INTRODUCTION

Obesity is a disorder of homeostasis resulting from the excessive supply of energy in relation to energy expenditure of the body. The development of the white adipose tissue constitutes an essential element of energetic homeostasis (1). Preadipocytes, which are the precursors of mature white adipose tissue cells, play an important role in preserving energetic homeostasis. Preadipocytes differentiate from precursor fibroblastic cells, i.e., from preadipocytes, during the entire ontogenetic life can result in the development of obesity related to the increase in the number as well as the volume of adipocytes. It was observed that the number of adipocytes is 4-fold higher in extremely obese patients as compared to slim individuals (2).

In the murine 3T3-L1 preadipocyte cell line, which is a good experimental model used in the majority of studies on adipocytes, the process of differentiation is similar to the one in human cells (3).

Adipogenesis is subject to neurohormonal regulation due to the presence of numerous receptors within adipose tissue cells. There is an inverse relationship between the number of autonomic nervous system axons and the influence on preadipocyte proliferation (4). It was proven that preadipocyte proliferation is inhibited by noradrenaline in vitro (5) and intensified by 2 receptor stimulation (6). Preadipocyte proliferation is also stimulated by thyroid hormones, glucocorticosteroids, estradiol, progesterone and the growth hormone (7, 8). Insulin-like growth factor I (IGF-I), tumor necrosis factor (TNF-α) and macrophage colony-stimulating factor (M-CSF) are among important paracrine factors stimulating preadipocyte proliferation (7, 9).

The discovery of melatonin receptors on adipose tissue cells gives grounds for considering the possibility of melatonin as a factor which influences energy storage through modulation of metabolism and adipocyte proliferation. To date only a few contradictory studies have been published on the influence of melatonin on preadipocytes. The aim of the present study is to evaluate the influence of melatonin at physiological and supraphysiological concentrations on the proliferation of 3T3-L1 murine preadipocytes after 3 and 24 hours of the experiment and to determine the participation of membrane melatonin MT2 receptors, and for the first time - MT3, in its melatonin action during a 24-hour experiment. The 3T3-L1 murine preadipocyte cell line were cultured with or without melatonin at 10^-3 and 10^-6 mol/L, with or without melatonin antagonists luzindole (10^-4 mol/L) and prazosin (10^-5 mol/L). Cell proliferation was determined by means of labeled [3H]-thymidine incorporation in the DNA of the cell. Melatonin at both physiological and supraphysiological concentrations has a stimulating effect on the number of 3T3-L1 preadipocytes. The application of luzindole inhibits the above effect of melatonin both at 10^-3 mol/L and 10^-6 mol/L concentrations (P<0.05). The presence of prazosin does not have a statistically significant influence on the effects of melatonin action. Summarizing, it has been proven that melatonin exerts a proproliferative effect on 3T3-L1 preadipocytes at physiological and supraphysiological concentrations, partially by MT2, and not by MT3 receptors.

Key words: melatonin, 3T3-L1 proliferation, preadipocytes, [3H]-thymidine, melatonin receptors, luzindole, prazosin, obesity
insomnias. Furthermore, melatonin administration may contribute to the management of jet lag (12).

Moreover, melatonin belongs to the endogenous antioxidants (13), exerts cytoprotective (14), analgesic (15), immunomodulative (16, 17) and oncostatic properties (16, 18). Melatonin is also used as an adjunct in the treatment of neoplasms, obesity, Alzheimer’s disease and cardiovascular diseases (19).

