Original Paper

Melatonin-Induced Changes in Cytosolic Calcium Might be Responsible for Apoptosis Induction in Tumour Cells

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Key Words

Melatonin • Apoptosis • ER-stress • Cancer • Calcium • Reactive oxygen species

Abstract

Background/Aims: Melatonin is a hormone transferring information about duration of darkness to the organism and is known to modulate several signaling pathways in the cells, e.g. generation of endoplasmic reticulum stress, oxidative status of the cells, etc. Melatonin has been shown to exert antiproliferative and cytotoxic effects on various human cancers. We proposed that this hormone can differently affect tumour cells and healthy cells. **Methods:** We compared the effect of 24 h melatonin treatment on calcium transport (by fluorescent probes FLUO-3AM and Rhod-5N), ER stress (determined as changes in the expression of CHOP, XBP1 and fluorescently, using Thioflavin T), ROS formation (by CellROX® Green/Orange Reagent) and apoptosis induction (by Annexin-V-FLUOS/propidiumiodide) in two tumour cell lines – ovarian cancer cell line A2780 and stable cell line DLD1 derived from colorectal carcinoma, with non-tumour endothelial cell line EA.hy926. **Results:** Melatonin increased apoptosis in both tumour cell lines more than twice, while in EA.hy926 cells the apoptosis was increased only by 30%. As determined by silencing with appropriate siRNAs, both, type 1 sodium/calcium exchanger and type 1 IP₃ receptor are involved in the apoptosis induction. Antioxidant properties of melatonin were significantly increased in EA.hy926 cells, while in tumour cell lines this effect was much weaker. **Conclusion:** Taken together, melatonin has different antioxidative effects on tumour cells compared to non-tumour ones; it also differs in the ability to induce apoptosis through the type 1 sodium/calcium exchanger, and type 1 IP₃ receptor. Different targeting of calcium transport systems in tumour and normal, non-tumour cells is suggested as a key mechanism how melatonin can exert its anticancer effects. Therefore, it might have a potential as a novel therapeutic implication in cancer treatment.

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Introduction

Melatonin (N-acetyl-5-methoxytryptamine) is a hormone primarily produced by the pineal gland, which displays characteristic daily and seasonal patterns of secretion, and plays an important role in sleep regulation [1, 2]. Large number of studies has demonstrated the protective role of melatonin and its metabolites against free radicals and oxidative stress [3-5]. Thus, melatonin acts as an antioxidant by scavenging free radicals and increasing the activity of antioxidant enzymes in the body. Melatonin has been consistently shown to suppress formation of reactive oxygen/nitrogen species (ROS/RNS) at the mitochondrial level, thereby protecting against oxidative or nitrosative damage to electron transport chain proteins. Moreover, it also limits lipid peroxidation in the inner membrane, thus favouring electron flux and ATP (adenosine triphosphate) production [6].

Melatonin has also been shown to exert antiproliferative and cytotoxic effects on human cancers, including colorectal cancer [7], neuroblastoma [8], hepatocarcinoma [9], lung cancer [10-12], breast cancer [13], prostate cancer [14, 15], and leukaemia [16, 17]. In a gastric cancer cell line it was already shown that proapoptotic effect of melatonin could be due to activation of a Caspase-dependent pathway and inhibition of the nuclear translocation of NF- κ B p65, two processes that are regulated by p38 and c-Jun N-terminal kinase (JNK) [18]. Nevertheless, in human MG-63 osteosarcoma cells melatonin's antiproliferative action was mediated by inhibition of the extracellular signal-regulated kinase (ERK1/2) signalling pathway rather than the p38, JNK, or Akt pathways [19]. However, the precise molecular mechanisms of melatonin action in cancer cells are still under investigation.

Several factors contribute to the mechanisms that make melatonin an interesting compound for targeting cancer cells. Among these, endoplasmic reticulum (ER) stress is of special importance. Previous studies have demonstrated that the ER is responsible for protein folding and trafficking, lipid synthesis, and the maintenance of calcium homeostasis in the cell. Melatonin acts cooperatively with ER stress to promote the apoptosis of cancer cells or inhibits ER stress to attenuate chemotherapy-associated side effects and chemoresistance. In rats with diethylnitrosamine-induced hepatocarcinogenesis, melatonin treatment significantly increases the expression of activating transcription factor 6 (ATF6), C/EBP homologous protein (CHOP), and binding immunoglobulin protein (BIP), which might further promote the incidence of apoptosis [20]. Apoptosis derived from unresolved ER stress has also been observed, indicating that a sustained ER stress response could contribute to tumour cell death [21]. Resistance to cell death is a distinctive characteristic of cancer. Apoptosis is one of the main mechanisms implicated in cell death, and its inactivation contributes to tumour progression and chemotherapy resistance [22]. Proapoptotic effects of melatonin [23, 24], and its effects in combination with ER stress inducers (e.g. tunicamycin) have been reported in different hepatocellular carcinoma cell lines [25]. Interestingly, melatonin has been shown to induce apoptosis under ER stress conditions in hepatoma cells via induction of C/EBP homologous protein (CHOP) and suppression of cyclooxygenase 2 (COX-2) [25]. Melatonin exerts antimetastatic effects on liver and breast cancers and also inhibits matrix metalloproteinase (MMP) activity [26].

