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Melatonin as a local regulator of human placental function

Abstract: Melatonin plays a critical role in a variety of mammalian reproductive processes not only acting on the central nervous system but also behaving as a peripheral physiologic regulator. To address the relevance of melatonin to the maintenance of pregnancy at the feto-maternal interface, we investigated the expression of two types of membrane melatonin receptors, MT_1 and MT_2, as well as arylalkylamine N-acetyltransferase (AA-NAT) and hydroxyindole-O-methyltransferase (HIOMT), the two enzymes required for the conversion of serotonin to melatonin, in the human placenta and the effect of melatonin on the release of human chorionic gonadotropin (hCG) from cultured human trophoblast cells. RT-PCR analysis and DNA sequencing revealed that transcripts of MT_1, MT_2, AA-NAT, and HIOMT were present in the first-trimester human placenta. We also found that melatonin significantly potentiated hCG secretion at optimal concentrations. These results suggest that melatonin may regulate human placental function in a paracrine/autocrine manner, providing evidence for a novel role in human reproduction.

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Introduction

Melatonin, the principal secretory product of the pineal gland, influences the timing of mammalian circadian rhythms [1, 2] and regulates reproductive changes that occur in response to variations in day length in seasonally breeding mammals [3, 4]. Although humans are not seasonal breeders, epidemiologic studies in several geographic areas point to a seasonal distribution in conception and birth rate [5, 6]. Sandyk et al. have postulated that low levels of serum melatonin during early pregnancy may be causally related to the development of spontaneous abortion [7]. To date, increasing instances of the following evidence substantiate the important role of melatonin in various processes of reproduction in mammals: (a) serum melatonin levels are high in early pregnancy [7, 8], (b) pinealectomy increases spontaneous abortions in the rat [9], (c) melatonin stimulates progesterone production [10], (d) melatonin inhibits prostaglandin synthesis [11], and (e) melatonin inhibits uterine contractility [12]. Also, in addition to the conventional hormonal action, melatonin has recently emerged as a possible local regulator in various types of tissues [13, 14]. However, the synthesis of melatonin, expression of its receptors, and its functional role at the feto-maternal interface, the human placenta in particular, remains poorly understood.

Melatonin exerts some of its versatile cellular effects including oncostatic [15], anti-aging [16], and immunomodulatory [17] properties on target tissues via its binding to receptors, although its antioxidant action [18] may be independent of its receptor system. Cloning studies in mammals have identified two distinct classes of melatonin receptors: the first class is the group of the membrane G protein-coupled receptors Mel1a and Mel1b [19–21], currently designated as MT_1 and MT_2, respectively [22], and the second one is the group of putative nuclear melatonin receptors belonging to the RZR/ROR orphan receptor subfamily [23, 24]. MT_1 is expressed in the hypothalamic suprachiasmatic nuclei and in the hypophyseal pars tuberalis, where it is presumably involved in circadian and reproductive responses [25]. In contrast, MT_2 is expressed in the retina and the brain, mediating some effects of melatonin on retinal function in mammals [25]. Melatonin receptors were also discovered in several other human tissues, including the kidney [26], granulosa cells of the ovarian follicle [27], and the prostate [28]. In addition, melatonin and its two synthesizing enzymes, arylalkylamine N-acetyltransferase (AA-NAT) and hydoroxyindole-O-methryltransferase (HIOMT), have been reported to be present in many extrapineal sites including retina, gastrointestinal tract, reproductive organs, skin, platelets, immune cells, and several brain regions [13, 29].

In this study, to address the role of melatonin to maintain pregnancy at the feto-maternal interface, we investigated the expression of melatonin receptors as well as AA-NAT, HIOMT and its physiologic function in the human placental tissue.

Material and methods

Tissue collection

Placental tissues of 10 wk gestation were obtained from patients who underwent dilatation and curettage for legal abortion. Gestational age was calculated from the last menstrual period, and ultrasonographic examination was also performed to determine the exact gestational period and eliminate an abnormal pregnancy. Approximately 100 mg of the tissue was immediately frozen in liquid nitrogen and then stored at −80°C until RNA extraction, and the rest were subjected to culture experiments. The study was approved by the local ethical committee and written informed consent was obtained prior to each investigation.