To date, only a few studies on the influence of melatonin on preadipocytes have been published. What is more, their results are contradictory. The first study was the one reported by us and it confirmed that melatonin administered at supraphysiological concentrations results in the increase in the number of 3T3-L1 preadipocytes after 24 and 48 hours (20). In subsequent studies, Maldonado et al. reported a dose-dependent stimulative influence of melatonin on adipogenesis in a murine fibroblast cell line (21). Similarly, Gonzales et al. described the stimulative influence of melatonin at physiological and supraphysiological concentrations on 3T3-L1 preadipocyte differentiation and adipogenesis and also on the increase in the expression of adipogenic transcription factors: peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer-binding protein α (C/EBPα) (22). Peroxisome proliferator activated receptors (PPARs) are transcriptional factors which are strongly involved in carbohydrate and lipid metabolism (23). Melatonin abolishes the inhibitory effect on 3T3-L1 preadipocyte differentiation induced by MCF-7 cells. Additionally, it reduces mRNA expression and the concentration of anti-adipogenic cytokines (TNF-α, IL-6 and IL-11) in 3T3-L1 cells in 3T3-L1 and MCF-7 cell co-culture (9).

On the other hand, however, Alonso-Vale et al. proved the inhibitory effect of melatonin at supraphysiological concentrations on adipocyte differentiation through the reduction in the expression of PPARγ and C/EBPα adipogenesis-regulating factors and late adipocyte differentiation markers, i.e., adiponectin, aP2, and perilipin (24).

The influence of melatonin on the proliferation depends on the cell type and concentrations. It was proven that melatonin at both physiological and pharmacological concentrations stimulates normal cell proliferation (25-31). Unlike normal cells, melatonin has an antiproliferative effect on cancer cells, mainly at pharmacological concentrations (18, 32-34).

There are three known types of membrane melatonin receptors, i.e., MT₁, MT₂ and MT₃ (35). Both the MT₁ and MT₂ receptors bind melatonin at picomolar concentrations and the MT₃ receptor - at nanomolar concentrations. Membrane melatonin receptors are widely distributed within cardiovascular, genitourinary, immune and central nervous systems as well as within the alimentary tract (15, 36-41). In light of studies describing the influence of melatonin on the adipose tissue metabolism and mammal body mass, the discovery of MT₁ and MT₂ receptors proved to be significant. These two types of receptors are located within human white and brown adipose tissues, on adipocyte surface of human PZ6 cell line and rat isolated adipocytes (10, 39, 40) and the MT₁ receptors are located on the surface of hamster adipocytes (41).

Due to lipid solubility, melatonin influences intracellular metabolism with the participation of calmodulin regardless of the participation of receptors (42).

The best known nonselective antagonist of MT₁ and MT₂ receptors is luzindole, with a 25-fold higher affinity to the MT₂ receptor. The application of luzindole abolishes proliferative effects of melatonin on murine splenocytes in vitro as well as in vivo, inhibits the antiproliferative effect of melatonin on the Ishikawa cell line of human endometrial cancer and antagonizes the protective activity of endogenous melatonin in rat cerulein-induced pancreatitis and inhibits the expression of melatonin-stimulated receptors for IL-2 and IL-2 synthesis in human lymphocytes (14, 28, 43-46). In the murine fibroblast line, luzindole has an inhibitory effect on melatonin-induced lipogenesis (21). Luzindole abolishes the stimulative effect of melatonin on 3T3-L1 preadipocyte differentiation by inhibiting mRNA expression for C/EBPα and PPARγ. It also abolishes the inhibitory effect of melatonin on the activity and expression of mRNA for aromatase in 3T3-L1 cells (22) and on the expression of anti-adipogenic cytokines (TNF-α, IL-6 and IL-11) in 3T3-L1 and MCF-7 cell co-culture (9).

MT₁ receptors were found on the surface of hamster adipocytes (41). There are both confirming and contradicting reports concerning the participation of the MT₁ receptor and prazosin in the melatonin action mechanism (15, 47, 48). Prazosin, a selective adrenergic α₁-receptor antagonist, is the only known selective MT₁ receptor antagonist (35, 38, 49). On their surface, adipocytes of white and brown adipose tissues contain adrenergic α₁, α₂, β₁, β₂ receptors (8, 50, 51).

