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Prolactin Response in Border–Leicester × Merino Ewes to Administration of Melatonin, Melatonin Analogues, a Melatonin Metabolite and 6-Methoxybenzoxazolinone

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Abstract

The effect of structural modifications of the melatonin molecule on plasma half-life of the analogues and basal prolactin secretion was studied in Border–Leicester × Merino ewes. Halogenation at position 6 and/or unsaturation of the 2,3-double bond of the melatonin molecule slightly lengthened the half-life of the analogues. Melatonin, 6-chloromelatonin, 2,3-dihydromelatonin and 6-chloro-2,3-dihydromelatonin decreased plasma prolactin to 31, 45, 54 and 48% of control levels respectively when administered daily (100 μg at 1600 h) for 21 days. The brain metabolite of melatonin, *N*-acetyl-*N'*-formyl-5-methoxykynurenamine, and the putative natural melatonin analogue, 6-methoxybenzoxazolinone, failed to affect prolactin levels when administered in a similar manner. These results indicate that certain structural modifications to the melatonin molecule can be tolerated biologically; however, the modifications reported here still did not prevent rapid clearance from the circulation.

Introduction

There is remarkable interest in the potential use of the pineal gland hormone, melatonin (*N*-acetyl-5-methoxytryptamine), for adjusting reproductive cycles in seasonally breeding animals (Lincoln 1983) and for resetting circadian rhythms in humans as a cure for jetlag (Arendt 1985). The current concept of melatonin action is that the duration of secretion during the scotophase interacts with the central nervous system and/or hypothalamus to alter basal and pulsatile hormone secretion (Bittman *et al.* 1983; Kennaway *et al.* 1983). Thus extended melatonin secretion during autumn in sheep decreases the oestradiol negative feedback sensitivity at the hypothalamus, allowing ovarian cycling to commence (Bittman *et al.* 1983). For melatonin to be used as a pharmacological agent it should therefore be administered at a specific time of day to mimic the extended secretion normally occurring during short daylength (Tamarkin *et al.* 1976; Kennaway *et al.* 1982). There is some evidence, however, that continuous delivery of melatonin is equally effective (Kennaway *et al.* 1982–83). Melatonin has a relatively short half-life (<30 min) in all animals studied (Kopin *et al.* 1961; Kennaway and Seamark 1980; Gibbs and Vriend 1981) and so there would be considerable advantages in the development of long-acting melatonin derivatives or analogues.

The primary site of melatonin metabolism is the '6' position which is hydroxylated by liver enzymes and subsequently conjugated to sulfate or glucuronide (Kopin *et al.* 1961; Kveder and McIsaac 1961; Taborsky *et al.* 1965). Melatonin is also metabolized in the brain by indole-2,3-dioxygenase to give rise to *N*-acetyl-*N'*-formyl-5-methoxykynurenamine (Hirata *et al.* 1974). Other minor metabolic

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pathways include demethylation to *N*-acetyl-5-hydroxytryptamine (Leone and Silman 1984) and deacetylation to 5-methoxytryptamine (Rogawski *et al.* 1979).

In this paper we report the synthesis of two new melatonin analogues, 2,3-dihydromelatonin and 6-chloro-2,3-dihydromelatonin. It was hypothesized that these compounds would escape metabolism by the liver and brain enzymes and thus have a prolonged half-life while retaining biological activity. Our biological assay involved the ability of the indoles to decrease basal prolactin levels in sheep exhibiting seasonally high levels of the hormone. In a previous study we showed that melatonin administration and light restriction decrease prolactin in an identical manner (Kennaway *et al.* 1982–83). The biological activities of the two new melatonin analogues and 6-chloromelatonin were compared with the same dose of melatonin. In addition, the prolactin-inhibiting activities of *N*-acetyl-*N'*-formyl-5-methoxykynurenamine and a putative natural analogue of melatonin, 6-methoxybenzoxazolinone (Sanders *et al.* 1981) were assessed.