RNA extraction and RT-PCR

Polyadenylated [poly(A)+] RNA was prepared with the Micro-Fast Track Kit (Invitrogen, Carlsbad, CA, USA) from human placenta during the first trimester. The amount of mRNA was determined by the DNA DipStick Kit (Invitrogen). All RNA preparations were additionally treated with DNase to eliminate residual genomic DNA contamination and integrity of RNA by agarose gel electrophoresis.

First-strand cDNA was generated by reverse transcription of poly(A)+ RNA (0.5–1.0 ng) from early human placenta at 42°C using Superscript reverse transcriptase (Perkin-Elmer Cetus, Norwalk, CT, USA), according to the manufacturer's instructions. PCR was performed on 2 μL aliquots of 20 μL first-strand cDNA reaction mixture using a set of consensus oligonucleotide primers of human MT_1 and MT_2 melatonin receptors, AA-NAT and HIOMT (Table 1). Each reaction cycle consisted of incubation at 99°C for 60 s, 55°C for 60 s (for MT_1 primers) or 59.6°C for 60 s (for MT_2 primers) or 49°C for 60 s (for AA-NAT primers) or 60.4°C for 60 s (for HIOMT primers), and 72°C for 90 s, for 35 cycles, with AmpliTaq DNA polymerase (Perkin-Elmer/Cetus, Foster City, CA, USA). The products were subjected to 1% agarose gel electrophoresis.

DNA sequencing

PCR products were run on agarose gels containing 5 μg/mL ethidium bromide. The PCR bands were isolated and sent along with the primers to Takara Biomedicals (Kusatsu City, Japan) for sequencing verification. After purification of DNA with glass powder, the products were verified by automated sequence analysis on an ABI 377 sequencer using big dye chemistry (Applied Biosystems, Foster City, CA, USA).

Cell culture

Fresh placental tissues were obtained as previously described [30]. The placenta was washed several times with ice-cold phosphate-buffered saline (10 mmol/L, pH 7.4) containing 1 mmol/L EDTA until blood was washed out. The villous tissue was separated from connective tissue and minced on ice. Commercially obtained crude collagenase (Sigma-Aldrich Fine Chemicals, St Louis, MO, USA) was further purified by chelating sepharose Fast Flow (Pharmacia, Uppsala, Sweden), to remove nonspecific protease activity. The villous tissue (2–3 g) was incubated with purified collagenase (original collagenase activity, 10,000 units) and DNase-1 (0.001%) at 37°C for 30 min in a shaking water bath. The mixture was filtered through cheesecloth, and enzymatic digestion was repeated. Pooled cells were filtered through a nylon mesh and centrifuged at 800 × g for 10 min. The precipitated cells were then washed twice with medium 199, containing 10% fetal bovine serum (Gibco Laboratories, Grand Island, NY, USA), 100 IU penicillin, and streptomycin 100 μg/mL, to remove collagenase. Cell viability, estimated by trypan blue exclusion, was more than 90%. Dispersed trophoblast cells were counted using a phase contrast microscope and a hemocytometer, then the cell number was adjusted to 2×10^5 cells/mL with culture medium. Cells were cultured for 2 days at 37°C in a humidified atmosphere of 5% CO_2 in air [22].

Treatment with melatonin

After 2 days of culturing, trophoblast cells were washed three times with medium 199 without fetal bovine serum. The cells were then incubated with increasing concentrations of melatonin (1–100 μM) for 4 hr at 37°C in 95% air and 5% CO_2, and the medium was collected and kept at −20°C until it was assayed. Melatonin was dissolved in ethanol and then was diluted with medium containing a diluted concentration of ethanol. The final ethanol concentration in medium was $< 0.01\%$.

