Vol. 203, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1994 Pages 416-421

MELATONIN INHIBITS LDL RECEPTOR ACTIVITY AND CHOLESTEROL SYNTHESIS IN FRESHLY ISOLATED HUMAN MONONUCLEAR LEUKOCYTES

Dirk Müller-Wieland, Bert Behnke, Kathrin Koopmann, and Wilhelm Krone

Klinik II und Poliklinik für Innere Medizin der Universität zu Köln
Cologne, Germany

Received July 5, 1994

Summary: There is some indirect evidence that the pineal hormone melatonin can suppress plasma levels of cholesterol in hypercholesterolemic rats. We have examined the effects of the hormone on cellular cholesterol metabolism in freshly isolated human mononuclear leukocytes. Incubation of cells for up to 20 h in a lipid-free medium resulted in an increase in the rate of cholesterol synthesis from [^{14}C]acetate and the high affinity accumulation and degradation of [^{125}I]labeled low density lipoprotein (LDL). Addition of melatonin in increasing concentrations to the incubation medium at zero time inhibited cholesterol synthesis and the specific accumulation and degradation of [^{125}I]labeled LDL; at a concentration of 100 μM, the inhibitions were 38 %, 42 %, and 48 %, respectively. Similar results were obtained using [^{14}C]mevalonate as precursor. Fatty acid synthesis was not altered under these conditions. In contrast to cholesterol, the synthesis of the first cyclic compound lanosterol was not affected by the pineal hormone. These results implicate that melatonin inhibits this pathway between lanosterol and cholesterol. The action of melatonin on LDL receptor activity appeared to be mediated by a decrease in the number of LDL receptors and not by a change in binding affinity. Pharmacological characterization of the potential melatonin receptor site using several analogs like tryptamine, 5-hydroxytryptamine, N-acetyl-5-hydroxtryptamine, 5-methoxytryptamine, and 6-chloromelatonin indicated that the 5-methoxy group is indispensible for the hormone action on cholesterol synthesis. The data provide evidence that melatonin can modulate cholesterol metabolism in human cells. © 1994 Academic Press, Inc.

Cellular cholesterol metabolism is affected by diet (1,2), lipoproteins (3,4), hormones (5-7), and drugs (8-11). Recent studies suggest, that the pineal hormone melatonin can influence cholesterol metabolism. Rats with diet-induced (12) or genetic severe hypercholesterolemia were treated with melatonin and plasma cholesterol levels were lowered significantly under this treatment (13). Another example could be an inverse relationship between urinary melatoninsulfate excretion and plasma LDL cholesterol levels in human beings[1]. We have examined whether melatonin can affect cellular cholesterol metabolism in human cells directly. The study shows, that the hormone inhibits LDL receptor activity and cholesterol synthesis in human mononuclear leukocytes.

[1]B. Behnke et al., unpublished observation.

0006-291X/94 $5.00
Copyright © 1994 by Academic Press, Inc.
All rights of reproduction in any form reserved.

Materials and Methods

Materials: Sodium [^{125}I], [^{14}C]acetate, [^{14}C]mevalonate, [^{3}H]cholesterol, and [^{3}H]palmitic acid were purchased from Amersham International (Amersham, U. K.). RPMI 1640 culture medium and penicillin/streptomycin mixture were obtained from Gibco (Glasgow, U. K.). Ficoll-Hypague (Lymphopreb) was purchased from Nyegaard & Co (Oslo, Norway). Conical (50 ml) plastic tubes were obtained from Falcon (Oxnard, USA) and 22-mm 12-well tissue culture clusters were from Costar (Cambridge, MA). Instant scintillation gel was purchased from United Technologies Packard (Downers Grove, IL), and prechanneled silic gel TLC plates were from Whatman, Chemica Separation (Clifton, NJ). Melatonin and its derivatives were purchased from Sigma, except 6-choromelatonin (Lilly, LY 99705).

Isolation and preparations of LDL and LPDS: Human LDL (1.019 - 1.063 g/ml) and human lipoprotein-deficient serum (LPDS, d > 1.215 g/ml) were isolated from plasma of normolipidemic healthy subjects by sequential ultracentrifugation (14). ^{125}I-labeled LDL was prepared by the iodine monochloride method of McFarlane (15) as modified by Bilheimer, Eisenberg, and Levy (16). After iodination, free iodine was removed by passage through a QAE-Sephadex A-50 anion exchange column and the LDL was sterilized by 0,45 μm Millipore filtration. Specific activity of the preparation was usually in the range between 80 and 150 cpm/ng. The concentration of LDL is expressed in terms of its protein content, which was determined by the method of Lowry et al. (17).

