0013-7227/99/$03.00/0
Endocrinology

Vol. 140, No. 2
Printed in U.S.A.

Daily melatonin administration at middle age suppresses male rat visceral fat, plasma leptin, and plasma insulin to youthful levels

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Abstract Human and rat pineal melatonin secretion decline with aging, whereas visceral fat and plasma insulin levels increase. Melatonin modulates fat metabolism in some mammalian species, so these aging-associated melatonin, fat and insulin changes could be functionally related. Accordingly, we investigated the effects of daily melatonin supplementation to male Sprague-Dawley rats, starting at middle age (10 months) and continuing into old age (22 months). Melatonin was added to the drinking water (92% of which was consumed at night) at a dosage (4 μg/ml) previously reported to attenuate the aging-associated decrease in survival rate in male rats, as well as at a 10-fold lower dosage. The higher dosage produced nocturnal plasma melatonin levels in middle-aged rats which were 15-fold higher than in young (4 months) rats; nocturnal plasma melatonin levels in middle-aged rats receiving the lower dosage were not significantly different from young or middle-aged controls. Relative (% of body wt) retroperitoneal and epididymal fat, as well as plasma insulin and leptin levels, were all significantly increased at middle age when compared to young rats. All were restored within 10 weeks to youthful (4 month) levels in response to both dosages of melatonin. Continued treatment until old age maintained suppression of visceral (retroperitoneal + epididymal) fat levels. Plasma corticosterone and total thyroxine (T4) levels were not significantly altered by aging or melatonin treatment. Plasma testosterone, insulin-like growth factor 1 (IGF-1) and total triiodothyronine (T3) decreased by middle age; these aging-associated decreases were not significantly altered by melatonin treatment. Thus, visceral fat, insulin and leptin responses to melatonin administration may be independent of marked changes in gonadal, thyroid, adrenal or somatotropin regulation. Since increased visceral fat is associated with increased insulin resistance, diabetes, and cardiovascular disease, these results suggest that appropriate melatonin supplementation may potentially provide prophylaxis or therapy for some prominent pathologies associated with aging.

The pineal gland secretes melatonin into the blood and (at least in some species) the cerebrospinal fluid, almost entirely at night (for review, see ref. (1)). This nocturnal melatonin secretion mediates photoperiodic entrainment of endogenous circadian rhythms and other physiologic functions. Melatonin is also reported to be a potent free radical scavenger, and has been suggested to have functions independent of photoperiod.

With aging, pineal melatonin biosynthesis declines; nocturnal plasma melatonin levels are significantly decreased by middle age (2). It has been hypothesized that the decreased circulating melatonin concentrations may lead to a variety of physiological changes associated with aging (3). This raises the possibility that nocturnal supplementation of melatonin may prevent or delay some of these changes

In species for which changes in photoperiod induce major physiological adaptations (e.g., hibernation, migration, or seasonal breeding), melatonin has a role in regulating energy balance and fat distribution (4). Although reproduction and physical activity of rats and humans do not exhibit this dramatic seasonality, human and Sprague-Dawley rat visceral fat (also called deep abdominal fat) levels increase with aging (5,6), i.e., opposite to the aging-associated decline in plasma melatonin concentrations. This suggests that declining pineal melatonin secretion could play a role in aging-associated visceral fat accumulation. Accordingly, we investigated the effect of daily melatonin supplementation on visceral fat and related endocrine parameters in middle-aged and older male Sprague-Dawley rats.

Received 08/20/98.

Materials and Methods

Animals. Male Sprague-Dawley rats from Harlan Sprague-Dawley, Inc. (Indianapolis, IN) were housed 2/cage in 14 h light/10 h dark, lights off at 0800 h. All procedures were performed under an institutionally approved protocol in accord with the NIH Guide for Care and Use of Laboratory Animals.

