

Expression of melatoninergic receptors in human placental choriocarcinoma cell lines

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BACKGROUND: Melatonin crosses the placenta and enters the fetal circulation. Moreover, experimental data suggest a possible influence of melatonin on placental function and fetal development in humans. To date, the expression and role of melatonin receptors in human placenta choriocarcinoma cell lines and in human term placental tissues remain to be elucidated. **METHODS AND RESULTS:** Results from RT-PCR, western blotting and confocal microscopy demonstrated that the MT1, MT2 and ROR α 1 melatonin receptors are expressed in the human term placental tissues and in choriocarcinoma cell lines JEG-3 and BeWo. Furthermore, enzyme-linked immunosorbent assay showed that 6-chloromelatonin (a melatonin agonist) inhibits, in a dose-dependent manner, forskolin-stimulated hCG- β secretion in JEG-3 ($P < 0.001$) and BeWo ($P < 0.05$) cells but had no effect on basal human chorionic gonadotrophin (hCG- β) levels. This effect of 6-chloromelatonin on forskolin-stimulated HCG- β secretion was abolished by pertussis toxin (PTX), suggesting that melatonin regulates hCG- β production by an action involving an inhibitory Gi/o protein. In PTX-treated BeWo cells, 6-chloromelatonin stimulated basal hCG- β secretion ($P < 0.001$). **CONCLUSION:** These results demonstrate, for the first time, the expression of melatonin receptors in human term placental tissues and in choriocarcinoma cells and suggest a possible paracrine/autocrine function for melatonin in human placenta.

Key words: human chorionic gonadotrophin/human choriocarcinoma cell lines/human term placental tissue/melatonin/melatonin receptors

Introduction

Melatonin (5-methoxy-*N*-acetyltryptamine), a lipophilic indoleamine, is mainly synthesized and released by the pinealocytes in the pineal gland (Underwood and Goldman, 1987). There is also evidence of its synthesis and release in periphery, including the gastrointestinal tract, reproductive organs, skin, platelets and cells of the immune system (Stefulj *et al.*, 2001). Melatonin secretion is cyclical, stimulated by darkness and inhibited by light (Weinberg *et al.*, 1979). This hormone has been shown to play key roles in the regulation of mammalian reproductive functions, including regulation of estrogen and progesterone production (Fiske *et al.*, 1984), inhibition of uterine contractility (Hertz-Eshel and Rahamimoff, 1965) and regulation of growth and functional activity of the ovary (Fernandez *et al.*, 1995; Graham *et al.*, 2004).

Currently, three mammalian melatonin receptors (MT1, MT2 and MT3) have been identified (reviewed in Witt-Enderby *et al.*, 2003). Human MT1 (Mell1a, ML1A or mt1) and MT2 (Mell1b or ML1B) melatonin receptors have been cloned, and both are members of the G-protein-coupled receptor (GPCR) superfamily (Reppert *et al.*, 1994, 1995). Although most MT1 and MT2 receptors are generally expressed in brain and retina, respectively, they are also widely distributed in a variety of

other tissues, including ovarian follicles, prostate, cells of the immune system and kidney (Reppert *et al.*, 1994, 1995; Naji *et al.*, 2004; Pozo *et al.*, 2004). The MT3 receptor subtype has radically different properties than those of MT1 and MT2. It has recently been identified as quinone reductase 2 (QR2), an enzyme involved in detoxification (Mailliet *et al.*, 2004). The MT3 receptor subtype is expressed in various tissues, including testes, brain, liver and kidney (reviewed in Witt-Enderby *et al.*, 2003). In humans, melatonin was also reported to bind and regulate gene transcription through the subfamily of putative nuclear melatonin retinoid orphan receptors/retinoid Z receptors (ROR/RZR) (Becker-Andre *et al.*, 1994). Members of the ROR/RZR subfamily include the products of three genes, ROR α (splicing variants ROR α 1, ROR α 2, ROR α 3 and RZR α), ROR β and ROR γ (reviewed in Smirnov, 2001). ROR α is expressed in a large number of tissues, including testes, kidney, brain and B-lymphocytes, and has been reported to regulate the immune response and development of the central nervous system (reviewed in Smirnov, 2001; Naji *et al.*, 2004; Pozo *et al.*, 2004).

The more generalized signalling mechanism proposed for MT1 and MT2 receptors is the inhibition of 3'-5'-cyclic adenosine monophosphate (cAMP) accumulation via a pertussis

toxin (PTX)-sensitive G protein, a process known to be mediated by Gi/o proteins, resulting in decreases in protein kinase A activity and in cAMP-response element-binding protein phosphorylation (for review, see Witt-Enderby *et al.*, 2003; Dubocovich and Markowska, 2005). MT1 receptors have also been shown to stimulate cAMP production through Gs protein to activate potassium channels, to stimulate inositol 1,4,5-trisphosphate (IP₃) production and cyclic guanosine monophosphate (cGMP) as well as to modulate protein kinase C (PKC) and phospholipase A₂ via Gβγ subunits liberated from Gi/o protein (Garcia-Perganeda *et al.*, 1997; Godson and Reppert, 1997; Jockers *et al.*, 1997; McArthur *et al.*, 1997; Chan *et al.*, 2002; Brydon *et al.*, 1999). This receptor can modulate the formation of arachidonic acid, stimulate c-Jun N-terminal kinase (JNK) activity and also modulate mitogen-activated protein kinases. Similar to MT1, MT2 receptors have been shown to stimulate IP₃ production. However, unlike MT1, MT2 receptor subtype activation decreases cGMP levels (Petit *et al.*, 1999).

Melatonin concentrations increase in maternal blood during pregnancy, reaching a maximum at term (Kivela, 1991), and decrease in cases of pre-eclampsia and intrauterine growth restriction versus normal pregnancies (Nakamura *et al.*, 2001). The presence of melatonin has been demonstrated in amniotic fluid (Kivela *et al.*, 1989). Moreover, melatonin crosses the placental barrier, enters the fetal circulation rapidly without biotransformation (Okatani *et al.*, 1998) and has been implicated in placental function and fetal development in animal models and human (e.g. Kennaway, 2000; Bishnupuri and Halder, 2001; Lee *et al.*, 2003; Iwasaki *et al.*, 2005).