Calcium is an important signalling molecule that modulates a variety of processes, including the induction of apoptosis. Moreover, depletion of calcium from the ER is an important feature of ER stress. The role of calcium in the action of melatonin is only partially elucidated. Melatonin reduces Ca^{2+} release and modulates the pancreatic responses induced by cholecystokinin-8 (CCK-8), which might be explained by the stimulation of Ca^{2+} transport from cells through the plasma membrane and subsequent Ca^{2+} reuptake into the endoplasmic reticulum [27, 28]. Melatonin might exert protective effects against oxidative stress, cytosolic calcium overload and mitochondrial damage in dexamethasone-induced neurotoxicity [8].

Based on this knowledge, we aimed to compare the proapoptotic effect of melatonin on tumour versus normal cell lines, examining its impact on ER stress, its induction of apoptosis and its antioxidant effects. Moreover, we studied the involvement of calcium and some calcium transporters (especially those that are known to be involved in the process of

apoptosis — type 1 sodium/calcium exchanger (NCX1) [29, 30] and type 1 IP₃ receptor [31]) in melatonin-induced apoptosis.

Materials and Methods

Cell lines and treatment

Experiments were performed on ovarian cancer cell line A2780 (Sigma Aldrich, USA), stable cell line derived from colorectal carcinoma DLD1 (Sigma Aldrich, USA) and endothelial EA.hy926 cell line (ATCC® CRL-2922™). Ovarian carcinoma cells and colon adenocarcinoma cells were incubated in RPMI-1640 medium (Sigma Aldrich, USA), supplemented with 2% L-glutamine and NaHCO₃, 10% fetal bovine serum, and penicillin/streptomycin antibiotics. EA.hy926 cells were incubated in DMEM medium, supplemented with 2% L-glutamine, 10% fetal bovine serum, and penicillin/streptomycin antibiotics. Cells were cultured in humidified atmosphere at 37°C and 5% CO₂. After plating, cells were treated for 24 h with melatonin (Sigma Aldrich, USA) in a final concentration 0.1, 1.0, and 10 μM.

Analysis of the gene expression by real-time PCR

Total RNA was isolated using TRI Reagent. Reverse transcription was performed using 1.0 μg of total RNAs and Ready-To-Go You-Prime First-Strand Beads with the pd(N6) primer (GE Healthcare Life Sciences, UK). The real-time PCR amplification and detection was carried out on the Applied Biosystems StepOne™ RealTime PCR Systems (Applied Biosystems, USA). SYBR Green Master Mix with ROX reference dye (ThermoFischer Scientific, USA), primers (10 pmol) and the reverse transcription product were mixed to a final volume of 10 μl. Master Mix with primers and template was separately loaded onto 96-well and 48-well plates. Plates were centrifuged to remove air bubbles in the wells. The PCR protocol consisted of 10 min 95°C initial denaturation, followed by 40 repeats of 15 s 95°C denaturation and 1 min 60°C annealing/elongation. The expression of target genes type 1 sodium-calcium exchanger (NCX1), inositol trisphosphate receptor type 1 (IP₃R1), inositol trisphosphate receptor type 2 (IP₃R2), inositol trisphosphate receptor type 3 (IP₃R3), plasma membrane Ca²⁺ ATPase (PMCA), CHOP, X-box binding protein 1 (XBP1) was normalized to the expression of housekeeping gene β-actin. For detection of β-actin, NCX1, PMCA and IP₃R1 the following primers were designed: human β-actin: 5'- ACA TCT GCT GGA AGG TGG AC - 3' (forward); 5'- TCC TCC CTG GAG AAG AGC TA - 3' (reverse); human NCX1: 5'- TCC CAT CTG TGT TGT GTT CGC - 3' (forward); 5'- TCA TCT TGG TCC CTC TCA TC - 3' (reverse); human PMCA: 5'- GTG AAG GCA GTG ATG TGG G - 3' (forward); 5'- CTT CAT CAT AGT GCG TGA GAT - 3' (reverse); human IP₃R1: 5'- TCT ATG AGC AGG GGT GAG ATG AG - 3' (forward); 5'- GGA ACA CTC GGT CAC TGG AT - 3' (reverse) [32]. For detection of IP₃R2, IP₃R3, CHOP, XBP1 expression the following primers were used: human IP₃R2: 5'- ATG CGT GTG TCC TTG GAT GC - 3' (forward) [33]; 5'- GTA GCA GAA GTA GCT GAT TG - 3' (reverse) [33]; human IP₃R3: 5'- AGT GAG AAG CAG AAG AAG G - 3' (forward) [34]; 5'- CAT CCG GGG GAA CCA GTC - 3' (reverse) [34]; human CHOP: 5'- GGA GCT GGA AGC CTG GTA TGA GG - 3' (forward) [35]; 5'- TCC CTG GTC AGG CGC TCG ATT TCC - 3' (reverse) [35]; human XBP1: 5'- CTG AGT CCG CAG CAG GTG - 3' (forward) [36]; 5'- AGT TGT CCA GAA TGC CCA ACA - 3' (reverse) [36].

