

Calorie restriction reduces pinealectomy-induced insulin resistance by improving GLUT4 gene expression and its translocation to the plasma membrane

Abstract: The present study aimed to investigate insulin sensitivity and GLUT4 expression protein in pinealectomized rats, as well as to determine the effects of melatonin and calorie restriction on the changes induced by pinealectomy. Wistar rats were pinealectomized (Pinx) or sham operated (Sham), and studied 30 days later. Melatonin replacement treatment (50 g/100 g body weight) was continued for 30 days after pinealectomy. Calorie restriction was performed by offering 60% of the standard food intake. In vivo insulin sensitivity was evaluated using the glucose disappearance constant (kITT) during an insulin tolerance test, and GLUT4 mRNA and protein were assessed by Northern and Western blotting, respectively. The in vitro effect of melatonin on GLUT4 protein content in plasma membrane was investigated in adipocytes isolated from intact rats. Compared with Sham rats, Pinx rats showed decreased kITT (40%), GLUT4 expression in white adipose tissue (WAT, ~70%), and unchanged GLUT4 expression in skeletal muscle. Melatonin treatment in Pinx rats restored the kITT and GLUT4 protein to control values. No in vitro effects of melatonin (10^{-9} M) upon GLUT4 protein were observed. Calorie restriction of Pinx rats increased their kITT value (~40%), total GLUT4 protein content (~240%) and its translocation to the plasma membrane (~80%) in WAT. The results show that pinealectomy, for lack of melatonin, decreased insulin sensitivity as well as GLUT4 gene expression. Calorie restriction improved insulin sensitivity in Pinx rats, and this was related to increased GLUT4 gene expression and insulin-induced GLUT4 translocation to the plasma membrane in WAT.

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Introduction

Central nervous system (CNS) control upon the energy balance was clearly demonstrated years ago [1], and in this process the sympathetic nervous system (SNS) seems to play a key role [2]. Recently, several neuro-hormonal systems have been described as being involved in energy balance and/or intermediary metabolism and homeostasis, acting directly or even throughout the SNS [3]. Most of these systems operate by balancing nutrient intake and energy expenditure, and may be related to the development of obesity [3]. The actions on energy expenditure involve changes in the non-shivering thermogenesis by brown adipose tissue and glucose disposal and shivering thermogenesis by skeletal muscle [4].

The pineal gland, through its secretory product melatonin, is believed to take part in the energy balance control of hibernating mammals [5]. In rats, the role of the pineal gland in the energy balance also has been reported [5, 6]. A diurnal rhythm in plasma glucose is known [7, 8], and it may be related to the diurnal rhythm of melatonin secretion

and/or a suprachiasmatic nucleus control upon the hepatic glucose production [8]. However, the peripheral mechanisms have not been clearly demonstrated.

Insulin resistance is a feature of pinealectomy in rats; this is shown in vivo by the presence of glucose intolerance, and in vitro by decreased adipose cell responsiveness to insulin [9, 10]. A direct effect of melatonin on sensitization of peripheral tissues to insulin action was also suggested by the observations that EC_{50} of insulin stimulated [3H]-2-deoxy-glucose uptake in isolated adipocytes previously incubated with melatonin shifted to the left [11]. However, the possibility that the pinealectomy-induced effects involve changes in CNS was not precluded. It is known that pinealectomy does not induce obesity [9, 10], indicating that induced insulin resistance is a direct consequence of ablation of the gland, probably mediated by the reduction in melatonin.

The main glucose transporter expressed in insulin-sensitive tissues is GLUT4 [12], and it plays a key role in the glucose utilization by these tissues. GLUT4 is involved in several states of altered glycaemic homeostasis such as

diabetes mellitus [13], obesity [14] and loss of weight [15]. In pinealectomized (Pinx) rats, reduction of GLUT4 protein content in white adipose tissue (WAT) was already reported as being involved in the decreased adipocyte sensitivity to insulin [9].

The present study was designed to clarify mechanisms involved in role of melatonin in glucose homeostasis, focusing on the effect of calorie restriction and melatonin replacement in Pinx rats, and using the GLUT4 protein as a molecular marker of the peripheral capacity of insulin-sensitive tissues to take up glucose.