A recent study demonstrated the local expression of the MT1 melatonin receptor gene in the rat placenta and showed that melatonin agonist decreased the expression of placenta lactogen-II (PL-II) mRNA in cultured late-pregnancy rat placental tissues (Lee *et al.*, 1999, 2003). Moreover, Iwasaki *et al.* (2005) have recently shown that mRNA transcripts of the melatonin-synthesizing enzymes and both MT1 and MT2 melatonin receptors are present in the first-trimester human placenta. The same authors showed that melatonin could stimulate human chorionic gonadotrophin (hCG) secretion from cultured first-trimester trophoblast cells. hCG, a member of the gonadotrophin family, is specifically synthesized in placental syncytiotrophoblastic cells (Hoshina *et al.*, 1982), plays key roles in pregnancy maintenance and sexual differentiation of the embryo and is a good marker of pregnancy and proper fetal development (Devoto *et al.*, 2002; Fujimoto *et al.*, 2002). The presence and function of melatonin receptors in human choriocarcinoma cell lines and human term placenta have never been studied. Herein we report, for the first time, that MT1, MT2 and RORα melatonin receptors are expressed in human term placental tissues and human choriocarcinoma cell lines. In addition, we demonstrated that 6-chloromelatonin significantly inhibits the forskolin-stimulated hCG-β secretion and has no effect on basal hCG-β levels in JEG-3 and BeWo cell lines.

Materials and methods

Human placental tissue and cell culture

The Human Subject Research Committee of participating hospital and Université de Moncton (Moncton, New Brunswick, Canada)

approved this project. Fresh placentas were obtained from normal full-term pregnancies (38–41-week gestation) from Dr Georges-L.-Dumont Hospital (Moncton, New Brunswick, Canada). The villous layers of trophoblastic tissues were dissected, washed with ice-cold 150 mM NaCl, frozen with dry ice and stored at –80°C until the time of protein and RNA preparation.

Human placental choriocarcinoma cell lines, JEG-3 and BeWo, and the breast cancer cell line, MCF-7, were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA). The JEG-3 cell line was maintained in Eagle's minimum essential medium (Invitrogen, Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT, USA), 2 mM L-glutamine, 1 mM sodium pyruvate, 110 U/ml of penicillin and 100 µg/ml of streptomycin (Invitrogen). The BeWo cell line was maintained in Ham's F-12K (Sigma, St Louis, MI, USA) supplemented with 10% FBS, 2 mM L-glutamine, 110 U/ml of penicillin and 100 µg/ml of streptomycin. The MCF-7 cell line was maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine, 110 U/ml of penicillin and 100 µg/ml of streptomycin. Cells were maintained in 75 cm² culture flasks at 37°C in a humidified atmosphere of 5% CO₂. Cells were split when they reached about 90–95% confluence using trypsin (Sigma).

RNA isolation, complementary DNA synthesis, RT-PCR amplification and sequencing

Total RNA was extracted from frozen placental tissue (three different placental tissues were used), rat brain, mouse brain and cell lines (JEG-3, BeWo and MCF-7) using the acid guanidium thiocyanate-phenol-chloroform extraction method with TRI Reagent according to manufacturer's instructions (Molecular Research Center, Cincinnati, OH, USA). Then, mRNA was isolated from total RNA with Oligotex Direct mRNA Mini kit for purification of poly A⁺ RNA from total RNA according to manufacturer's instructions (Qiagen, Mississauga, Ontario, Canada). mRNA was quantified using spectrophotometric measurement at 260 nm using the Spectramax 383 Plus (Molecular Devices, Sunnyvale, CA, USA). Protein contamination was monitored by A₂₆₀/A₂₈₀ ratio.

mRNA (0.1–0.5 µg) was incubated at 65°C for 10 min with oligo(dT) primers (Invitrogen), diluted with distilled H₂O to a final volume of 27 µl and then chilled on ice. The other components were added to a final volume of 40 µl [8 µl of 5× first-strand buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl; Sigma), 15 mM MgCl₂ (Roche Molecular Biochemicals, Laval, Québec, Canada) and 50 mM dithiothreitol (Sigma)], 1.25 mM dNTPs (mixture of dATP, dCTP, dGTP and dTTP at 25 mM; Promega, Madison, WI, USA), 10 U RNase inhibitor (Invitrogen) and 200 U SuperScript II reverse transcriptase (Invitrogen). This first-strand complementary DNA (cDNA) was used immediately for PCR amplification or stored at –20°C until further use. PCR reactions were performed using the specific primer set described (see *Primer design*). PCR reactions contained 1× PCR buffer (Roche Molecular Biochemicals), 2.5 µM MgCl₂, 0.25 mM dNTPs, 0.4 µM of each primer, 2 U AmpliTaq Gold (Roche Molecular Biochemicals), 4% dimethyl sulfoxide (Sigma) and 2 µl of first-strand cDNA to a final volume of 25 µl. MT1 PCR was carried out as follows: samples were denatured for 8 min at 94°C, followed by 40 cycles of 30 s denaturing at 94°C, 45 s annealing at 56°C and 50 s extension at 72°C with a final extension of 7 min at 72°C. For MT2, samples were denatured for 8 min at 94°C, followed by 40 cycles of 30 s denaturing at 94°C, 45 s annealing at 58°C, 30 s extension at 72°C, and a final extension of 7 min at 78°C. For RORα1, samples were denatured for 8 min at 94°C, followed by 40 cycles of 30 s denaturing at 94°C, 30 s annealing at 61°C and 30 s extension at 72°C. A final extension of 7 min at 72°C concluded the PCR. PCR fragments

were separated by gel electrophoresis on a 2% agarose gel (Invitrogen) containing ethidium bromide (Sigma), and Chemigenius² (SynGene, Frederick, MD, USA) was used to visualize DNA bands with UV light and to generate digital images of the gels. MCF-7 breast cancer cell line, whole rat brain and whole mouse brain were used as positive controls for MT1, MT2 and ROR α receptors, respectively (Wan *et al.*, 1999; Ram *et al.*, 2002; Naji *et al.*, 2004), whereas water was used as negative control. The same controls were used for western blot analysis.