Measurement of human chorionic gonadotropin in medium

The effects of melatonin on human chorionic gonadotropin (hCG) secretion by trophoblast cells obtained at 10 wk gestation were quantified by an enzyme-linked immunosorbent

Table 1. Primers used for RT-PCR

Gene	Accession No.	Primer sequences	PCR product length (bp)
MT_1	U14108	Forward: 5′-CATCAACCGCTACTGCTACA-3′ Reverse: 5′-AGGACAAAAAACCACAAACAT-3′	372
MT_2	U25341	Forward: 5′-CCCCTACCCGCTAATCCTCG-3′ Reverse: 5′-GCTCTCTGCCTTGGCTTTCCTG-3′	439
AA-NAT	AF067408	Forward: 5′-CAAATCAGAAGGGAACAGTA-3′ Reverse: 5′-TGGATGACAAATAGACAAGA-3′	424
HIOMT	U11090	Forward: 5′-GGGCGTGTTTGACCTTCTCG-3′ Reverse: 5′-GCTCGCCCTCGGACCTGTAG-3′	365

assay (Mochida Co., Tokyo, Japan) using monoclonal antibody specific for the beta-subunit that recognizes both native and beta-subunit of hCG. Intra- and inter-assay coefficients of variation in conditioned media were $< 12\%$. Results were expressed using the Second International hCG Standard.

Statistical analysis

Statistical analyses were performed by ANOVA using the Statview 4.5 program. A P-value of < 0.05 was considered significant.

Results

Messenger RNA isolated from human first-trimester placenta was analyzed by RT-PCR for the presence of MT_1 and MT_2 transcripts. As shown in Fig. 1A, transcripts of MT_1 and MT_2 receptors were detected with the predicted sizes of the PCR products and no products deriving from genomic DNA templates were obtained (data not shown). Verification of each RT-PCR product was performed by DNA sequencing and it turned out to be 100% identical to the corresponding melatonin receptor gene. Transcripts of AA-NAT and HIOMT were also present with the predicted sizes in the first-trimester human placenta (Fig. 1B). DNA sequencing revealed that the RT-PCR products were 100% identical to AA-NAT and HIOMT genes.

The presence of the melatonin receptors and its synthesizing enzymes in the first-trimester human placenta prompted us to examine the effect of melatonin on hCG release from trophoblast cells in culture. As shown in Fig. 2, melatonin stimulated hCG secretion from the cultured trophoblast cells isolated from the first-trimester human placenta. The stimulating effects of 10 and 100 μM melatonin were significant ($P < 0.01$ and $P < 0.05$, respectively).

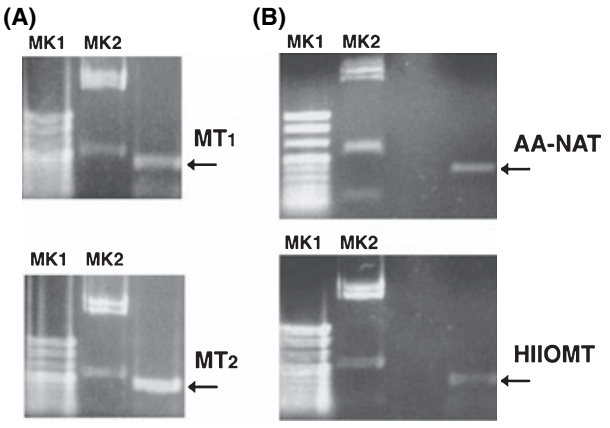

Fig. 1. RT-PCR analysis of the MT_1 and MT_2 melatonin receptors (A) and melatonin synthesizing enzymes AA-NAT and HIOMT (B). RT-PCR was performed on poly(A)+ mRNA from the first-trimester human placenta as described in Material and methods. PCR products visualized by ethidium bromide staining are shown. The size of MT_1 and MT_2 RT-PCR products were 372 and 439 bp, respectively (A). The size of AA-NAT and HIOMT RT-PCR products were 424 and 365 bp, respectively (B). MK1, Takara molecular weight marker VIII; MK2, Hind III marker.