Material and methods

Animals treatment

Male Wistar rats (body weight ~100 g) were pinealectomized or sham operated (Sham) as previously reported [9, 10] and studied 30 days later. The animals were kept on a 12:12 hr light–dark cycle (lights on at 06:00 hr), and fed with standard rodent chow (NUVILAB, Nuvital, Brazil; containing calories of 59% carbohydrate, 9.5% lipid, and 31.5% protein). Food intake was monitored daily in Sham and Pinx rats, and groups of calorie-restricted rats were prepared by offering them 60% of the amount of food intake measured in the ad libitum fed rats. Additional group of Pinx rats was daily injected with melatonin (Pinx + Mel) or vehicle solution (Pinx) for 30 days after pinealectomy. Melatonin was dissolved in ethanol (1 mg/40 μ L) and further diluted in saline (0.09% NaCl wt/vol) to give a final concentration of 100 μ g/mL (0.4% ethanol). Vehicle solution was 0.4% ethanol saline. The solutions were freshly prepared, and daily intraperitoneal (i.p.) injections (50 μ g/100 g body weight of melatonin, or the same volume of vehicle solution) were given at 18:00 hr. On the day of the experiments, all procedures were performed under anesthesia (sodium pentobarbital, 50 mg/kg i.p.), except pineal gland withdrawal, which was performed after decapitation. The experimental protocol (protocol # 017/2000) was approved by the Ethical Committee for Animal Research (CEEA) of the Institute of Biomedical Sciences, University of São Paulo.

General characteristics of the animals

Body weight and food intake were monitored daily. Before being killed, blood samples were taken from inferior vena cava for plasma analysis of glucose, insulin and albumin concentration. Epididymal WAT and gastrocnemius muscle (skeletal muscle) were removed from anesthetized animals, weighed, immediately frozen in liquid nitrogen, and stored at -70°C for further analysis of GLUT4. The animals were killed by anesthetic overdose, and all procedures were performed at noon, after 6 hr of food deprivation. Plasma samples were assayed for glucose by the glucose oxidase method (Analisa Diagnostica, Belo Horizonte, Brazil), for insulin by RIA (Coat-A-Count; Diagnostic Products Corporation, Los Angeles, CA, USA), and for albumin (Analisa Diagnostica). The glucose/insulin ratio (G/I) was calculated as previously described [16], and the insulin tolerance test was performed as described below.

Intravenous insulin tolerance test (ivITT)

A silastic catheter was implanted into the right jugular vein of the animals for insulin injection and blood sampling. The test was performed under anesthesia and began 15 min after catheterization in order to reduce the stress response resulting from the surgical procedures. Regular insulin (Iolin; Biobras, Montes Claros, MG, Brazil) pulse (0.75 U/kg) was injected as a bolus and 0.4 mL blood samples were collected at 0 (just before injection), 4, 8, 12 and 15 min after injection and the glucose concentration was measured using a glucometer (Precision QID; Abbott Laboratories Medicine Products, Bedford, MA, USA). The results were analyzed by comparing the glucose disappearance constant (kITT) from 0 to 15 min of the test.

Chromatographic detection of melatonin

Groups of ad libitum and calorie-restricted animals were killed by decapitation at 17:45, 20:00, 22:00, 24:00, 03:00 and 05:45 hr. The pineal glands were removed and kept at -70°C for later determination of their melatonin content. Melatonin was assayed by high-performance liquid chromatography as previously described [17]. Briefly, each gland was sonicated in a solution of perchloric acid, 0.02% ethylenediaminetetraacetic acid (EDTA) and 0.02% sodium disulfide, centrifuged, and the clear supernatant was injected (20 μ L loop) into the chromatographic system (Waters, Milford, MA, USA) using 30% methanol in acetate buffer as the mobile phase. The total run time was 10 min and the elution time was about 6 min.

In vitro studies in isolated adipocytes

Adipocytes were isolated from intact (non-sham operated) ad libitum-fed rats as previously described [9, 11]. Briefly, the epididymal fat pads were excised, weighed, minced with fine scissors, and poured into a flask containing 4 mL of EHB buffer [Earle's salts 25 mM, Hepes (hydroxy-ethyl-piperazine-ethane-sulfonic acid), 4% BSA (bovine serum albumin)], 5 mM glucose, 1.25 mg type II collagenase, pH 7.4 at 37°C . The mixture was incubated for 30 min at 37°C in an orbital shaker (New Brunswick Scientific Co., Inc., Edison, NJ, USA). The isolated adipocytes were filtered through a fine plastic mesh, washed three times with 25 mL EHB (20 mM Hepes, 1% BSA, no glucose, 2 mM sodium pyruvate, 4.8 mM NaHCO_3), pH 7.4 at 37°C and resuspended up to a final cell concentration of 20% (v:v). Adipocyte viability was tested with trypan blue, and cell counting was performed as described elsewhere [18]. The 20% adipocyte suspension was divided (10 mL aliquots) and incubated either with vehicle (Bs), 10^{-8} M insulin plus vehicle (Ins) or 10^{-8} M insulin plus 10^{-9} M melatonin (Ins + Mel), at 37°C for 30 min in a water bath and the assay was interrupted by ice-cooling the tubes. The cell suspension was immediately washed three times in buffer free of insulin and melatonin, and finally resuspended in 10 mM Tris-HCl, 1 mM EDTA, and 250 mM sucrose, pH 7.4 buffer. The cells were disrupted by sonication, and fractionated to obtain a plasma membrane fraction as described below. In another study, 20% adipocyte

suspension was divided into 20 mL aliquots and incubated with vehicle (V) or 10^{-9} M melatonin at 37°C in atmosphere of O₂:CO₂ (95:5, v/v) for 4 hr. Thereafter, the cell suspension was washed, divided into two tubes, and additionally incubated for 30 min with or without 10^{-8} M insulin. After this incubation, the cell suspensions were processed as described above. Three to four different experiments were performed, and the plasma membrane fractions were used for GLUT4 protein analysis.