Western blot analysis

Cells were scraped and resuspended in 10 mM Tris-HCl, pH 7.5, 1 mM phenylmethyl sulfonylfluoride (PMSF, Serva, Heidelberg, Germany), protease inhibitor cocktail tablets (Complete EDTA-free, Roche Diagnostics, Mannheim, Germany) and subjected to centrifugation for 10 min at 3000 x g at 4°C. The pellet was resuspended in Tris-buffer containing the 50 μM CHAPS (3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate, Sigma Aldrich, USA), and then incubated for 20 min at 4°C. Protein concentration in lysate was determined by the method of Lowry et al. [37]. 5-90 μg of protein extract from each sample was separated by electrophoresis on 8-16% and 4-12% SDS polyacrylamide gradient gels (Amersham Biosciences, UK), and proteins were transferred to the Hybond-PVDF membrane (Amersham Biosciences, UK) using semidry blotting (Owl, Inc., USA). Membranes were blocked in 5% non-fat dry milk in Tris-Buffered Saline with Tween 20 (TBS-T) overnight at 4°C and then incubated for 1 h with appropriate primary antibody. Following washing, membranes were incubated with secondary antibodies to mouse or rabbit IgG conjugated to horseradish peroxidase for 1 h at room temperature. An enhanced chemiluminescence

detection system (LuminataTM Crescendo Western HRP Substrate, Millipore) was used to detect bound antibody. The optical density of individual bands was quantified using PCBAS 2.0 software. Antibodies raised against the following proteins were used: rabbit polyclonal antibody to NCX1 (π 11-13, Swant, Bellinzona, Switzerland), mouse monoclonal antibody to β -actin (Abcam, Cambridge, UK) and rabbit polyclonal antibody to IP₃R1 (Sigma, USA).

Determination of the levels of cytosolic [Ca²⁺]_i by Fluo-3AM fluorescent dye

This method was already described in Lencesova et al. [38]. Briefly, A2780, DLD1, and EA.hy926 cells were plated on 24-well plate at density of 4×10^4 . After 24 h of treatment, cells were washed with 1 ml of serum-free medium and loaded with 2 μ M Fluo-3AM (4-(6-acetoxymethoxy-2, 7-dichloro-3-oxo-9-xanthenyl)-4'-methyl-2, 2'-(ethylendioxy)dianiline-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl ester) (Sigma-Aldrich, USA) in the presence of 0.5% pluronate (Sigma-Aldrich, USA) in serum-free medium, for 30 min at 37°C, 5% CO₂, in dark. Afterwards, cells were washed with 1 ml of serum-free medium, and fluorescence was measured on the fluorescence scanner (BioTek, Germany) at λ_{ex} 560 nm and λ_{em} 526 nm. Results were calculated as the arbitrary units of fluorescence.

Fluorescence

Cells were plated on poly-L-lysine (10 mg mL⁻¹; Sigma-Aldrich, St. Luis, MD, USA)-coated coverslips in Sarstedt 24-well plates in 1 mL of medium with 10% of fetal bovine serum and mixture of streptomycin and penicillin. Cells were incubated in a humidified atmosphere of 5% CO₂ air at 37°C. After the melatonin treatment, cells were washed three times with 1 ml of 0.1 M PBS (phosphate buffers saline, pH 7.4). Cell ROX Green reagent (Invitrogen, USA) was used to visualize ROS. The cells were stained with reagent (5 μ M) and incubated at 37°C for 30 min. Afterwards, cells were washed with PBS. The intensity of CellROX Green fluorescence is proportional to the level of free radical oxidation. In order to determine ER stress, Thioflavin T in PBS at a final concentration 50 μ M was used according to procedure described by Beriault and Werstuck [39]. Hoechst 33258 (50 μ g.mL⁻¹, Sigma Aldrich, USA) was used to counterstain nuclei. All specimens were imaged on Nikon Eclipse Ti-S/L100 epifluorescence microscope equipped with appropriate set of excitation and emission filters (Nikon, Japan).

