Effect of Reversing Dark-Light Cycles on Normal Diurnal Variation and Related Metabolic Disturbance in Rats

Kuang-Chung Shih1,4, Liahng-Yirn Liu2, Ching-Fai Kwok3, Chii-Min Hwu3, Chi-Chang Juan2, Yung-Pei Hsu4, and Low-Tone Ho2,4

1Section of Endocrinology and Metabolism, Department of Medicine
Tri-Service General Hospital, National Defense Medical Center
2Department of Physiology
National Yang-Ming University
3Section of Endocrinology and Metabolism
Department of Medicine
Taipei Veterans General Hospital
and
4Department of Medical Research and Education
Taipei Veterans General Hospital
Taipei, Taiwan, R.O.C.

Abstract

Diurnal variation of glucose tolerance and insulin action was studied in male Sprague-Dawley rats with a normal or reversed light-dark cycle. A series of experiments conducted was at 12 AM and 12 PM in the two groups. All measurements were separated by a recovery period of at least 3 days and preceded by a 16-hour fast. Glucose tolerance and insulin action were measured by both an oral glucose tolerance test and intraperitoneal insulin tolerance test. Normal light-dark cycle rats had significantly (*P* < 0.05) greater insulin sensitivity at 12 PM than at 12 AM, whereas reversed light-dark cycle rats had the opposite results (*P* < 0.05). Rats in the normal light-dark cycle group had a significantly higher growth hormone concentration at 12 AM than at 12 PM, whereas rats in the reversed group had the opposite results. Measurement of insulin-stimulated glucose uptake of isolated adipocytes preincubated with or without 100 ng/ml growth hormone at 37°C for 5 hours revealed that approximately 30% of insulin-stimulated glucose uptake was suppressed when adipocytes were treated with growth hormone. These results indicate that male rats exhibit significant diurnal variation of glucose tolerance and insulin sensitivity, and suggest that the concomitant diurnal variation of growth hormone may have a superimposed and amplifying effects on this variation.

Key Words: Insulin sensitivity, diurnal variation, and growth hormone

Introduction

Maintaining glucose homeostasis is essential for daily functioning. Disturbances in glucose availability and insulin action have severe consequences and lead to disease, including obesity and type 2 diabetes mellitus. The body has developed several control mechanisms to maintain plasma glucose concentrations within strict physiological boundaries. The daily fluctuations in plasma glucose concentrations are not random but describe a clear 24-hour rhythm. Fluctuations in plasma glucose concentrations result from food intake, hepatic glucose production, and/or changes in glucose tolerance (the relative amount of glucose taken up by peripheral tissues). In addition, increased glucose tolerance (i.e. a higher glucose uptake) results from various processes, such as a higher amount of insulin secreted from the pancreatic β-cells.
(i.e., insulin response), an increase in (non)-insulin-mediated glucose transporters, and/or a higher availability and sensitivity of the insulin receptor (often referred to as insulin sensitivity). Humans show high glucose output and insulin requirements in the early morning hours (2, 31), suggesting anticipation of glucose metabolism to the upcoming activity period. Diurnal variations in glucose tolerance have also been described; oral glucose, intravenous glucose infusions, or consumption of a meal result in a markedly higher plasma glucose response in the evening than in the morning. Diminished insulin sensitivity and decreased insulin responses to a glucose load have both been suggested to cause reduced glucose tolerance later in the day (3, 15, 32). Previous studies in our laboratory also found similar diurnal variation in glucose tolerance and insulin sensitivity to a glucose load (25, 33).

Taking into account the 12-hour shift due to the different activity patterns (i.e., humans are day-active; rodents, such as mice and rats, are night-active), rodents show similar diurnal variations in hormones involved in glucose metabolism and in energy substrates. Plasma concentrations of glucose and insulin are higher in the dark period than in the light period (22). The feeding schedule is a key factor influencing the diurnal variations in plasma glucose and insulin responses to an oral glucose load in rats, which may occur in the day-time or night-time (11). There have been few reports on the relationship between growth hormone and diurnal variation in glucose tolerance or insulin sensitivity to glucose in rats. The mechanism responsible for the growth hormone-induced diurnal variation in glucose tolerance and insulin sensitivity remains unclear. The purpose of this study was to determine the diurnal changes in glucose tolerance and insulin sensitivity in normal and reversed light-dark cycle rats using an oral glucose tolerance test and an intraperitoneal insulin tolerance test. Our results showed that light-dark cycle, food intake, and growth hormone are all important factors controlling the diurnal variation of glucose tolerance and insulin sensitivity in rats.