Melatonin administration. Melatonin (Sigma Chemical Co., St. Louis, MO) dissolved in ethanol was added to the drinking water; the final ethanol concentration was 0.01% for both vehicle- and melatonin-treated rats. The water bottles were covered with aluminum foil and fresh solutions prepared twice weekly. Two experiments were conducted; in the first the melatonin concentration was 4 μg/ml and in the second 0.4 μg/ml, with melatonin administration initiated at 10 months of age. Both experiments also included young (4 months old at sacrifice) rats receiving 0.01% ethanol vehicle in their water.

Melatonin assessment. Blood samples for melatonin determination were collected into heparinized capillary tubes after nicking the tip of the tail at the midpoint of the dark period, one week before sacrifice. Plasma was stored at −70°C.

Sacrifice and dissection. The rats were sacrificed by decapitation at the end of the dark period; blood was collected and plasma stored at −70°C. Retroperitoneal (including perirenal) and epididymal fat pads were dissected and weighed.

Radioimmunoassay (RIA). Melatonin was quantitated as previously described (7) using the Guildhay antiserum which has been well characterized for determination of melatonin concentrations in rat plasma (8). Testosterone, total T3 and total T4 were quantitated with Coated-Tube Radioimmunoas-

say kits from Diagnostic Systems Laboratories, Inc. (Webster, TX). Leptin and insulin were quantitated with Rat Leptin and Insulin RIA kits from Linco Research, Inc. (St. Charles, MO). IGF-1 was quantitated with a Rat IGF-1 homologous double antibody RIA kit from Diagnostic Systems Laboratories, Inc. Total corticosterone was quantitated as previously described (9). For each hormone, all samples were assayed in a single RIA. Intra-assay coefficients of variation were all <10 %.

Statistical analyses. Comparisons between two groups were performed by t-test and between three groups by one-way analysis of variance followed by Newman-Keuls multiple comparisons, unless otherwise noted. $p<0.05$ was considered significant. Data are presented as mean ± SEM.

Results

In the high-dosage experiment (4 μg melatonin/ml of water) the rats drank 92±3% of their total daily water during the dark period; this % nocturnal consumption was not significantly different between melatonin *vs* control treatments. Consumption pattern was not evaluated in the lower dosage study. Low-dosage melatonin administration provided approximately 16-20 μg melatonin/day (30-40 μg melatonin/kg body wt/day). If the rats drank a consistent amount during each hour of the dark period, this would correspond to approximately 1.5-1.8 μg/h (2.8-3.7 μg/kg body wt/h) through the 10 h scotophase. High dosage melatonin provided 10-fold greater daily melatonin administration.

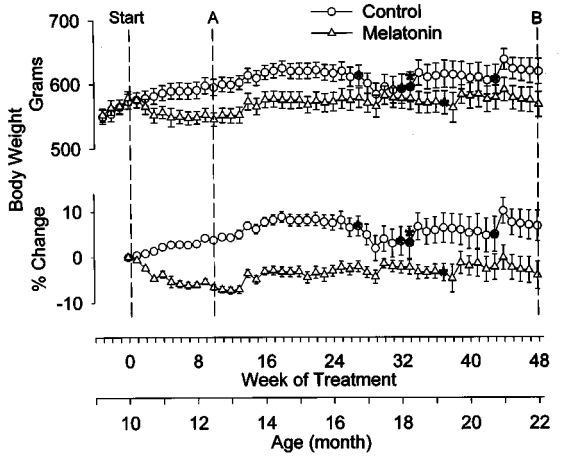

Fig. 1. Long-term melatonin administration from middle to old age. At 10 months of age, 18 rats received melatonin in their drinking water (4 μg/ml), and 22 received vehicle control. Some rats (8/treatment) were sacrificed at the end of the dark period after 10 weeks treatment (point 'A' in the figure); the remaining rats (10 melatonin, 14 control) received treatments for 48 weeks before sacrifice at 22 months of age (point 'B'). Each asterisk indicates that a rat died.

As illustrated in Fig. 1, body weights of rats receiving relatively high dosage melatonin starting in middle age were significantly ($p<0.05$) less than vehicle-treated rats at both 10 and 48 weeks of treatment. One of the 10 long-term melatonin-treated rats and 5 of the 14 long-term vehicle-treated control rats died during the study; these proportions were not significantly different (Fisher Exact Test, $p = 0.34$).