Membrane preparation from tissue sample and cell suspension

Two subcellular membranes fractions were prepared as previously described [14, 15, 19]. Briefly, WAT was homogenized using a Polytron (Brinkmann Instruments, Westbury, NY, USA) for 30 s at 4°C in 10 mM Tris-HCl, 1 mM EDTA, and 250 mM sucrose, pH 7.4 buffer. Homogenates from WAT and adipocyte cell suspension were centrifuged at 3000 g for 15 min. Fat cakes were discarded, and the infranatant, a fat-free extract, was centrifuged at 12,000 g for 15 min. The pellet was resuspended as a plasma membrane fraction. The supernatant was centrifuged at 28,000 g for 15 min, the pellet was discarded and the supernatant was centrifuged at 146,000 g for 75 min. The final pellet was resuspended as a microsomal fraction. This procedure was previously checked by measuring the plasma membrane marker 5'-nucleotidase enzyme activity [20]. Skeletal muscle was processed differently [14, 15, 19]. The tissue was homogenized in the same buffer, and centrifuged at 1000 g for 10 min. The supernatant was saved. The pellet was resuspended in 1/3 of the initial volume, and centrifuged again at 1000 g for 10 min. Both supernatants were mixed and submitted to a 150,000 g centrifugation for 75 min. The final pellet was resuspended as a total membrane fraction. The total protein concentration of membrane samples was assayed by Lowry's method, and used for GLUT4 protein analysis by Western blotting.

RNA extraction

Total RNA was extracted from the 0.1 g of skeletal muscle or 0.5 g of WAT tissue samples, using Trizol[®] reagent (Gibco BRL, Grand Island, NY, USA) according to manufacturer's instructions.

Western blotting

Immunoblotting was carried out as previously described [14, 15, 19]. Briefly, equal amounts of membrane sample proteins were solubilized in Laemmli's sample buffer, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10%), and electrophoretically transferred to nitrocellulose membrane. After blocking with BSA in phosphate-buffered saline, the sheets were subsequently incubated with the antiserum anti-GLUT4 during 3 hr at 37°C, washed five times, and incubated with [¹²⁵I]protein-A (Amersham Biosciences, Amersham, UK) during 2 hr at room temperature. Then, the membranes were washed and exposed to X-ray film for 5 days. The blots were quantified by densitometry (Image Master 1D[®],

Pharmacia Biotech, Upsalla, Sweden), and the results were expressed as arbitrary units in microgram total protein subjected to electrophoresis. Taking into account the total protein yield, and considering the respective tissue weights, results were also expressed as arbitrary units per gram tissue.

Northern blotting

RNA samples (20 µg) were first separated by electrophoresis in 1.2% agarose gel containing 2.2 M formaldehyde, and then blotted onto a nylon membrane by neutral capillary transfer for 18 hr. After fixing the membrane and blocking with prehybridization buffer (0.5 M NaPO₄, pH 6.8, 1% SDS, 1% BSA, 1 mM EDTA, 100 µg/mL denatured salmon sperm DNA) for 3 hr at 65°C, hybridization was carried out by adding a [³²P]-labeled rat GLUT4 cDNA probe ($1-2 \times 10^6$ cpm/mL) and further incubation for 18 hr at 65°C. Then, the membrane was washed [two washes with washing buffer 1 (0.1% SDS, 2 × SSC) for 5 min at 25°C, and one wash with washing buffer 2 (1% SDS, 0.1 × SSC) for 15 min at 50°C] and exposed to autoradiography. After that, the membrane was stripped and rehybridized with [³²P]-labeled cDNA probes of human β-actin, followed by another autoradiography. The blots were analyzed by densitometry (Image Master 1D[®]; Pharmacia Biotech), and the obtained arbitrary units for GLUT4 were normalized by the corresponding β-actin value, and expressed as arbitrary units per microgram total RNA.

Data analysis

All values are reported as mean ± S.E.M., and the number of animals or of experiments is indicated in tables and figures. Data were analyzed by Student's *t*-test or by ANOVA (Student-Newman-Keuls as a post-test) to compare two or more groups, respectively.

Results

Pinx rats did not become obese considering their body weight and epididymal fat pad mass (Table 1). On the contrary, Pinx rats had increased plasma insulin ($P < 0.05$), decreased G/I index ($P < 0.01$) and decreased glucose disappearance constant (kITT) during the insulin tolerance test ($P < 0.05$), clearly showing the insulin resistance condition (Table 1).