Isolation and incubation of mononuclear leukocytes: Peripheral blood mononuclear leukocytes were isolated from heparinized blood obtained from healthy subjects, after an overnight fast, by the method of Böyum (18), as described previously (7). The incubation of cells for the determination of LDL receptor activity and cholesterol synthesis was performed in RPMI 1640 medium without and with 4 % (v/v) LPDS, respectively.

Measurement of LDL receptor activity: At zero time and after incubation periods as indicated in results and the figure legends, 50 μl of 20 % BSA in RPMI 1640 and [^{125}I]labeled LDL were added to the cells with and without a 25-fold excess of unlabeled LDL. Cells were incubated for an additional 6 h period at 37°C before the incubation was stopped in ice. Mononuclear cells were overlayered on a solution of 80 mg/ml BSA and sedimented by centrifugation. From aliquots of the supernatant, the content of [^{125}I]labeled trichloroacetic acidsoluble material was determined (degradation) (3). The cell pellet was washed three times to determine the total cellular content of [^{125}I]labeled LDL (cellular accumulation) (3). Specific accumulation and degradation were defined as the respective differences of values with and without excess of unlabeled LDL and were expressed as nanograms of [^{125}I]labeled LDL protein that was associated with $2*10^6$ cells or degraded to acidsoluble non-iodine material per $2*10^6$ cells.

Determination of the incorporation rate of [14C]acetate and [14C]mevalonate into sterols and fatty acids: At zero time and after incubation periods as indicated in results and the figure legends, 12.5 μl of [2-^{14}C] acetate (2,5 μCi/mmol) was added to each dish. After 3 additional hours at 37°C, the incubation was stopped by transferring the cells to 8.5 ml chloroform-methanol 1:2 (v/v). [^{3}H]cholesterol and [^{3}H]palmitic acid were added as internal standards and lipids were extracted by the method of Bligh and Dyer (19) and saponified with methanolic potassium hydroxide (2M) for 1 h at 70°C. The potassium salts of the lipids were acidified with 1 ml of 37 % HCl and the fatty acids were extracted three times with 2 ml n-heptane (20). The recovery of [3-H]palmitic acid was used to correct for procedural losses of [14-C]acetate incorporated into saponifiable lipids. The nonsaponifiable fraction was extracted three times with 2,5 ml heptane and the extracts were combined. An aliquot, 1.5 ml, was counted, after adding 10 ml of Insta-Gel scintillation fluid, to determine the incorporation of [^{14}C]acetate or [^{14}C]mevalonate into sterols. The rest of the extract was evaporated at 70°C and dissolved in dichlormethane. [^{14}C]Squalene, [^{14}C]lanosterol, and [^{14}C]cholesterol were separated by thin-layer chromatography (4). The recovery of [^{3}H]cholesterol was used to correct for procedural losses of synthesized [^{14}C]cholesterol.

Results and Discussion

Freshly isolated human mononuclear leukocytes were used in this study since they reflect the in vivo milieu (21), catabolize LDL after binding to high affinity receptors (3), and are able to synthesize cholesterol (4, 22). Work from our laboratory has shown that LDL receptor activity and sterol synthesis in human mononuclear leukocytes is under hormonal control. Insulin stimulates (23, 24) and catecholamines (7, 24) as well as prostaglandins (22) suppress both pathways.

Freshly isolated human mononuclear leukocytes exhibit a low number of high affinity binding sites for LDL (3) and therefore have a relatively low ability to degrade [^{125}I]labeled LDL. Incubation of cells in a lipid-depleted serum leads to 3 - 4 fold increased ability of these cells to bind (3) and accordingly to accumulate and degrade [^{125}I]labeled LDL was after an

