Melatonin inhibits fatty acid-induced triglyceride accumulation in ROS17/2.8 cells: implications for osteoblast differentiation and osteoporosis

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1 Department of Cellular and Structural Biology, University of Texas Health Science Center at San Antonio, Texas; and 2 Geriatric Research Education and Clinical Center, South Texas Veterans Health Care System, Audie L. Murphy Division, and Division of Geriatrics and Gerontology, Department of Medicine, University of Texas Health Science Center at San Antonio, Texas

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Sanchez-Hidalgo M, Lu Z, Tan D-X, Maldonado MD, Reiter RJ, Gregerman RI. Melatonin inhibits fatty acid-induced triglyceride accumulation in ROS17/2.8 cells: implications for osteoblast differentiation and osteoporosis. Am J Physiol Regul Integr Comp Physiol 292: R2208–R2215, 2007. First published March 22, 2007; doi:10.1152/ajpregu.00013.2007.—Melatonin is produced not only by the pineal gland but by cells of the bone marrow. Moreover, melatonin is known to promote osteogenic differentiation in several cell line models and in multipotential bone marrow mesenchymal stem cells. Fatty acids have been independently shown to direct such cells to acquire the phenotype and molecular characteristics of adipocytes. To examine the effect of melatonin on intracellular triglyceride accumulation, an indicator of adipogenic differentiation in the rat osteoblast-like ROS17/2.8 cell line, cells were incubated with added oleic acid (100 μM), fixed and stained with Oil Red O. Cellular lipid accumulation was quantitated by an Oil Red O method highly specific for triglycerides and expressed as a triglyceride accumulation index (TGAI). Melatonin in nanomolar concentrations inhibited oleic acid-induced triglyceride accumulation. To identify the mechanism by which melatonin reduces triglyceride accumulation, cells were incubated with the two melatonin receptor antagonists, luzindole and S20928, or forskolin, a stimulator of adenyl cyclase and CAPM production. These compounds prevented the inhibitory effect of melatonin on triglyceride accumulation, indicating that melatonin acts through known melatonin receptor-mediated mechanisms. In view of the previously demonstrated positive effects of melatonin in promoting osteoblastic differentiation in ROS17/2.8 cells and their reciprocal adipogenic differentiation induced by fatty acids, our observations may serve to relate the known age-related decreases of melatonin production, the shift in the bone marrow toward an adipogenic line of cell development, and the development of osteoporosis during aging.

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carboxamide) were used as MEL receptor antagonists. LZD was from Sigma-Aldrich (St. Louis, MO), and S20928 was a kind gift of Dr. David E. Blusk (Bassett Research Institute, Cooperstown, NY). Both receptor antagonists were prepared as stock solutions (100 mM in DMSO) and stored in small aliquots at −20°C. Forskolin (FSK) from LC Laboratories (Woburn, MA) was dissolved in DMSO at a concentration of 10 mM and stored at −20°C. In experiments indicated, FSK was added into culture media containing MEL immediately before use.

Appropriate concentrations of reagents cited were obtained by diluting stock solutions in DMEM containing 5% FBS and were added 2 h before OA. OA (final concentration in the culture medium 100 μM) was added from a stock solution (100 mM) in ethanol, and cells were incubated for 24 h. The final concentration of ethanol did not exceed 0.1%.

Control cultures received an equivalent quantity of DMSO (vehicle for MEL, LZD, S20928, and FSK) and/or ethanol (vehicle for OA).

Cell culture. ROS17/2.8 cells, an osteoblast-like cell line that can undergo differentiation into adipocyte-like cells, were seeded at 5,000/well in 48-well plates and cultured in DMEM supplemented with 10% FBS containing the antibiotics streptomycin (100 μg/ml) and penicillin G (100 units/ml) at 37°C in a humidified atmosphere of 95% air-5% CO2. Medium was changed every 3 days. At 90% confluence, the medium was aspirated, discarded, and replaced by DMEM containing 5% FBS (assay medium), and the cells were treated according to the selected protocol.

