Anti-Cancer Drugs, 3, pp. 623–628

A possible modulatory influence of melatonin on representative phase I and II drug metabolizing enzymes in 9,10-dimethyl-1,2-benzanthracene induced rat mammary tumorigenesis

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The oncosuppressive effect of melatonin on 9,10-dimethyl-1,2-benzanthracene (DMBA) induced rat mammary tumorigenesis led us to assess its possible modulatory influence on representative hepatic and mammary drug metabolizing enzymes in DMBA treated female Holtzman rats, reared in short and long photoperiods. Melatonin treated rats in either photoperiod showed a significant induction in hepatic and mammary levels of glutathione (GSH) and cytosolic activities of glutathione S-transferase (GST) when compared with the corresponding controls, along with a significant drop in hepatic microsomal contents of cytochromes b_5 and P450. This induction of GSH and GST, and depletion of cytochromes b_5 and P450 by melatonin may possibly be related to its anticarcinogenic potential in this tumor model.

Key words: Cytochrome b_5, cytochrome P450, glutathione, glutathione S-transferase, mammary carcinogenesis, melatonin.

Introduction

Melatonin, the most widely investigated pineal indoleamine, is known to inhibit 9,10-dimethyl-1,2-benzanthracene (DMBA) induced rat mammary tumorigenesis.[1–5] In a recent study of ours,[6] wherein we comprehensively analyzed the influence of melatonin on the two major components of this carcinogenic process, i.e. the initiation and promotion phases, melatonin was seen to act both as an anti-initiating and anti-promoting agent in animals with intact pineals; however, in pineal-ablated animals, only an anti-promoting effect of melatonin was observed.

DMBA, a polycyclic aromatic hydrocarbon, is an indirect carcinogen that requires host metabolism to yield its ultimate carcinogenic form.[7] The initiation of the carcinogenic response is complete within 24 h following the intragastric administration of DMBA,[8] since by this time the binding of the carcinogen to mammary parenchymal cell DNA and protein is complete, and unreacted DMBA is no longer detectable in the gland.[9] This raises the possibility that one of the mechanisms by which melatonin may have been exerting its oncosuppressive effect on the initiation phase of DMBA induced mammary carcinogenesis in intact animals could be by modifying the metabolism of DMBA. Hence, in this preliminary study, we have examined the modulatory influence of short-term treatment of melatonin, administered to female Holtzman rats reared in varying photoperiods, on enzymes responsible for the biotransformation of DMBA, i.e. on representative phase I and II drug metabolizing enzymes, both in the mammary gland, which is the primary target organ for DMBA,[10,11] and in the liver, which though a non-target organ for DMBA carcinogenesis is one that is important for the metabolism of this carcinogen.[7,12]

Materials and methods

Animals

Randomly bred, female Holtzman rats, obtained from the Animal House of the Cancer Research Institute, Tata Memorial Centre, Bombay, India, were used for this study. The animals were reared in short [light:dark schedule (L:D) 10:14] and long (L:D 24:0) photoperiods, and were housed (four or five animals per plastic cage) in air-conditioned rooms with controlled temperature (22 ± 2°C) and

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humidity (60%). They had free access to food (standard diet) and drinking water. The cages were rotated from time to time to ensure uniform light exposure.

Melatonin preparation and administration

Based on a pilot experiment designed to assess daily water consumption of Holtzman females, 5 mg of melatonin (Sigma, St Louis, MO) was dissolved in ethanol:drinking water (1:500 ml) and the solution orally administered to the experimental animals in dark bottles, round the clock, achieving a dosing concentration of 200 μg melatonin/rat/day. This dose was selected because it was seen to exert a suppressive effect on the incidence of mammary tumors in our earlier study.[6] The stock melatonin solution was stored in amber-colored bottles in the dark at 4°C and was freshly prepared every alternate day. The control groups of animals were administered only the vehicle, i.e. ethanol:drinking water (1:500 ml).

Carcinogen preparation and administration

Crystalline DMBA (Sigma) was dissolved in sesame oil at a concentration of 10 mg/ml by constant stirring in the dark. A single intragastric (i.g.) instillation of 10 mg DMBA (i.e. 10 mg DMBA/ml sesame oil/rat) was then administered to animals on day 55 of age.