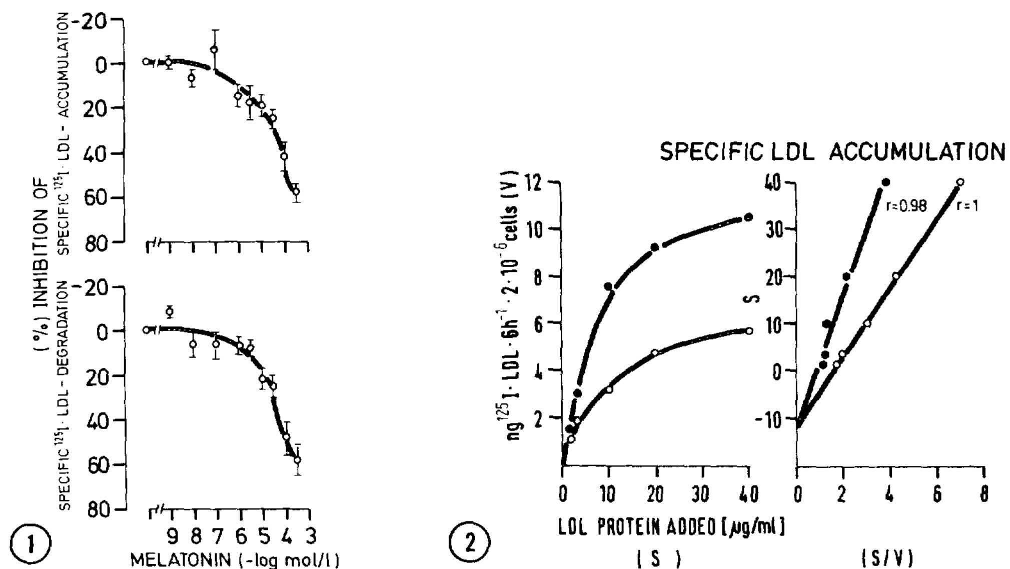

Fig. 1. Log concentration-effect curves of melatonin on the rate of accumulation and degradation of [^{125}I]labeled LDL with high affinity on freshly isolated human mononuclear leukocytes. Melatonin was added at various concentrations at the beginning of the incubations. Cells were incubated at 37°C in RPMI 1640 with or without hormone for 20 h. [^{125}I]labeled LDL (10 µg protein/ml) was then added and accumulation and degradation assays were performed as described in Methods. Values without hormone at 20 h (18 +/- 3ng/2*10^6 cells per 6 h for specific accumulation, and 78 (+/- 6 ng)/2*10^6 cells per 6 h for specific degradation) were defined as 0 % inhibition. Each point represents mean +/- SD of four experiments performed in triplicate.

Fig. 2. Effects of melatonin on the degradation of [^{125}I]labeled LDL with high affinity on freshly isolated human mononuclear leukocytes as a function of the [^{125}I]labeled LDL concentration. Cells were incubated at 37°C in RPMI 1640 with or without melatonin for 20 h. [^{125}I]labeled LDL was then added at the concetrations indicated for 6 h at 37°C. Assays were performed as described in Methods. Values represents means of three experiments that were performed in triplicate; (●) without hormone, (O) melatonin. The curves on the right represent reciprocal plots of the data of the curves on the left. The slope of the lines equals apparent V_{max} for degradation. The points of the intersection with the y axis equal appparent -K_m.

incubation of 20 h (data not shown). Melatonin added in increasing concentrations to the incubation medium at zero time inhibited the specific accumulation and degradation of [^{125}I]LDL. The hormone yielded sigmoidal log concentration-effect curves which are shown in Fig. 1. Melatonin at a concentration of 100 μM reduced specific LDL accumulation by 42 % and degradation by 48 %. To determine whether the suppression of LDL receptor activity by melatonin is caused by a decreased number of LDL receptors or by a decreased affinity, LDL concentration curves were performed in the presence or absence ot 100 μM melatonin (Fig. 2). Increasing concentrations of [^{125}I]labeled LDL in the incubation medium led to a saturation of its degradation. In the presence of melatonin, the degradation rate was lower at all concentrations used. Double reciprocal plots (25) of the data indicate that the main effect of melatonin was to produce a decrease in maximum velocity (V_{max}) of LDL degradation and no change was observed in the Michaelis constant (K_m) of degradation, reflecting a decreased number of cell surface receptors.

Sterol synthesis in freshly isolated human mononuclear leukocytes was determined by the incorporation rate of [^{14}C]acetate or [^{14}C]mevalonate into nonsaponifiable lipids. Fractionation of the nonsaponifiable lipids by thin-layer chromatography showed three radiolabeled products corresponding in mobility to control samples of cholesterol, lanosterol, and squalene (4, 22). Incubation of freshly isolated human mononuclear leukocytes in serumfree culture medium resulted in a 4-fold (240 vs. 959 dpm/h/10^6cells) increase of [^{14}C]acetate incorporation rate into sterols and in a 2.7-fold (436 vs. 1171 dpm/h/10^6 cells)