Materials and Methods

Reagents

Collagenase was obtained from Worthington Biochemical (Freehold, NJ, USA). Humulin R was from Lilly Deutschland GmbH (Giessen, Germany). Chloroform, n-butanol, n-haptane, methanol, boric acid and ethanol were from Merck KGaA (Darmstadt, Germany). Silicone oil was from General Electric (Waterford, NY, USA). Potassium dihydrogen phosphate (KH₂PO₄), sodium chloride (NaCl), magnesium sulfate (MgSO₄), potassium chloride (KCl), calcium chloride dihydrate (CaCl₂·2H₂O), sodium bicarbonate (NaHCO₃), bovine serum albumin (BSA)-free fatty acid, pyruvic acid,argaric acid, trisodium citrate dihydrate (C₆H₅Na₃O₇·2H₂O), cupric nitrate (Cu(NO₃)₂), triethanolamine (C₆H₁₅NO₃), 1-(2-thiozolylazo)-2-naphthol (TAN), human gamma globulin, polyethylene glycol and all other reagents and chemicals were from Sigma Chemical Co. (St. Louis, MO, USA). Rat GH [¹²⁵I] assay system, (3-iodotyrosyl) insulin (1800 Ci/mmol) and 2-deoxy-D-[¹³⁵I]-glucose ([¹H] 2-DG) (11 Ci/mmol) were from Amersham (Little Chalfont, UK). The Coat-A-Count rat corticosterone kit was from Diagnostic Products Corporation (Los Angeles, CA, USA). Other insulin radioimmunoassay (RIA) materials were prepared in our own laboratory.

Animal Model

Male Sprague-Dawley rats obtained from the Animal Center of National Yang-Ming University at the age of 3 weeks were housed four per cage in two rooms under constant conditions of temperature (20-22°C) and lighting (12 h light - 12 h dark). All animals had access to regular rat chow and water ad libitum except when scheduled for testing and for sacrifice. The food and water intakes were determined by measuring weight change of chow and volume change of water at 4-hour interval. In order to facilitate handling, one of the rooms had an inverted light-dark cycle (light from 6 PM to 6 AM and dark from 6 AM to 6 PM) while the other room had the normal cycle. The animals were kept in these rooms for 1 month before experiments began in order to allow adjustment to these light-dark cycles. All procedures were performed in accordance with the Taiwan Government Guide for the Care and Use of Laboratory Animals, and the protocol was approved by the Animal Welfare Committee of Taipei Veterans General Hospital.

Experimental Protocols

After a week of habitation, rats were divided into two groups: the normal light-dark cycle group and the reversed light-dark cycle group. The animals were then kept in these rooms for an additional 1 month before experiments began in order to allow adjustment to the different light-dark cycles. The normal or reversed 12 h light-dark cycle was maintained throughout the experiments, which were performed on the same day at 12 AM and 12 PM with only one experiment in each group on a given day. Each experiment in each group was separated by an interval of at least 3 days, and preceded by a 16-h fast. The diurnal variation of glucose tolerance and insulin sensitivity were measured with an oral glucose tolerance test and intraperitoneal insulin tolerance test at 12 AM and at 12 PM in both groups.
The levels of plasma glucose, free fatty acid, insulin, growth hormone and corticosterone were measured in both the normal light-dark cycle and reversed light-dark cycle groups at 12 AM and at 12 PM. In another experiment, insulin-stimulated glucose uptake of isolated adipocytes from rats was measured after pre-incubation both with and without 100 ng/ml growth hormone at 37°C for 5 hours.

**Oral Glucose Tolerance Test and Intraperitoneal Insulin Tolerance Test**

Glucose solution (2 g/ml/kg body weight) was administered by stomach gavage through a plastic catheter attached to a syringe. Blood was collected and plasma glucose and insulin levels were measured at 30, 60, 90 and 120 min after glucose administration. Insulin (0.5 U/kg) was injected into the peritoneum by syringe. Plasma glucose and insulin levels were determined at 30, 60, 90, 120 and 180 min after insulin administration.