As seen in Fig. 1, GLUT4 gene expression was reduced in WAT of Pinx rats. The GLUT4 mRNA and protein contents were reduced ($P < 0.05$) by ~70% in Pinx rats. On the contrary, both the GLUT4 protein and mRNA were unchanged in skeletal muscle of Pinx rats (Table 1).

The metabolic and hormonal changes observed in Pinx rats were completely restored after 30-day melatonin treatment (Table 1), and this was accompanied by an increase ($P < 0.05$) in membrane GLUT4 protein content in WAT, to a value similar to Sham rats (Fig. 2A).

Although chronic *in vivo* treatment of Pinx rats with melatonin restored the impaired GLUT4 protein expression in WAT, no melatonin effect was observed upon GLUT4

Parameter	Sham	Pinx	Pinx + Mel
Body weight (g) (n = 10)	193 ± 5	206 ± 7	206 ± 8
WAT weight (g) (n = 4)	1.38 ± 0.15	1.26 ± 0.13	1.09 ± 0.06
Skeletal muscle weight (g) (n = 4)	0.81 ± 0.06	0.74 ± 0.16	0.78 ± 0.12
Glycemia (mg/dL) (n = 10)	119 ± 5	108 ± 6	119 ± 2
Insulinemia (μ U/mL) (n = 6 to 10)	21.8 ± 4.6	61.3 ± 13.9*	28.7 ± 5.2
Glucose/insulin index (n = 6 to 10)	5.44 ± 0.65	1.70 ± 0.24**	4.14 ± 0.41
kITT (%/min) (n = 7)	4.51 ± 0.30	2.91 ± 0.32*	3.80 ± 0.21
Skeletal muscle GLUT4 mRNA (AU/ μ g total RNA) (n = 4)	3575 ± 388	4231 ± 634	–
Skeletal muscle GLUT4 protein (AU/ μ g total protein) (n = 4)	31.8 ± 1.79	33.9 ± 4.02	–

Data are mean ± S.E.M. The number of animals in parentheses. WAT, epididymal white adipose tissue; kITT, glucose disappearance constant obtained in the insulin tolerance test; AU, arbitrary units. * $P < 0.05$ and ** $P < 0.01$ vs. Sham and Pinx + Mel; ANOVA (Student–Newman–Keuls as a post-test).

Table 1. General characteristics of sham-operated (Sham), vehicle-treated pinealectomized (Pinx) and melatonin-treated Pinx (Pinx + Mel) rats

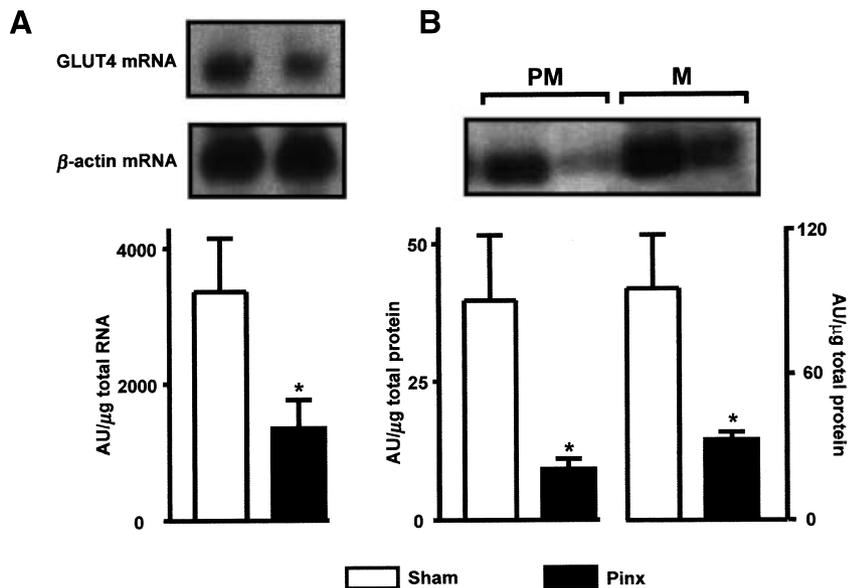


Fig. 1. GLUT4 gene expression in white adipose tissue (WAT) of sham-operated (Sham) and pinealectomized (Pinx) rats. (A) GLUT4 mRNA analysis. Top: representative autoradiograms from GLUT4 and β -actin mRNAs are shown. Bottom: bars are mean ± S.E.M. of four animals processed in four different experiments. (B) GLUT4 protein analysis in plasma membrane (PM) and microsomal (M) fractions of WAT. Top: representative autoradiogram of PM and M fractions is shown. Bottom: bars are mean ± S.E.M. of four animals processed in four different experiments. AU, arbitrary units. * $P < 0.05$ vs. Sham; Student's *t*-test.

protein in adipocytes isolated from intact rats. The plasma membrane GLUT4 from isolated adipocytes incubated for 30 min (Fig. 2B) increased by 63% after insulin ($P < 0.01$), and by 42% with insulin plus melatonin ($P < 0.05$). Furthermore, in isolated adipocytes, 4 hr of preincubation with vehicle or melatonin did not change the 30-min insulin effect on PM GLUT4 content (Fig. 2C).