Immunofluorescence

EA.hy926 and DLD1 grown on glass coverslips were fixed in ice-cold methanol. Non-specific binding was blocked by incubation with PBS containing 3% BSA for 60 min at room temperature. Cells were then incubated with primary antibody diluted in PBS with 1% BSA for 1 h at 37°C. In these experiments, the anti-NCX1 rabbit polyclonal antibody p11-13 (1:200 dilution, Swant, Switzerland) against the full length canine cardiac NCX1 was used. This antibody recognizes all splice variants of NCX1, does not cross react with other NCX isoforms. Afterwards, cells were washed four times with 1xPBS with 0.02% TWEEN (Sigma Aldrich, USA) for 10 min, incubated with Alexa Fluor-488 donkey anti-rabbit IgG (1:1000 dilution, Thermo Fisher Scientific, USA) in PBS-BSA for 1 h at 37°C and washed as previously. Also, to determine type 1 IP₃ receptor mouse monoclonal antibody to synthetic peptide, corresponding to amino acids near the C-terminus of the IP₃ receptor (1:100 dilution, Calbiochem, Darmstadt, Germany) was used. Cells were washed four times with 1xPBS with 0.02% TWEEN (Sigma Aldrich, USA) for 10 min, incubated with Alexa Fluor-594 goat anti-mouse IgG (1:1000 dilution, Thermo Fisher Scientific, USA) in PBS-BSA for 1 h at 37°C and washed as previously. Finally, cells were mounted onto slides in mounting medium with DAPI (Sigma Aldrich, USA), analyzed by fluorescence microscopy (Axio Scope.A1, Zeiss, Germany) using EC Plan-Neofluar 100x/1.3 oil objective. Images of all samples were acquired at the same microscope setup.

Detection of apoptosis with Annexin-V-FLUOS

After the melatonin treatment, A2780, DLD1, and EA.hy926 cells were gently scraped and pelleted at 100 x g for 5 min. Cells were then washed with 1 ml of PBS and cell pellet was resuspended in 200 μ l of Annexin-V-FLUOS/propidium iodide labeling solution (Roche Diagnostics, USA) and incubated at a room temperature in dark for 20 min according the manufacturer's protocol. After the incubation, samples were diluted with 400 μ l PBS, placed on ice and measured on BD FACSCanto II flow cytometer (Becton Dickinson, Ann Arbor, USA).

Cellular oxidative stress by CellROX® Orange Reagent

Cellular oxidative stress was considered by measuring of ROS using CellROX® Orange Reagent (Thermo Fisher Scientific, USA). Cells were plated 24 h before the experiment in 24 well plates. After that, cells were treated by different concentrations of melatonin (10 μ M, 1 μ M, and 100 nM) for 24 h in serum free medium. As a positive control, pyocyanine was used (50 μ M for 4 h, Sigma Aldrich, USA). Treated cells were washed with serum free medium and incubated with CellROX® Orange Reagent at 5 μ M final concentration in well for 30 min at 37°C in the dark in CO₂ incubator. After removing of fluorescent dye, cells were washed twice with 1x PBS (pH 7.4) and fluorescence was measured at 545 nm excitation and 565 nm emission wavelength on Synergy fluorescence reader (BioTec, Germany). Results were expressed as fluorescence arbitrary units.

Gene silencing

Cells were grown in 6-well plates in RPMI with 10% FBS. Transfection of siRNAs was performed with DharmaFECT1 transfection reagent (Dharmacon, Thermo Scientific, USA) as described previously Hudecova et al. [40]. ONTARGET plus SMART pool human ITPR1 and NCX1 siRNAs (Dharmacon, Thermo Scientific, USA) were applied to the final concentration of 100 pmol per well for 48 h. The same procedure was performed with ON-TARGET plus Non-targeting Pool, which serves for the determination of baseline cellular responses in RNAi experiments. Silencing was performed for 48 h. After the first 24 h of silencing, melatonin (10 μ M) was applied for additional 24 h. All groups of cells were then harvested and used in further experiments.

Statistical analyses

The results are presented as mean $\pm$ S.E.M. Each value represents an average of at least 6 wells from at least three independent cultivations of each type of cells. Statistical differences among groups were determined by ANOVA. Statistical significance * - $p < 0.05$ was considered to be significant, ** $p < 0.01$, *** $p < 0.001$. For multiple comparisons, an adjusted t-test with p values corrected by the Bonferroni method was used (Instat, GraphPad Software).

Results

In ovarian A2780 tumour cells and also in DLD1 colorectal carcinoma cells, melatonin decreased the cytosolic calcium concentration in a dose-dependent manner (Fig. 1A, B). The most pronounced decrease was observed at the melatonin concentration 10 μ M (in A2780 cells: from 4.1 ± 0.2 a.u. in control group vs. 3.0 ± 0.1 a.u. in melatonin treated group; in DLD1 cells from 4.03 ± 0.05 a.u. in control group vs. 2.77 ± 0.10 a.u. in melatonin treated group). However, melatonin did not cause any changes in cytosolic calcium in the normal

