Melatonin Attenuates Estradiol-Induced Oxidative Damage to DNA: Relevance for Cancer Prevention

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Estrogens exert pro-oxidative effects and have been shown to damage DNA, potentially leading to cancer. Melatonin is a well-known antioxidant, free radical scavenger, and oncostatic agent. Changes in the levels of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo), an index of DNA damage, and the levels of malondialdehyde + 4-hydroxyalkenals, an index of lipid peroxidation, were measured in kidneys, liver, and testes from hamsters treated with E_2 (75 mg/kg body wt) and were collected 3 or 5 hr later. Other animals were treated with melatonin (15 mg/kg body wt, 30 min before and 120 min after E_2 treatment) or were given both compounds. Additionally, lipid peroxidation was measured in liver homogenates exposed to ferrous sulfate (15 μM) *in vitro*. E_2 treatment caused an increase in 8-oxodGuo levels in kidneys collected 5 hr after E_2 administration, and in liver 3 hr after estrogen treatment. Melatonin completely prevented E_2-induced DNA damage in both organs. Melatonin alone or when given with E_2 and examined 3 hr later decreased the base level of 8-oxodGuo in testes. A tendency for a reduction in *in vivo* lipid peroxidation was observed after treatment of hamsters with either melatonin, E_2, or both compounds, with a statistically significant decrease being measured in the liver following E_2 administration. *In vitro* exposure to iron significantly enhanced lipid peroxidation in hepatic homogenates from untreated, melatonin-treated, or E_2-injected hamsters; in the hepatic homogenates of hamsters given both E_2 and melatonin, ferrous sulfate failed to augment lipid peroxidation. Our results confirm the dual actions of estrogens relative to oxidative damage, i.e., estrogen increases oxidative destruction of DNA while reducing lipid peroxidation. Melatonin had antioxidative actions in reducing oxidative damage to both DNA and to membrane lipids. Melatonin completely prevented the damaging action of E_2 on DNA and synergized with the steroid to reduce lipid peroxidation. [Exp Biol Med Vol. 226(7):707–712, 2001]

Key words: DNA damage; lipid peroxidation; kidney; liver; melatonin; estrogen; hamster

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On the basis of biological studies in animals, some estrogens are known to be carcinogenic (1, 2). Estrogen administration to rodents results in the initiation of tumors in several organs (3). The induction of renal tumors in Syrian hamsters due to chronic exposure to estrogens is an extensively studied model of carcinogenesis, and estrogen-induced free radicals are thought to play a tumor-initiating role in this process (4).

Estrogens have dual actions in reference to their oxidative effects. In addition to their pro-oxidative effects on DNA, estrogens also possess antioxidative properties with respect to several *in vivo* and *in vitro* processes (5, 6). Estrogens, because of their beneficial effects, are commonly used in a number of clinical conditions. Because of the well-documented pro-oxidative properties of estrogens (1–5, 7), however, the use of antioxidants in combination with estrogens may prove beneficial.

Melatonin, the chief indoleamine produced by the pineal gland, is a well-known antioxidant and free radical scavenger (8–16). The molecule has been shown to be highly effective in protecting against oxidative damage caused by a variety of carcinogens, e.g., ferric nitrilotriacetate (17), ionizing radiation (16), δ-aminolevulinic acid (18), etc. Mechanisms involved in the protective effects of melatonin against oxidative stress are complex and involve direct free radical scavenging and indirect antioxidative actions of the molecule (10).

The aim of the present study was to determine the effects of E_2 and melatonin and their interactions on parameters related to oxidative damage to DNA and cellular membranes in hamster kidney, liver, and testes.

Materials and Methods

Chemicals. RNase A and T_1, proteinase K, nuclease P_1, and alkaline phosphatase were purchased from Boehringer Mannheim (Indianapolis, IN). The LPO-586 kit for lipid peroxidation was obtained from Calbiochem (La Jolla, CA), 1,3,5[10]-estratriene-3,17β-diol (17β-estradiol, [E_2]), ferrous sulfate, and hydrogen peroxide were from Sigma

(St. Louis, MO). Pure melatonin was a gift from Helsinn Chemicals SA (Biasca, Switzerland). Other chemicals used were of analytical grade and came from commercial sources.