To verify the effect of calorie restriction on pinx-induced insulin resistance, Sham and Pinx rats were submitted to 40% food restriction. Table 2 shows the characteristics of these animals. The 1-month body weight gain was similar in Sham and Pinx rats (~100 g), and the daily food intake was the same in these animals (data not shown). The calorie restriction reduced ($P < 0.05$) the body weight in both Sham and Pinx rats, although the body weight loss in Pinx rats was 35% ($P < 0.05$) less than the observed in Sham rats. Additionally, the WAT weight, which was reduced ($P < 0.05$) in Sham calorie-restricted rats, was unchanged in Pinx calorie-restricted rats. Increased insulinemia and decreased G/I index and kITT observed

in Pinx ad libitum rats were reversed by calorie restriction (Table 2).

The plasma albumin concentration (Table 3) was similar in Sham and Pinx fed ad libitum or subjected to calorie-restriction, showing that the calorie restriction did not induce undernutrition. Besides, in Sham ad libitum and Sham calorie-restricted rats, the pineal gland content of melatonin (Table 3) was unchanged by the analysis of both the highest expected content measured at 24:00 hr and the area under the curve over a 12-hr period.

To investigate the key step of insulin-induced glucose utilization, the GLUT4 protein content was measured in tissues from calorie-restricted Sham and Pinx rats. In skeletal muscle, the GLUT4 protein content was similar in all groups, either expressed per microgram total electrophoresed protein or per gram tissue (Table 3).

On the contrary, the GLUT4 protein in WAT changed expressively. In the plasma membrane (Fig. 3A) and microsomal fraction (Fig. 3B) of Pinx calorie-restricted rats the GLUT4 protein content, expressed in microgram