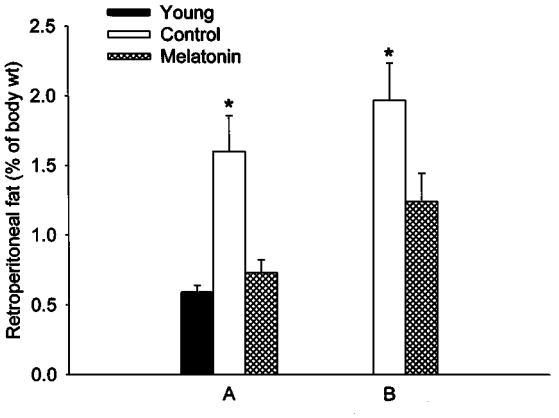

Fig. 2. Effect of aging and chronic melatonin treatment on relative retroperitoneal fat content. 'A' represents 10 weeks treatment, corresponding to time point 'A' in Fig. 1. In addition to the middle-aged vehicle- and melatonin-treated rats represented in Fig. 1, vehicle-treated young (4 month old) rats were also sacrificed at this time. 'B' represents 48 weeks treatment, corresponding to 'B' in Fig. 1. Each bar represents the mean ± SEM of 8-9 rats/treatment. *$p<0.05$.

After 10 weeks, relative (% of body weight) retroperitoneal fat in vehicle-treated middle-aged controls was 171% greater than in young rats (Fig. 2). In contrast, middle-aged rats which received melatonin for 10 weeks had relative retroperitoneal fat mass equivalent to the young rats. After 48 weeks of treatment, relative retroperitoneal fat in control rats remained 58% greater than in melatonin-treated rats (Fig. 2). Changes in relative epididymal fat mass (data not shown) at 10 weeks of treatment were consistent with the changes in retroperitoneal fat, i.e., 46% greater in controls than in young rats (1.3±0.1 *vs* 0.9±0.1% of body wt, respectively; $p<0.05$) and restored to that in young rats (0.9±0.1%) in response to 10 weeks melatonin treatment. After 48 weeks of treatment the difference between relative epididymal fat levels in vehicle- *vs* melatonin-treated rats (1.4±0.1 *vs* 1.2±0.1% of body wt, respectively) was no longer statistically significant ($p=0.18$), although the combined (retroperitoneal + epididymal) visceral fat mass did remain significantly suppressed (29%, $p<0.05$).

Changes in plasma leptin levels in response to melatonin treatment at middle age paralleled the changes in relative retroperitoneal and epididymal fat mass, i.e., they were increased at middle age (i.e., at 'A' in Figs. 1 and 2) and restored to youthful levels in response to melatonin (Table 1). Changes in plasma insulin levels tended ($p=0.07$) to follow the same pattern (Table 1). Plasma testosterone, IGF-1 and T3 decreased with age, but these changes were not significantly altered by melatonin treatment. Plasma corticosterone and T4 were not significantly altered by age or melatonin treatment (Table 1).

Table 1. Effect of aging and melatonin treatment on hormones involved with energy regulation and body composition

Hormone	Units	Young	Middle Age Control	Middle Age Melatonin
Leptin	ng/ml	3.48±0.27	8.28±1.48b	3.27±0.45
Insulin	ng/ml	2.75±0.25	3.48±0.53	2.57±0.25
Testosterone	ng/ml	1.95±0.26	0.67±0.08a	0.68±0.23a
Corticosterone	ng/ml	26.6±11.5	12.2±3.8	20.7±7.0
T3	ng/ml	56.1±2.1	36.0±3.4a	36.3±2.9a
T4	ng/ml	0.87±0.11	0.93±0.04	0.99±0.03
IGF-1	µg/ml	1.82±0.05	1.66±0.04a	1.55±0.04a

Data represent the mean ± SEM of 8 rats/group. a: $p<0.05$ *vs* Young. b: $p<0.05$ *vs* Young and *vs* Melatonin.