Effect of MEL on triglyceride accumulation. To assess the effects of MEL on triglyceride (TG) accumulation, cells were incubated in the absence or presence of added OA for 24 h in assay medium after the treatment with MEL for 2 h. Experiments were performed using four conditions: (1) vehicle control; (2) 100 nM MEL + vehicle for OA; (3) 100 μM OA + vehicle for MEL; and (4) 100 nM MEL+100 μM OA.

Effects of LZD and S20928 on TG accumulation. In an attempt to elucidate the mechanism of action by which MEL influences TG accumulation, 90% confluent ROS17/2.8 cells were treated for 2 h in assay medium with MEL antagonists, either LZD (10 μM) or S20928 (10 μM); then incubated for an additional 2 h with or without added MEL (100 nM); thereafter, the cells were incubated for 24 h in the presence or absence of 100 μM OA.

Effect of FSK on TG accumulation. To characterize further whether the inhibitory effect of MEL on TG accumulation is a cAMP-mediated event, cells were incubated with 10 μM FSK for 2 h in the absence or presence of 100 nM MEL, and then incubated for an additional 24 h in the absence or presence of added 100 μM OA.

Quantitation of TG by ORO staining. The ORO staining method as described previously (16, 18, 30) or adapted by us with identified minor modification, is highly specific for TG (18, 30). Similarly, the instructions of the manufacturer were followed with minor variations. The cell number is estimated by the amount of dye adsorbed by the cells in each well. The cells in the ORO-extracted wells were washed three times with isopropanol following the initial isopropanol extraction, air dried, and exposed to 200 μl/well of proprietary Cell Staining Solution (SuperArray) for 30 min. The Staining Solution was then aspirated and discarded, and the stained cells were washed five times with distilled water to eliminate the unasorbed dye. The adsorbed dye was then extracted with 400 μl 1% SDS for at least 4 h (or overnight) and the absorbance values of the wells were measured spectrophotometrically at 595 nm using a plate reader (Perkin Elmer HTS 7000 BioAssay Reader).

TG accumulation index calculation. Using the absorbance values obtained for ORO and cell stain solution the TG accumulation index (TGA1) was calculated as follows based on Laughton (18): TGA1 = L/C, where L is the absorbance at 510 nm due to ORO stain (lipid); C is the absorbance at 595 nm due to cell stain (relative cell numbers). For example, L = 0.121, C = 1.325, TGA1 = L/C = 0.121/1.325 = 0.091.

Statistical analysis. Values in the figures are expressed as arithmetic mean ± SE. The data were evaluated using GraphPad Prism (San Diego, CA) ver. 2.01 software. The statistical significance of any difference in each parameter among the groups was evaluated by one-way ANOVA followed by Tukey’s test. P values of <0.05 were considered statistically significant. The figures shown are representative of at least four experiments performed on different days and are expressed as TGA1.

RESULTS

Morphological changes of the cells and their lipid droplets. When the ROS17/2.8 cells are treated with OA for 24 h, they change in shape from elongate ("spindle shaped") to round, as ORO stainable lipid accumulates in the cytoplasm. The cell nuclei do not stain with ORO and are usually centrally located, about 25 μm in diameter, and occupy most of the cytoplasm, appearing as "empty" spaces (Fig. 1). The lipid droplets at 24 h after the addition of OA are from 1–2 μm in diameter (Fig. 1B), somewhat larger than the 1-μm size of ordinary lipogenic (nonadipogenic) lipid droplet (3). Quantitative TG measurements reported here were made at this time. However, if the OA-treated cultures were carried on for up to 6 days (3 additions of fresh medium containing OA at 100, 250, and 500 μM), droplet size increased to 3–4 μm (Fig. 1C), larger than ordinary lipogenic droplets but not as large as those of differ-
entiated 3T3 L1 preadipocytes or those differentiated from the stromal-vascular portion of adipose tissue.

