Inhibitory effects of melatonin on sulfatase and 17ß-hydroxysteroid dehydrogenase activity and expression in glioma cells

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Abstract. Melatonin interacts with estradiol at the estrogen receptor level in different kinds of neoplasias and also regulates the expression and the activity of some enzymes involved in the biosynthesis of estrogens in peripheral tissues. Glioma cells express estrogen receptors and have the ability to synthesize estrogens locally. Since melatonin inhibits the growth of C6 cells, and this indoleamine has been demonstrated to be capable of decreasing aromatase expression and activity in these cells, the aim of the present study was to analyze whether the regulation of the sulfatase, the enzyme that catalyzes the rate-limiting step in the conversion of estrogen sulfates to estrogens, and 17ß-hydroxysteroid dehydrogenase, the enzyme which converts the relatively inactive estrone to the most potent 17ß-estradiol, could be involved in the inhibition of glioma cell growth by melatonin. We found that melatonin decreases the growth of C6 glioma cells and reduces the sulfatase and 17ß-hydroxysteroid dehydrogenase activity. Finally, we demonstrated that melatonin downregulates sulfatase and 17ß-hydroxysteroid dehydrogenase mRNA steady state levels in these glioma cells. By analogy to the implications of these enzymes in other forms of estrogen-sensitive tumors, it is conceivable that their modulation by melatonin may play a role in the growth of glioblastomas.

Introduction

The role of melatonin, the main secretory product of the pineal gland, as a regulator of neoplastic cell growth has been under intensive study during the last few decades, particularly on hormone-dependent tumors and especially on estrogen-dependent breast cancer (1-5). Experimental manipulations activating the pineal gland, or the administration of melatonin, reduce the incidence and growth rate of chemically-induced mammary tumors in rodents, while pinealectomy or situations which implicate a reduction of melatonin production usually stimulate mammary carcinogenesis (1,2,6). Melatonin reduces the estrogen-mediated development of breast cancer on the basis of two different mechanisms: indirectly, by the down-regulation of the neuroendocrine reproductive axis and the consequent reduction of pituitary and gonadal hormones responsible for the normal and pathological growth of the mammary gland, and, on the other hand, by a direct interaction with the estrogen-response pathway at the tumor cell level (5-8). It has been demonstrated that melatonin counteracts the effects of estrogens on mammary tumoral cells, thus behaving as a selective estrogen receptor modulator (SERM). Recently, melatonin has been described as also being able to regulate the activity of some enzymes (aromatase, sulfatase, 17ß-hydroxysteroid dehydrogenase, estrogen sulfotransferase) responsible for the local synthesis of estrogens in cultured human breast cancer cells, thus behaving as a selective estrogen enzyme modulator (SEEM) (7,9-11).

In the brain, estrogens act as a mitogen and trophic factor that influences neuronal development (12). Estrogens have well known cytoprotective effects in neural cells and it has been reported to have also biological effects on glial cells, although these have been less studied (13,14). In addition to gonadal estrogens of females, estrogens are also synthesized in the brain and these locally synthesized estrogens are responsible for much of the estrogenic signalling activity in the developing brain. As in other estrogen-responsive tissues, in the estrogen-sensitive cells of the nervous system, the binding of estradiol at membrane-associated and intracellular estrogen receptors regulates the activity of various growth factor-like signalling pathways and the transcription of estrogen-responsive genes (15,16). Neuroactive estrogens play a main role not only in normal physiological states but also in the pathogenesis of several brain diseases. Glioblastoma is the most frequent primary malignant CNS tumor in adults. These tumors express estrogen receptors and also express some of the enzymes involved in the local synthesis of

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estrogens (17-20). Expression of aromatase in the brain is involved in the regulatory effects of androgens, via its conversion to estrogens, on neural differentiation, maturation, neural plasticity, neuroendocrine functions and sexual behaviour (21). There is some evidence that tamoxifen, one of the estrogen receptor modulators most frequently used for treatment and also for prevention of breast cancer, arrests the growth of some glioblastomas and it has neuroprotective actions (13). Recently, different sulfatase activity levels have been described in human brain tumors, glioblastomas, pituitary adenomas, meningiomas and astrocytomas (20).