Materials and Methods

Synthesis of the Melatonin Analogues

2,3-Dihydromelatonin and 6-chloro-2,3-dihydromelatonin were prepared by reduction of the respective unsaturated compounds with triethylsilane (Lanzilotti *et al.* 1979). Melatonin (2 mmol, 464·6 mg) was slowly added to trifluoroacetic acid (7 ml) in a flask immersed in an ice–water bath. The mixture (under a stream of nitrogen gas) was then heated in a silicone oil bath to a temperature of 50–60°C and triethylsilane (4 mmol, 0·62 ml) was added via a syringe and a septum inlet. Progress of the reaction was monitored at intervals by thin-layer chromatography on silica-gel plates developed in ethyl acetate and the reaction was found to be complete after heating for 64 h. Aqueous potassium hydroxide was added to the cooled reaction flask until the mixture was pH 9. The product was extracted with ethyl acetate (4 × 15 ml) and dried over potassium carbonate. Dry HCl gas was bubbled for 5 min into the filtered solution which was kept in a cooling bath. The hydrochloride salt precipitated out of solution and was filtered under nitrogen. The product (hygroscopic) was dried in a vacuum dessicator for 4 h (yield 70%). Mass spectrum ($C_{13}H_{18}N_2O_2$: 233 M^+). ^{13}C n.m.r. ($CDCl_3$) 8 (ppm), 23·02, 33·75, 37·49, 40·20, 53·58, 55·91, 110·30, 110·84, 112·46, 134·03, 145·13, 153·42, 170·49.

6-Chloro-2,3-dihydromelatonin was prepared as the free base using the same procedure. Mass spectrum ($C_{13}H_{17}N_2O_2Cl$: 268, 270 M^+).

6-Methoxy-2-benzoxazolinone was prepared using 5-methoxy-2-aminophenol (0·02 mol, 3 g) (Richey *et al.* 1975) and 1,1'-carbonyldiimidazole (0·03 mol, 5·3 g). The mixture was refluxed in dry tetrahydrofuran (80 ml) in a nitrogen atmosphere for 6 h and the reaction progress monitored by silica-gel, thin-layer chromatography in chloroform. The cooled solution was concentrated in a rotary evaporator and the residue partitioned between a 3 M HCl–chloroform solution (50 ml–50 ml). The aqueous layer was extracted with chloroform (2 × 40 ml) and the combined chloroform solution was washed with water (2 × 40 ml), saturated sodium chloride (2 × 40 ml) and dried over anhydrous magnesium sulfate. The concentrated product was recrystallized from dichloromethane (m.p. 154–155°C, lit. 154–155°C, Sonissman *et al.* 1957). The yield was 65%. Infrared spectrum (nujol) 3300–3060 cm^{-1} (N–H), 1790 (C=O, carbamate), 1610 (C=C). Mass spectrum ($C_8H_7NO_3$: 165·04 (M^+, 100%), 150 (M^+-CH_3, 40%), 122 (7%), 109 (20%).

N-Acetyl-*N'*-formyl-5-methoxykynurenamine was synthesized by ozonolysis of melatonin as described by Hirata *et al.* (1974). 6-Chloromelatonin was synthesized as previously described (Hugel 1983). Melatonin was purchased from Sigma (St Louis, Missouri, U.S.A.).

Animal Studies

Disappearance of immunoreactive melatonin, 6-chloromelatonin 2,3-dihydromelatonin and 6-chloro-2,3-dihydromelatonin from plasma was studied in two Merino ewes. On successive days 100 μg of each compound was injected subcutaneously and blood samples obtained at 15, 30, 60, 90, 120 and 180 min after injection. Differences in cross-reactivities of the analogues with the antibody required that different volumes of plasma be used (0·5 ml for melatonin, 1 ml for 6-chloromelatonin and 2 ml for 2,3-dihydro and 6-chloro-2,3-dihydromelatonin. The plasma was extracted with 5 vol. of

dichloromethane–hexane (1 : 1) after addition of 1 vol. 0·5 M borate buffer, pH 9·6. Thereafter the radioimmunoassay procedure of Earl *et al.* (1985), which uses the G280 antibody, was followed. Previous work had indicated up to 40% cross-reactivity by compounds of the type used in the present study (Kennaway 1983). Each indole was measured against its own extracted standard curve. Sensitivities for melatonin, 6-chloro-, 2,3-dihydro-, and 6-chloro-2,3-dihydromelatonin estimations were approximately 60, 600, 500 and 750 pmol/l, respectively.