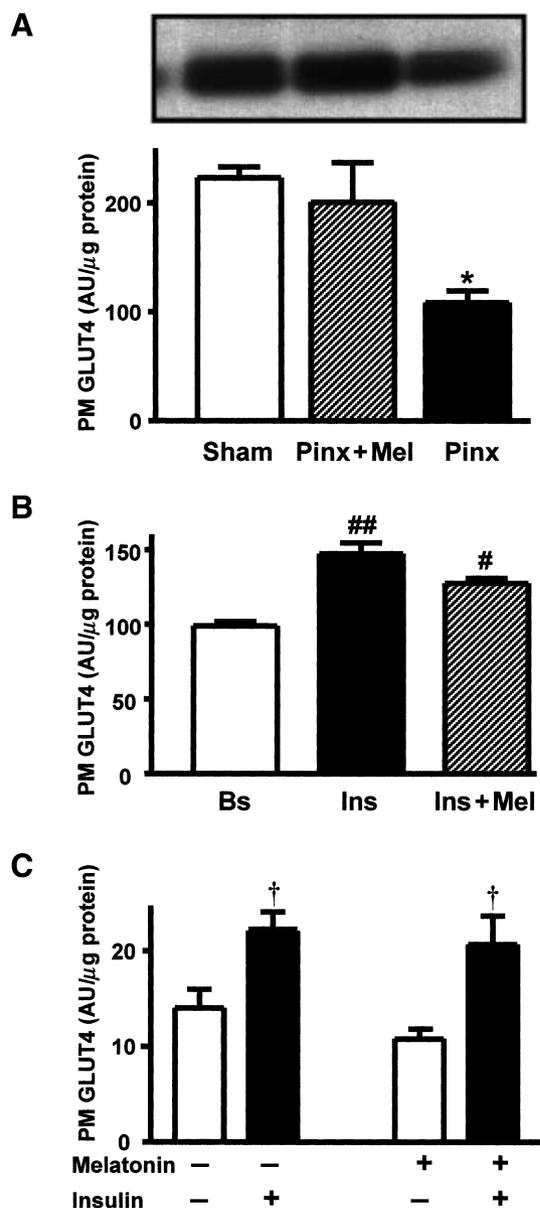


Fig. 2. GLUT4 protein analysis in plasma membrane fraction (PM) from white adipose tissue (WAT) or isolated adipocytes. (A) PM GLUT4 protein content in WAT of Sham-operated (Sham), vehicle-treated pinealectomized (Pinx) and melatonin-treated Pinx (Pinx + Mel) rats. Top: representative autoradiogram is shown. Bottom: bars are mean \pm S.E.M. of three animals processed in three different experiments. (B) PM GLUT4 protein content in adipocytes isolated from intact rats and incubated for 30 min in buffer (Bs) with 10^{-8} M insulin (Ins) or with 10^{-8} M insulin plus 10^{-9} M melatonin (Ins + Mel). (C) PM GLUT4 protein content in adipocytes isolated from intact rats, and preincubated for 4 hr without (-) or with (+) 10^{-9} M melatonin. After that, the cells were incubated for additional 30 min without (-) or with (+) 10^{-8} M insulin. All graphs are mean \pm S.E.M. of three different experiments. AU, arbitrary units. * $P < 0.05$ vs. Sham and Pinx + Mel; # $P < 0.05$ and ## $P < 0.01$ vs. Bs; † $P < 0.05$ vs. respective basal value; ANOVA (Student–Newman–Keuls as a post-test).

total electrophoresed protein, was similar to that observed in Sham ad libitum or calorie-restricted rats, showing that calorie restriction restored the reduced GLUT4 protein content observed in Pinx ad libitum rats ($P < 0.05$).

When the GLUT4 content was analyzed per gram tissue, different results were observed. Compared with Sham ad libitum rats, the plasma membrane GLUT4 per gram tissue remained reduced ($P < 0.05$) by 34% in Pinx ad libitum rats, and the calorie restriction restored GLUT4 content (Fig. 4A). Additionally, the plasma membrane GLUT4 per gram tissue was $\sim 30\%$ higher ($P < 0.05$) in Sham calorie-restricted rats when compared with Sham ad libitum rats, showing that calorie restriction induced an increase in GLUT4 translocation to the plasma membrane (Fig. 4A). However, the microsomal GLUT4 expressed per gram tissue was differently modulated. The microsomal GLUT4 per gram tissue in Pinx calorie-restricted rats increased to a value higher ($P < 0.05$) than that observed in Pinx ad libitum and Sham ad libitum or calorie-restricted rats (Fig. 4B). Finally, the plasma membrane GLUT4 per gram tissue related to the respective mean plasma insulin concentration at the moment of killing (Fig. 4C) confirmed a reduction ($P < 0.001$) of 55% in Pinx ad libitum compared with Sham ad libitum rats, and the calorie restriction increased these values by $\sim 60\%$ ($P < 0.001$) in Sham and by $\sim 100\%$ ($P < 0.01$) in Pinx rats.

Discussion

Impaired glucose homeostasis in Pinx [6, 21, 22] and diabetic [23] rats has already been reported, but the mechanisms and the true role of melatonin in this phenomenon have not been established. Herein, we found pinx of adult rats for 1 month is followed by clear insulin resistance condition, which involves impaired GLUT4 gene expression in WAT without modifying gene expression in skeletal muscle. The impaired GLUT4 expression seems to be transcriptionally regulated, as both mRNA and protein are stoichiometrically reduced. Although, in the present study, GLUT4 expression was unchanged in skeletal muscle, a small reduction of the protein content in skeletal muscle was reported in older Pinx rats [10]. This may be understood by considering that skeletal muscle development is an important stimulator of GLUT4 protein content [19] which occurs early in life, and thus the pinx effect may be masked by the tissue developmental control in younger rats.

As the pinx-induced insulin resistance involves reduction of GLUT4 expression only in WAT, the effect of 30-day melatonin replacement on plasma membrane GLUT4 content, which is the key step for tissue glucose disposal, was investigated in WAT. The results showed that melatonin replacement restored the GLUT4 protein content, as well as the in vivo insulin sensitivity. These data clearly establish a physiological role for melatonin in glucose homeostasis, which includes modulation of GLUT4 gene expression.

On the contrary, the melatonin effect upon plasma membrane GLUT4 protein content in isolated adipocytes of intact ad libitum fed rats was also investigated, and no effects were observed either after short (30 min) or long-term

Table 2. General characteristics of sham-operated (Sham) and pinealectomized (Pinx) rats fed ad libitum or subjected to calorie restriction

Parameter	Sham		Pinx	
	Ad libitum	Calorie-restricted	Ad libitum	Calorie-restricted
Δ Body weight (g) (n = 10)	101 ± 4	36 ± 5*	100 ± 5	58 ± 3*
WAT weight (g) (n = 4)	1.38 ± 0.10	0.96 ± 0.04*	1.27 ± 0.09	1.26 ± 0.08
Skeletal muscle weight (g) (n = 4)	0.81 ± 0.06	0.65 ± 0.09	0.74 ± 0.16	0.88 ± 0.19
Glycemia (mg/dL) (n = 10)	119 ± 5	130 ± 6	108 ± 6	117 ± 6
Insulinemia (μU/mL) (n = 10)	21.8 ± 4.6	17.1 ± 3.3	61.3 ± 13.9*	17.1 ± 2.9
Glucose/insulin index (n = 10)	5.44 ± 0.65	7.60 ± 0.89	1.70 ± 0.24**	6.80 ± 0.76
kITT (%/min) (n = 7–12)	4.51 ± 0.30	4.88 ± 0.41	2.91 ± 0.32*	4.01 ± 0.39

Data are mean ± S.E.M. The number of animals in parentheses. WAT, epididymal white adipose tissue; kITT, glucose disappearance constant obtained in the insulin tolerance test. Δ Body weight, 1-month difference body weight. * $P < 0.05$ and ** $P < 0.001$ vs. all groups; ANOVA (Student–Newman–Keuls as a post-test).