Melatonin has been shown to have neuroprotective effects in a large number of different experimental models (22-27) and there is evidence suggesting that melatonin may affect glioblastoma growth. In a clinical trial in which melatonin was administered to patients being treated with radiation therapy for glioblastoma, a significant increase in one-year survival was seen (28). C6 glioma cells express both of the protein-coupled melatonin receptor subtypes, MT_1 and MT_2 and when they are treated with physiological or higher concentrations of melatonin, a significant induction of relative glial cell line-derived neurotrophic factor has been detected (23). This finding has suggested that induction of the glial cell line-derived neurotrophic factor is involved in physiological neuroprotection by melatonin. High doses of valproic acid, a potent anti-epileptic, induce both upregulation of melatonin receptors and expression of neurotrophic factors in C6 glioma cells (25,29). It has been also reported that melatonin at millimolar concentrations reduces, both *in vitro* and *in vivo*, the growth of C6 glioma cells (26,30). This inhibition has been related to reduced intracellular peroxides and a subsequent inhibition of the activity of important intracellular effectors, such as Akt and nuclear factor (NF)-κB and protein kinase C (26,30). In addition to the beneficial effects of providing direct antioxidant protection to glioma cells, melatonin may enhance neuroprotection against Aß-induced neurotoxicity by attenuating Aß25-35 or Aß1-42-induced apoptosis and promoting the survival of glial cells (24).

Although C6 glioma cells express both estrogen receptor and estrogen enzymes, such as aromatase and sulfatase (19,31), and have, therefore, the possibility to produce estrogens from androgen precursors and from estrogen sulfates, which may act as paracrine or autocrine factors to promote tumor cell growth, the significance of this fact is not very well determined. In other estrogen-dependent tumors, such as mammary tumors, the inhibition of aromatase activity by aromatase inhibitors is currently one of the first therapeutic strategies used against the growth of these tumors (32) and the inhibition of sulfatase activity offers a potential new additional option of endocrine therapy, initially in patients whose disease has progressed after prior aromatase inhibitor therapy. Since melatonin inhibits the growth of C6 cells (26,30,33), and this indoleamine has been demonstrated to be capable of decreasing aromatase expression and activity in glioma cells (33) and other tumor cell lines (9), our objective in the present study was to analyze the possible effects of melatonin on the local synthesis of estrogens in C6 glioma cells, particularly through the modulation of sulfatase and 17ß-hydroxysteroid dehydrogenase activity and expression.

Material and methods

Cells and culture conditions. Rat glioma cells (C6) were a kind gift from Dr Carmen Rodríguez from the Department of Structural and Cellular Biology of the University of Oviedo, Spain. They were maintained as monolayer cultures in 75 cm^2 plastic culture flasks in Dulbecco's modified Eagle's medium (DMEM)/Ham's Nutrient mixture F-12 (Sigma-Aldrich, Madrid, Spain) supplemented with 10% fetal bovine serum (FBS) (Gibco, France), 20 U/ml penicillin and 20 μg/ml streptomycin (Sigma-Aldrich), at 37˚C in a humid atmosphere containing 5% CO_2. Cells were subcultured every 3-4 days by suspension in 5 mM Na_2-EDTA in PBS (pH 7.4) at 37˚C for 5 min.

Before each experiment, stock subconfluent monolayers (80%) of glioma cells were incubated with 5 mM Na_2-EDTA in PBS (pH 7.4) at 37˚C for 5 min, resuspended in DMEM/HAM F12 mixture supplemented with 10% FBS and passed repeatedly through a 25-gauge needle to produce a single cell suspension. Cell number and viability were determined by staining a small volume of cell suspension with 0.4% trypan blue saline solution and examining the cells in a hemocytometer.