It is suggested that melatonin treatment can result in an increase in adipose tissue mass (42, 52-54) and a reduction in body mass (55). The mechanism of melatonin participation in the body mass regulation has not yet been fully understood. According to some authors, it results from the stimulation of melatonin receptors in the central nervous system and the modulation of the autonomic nervous system activity and food intake modification (53), whereas others authors conclude that it is related to a direct action of melatonin on adipocytes (10, 40, 41). In the case of hibernating animals, an increase in adipose tissue mass is thought to be related to melatonin and the seasonal rhythm (52-54). A different effect of melatonin on autonomic nervous system activation is suggested, i.e., stimulative in the case of brown adipose tissue cells and, most probably, inhibitory in the case of white adipose tissue cells. The examination performed on adipocytes of the brown adipose tissue of the golden hamster showed that melatonin reduces adipose tissue mass, probably through the activation of thermogenesis (53). In humans and rats the amount of visceral adipose tissue increases with age whereas nocturnal melatonin concentrations decrease (56). Melatonin reduces the body mass in diet-induced obesity in rats (55).

Cell growth is inhibited by high concentrations of reactive oxygen species (ROS) and induced by low ROS concentrations due to the stimulation of the proliferation process (57). It was proven that ROS produced in mitochondria limit oxidative phosphorylation within the respiratory chain, which acts as a signal inhibiting the proliferation of 3T3-L1 preadipocytes. When ROS production is limited, e.g., after the application of antioxidants, an increased proliferation of these cells is obtained (58). Paradoxically, both oxidants, H₂O₂ and antioxidants, e.g., alpha-tocopherol may have similar effects on cell growth (59). The application of antioxidants such as vitamins E, C and A, or N-acetyl-L-cysteine inhibits cell proliferation and the application of butylated hydroxyanisole, trolox or propofol stimulates 3T3-L1 cell proliferation (60, 58).

The aim of the present study is to evaluate the influence of melatonin at physiological and supraphysiological concentrations on the proliferation of the 3T3-L1 murine preadipocyte after 3 and 24 hours of the experiment and to determine the participation of membrane melatonin MT₂ receptors, and for the first time - MT₃, in melatonin influence on cell proliferation in the 3T3-L1 murine preadipocyte culture during a 24-hour experiment.

**MATERIALS AND METHODS**

**Cell culture**

The 3T3-L1 murine preadipocyte cell line obtained from murine 3T3 fibroblasts is the precursor of mature adipose tissue...
cells. A suspension of the cells at a density of $1 \times 10^6$ was cultured in culture flasks (25 cm$^2$) in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 4 mmol/L L-glutamine, 10% fetal bovine serum (FBS), 100 U/mL penicillin and 0.1 mg/mL streptomycin in standard conditions, i.e., 5% CO$_2$ at 37°C.

Reagents

The 3T3-L1 cells were obtained from the American Type Culture Collection, Rockville, USA (ATCC). The following were used in the experiment: melatonin, luzindole, and prazosin - Sigma Chemical Co. (St. Louis, MO, USA) whereas trypsin-EDTA, Dulbecco's Modified Eagle's Medium (DMEM) supplemented with L-glutamine, fetal bovine serum (FBS), trypan blue were purchased from Bio-Whittaker (Verviers, Belgium). Penicillin and streptomycin were obtained from Gibco (Paisley, Scotland, UK) and [3H]-thymidine from Amersham (Aylesbury, UK). Melatonin was dissolved in ethanol 95% (0.001%) directly prior to the experiment and diluted in the medium to obtain a supraphysiological concentration (10$^{-3}$ mol/L) and then a physiological concentration (10$^{-6}$ mol/L). Melatonin solution was not added to the control group cells. Cells were only treated with 0.001% ethanol solution. Luzindole and prazosin were also dissolved in 0.2 mL of ethanol (95%) and diluted with the medium to the final concentration of 10$^{-4}$ and 10$^{-5}$ mol/L, respectively.

The concentrations of luzindole and prazosin given were selected according to the preliminary study and our previous experiments. The abovementioned concentrations have been chosen due to their optimal effects. Similar amounts were also used in the studies conducted by previous authors.