Nucleotide sequences for MT1 and MT2 melatonin receptors were analysed by the dideoxynucleotide chain termination method of Sanger *et al.* (1977) using ThermoSequenase (Amersham Biosciences, Piscataway, NJ, USA). DNA sequences obtained were analysed using ALFwin Sequence Analyser 2.00 (Amersham Pharmacia Biotech).

Primer design

Primers were designed with Primer Premier 5.0 (Premier Biosoft International, Palo Alto, CA, USA) based on the full human MT1, MT2 and ROR α 1 receptor cDNA sequences (GenBankTM Accession No. NM_005958, No. NM_005959 and No. U04897, respectively). Online BLAST was used to search for homologies to other gene sequences. Primers were synthesized by Invitrogen (Carlsbad, CA, USA). The primer set, sense primer (5'-CGTTGGTGCTGATGTCG-3', nucleotides 323–339) and antisense primer (5'-AGTTTGGGTTTGGGTC-3', nucleotides 748–764), was used to amplify the human MT1 melatonin receptor. Human MT2 melatonin receptor was amplified using the primer set, sense primer (5'-CAACTGCTGCGAGGCG-3', nucleotides 127–142) and antisense primer (5'-GGCGGTGGTGA CGATG-3', nucleotides 244–259). The following primers were used to amplify human ROR α 1-melatonin receptor, sense primer (5'-GGA AGAGCTCCAGCAGATAACG-3', nucleotides 978–998) and antisense primer (5'-GCTGACATCAGTACGAATGCAG-3', nucleotides 1372–1393).

Protein isolation and western blot analysis

Human placental tissue samples from normal full-term pregnancies, rat brain and mouse brain were centrifuged 10 min at 3000 *g* in ice-cold lysis buffer [17 mM Tris–HCl pH 7.3 (Fisher Scientific, Nepean, Ontario, Canada) containing 0.144 M NH₄Cl (Sigma)] to eliminate lymphocytes that express melatonin receptors (Pozo *et al.*, 1997). Tissues were homogenized using a Potter–Elvehjem homogenizer (20 strokes, on ice). Total proteins were extracted from the placental tissue pellet, rat brain, mouse brain and cell lines (JEG-3, BeWo and MCF-7) using the acid guanidium thiocyanate-phenol-chloroform extraction method with TRI Reagent according to manufacturer's instructions (Molecular Research Center). Membrane proteins from JEG-3 and BeWo cell lines were extracted with ProteoExtract® Native Membrane Protein Extraction Kit as directed by the manufacturer (EMD Biosciences, San Diego, CA, USA). Protein concentration of each sample was assayed by the BCA protein Assay Reagent according to manufacturer's instructions (Pierce Biotechnology, Rockford, IL, USA).

Proteins samples were heated at 95°C for 5 min in 2× sodium dodecyl sulphate (SDS) gel-loading buffer [100 mM Tris–HCl pH 6.8, 200 mM β -mercaptoethanol, 20% glycerol, 4% SDS and 0.2% bromophenol blue (Sigma)]. Proteins were fractionated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) on 12% separating gels with 4.4% stacking gels. Proteins were then transferred to nitrocellulose membranes (Amersham Biosciences). Blots were blocked in blocking buffer [20 mM Tris–HCl (pH 7.4) containing 150 mM NaCl, 0.02% (v/v) Tween 20 (TBST;tris-buffered saline with Tween-20) and 5% non-fat dry milk (Sigma)]. Western blot analysis was carried

out using a 1:250 dilution of specific goat affinity-purified polyclonal antibody against MT1 (sc-13179), MT2 (sc-28453) and ROR α 1 (sc-6062) (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) in blocking buffer for 1 h at room temperature. After TBST washing procedure, the blots were incubated with 1:1000 horse-radish peroxidase-labelled specific antibodies (Sigma) in TBST for 45 min at room temperature. Immunodetection was performed using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology). The affinity-purified polyclonal antibodies were directed against a peptide mapping within an internal region of MT1 or MT2 melatonin receptors of human origin or against a peptide mapping at the C-terminus of ROR α 1 of human origin.

Immunofluorescence analysis

Cells in culture were grown to confluence, trypsinized and plated at 1×10^5 cells per well of a 6-well culture plate containing coverslips. After 24 h, cells were washed three times with phosphate-buffered saline (PBS) Ca/Mg (Sigma) and fixed with freshly prepared 10% formaldehyde at room temperature for 1 h. Cells were washed three times with PBS Ca/Mg and permeabilized with 0.2% Triton X-100 (Fisher Scientific) in PBS Ca/Mg for 2 min on ice. Cells were then washed three times with wash buffer [0.5% rabbit serum (Sigma), 0.05% Tween 20 in PBS Ca/Mg]. After non-specific antigens were blocked for 1 h at room temperature with 1% rabbit serum and 0.1% Tween 20 in PBS Ca/Mg, the samples were incubated with primary antibodies. Goat purified polyclonal anti-MT1 (10 μ g/ml), anti-MT2 (12 μ g/ml) or anti-ROR α 1 (12 μ g/ml) was diluted in blocking buffer at 4°C in a moist chamber overnight. The negative control comprised only blocking buffer. Sample wells were then washed three times with wash buffer and incubated with Alexa Fluor 488 rabbit anti-goat immunoglobulin G (Invitrogen) at a 4 μ g/ml dilution in blocking buffer for 1 h at 37°C. Cells were then washed three times with PBS Ca/Mg, coverslips were briefly air dried and a drop of Slowfade Gold anti-fade reagent (Invitrogen) was added to cells, which were then mounted on glass slides. Immunofluorescence was analysed at 600× with a Nikon Eclipse E800 microscope equipped with a BioRad Radiance 2000 confocal imaging system (Bio-Rad Laboratories, Hercules, CA, USA). Fluorochrome excitation was achieved using a 10 mW Argon–Krypton laser for 488 nm (Alexa Fluor 488). Images were obtained using appropriate filters, through the sequential scanning mode of the LaserSharp software (Bio-Rad Laboratories). Representative images correspond to the confocal slice in the middle of the cell.

