treatment of adipocytes with melatonin inhibits isoproterenol-induced lipolysis,[6] we must consider melatonin's lack of a direct effect on adipocytes as one of the causes of the low anabolic state observed in this study.

In conclusion, our study demonstrated that pineal ablation provoked insulin resistance (as measured by the 2DGU test) in rats, which was impaired throughout the fasting period. Pinealectomy also intensified the activity of the HPA axis and reduced plasma leptin levels in 36-hour fasted rats. Fasting and pinealectomy together inhibited insulin-induced triacylglycerol synthesis, and stimulated oxidative activity, reducing the anabolic state of the adipocytes. These data reinforce the hypothesis that the pineal gland modulates adipocyte metabolism, and that its absence alters metabolic adaptation to fasting in rats. Taken together, all of these features compose the syndrome of pineal failure.

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