To test the biological activity of the compounds 40 3-year-old Border–Leicester × Merino ewes were randomly assigned to eight treatment groups in November 1984. The sheep were brought into an outside race and jugular venous blood samples obtained by venepuncture with vacutainers (Becton-Dickinson Ltd) every 30 min for 5 h. The next day the ewes were again brought in from the field and injected at 1600 h with 1 ml saline (group I), 100 μg melatonin (group 2), 100 μg 6-chloro-melatonin (group 3), 100 μg 2,3-dihydromelatonin (group 4), 100 μg 6-chloro-2,3-dihydromelatonin (group 5), 100 μg *N*-acetyl-*N'*-formyl-5-methoxykynurenamine (group 6), and 100 or 300 μg 6-methoxybenzoxazolinone (groups 7 and 8). All compounds were in 1 ml saline and were injected subcutaneously except group 6 animals which were injected intravenously. Injections continued daily at 1600 h for 21 days. On day 21 all animals were again sampled every 30 min for 5 h.

Plasma prolactin was assayed in all blood samples using the radioimmunoassay method of Kennaway *et al.* (1981). Sensitivity was <3 ng/ml and inter- and intraassay coefficients of variation were always <12% at 36 and 134 ng/ml. Prolactin levels within a sampling period were analysed by one-way analysis of variance (ANOVA) to test whether there were any changes in secretion during the day. The means of the 10 samples for each sheep were then analysed by two-way ANOVA to test for treatment effects.

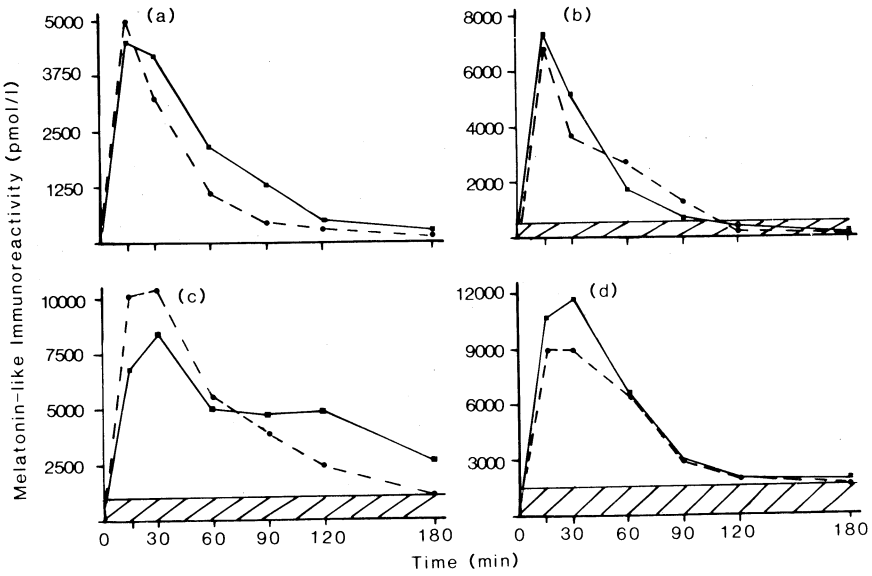

Fig. 1. Plasma melatonin-like immunoreactivity in two ewes injected subcutaneously with 100 μg of (*a*) melatonin, (*b*) 6-chloromelatonin, (*c*) 2,3-dihydromelatonin, and (*d*) 6-chloro-2,3-dihydro-melatonin. The values of the immunoreactivity are relative to standard compounds run through the assay. The hatched horizontal area represents the limit of assay sensitivity for the compounds.

Results

Injection of 100 μg melatonin resulted in peak plasma levels of immunoreactive melatonin within 15 min; thereafter melatonin disappeared from blood with an approximate half-life of 30 min (Fig. 1). 6-Chloromelatonin levels similarly peaked at 15 min and decreased with a half-life of 15–30 min. When 2,3-dihydromelatonin

and 6-chloro-2,3,-dihydromelatonin were injected the levels of immunoreactivity were approximately double those of the true indole compounds and the half-life appeared to be extended such that there was detectable immunoreactivity between 120 and 180 min post-injection.

