Melatonin Modulates The Oxidant–Antioxidant Imbalance During N-Nitrosodiethylamine Induced Hepatocarcinogenesis In Rats

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Received March 11 2005, Revised June 6 2005, Accepted June 7 2005, Published August 12 2005

ABSTRACT. **PURPOSE:** Melatonin, the principle hormone of pineal gland plays an important role in several biological processes. The effects of melatonin on hepatic marker enzymes [aspartate and alanine transaminases (AST and ALT)], lipid peroxides [thiobarbituric acid reactive substances (TBARS)] and antioxidants [reduced glutathione (GSH), glutathione peroxidase (GPx) and glutathione-S-transferase (GST)] during N-nitrosodiethylamine (NDEA) – induced hepatocarcinogenesis in rats were studied. **METHODS:** Male albino Wistar rats of body weight 150-170 g were divided into four groups of six animals each. Group I animals served as control, Group II animals received single intraperitoneal injection of NDEA at a dose of 200 mg/kg body weight followed by weekly subcutaneous injections of CCl_4 at a dose of 3 mL/kg body weight. Group III animals were treated as in Group II and melatonin (5 mg/kg body weight) was administered intraperitoneally. Group IV animals received melatonin alone at the same dose as Group III animals. **RESULTS:** A significant increase in the activities of serum AST and ALT was observed in NDEA treated rats when compared with control animals. Melatonin administered rats showed a significant decrease in the activities of these enzymes when compared with NDEA treated animals. In the liver of NDEA-treated animals, decreased lipid peroxidation associated with enhanced antioxidant levels was observed. Administration of melatonin positively modulated these changes. **CONCLUSION:** Our results indicate that melatonin exerts chemopreventive effect by restoring the activities of hepatic marker enzymes and reversing the oxidant-antioxidant imbalance during NDEA-induced hepatocarcinogenesis.

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INTRODUCTION

Hepatocellular carcinoma is the fifth most common cancer in the world (1, 2). The major risk factors of this disease include hepatitis B and C viral infections, exposure to nitrosocompounds and aflatoxin B_1 and alcoholic liver disease (3, 4). A number of endogenous and exogenous cancer risk factors generate oxygen free radicals *in vivo* (5, 6) and initiate carcinogenic process (7). N-Nitrosodiethylamine (NDEA) is one of the most important environmental hepatocarcinogens that has been reported to generate free radicals to exert its carcinogenic effects (8) and it has been widely used in the field of experimental hepatocarcinogenesis (9,10).

Several antioxidants with different efficacies protect against oxidative abuse caused by wide range of carcinogens including NDEA (11). Melatonin, N-acetyl-5-methoxytryptamine is a pineal gland hormone. It is a direct free radical scavenger, an indirect antioxidant and an immunomodulatory agent (12). It holds the unique position of being the only known chronobiotic regulator of neoplastic cell growth (13, 14). Both *in vitro* and on different animal models of tumorgenesis, melatonin has been found to inhibit neoplastic growth and to delay tumor progression (15, 16). The present study was designed to analyse the effect of melatonin on hepatic marker enzymes (aspartate and alanine transaminases), lipid peroxides (thiobarbituric acid reactive substances); glutathione and its dependent enzymes (glutathione peroxidase and glutathione-S-transferase) during NDEA- induced hepatocarcinogenesis in rats.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (150-170 g) obtained from Central Animal House, Rajah Muthiah Medical College, Annamalai University, Annamalainagar were used in the present study. Commercial pellet diet (Kamdhenu agencies, Bangalore, India) and water were made available to the animals *ad libitum*. Animals were maintained in a controlled environment (temperature 30 ± 3°C) with 12 h light/dark cycles in an experimental room simulating natural conditions. In Annamalainagar (11°24′N, 79°42′E), the light dark (LD) cycle is almost 12:12 h throughout the year (17). The animals were maintained in accordance with the guidelines of National Institute of Nutrition, Indian Council of Medical Research,

Hyderabad, India and approved by the ethical committee, Annamalai University.

Chemicals

NDEA was purchased from Sigma Chemical Co., USA. Melatonin was purchased from Sisco Research Laboratories, Mumbai. All other chemicals used in the study were of analytical grade.

Experimental induction of hepatocarcinogenesis

One gram of NDEA was dissolved in 1mL of saline and administered to each rat at a dose of 200 mg/kg body weight once intraperitoneally (18), followed by subcutaneous injections of carbon tetrachloride (CCl_4) [3 mL/kg body weight/week] for 6 weeks (10).

Experimental design

Animals were divided into four groups of six animals each. Group I rats served as controls, Group II rats received single intraperitoneal injection of NDEA (200 mg/kg body weight) followed by weekly subcutaneous injections of CCl_4 (10). Animals in Group III were treated as in Group II, in addition melatonin [5 mg/kg body weight] (19) was administered intraperitoneally throughout the experimental period of 20 weeks. Group IV animals received melatonin alone at the same dose as group III animals.