Animals. The procedures used in the study were approved by the Institutional Animal Care and Use Committee. Fifty male Syrian hamsters (*Mesocricetus auratus*; weighing 110–120 g) were used in the study. They were housed in Plexiglas cages (three animals per cage) in a windowless room with automatically regulated temperature (22° ± 2°C) and lighting (14-hr light/10-hr dark, with lights on from 0600 to 2000 hr). The animals received standard chow and water *ad libitum*. After 1 week of acclimatization, the hamsters were randomly divided into six groups with eight (Groups I–IV) or nine (Groups V–VI) animals per group. The animals of groups III through VI were injected with E_2 (a single dose of 75 mg/kg body wt) suspended in corn oil. Hamsters of Groups II, IV, and VI were injected with melatonin (15 mg/kg body wt) in freshly prepared 0.9% NaCl/ethanol (v/v, 20/1) 30 min before and 120 min after the treatment with E_2 or, in case of Group II, with 0.9% NaCl/ethanol. The control hamsters, which did not receive either E_2 or melatonin, were injected with their solvents, i.e., 0.9% NaCl/ethanol (v/v, 20/1) or corn oil at the time points mentioned above. Injections of E_2 were performed at 1100 hr. All substances were administered intraperitoneally in the volume of 0.5 ml/injection.

Hamsters were sacrificed by decapitation 3 (Groups III and IV) or 5 hr (Groups I, II, V, and VI) after E_2 treatment. The kidneys, liver, and testes were collected, frozen on solid CO_2, and stored at −80°C until assay.

Measurement of 8-Oxo-7,8-Dihydro-2′-Deoxyguanosine (8-OxodGuo). DNA was isolated and purified as described previously (17) with minor modifications. Briefly, 170 mg of kidney, liver, or testis were homogenized in 1 ml of ice-cold buffer (0.1 M NaCl, 10 mM EDTA, 10 mM 2-mercaptoethanol, and 0.5% Triton X-100, pH 8.0), centrifuged at 4°C for 10 min at 1000*g*, and the resulting pellets were resuspended in 0.5 ml of lysis solution (120 mM NaCl, 10 mM Tris, 1 mM EDTA, and 0.5% SDS, pH 8.0) with 20% butylated hydroxytoluene. RNA and protein were digested by incubation with RNase or proteinase K at 55°C for 30 or 60 min, respectively. After extraction by successive mixing with saturated phenol, a mixture of phenol:chloroform:isoamyl-alcohol (25:24:1), and then a mixture of chloroform:isoamyl-alcohol, DNA was precipitated by the addition of 5 volumes of ethanol (−20°C).

The isolated DNA (100–500 μg) was dissolved in 200 μl of 20 mM sodium acetate (pH 5.0), denatured by heating at 95°C for 5 to 10 min, and cooled on ice. The DNA samples were digested to nucleotides by incubation with 12 units of nuclease P_1 at 37°C for 30 min. Next, after adding 20 μl of 1 M Tris-HCl (pH 8.0) and 4 units of alkaline phosphatase, the samples were incubated at 37°C for 1 hr. The resulting deoxynucleoside mixture was filtered through a Millipore filter (0.22 μm) and analyzed by means of HPLC-electrochemical detection (ECD) system. An ESA HPLC system equipped with eight channels CoulArray 5600 EC detector was used. Waters column ODS 3 (Partisil, 5 μm, 4.6 × 250 mm i.d.); eluent, 10% aqueous methanol containing 12.5 mM citric acid, 25 mM sodium acetic acid, 30 mM sodium hydroxide, and 10 mM acetic acid at a flow rate of 1 ml/min. The quantities of 8-oxodGuo and 2′-deoxyguanosine (dGuo) were measured using different channels and two oxidative potentials (300 and 900 mV, respectively). The results are expressed as the ratio of 8-oxodGuo to dGuo × 10^5.

***In Vitro*-Induced Lipid Peroxidation.** Approximately 100 mg of liver tissue from untreated hamsters (Group I) and from animals given melatonin (Group II), E_2 (Group V), or given both E_2 and melatonin (Group VI) were homogenized (Euro Turrax T20B homogenizer) in ice-cold 50 mM Tris buffer (pH 7.4; 10%, w/v). Two aliquots of homogenates from each animal were incubated for 1 hr in a water bath at 37°C, one in the presence of ferrous sulfate (15 μM) and hydrogen peroxide (H_2O_2, 0.1 mM) to generate free radicals, and the other in the absence of these compounds.