Hormone assays and statistics

Human choriocarcinoma cell lines JEG-3 and BeWo were plated at 7.5×10^4 cells/well in 24-well cell culture plates and incubated for 6 h at 37°C in 10% FBS culture medium for trypsin deactivation. Then, medium was removed and replaced with 0.1% FBS culture medium, and plates were further incubated for 18 h at 37°C for adherence. In the experiment with forskolin, medium was then replaced with 1 ml of fresh culture medium (with 0.1% FBS) with 6-chloromelatonin (10 pM, 1 nM, 100 nM or 10 μ M) or solvent alone (control, 71 nM Me₂SO) in the absence or presence of forskolin (10 μ M) (Sigma). Cells were incubated for 72 h at 37°C, and then media were removed and kept at –20°C until further analysis. In the experiment with PTX (Calbiochem, San Diego, CA, USA), media were removed and cells were pretreated for 6 h with 1 ml of fresh culture medium (with 0.1% FBS) containing either 200 ng/ml of PTX or water (solvent alone). Following pretreatment, cells were washed twice with 0.1% FBS culture medium. Cells were then treated with 10 μ M 6-chloromelatonin, 10 μ M forskolin or solvent alone (71 nM Me₂SO). Cells were incubated for 72 h at 37°C, and then media were removed and kept at –20°C until

further analysis. Protein content per well was assayed using the BCA Protein Assay. Protein content was equivalent between samples (<10% variation). hCG- β concentration in the medium was measured in triplicate by enzyme-linked immunosorbent assay using KTSP-4951 kits obtained from Mediacorps (Montréal, Québec, Canada). The sensitivity of the assay was 2.5 U/l, and cross-reactivity was less than 0.15% with human luteinizing hormone (LH), 0.001% with human growth hormone (GH), 0.001% with human thyroid-stimulating hormone (TSH), 0.001% with human follicle-stimulating hormone (FSH) and 0.04% with human prolactin. All data are presented as mean \pm SEM of at least three experiments with different cell-line passages performed in triplicate. Statistical significance was determined by one-way analysis of variance with Newman-Keuls post-testing using Prism version 4.0 (GraphPad, San Diego, CA, USA). A probability value of $P < 0.05$ was considered statistically significant.

Results

Expression of MT1, MT2 and ROR α 1 receptor mRNA and protein in the human placental tissues and in JEG-3 and BeWo choriocarcinoma cell lines

RT-PCR amplification of the single-strand cDNA using specific primers yielded the expected single amplicon of 442 bp (MT1; Figure 1A), 133 bp (MT2; Figure 1B) and 417 bp (ROR α 1; Figure 1C). The identity of MT1 and MT2 receptor PCR products was confirmed by direct automated DNA sequencing (data not shown). Sequence comparison using

online BLAST revealed that all amplicon sequences were identical to the already published sequences for the human MT1 (GenBank™ Accession No. NM_005958) and MT2 (GenBank™ Accession No. NM_005959)-melatonin receptors and not similar to any other gene.

Western blot analysis was used to determine melatonin receptor protein expression in human placental tissues and human placental choriocarcinoma cell lines. Optimal antibody signal was initially determined by separating increasing concentrations of total proteins by SDS-PAGE and immunoblotting using specific goat purified polyclonal (anti-MT1, anti-MT2 or anti-ROR α 1) antibodies. Densitometric analysis of these immunoblots showed that the signal was proportional to the amount of protein (JEG-3, BeWo and human placental tissue) applied to gel within a range of 20–150 μ g (correlation coefficients ≤ 0.98) for all the three antibodies (data not shown). To compare the chemiluminescence profiles of each cell line and placental tissue on the same gel, we carried out all studies with 75 μ g of total protein that gave the optimal antibody signal. Chemiluminescence revealed a single band of 37 kDa for JEG-3 and BeWo cell lines and human placental tissue proteins for MT1, similar to that of the MCF7 cell line serving as positive control (Figure 1D). In the case of MT2, chemiluminescence revealed two major bands of 40 and 36 kDa for JEG-3 and BeWo cell lines, whereas native tissues, human placental tissue and rat brain (positive control) demonstrated two major bands

