Effects of melatonin ingestion on cAMP and cGMP levels in human plasma

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Abstract

The effects of daytime melatonin treatment (0.3 mg) on cAMP and cGMP levels in platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were investigated in 14 normal human subjects (age $\pm$ s.e.m. 26.2 ± 3.2 years). Plasma levels of cAMP, cGMP and melatonin were measured before and at intervals for 3 h after the treatment was administered at 1300 h.

Plasma melatonin concentrations reached peak levels 1 h after the treatment (mean $\pm$ s.d. 182.3 ± 43.5 pg/ml). The mean areas under the curve (AUC) for the time-cGMP concentration curves in PPP and in PRP were significantly increased after melatonin treatment compared with those observed after placebo treatment ($P=0.001$). No significant difference in cGMP levels was observed

between PPP and PRP. Increase in self-reported sleepiness after melatonin treatment positively correlated with increase in plasma cGMP levels ($r=0.92$). The mean AUC for the time-cAMP concentration in PRP, but not in PPP, was increased 1 h after melatonin treatment compared with that observed after placebo treatment, but not thereafter. No correlation between individual PRP or PPP cAMP levels and subjective sleepiness was observed. These results demonstrate a stimulating effect of melatonin treatment on plasma cGMP levels in humans and suggest a correlation between the increase in circulating cGMP levels and the sleep-promoting effect of the pineal hormone.

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Introduction

In laboratory animals and in humans the pineal hormone melatonin is reported to influence several important physiological functions, including circadian rhythmicity, sleep and reproductive function (Lewy *et al.* 1996, Zhdanova *et al.* 1997, Reiter 1998). Melatonin receptors or binding sites have been identified in a variety of central neural sites and in peripheral tissues, e.g. the supra-chiasmatic nuclei of the hypothalamus, the thalamus, the pars tuberalis of the pituitary gland, the retina and blood lymphocytes and platelets (Dubocovich 1984, Vanecek *et al.* 1987, Weaver *et al.* 1988, Williams & Morgan 1988, Niles & Hashemi 1990, Vacas *et al.* 1992).

The physiological functions and the mechanisms of melatonin's action in any of these diverse tissues remain obscure. However, in most of the tissues studied the hormone acutely inhibits intracellular cAMP accumulation through a G-protein coupled receptor (Reppert *et al.* 1994). The high lipid solubility of melatonin provides for the ready penetration of biological membranes by this amine, suggesting that its effects may be exerted not only at the membrane level but also intracellularly.

The marked diversity that has been observed in the behavioral effects of melatonin in different individuals

(Zhdanova & Wurtman 1997) might be attributable not only to variations in its circulating concentration, but also to the relative sensitivity of different target cells to the pineal hormone. The concurrent assessment of both a behavioral and a cellular response to the hormone may facilitate understanding of the mode of action and inter-individual variability in melatonin's effects. Since platelets were reported to be affected by melatonin treatment, we sought to examine the possibility that intracellular effects of melatonin in humans might be revealed by monitoring changes in the level of cyclic nucleotides in platelet-rich plasma (PRP) and platelet-poor plasma (PPP) samples obtained at intervals after the daytime administration of a low oral dose of melatonin (0.3 mg) that tends to induce circulating melatonin levels within the physiological range (Dollins *et al.* 1994).

Materials and Methods

Fourteen healthy volunteers (13 males, 1 female; mean age $\pm$ s.e.m. 26.2 ± 3.2 years) participated in a double-blind placebo-controlled study after giving informed consent. The experimental protocol and the Subject's Informed Consent Form were approved by the

Massachusetts Institute of Technology Committee on the Use of Humans as Experimental Subjects. Subjects underwent a physical examination and their urine and blood analyses were within the normal range. All of the subjects indicated that they were drug-free for at least 2 weeks prior to entering the study. They were asked to refrain from alcohol or caffeine consumption for 24 h prior to each test session, and to not take any medications during the entire period of the study.

Volunteers reported to the Clinical Research Center at 1000 h on the morning of each test session. A catheter with a saline lock was established in the subject's forearm vein 1 h before the first blood sample was collected and a light uniform lunch was served at noon. In test sessions, separated by at least 1 week without treatment, either a placebo (microcrystalline cellulose) or a 0.3 mg dose of melatonin (Nestle Co., Vevey, Switzerland) was administered orally in a gelatin capsule at 1300 h. The environmental light intensity was held constant on both test days at 250 lx.