Fig. 1. Changes in levels of the cytosolic calcium in A2780 (A), DLD1 (B) and EA.hy926 (C) cells due to the melatonin treatment. In tumour cell lines A2780 and DLD1, melatonin decreased levels of cytosolic calcium in a concentration-dependent manner. In normal endothelial cell line EA.hy926, melatonin did not change levels of the cytosolic calcium. cont – untreated control, M0.1 – melatonin treatment (final concentration 0.1 μ M), M1 – melatonin treatment (final concentration 1 μ M), M10 – melatonin treatment (final concentration 10 μ M), for 24 h. Results are displayed as mean $\pm$ S.E.M. Each column represents an average of 6–9 samples from three independent cultivations. Statistical significance * represents $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Fig. 2. Changes in levels of the NCX1 expression in A2780 (A, B), DLD1 (C, D) and EA.hy926 (E, F) cells due to the melatonin treatment. In tumour cell lines A2780 and DLD1, melatonin increased NCX1 mRNA (B, D) and protein (typical Western blots – A, C) by a concentration-dependent manner. In normal endothelial cell line EA.hy926, melatonin also increased NCX1 mRNA (F) and protein (E), although this increase was not so pronounced as in tumour cells. cont – untreated control, M0.1 – melatonin treatment (final concentration 0.1 μ M), M1 – melatonin treatment (final concentration 1 μ M), M10 – melatonin treatment (final concentration 10 μ M), for 24 h. Results are displayed as mean $\pm$ S.E.M. Each column represents an average of 6–9 samples from three independent cultivations. Statistical significance * represents $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Fig. 3. Changes in levels of the IP₃R1 expression in A2780 (A, B), DLD1 (C, D) and EA.hy926 (E, F) cells due to the melatonin treatment. In tumour cell lines A2780 and DLD1, melatonin increased IP₃R1 mRNA (B, D) and protein (typical Western blots – A, C) by a concentration-dependent manner. In normal endothelial cell line EA.hy926, melatonin did not cause any changes in IP₃R1 mRNA (F) and protein (E). cont – untreated control, M0.1 – melatonin treatment (final concentration 0.1 μ M), M1 – melatonin treatment (final concentration 1 μ M), M10 – melatonin treatment (final concentration 10 μ M), for 24 h. Results are displayed as mean $\pm$ S.E.M. Each column represents an average of 6–9 samples from three independent cultivations. Statistical significance * represents $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

endothelial cell line EA.hy926 (Fig. 1C). The decrease in cytosolic calcium might be due to increased expression or activity of NCX1 or PMCA, or to calcium release from the ER, presumably through IP₃ receptors. Therefore, we tested the expression of NCX1 (Fig. 2). We observed melatonin-induced up-regulation of NCX1 mRNA in all three cell lines, although in A2780 and DLD1 tumour cells the increase was much greater (approximately three-fold, when 10 μ M melatonin treatment was applied for 24 h; Fig. 2B, D). In EA.hy926 cells, melatonin induced increase was less than two-fold when treated with 10 μ M melatonin (Fig. 2F). The up-regulated expression of NCX1 was verified by Western blot analysis, where the melatonin-induced increase was determined relative to β -actin in all three cell lines (Fig. 2,

Fig. 4. Determination of NCX1 (left) and IP₃R1 (right) in EA.hy926 and DLD1 cells in response to the melatonin treatment (10 μ M) as determined by the fluorescence microscopy. For NCX1, primary antibody anti-rabbit NCX1 and Alexa Fluor-488 labeled secondary antibody were used. Intensity of green fluorescence indicates amount of NCX1. For IP₃R1, primary antibody anti-mouse IP₃R1 and Alexa Fluor-594 labeled secondary antibody was used. Intensity of red fluorescence indicates amount of NCX1. cont – untreated control, M10 – melatonin treatment (final concentration 10 μ M) for 24 h, Inset represents the negative control. Bar represents 10 μ m.

Fig. 5. Changes in levels of the ER stress markers in A2780 (A,B), DLD1 (C,D) and EA.hy926 (E,F) cells in response to the melatonin treatment. In tumour cell lines A2780 and DLD1, melatonin increased CHOP and XBP1 mRNA by a concentration-dependent manner. In normal endothelial cell line EA.hy926, melatonin did not cause any changes in mRNA of ER stress markers (XBP1 and CHOP). cont – untreated control, M0.1 – melatonin treatment (final concentration 0.1 μ M), M1 – melatonin treatment (final concentration 1 μ M), M10 – melatonin treatment (final concentration 10 μ M) for 24 h. Results are displayed as mean $\pm$ S.E.M. Each column represents an average of 6–9 samples from three independent cultivations. Statistical significance * represents $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

A, C, E); and also by immunofluorescence in the EA.hy926 and DLD1 cell lines (Fig. 4, left). In addition, we measured plasma membrane Ca^{2+} ATPase (PMCA) gene expression and did not observe any changes due to melatonin treatment (not shown). Nevertheless, we observed a significant increase in type 1 IP₃Rs in the melatonin-treated A2780 and DLD1 tumour cell

Fig. 6. Changes in the ER stress in A2780, DLD1 and EA.hy926 cells in response to the melatonin treatment, as determined by the fluorescent microscopy by thioflavin (green). Nuclei of the cells are stained by DAPI (blue). cont – untreated control, M10 – melatonin treatment (final concentration 10 μ M) for 24 h, NC – negative control. Bar represents 50 μ m.