Measurement of steroid sulfatase (STS) activity. STS activity in C6 glioma cells was assayed by the formation of estrone from a labelled substrate ([6,7-^{3}H(N)]-estrone sulfate ammonium salt) (34). C6 glioma cells were seeded onto six-well plates ($1.5x10^5$ cells/well) in DMEM/HAM F12 mixture supplemented with 10% FBS. Two days later, the media were replaced with fresh ones (1 ml per plate) containing 2 nM [6,7-^{3}H(N)]-estrone sulfate ammonium salt (NEN Life Science Products, Boston, MA, USA) (57.3 Ci/mM) in the presence of either 1 nM, 1 μM, 1 mM melatonin or its diluent (ethanol at a final concentration <0.001%). After 24 h of incubation, culture dishes were placed on ice for 15 min to condense any water vapour and the media were transferred to tubes containing 5 ml of toluene, vortexed and centrifuged at 1000 x g for 10 min. The resulting organic phase was added to vials with scintillation cocktail and counted in a beta counter. The amount of radioactivity measured in the [^{3}H]-toluene was corrected by subtracting the blank values from each sample, obtained by incubating dishes containing medium but no cells with the tritiated estrone sulfate. The values were also corrected by taking into account the fractional retention of tritium throughout the procedure of incubation and processing, utilizing parallel dishes containing medium plus known amounts of [^{3}H] estrone (NEN Life Science Products) through incubation and assay. The fractional retention of tritium throughout the incubation and processing of samples was always >92%.

Measurement of 17ß-hydroxysteroid dehydrogenase type 1 (17ß-HSD1) activity. Activity of 17ß-HSD1 was assayed in C6 glioma cells by the formation of estradiol from a labelled substrate [2,4,6,7-^{3}H(N)]-estrone (35). C6 glioma cells were cultured in six-well plates ($1.5x10^5$ cells/well) in DMEM/HAM F12 mixture supplemented with 10% FBS. After 48 h, the media were changed for fresh ones (1 ml per plate) containing 2 nM [2,4,6,7-^{3}H(N)]-estrone (NEN Life Science

Table I. Primers used for amplification of mRNA transcripts of STS, 17ß-HSD1 and S14 (control).

mRNA	Sequence	bp	nM	T
STS				
Forward:	5'-ACACAGCGCCTGGCAAGC-3'	136	100	60˚C
Reverse:	5'-CCCCATGCAGGCTCTGCC-3'		200	60˚C
17ß-HSD1				
Forward:	5'-AAGTCCATTGGGGTGTCC-3'	189	200	58˚C
Reverse:	5'-ACGGTGGCTTCCCAGAGC-3'		200	58˚C
S14				
Forward:	5'-TCACCGCCCTACACATCAAAC-3'	159	100	
Reverse:	5'-TCCTGCGAGTGCTGTCAGAG-3'		100	

bp, product size (base-pairs); nM, primers concentration; T, annealing temperature. Forward, sense forward primer; Reverse, antisense reverse primer.

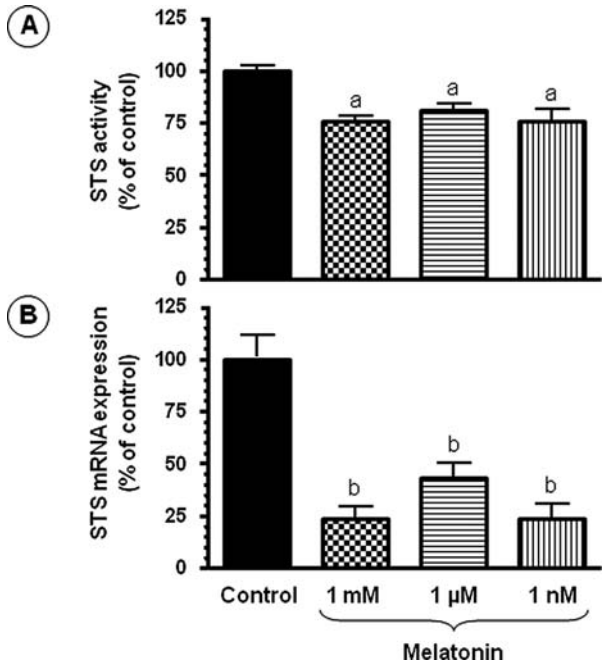

Figure 1. (A) Effects of melatonin (1 mM, 1 μM and 1 nM) or its diluent (ethanol at 0.001% final concentration) on basal STS activity of C6 glioma cells, determined after 24 h of incubation with the indolamine. (B) Effects of melatonin (1 mM, 1 μM and 1 nM) or its diluents on the expression of mRNA STS in glioma cells. Cells were incubated with melatonin or ethanol (control) for 2 h. Total mRNA was isolated from C6 cells and reverse transcribed. cDNA was subjected to PCR using specific primers for STS or S14. Data are expressed as the percentage of the control group (mean ± SEM). a, $p<0.05$ vs. control; b, $p<0.01$ vs. control.