Blood samples, 6 ml each, were drawn into polystyrene tubes containing 10% acid citrate dextrose anticoagulant solution (137 mM sodium citrate, 65 mM citric acid, 104 mM glucose). Blood samples were collected prior to treatment and at hourly intervals for 3 h after the treatment. The subjects were asked to report any changes in their subjective experience of sleepiness. Prior to each blood withdrawal, subjects were asked to report their sleepiness as either (1) decreased, (2) the same or (3) increased, relative to the pretreatment condition, on a three-score scale. Subjects were seated throughout the period of testing and they remained awake. Each of the subjects participated in two test sessions (at least a week apart) where they received either placebo or melatonin, half of the subjects receiving placebo first.

PRP preparation

PRP was obtained after blood samples were centrifuged at 200 *g* for 5 min, 'low brake', at room temperature. Reactions were terminated immediately in two 100 µl and two 200 µl aliquots of PRP by the addition of 1 ml ice-cold ethanol in order to measure baseline levels of cAMP and cGMP in the PRP. Triplicate 100 µl aliquots of PRP were used to obtain platelet counts (Cell-DYN 3000, Abbott Diagnostics, Santa Fe Springs, CA, USA) and, thereby, to document the platelet concentration in each PRP sample. Platelet counts varied among the different subjects between 250 and 450 × 10³/µl PRP, and were typically consistent in a set of replicate samples collected from the same subject. If we found more than a 10% difference between platelet counts in the same set of triplicate aliquots the results were not accepted and these PRP samples were not used to measure cyclic nucleotide concentrations.

After PRP samples were collected, the blood was further centrifuged for 5 min at 4000 *g*, the plasma transferred into Eppendorff tubes, then centrifuged at 13 000 *g* for 5 min to remove all remaining cells and yield PPP. Duplicate 100 µl and 200 µl PPP aliquots were inactivated in 1 ml ice-cold ethanol for cAMP and cGMP measurements. All inactivated PRP and PPP samples were centrifuged at 13 000 *g*, the supernatant was collected, lyophilized, reconstituted using the cAMP/cGMP assay buffer, and stored frozen at -20 °C until assayed for cyclic nucleotides. A 500 µl aliquot of each PPP sample was stored at -20 °C until assayed for melatonin concentration.

In one subject, the variation among the platelet counts observed within the sets of triplicate aliquots of the samples collected in one of the test sessions repeatedly exceeded 10%. For this reason his PRP data were excluded from the analysis and PRP results reflect data collected from 13 subjects only.

Assays

Both cAMP and cGMP concentrations in plasma samples were measured using [¹²⁵I]cAMP and [¹²⁵I]cGMP non-acetylation RIA kits (Amersham International, Amersham, Bucks, UK). Interassay coefficients of variation were less than 12.5%. In the event that an intra-assay coefficient of variation exceeded 8%, the assay was repeated. Standard cyclic nucleotide concentrations were repeatedly tested through each assay procedure in order to ensure the stable performance of the assay.

Plasma melatonin concentrations were also measured using a RIA kit (Buhlmann Laboratories, Allschwil, Switzerland). Melatonin extraction was accomplished using C18 columns. The limit of detection of the melatonin assay was 0.5 pg/ml. The intra-assay coefficients of variation for control samples were 7.5% (11 pg/ml) and 7.3% (25 pg/ml); the corresponding interassay coefficients of variation were 12.1 and 15.7%.

Measurements of cAMP in PRP serially diluted with PPP established that platelet cAMP estimates (PPP cAMP concentration subtracted from PRP concentration) were proportional to the number of platelets counted. PPP samples, processed as described above, contained near zero platelets.

Statistics

Areas under the curves (AUC) for plasma cGMP and cAMP concentrations were calculated for two periods: a 3-h period after melatonin treatment and a 3-h period after placebo treatment, from 1300 h to 1600 h. A relationship between individual melatonin levels (peak level or AUC) or reported sleepiness (individual AUC for sleepiness scores over a 3-h period) and individual changes in cyclic nucleotide levels were analyzed using Pearson's correlation

Figure 1 Group mean plasma melatonin levels (S.E.M.) for 3 h after ingestion of 0.3 mg melatonin at 1300 h ($n=14$).

coefficient. For this comparison, changes in the individual cyclic nucleotide levels for each individual were expressed as AUC for the cyclic nucleotide concentration over a 3-h period, with each data point of such curves representing a difference between cyclic nucleotide levels at a particular time and a baseline level prior to ingestion of placebo or a 0.3 mg dose of melatonin (delta AUC). Statistical comparison of peak melatonin levels after placebo or melatonin treatment, and of the AUC for plasma cGMP and cAMP after placebo or melatonin treatment, was performed using a two-tailed paired t -test. The minimum statistical significance level was $P<0.05$. The results are presented as the means $\pm$ S.E.M. Since cGMP levels in PRP and PPP were almost identical, and only 13 sets of data were available for PRP, all the statistical analysis was performed using PPP cGMP concentrations, which will be further referred to as plasma cGMP levels.