**MEL decreases TG accumulation and therefore inhibits adipogenesis in ROS17/2.8 cells in the absence of added OA.** Using TGAI for the quantitation of intracytoplasmatic TG accumulation, we examined the potential role of MEL as a regulator of TG accumulation in the ROS17/2.8 cell line. Intracytoplasmic TG accumulation can be considered to be an index of adipocytic differentiation in this cell line in which it has been shown that fatty acids drive the adipogenic differentiation program as shown by increased aP2 and decreased alkaline phosphatase (10). A more extensive demonstration of the ability of fatty acids to do the same was also shown by others using a bone marrow-derived multipotential cell line, in which fatty acids were shown to induce adipogenesis using multiple markers [see (1) and DISCUSSION].

Treatment of the cells with MEL at a concentration of 100 nM significantly reduced intracellular TG accumulation (Fig. 2, A and B) and therefore inhibited adipogenesis. There was no difference in TG accumulation between cells cultured with DMSO and cells that received no treatment (data not shown). MEL significantly decreased TG accumulation in the cells (TGAI) from 0.402 \( \pm \) 0.025 (vehicle controls) to 0.288 \( \pm \) 0.016 (MEL treated). This represents a decrease of TG in the MEL-treated cells of 36% (\( P \) < 0.01).

**MEL also decreases TG accumulation even in the presence of added OA.** Fig. 2C shows that, after 24 h of incubation with 100 \( \mu \)M OA, the control cells increased intracytoplasmic lipids, which were manifested as lipid droplets of varying sizes. When OA-incubated cells were grown in a medium containing 100 \( \mu \)M MEL which was added 2 h before OA induction, the cells accumulated significantly less lipids than did the OA controls (Fig. 2D). Melatonin (TGAI 1.254 \( \pm \) 0.04) prevented the OA-induced lipid accumulation (1.585 \( \pm \) 0.07), \( P \) < 0.01.

To select a dose of MEL, a dose-response study (1 nM to 1 mM) was performed in the presence of 100 \( \mu \)M OA (Fig. 3).
A minimal effect could possibly be seen at a concentration as low as 1 nM. An exact determination of the dose that produced maximal effect was not made, but 50 nM already seemed to produce a maximal effect; 100 nM concentration for MEL was selected for the studies performed here. Very high concentrations of MEL (100 nM to 1 mM) were less effective, possibly because of desensitization (down-regulation) of the melatonin receptors (29). The effective dose of melatonin for inhibition of TG accumulation (50–100 nM) approximates that for induction of osteoblastogenesis (alkaline phosphatase) in human adult mesenchymal stem cells (50 nM; Ref. 29).

**Inhibitory effect of MEL on TG accumulation is blocked by LZD and S20928.** To determine the potential involvement of MEL receptors relative to MEL’s action on TG accumulation, we assessed the ability of two MEL receptor antagonists, 10 µM LZ and 10 µM S20928, to antagonize the inhibitory effect of MEL on TG accumulation. The results obtained showed that cells treated with both LZD (Fig. 4, 5) and S20928 (Fig. 6, 7) did significantly change the response to MEL. After incubation with either LZD or S20928 for 2 h, cells had levels of TG equivalent to those of control cells either in the absence (Fig. 4, 6) or presence (Fig. 5, 7) of added OA. Thus, the additions of S20928 or LZD completely reversed the inhibition by MEL and restored TG accumulation, indicating that the MEL receptor is involved, as it is in adipocytes (7). Details of melatonin-receptor interactions have been recently reviewed (11, 12, 25).

![Fig. 3. Effect of MEL on OA-induced TG accumulation. Data are expressed as the means ± SE of five independent experiments performed in duplicate/triplicate; n = 14. *P < 0.05, **P < 0.01, and #P = 0.05 vs. OA.](http://ajpregu.physiology.org/)

![Fig. 4. Luzindole (LZD) blocks the inhibitory effect of MEL on TG accumulation. Cells were treated with LZD in the presence or absence of MEL. After incubation with LZD for 2 h, MEL was added to assay medium. Cells treated with vehicle control, 10 µM LZ, 100 nM MEL, and 10 µM LZ + 100 nM MEL for 24 h. Cells treated with MEL showed the TGAI was statistically different vs. control and LZD. Cells treated with LZD + MEL were not statistically different vs. control and LZD but showed a tendency toward a blockade of MEL inhibitory effect. The inhibitory effect of MEL on TG accumulation was clearly blocked by LZD. Data are expressed as mean TGAI ± SE; n = 25–46. **P < 0.01 vs. control and +++P < 0.001 vs. LZD. #P = 0.079 vs. MEL. NS P > 0.05 vs. control and LZD.](http://ajpregu.physiology.org/)