Products) (100 Ci/mM) in the presence of either melatonin (1 nM, 1 μM, 1 mM) or its diluent (ethanol at a final concentration <0.001%). After 30 min of incubation, the media were transferred to tubes containing 4 ml of diethyl ether, vortexed and centrifuged at 800 x g for 5 min. The aqueous phase was frozen and the resulting organic phase was decanted and evaporated in tubes containing 50 μg of estradiol. The residue was resuspended in diethyl ether and separated by TLC using dichloromethane/ethyl acetate (4:1; v/v). Once the spots had been visualised, excised and eluted with methanol, they were counted in a liquid scintillation counter. Values were corrected for blanks and tritium recovery (>82%) as described for STS activity.

Measurement of mRNA expression of STS and 17ß-HSD1. Expression of the mRNA from both enzymes was carried out by real-time PCR in C6 glioma cells. The total cellular RNA was purified with the Nucleospin RNA II kit (Macherey-Nagel GmbH & Co. Düren, Germany) following the manufacturer's instructions. Integrity of RNA was assessed by electrophoresis in ethidium bromide-stained 1.2% agarose-Tris-borate EDTA gels. The absorbance ratio A260 nm/A280 nm was >1.8. For cDNA synthesis, 1 μg of total RNA was denaturated at 65˚C for 10 min and reverse-transcribed 50 min at 45˚C with the cDNA synthesis kit (Bioline) in a final volume of 20 μl in the presence of 500 ng of oligo (dT)12-18 primer. Real-time PCRs (MX3000 Stratagene) were performed using Brilliant SYBR Green PCR master mix (Stratagene) following the manufacturer's instructions. The sets of human oligonucleotides (Sigma Genosys Ltd., Cambridge, UK) used as primers are indicated in Table I. S14 mRNA expression was used as a control. PCRs were performed for 40 cycles for quantitative analysis using the annealing temperature indicated in Table I during 60 sec, the extension being carried out at 72˚C for 45 sec and the denaturation at 95˚C for 30 sec. Each product was electrophoresed on ethidium bromide-stained 2% agarose-Tris-borate gels to corroborate that the product amplified corresponded to the adequate length. Finally, the mRNA expression levels of target genes were normalized to S14 expression. Melting curves were performed by using dissociation curve to verify that only a single product was amplified.

Statistical analysis. The data on sulfatase and 17ß-HSD1 activity and expression are expressed as the mean ± standard errors of the mean (SEM). Statistical differences between groups were processed by one way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. Results were considered as statistically significant at $p<0.05$.

Results

We first studied the effects of melatonin on STS activity and mRNA expression. The sulfatase activity of C6 glioma cells incubated with tritiated estrone sulphate was estimated by the formation of tritiated estrone. Physiologic (1 nM) and higher (1 mM and 1 μM) concentrations of melatonin induced a significant (20%, $p<0.05$) inhibition of the STS activity of C6 glioma cells and were effective at reducing the conversion of estrone sulfate to estrone (Fig. 1A).

The mRNA expression of STS was also inhibited by physiologic (1 nM) and supraphysiologic (1 mM and 1 μM) concentrations of melatonin in glioma cells (Fig. 1B). In a second set of experiments, the effects of melatonin on the 17ß-HSD1 activity and mRNA expression were studied. The effect of melatonin on reductive 17ß-HSD1 activity is shown