Table 3. Pineal melatonin content, plasma albumin concentration and skeletal muscle GLUT4 protein in sham-operated (Sham) and pinealectomized (Pinx) rats fed ad libitum or subjected to calorie restriction

Parameter	Sham		Pinx	
	Ad libitum	Calorie restricted	Ad libitum	Calorie restricted
Melatonin at 24:00 hr (ng/gland) (n = 15)	3.19 ± 0.33	2.98 ± 0.29	–	–
Melatonin/12 hr [(ng/gland)/12 hr] (n = 15)	9.33 ± 0.80	8.97 ± 0.60	–	–
Plasma albumin (g/dL) (n = 12–15)	2.73 ± 0.04	2.70 ± 0.05	2.91 ± 0.04	2.80 ± 0.09
Skeletal muscle GLUT4				
AU/μg total protein (n = 4)	31.8 ± 1.8	33.4 ± 7.8	33.9 ± 10.2	39.5 ± 11.0
AU/g tissue (n = 4)	308 ± 21	398 ± 29	351 ± 27	380 ± 57

Data are mean ± S.E.M. The number of animals in parentheses. Pineal melatonin content was measured during night, and results are melatonin at 24:00 hr and area under the curve at six points over a 12-hr period. AU, arbitrary units.

(4.5 h) incubations, with or without insulin. These data indicate that, in acutely treated normal adipocytes, melatonin does not modify the tissue insulin responsiveness as suggested by studies on insulin-stimulated 2-DG uptake [11]. Taken together, the results indicate that melatonin has an important physiological role in the glucose homeostasis, which involves GLUT4 protein modulation, and can only be observed in chronically Pinx rats. In contrast, 14-day melatonin-treated human brown adipocyte cell line (PAZ6) had lower GLUT4 mRNA expression; however, in this study, melatonin treatment was continuous rather than intermittent [24].

Conversely, the acute effect of a single melatonin injection was previously investigated in rats [25], mice [26] and postmenopausal women [27], and indubitably caused a reduction in insulin sensitivity. These studies confirm that the physiological effect of melatonin in improving insulin sensitivity, reported here, may not be achievable in acute treatments.

Interesting results were observed in calorie-restricted rats, once the effects obtained in Pinx rats were different from the observed in Sham rats. The body weight loss in Pinx calorie-restricted rats was lower than in Sham calorie-restricted rats, and this seems to be caused by reduced fat mass loss. All parameters of in vivo insulin sensitivity were improved in Pinx calorie-restricted, whereas in Sham rats the increase in insulin sensitivity was not so evident. The increased insulin sensitivity of Pinx calorie-restricted rats may be a consequence of improved GLUT4 protein content

and translocation to the plasma membrane, which may contribute to higher adipocyte lipid accretion, explaining the lower fat mass loss of these rats. Weight loss improves GLUT4 gene expression and/or translocation, especially in obese insulin resistant subjects [13–15]. Although Pinx rats were not obese by morphometric analysis, they showed a clear metabolic obese condition, in which hyperinsulinemia may be present [10] not only as a consequence of insulin resistance, but also as a primary effect of pinx. In fact, glucose-induced insulin secretion in pancreatic islets isolated from Pinx rats was reported as increased [28]. Considering that insulin hypersecretion plays an important role in establishing insulin resistance and reduction of GLUT4 protein in Pinx rats, decreased insulin secretion by calorie restriction [29] reduced the development of insulin resistance after Pinx.

In summary, the results show that melatonin plays a physiological role in glycemic homeostasis, which involves the GLUT4 protein. Pinx, for lack of melatonin, decreases insulin sensitivity as well as GLUT4 gene expression. Calorie restriction improves insulin sensitivity of Pinx rats, and this seems to be related to the increased GLUT4 gene expression and insulin-induced translocation to the plasma membrane in WAT.