![Fig. 5. LZD blocks the inhibitory effect of MEL on OA-induced TG accumulation. The experimental protocol was performed as indicated in METHODS. The selected protocol was the following: control (OA) (A), LZD + OA (B), MEL + OA (C), and LZD + MEL + OA (D). Microscopic examination showed that the induced TG accumulation by OA, 2 h after LZD administration, reflected no important changes in relation to stained lipid droplets in the presence or absence of MEL. Data are expressed as means ± SE; n = 21–46. **P < 0.01 vs. OA and vs. LZD + MEL + OA; +++P < 0.001 vs. OA + LZD. NS P > 0.05 vs. OA.](http://ajpregu.physiology.org/)

![Fig. 6. S20928 blocks the effect of MEL on TG accumulation. Cells were incubated with S20928 for 2 h. Then, 100 nM MEL was added in the wells indicated. Data showed S20928 prevented the inhibitory effect of MEL on TG accumulation. Data are expressed as mean TGAI ± SE; n = 10–72. **P < 0.01 vs. control. NS P > 0.05 vs. control. #P > 0.05 vs. MEL, failed to show a statistical difference, possibly due to the relatively small number of S20928 + MEL samples (n = 10).](http://ajpregu.physiology.org/)
FSK enhanced lipid accumulation and blocked MEL’s inhibitory effect on lipid accumulation in both the absence and presence of added OA.

Taken together, these observations on melatonin receptor blockade and its reversal with forskolin support the idea that the mechanism by which MEL acts to reduce TG accumulation in the ROS17/2.8 cell line is via a specific MEL receptor-mediated event rather than by a nonspecific action.

**DISCUSSION**

The present observations complement others that point to a possible important role for melatonin in osteogenesis (8, 25, 42). As outlined below, information that supports this hypothesis is derived from literature employing a number of different cell types studied in vitro, in intact animals, and under widely varying experimental conditions. We postulate from our results that melatonin’s action is through its ability to inhibit cellular fatty acid accumulation, which in cells with both adipocytic and osteoblastic potential, antagonizes the osteoblastic pathway (1, 10).

Much of the experimental work on osteogenesis has used cell lines that serve as models for this process, e.g., ROS17/2.8 and MC3T3 cells, rodent cell lines derived from neoplastic or normal tissues (34). PPAR-γ is the single gene that is most closely linked in many studies to adipogenesis. Recently, experimental work on the osteoblastic-adipocytic relationship has used human and rodent bone marrow-derived stromal/stem cells, as well as cell lines derived from these by stable transfection with the PPAR-γ2 gene (UAMS-33 cells) (22). Gene deletion studies reveal a reciprocal role of PPAR-γ2 in osteogenesis, as well as adipogenesis (2).

Classical studies have established that in normal developing and mature adipocytes, TG are synthesized from circulating lipoproteins, which, of course, contain TG as part of their structure. Lipoprotein lipase (LPL), expressed in both early developing and mature adipocytic cells, hydrolyzes the TG to

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*Fig. 7. S20928 blocks the inhibitory effect of MEL on OA-induced TG accumulation. Two hours before MEL addition, 10 μM S20928 was added to the media. OA was then added. After 24 h of incubation with OA, S20928-treated cells in the presence or absence of MEL did not manifest changes in intracellular lipid accumulation. The inhibitory effect of MEL was prevented by S20928 addition. OA (A), MEL+OA (B), S20928+OA (C), and S20928+MEL+OA (D). Values are represented as means ± SE. *P < 0.05 vs. OA; **P < 0.05 and ***P < 0.001 vs. MEL+OA. Original magnification ×500.*
fatty acids and 2-monoacylglycerol, both of which are taken up and reesterfied to TG. Nonesterified fatty acids from the circulation are also taken up by the cells. LPL, in addition to its hydrolytic function that releases fatty acids from TG, is also believed to facilitate the cellular uptake of lipoproteins in a “bridging” function with apolipoprotein CII (21). In the absence of LPL, fat cells, at least in mice, are maintained by de novo fat cell endogenous fatty acid synthesis (41). However, strong evidence is now available that another mechanism is normal operative downstream from LPL to promote fatty acid transport into cells. Although very rapid but passive mechanisms have long been thought to be largely responsible for fatty acid entry, a consensus has emerged that the major mechanisms have long been thought to be largely responsible for fatty acid entry, a consensus has emerged that the major mechanism distal to LPL is vested in the number of specific fatty acid transport proteins that enhance this process (15).