In the second experiment, administration of 10-fold lower dosage melatonin to middle-aged rats again suppressed ($p<0.01$) body weight (starting weight for both groups: 544±19 g; weight at time of sacrifice after 10 weeks treatment: 585±8 and 544±7 g for the control *vs* melatonin-treated rats, respectively; 10 rats/treatment). Relative retroperitoneal fat, plasma leptin and non-fasting plasma insulin levels in the middle-aged controls were again all increased relative to young rats, and 10 weeks of lower dosage melatonin again restored more youthful levels of each (Fig. 3).

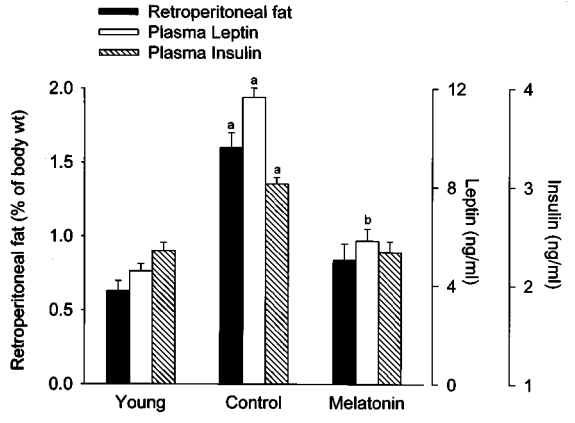

Fig. 3. Effects of aging and low dosage (0.4 µg/ml of drinking water) melatonin administration on relative retroperitoneal fat, plasma leptin, and non-fasting plasma insulin levels. Each bar represents the mean ± SEM of 10 rats/treatment. a: $p<0.01$ *vs* Young and *vs* Melatonin. b: $p<0.05$ *vs* Young.

Changes in relative epididymal fat mass again paralleled those in retroperitoneal fat, i.e., 34% greater in vehicle-treated middle-aged controls than in young rats or after melatonin treatment (1.4±0.1 *vs* 1.0±0.1 and 0.9±0.1% of body wt, respectively; $p<0.05$). Plasma testosterone levels in middle-aged controls were 54% lower than in young rats ($p<0.05$), and this decline was not significantly altered by melatonin treatment (1.30±0.34, 0.60±0.10, and 0.43±0.06 ng/ml for young, control and melatonin-treated groups, respectively). Plasma corticosterone levels were not significantly altered by aging or by melatonin treatment (10.2±4.2, 6.6±3.3, and 9.3±3.7 ng/ml for young, middle-aged control and middle-aged melatonin-treated rats, respectively).

Melatonin concentrations in plasma collected at the midpoint of the dark period from young, middle-aged control, and low dosage melatonin-treated rats from the second experiment, as well as from middle-aged rats receiving high dosage melatonin in the first experiment, were not normally distributed. Consequently, medians were evaluated by Kruskal-Wallis ANOVA of ranks followed by Dunn's multiple comparisons. The median melatonin concentration in high-dosage melatonin-treated rats (2,766 pg/ml) was significantly ($p<0.01$) higher than in young, middle-aged control, and middle-aged low-dosage melatonin-treated rats (189, 73, and 338 pg/ml, respectively), which were not significantly different. When plasma melatonin levels in only the young, middle-aged control, and middle-aged low-dose melatonin-treated rats were compared, the differences were still not significant; however there was a trend ($p=0.07$) for the middle-aged vehicle-treated rats to have slightly lower and middle-aged melatonin-treated rats to have slightly higher levels than the young rats.

Discussion

These results demonstrate that visceral fat, plasma leptin (a hormone produced by fat cells), and non-fasting plasma insulin levels all increased by middle age in male Sprague-Dawley rats. Daily supplementation with either high-dosage melatonin to markedly increase nocturnal plasma melatonin levels, or low-dosage melatonin which did not significantly alter nocturnal plasma melatonin levels, both suppressed all of these parameters to youthful levels within 10 weeks.