Experimental protocol

The cells were cultured with or without melatonin at 10$^{-3}$ and 10$^{-6}$ mol/L, with or without melatonin antagonists, i.e., luzindole (10$^{-4}$ mol/L) and prazosin (10$^{-5}$ mol/L) for 3 and 24 hours without any change of medium. Luzindole (10$^{-4}$ mol/L) and prazosin (10$^{-5}$ mol/L) were added 30 min before subsequent exposure of the cells to melatonin. After the experiment, the medium was collected, centrifuged and frozen for examination. Cells were trypsinized (2–3 mL of 0.25% Trypsin-EDTA solution) and the number of cells was estimated by direct staining performed for 15 min. The proportion of dead cells was counted/mL. Cell viability was evaluated by means of trypan blue staining performed for 15 min. The results were presented as cell count/mL. The vitality of the cells were determined by coloring them with tryptan blue for 15 min; n=5.

Statistical analysis

The data were collected in the Microsoft Excel spreadsheet. After the preliminary calculation, the data were transferred to a database of StatSoft STATISTICA 7.0, by means of which the analyses were conducted. The conformity of the distribution of continuous variables to the Gaussian distribution was verified with the Kolmogorov-Smirnov test. The evaluation of the significance of statistical difference between the means was performed with parametric Student's t-test and non-parametric tests, i.e., the Mann-Whitney U test for unrelated variables and the Wilcoxon signed-rank test for related variables, depending on the conformity of the results to the Gaussian distribution. The results were given as arithmetic means ± standard deviation. Statistical significance was defined as $P<0.05$.

RESULTS

The influence of melatonin on cell count

Melatonin at both the physiological and supraphysiological concentration has a stimulative effect on the number of 3T3-L1 preadipocytes. An increase by 12% and 34% in cell count was observed compared to the controls at 10$^{-3}$ mol/L and 10$^{-6}$ mol/L concentrations ($P<0.05$), respectively. The application of luzindole inhibits the above effect of melatonin both at the 10$^{-3}$ mol/L and 10$^{-6}$ mol/L concentration ($P<0.05$). The presence of prazosin does not have a statistically significant influence on the effects of

<table>
<thead>
<tr>
<th>Cell count (10$^3$/mm$^3$)</th>
<th>Start point</th>
<th>After 24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>250 ± 15</td>
<td>350 ± 40</td>
</tr>
<tr>
<td>Luzindole</td>
<td>225 ± 10</td>
<td>330 ± 25</td>
</tr>
<tr>
<td>Prazosin</td>
<td>200 ± 10</td>
<td>270 ± 10</td>
</tr>
<tr>
<td>Melatonin 10$^{-3}$ mol/L</td>
<td>250 ± 15</td>
<td>380 ± 30</td>
</tr>
<tr>
<td>Melatonin 10$^{-3}$ mol/L + luzindole</td>
<td>350 ± 25</td>
<td>465 ± 30</td>
</tr>
<tr>
<td>Melatonin 10$^{-3}$ mol/L + prazosin</td>
<td>400 ± 30</td>
<td>600 ± 40</td>
</tr>
<tr>
<td>Melatonin 10$^{-6}$ mol/L</td>
<td>350 ± 30</td>
<td>610 ± 45</td>
</tr>
<tr>
<td>Melatonin 10$^{-6}$ mol/L + luzindole</td>
<td>300 ± 20</td>
<td>450 ± 35</td>
</tr>
<tr>
<td>Melatonin 10$^{-6}$ mol/L + prazosin</td>
<td>175 ± 10</td>
<td>300 ± 25</td>
</tr>
</tbody>
</table>

Cell count at the beginning of the experiment and 24 hour after were presented as mean values ± S.D. in thousands per mm$^3$ (10$^3$/mm$^3$). The vitality of the cells were determined by coloring them with tryptan blue for 15 min; n=5.
melatonin action (Table 1, Fig. 1). The number of dead cells
determined by means of blue trypan staining did not exceed 3%.