Therefore, the major experimental animal groups were (i) L:D 10:14 Intact and (ii) L:D 24:0 Intact with each group having the following subgroups:

(i) Melatonin (200 μg/rat/day, orally) from day 48 to 58 of age.
(ii) Melatonin vehicle (ethanol:drinking water, 1:500 ml) from day 48 to 58 of age.
(iii) DMBA (10 mg/ml sesame oil/rat, i.g.) on day 55 of age.
(iv) DMBA plus melatonin.

Necropsy and collection of tissue samples

Animals were sacrificed on day 58 of age by cervical dislocation. Liver and inguinal mammary glands were immediately removed, rinsed with ice-cold isotonic (1.15%) potassium chloride (KCl), blotted dry, weighed, and homogenized in ice-cold 1.15% KCl to obtain a 20% suspension.

A part of this tissue homogenate was kept aside and the rest was centrifuged at 9500 *g* for 15 min to remove nuclei, debris and mitochondria. The supernatant was then spun at 105 000 *g* for 60 min. The resulting soluble supernatant (cytosolic fraction) was collected and the microsomal pellet was suspended in 1.15% KCl to give a protein concentration of 10 mg/ml. All the above procedures were carried out at 0–4°C.

Biochemical determinations

The reduced glutathione (GSH) content was assayed in liver and mammary tissue homogenates using 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), according to the method of Moron *et al.*[13] The assay was performed within 1 h of sacrificing the animals so as to avoid any errors due to oxidation of GSH. Glutathione S-transferase (GST) activity was measured kinetically in the hepatic and mammary post-105 000 *g* cytosolic fraction by the method of Habig *et al.*[14] using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. Cytochromes b_5 and P450 were determined in liver microsomal pellets by the method of Omura and Sato[15] using millimolar extinction coefficients of 171 and 91 mM^{-1}, cm^{-1}, respectively. Protein concentrations were determined according to the method of Lowry *et al.*[16] using bovine serum albumin as standard.

It is important to mention here, that in spite of repeated attempts, we failed to obtain sufficient amounts of microsomal pellet from mammary gland tissue, due to which the estimation of mammary cytochromes b_5 and P450 was discontinued.

Statistical analyses

Statistical comparisons between any two groups were made using Student's *t*-test. A level of significance of $p < 0.05$ (two-tailed) was chosen. All values are expressed as mean ± SEM.

Results

Tables 1–4 show the effect of oral melatonin, administered in the initiation phase of DMBA induced mammary tumorigenesis to intact animals reared in varying photoperiods, on key phase I and II xenobiotic metabolizing enzymes.

Table 1. Effect of melatonin on hepatic phase I and II drug metabolizing enzymes in L:D 10:14 intact rats

Treatment	GSH*	GST**	Cytochrome b_5***	Cytochrome P450***
Melatonin	6131.80 ± 202.89	204.17 ± 23.53	0.17 ± 0.04	0.29 ± 0.05
Melatonin vehicle	4922.03 ± 297.88[a3]	122.31 ± 4.91[a1]	0.40 ± 0.13[a1]	0.58 ± 0.05[a3]
DMBA	5441.88 ± 177.53[a1]	175.36 ± 14.70[b1]	0.45 ± 0.18[a1]	0.80 ± 0.15[a3]
DMBA + melatonin	6108.37 ± 174.96[b3,c1]	239.40 ± 27.62[b1]	0.34 ± 0.05[a1]	0.35 ± 0.05[b1,c2]

Results are mean ± SEM of at least six animals.
Units: *nmol/g tissue; **nmol CDNB conjugated/min/mg protein; ***nmol/mg protein.
[a] Statistically significant as compared with metatonin.
[b] Statistically significant as compared with metatonin vehicle.
[c] Statistically significant as compared with DMBA.
[1] $p < 0.05$; [2] $p < 0.01$; [3] $p < 0.005$.

Table 2. Effect of melatonin on hepatic phase I and II drug metabolizing enzymes in L:D 24:0 intact rats

Treatment	GSH*	GST**	Cytochrome b_5***	Cytochrome P450***
Melatonin	5897.20 ± 513.43	214.49 ± 15.39	0.07 ± 0.02	0.27 ± 0.06
Melatonin vehicle	4458.26 ± 81.80[a1]	145.58 ± 12.32[a2]	0.32 ± 0.06[a3]	0.72 ± 0.04[a3]
DMBA	5364.00 ± 434.50	182.62 ± 13.09	0.41 ± 0.03[a3]	0.81 ± 0.05[a3]
DMBA + melatonin	5441.41 ± 328.71[b2]	269.60 ± 13.99[b3,c2]	0.20 ± 0.07[c1]	0.36 ± 0.11[b2,c2]

Results are mean ± SEM of at least six animals.
Units: *nmol/g tissue; **nmol CDNB conjugated/min/mg protein; ***nmol/mg protein.
[a] Statistically significant as compared with metatonin.
[b] Statistically significant as compared with metatonin vehicle.
[c] Statistically significant as compared with DMBA.
[1] $p < 0.02$; [2] $p < 0.01$; [3] $p < 0.001$.