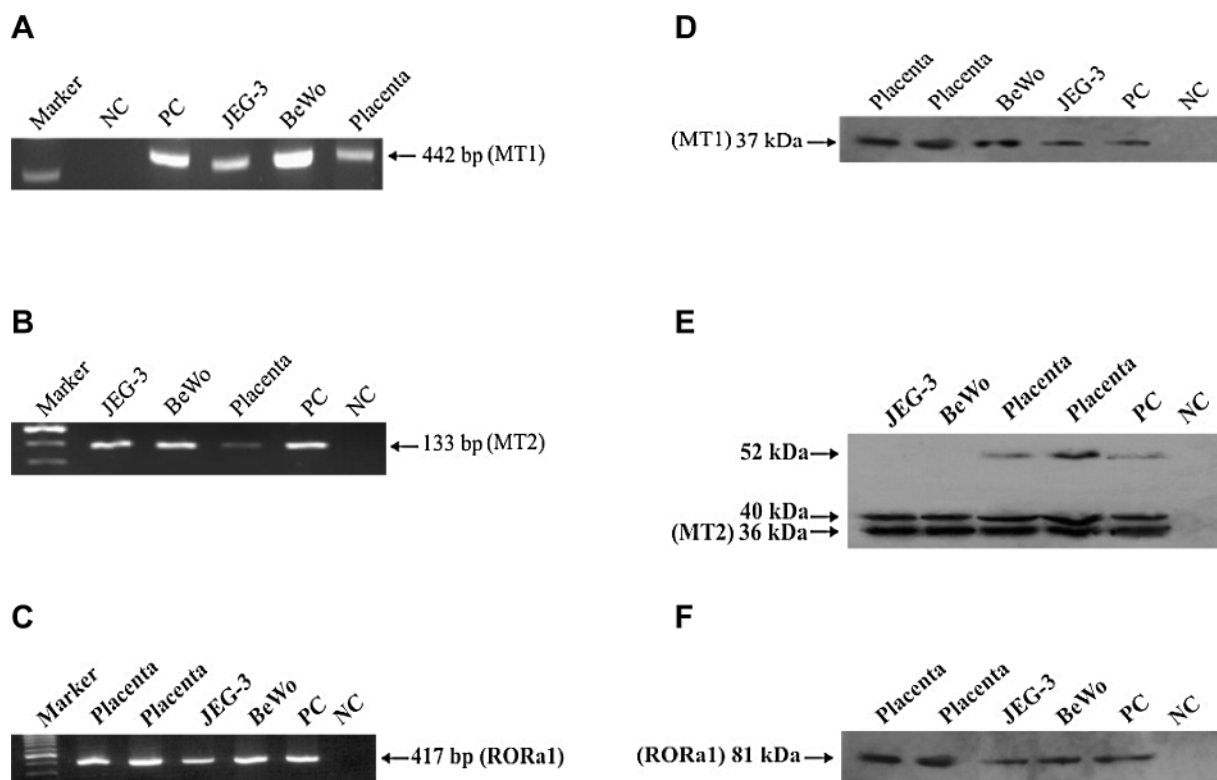


Figure 1. Expression of MT1, MT2 and ROR α 1 melatonin receptor mRNA and protein in human placental tissues and in JEG-3 and BeWo human placental choriocarcinoma cell lines. mRNAs were amplified by RT-PCR with (A) MT1-, (B) MT2- or (C) ROR α 1-specific primers and visualized in agarose gels by ethidium bromide staining. Western blots were hybridized with specific, anti-MT1 (D), anti-MT2 (E) or anti-ROR α 1 (F) antibodies. Marker, 100 bp ladder; PC (positive control), MCF-7 breast cancer cell line (MT1), rat brain (MT2) and mouse brain (ROR α 1), respectively; NC (negative control), water. Representative results from three experiments are shown.

of 40 and 36 kDa and a faint band of 52 kDa (Figure 1E). Western blot analysis for the nuclear ROR α 1 receptor reveals a single band of 81 kDa for JEG-3 and BeWo cell lines, and human placental tissue and mouse brain (positive control) exhibit a similar recognition profile (Figure 1F). Similar results were observed with nuclear proteins (data not shown).

Immunofluorescence identification of MT1, MT2 and ROR α 1 receptor in human placental choriocarcinoma cell lines JEG-3 and BeWo

Confocal microscopy analysis confirmed the presence of the MT1 (Figure 2C and D), MT2 (Figure 2E and F) and ROR α 1 (Figure 2G and H) receptors in both JEG-3 and BeWo cells. A specific plasma membrane and cytoplasmic immunoreactivity was observed for MT1 and MT2 receptors (Figure 2C–F). This immunoreactive staining pattern is typical for GPCRs (Mongan and Grubb, 2004). In addition, nuclear staining was observed for MT2 antibody (Figure 2E–F). As expected,

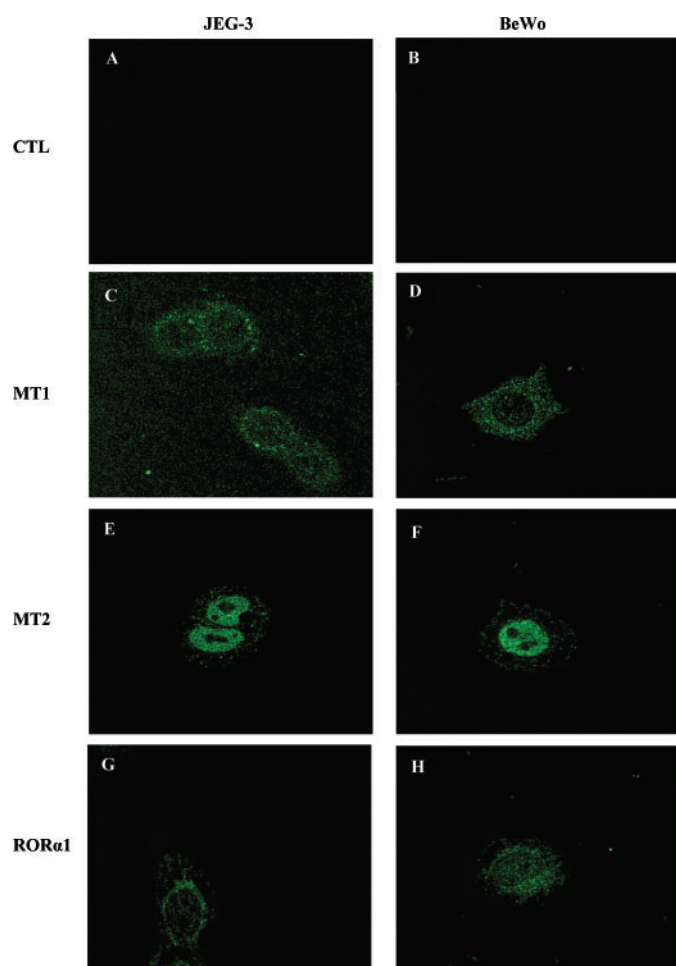


Figure 2. Confocal microscopy analysis of MT1, MT2 and ROR α 1 receptor proteins in human placental choriocarcinoma cell lines. JEG-3 and BeWo choriocarcinoma cell lines were analysed using the goat purified polyclonal anti-MT1 antibody (C and D), anti-MT2 antibody (E and F), anti-ROR α 1 (G and H) or, as negative control, without primary antibody (A and B). These pictures are representative of three different confocal microscopy analyses at 600 \times magnification for both cell lines.