Measurement of Products of Lipid Peroxidation. The concentrations of malondialdehyde + 4-hydroxyalkenals (MDA + 4-HDA), as an index of the degree of lipid peroxidation, were measured in liver homogenates after *in vitro*-induced lipid peroxidation, and in homogenates of kidneys, liver, and testes obtained after treatment of the hamsters *in vivo*. Homogenates were centrifuged at 3000*g* for 10 min at 4°C. The obtained supernatant was mixed with 650 μl of methanol:acetonitrile (1:3, v/v) solution containing *N*-methyl-2-phenylindole and was then vortexed. After adding 150 μl of 15.4 M methanesulfonic acid, incubation was carried out at 45°C for 40 min. The concentration of MDA + 4-HDA was measured spectrophotometrically at the absorbance at 586 nm using a solution of 10 mM 4-hydroxynonenal as standard. The level of lipid peroxidation is expressed as the amount of MDA + 4-HDA (nanomoles) per milligram of protein.

Measurement of Protein. Protein was measured using the method of Bradford (19), with bovine serum albumin as the standard.

Statistical Analyses. Results are expressed as means ± SE. The data were statistically analyzed using a one-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls test. Statistical significance was determined at a level of <0.05.

Results

Changes in 8-oxodGuo levels in hamster kidney are presented in Figure 1. Treatment of animals with E_2 resulted in the increase in 8-oxodGuo levels in kidneys collected 5 hr after steroid administration; when melatonin was given as a cotreatment, it completely prevented the elevation in this index of DNA damage. A similar tendency with regard to

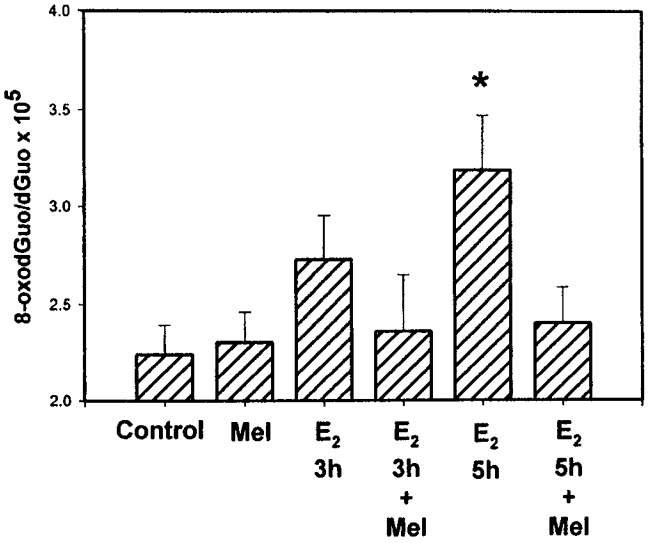

Figure 1. The level of 8-oxodGuo in kidneys of hamsters treated only with 17β-estradiol (75 mg/kg body wt) and collected 3 or 5 hr later (E_2 [3 hr] or E_2 [5 hr], respectively), treated only with melatonin (Mel, 15 mg/kg body wt 30 min before and 120 min after the time point of E_2 treatment), or treated with both E_2 and melatonin and collected 3 or 5 hr after E_2-treatment (E_2 [3 hr] + Mel or E_2 [5 hr] + Mel, respectively]. Data are expressed as the ratio of 8-oxodGuo/dGuo x 10^5. Bars represent means ± SE. Eight or nine hamsters per group. *$P < 0.05$ versus Control, versus Mel, versus E_2 (3 hr) + Mel, and versus E_2 (5 hr) + Mel.

the damaging effects of E_2 and the protective effects of melatonin on nuclear DNA was found in kidneys collected 3 hr after E_2 treatment; however, the observed increase in 8-oxodGuo level did not reach statistical significance.

DNA damage in liver related to E_2 treatment was observed in tissue collected 3 hr after exposure to the steroid (Fig. 2). The increase in hepatic 8-oxodGuo levels was prevented in hamsters treated with both melatonin and E_2. The levels of 8-oxodGuo in liver collected 5 hr after E_2 treatment were not significantly different from those observed in the controls.

Two injections of melatonin (15 mg/kg body wt/dose) into animals that were not treated with E_2 did not change 8-oxodGuo levels in kidneys and liver (Figs. 1 and 2). On the contrary, melatonin treatment reduced 8-oxodGuo levels in testes of hamsters injected only with the indoleamine or those of hamsters treated with both melatonin and E_2 and examined 3 hr later (Fig. 3). The levels of oxidatively damaged DNA in testes of animals treated only with E_2 were similar to those observed in the control hamsters.

Lipid peroxidation was not augmented by any of the *in vivo* treatments in either the kidneys, liver, or testes. On the contrary, the levels of lipid peroxidation products after treatment with either melatonin or E_2 or both compounds exhibited tendencies to decrease in all organs (Fig. 4). A significant reduction in the concentration of MDA + 4-HDA was observed in the liver of hamsters treated with E_2 or E_2 plus melatonin (Fig. 4).