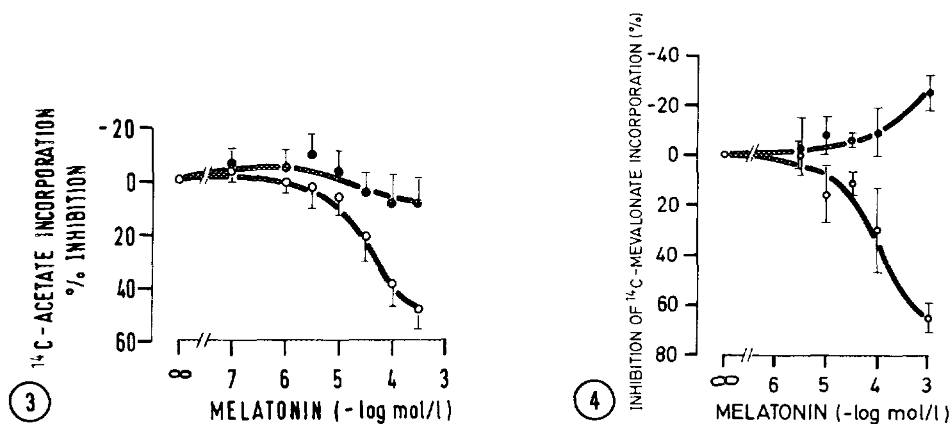

Fig. 3. Effects of melatonin on the synthesis of sterols (0) and fatty acids (●) from [^{14}C]acetate in freshly isolated human mononuclear leukocytes. Melatonin was added in increasing concentrations at zero time. Cells were incubated for 20 h, labeled with [^{14}C]acetate, harvested and the lipids extracted as described in Methods. The control was defined as difference between sterol or fatty acid synthesis at 0 h and 20 h without hormone (0 % inhibition). Values are means +/- SD of three separate experiments performed in triplicate.

Fig. 4. Effects of melatonin on the synthesis of lanosterol (●) and cholesterol (0) from [^{14}C]mevalonate in freshly isolated human mononuclear leukocytes. Melatonin was added at zero time and cells were incubated as outlined in Methods. Values are means +/- SD of three separate determinations, performed in duplicate.

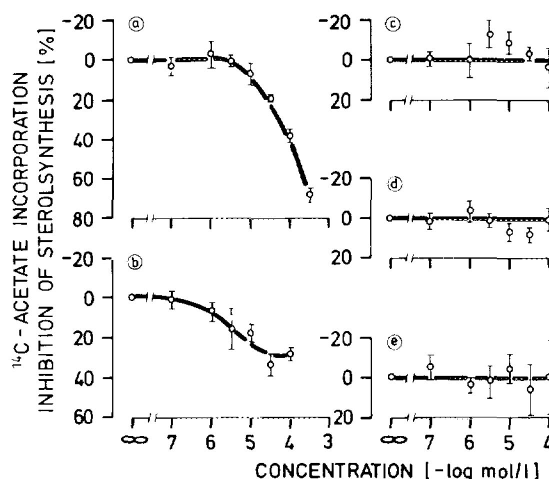

Fig. 5. Effects of melatonin analogues on sterol synthesis in human mononuclear leukocytes: a) 6-chlormelatonin, b) 5-methoxytryptamine, c) tryptamine, d) serotonin, e) N-acetyl-serotonin. For further details see legend to figure 3. Values are means +/- SD of three to five experiments performed in duplicate.

induction of fatty acid synthesis after 20 h. Increasing concentrations of melatonin added to the incubation medium at zero time inhibited the induction of sterol synthesis from [^{14}C]acetate, yielding a sigmoidal log concentration-effect curve (Fig. 3), the inhibition being 38 % at a concentration of 100 µM. The rate of fatty acid synthesis was not altered under these experimental conditions (Fig. 3). Identical experiments using [^{14}C]mevalonate as precursor substrate yielded similar results (Fig. 4). Melatonin inhibited cholesterol synthesis up to 30 % at a concentration of 100 µM. In contrast to cholesterol synthesis of the first cyclic compound lanosterol was not affected (Fig. 4), indicating that melatonin affects cholesterol synthesis between lanosterol and cholesterol. Pharmacological characterization of the melatonin receptor mediating the effects on sterol synthesis revealed that tryptamine, 5-hydroxytryptamine (serotonin), and N-acetyl-5-hydroxytryptamine had almost no influence on this pathway, whereas 5-methoxytryptamine and 6-chloromelatonin mimicked the hormone action (Fig. 5). At a concentration of 100 µM 6-chloromelatonin suppressed sterol synthesis by 38 % and 5-methoxytryptamine by 28 %. All hormone analogues had no effect on fatty acid synthesis (data not shown). Since only melatonin, 5-methoxytryptamine, and 6-chloromelatonin suppressed sterol synthesis it can be concluded that the 5-methoxy group is responsible for mediating the hormone effects. The pharmalogical profile corresponds to binding characteristics proposed to be typical for a melatonin receptor subtype ML-1 (26, 27). Since high concentrations of the hormone were used in our experiments, we cannot draw conclusions about the possible physiological relevance especially in relation to the circadian rhythm of melatonin secretion (28) and cholesterol synthesis (29, 30). However, melatonin can be secreted by monocytes directly (31), possibly acting para- and/or autocrine at much higher concentrations than anticipated from mere plasma levels. Furthermore, treatment of rats with

high doses of melatonin suppressed plasma levels of cholesterol in hypercholesterolemic animals (12, 13) and our study might provide some insights into the possible mechanisms. The results provide direct evidence that melatonin can potentially affect cellular metabolism in human cells.