Fig. 7. Melatonin treatment increases apoptosis in A2780, DLD1, but very weakly in EA.hy926 cells, as determined by Annexin V-Fluos. In tumour cell lines A2780 and DLD1, melatonin increased apoptosis approximately twice, but in normal endothelial cell line EA.hy926 only 1.3-times. cont – untreated control, M0.1 – melatonin treatment (final concentration 0.1 μ M), M1 – melatonin treatment (final concentration 1 μ M), M10 – melatonin treatment (final concentration 10 μ M,) for 24 h. Results are displayed as mean $\pm$ S.E.M. Each column represents an average of 6 -9 samples from three independent cultivations. Statistical significance * represents $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

lines (Fig. 3A, B, C, D), but not in EA.hy926 endothelial cells (Fig. 3 E, F) that was determined on both, mRNA (Fig. 3, B, D, F) and protein (Fig. 3, A, C, E) level. Immunofluorescence assays using IP₃R1 antibody did not show any differences in the IP₃R1 signal (red) in EA.hy926 cells after melatonin treatment, but a rapid increase in the IP₃R1 signal was observed in melatonin treated DLD1 cells (Fig. 4, right). Since it is known that depletion of calcium from the ER (also through IP₃Rs) causes ER stress, we measured markers of ER stress – CHOP (Fig.5 B, D, F) and XBP1 (Fig.5 A, C, E) in all three types of cells. ER stress (determined by increased expression of CHOP and XBP1) was evident in A2780 and DLD1 cells (especially, at melatonin concentration 10 μ M), but not in EA.hy926 cells (Fig. 5 E, F), where no changes in the expression of CHOP and XBP1 were determined in control and melatonin treated

Fig. 8. Involvement of the NCX1 and IP₃R1 in the melatonin-induced apoptosis in A2780 and DLD1 cells. Group of A2780/DLD1 cells was silenced by either NCX1 or IP₃R1 and subsequently treated with 10 μ M melatonin for 24 h. Afterwards, apoptosis was determined by Annexin V-Fluos. cont – untreated control, M10 – melatonin treatment, siNCX – cells silenced with NCX1 siRNAs, M10siNCX

– cells silenced with NCX1 siRNAs and treated with melatonin, scr – cells silenced with ON-TARGETplus Non-targeting Control Pool (negative control siRNAs), scrM10 – cells silenced with ON-TARGETplus Non-targeting Control Pool and treated with melatonin, siIP1 – cells silenced with IP₃R1 siRNAs, M10siIP1 – cells silenced with IP₃R1 siRNAs and treated with melatonin. Results are displayed as mean $\pm$ S.E.M. Each column represents an average of 3-6 samples. Statistical significance compared to control - *** $p < 0.001$ and compared to melatonin-treated group + represents $p < 0.05$ and ++ $p < 0.01$.

cells. Melatonin-induced ER stress in A2780 and DLD1 cells was verified by fluorescence microscopy using Thioflavin T (Fig. 6, green signal). Cell nuclei are stained in blue with DAPI (Fig. 6, blue signal). ER stress is often connected with apoptosis induction. Therefore, we measured apoptosis in A2780, DLD1 and EA.hy926 cells after the melatonin treatment (Fig. 7). The increase in apoptosis after melatonin treatment (10 μ M for 24 h) in DLD1 and A2780 cells was more than two-fold, while in EA.hy926, apoptosis was only elevated 1.4-fold. To determine if the melatonin induced changes in the expression of NCX1 and IP₃R1 in A2780 and DLD1 cells might affect apoptosis induction, we silenced genes for these two proteins and measured apoptosis in all types of cells after melatonin treatment (Fig. 8). In DLD1 cells, knockdown with NCX1 siRNA decreased melatonin-induced apoptosis from $25.24 \pm 1.04\%$ to $14.51 \pm 1.23\%$ and also IP₃R1 siRNA partially prevented melatonin-induced apoptosis (to $19.13 \pm 0.33\%$) compared with non-siRNA cells treated with melatonin. Similarly in A2780 cells, lower level of melatonin-induced apoptosis was observed in groups, in which NCX1 (from $21.50 \pm 0.46\%$ to $15.44 \pm 1.23\%$) or IP₃R1 (to $16.22 \pm 0.43\%$) was silenced compared with non-siRNA cells treated with melatonin. Since apoptosis in melatonin-treated cells knockdown with NCX1 and IP₃R1 siRNA was higher than in control, untreated groups ($9.03 \pm 0.27\%$ in DLD1 cells and $9.95 \pm 0.35\%$ in A2780 cells), it becomes clear that these two calcium transport systems contribute only partially to the mechanism of melatonin-induced apoptosis. The efficiency of the NCX1 silencing was approximately 80–90%, while that of siIP₃R1 silencing was only 50–60%. The antioxidant effects of melatonin were determined by total ROS detection (Fig. 9). While in normal EA.hy926 cells total ROS content decreased on melatonin treatment in a concentration-dependent manner (from 14.8 ± 0.2 RFU/ μ g protein in control group to 4.7 ± 0.2 RFU/ μ g protein in group treated with 10 μ M melatonin), ROS decrease in A2780 and DLD1 tumour cells was much lower (from 25.3 ± 0.2 RFU/ μ g protein