As can be seen from Tables 1 and 2, melatonin treatment to intact animals reared in short and long photoperiods brought about a significant elevation in hepatic phase II enzymes, i.e. in the cytosolic activity of GST (L:D 10:14, 67% increase; L:D 24:0, 47% increase) and in the levels of GSH (L:D 10:14, 25% increase; L:D 24:0, 32% increase), when compared with the corresponding vehicle treated controls. Further, the treated animals showed a significant drop in hepatic phase I enzymes, i.e. in the liver microsomal contents of cytochrome b_5 (L:D 10:14, 58% reduction; L:D 24:0, 81% reduction) and cytochrome P450 (L:D 10:14, 50% reduction; L:D 24:0, 63% reduction), when compared with vehicle controls. When animals administered only DMBA were compared with those administered both DMBA and melatonin, the latter group showed a significant increase in the hepatic levels of GSH in L:D 10:14 animals (12% increase) (Table 1) and in the hepatic cytosolic activity of GST in L:D 24:0 animals (48% increase) (Table 2); also, there was a significant reduction in the hepatic microsomal content of cytochrome b_5 in L:D 24:0 animals (51% reduction) (Table 2) and in the hepatic cytochrome P450 content in animals reared in both photoperiods (L:D 10:14, 56% reduction; L:D 24:0, 59% reduction) (Tables 1 and 2).

Tables 3 and 4 represent the effect of melatonin on GSH levels and cytosolic GST activity in the mammary glands of intact groups of animals reared in short and long photoperiods. As is evident, melatonin treatment brought about a significant elevation in both parameters as compared with the respective vehicle controls, i.e. in terms of GSH

Table 3. Effect of melatonin on mammary phase II drug metabolizing enzymes in L:D 10:14 intact rats

Treatment	GSH*	GST**
Melatonin	254.60 ± 14.74	14.50 ± 1.16
Melatonin vehicle	150.75 ± 11.71[a3]	7.34 ± 1.49[a2]
DMBA	161.10 ± 6.04[a3]	9.53 ± 1.01[a1]
DMBA + melatonin	186.72 ± 9.10[a2,b1,c1]	18.39 ± 1.65[b2,c2]

Results are mean ± SEM of at least six animals.
Units: *nmol/g tissue; **nmol CDNB conjugated/min/mg protein.
[a] Statistically significant as compared with metatonin.
[b] Statistically significant as compared with metatonin vehicle.
[c] Statistically significant as compared with DMBA.
[1] $p < 0.05$; [2] $p < 0.01$; [3] $p < 0.001$.

Table 4. Effect of melatonin on mammary phase II drug metabolizing enzymes in L:D 24:0 intact rats

Treatment	GSH*	GST**
Melatonin	169.28 ± 6.62	20.11 ± 0.65
Melatonin vehicle	109.95 ± 12.56[a2]	11.74 ± 0.46[a3]
DMBA	190.98 ± 35.56[b1]	22.75 ± 3.53[b3]
DMBA + melatonin	184.94 ± 3.68[b3]	17.36 ± 0.53[a1,b3]

Results are mean ± SEM of at least six animals.
Units: *nmol/g tissue; **nmol CDNB conjugated/min/mg protein.
[a] Statistically significant as compared with metatonin.
[b] Statistically significant as compared with metatonin vehicle.
[1] $p < 0.05$; [2] $p < 0.01$; [3] $p < 0.001$.

levels (L:D 10:14, 69% increase; L:D 24:0, 54% increase) and GST activity (L:D 10:14, 98% increase; L:D 24:0, 71% increase). On comparison of the DMBA treated group with the one administered both DMBA and melatonin, it was seen that in the case of L:D 10:14 animals, the latter group showed a significant increase in mammary GSH levels (16% increase) and in GST activity (93% increase) (Table 3).