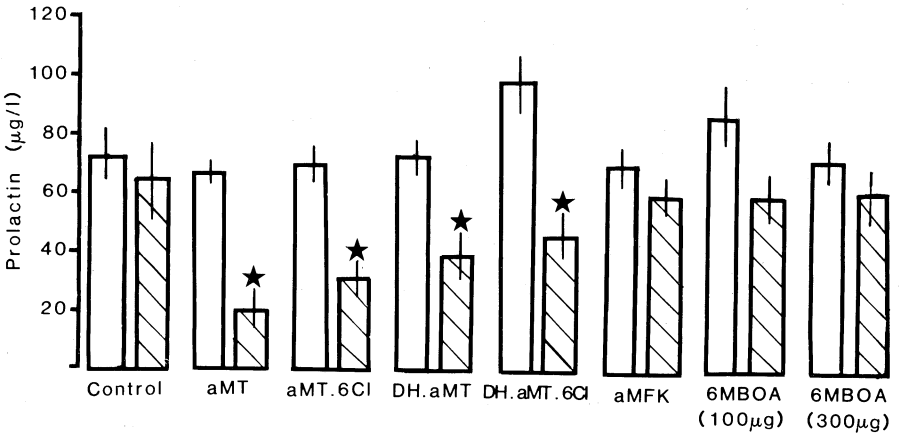

Fig. 2. Plasma prolactin levels in ewes before and 21 days after daily injections of saline control, melatonin (aMT) 6-chloromelatonin (aMT.6Cl), 2,3-dihydromelatonin (DH.aMT), 6-chloro-2,3-dihydromelatonin (DH.aMT.6Cl), *N*-acetyl-*N'*-formyl-5-methoxykynurenamine (aMFK) and 6-methoxybenzoxazolinone (6-MBOA). The mean of 10 successive 30-min samples was obtained and group mean + s.e.m. determined. The open histograms are the prolactin values for the pretreatment period and the hatched histograms the post-treatment period. * Values of prolactin which were significantly less ($P < 0{\cdot}05$) following treatment.

Prolactin levels did not change significantly with time during the sampling periods. However, there were occasional high values which presumably were the result of the stress of venepuncture. The mean of the 10 samples was therefore used to estimate basal secretion. Fig. 2 shows the group mean prolactin levels 1 day before and 21 days after daily drug administration. Melatonin, 6-chloromelatonin, 2,3-dihydromelatonin and 6-chloro-2,3-dihydromelatonin significantly decreased ($P < 0{\cdot}05$) prolactin levels to 31, 45, 54 and 48% of control levels, respectively. Treatment with 100 or 300 μg per day of 6-methoxybenzoxazolinone or *N*-acetyl-*N'*-formyl-5-methoxykynurenamine had no significant effect on prolactin levels.

The day after treatment ceased, rams were run with the ewes, with 35 out of 40 ewes becoming pregnant. No treatment effects were noted and a total of 37 lambs were identified at 70 days by ultrasound sonography.

Discussion

Because melatonin is metabolized by liver hydroxylases and brain indole 2,3-dioxygenase, synthetic melatonin analogues were used in an attempt to by-pass such metabolism and so enable the estimation of their half-lives. The doses of analogues used were known to be biologically active but the amounts used precluded the use of chemical methods of quantitation due to lack of sensitivity. A radio-immunoassay approach was therefore used. Although the true identity of the immunoreactivity following analogue injection is not known, the specificity of the antibody has been extensively investigated (Kennaway 1983). 6-Hydroxylated and 6-sulfated melatonin, 5-methoxytryptamine and 5-methoxyindoleacetic acid all cross-

react less than 0·02%. Data from the present study does not indicate that a substantial extension of half-life was achieved in the sheep by chlorination of melatonin in the 6-position. It is possible that chlorine substitution is inadequate for protecting effectively against hydroxylation. Further chemical manipulation of the melatonin molecule such as unsaturation of the 2,3-double bond alone or in combination with the 6-chloro substitution resulted in an apparent slight decrease in the half-life. Flaugh *et al.* (1979) reported that 6-chloromelatonin had a half-life of 27 min compared with 12–15 min for melatonin when administered intravenously to rats. No experimental details were given but in view of the amounts used elsewhere in that study (1–4 mg per rat) this time of 27 min may be an overestimate of the true half-life expected when using more 'physiological' doses (Gibbs and Vriend 1981).