**The influence of melatonin on [3H]-labeled-thymidine
 incorporation in the cells**

A stimulative effect of melatonin on [3H]-thymidine
incorporation in 3T3-L1 preadipocytes was observed at both
concentrations and time intervals as compared to the control. It has
to be noted that the 10^-9 mol/L concentration has a stronger effect
compared to 10^-3 mol/L concentration both after 3 hours (133% vs.
117%, respectively; P<0.05) and 24 hours (209% vs. 105%;
P<0.05). The stimulative effect of melatonin on [3H]-thymidine
incorporation in the cells at 10^-9 mol/L concentration is expressed
much more strongly during a 24-hour incubation compared to a
3-hour incubation (209% vs. 133%; P<0.05) (Fig. 2).

The increase in labeled [3H]-thymidine incorporation in the
DNA of the cells compared to the control was observed after the
application of melatonin at 10^-3 mol/L concentration after 24 hours
(4.9% increase; P<0.05). The application of luzindole with
melatonin resulted in the inhibition of cell proliferation by 7%
(P<0.05) compared to the application of melatonin independently.
The presence of prazosin did not have a statistically significant
influence on the effects of melatonin action (Fig. 3).

There was a significant, over two-fold increase in the labeled
[3H]-thymidine incorporation in the DNA of the cells compared to
the control at 10^-9 mol/L concentration (109.4% increase;
P<0.05) during a 24-hour exposure. The above melatonin action
was partially inhibited by about 34% due to the application of
luzindole (P<0.05). However, the presence of prazosin did not
have a statistically significant influence on the effects of
melatonin action (Fig. 4).

In summary, the application of melatonin at both
concentrations stimulated the 3T3-L1 cell proliferation. It has to
be noted that the physiological concentration had a stronger
effect compared to supraphysiological concentrations.

**DISCUSSION**

In the conducted experiment, melatonin at physiological (10^-9
mol/L) and supraphysiological (10^-3 mol/L) concentrations had a
stimulative effect on the 3T3-L1 cell proliferation during 3- and
24-hour incubation. The maximum proliferation increase was

![Fig. 1. Influence of a 24-hour incubation with
melatonin (10^-3 mol/L and 10^-9 mol/L) and
melatonin receptors antagonists - luzindole (10^-4
mol/L) and prazosin (10^-5 mol/L) on 3T3-L1
preadipocyte cell count. The values are presented
as percent of increase in cell count. The initial
number of cells in particular groups were claimed
as 100%. n=5; * p<0.05 vs. control; # p<0.05 vs.
melatonin 10^-3 mol/L; " p<0.05 vs melatonin 10^-9
mol/L.]

![Fig. 2. Influence of 10^-3 mol/L and physiological
concentration (10^-9 mol/L) of melatonin on
[3H]-thymidine incorporation in 3T3-L1
preadipocytes, during 3-hour and 24-hour
incubation. The values are presented as percents
± S.D. The initial number of cells in particular
groups were claimed as 100%. n=5; * p<0.05 vs.
control; # p<0.05 vs melatonin 10^-3 mol/L.]


obtained during long exposure using the physiological concentration. Membrane receptors, mainly MT₂, participate in the above melatonin action.

Melatonin at 10⁻³ mol/L and 10⁻⁹ mol/L concentrations had a stimulative effect on the number of 3T3-L1 preadipocytes. The highest increase in cell count by 34% compared to the control was observed at the physiological concentration. Comparing the 3- and 24-hour proliferative effect of melatonin it was observed that physiological concentrations of melatonin resulted in over a two-fold increase in [³H]-thymidine incorporation in the cells after 24 hours and only a 33% increase after 3 hours. However, in the instance of supraphysiological concentrations an increase by 4.8% and 17% was observed, respectively. The above results may be related to the fact that a full cell cycle of 3T3-L1 preadipocytes lasts about 18 hours and in the initial phase of the preadipocyte cell cycle - between G₁ and S phases - due to a cell-to-cell contact, their growth stops and one or two cell divisions occur (the phenomenon known as clonal expansion) (2).

In our previous study on the influence of melatonin on 3T3-L1 preadipocytes, it was proven that melatonin at high, i.e., 10⁻⁶ mol/L and 10⁻³ mol/L concentrations caused a significant increase in the number of cells, which was directly proportional to the exposure time and inversely proportional to the dose (20). The increase was from 6 to 50% as assessed by the direct method.