In the studies, we report here that melatonin clearly inhibits the accumulation of cellular TG from fatty acid (OA) by ROS17/2.8 cells. In our cell culture system, the basal medium containing FBS supplies some fatty acids and lipoproteins, but Ten micromoles forskolin showed a statistically significant increase in TG accumulation either in the presence or absence of melatonin vs. control group, preventing the inhibitory effect of melatonin on lipid uptake. Data are expressed as means ± SE. *P < 0.05 and **P < 0.01 vs. control, +++P < 0.001 vs. MEL.

The accumulation of TG indicates adipogenic differentiation of our ROS17/2.8 cells (10); the latter’s inhibition by melatonin would thus promote the osteogenic differentiation pathway. Also relevant to the issue of fatty acid regulation of adipogenesis in cells with both osteogenic and adipogenic potential are results using F/STRO-1(+) cells (an immortalized tripotential human fetal bone marrow stromal cell line) showing that fatty acids drive the adipogenic differentiation pathway (1). A variety of adipogenic markers was used in this study (PPARγ2, CEBPα, LPL, G3PDH, leptin) in addition to aP2 that was also measured by others (10). Linoleic acid was the most potent of the three fatty acids tested (linoleic, oleic, and palmitic). Moreover, and importantly, these workers concluded that: “... differentiation of this ... cell into one lineage restricts expression of other lineage specific genes” (1). Thus, in marrow differentiation) medium (20). Although at that time, we attributed the differentiation action to a protein factor(s) in both serum and fat cell conditioned medium, subsequent work unequivocally showed that the adipogenic effect of both was attributable to fatty acids, although a role for a protein acting in concert with the fatty acids has not been excluded (Z. Lu, Z. and R. I. Gregerman, unpublished data). Very recently, oleic acid (or a mixture of oleic, palmitic, and linoleic acids) has been shown to induce adipogenesis in 3T3-L1 preadipocytes in a study that includes not only TG accumulation but a pattern of expression of adipocyte mRNA markers that is similar to but different in time course from that which is produced by the “differentiation cocktail” (dexamethasone, IBMX, and insulin), frequently used to induce the conversion of preadipocytes to adipocyte-like cells in culture (44).

Preadipocytes of the stromal-vascular (S-V) fraction of human and rat adult adipose tissue are now known to be multi-potential cells which, like bone marrow mesenchymal stem cells, under appropriate stimulation can develop along one of several pathways (adipocytic, osteoblastic, myoblastic, chondroblastic, and others) (14, 38, 45). Moreover, bone marrow mesenchymal stem cells serve as the progenitors of both bone marrow osteoblastic cells and the adipocytes of the bone marrow (5, 14, 23).

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stromal cells, preadipocytes, and ROS17/2.8 cells that we have used, normal fatty acids at physiological concentrations induce adipogenic differentiation, as judged by the mRNA expression patterns and their corresponding protein products, as well as by the criterion of triglyceride-containing lipid droplet accumulation.

Melatonin’s effect on osteoblastic cells can be viewed in this context (34). The presumption was made in that key study that melatonin directly stimulates osteogenesis in two cell lines (34). However, we would propose that the mechanism of melatonin’s effect on osteogenesis may at least, in part, be produced by its inhibitory action on fatty acid uptake acting through the reciprocal nature of the osteogenesis/adipogenesis differentiation pathways and thus favoring the osteogenic pathway of cell development (1, 14). The precise regulatory mechanisms involved which determine the direction of these pathways are at present unknown but are clearly under genetic control (2).