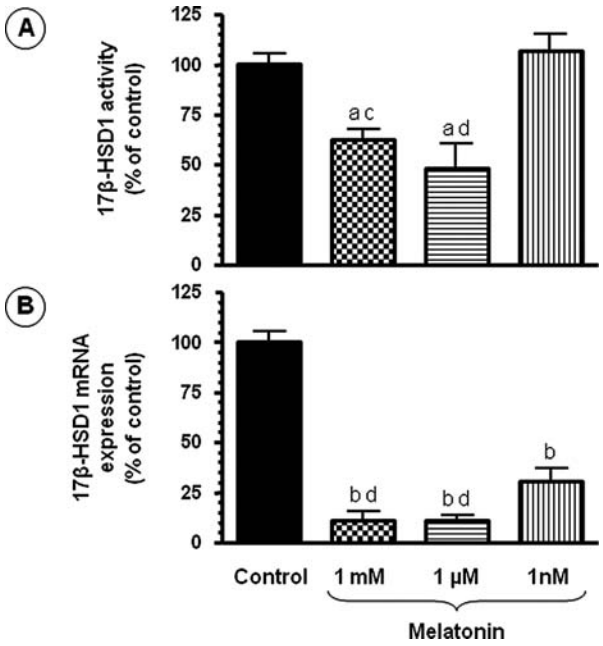

Figure 2. (A) Activity of 17ß-HSD1 in glioma cells after 24 h of incubation with melatonin (1 mM, 1 μM or 1 nM) or the diluent (ethanol at 0.0001% final concentration). (B) mRNA expression of 17ß-HSD1 after 2 h of incubation with melatonin or ethanol (control). Data are expressed as the percentage of the control group (mean ± SEM). a, $p<0.05$ vs. control; b, $p<0.001$ vs. control; c, $p<0.05$ vs. melatonin 1 nM; d, $p<0.01$ vs. melatonin 1 nM.

in Fig. 2A. The production of estradiol from estrone was significantly reduced by melatonin (1 mM and 1 μM).

In order to determine whether this inhibitory effect over 17ß-HSD1 activity was correlated to a downregulation of the enzyme expression, we measured the expression of this enzyme in C6 glioma cells incubated for 2 h with either melatonin (1 mM, 1 μM and 1 nM) or vehicle. Physiologic concentrations of melatonin (1 nM) induced a significant (70%, $p<0.001$) inhibition of the expression of 17ß-HSD1 (Fig. 2B). Higher concentrations of melatonin (1 mM and 1 μM) were also effective at reducing (90%, $p<0.001$) the conversion of estrone to estradiol.

Discussion

Estrogens are major regulators of normal brain development, growth, maturation and function in both males and females. Estradiol is also a well-known carcinogen that plays a central role in the etiology and progression of hormone-responsive tumors (15,16). Sex differences in brain tumor incidence suggest that hormonal factors may play a role in the etiology of this type of tumor and there is evidence that hormones related to the female reproductive function may be associated with a risk of brain cancer (36). The incidence of glioma is 1.5 times greater in men than in women and it has been described as an inverse relationship between younger age at menarche (among postmenopausal women), cumulative number of menstrual cycles over a lifetime, fewer months of breast-feeding and the use of hormone replacement therapy and the risk for glioma (36,37). The brain is well equipped with enzymes necessary for estrogenic hormone biosynthesis and steroids or their precursors can be metabolized locally in the brain to derivates which can affect brain function and may have an important clinical significance (38). Previous studies have shown that human brain and human neuronal cell lines secrete estrogens (38) and that estrogens are locally synthesized in the brain mainly from androgens by cytochrome P450 aromatase (CYP19) and from estrogen sulfates by sulfatase (19,20).

Melatonin, the main pineal hormone, inhibits the growth of C6 rat glioma cells both *in vitro* and *in vivo*, and this inhibition has been mainly related with the antioxidant abilities of this indolamine (26,30). In other models of estrogen-sensitive tumors, such as breast cancer, melatonin oncostatic actions seem to be mainly based on its interaction with the tumor cell estrogen-response pathway (1,2,7,39). Melatonin interacts with estradiol at the estrogen receptors level in the mammary tumor cells and also regulates the expression and the activity of some enzymes involved in the biosynthesis of estrogens in peripheral tissues (7,9,11,40). Melatonin reduces the activity and expression of aromatase, sulfatase and 17ß-hydroxysteroid dehydrogenase and increases the activity and expression of estrogen sulfotransferase in breast tumors, protecting mammary tissue from excessive estrogenic effects (40). Thus, a single molecule has both SERM and SEEM properties, one of the main objectives desired for the breast antitumor drugs (6,7,9,40,41). For that reason, since we know that melatonin downregulates aromatase activity and expression in C6 glioma cells (33), we decided to address whether the modulation of the local biosynthesis of estrogens by melatonin in C6 glioma cells is also mediated through the regulation of sulfatase and 17ß-hydroxysteroid dehydrogenase activity and expression.