At the end of experimental period of 20 weeks, the animals were sacrificed by cervical dislocation; blood samples and liver tissue were used for analysis.

Preparation of tissue homogenate

10% tissue homogenate was prepared using an appropriate buffer for the estimation of lipid peroxides and antioxidants.

BIOCHEMICAL INVESTIGATIONS

Serum aspartate and alanine transaminases (AST and ALT)

The activities of these enzymes were estimated by the method of Reitman and Frankel (20). 0.2 mL of serum was added to 1 mL of phosphate buffer containing substrate, mixed and incubated for 60 minutes for AST and 30 minutes for ALT at 37°C. Then 1 mL of dinitrophenylhydrazine was added and incubated for 20 minutes at room temperature and 10 mL of 0.4% sodium hydroxide was added, mixed well and after five minutes, read at 550 nm. Blank and a series of standards were processed similarly.

Thiobarbituric acid reactive substances (TBARS)

TBARS were assayed by the method of Niehaus and Samuelsson (21). To 1 mL of tissue homogenate, 2 mL of TCA-TBA-HCl reagent was added, mixed thoroughly and kept in boiling water bath for 15 minutes. The precipitate was removed by centrifugation. A series of standards was processed similarly and the absorbance was read at 535 nm.

Reduced glutathione (GSH)

GSH was estimated by the method of Ellman (22). 0.5 mL of tissue homogenate was precipitated with 2 mL of 5% TCA. After centrifugation, 1 mL of supernatant was taken and added 0.5 mL of Ellman's reagent (19.8 mg of 5,5' dithio(*bis*)nitrobenzoic acid in 100 mL of 1% sodium citrate) and 3 mL of phosphate buffer. Standards were treated in a similar way and the colour developed was read at 412 nm.

Glutathione peroxidase (GPx)

GPx activity was measured by the method of Rotruck *et al.* (23). To 0.2 mL of buffer, 0.2 mL of EDTA, 0.1 mL of sodium azide and 0.5 mL of tissue homogenate were added. To that mixture, 0.2 mL of glutathione solution and 0.1 mL of hydrogen peroxide were added. The contents were mixed well and incubated at 37°C for 10 minutes along with the control tubes containing all the reagents but no enzyme. After 10 minutes, the reaction was arrested by the addition of 0.4 mL of 10% TCA. 0.2 mL of tissue homogenate was added to the control tubes. The tubes were centrifuged and supernatant was assayed for glutathione content by adding Ellman's reagent.

Glutathione-S-transferase (GST)

GST activity was measured by the method of Habig *et al.* (24). The reaction mixture containing 1 mL of buffer, 0.1 mL of 1-chloro-2, 4-dinitrobenzene (CDNB), 0.1 mL of homogenate and 1.7 mL of distilled water was incubated at 37°C for 5 minutes. The reaction was then started by the addition of 1 mL of glutathione. The increase in absorbance was followed for 3 minutes at 340 nm. The reaction mixture without the enzyme was used as blank.

Statistical analysis

Statistical analysis was carried out by analysis of variance (ANOVA) and the groups were compared using Duncan's Multiple Range Test (DMRT).

RESULTS

The activities of serum aspartate and alanine transaminases were found to be significantly higher in NDEA plus CCl_4 treated rats when compared with control animals and lower in the animals treated with NDEA plus CCl_4 as well as melatonin when compared with NDEA plus CCl_4 treated animals. The animals treated with melatonin alone showed no significant change in the activities of transaminases (Table 1).

The levels of thiobarbituric acid reactive substances in the liver of NDEA plus CCl_4 treated animals were significantly decreased when compared with control animals. NDEA plus CCl_4 as well as melatonin treated animals showed significantly increased levels of TBARS when compared with NDEA and CCl_4 treated animals.

Table 1. Changes in activities of serum aspartate and alanine transaminases (AST and ALT)

	Control	NDEA	NDEA + Melatonin	Melatonin
AST (IU/L)	92.15 ± 5.40[a]	240.32 ± 22.36[b]	130.33 ± 8.50[c]	86.16 ± 4.75[a]
ALT (IU/L)	38.66 ± 2.50[a]	125.39 ± 6.44[b]	54.12 ± 2.60[c]	36.19 ± 1.41[a]

ANOVA followed by Duncan's multiple range test.
Values not sharing a common superscript (a, b, c) differ significantly at $p \leq 0.05$

The animals treated with melatonin alone did not show any significant change in the levels of TBARS (Table 2).