**Blood Sample Collection**

Blood samples were collected at 12 AM and at 12 PM in both groups, with each measurement performed after a recovery interval of at least 3 days. Blood samples were collected by the tail-bleeding method. After the tail tip was severed without anesthesia using a sharp razor blade, approximately 300 µl free-flowing blood was collected into a 1.5 ml polyethylene heparin-coated microcentrifuge tube and kept on ice. The blood loss did not exceed 7 ml/kg (23). Plasma was separated by centrifugation and stored at -20°C until assayed.

**Isolation of Adipocytes**

Male Sprague-Dawley rats weighing 340-380 g were sacrificed by decapitation, and the epididymal fat pads were collected. Isolated adipocytes were obtained using the method of Rodbell (24) with some minor modifications (5) by shaking (100 rpm) finely minced tissue at 37°C for 1 h in Krebs-Ringer bicarbonate (KRB) buffer (118 mM NaCl, 5 mM KCl, 1.2 mM MgSO4, 1.2 mM KH2PO4, 1.3 mM CaCl2, 2H2O, 25 mM NaHCO3, pH 7.4) containing 1 mM pyruvate, 1% BSA, and 0.1% collagenase. The cell suspension was then filtered through nylon mesh (400 µm), centrifuged at 100 rpm for 1 min, and washed twice with the same buffer solution without collagenase at 37°C. Finally, the supernatant layer of isolated adipocytes was harvested, diluted threefold with the same collagenase-free buffer solution, and used in experiments. The cell number was counted after an aliquot of diluted cell suspension was fixed in a collidine buffer containing 2% osmium tetroxide (6).

**Measurement of Glucose Uptake by Adipocytes**

Glucose uptake by isolated adipocytes was determined by measuring 2-DG transport into the cells using the method of Garvey et al. (9) with minor modifications (16). Briefly, aliquots (400 µl) of isolated adipocytes with a predetermined cell number were mixed with increasing concentrations of insulin (0 to 50,000 pM) in 100 µl KRB buffer containing 1 mM pyruvate and 1% BSA. After the mixtures were incubated at 37°C for 30 min, 50 µl [3H] 2-DG (to a final concentration: 50 mM) was added and the incubation was continued for 3 min. The incubation was terminated by addition of 200 µl unlabeled 2-DG in KRB solution (500 mM). After thorough mixing, 300 µl of the mixture was transferred to a centrifuge tube containing 200 µl silicone oil and the cellular layer was separated by centrifugation at 1,000 rpm for 1 min. The radioactivity retained by the adipocytes was measured by a liquid scintillation counter.

**Measurement of Plasma Glucose, Free Fatty Acid, Insulin, Growth Hormone and Corticosterone**

Plasma glucose level in 25 µl of plasma was measured by a glucose analyzer (Model 23A, Yellow Springs Instrument, Yellow Springs, OH, USA). Plasma free fatty acid levels were determined by the fluorometric method described by Noma et al. (20). Standard curves were made from margaric at sequential concentrations from 0.1 to 1 mM. Each plasma sample (100 µl) was transferred to a glass-stoppered test tube containing 3 ml of extraction solvent [chloroform : heptane : methanol = 28 : 21 : 1 (v/v)] and 1 ml of copper reagent [0.75 g Cu(NO3)2, 3.25 g C6H15NO3, 25.0 g NaCl with distilled water to 100 ml] was then added. The tube was immediately stoppered and shaken mechanically for 2-5 min, then centrifuged at 2,000 rpm for 5 min. 1 ml of the upper phase was transferred to a second test tube containing 0.25 ml of TAN solution (10 mg of TAN dissolved in 100 ml of ethanol). After careful mixing for 5 minutes, the greenish blue color developed immediately and was measured at 570 nm against a reagent blank.

Plasma insulin concentration was determined by an RIA technique developed in our laboratory (13). The sensitivity of the assay was around 2.5 µU/ml. The coefficients of variation for inter-assay and intra-assay are 9% and 7%, respectively. The intra-assay precision (ratio) was calculated for each of two samples from the results of several replicates in a single run or assayed on several different days. The inter-assay variation coefficient showed substantial lot-to-lot variability. It had decreased inter-assay precision only in procedures that were subject to considerable between-run variation, as shown by
significant matrix lot-to-lot variability.

Plasma growth hormone concentration was measured with a commercially available RIA kit from Amersham (Little Chalfont, UK). Standard solutions were made from the rat growth hormone assay system at sequential concentrations from 1.6 to 100 ng/ml. Plasma samples and the following agents were added in order: goat anti-rat growth hormone serum, $^{125}$I-rat growth hormone. The mixture was vortexed and then incubated at 25°C for 16 h. Donkey anti-goat IgG serum was then added and the mixture was vortexed, incubated for 10 min, and then centrifuged at 2,000 rpm at 25°C for 10 min. A gamma counter was used to count the precipitates. The inter-assay coefficient of variation was 3% and the intra-assay coefficient of variation was 3%.