Results

Mean plasma melatonin levels before and 3 h after placebo treatment were 1.3 ± 0.7 and 1.6 ± 0.8 pg/ml respectively. Peak melatonin levels were typically reached within 1 h after melatonin treatment; the mean melatonin level at this time was 182 ± 10.4 pg/ml (Fig. 1).

Plasma concentration of cAMP in PRP was significantly higher than in PPP at all time points tested, including baseline levels before placebo or melatonin treatment (PRP: 1864 ± 52.1 and 1872 ± 54.4 fmol/100 μ l; PPP: 916 ± 76.3 and 932 ± 79.1 fmol/100 μ l). cGMP levels in PRP and PPP were remarkably similar at baseline (PRP: 245 ± 14.3 and 239 ± 11.2 fmol/100 μ l; PPP: 246 ± 17.4 and 251 ± 15.6 fmol/100 μ l) and remained almost identical in PRP and PPP after placebo or melatonin treatment.

No significant changes in the concentration of both cyclic nucleotides were observed during a 3-h period after the placebo treatment. One hour after melatonin

Figure 2 Group mean cAMP levels (S.E.M.) in PRP for 3 h after ingestion of placebo (white) or 0.3 mg melatonin (black) at 1300 h; $*P<0.05$ ($n=13$).

treatment PRP cAMP levels were transiently, but significantly ($P<0.05$), augmented (Fig. 2). No changes in PPP cAMP levels were observed. Plasma cGMP concentration gradually increased during the 3-h period of testing following melatonin ingestion (Fig. 3). The mean AUC for the time–cGMP concentration curve was significantly increased after melatonin treatment compared with that observed after placebo treatment (1210 ± 223 vs 995 ± 226 fmol/100 μ l h; $P=0.001$). Individual changes in plasma cGMP or PRP cAMP levels did not correlate with the individual peak plasma melatonin levels or melatonin AUC after the hormone ingestion ($r=0.31$).

Placebo treatment did not cause a significant change in self-reported sleepiness until the third hour of testing. No significant correlation between cyclic nucleotide levels and sleepiness at the end of the placebo session was observed ($r=0.27$). After melatonin treatment, significant correlation was observed between the AUC of the increase in individual plasma cGMP levels relative to baseline (delta cGMP AUC) and sleepiness AUC ($r=0.92$) (Fig. 4). No such correlation was observed for PRP or PPP cAMP (PRP: $r=0.22$). A significant increase in subjectively assessed sleepiness occurred 1 h after the hormone ingestion and 3 h after the administration of placebo (Fig. 3). No correlation was observed between individual baseline plasma cGMP or cAMP levels and the magnitude of a sleep-promoting effect of melatonin treatment or changes in plasma cGMP levels after the treatment.

Discussion

Our results demonstrate that the oral administration of melatonin to human adults, using a dose that increases the circulating melatonin concentration within the physiological range, substantially elevates the concentration of cGMP in plasma. The initiation of this increase can be detected 1 h after daytime melatonin treatment, coincident with the peak levels of circulating melatonin achieved. The rise in plasma cGMP concentrations

Figure 3 Columns: group mean cGMP levels (S.E.M.) in PPP for 3 h after ingestion of placebo or 0.3 mg melatonin at 1300 h. Lines: group mean sleepiness scores (S.E.M.) for 3 h after ingestion of placebo or 0.3 mg melatonin at 1300 h; * $P < 0.05$ ($n = 14$).

continues for at least 2–3 h after melatonin is administered, while the circulating melatonin levels are gradually declining. The limited period of our observations precludes an estimate of the maximum duration of the cGMP stimulating effect. The period of increased plasma levels of cGMP after melatonin treatment and the magnitude of such increase positively correlated with the increase in subjectively reported sleepiness. The moderate increase in cAMP levels in PRP that was observed in some of the subjects within 1 h after melatonin treatment was not correlated with either the individual subject's sleepiness after the melatonin administration, nor with the magnitude of his peak circulating melatonin level.