Table 2. Changes in the levels of TBARS and GSH and activities of GPx and GST in liver of experimental animals

	Control	NDEA + CCl_4	NDEA + CCl_4 + Melatonin	Melatonin
TBARS (mmoles x 10^{-3}/mg protein)	0.903 ± 0.072[a]	0.148 ± 0.031[b]	0.517 ± 0.049[c]	0.911 ± 0.016[a]
GSH (mg/mg protein)	0.128± 0.07[a]	0.150 ± 0.09[b]	0.134 ± 0.09[c]	0.126 ± 0.08[a]
GPx (U^A/mg protein)	11.76 ± 0.71[a]	21.50 ± 1.78[b]	15.26 ± 0.93[c]	12.80 ± 0.71[a]
GST (U^B/mg protein)	690.53 ± 26.83[a]	1070.61 ± 44.72[b]	820.19 ± 35.77[c]	671.31 ±26.87[a]

U^A : μmoles of GSH utilized/min
U^B : μmoles of CDNB-GSH conjugate formed/min
ANOVA followed by Duncan's multiple range test
Values not sharing a common superscript (a, b, c) differ significantly at $p \leq 0.05$

The levels of GSH and the activities of glutathione peroxidase and glutathione-S-transferase in liver of NDEA plus CCl_4 treated animals were significantly higher when compared with control animals. Administration of melatonin restored the levels of GSH and the activities of GPx and GST to near normal levels. The animals treated with melatonin alone showed no significant change in the levels of GSH and in the activities of GSH related enzymes (Table 2).

DISCUSSION

NDEA undergoes metabolic activation by cytochrome P450 enzymes to reactive electrophiles that are cytotoxic, mutagenic and carcinogenic (25). Serum transaminases are sensitive indicators of hepatic injury (26). Several reports have shown an increase in the activities of AST and ALT during NDEA-induced hepatocarcinognesis (27, 28). Elevated activities of serum AST and ALT observed in NDEA and CCl_4 treated rats may be due to NDEA induced hepatic damage and subsequent leakage of these enzymes into circulation. It has been reported that NDEA is transported through blood and it causes hepatic injury (8). Administration of melatonin restored the activities of these enzymes to near normal values, which may be attributed to the hepatoprotective role of melatonin.

Although, several studies have demonstrated increased lipid peroxidation in liver during NDEA-induced hepatocarcinogenesis (27,29,30); in primary colon tumor (31) and in breast cancer tissue (32), we

observed decreased lipid peroxidation in the liver of NDEA plus CCl_4 treated rats, as evidenced by thiobarbituric acid reactive substances. Oxidant-antioxidant balance plays a significant role in the pathogenesis of cancer (32). Tumor cells generally display low levels of lipid peroxidation (33) which in turn can stimulate cell division and promote tumor growth (34, 35). It has been reported that mild oxidative stress stimulates while excessive oxidative stress inhibits cell growth (36, 37). Slater et al. proposed that rapidly dividing cells tend to set an oxidant-antioxidant status favourable to their growth (38). The decrease in lipid peroxidation observed in NDEA plus CCl_4 treated animals may be due to stimulation of cell division and tumor promotion. Lipid peroxidation has been found decreased in NDEA-induced hepatoma (39). In the present study, melatonin administration modulated the lipid peroxide levels to near normal levels that may be attributable to its inhibitory action on tumor cell proliferation. It has been well known that melatonin controls tumor cell proliferation (40, 41).

A significant increase in GSH content and activities of GSH related enzymes, glutathione peroxidase and glutathione-S-transferase was observed in NDEA plus CCl_4 treated rats, which may be due to over expression of these antioxidants during enhanced cell proliferation. Huang et al. have reported that GSH is increased in human hepatocellular carcinoma and this increased GSH level facilitates the growth of liver cancer cells (42). A number of studies have demonstrated an enhanced expression of the various GST isoforms in cancer tissue in comparison to normal tissue (43, 44). Ferruzzi *et al.* have reported that GSH concentration is higher in tumor than in normal tissues (45).

Administration of melatonin reverted the NDEA induced alterations in GSH and GSH related enzyme activities in liver. Sugie *et al.* reported that melatonin inhibited development of hepatocellular adenomas and carcinomas induced by diethylnitrosamine and phenobarbital in both initiation and promotion phases (46). In conclusion, melatonin would exert a chemopreventive effect by restoring the activities of hepatic marker enzymes and reversing the oxidant-antioxidant imbalance during hepatocarcinogenesis.

ACKNOWLEDGMENTS

The financial assistance from UGC, New Delhi in the form of a research project [F.3-68/2002(SR-II)] to Dr. P. Subramanian and senior research fellowship from ICMR, New Delhi to Ms. K.B. Dakshayani [3/2/2/49/2004/NCD-III] is gratefully acknowledged.

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