Plasma corticosterone concentration was measured using an RIA kit from Diagnostic Products Corporation (Los Angeles, CA, USA). Standard solutions were made from Coat-A-Count rat corticosterone in sequential concentrations from 20 to 2,000 ng/ml. The following agents were added in order to the plasma sample tube: antibody to corticosterone, $[^{125}$I] corticosterone. The mixture was then vortexed and incubated at 25°C for 2 h. A gamma counter was used to count the precipitates. The sensitivity of the assay was around 20 ng/ml.

**Statistical Analysis**

All values are expressed as mean ± SEM. Two means were compared using Student’s t-test. When there were more than two means, significant differences between means were determined by analysis of variance. A P level less than 0.05 was considered statistically significant.

**Results**

**Food Intake and Water Intake**

Fig. 1 shows the 24-h profiles of food intake (left panel) and water intake (right panel) in rats with normal and reversed light-dark cycles. As anticipated, food intake (5.05 ± 0.35 vs. 1.98 ± 0.47 g/100 g BW, P < 0.01) and water intake (9.60 ± 1.02 vs. 2.36 ± 0.70 ml/100 g BW, P < 0.01) in the normal light-dark cycle rats were significantly heavier during the dark cycle than during the light cycle (n = 8). Similar diurnal variation of food intake (5.58 ± 0.51 vs. 1.85 ± 0.84 g/100 g BW, P < 0.01) and water intake (9.45 ± 0.99 vs. 1.31 ± 0.43 ml/100 g BW, P < 0.01) during the dark cycle was also observed in the reversed light-dark cycle rats (n = 8).

**Oral Glucose Tolerance Test**

Fig. 2 shows the plasma glucose and insulin response to oral glucose tolerance test in the normal light-dark cycle rats (n = 12). As shown in the left panel, plasma glucose levels were higher at 12 AM than at 12 PM, 1 PM, 1:30 PM and 2 PM. As shown in the right panel, plasma insulin levels were higher at 12 AM than at 12 PM, 1:30 PM and 2 PM. These suggest the glucose tolerance and insulin sensitivity to oral glucose tolerance test were greater at 12 PM than at 12 AM in normal light-dark cycle rats. Fig. 3 shows the plasma glucose and insulin response to oral glucose tolerance test in the reversed light-dark cycle rats (n = 12). As shown in the left panel, plasma glucose levels were higher at 12 AM than at 12 AM, 1:30 AM and 2 AM. As shown in the right panel, plasma insulin levels were higher at 12 AM than at 12 AM, 1 AM and 1:30 AM. These suggest the glucose tolerance and insulin sensitivity to an oral glucose tolerance test were greater at 12 AM than at 12 PM in the reversed light-dark cycle rats. In summary, both normal and reversed light-dark cycle rats showed greater insulin sensitivity during the dark cycle (12 PM).

**Intraperitoneal Insulin Tolerance Test**

Fig. 4 shows the plasma glucose response to intraperitoneal insulin tolerance test in normal (n = 8) and reversed light-dark cycle rats (n = 8). As shown in the left panel, following insulin injection 30 min after the test in the normal light-dark cycle rats, plasma glucose levels were higher at 12 AM than at 12 PM. As shown in the right panel, the opposite results were obtained following insulin injection 30 min after the test in reversed light-dark cycle rats. The glucose tolerance and insulin sensitivity were better at 12 PM than at 12 AM in normal light-dark cycle rats and the opposite finding were made in reversed light-dark cycle rats.

**Plasma Glucose, Free Fatty Acid, Insulin, Growth Hormone and Corticosterone**

Plasma glucose, free fatty acid, insulin, growth hormone, and corticosterone were measured at 12 AM and at 12 PM in both the normal (n = 8) and reversed (n = 8) light-dark cycle rats (Table 1). Growth hormone level was higher at 12 AM than at 12 PM in the normal light-dark cycle rats and the opposite findings were made in reversed light-dark cycle rats. However, there was no significant difference between the normal and reversed groups in the levels of plasma glucose, insulin, corticosterone and free fatty acid.