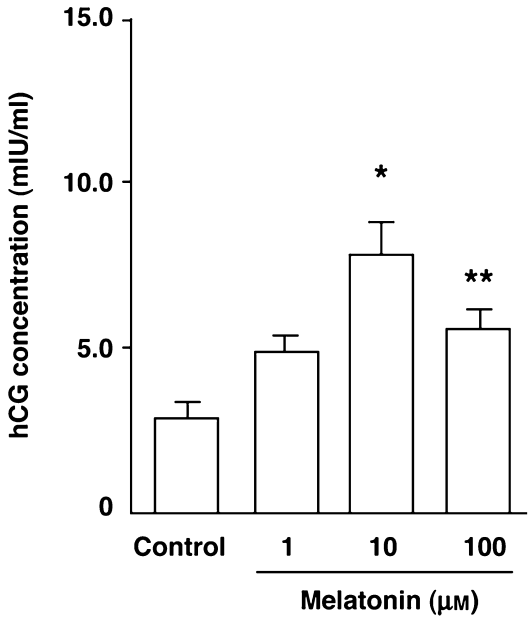

Fig. 2. Effects of melatonin on hCG secretion from human trophoblast cells in culture. The trophoblast cells were isolated from the human placenta of 10 wk gestation and cultured for 2 days without or with melatonin at various concentrations (1–100 μM). Each bar indicates the mean + S.E.M. $*P < 0.01$, versus control, $**P < 0.05$, versus control.

Discussion

As on date, a large number of melatonin-producing cells have been identified in many extrapineal organs [29]. Besides, the main property of melatonin as a universal regulator of biologic rhythms, and its wide spectrum of biologic activities have been demonstrated. These findings collectively substantiate the possible role of extrapineal melatonin as a paracrine signal molecule for local coordination of intercellular functions [29]. Locally produced (paracrine) melatonin may contribute to numerous effects attributable to melatonin, either by itself or by interfering with the endocrine effects of this agent. Furthermore, local tissue concentrations of melatonin may attain much higher values than what was hitherto regarded as the physiologic level. This issue might be particularly important in view of the antioxidative features of melatonin [18]. Therefore, studies on the physiologic relevance of extrapineal melatonin are important.

During pregnancy in rats, melatonin level in the plasma is maintained by the circadian rhythm, where it is significantly higher at night than in the daytime [8, 31], while its placental level is constant throughout the day [31]. The melatonin concentration in the rat placental tissue is about seven times higher than that in the plasma [31]. In this study, we show the existence of melatonin-synthesizing key enzymes in the first-trimester human placenta. Collectively, these data suggest that melatonin may be locally and actively produced at the feto-maternal interface, particularly, at the placental site and that locally produced melatonin, and not the circulating one, dominantly

determine the placental melatonin concentration. To address the role of placental melatonin, we then demonstrated the presence of melatonin receptors in the human placenta and the potential of melatonin to stimulate hCG release from human trophoblast cells in culture. Taken together, we here postulate that melatonin may act as a local regulator of placental function in an autocrine/paracrine manner.

Melatonin has been implicated in placental function. Mel1a (MT_1) is spatially and temporally expressed in the rat placenta, which, in turn, negatively regulates the expression of placental lactogen-II gene [32]. As the level of placental melatonin is constant throughout a day [31], circadian regulation of placental function by melatonin may largely depend on the expression levels of placental MT_1. Melatonin has also been demonstrated to exert an antioxidant action to prevent oxidative mitochondrial damage induced in rat placenta by ischemia and reperfusion [33]. We here provide evidence for the first time suggesting the production of extrapineal melatonin in the human placenta and its novel role to simulate hCG release.

It is well known that melatonin can inhibit cAMP signaling via coupling of MT_1 and MT_2 to pertussis toxin-sensitive G proteins [25]. Intracellular accumulation of cAMP, in general, results in the enhancement of hormone synthesis and secretion [34]. Indeed, Feinman et al.[35]. demonstrated that 8-bromo-cAMP, but not 8-bromo-cGMP, provokes a dose-dependent increase in the secretion of hCG in human cytotrophoblasts in culture This paradigm, however, appears to be incompatible with our present data in that melatonin stimulated hCG release.