Administration of melatonin daily for 21 days resulted in a significant decrease in prolactin levels. If sheep are placed in the dark at 1600 h under similar conditions as those used in this study, prolactin levels decrease (Kennaway *et al.* 1982–83). It is thought that the rise in melatonin following injection and the subsequent fall of endogenous melatonin at dawn provides the animals with the same hormonal stimulus as short daylength. The three melatonin analogues we have tested resulted in decreased prolactin secretion and thus presumably acted through the same mechanism as melatonin.

6-Chloromelatonin has been tested for endocrine activity previously by Flaugh *et al.* (1979) and Clemens *et al.* (1980). Those authors found that chlorination of melatonin in the 6-position led to slightly enhanced anti-ovulatory activity in rats. In addition Clemens *et al.* (1980) and Martin *et al.* (1980) found that 6-chloro- and 6-fluoromelatonin maintained the anti-LH activity and anti-LHRH effects of melatonin in rats. Similarly a comprehensive study of melatonin analogues by Frohn *et al.* (1980), utilizing the pigmentation response of the pencil fish, showed that 6-chloromelatonin retained full biological activity while 2,3-dihydromelatonin was approximately 10-fold less potent than melatonin. In our experiment 2,3-dihydromelatonin and 6-chloro-2,3-dihydromelatonin retained biological activity in that prolactin secretion was depressed after 21 days treatment. There was, however, no apparent advantage in the apparent higher blood levels or the slightly extended half-life of these compounds.

The rationale for synthesizing the 2,3-dihydromelatonins was that we did not expect them to be metabolized by indole-2,3-dioxygenase. It has been previously hypothesized that the products of this enzyme and formamidase, *N*-acetyl-*N'*-formyl-5-methoxykynurenamine (aFMK) and *N*-acetyl-5-methoxykynurenamine (aMK) may participate in the actions of melatonin (Kelly *et al.* 1984). In this respect aMK was found to be a very potent *in vitro* inhibitor of prostaglandin synthase, being more potent than melatonin or aspirin. In the present study aFMK, when administered intravenously to sheep (100 μg per day), failed to mimic the actions of melatonin. This finding together with the partially preserved activity of the unsaturated melatonin analogues do not support the hypothesis that melatonin must be metabolized by indole-2,3-dioxygenase in order to decrease prolactin levels. At this stage, however, we know little about the bioavailability and further metabolism of aFMK when injected into sheep, nor is it known whether unsaturated melatonin analogues bind to the putative melatonin receptors. Further work is therefore required before this attractive hypothesis is totally discarded.

The final compound tested in this study was 6-methoxybenzoxazolinone (6-MBOA) a compound isolated from freshly sprouted winter wheat (Sanders *et al.* 1981). When fed or injected into voles, mice or rats, 6-MBOA has a stimulatory effect upon the reproductive axis: uterine weight increases and the number of ova shed is increased (Sanders *et al.* 1981; Butterstein *et al.* 1985). 6-MBOA possesses remarkable structural similarities to melatonin and so could potentially be either an agonist or antagonist of melatonin. When injected daily at 1600 h at doses of 100 and 300 μg, however, 6-MBOA was totally devoid of prolactin-inhibiting activity. Our study did not address the question of antagonism of melatonin action.

In conclusion we have shown that modification of melatonin, involving halogenation at position 6 and/or unsaturation of the 2,3-double bond results in some protection from metabolism and retention of almost full biological activity. Under the experimental conditions we used neither the melatonin metabolite aFMK nor 6-MBOA were able to decrease prolactin levels. None of these compounds improved or decreased subsequent fertility or fecundity in the ewes.

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