**Glucose Uptake by Adipocytes**

Insulin-stimulated glucose uptake was measured
Fig. 1. The 24-hour profiles of food intake (left panel) and water intake (right panel) were measured on the 7th day of the adaptation period in rats with a normal light-dark cycle (■, n = 8) and a reversed light-dark cycle (●, n = 8). Data are expressed as mean ± SEM; *P < 0.05 (normal cycle vs. reversed light-dark cycle), ***P < 0.001 (normal vs. reversed light-dark cycle).

Fig. 2. Plasma glucose (left panel) and insulin (right panel) responses to oral glucose tolerance test in normal light-dark cycle (n = 12, 12 AM: ○○, 12 PM: ●●). Data are expressed as mean ± SEM; *P < 0.05 (AM vs. PM), **P < 0.01 (AM vs. PM).

Fig. 3. Plasma glucose (left panel) and insulin (right panel) responses to oral glucose tolerance test in rats with a reversed light-dark cycle (n = 12, 12 PM: ○○, 12 AM: ●●). Data are expressed as mean ± SEM; *P < 0.05 (AM vs. PM).

in isolated rat adipocytes after preincubation with or without 100 ng/ml growth hormone at 37°C for 5 h (Fig. 5). Insulin sensitivity varied depending on the insulin-stimulated glucose uptake by adipocytes. Approximately 30% of insulin-stimulated glucose uptake was suppressed when adipocytes were treated with growth hormone (Fig. 5).

Discussion

In this study, the plasma glucose and insulin responses to an oral glucose tolerance test showed diurnal variation in rats with both normal (Fig. 2) and reversed light-dark cycles (Fig. 3). Both normal and reversed light-dark cycle rats showed greater insulin
sensitivity during the dark cycle, as measured by a decrease in plasma glucose by intraperitoneal insulin tolerance test (Fig. 4). In rats with a normal light-dark cycle, the level of growth hormone was higher at 12 AM than at 12 PM, while rats with a reversed light-dark cycle had the opposite results. The greater insulin sensitivity (oral glucose tolerance test and intraperitoneal insulin tolerance test) during the dark cycle in both groups of rats suggests that insulin sensitivity was related to diurnal variation of growth hormone. In order to investigate the mechanism responsible for diurnal variation in insulin sensitivity, insulin-stimulated glucose uptake was measured in isolated adipocytes with and without growth hormone preincubation. Approximately 30% of insulin-stimulated glucose uptake (Fig. 5) was found to be suppressed when adipocytes were pretreated with growth hormone. These results indicate that growth hormone has superimposed roles upon the diurnal variation of glucose tolerance and insulin sensitivity.

In the present study, the responses of plasma glucose and insulin were undoubtedly influenced by variations in feeding patterns although it was difficult to measure the extent of such variation. It has been demonstrated that evolved animals are able to synchronize cycles of eating and fasting with several diurnal rhythms, such as the rhythmic changes of urinary excretion of water and potassium (29), plasma corticosterone level (28, 30), sensitivity of glucose metabolism to insulin in rat soleus muscle (17), melatonin (18), B apolipoprotein (19) and plasma vasopressin and osmolality levels (10). Thus, it is likely that the sensitivity of pancreatic β-cells to glucose is higher during the feeding time in the diurnal cycle. The mechanisms which control the glucose sensitivity of β-cells however remain unclear. The present results are consistent with findings of daily rhythmic changes in the rates of absorption of saccharides in rat small intestine (27).

Insulin resistance leads to a heightened pancreatic insulin response to glucose ingestion, resulting in the need for abnormally increased insulin secretion to maintain glycemic control. Insulin secretion eventually becomes insufficient to compensate for the insulin resistance, resulting in an inability to maintain euglycemia. Therefore, insulin resistance can lead to hyperglycemia and further complications in a vicious cycle. In the present study, normal light-dark cycle rats had higher plasma glucose and insulin levels at 12 AM than at 12 PM (Fig. 2). By contrast, in reversed light-dark cycle rats, the plasma glucose and insulin levels were higher at 12 PM than at 12 AM (Fig. 3).