To date it, has been shown that melatonin at physiological and pharmacological concentrations stimulates normal cell proliferation, i.e., human osteoblasts (25), keratinocytes (26), dermal fibroblasts (27), murine splenocytes (28), rat hippocampal dentate gyrus cells (29), human mesenchymal stem cells (30), and neural stem cells (31). On the other hand, there are reports on an antiproliferative effect of melatonin on cells from rat thymus, brain, lung and kidney homogenates (61).
Adipocytes are produced from pluripotent mesenchymal stem cells. Mesenchymal cells differentiate into adipoblasts, preadipocytes, and then adipocytes. Numerous transcription factors participate in the differentiation into mature adipocytes. These are the following factors: CCAAT/enhancer-binding protein β (C/EBPβ), CCAAT/enhancer-binding protein δ (C/EBPδ), PPARγ, C/EBPα and cAMP response element-binding protein (CREB) (62).

The majority of the mentioned studies indicate proproliferative effects of melatonin on normal cells, including 3T3-L1 preadipocytes, which is consistent with our results. Maldonado et al. described a dose-dependent stimulating effect of melatonin on adipogenesis in the murine fibroblast line (21). Interesting studies were reported by Gonzalez et al. and Alvarez-Garcia et al., where 3T3-L1 cells are presented as a model of fibroblasts of adipose tissue surrounding breast carcinoma. It was shown that melatonin at physiological and supraphysiological concentrations stimulates the 3T3-L1 preadipocyte differentiation and adipogenesis, as well as the expression of C/EBPα and PPARγ transcription factors. Melatonin abolishes the inhibitory influence on 3T3-L1 preadipocyte differentiation induced by MCF-7 cells in 3T3-L1 and MCF-7 cell co-culture. Additionally, it reduces the expression of mRNA and the concentration of anti-adipogenic cytokines (TNF-α, IL-6 and IL-11) in 3T3-L1 and MCF-7 cell co-culture (9).

On the other hand, Alonso-Vale et al. (24) demonstrated the inhibitory effect of melatonin at supraphysiological concentrations on adipocyte differentiation by the reduction in the expression of adipogenic transcription factors (PPARγ and C/EBPα) and late adipocyte differentiation markers, i.e., adiponectin and perilipin.

Based on the results in the literature on the presence of membrane melatonin receptors on the surface of white adipose tissue adipocytes, a melatonin receptor antagonist - luzindole - was used in the present experiment. In the conducted study, melatonin influences preadipocyte proliferation partly by MT, and MT2 membrane receptors, which was proven by the ability of luzindole to inhibit the effects of melatonin, particularly by the MT2 receptor. This is due to the fact that luzindole, which is a nonselective antagonist of MT1 and MT2 melatonin receptors, has the highest, i.e., about 25-fold affinity to the MT2 receptor. Luzindole antagonizes the influence of melatonin at physiological concentrations on 3T3-L1 cell proliferation most powerfully, i.e., by about 34%. However, it does not abolish its action entirely, which may show that other melatonin receptors, e.g., MT1 membrane receptor or a nuclear receptor participate in this process. Similarly, Gonzalez et al. (22) showed that luzindole abolishes the inhibitory effect of melatonin on 3T3-L1 preadipocytes by the inhibition of mRNA expression for PPARγ and C/EBPα increased by melatonin to the level present in the control group. Additionally, luzindole abolished the inhibitory effect of melatonin on the activity and expression of mRNA for aromatase in 3T3-L1 cells. The above action proves that MT2 receptors participate in the mechanism of melatonin action on such cells (22).

Luzindole abolishes the inhibitory effect of melatonin on the expression of anti-adipogenic cytokines (TNF-α, IL-6 and IL-11) in 3T3-L1 and MCF-7 cell co-culture (9).