It is important to note that melatonin has been reported to have a direct inhibitory effect on fatty acid uptake into the cells of perfused adipose tissue. When melatonin at nanomolar concentrations was infused into the inguinal fat pads of rats, fatty acid uptake was completely inhibited (36). The inhibitory effect was reversed by two of the same MEL receptor antagonists, LZD and S20928, that were effective in our present cell studies and by FSK. The authors postulated that their results were due to inhibition by melatonin of a fatty acid transporter into the adipose cells but could only speculate on which of several possible known fatty acid transporters might be involved. Since then, FATP-1 has been considered to be the major fatty acid transporter of adipocytes.

A number of other reports have linked melatonin to adipose tissue metabolism and obesity. It is possible that some of these observations may relate to the reported inhibition of fatty acid uptake into adipose tissue (above). However, other melatonin-mediated mechanisms are probably involved. In one study in which rats consumed a high fat diet, the amount of food consumed was the same in both of the animals given melatonin and the controls, yet the melatonin-treated group lost more weight. No obvious explanation was available, although a small increase in physical activity of the melatonin-treated animals was observed (28, 43). However, in an earlier study, the latter group noted that melatonin’s action on body weight might be a consequence of inhibition of lipid synthesis (43). Several other studies in rodents also indicate that administered melatonin reduces body weight gain and decreases visceral fat accumulation, as well as having effects on adipocyte production of leptin (27, 28, 31). Other aspects of a possible role for melatonin in adipose tissue lipolysis, lipogenesis, and the effects of exercise training have been recently reviewed (6). Melatonin is also involved in seasonal changes in adiposity [Syrian and Siberian hamsters (40)], although the mechanisms are complicated and presumably involve more than a simple relationship between the pineal, melatonin release, and white adipose tissue (4).

Important observations relative to a possible role for melatonin in the regulation of bone cell differentiation include the state-of-the-art (HPLC and mass spectroscopy) identification of melatonin in bone marrow. Two reports are in agreement that melatonin is present in high concentrations in bone marrow cells; overall, the amount in marrow is two orders of magnitude greater than that in serum (9, 37). Interestingly, several observations strongly suggest that marrow melatonin does not originate in the pineal gland. Indeed, melatonin is synthesized even in a variety of cell lines. The precise type of cell that produces melatonin in the marrow is not known (37). Other aspects of the melatonin-marrow cell interrelationship have been reviewed in detail (8).

As further regards melatonin’s role in osteogenesis, several interesting biological generalizations are worth noting. An apparent shift in the dominance of bone marrow adipocyte development over net osteoblastic activity is associated with aging in all vertebrates studied. Remarkably, this shift is accompanied by a concurrent drop in endogenous melatonin production (32, 33, 35), although an age-related loss specifically of “marrow” melatonin production or levels has not been studied. An extreme form of bone loss is seen, of course, in the clinical condition of osteoporosis in humans, in which bone loss is so severe that bone fractures occur without obvious trauma, or with ordinarily trivial trauma, and vertebral deformities (compression or collapse) do alter skeletal spinal integrity. Similarly, pinealectomized animals (salmon, chicken, mice) that have a relative melatonin deficiency also develop spinal curvature and bone demineralization not unlike that seen in osteoporosis in the human (13, 24, 26, 39).

In summary, we wish to propose that the melatonin-fatty acid interrelationship may have implications for bone marrow stromal cell differentiation. This view is taken in the context of what is known about the ability of fatty acids to drive ROS17/2.8 cells and other multipotential cells capable of both osteogenic and adipogenic differentiation toward a pattern of adipogenic gene expression (1, 10), and the evidence that melatonin may well be a regulator of the opposite, i.e., osteoblastic development (34). Our findings have implications for further exploring our understanding of the mechanisms involved in the relative dominance of the adipocytic over the osteoblastic pathway with aging, during which loss of bone matrix occurs and in which adipocytes come to occupy an increasing amount the bone marrow (8, 14). Pharmacological or other molecular manipulation of the melatonin-fatty acid-adipogenesis relationship in bone could conceivably yield additional drug targets for therapeutic interventions in osteoporosis.

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