Discussion

The decreased susceptibility of the mammary epithelium to the potent indirect carcinogen DMBA, following melatonin treatment to intact animals in the initiation phase,[6] could be due to a modulation in the metabolism of DMBA to its putative reactive intermediates.[17]

Different classes of anticarcinogens act by different mechanisms.[18,19] One of the mechanisms involves an alteration in the metabolic fate of the carcinogen, i.e. a modulation in the activities of either and/or both phase I (or activating) and phase II (or detoxifying) xenobiotic metabolizing enzymes. The mutagenic and carcinogenic activities of DMBA are dependent upon its bioactivation by the phase I enzymes, especially the cytochrome P450-dependent mono-oxygenase enzyme system, to its ultimate reactive metabolite, i.e. 3,4-dihydrodiol-1,2-epoxide. Simultaneous detoxification of DMBA occurs in the presence of phase II enzymes, one of the reactions being GSH conjugation which is catalyzed by the phase II enzyme, GST. Another important aspect to be considered in the present study is the metabolism of melatonin. The primary site of melatonin degradation is the liver, where under the influence of cytochrome P450 dependent mono-oxygenases[20,21] melatonin is rapidly oxidized at the 6-position to generate 6-hydroxymelatonin. This is subsequently conjugated with sulfate (70–80%) or glucuronide (5%) by liver microsomes[22–25] and excreted in the urine, 6-sulfatoxymelatonin being the major urinary metabolite.[26] These data lend further credence to the assumption that melatonin may have a modulatory effect on the metabolism of DMBA, especially via the cytochrome P450-dependent mono-oxygenase enzyme system. In fact, in recent studies by Bartsch *et al.*[27,28] it was observed that, during the phase of mammary tumor induction by DMBA, there was a significant depression of nocturnal plasma melatonin concentration, which was attributed not to a reduction in pineal melatonin biosynthetic activity but to enhanced peripheral metabolism of melatonin by the liver.

From the results of our present study, it can be seen that treatment of intact rats with melatonin in the initiation phase brought about a significant elevation in the hepatic and mammary levels of GSH and cytosolic activity of GST in intact rats reared in short and long photoperiods, as compared with their corresponding vehicle controls. Simultaneously, there was a significant depletion in the hepatic microsomal levels of cytochromes b_5 and P450 in intact rats reared in both photoperiods, as compared with the respective controls.

These results suggest a modulating effect of short-term melatonin treatment on enzymes responsible for the metabolism of DMBA, both in the liver and mammary gland, i.e. on hepatic levels of the phase I enzymes, cytochromes b_5 and P450, and on hepatic and mammary levels of the phase II enzymes, GSH and GST. The decrease in cytochromes b_5 and P450, and the increase in GSH and GST brought about by melatonin may lead to decreased activation and increased detoxification of DMBA, thus resulting in decreased formation of reactive intermediates and a consequent reduction in the initiation of carcinogenesis, as seen in our previous study.[6] That the oncosuppressive effect of melatonin was observed only in intact animals and not in pineal-ablated animals in our earlier study[6] suggests the absolute requirement of the intact pineal for melatonin to exert its effect on the initiation phase of DMBA induced mammary cancer. This could perhaps be due to the fact that the pineal gland secretes compounds other than melatonin which are possibly oncostatic,[29] the absence of which prevents the action of short-term exogenous melatonin treatment from being manifested. This, however, does not seem to hold true in the case of chronic and prolonged melatonin

administration, probably because of its ability to counteract the need for these endogenous pineal secretory compounds, as a result of which its effect is pronounced even in pinealectomized animals.

In a very recent study, Blask *et al.*[30] have attributed an anti-promoting but not an anti-initiating action to melatonin in both DMBA and *N*-nitroso-*N*-methylurea induced rat mammary tumor models. Though we agree with them as to the anti-promoting effect of melatonin in the DMBA model,[6] we seem to contradict the initiation phase data of their DMBA study (Blask *et al.*, unpublished data). It is pertinent to mention that the strain of animals used, the dose and the route of melatonin administration, and, most importantly, the dose of carcinogen used, play a major role in carcinogenesis experiments.[31]

Conclusions

In conclusion, the results of this investigation provide evidence for the first time that melatonin has a modulatory influence on enzymes responsible for the metabolism of DMBA, suggesting that this could be one of the mechanisms by which it exerts its anti-initiating effect in this tumor model.[6] These preliminary findings need to be further substantiated by examining the direct effect of melatonin treatment on the metabolites of DMBA.

Acknowledgements

The technical assistance rendered by Mr GK Kapse and the excellent typing of the manuscript by Mrs Premlata M Kotenkar are gratefully acknowledged.

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(Received 11 August 1992; accepted 14 September 1992)