Numerous studies have proven that luzindole inhibits the effects of melatonin action on other cells (28, 44-46). Our previous experiment showed that luzindole partly abolishes the stimulatory effect of melatonin at pharmacological concentrations on the increase in the 3T3-L1 cell count, estimated by the direct counting (20). The authors of the above studies agree that luzindole at the concentrations similar to the above has an inhibitory effect on proproliferative melatonin action with the participation of MT2 receptor.

To date, in the available literature there have not been any studies on the presence of the MT1 receptor in white adipose tissue cells. In the present study, prazosin, which is a selective MT1 receptor antagonist, was used. It is also a well-known selective antagonist of 1-adrenergic receptors. The MT1 membrane receptor, a 26 kDa protein, is a homolog of flavoenzyme - human quinone reductase 2 (QR2, EC 1.6.99.2). The receptor does not act through G proteins and is characterized by a low affinity to melatonin. The role of the MT1 receptor in intracellular transmission is to stimulate phosphatidylinositol (63). The receptor is present in the liver, kidney, brain, heart, adipose tissue, skeletal muscles and lungs of the hamsters and the mice (37, 52, 63), and on the surface of hamster adipocytes (42). The only known selective MT1 receptor antagonist is prazosin - a selective antagonist of α1 adrenergic receptors (35, 38, 49). White and brown adipose tissue adipocytes have adrenergic receptors, i.e., α1, α2, β1, β2 on their surface (8, 51, 52). There are both confirming and contradictory reports on the participation of the MT1 receptor and prazosin in melatonin action mechanism (15, 47, 48).

In the present study, the influence of prazosin on proproliferative effect of melatonin action has not been observed. Few in vivo and in vitro studies show an inhibitory effect or lack of the effect of prazosin and the participation of MT1 receptor on melatonin action (47, 64, 65). Podda et al. showed that melatonin inhibits the neural activity within rat central vestibular nucleus in vitro and that the application of prazosin did not antagonize this effect (64). On the other hand, Souze et al. (47) proved that prazosin suppresses stimulative effects of melatonin on the cell growth and tyrosinase activity in the human melanoma cell line (47). Similarly, Santagostino-Barbone et al. showed that prazosin at the 0.3 μmol/L concentration inhibits the melatonin-induced contraction of colonic smooth muscle isolated from guinea pigs by about 30% compared to the maximum melatonin effect (65). The absence of prazosin action in the present study may be explained by a potential absence of MT1 receptor on the surface of 3T3-L1 preadipocytes.

The discovery of melatonin receptors on adipose tissue cells gives grounds for considering the possibility of melatonin acting as a factor influencing energy storage through the modulation of metabolism and adipocyte proliferation (10). Melatonin inhibits, rather than stimulates, the influence of the autonomic nervous system on white adipose tissue cells. Therefore, it can stimulate not only lipid storage, but also adipocyte proliferation (53).

Few study results on the relationship between melatonin and adipocyte metabolism are contradictory. On the one hand, Brydon et al. proved that the exposure of human PAZ6 adipocytes to melatonin resulted in the decrease in the amount of mRNA, GLUT-4 receptor protein count, and glucose uptake in these cells (10). Conversely, Alonso-Vale et al. (11) found that melatonin results in the increase in adipocyte sensitivity to insulin. Moreover, melatonin at physiological concentrations (10-9 mol/L) resulted in an over two-fold increase in glucose uptake in the murine C2C12 skeletal muscle cell line, accelerated phosphorylation of insulin receptor substrate (1 IRS-1), and resulted in the increase in phosphoinositide 3-kinase (PI3-kinase) through the participation in glucose homeostasis (66). Additionally, it is suggested that there exists a relationship between the development of diabetes and the decrease in melatonin concentration in elderly individuals and in individuals exposed to light at night (67).

It has been noticed that changes in adipose tissue mass in hibernating animals are most likely related to the regulation of seasonal rhythms, for which melatonin is responsible. The increase in body mass in these animals starts from July when the day length and the amount of light begin to decrease and
melatonin secretion increases so that the maximum body mass is gained in September (41). In rats and humans the amount of visceral adipose tissue increases with age, whereas nocturnal melatonin concentrations decrease (56). It was proven that in shift workers who experience disturbances of day-night and sleep-wakefulness rhythms, which are related to melatonin production disturbances, there is a co-occurrence of disturbances in the body mass regulation, along with hypersonnia and the excessive demand for sweets (67).