ROR α 1 receptor immunofluorescence revealed a specific peri-nuclear and nuclear immunoreactive pattern (Figure 2G and H). Moreover, faint cytoplasmic and plasma membrane staining was also observed. No staining was observed in the absence of the primary antibody for all the three receptors (Figure 2A and B).

Characterization of the effect of melatonin on HCG- β secretion in JEG-3 and BeWo cell lines

Figure 3A and B shows the effects of melatonin agonist, 6-chloromelatonin, on hCG- β release in JEG-3 and BeWo human placental choriocarcinoma cell lines, respectively. Incubation of both choriocarcinoma cell lines with increasing doses of 6-chloromelatonin did not affect basal hCG- β production, whereas it did inhibit forskolin-stimulated hCG- β release in a dose-dependent manner. hCG- β release was stimulated 54.45 ± 3.05 - and 25.01 ± 1.11 -fold by forskolin (10 μ M) in JEG-3 and BeWo, respectively. The maximal inhibition was obtained with 10 μ M 6-chloromelatonin (JEG-3 and BeWo). At this concentration, forskolin-stimulated hCG- β release was inhibited by $63.89 \pm 10.33\%$ ($P < 0.001$) and by $40.50 \pm 10.38\%$ ($P < 0.05$) in JEG-3 and BeWo, respectively. Figure 4A and B shows that pretreatment with PTX completely abolishes the ability of 6-chloromelatonin to suppress forskolin-induced HCG- β secretion in JEG-3 and BeWo cell lines. We observed that PTX had no effect on forskolin-induced hCG- β secretion but abolished the inhibitory effect of melatonin on forskolin-stimulated hCG- β in both JEG-3 and BeWo cells (Figure 4). However, unlike the absence of effect of 6-chloromelatonin on basal hCG- β secretion observed in non-treated cells, in PTX-pretreated cells, 6-chloromelatonin had a significant stimulatory effect on basal hCG- β in BeWo cells ($40.52 \pm 14.28\%$, $P < 0.001$). No effects were seen on cell viability by any treatment, as demonstrated by trypan blue exclusion (data not shown).

Discussion

Expression of melatonin receptors

An important question addressed in the present study was whether the choriocarcinoma cells and human term placenta expressed melatonin receptors. Using RT-PCR, western blot and confocal microscopy analysis, we showed the expression of the MT1, MT2 and ROR α 1 melatonin receptors in JEG-3 and BeWo human choriocarcinoma cell lines as well as in human normal term placental tissues. Our novel observations of the expression of both MT1 and MT2 melatonin receptors in human term placental tissues and choriocarcinoma cells are consistent with the recent findings of MT1 and MT2 melatonin receptors mRNA in human first-trimester placenta (Iwasaki *et al.*, 2005). However, the expression of MT1 and MT2 melatonin receptor proteins in human first-trimester placenta, as well as melatonin receptor expression by term trophoblast cells, remains to be investigated.

Although still somewhat controversial, melatonin has been identified as a specific ligand for the ROR α nuclear receptor (Becker-Andre *et al.*, 1994). The ROR α gene, a member of the

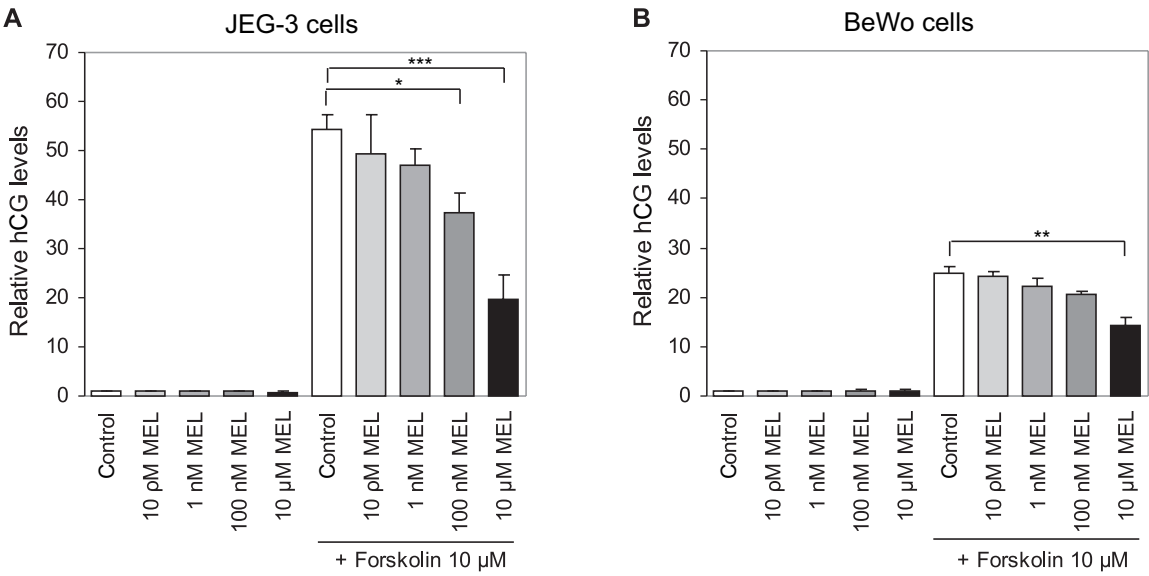


Figure 3. Effect of 6-chloromelatonin on HCG-β secretion in human placental choriocarcinoma cells lines. JEG-3 (A) and BeWo (B) cell lines were treated for 72 h with 10 pM, 1 nM, 100 nM, 10 μM 6-chloromelatonin (MEL) or solvent alone (control), in the presence or absence of forskolin (10 μM). Results shown are mean ± SEM (control, taken as 1.0) (*n* = at least three independent experiments with each sample run in triplicate; **P* < 0.05, ***P* < 0.01, ****P* < 0.001).