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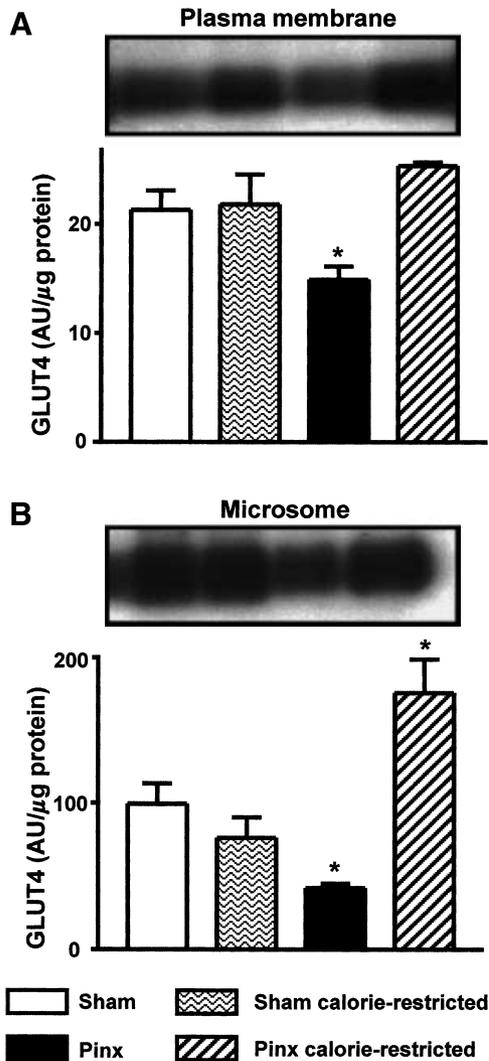


Fig. 3. GLUT4 protein content in white adipose tissue (WAT) of sham-operated (Sham) and pinealectomized (Pinx) rats fed ad libitum or subjected to calorie restriction. (A) GLUT4 protein analysis in plasma membrane fraction. Top: representative autoradiogram is shown. Bottom: bars are mean \pm S.E.M. of four animals processed in four different experiments. (B) GLUT 4 protein analysis in microsomal fraction. Top: representative autoradiogram is shown. Bottom: bars are mean \pm S.E.M. of four animals processed in four different experiments. AU, arbitrary units. * $P < 0.05$ vs. all groups; ANOVA (Student–Newman–Keuls as a post-test).

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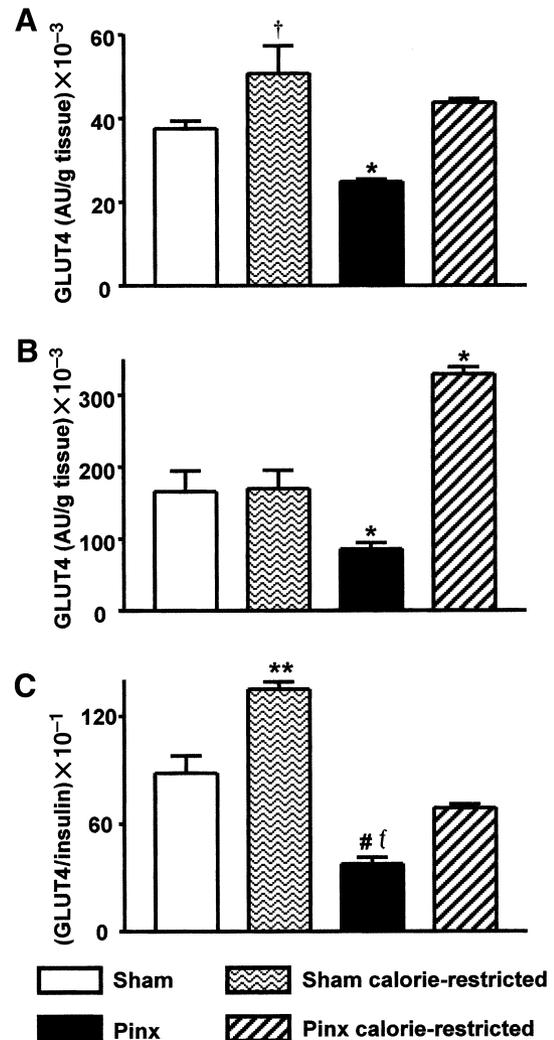


Fig. 4. GLUT4 protein analysis in white adipose tissue (WAT) of sham-operated (Sham) and pinealectomized (Pinx) rats fed ad libitum or subjected to calorie restriction. (A) GLUT4 protein content in plasma membrane fraction expressed per gram tissue. (B) GLUT4 protein content in microsomal fraction expressed per gram tissue. (C) GLUT4 protein content in plasma membrane fraction expressed per gram tissue, and related to the respective mean of plasma insulin concentration. All data are mean \pm S.E.M. of four animals processed in four different experiments. AU, arbitrary units. [†] $P < 0.05$ vs. Sham ad libitum; * $P < 0.05$ vs. all groups; ** $P < 0.001$ vs. all groups; [#] $P < 0.001$ vs. Sham ad libitum and Sham calorie-restricted; ^f $P < 0.01$ vs Pinx calorie-restricted; ANOVA (Student–Newman–Keuls as a post-test).

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