Fig. 9. Determination of ROS decreases in tumour A2780 and DLD1 cells and normal epithelial EA.hy926 cells due to the melatonin treatment. In EA.hy926 cells, melatonin presents its antioxidant effect through a decrease of ROS in a concentration-dependent manner. Antioxidant effect of melatonin was much smaller in tumour cell lines A2780 and DLD1. cont – untreated control, M0.1 – melatonin treatment (final concentration 0.1 μ M), M1 – melatonin treatment (final concentration 1 μ M), M10 – melatonin treatment (final concentration 10 μ M,) for 24 h. Results are displayed as mean $\pm$ S.E.M. Each column represents an average of 3–6 samples from two independent cultivations. Statistical significance * represents $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Fig. 10. Determination of ROS in A2780, DLD1 and EA.hy926 cells in response to the melatonin treatment as determined by the fluorescent microscopy using CellROX Green reagent. Intensity of green fluorescence indicates amount of ROS. cont – untreated control, M10 – melatonin treatment (final concentration 10 μ M) for 24 h, NC – negative control. Bar represents 50 μ m.

in control group to 17.7 ± 0.17 RFU/ μ g protein in group treated with 10 μ M melatonin in A2780 cells and from 18.2 ± 0.2 RFU/ μ g protein to 15.2 ± 0.3 RFU/ μ g protein in DLD1 cells). These results were verified by fluorescent microscopy using CellROX Green reagent (Fig. 10), where the fluorescent signal after melatonin treatment was much lower in EA.hy926 than in A2780 and DLD1 cells.

Discussion

In this study, we have shown that melatonin acts differently on A2780 (derived from ovarian cancer) and DLD1 (derived from colorectal carcinoma) tumour cells, compared with cells of the normal endothelial EA.hy926 cell line. In tumour cell lines, melatonin was able to decrease levels of cytosolic calcium primarily through increased expression of NCX1, generate ER stress, and induce apoptosis *via* calcium release from the ER as a consequence of increased expression of IP₃R1. However, in normal EA.hy926 cells, melatonin acts mainly as an antioxidant agent, thus decreasing the amount of total ROS.

ROS species produced by oxidative stress have been detected in various cancers due to their high metabolic activity, and these ROS may promote many processes that maintain an aggressive phenotype [41]. Anti-oxidant compounds are generally considered as a protective factor against cancer [42]. Melatonin is well known as a potent scavenger of ROS such as hydroxyl radicals, peroxy radicals, superoxide anion radicals and hydrogen peroxide [43-47]. In our experiments we detected higher levels of ROS in tumour cells (A2780 – 25.3±0.2 RFU/ µg prot., DLD1 – 18.2±0.2 RFU/µg prot.) compared with non-tumour endothelial EA.hy926 cells (14.8±0.2 RFU/ µg prot.). Nevertheless, a melatonin induced decrease in ROS was robust in non-cancer EA.hy926 cells (app. 68% at 10 µM melatonin compared with the untreated control), but relatively weak in both types of cancer cells (approximately 30% in A2780 around 16.5% in DLD1 with 10 µM melatonin treatment compared with untreated controls), indicating that melatonin acts differently in tumour cells compared with normal cells. This difference is interesting both from the theoretical and therapeutic points of view, but the mechanisms behind it are not understood. In normal cells, the products of several tumour suppressor genes counteract oxidative stress and maintain the redox balance by prevention of lipid peroxidation and oxidative damage to DNA and proteins [48]. In the absence of wild-type tumour suppressor genes, such as *TP53*, cancer cells switch off several antioxidative pathways, leading to ROS accumulation [49].