The relationship between melatonin and cGMP levels has attracted the attention of a number of researchers. A 2- to 3-fold increase in guanylate cyclase activity was observed in *in vitro* experiments involving the rat anterior

Figure 4 Relationship between individual AUC of the increase in plasma cGMP relative to baseline (delta cGMP AUC) and sleepiness AUC for 3 h after ingestion of placebo or 0.3 mg melatonin at 1300 h ($n = 14$).

pituitary, thyroid, testis, ovary, liver and small intestine in the presence of a near physiological, 1 nM, melatonin concentration (Vesely 1981). Similarly, an increase in cGMP accumulation was reported in the rat medial basal hypothalamus (Vacas *et al.* 1981), in the hamster's retina (Faillace *et al.* 1996), and in murine mammary glands (Cardinali *et al.* 1992) incubated with melatonin. A significant increase in cGMP concentration in the cerebrospinal fluid (CSF) was found in both rabbits and humans after they were treated with high pharmacological doses of melatonin (Rudman 1976, Young *et al.* 1984).

Interestingly, while in both of these earlier CSF studies (Rudman 1976, Young *et al.* 1984) cAMP measurements were made, neither reported a significant effect of melatonin treatment on cAMP concentrations in CSF. We, similarly, did not observe any significant changes in PPP cAMP after the melatonin treatment. It suggests that if changes in the intracellular cAMP levels occur as a result of the *in vivo* melatonin treatment, they are not

significantly reflected in such extracellular fluids as plasma or CSF. Though we have observed a clear increase in PRP cAMP levels 1 h after melatonin treatment in some of our subjects, the magnitude of the increase was modest. There are numerous observations in diverse tissues and brain structures studied *in vitro* that show an acute inhibition of cAMP accumulation as a result of melatonin treatment (Morgan *et al.* 1994). This effect is well described and linked to the hormone's interaction with the specific G-protein coupled receptors (Reppert *et al.* 1994). In contrast, an increase in cAMP accumulation was reported in other *in vitro* studies involving pars tuberalis cells (Hazlerigg *et al.* 1993) or rat Leydig cells (Valenti *et al.* 1995) after a prolonged, several hour, exposure of these tissues to melatonin. The differences in these observations may be attributable to a number of methodological considerations or possible downstream processes that may occur. A variability in the time course and in the magnitude of the adenylyl cyclase suppression, its recovery and possible further sensitization after exposure to melatonin may be the crucial factors in the development of particular systemic and behavioral effects of the pineal hormone *in vivo*. An increase in cGMP concentration as a result of melatonin treatment could also affect the cAMP levels by modulating the phosphodiesterase-3 activity. These results call for further detailed investigation of the dynamics of cAMP responses to melatonin treatment in *in vivo* experiments in animals and in human subjects.

The continued increase in concentrations of circulating cGMP observed in our subjects, following treatment with a physiological dose of melatonin, suggests that this effect might represent a normal physiological response to melatonin in humans. The increase in cGMP in the blood stream reflects an increased efflux of this intracellular second messenger. It may result from an increase in the intracellular cGMP production, an inhibition in its degradation, or an increase in its active release from the cells. It is not clear at this time specifically which cells might contribute to the observed phenomenon. As a first attempt to address the question of possible intracellular changes in *in vivo* cGMP concentrations we chose to test platelets, since an increase in cGMP concentration can reduce their activation (Waldmann & Walter 1989) and melatonin was also shown to inhibit platelet aggregation (Vacas *et al.* 1991). However, our data did not reveal a significant difference in cGMP levels between PRP and PPP, independent of the treatment administered. Thus, platelets do not substantially accumulate cGMP under the conditions tested. It is possible, though, that an increased intracellular cGMP production in platelets was compensated for by an increased cGMP efflux, as it has been shown in platelet activation studies (Wu *et al.* 1993).

The fact that there was a significant positive correlation between an increase in subjective sleepiness after melatonin treatment and an increase in cGMP concentrations suggests that these two effects of melatonin

might be related. Such a hypothesis is further substantiated by our recent finding that there is a significant daily variation in human plasma cGMP levels with an increase concurrent with the habitual nighttime hours of sleep (Zhdanova *et al.* 1999). Further studies involving larger numbers of human subjects, and studies in other diurnal animals that are sensitive to sleep-promoting effects of melatonin (Zhdanova *et al.* 1998), are needed in order to establish and explore the peripheral and central mechanisms of this possible relationship between the molecular and behavioral parameters of melatonin's action.

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