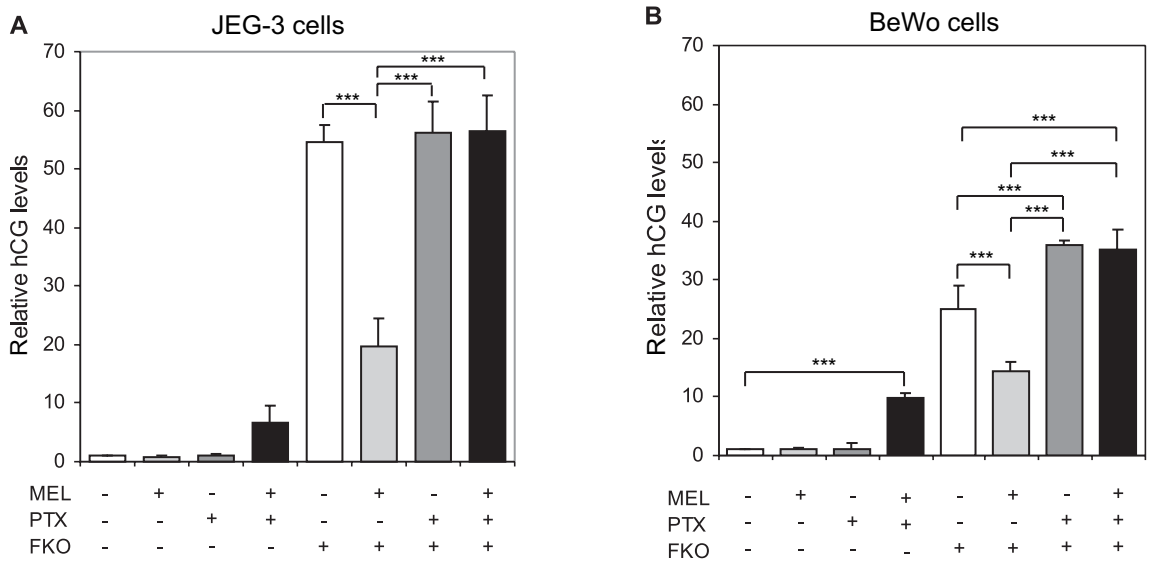


Figure 4. Effect of pertussis toxin (PTX) and 6-chloromelatonin on HCG-β secretion in human placental choriocarcinoma cell lines. JEG-3 (A) and BeWo (B) cell lines were pretreated with 200 ng/ml PTX or solvent alone (control; (-)) for 6 h and then treated for 72 h with 10 μM (MEL), 10 μM forskolin (FKO) or solvent alone (control; (-)). Results shown are mean ± SEM (control, taken as 1.0) (*n* = at least three independent experiments with each sample run in triplicate; ****P* < 0.001).

ROR/RZR family, is ubiquitously expressed and is thought to regulate gene transcription. Some researchers have questioned the validity of the melatonin-binding studies to ROR/RZR receptors and further suggest that these receptors may not be the correct or the only nuclear receptors for melatonin (Giguere, 1999). These findings add complexity to the known molecular signalling mechanisms of melatonin action and remain to be investigated in the human placental system. However, our finding of both membrane and nuclear melatonin

receptors in choriocarcinoma cells and human term placental tissues supports the hypothesis that the trophoblast cells of human placenta may be a direct target of melatonin. Western blot analysis of MT2R antibody (Figure 1E) revealed a different profile of recognition, with choriocarcinoma cells demonstrating only two major protein bands at 36 and 40 kDa and human placental tissue presenting an additional band at 55 kDa. We previously observed a similar phenomenon in western blot analysis of 5-HT_{2A} serotonin receptor

in human term placenta and human choriocarcinoma cell lines JEG-3 and BeWo (Sonier *et al.*, 2005). We hypothesize that differences in protein phosphorylation and glycosylation, which might occur between normal (placental tissue and rat brain tissue) and neoplastic cells (JEG-3 and BeWo), most likely explain our results. The multiple bands observed with MT2 antibody could be attributed to such differences in protein post-translational modifications or receptor dimerization (Prada *et al.*, 2005). One study has demonstrated that MT2 melatonin receptor, in *Xenopus* tectal cells, is expressed primarily as a dimeric complex (85 kDa) and as a glycosylated monomer (45 kDa) (Prada *et al.*, 2005). Moreover, the cloned MT2 receptor (Reppert *et al.*, 1995) has a predicted molecular weight of 40 kDa, not including post-translational modifications, which is similar to that reported for the deglycosylated receptor (38 kDa) (Prada *et al.*, 2005), suggesting that the bands we observed at 36 and 40 kDa correspond to the MT2 receptors.

Confocal microscopy experiments suggest that MT2 receptors in JEG-3 and BeWo cells are not only in plasma membranes, as nuclear staining was also observed for MT2 antibody. All cellular activities of the melatonin GPCR are currently thought to be mediated through their interactions with specific receptors located on the cell surface. However, over the past few years, GPCRs have been shown to reside not only in the plasma membrane but also in the nuclei and the nuclear envelope. There is now evidence demonstrating that AT1-angiotensin II (Lu *et al.*, 1998), prostaglandin (Bhattacharya *et al.*, 1998), endothelin (Boivin *et al.*, 2003) and leukotriene D4 (LTD4) (Nielsen *et al.*, 2005) receptors localize to the nuclear/perinuclear region. For example, the expression of the LTD4 receptor CysLT1, a GPCR, is not only in the plasma membrane but also in the nuclei of both normal colon epithelial cells and colon cancer cells (Nielsen *et al.*, 2005). These investigators suggest that the nuclear location of CysLT1 is not characteristic of an internalized receptor that is about to be degraded or recycled to the plasma membrane. This could indicate that signalling by a GPCR can occur in a novel location, providing the receptor can be directly accessed by its ligand in the nuclei. This hypothesis is particularly interesting for MT receptors, because melatonin is a lipophilic molecule that could easily cross the plasma membrane. However, the presence and possible role of the MT2 receptors on perinuclear membrane and in the nuclei in choriocarcinoma cells remain to be confirmed.