In stark contrast to ROS, Ca²⁺ exists in only one biologically relevant form that undergoes neither catabolic degradation nor anabolic synthesis (for a review, see Görlach et al. [50]). Interactions between ROS and calcium signalling can be considered as bidirectional, wherein ROS can regulate cellular calcium signalling, while calcium signalling is essential for ROS production [51]. Thus, in addition to calcium regulation of ROS generation, redox state and ROS have been shown to modulate the activity of a variety of Ca²⁺ channels, pumps, and exchangers [50]. In our study, melatonin decreased cytosolic calcium in a concentration dependent manner in DLD1 and A2780 tumour cells. However, such a melatonin-induced decrease in cytosolic calcium was not observed in endothelial EA.Hy926 cells. A similar melatonin-induced decrease in cytosolic calcium has already been described in mouse pancreatic acinar cells [28] and human neuroblastoma SH-SY5Y cells [8]. The effect of melatonin could be explained on the basis of stimulated Ca²⁺ transport out of the cell through the plasma membrane and by stimulation of Ca²⁺ reuptake into the ER. Generally, two major extrusion systems are responsible for the calcium extrusion from the cell — plasma membrane ATPase (PMCA) and NCX. We have shown that melatonin significantly increased the expression of NCX1 (major form of NCX), but did not change the expression of PMCA (not shown). Melatonin-induced up-regulation of NCX1 has not previously been described in cancer cells. Nevertheless, it was described in the prevention of pancreatic damage [52], since melatonin was able to reduce pancreatic damage via the up-regulation of NCX expression, which can alleviate calcium overload in pancreatic acinar cells. Taken together, melatonin might exert protective effects against cytosolic calcium overload.

Besides NCX, some other transporters have been shown to be affected by melatonin. Celik and Naziroglu [53] observed a modulatory role of intracellular and extracellular melatonin on Ca²⁺ influx and apoptosis through a transient receptor potential melastatin – like 2 (TRPM2) channel in transfected Chinese hamster ovary (CHO) cells. Also, melatonin activates phospholipase C to generate IP₃ and open ER-localised IP₃-sensitive Ca²⁺ channels in the malaria parasite *Plasmodium falciparum* [54]. We have shown a robust up-regulation

in the expression of IP₃R1 in A2780 and DLD1 tumour cells, but not in epithelial EA.hy926 cells. Expression of other types of IP₃Rs — IP₃R2 and IP₃R3 — was not changed (not shown). Release of calcium through IP₃R1 is tightly associated with the induction of apoptosis via the inner, mitochondrial pathway [55]. Therefore, we studied the effect of melatonin on potential apoptosis induction in two different tumour cell lines — A2780 and DLD1. To compare the effect of melatonin on non-tumour cells, we used endothelial EA.hy926 cells. Melatonin-induced apoptosis was much more pronounced in A2780 and DLD1 tumour cells than in endothelial EA.hy926 cells. The ability of melatonin to induce apoptosis has already been described in cancer cells, e.g. hepatoma cells [24], but also, to a limited extent, in non-tumour cells.

Moreover, melatonin promotes the apoptosis induced by other compounds, e.g. sorafenib in hepatocellular carcinoma [56], cisplatin in hepatocellular carcinoma [57], fluorouracil in oesophageal squamous cell carcinoma [58], etc. Although diverse mechanisms for melatonin-induced apoptosis have been suggested, many of them have the same denominator — ER stress. Activation of the mitochondrial pathway of apoptosis is deeply dependent on calcium release from the ER due to ER stress [55, 59]. We have shown that in tumour cells, melatonin is able to induce ER stress and activate the apoptotic process. This observation was already made in rats with diethylnitrosamine-induced hepatocarcinogenesis [20]. However, ER stress markers were not elevated in non-tumour EA.hy926 cells. The protective effects of melatonin against ER stress have already been described in some non-tumour cell lines. The protective effect of melatonin against ER stress induced by kainic acid in neurons was described by Xue et al. [60]. This effect might be associated with the modulation of intracellular Ca²⁺ levels and cellular homeostasis. Accumulating evidence emphasises the contribution of melatonin towards the maintenance of ER and mitochondrial homeostasis, which makes melatonin a promising pharmacological agent against neurodegenerative diseases [5, 61].

Multiple mechanisms underlying melatonin's ability to counteract tumour growth have been described up to now. These include, but are not limited to, its various antioxidant effects, modulation of the cell cycle, induction of apoptosis, inhibition of telomerase activity, ability to antagonise metastasis, prevention of circadian disruption, anti-angiogenesis, epigenetic effects, inhibition of growth factor uptake, stimulation of cell differentiation, and activation of the immune system [62, 63]. Based on our results we can conclude that melatonin acts differently in tumour cells than in normal endothelial cells, probably due to reorganisation of signalling pathways in tumour cells. Besides the known effects of melatonin as an ROS scavenger, its modulation of intracellular calcium might significantly participate in deciding the fate of tumour cells and inducing apoptosis. Different targeting of calcium transport systems might explain some mechanisms whereby melatonin can exert its anticancer effects and therefore represent potential novel therapeutic compound for cancer treatment. Thus, a potential therapeutic or clinical relevance of drugs that affect the level of cytosolic calcium in combination with melatonin should be further studied.

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Disclosure Statement

The authors declare no conflict of interest regarding the publication of this paper.

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