In previous studies (26,30,33) a strong inhibitory effect induced mainly by millimolar concentrations of melatonin on C6 glioma cells growth has been described. In the same way, high concentrations of melatonin are also necessary to obtain oncostatic actions in neuroblastoma cells and other kinds of tumor cells (42,43). Melatonin is a highly lipid-soluble indolamine which may easily cross the blood-brain barrier, and there is evidence that melatonin concentration in the cerebrospinal fluid is higher than in blood (44). These high concentrations of melatonin in the cerebrospinal fluid may contribute towards explaining why high levels of melatonin are necessary to inhibit glioblastoma cell proliferation.

As occurs in other estrogen-responsive tissues, in the estrogen-sensitive cells of the normal brain and brain tumors, the binding of estradiol to membrane-associated and intracellular estrogen receptors allows these nonneuronal cells of the central nervous system to respond to estrogens (15-18); thus, glioma cells are able to respond significantly to estradiol in the culture (33). In addition, previous studies have also shown that the human brain, human neuronal cell lines and glioma cell lines express aromatase and sulfatase and are, therefore, capable of producing estrogens from androgens or estrogen sulfates (19,20,38).

The present study demonstrates that melatonin, at physiologic and pharmacological concentrations, reduces the synthesis of estrogens in C6 glioma cells, through the

inhibition of sulfatase, the enzyme that catalyzes the rate-limiting step in the conversion of estrogen sulfates to estrogens. These results are supported by two types of experiments that we carried out. In the first series of experiments, we directly quantified the sulfatase activity of glioma cells, finding that this was reduced by melatonin. Then, in the other set of experiments, we demonstrated that physiologic and pharmacological concentrations of melatonin also induced a remarkable decrease in the expression of mRNA sulfatase of C6 glioma cells. To our knowledge this is the first time that the influence of melatonin on sulfatase activity and expression of glioblastoma cells has been established. In fact, the antisulfatase activity of melatonin in other cells has been solidly described recently (7,40). Melatonin, at physiologic concentrations, modulates the synthesis and transformation of biologically active estrogens in MCF-7 human breast cancer cells through the inhibition of sulfatase activity and expression (7,40).

The last step of steroidogenesis in peripheral tissues is the conversion of the weak estrone to the potent biologically active estradiol by the activity of 17ß-HSD1 (7,40,45). We reported herein that the production of estradiol from estrone in C6 glioma cells decreases (2-fold) in the presence of 1 μM melatonin. Furthermore, physiological or pharmacological doses of melatonin also decreased 17ß-HSD1 expression (90% inhibition of 17ß-HSD1 expression with 1 mM and 1 μM melatonin). The effects of melatonin on the steroidogenic activity of hamster testes, consistent with an inhibition of 17ß-HSD activity and/or expression, have been previously described (46). Antiestrogens such as tamoxifen, 4-hydroxytamoxifen or ICI 164384 can also inhibit by competition the 17ß-HSD1 effects in both rat mammary and human breast tumor tissues (45). This is also the first time that an inhibitory effect of melatonin on 17ß-HSD1 activity and expression in glioma cells has been demonstrated. In this way, it was our group that recently described, in the first instance, that melatonin downregulates 17ß-HSD1 activity and expression in other estrogen-responsive tissues, such as breast cancer (40).

Our results indicate that estrogens locally synthesized by sulfatase and 17ß-HSD1 in glioblastoma cells may act as autocrine or paracrine factors and melatonin is able to inhibit this local production of estrogens by decreasing the activity and expression of both enzymes. The significance of this melatonin action on sulfatase and 17ß-HSD1 in glioblastoma needs to be determined. There is little information about the capacity of C6 glioma cells to grow in the presence of sulfatase and 17ß-HSD1 inhibitors, however, by analogy to the implications of these enzymes in other forms of estrogen-sensitive tumors, such as breast tumors, it is conceivable that the modulation of the activity and expression of sulfatase and 17ß-HSD1 by melatonin may play a role in the growth of glioblastomas.

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