It is important to note that melatonin affects numerous signal transduction pathways other than the cAMP-dependent pathway [36]. For instance, there are two distinct and parallel pathways of melatonin-mediated signal transduction: one inhibits adenyl cyclase thereby leading to an increase in intracellular cAMP levels and the other potentiates phospholipase activation [21]. Furthermore, different intracellular events elicited by melatonin depend on the cell types [36]. A recent intriguing report demonstrated that forskolin-induced cAMP accumulation is inhibited by melatonin in nonpregnant uterine myocytes, while, in pregnant uterine myocytes, intracellular cAMP levels are not changed but rather significantly increased by treatment with high concentrations of melatonin [37]. This finding supports our data that high concentration of melatonin used in this study provoked a significant increase in hCG production from trophoblast cells.

In conclusion, transcripts of the melatonin-synthesizing enzymes and MT_1 and MT_2 melatonin receptors are present in the first-trimester human placenta. Furthermore, melatonin potentiates hCG secretion from cultured trophoblast cells isolated from the first-trimester human placenta. Taken together, these data suggest that melatonin may behave as a local regulator of placental function in an autocrine/paracrine manner.

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References

1. Reiter RJ. Pineal melatonin: cell biology of its synthesis and of its physiological interactions. Endocr Rev 1991; **12**:151–180.
2. Arendt J. Melatonin and the pineal gland: influence on mammalian seasonal and circadian physiology. Rev Reprod 1998; **3**:13–22.
3. Tamarkin L, Baird CJ, Almeida OF. Melatonin: a coordinating signal for mammalian reproduction? Science 1985; **227**:714–720.
4. Reiter RJ. The pineal and its hormones in the control of reproduction in mammals. Endocr Rev 1980; **1**:109–131.
5. Cagnacci A, Volpe A. Influence of melatonin and photoperiod on animal and human reproduction. J Endocrinol Invest 1996; **19**:382–411.
6. Rojansky N, Brzezinski A, Schenker JG. Seasonality in human reproduction: an update. Hum Reprod 1992; **7**:735–745.
7. Sandyk R, Anastasiadis PG, Anninos PA et al. The pineal gland and spontaneous abortions: implications for therapy with melatonin and magnetic field. Int J Neurosci 1992; **62**:243–250.
8. Kivela A. Serum melatonin during human pregnancy. Acta Endocrinol (Copenh) 1991; **124**:233–237.
9. Guerra MO, Silva NO, Guimaraes CS et al. Pinealectomy and blindness during pregnancy in the rat. Am J Obstet Gynecol 1973;**115**:582–583.
10. MacPhee AA, Cole FE, Rice BF. The effect of melatonin on steroidogenesis by the human ovary in vitro. J Clin Endocrinol Metab 1975; **40**:688–696.
11. Gimeno MF, Landa A, Sterin-Speziale N et al. Melatonin blocks in vitro generation of prostaglandin by the uterus and hypothalamus. Eur J Pharmacol 1980; **62**:309–317.
12. Hertz-Eshel M, Rahamimoff R. Effect of melatonin on uterine contractility. Life Sci 1965; **4**:1367–1372.
13. Stefulj J, Hortner M, Ghosh M et al. Gene expression of the key enzymes of melatonin synthesis in extrapineal tissues of the rat. J Pineal Res 2001; **30**:243–247.
14. Naji L, Carrillo-Vico A, Guerrero JM et al. Expression of membrane and nuclear melatonin receptors in mouse peripheral organs. Life Sci 2004; **74**:2227–2236.
15. Cos S, Fernandez R, Guezmes A et al. Influence of melatonin on invasive and metastatic properties of MCF-7 human breast cancer cells. Cancer Res 1998; **58**:4383–4390.
16. Reiter RJ. The ageing pineal gland and its physiological consequences. Bioessays 1992; **14**:169–175.
17. Calvo JR, Rafii-el-Idrissi M, Pozo D et al. Immunomodulatory role of melatonin: specific binding sites in human and rodent lymphoid cells. J Pineal Res 1995; **18**:119–126.
18. Reiter RJ, Tan DX, Mayo JC et al. Melatonin as an antioxidant: biochemical mechanisms and pathophysiological implications in humans. Acta Biochim Pol 2003; **50**:1129–1146.
19. Ebisawa T, Karne S, Lerner MR et al. Expression cloning of a high-affinity melatonin receptor from *Xenopus* dermal melanophores. Proc Natl Acad Sci USA 1994; **91**:6133–6137.
20. Reppert SM, Godson C, Mahle CD et al. Molecular characterization of a second melatonin receptor expressed in human retina and brain: the Mel1b melatonin receptor. Proc Natl Acad Sci USA 1995; **92**:8734–8738.
21. Godson C, Reppert SM. The Mel1a melatonin receptor is coupled to parallel signal transduction pathways. Endocrinology 1997; **138**:397–404.