Effect of melatonin on hCG- β secretion

In this study, we intended to investigate whether melatonin influences the secretion of hCG- β , a hallmark of trophoblast. Two choriocarcinoma cell lines, BeWo and JEG-3, were used as *in vitro* models for human trophoblast; both cells secrete hCG- β (Mauschitz *et al.* 2000; Mandl *et al.*, 2002). The experiments were performed in 0.1% FBS to avoid serum influences. So far, interest in MT1 and MT2 receptor signalling has mainly focused on the adenylyl cyclase pathway. Several studies in various tissues demonstrated that melatonin can inhibit cAMP secretion via PTX-sensitive G proteins (Gi/o)

coupled to MT1 and MT2 receptors (reviewed in Dubocovich and Markowska, 2005; Witt-Enderby *et al.*, 2003). Intracellular accumulation of cAMP, in general, results in the enhancement of hormone synthesis and secretion (Kebabian, 1992; Mei *et al.*, 2002). Indeed, it has been demonstrated that cAMP increases hCG- β production in cultured trophoblast cells from human placenta and human choriocarcinoma cell lines (Chou *et al.*, 1978; Feinman *et al.*, 1986). Interestingly, in cultured human uterine myocytes of term pregnancies, melatonin has been shown to stimulate basal cAMP levels, whereas it had no effect on forskolin-stimulated cAMP accumulation (Schlabritz-Loutsevitch *et al.*, 2003). In the same study, however, authors also reported that in uterine myocytes from non-pregnant women, melatonin had no effect on basal cAMP levels but did cause a decrease in forskolin-stimulated cAMP accumulation. Also, the ability of melatonin to decrease forskolin-stimulated cAMP levels in pars tuberalis has been described (reviewed in Vanacek, 1998). These are compatible with our present result, showing for the first time, that the melatonin agonist, 6-chloromelatonin, inhibits forskolin-stimulated hCG- β secretion in a dose-dependent manner, whereas it has no effect on basal hCG- β secretion in JEG-3 and BeWo cell lines. Forskolin is a labdane diterpenoid with adenylyl cyclase-activating properties (de Souza *et al.*, 1983). Moreover, our results showing that the pretreatment of JEG-3 and BeWo cells with PTX abolished the effect of melatonin suggest that the effect of melatonin on forskolin-stimulated hCG- β secretion is mediated, in part, by a PTX-sensitive G protein, Gi/o. The higher secretion of hCG- β from the JEG-3 than the BeWo cell line could be attributed to their relative differentiation states; BeWo cell line is poorly differentiated, whereas JEG-3 has a more highly differentiated phenotype (Aplin *et al.*, 1992; Mandl *et al.*, 2002).

Hormonal activation of a receptor often results in the stimulation of multiple signalling pathways. Coupling to PTX-sensitive Gi proteins is responsible for melatonin's inhibitory effect on intracellular cAMP, and coupling to PTX-insensitive Gq/11 proteins activates a second pathway, the mobilization of Ca²⁺ from intracellular stores and activation of PKC via phospholipase C (PLC). Therefore, the significant stimulatory effect of 6-chloromelatonin on basal hCG- β secretion observed in PTX-pretreated BeWo cells could be due to the coupling of MT1 and/or MT2 receptors to the Gq/11 signalling pathway. In fact, it has been demonstrated that cAMP, PKC and calcium increase hCG- β production in cultured trophoblast cells from human placenta and human choriocarcinoma cell lines (Chou *et al.*, 1978; Feinman *et al.*, 1986). The intrinsic property of PTX to ADP ribosylate, the α -subunit of the intact G $\alpha\beta\gamma$ heterotrimer protein and not the free α -subunit (Gierschik, 1992), excludes the possibility that the stimulation of hCG- β secretion by 6-chloromelatonin in PTX-treated BeWo cells could be explained by coupling of PTX-sensitive Gi protein $\beta\gamma$ -subunits to PLC (Godson and Reppert, 1997). Which signalling pathways are involved in melatonin effects on hCG- β production in choriocarcinoma cell lines as well as in normal trophoblast cells remains to be investigated.

In accord with our present data, a recent study indicated that melatonin, coupled to MT1 receptors, negatively regulates the expression of PL-II in the late-pregnancy rat placenta (Lee *et al.*, 2003). However, our results appear to be incompatible with those of a recent study in which melatonin stimulates basal HCG secretion in cultured trophoblast cells from first-trimester human placenta (Iwasaki *et al.*, 2005). These divergent observations can be explained by differences in the cell conditions (e.g. signalling machinery) used in both studies [normal first-trimester trophoblast cells versus choriocarcinoma cell lines (tumour trophoblast)]. We have previously shown a significant change in placental D₂-dopamine receptor/PTX-sensitive G-protein interaction in pathological placentas (Vaillancourt *et al.*, 1997). Furthermore, it has been shown that melatonin elicited different intracellular events dependent on the cell types (reviewed in Smirnov, 2001; Witt-Enderby *et al.*, 2003). Determining the melatonin effects on hCG secretion in normal and abnormal placentas during pregnancy will require further studies.

Taken together, this study demonstrates for the first time that MT1, MT2 and ROR α 1 melatonin receptors are expressed in normal human term placenta and in human placental choriocarcinoma cell lines JEG-3 and BeWo. Our results also show that in choriocarcinoma cells, an *in vitro* model for human trophoblast, melatonin regulates hCG- β production by an action involving a PTX-sensitive and a PTX-insensitive G-protein and melatonin GPCR (MT1 and/or MT2). In conclusion, the present study points to an important role for melatonin in human placental autocrine/paracrine function and, as a consequence, on fetal development.

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