Acknowledgment

This work was supported by Deutsche Forschungsgemeinschaft (SFB 232).

References

1. Leren, T.P., Hjermann, I., Maartmann-Moe, K., Beckmann, S.L., Leren, P., and Berg, K. (1985) Acta Med. Scand. 218: 41 - 49
2. Mistry, P., Miller, N.E., Laker, M., Hazzard, W.R., and Lewis, B. (1981) J. Clin. Invest. 67: 493 - 502
3. Ho, Y.K., Brown, M.S., Bilheimer, D.W., and Goldstein, J.L. (1976) J. Clin. Invest. 58: 1465 - 1474
4. Ho, Y.K., Faust, J.R., Bilheimer, D.W., Brown, M.S., and Goldstein, J.L. (1977) J. Exp. Med. 145: 1531 - 1549
5. Chait, A., Bierman, E.L., and Albers, J.J. (1979). J. Clin. Invest. 64: 1309 - 1319
6. Edwards, P.A., Lemongello, D., and Fogelman, A.L. (1979) J. Lipid Res. 20: 2-7
7. Krone, W., Hildebrandt, F., and Greten, H. (1982) Eur. J. Clin. Invest. 12: 467 - 470
8. Betteridge, D.J., Krone, W., Reckless, J.P.D., Galton, D.J. (1978) Lancet II: 1342 - 1343
9. McNamara, D.J., Davidson, N.O., and Fernandez, S. (1980) J. Lipid Res. 21: 65 - 71
10. Sundberg, E.E., and Illingworth, D.R. (1983). Proc. Natl. Acad Sci. USA 80: 7631 - 7635
11. Krone, W., Müller-Wieland, D., and Greten, H. (1985) J. Cardiovasc. Pharmacol. 7: 1134 - 1137
12. Mori, N., Aoyama, H., Murase, T., Mori, W. (1989) Acta Pathol. Jpn. 39: 613 - 618
13. Aoyama, H., Mori, N., and Mori, W. (1988) Atherosclerosis 69: 269 - 272
14. Havel, R.J., Eder, H.A., and Bragdon, J.H. (1955)J. Clin. Invest. 63: 1345 - 1353
15. McFarlane, A.S. (1958) Nature (London) 182: 53 - 57
16. Bilheimer, D.W., Eisenberg, S., and Levy, R.I. (1972) Biochim. Biophys. Acta 260: 212 - 221
17. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193: 265 - 275
18. Böyum, A. (1968) Scand. J. Clin. Invest. 97 (Suppl 121): 77 - 89
19. Bligh, E.G., and Dyer, W.L. (1959) Can. J. Biochem. Physiol. 37: 911 - 917
20. Shapiro, D.J., and Rodwell, V.W. (1972) Biochemistry 11: 1042 - 1045
21. Berger, S.L. (1979) Methods Enzymol. 58: 486 - 492
22. Krone, W., Klass, A., Nägele, H., Behnke, B., and Greten, H. (1988) J. Lipid Res. 29: 1663 - 1669
23. Krone, W., and Greten, H. (1984) Diabetologia 26: 366 - 369
24. Krone, W., Nägele, H., Behnke, B., and Greten, H. (1988) Diabetes 37: 1386 - 1391
25. Riggs, P.S. (1963) MIT Press, Cambridge, MA: 276
26. Dubocovich, M.L. (1988) FASEB J. 2: 2765 - 2773
27. Kennaway, D.J., and Hugel, H.M. (1992) Mol. Cell. Endocrinol. 88: C1 - C9
28. Utiger, R.D. (1992) N. Engl. J. Med. 327: 1377 - 1379
29. Back, P., Hamprecht, B., and Lynen, F. (1969) Arch. Biochem. Biophys. 133: 11 - 21
30. Edwards, D.J., and Rodwell, V.W. (1972) Biochemistry 11: 1042 - 1045
31. Finocchiaro, L.M.E., Arzt, E.S., Fernandez-Casklo, S., Crisudo, M., Finkielman, S., Nahmod, V.E. (1988) Interferon Res. 8: 705 - 716