22. Dubocovich ML, Cardinali DP, Delagrange P et al. The IUPHAR Compendium of Receptor Characterisation and Classification, 2nd edn. IUPHAR Media, London, 2000; pp. 187–193.
23. Becker-Andre M, Andre E, DeLamarter JF. Identification of nuclear receptor mRNAs by RT-PCR amplification of conserved zinc-finger motif sequences. Biochem Biophys Res Commun 1993; **194**:1371–1379.
24. Becker-Andre M, Wiesenberg I, Schaeren-Wiemers N et al. Pineal gland hormone melatonin binds and activates an orphan of the nuclear receptor superfamily. J Biol Chem 1994; **269**:28531–28534.
25. von Gall C, Stehle JH, Weaver DR. Mammalian melatonin receptors: molecular biology and signal transduction. Cell Tissue Res 2002; **309**:151–162.
26. Song Y, Chan CW, Brown GM et al. Studies of the renal action of melatonin: evidence that the effects are mediated by 37 kDa receptors of the Mel1a subtype localized primarily to the basolateral membrane of the proximal tubule. FASEB J 1997; **11**:93–100.
27. Yie SM, Niles LP, Younglai EV. Melatonin receptors on human granulosa cell membranes. J Clin Endocrinol Metab 1995; **80**:1747–1749.
28. Gilad E, Laudon M, Matzkin H et al. Functional melatonin receptors in human prostate epithelial cells. Endocrinology 1996; **137**:1412–1417.
29. Kvetnoy I. Extrapineal melatonin in pathology: new perspectives for diagnosis, prognosis and treatment of illness. Neuro Endocrinol Lett 2002; **23**(Suppl. 1):92–96.
30. Iwashita M, Watanabe M, Adachi T et al. Effect of gonadal steroids on gonadotropin-releasing hormones stimulated human chorionic gonadotropin release by trophoblast cells. Placenta 1989; **10**:103–112.
31. Nakazawa K, Kanakura Y, Kometani K et al. Study on melatonin in human and rat placental tissue. Trophoblast Res 1999; **13**:467–474.
32. Lee CK, Moon DH, Shin CS et al. Circadian expression of Mel1a and PL-II genes in placenta: effects of melatonin on the PL-II gene expression in the rat placenta. Mol Cell Endocrinol 2003; **200**:57–66.
33. Okatani Y, Wakatsuki A, Shinohara K et al. Melatonin protects against oxidative mitochondrial damage induced in rat placenta by ischemia and reperfusion. J Pineal Res 2001; **31**:173–178.
34. Taylor SS, Buechler JA, Yonemoto W. cAMP-dependent protein kinase: framework for a diverse family of regulatory enzymes. Annu Rev Biochem 1990; **59**:971–1005.
35. Feinman MA, Kliman HJ, Caltabiano S et al. 8-Bromo-3′,5′-adenosine monophosphate stimulates the endocrine activity of human cytotrophoblasts in culture. J Clin Endocrinol Metab 1986; **63**:1211–1217.
36. Vanecek J. Cellular mechanisms of melatonin action. Physiol Rev 1998; **78**:687–721.
37. Schlabritz-Loutsevitch N, Hellner N, Middendorf R et al. The human myometrium as a target for melatonin. J Clin Endocrinol Metab 2003; **88**:908–913.