The amount of melatonin produced in the circadian rhythm is also age-dependent. The highest nocturnal melatonin concentration is noted in children, and the lowest in the elderly. Human brown adipose tissue is most developed in children, together with the highest melatonin concentrations; the amount of the tissue decreases with age and along with an accompanying decrease in melatonin secretion (68).

Since melatonin was found to reduce body mass in diet-induced rat obesity (55), and considering the role of melatonin in maintaining energetic balance of the body, attention has been paid to the relationship between melatonin and leptin. Melatonin at physiological concentrations was observed to increase mRNA expression for leptin in murine adipocytes during simultaneous exposure to insulin, acting through the MT_1 membrane receptor (39). Also, a simultaneous decrease in both melatonin and leptin concentrations in night shift workers was observed (69). The increase in leptin concentration with a simultaneous decrease in melatonin concentration in middle-aged rats seems to be interesting and can be connected to the pathogenesis of age-related obesity, where leptin concentrations correlate positively with the amount of the adipose tissue (70).

The above mentioned controversial literature reports on varying influence of melatonin on the adipose tissue metabolism may partly result from a disparate behavior of the hormone in vivo and in vitro. In vitro conditions cannot precisely correspond to in vivo conditions, in which melatonin is inactivated in the liver within an hour from its application with the omission of significant neuroendocrine systems.

The present study has shown that melatonin is capable of stimulating preadipocyte proliferation. A direct relationship between the influence of produced ROS and cell proliferation has so far been observed in endothelial, smooth muscle and prostate cells, different neoplastic cell lines, fibroblasts and macrophages (59, 60, 71, 72). Paradoxically, both oxidants and antioxidants can exert similar effects on cell growth. High ROS concentrations both during short- and long-lasting exposure. These effects were accompanied by the stimulation of antioxidative enzymes, mainly mitochondrial (MnSOD) and peroxisomal (CAT), which was shown in our previous study (48). The same effects with the application of oxidants and antioxidants were produced by an experiment on a human preadipocyte cell culture from the subcutaneous adipose tissue of slim individuals (58).

The application of antioxidants alone to a human fibroblast culture without factors triggering the ROS production resulted in a dose-dependent inhibition of tritium-labeled thymidine incorporation (57). Similarly, it was shown that the inhibition of the ROS production by the application of such antioxidants as vitamins E, C and A, or N-acetyl-L-cysteine reduced cell proliferation (60). These results, which are not consistent with our observations, may be related to the differences connected with the usage of different antioxidants, different cell lines, experimental models and doses. These results can also be related to the fact that depending on their concentrations, antioxidants can act as oxidants and have similar effects on cell proliferation.

Thus, the above studies indicate a relationship between oxygen metabolism and preadipocyte number along with the amount of the adipose tissue, which is closely related to the change in body mass occurring in obesity. Moreover, we have proven in our previous work that melatonin precursor, serotonin, influences the cardiovascular system and modulates sympathetic activity (73). Based on these observations, melatonin can be considered to be among the factors capable of influencing the adipose tissue mass regulation.

Our observations from the present study confirm that melatonin at physiological and supraphysiological concentrations stimulates the 3T3-L1 preadipocyte proliferation. It has to be noted that physiological concentrations of melatonin are more effective. The present experiment has, for the first time, assessed the MT_1 receptor participation through the application of prazosin. The action of melatonin has not been confirmed in this mechanism. It has been proven that melatonin has a proproliferative effect on 3T3-L1 preadipocytes, partially by MT_2 receptors, and that prazosin has no effect on the proliferation. This proves that melatonin receptors, primarily MT_1, participate in the proproliferative melatonin effect.

Conflict of interests: None declared.

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Received: October 27, 2013
Accepted: January 9, 2014

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