Table 1. Plasma glucose, free fatty acid, insulin, growth hormone, and corticosterone were measured at 12 AM and at 12 PM in rats with normal and reversed light-dark cycles

<table>
<thead>
<tr>
<th></th>
<th>Rats with normal light-dark cycle (n = 8)</th>
<th>Rats with reversed light-dark cycle (n = 8)</th>
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<tbody>
<tr>
<td></td>
<td>12 AM</td>
<td>12 PM</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>115.1 ± 4.0</td>
<td>107.5 ± 5.2</td>
</tr>
<tr>
<td>Insulin (µU/ml)</td>
<td>37.1 ± 6.6</td>
<td>40.4 ± 6.9</td>
</tr>
<tr>
<td>Growth hormone (ng/ml)</td>
<td>32.1 ± 7.8*</td>
<td>16.1 ± 2.4</td>
</tr>
<tr>
<td>Corticosterone (ng/ml)</td>
<td>175.3 ± 39.9</td>
<td>166.7 ± 22.5</td>
</tr>
<tr>
<td>Free fatty acid (µM)</td>
<td>120.5 ± 34.7</td>
<td>102.5 ± 25.2</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM; *Indicates significant difference between 12 AM and 12 PM. (P < 0.05)

Fig. 4. Plasma glucose response to intraperitoneal insulin tolerance test in rats with normal (left panel, n = 8, 12 AM: ○–○, 12 PM: ●–●) and reversed (right panel, n = 8, 12 PM: ○–○, 12 AM: ●–●) light-dark cycles. Data are expressed as mean ± SEM; * P < 0.05 (AM vs PM).
The diurnal variation in plasma glucose and insulin responses in rats suggests that some endogenous mechanism probably participates in the diurnal changes.

Growth hormone has both acute and chronic effects on carbohydrate metabolism. The acute effects of growth hormone are referred to as insulin-like because growth hormone decreases blood glucose concentration, stimulates glucose uptake by muscle in vitro, and stimulates glucose uptake and lipogenesis in isolated adipocytes. The insulin-like effects are transient, however, and the chronic anti-insulin effects of growth hormone develop within a few hours. The anti-insulin effects of growth hormone include increased blood glucose concentration, inhibition of glucose uptake, stimulation of lipolysis, and insulin resistance. Acute and chronic elevation of circulating growth hormone causes insulin resistance and hyperinsulinemia in rats. Smith et al. demonstrated that the primary site of growth hormone action in inducing insulin resistance is at the insulin receptor. Growth hormone has also been shown to decrease insulin receptor autophosphorylation and receptor tyrosine kinase activity. This decreased tyrosine kinase activity of the insulin receptor is then transmitted to downstream components of the insulin-signal pathway. The insulin-like effects of growth hormone are readily demonstrable at hormone concentrations of 100 ng/ml or less. Growth hormone treatment decreases adipose tissue sensitivity to insulin. However, the exact molecular mechanism involved remains unclear. Castro et al. (4) reported that chronic cultures of adipocytes showed similar changes in insulin receptor substrate-1 and insulin receptor substrate-2 concentration and phosphorylation to those observed for liver and muscle after long-term in vivo treatment with growth hormone. Their data suggest that chronic growth hormone treatment alters the early steps of the insulin signal transduction pathway, and may explain the changes in adipose tissue sensitivity to insulin. However, Furuhata et al. demonstrated that growth hormone deficiency in transgenic rats led to impairment in at least the early steps of insulin signaling in the liver with a resultant defect in glucose metabolism. The molecular events associated with growth hormone treatment included tissue specific changes in the function of insulin receptor and insulin receptor substrate-1 suggesting the liver to be the primary site of insulin resistance. However, ghrelin (endogenous agonist of the growth hormone secretagogue receptor) appears to directly potentiate adipocyte insulin-stimulated glucose uptake in selective adipocyte populations. Ghrelin may play a role in adipocyte regulation of glucose homeostasis.

The present study found significant differences in growth hormone concentrations at 12 AM and 12 PM in rats with normal and reversed light-dark cycles. No significant difference, however, was found between levels of plasma free fatty acid and corticosterone in these rats. In conclusion, the main results of this study are as follows: (1) male Sprague-Dawley rats exhibit diurnal variation of glucose tolerance and insulin sensitivity, with greater tolerance and lower insensitivity at 12 PM than at 12 AM in parallel with the greater food intake during the dark cycle; and (2) the effect of the inhibition of insulin-stimulated glucose uptake by growth hormone may have a superimposed and amplifying effect on the diurnal variation of growth hormone.

Acknowledgments

This study was supported by grants from the National Science Council, National Health Research Institute, and Taipei Veteran General Hospital; Taiwan, R.O.C. We wish to thank the skillful technical assistance from P.C. Yu, Y.C. Lee and S.H. Cheng.

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