There have been contradictory reports regarding the potential role of melatonin in regulating body weight and metabolism in young rats (reviewed in ref. (10)). However, pinealectomy, which decreases but does not eliminate circulating melatonin, has been demonstrated to increase body weight and food consumption in rats (10). Thus, it would be reasonable to hypothesize that aging-associated decreases in pineal melatonin secretion may similarly lead to increased body weight and fat stores in aging rats, consistent with our results, and that supplementation of melatonin to restore or exceed youthful levels would reverse these changes, as we have demonstrated. No marked differences in food or water consumption were detected in the present study; however the rats were housed 2/cage and fed pelleted chow so it was not possible to reliably assess small individual changes. The possibility that melatonin may effect food and water consumption by individually housed rats receiving powdered diet is being investigated. Preliminary results from investigations with previously untreated middle-aged rats suggest that addition of 0.4 µg/ml melatonin to the drinking water does not acutely alter water consumption, such as might occur in response to altered taste. For example, addition and subsequent removal of melatonin on sequential days did not alter daily water consumption (36.1±1.0, 37.7±1.0, and 36.8±0.7 ml/rat/day on the day preceding melatonin, day of melatonin, and day following re-

moval of melatonin, respectively; n=24). Moreover, the rats did not discriminate between control and melatonin-containing water during two-bottle choice testing (50.5±2.5 *vs* 49.5±2.5% of total consumption for control *vs* melatonin, respectively, n=9). These studies will be presented in detail elsewhere.

The rats in the present study received approximately 92% of their exogenous melatonin during the dark period, i.e. at the same time as most endogenous pineal melatonin secretion. Thus, circadian rhythmicity of melatonin exposure was presumably maintained. The changes in body weight in response to this treatment were essentially identical to those which we have recently demonstrated in similar studies with drinking water containing the same dosage of melatonin available only during the first 8 h of the dark period (e.g., ref. (11) and additional studies to be presented elsewhere).

The first experiment used a model (i.e., addition of melatonin to the drinking water) and dosage (4 μg/ml) of melatonin administration reported to attenuate the aging-associated decline in survival rate in male rats (12). The second experiment used a model and dosage (0.4 μg/ml) reported to delay reproductive aging in female rats (13). These 10-fold different high and low dosages, producing nocturnal plasma melatonin levels that were either extremely elevated or similar to those of young animals, respectively, produced essentially identical effects on visceral fat mass, plasma insulin and plasma leptin. This suggests that both the threshold and saturation of the melatonin responses occur in the physiological range for circulating melatonin. The non-dose-dependent nature of this response to widely varying (15-fold) higher plasma melatonin levels also argues against mediation by non-specific mechanisms such as sedation or toxicity, neither of which were discernible. Indeed, preliminary results from a follow-up study in our laboratory reveal that low-dosage (0.4 μg/ml of water) melatonin administration produced a small increase (19%, $p<0.05$, n=17-19/treatment) in daily ambulatory locomotor activity recorded as sequential infrared beam interruptions, achieving more youthful activity levels.

Melatonin administration did not significantly alter plasma testosterone, T3, T4, corticosterone or IGF-1 levels. This suggests that the visceral fat, insulin, and leptin responses to melatonin treatment may be independent of marked changes in gonadal, thyroid, adrenal or somatotropin regulation.

Although hyperinsulinemia and increased visceral fat are commonly associated during aging of both rats and humans (5), the cause-effect relationship between these factors is not clear. However, a recent study indicated that in male Sprague-Dawley rats it was the aging-associated increase in hepatic (rather than peripheral) insulin resistance that was the major determinant of hyperinsulinemia, and that increased visceral adiposity played a major role in inducing hepatic insulin resistance (5). This suggests that interventions which prevent accumulation of visceral fat will likely improve carbohydrate metabolism during aging. Increased visceral fat has been demonstrated to be associated with not only insulin resistance and non-insulin-dependent diabetes mellitus, but also cardiovascular disease (14). The present results thus further suggest that appropriate melatonin supplementation may potentially provide prophylaxis or therapy for some of these prominent pathologies associated with aging.

Acknowledgement

Supported by the Department of Veterans Affairs.

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