In the *in vivo/in vitro* study, lipid peroxidation in hepatic homogenates from untreated, melatonin-treated, or E_2-injected hamsters increased during incubation with ferrous sulfate. However, iron exposure did not augment significantly the concentration of MDA + 4-HDA in the liver of E_2-treated hamsters when they also had been given melatonin (Fig. 5).

Discussion

The damage to nuclear DNA within hours after a single injection of a relatively large dose of E_2 as observed in

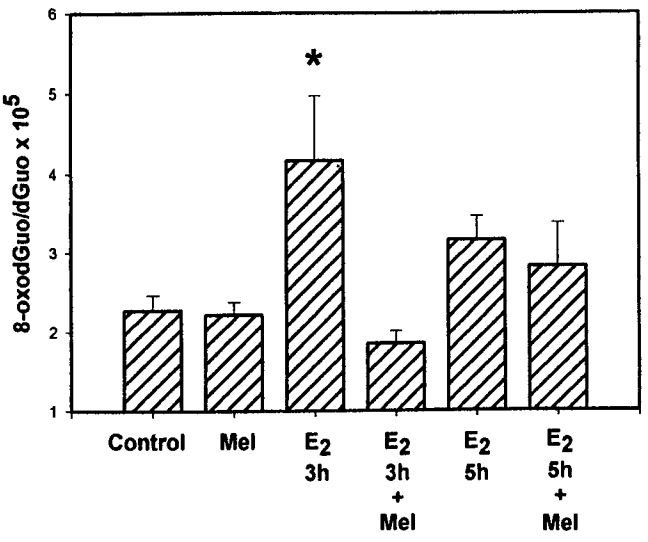

Figure 2. The level of 8-oxodGuo in liver of hamsters treated only with 17β-estradiol (75 mg/kg body wt) and collected 3 or 5 hr later (E_2 [3 hr] or E_2 [5 hr], respectively), treated only with melatonin (Mel, 15 mg/kg body wt 30 min before and 120 min after the time point of E_2 treatment), or treated with both E_2 and melatonin and collected 3 or 5 hr after E_2-treatment (E_2 [3 hr] + Mel or E_2 [5 hr] + Mel, respectively]. Data are expressed as the ratio of 8-oxodGuo/dGuo x 10^5. Bars represent means ± SE. Eight or nine hamsters per group. *$P <$ 0.05 verus Control, versus Mel, and versus E_2 (3 hr) + Mel.

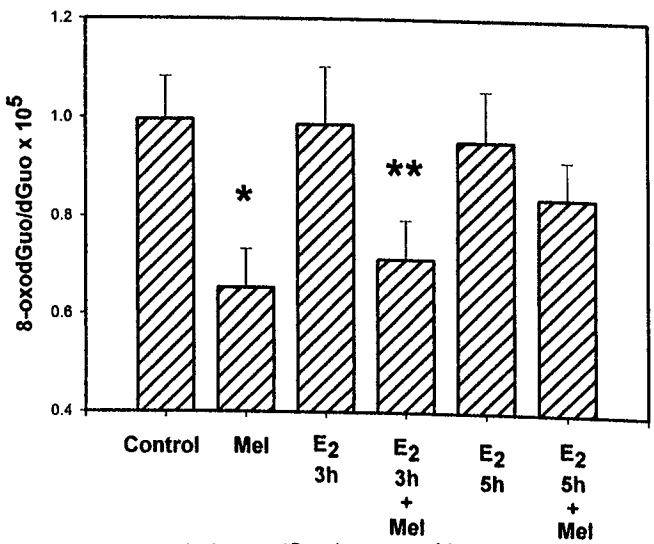

Figure 3. The level of 8-oxodGuo in testes of hamsters treated only with 17β-estradiol (75 mg/kg body wt) and collected 3 or 5 hr later (E_2 [3 hr] or E_2 [5 hr], respectively), treated only with melatonin (Mel, 15 mg/kg body wt 30 min before and 120 min after E_2 treatment), or treated with both E_2 and melatonin and collected 3 or 5 hr after the time point of E_2 treatment (E_2 [3 hr] + Mel or E_2 [5 hr] + Mel, respectively]. Data are expressed as the ratio of 8-oxodGuo/dGuo x 10^5. Bars represent means ± SE. Eight or nine hamsters per group. *$P < 0.05$ versus Control, versus E_2 (3 hr), and versus E_2 (3 hr), **$P < 0.05$ versus Control.

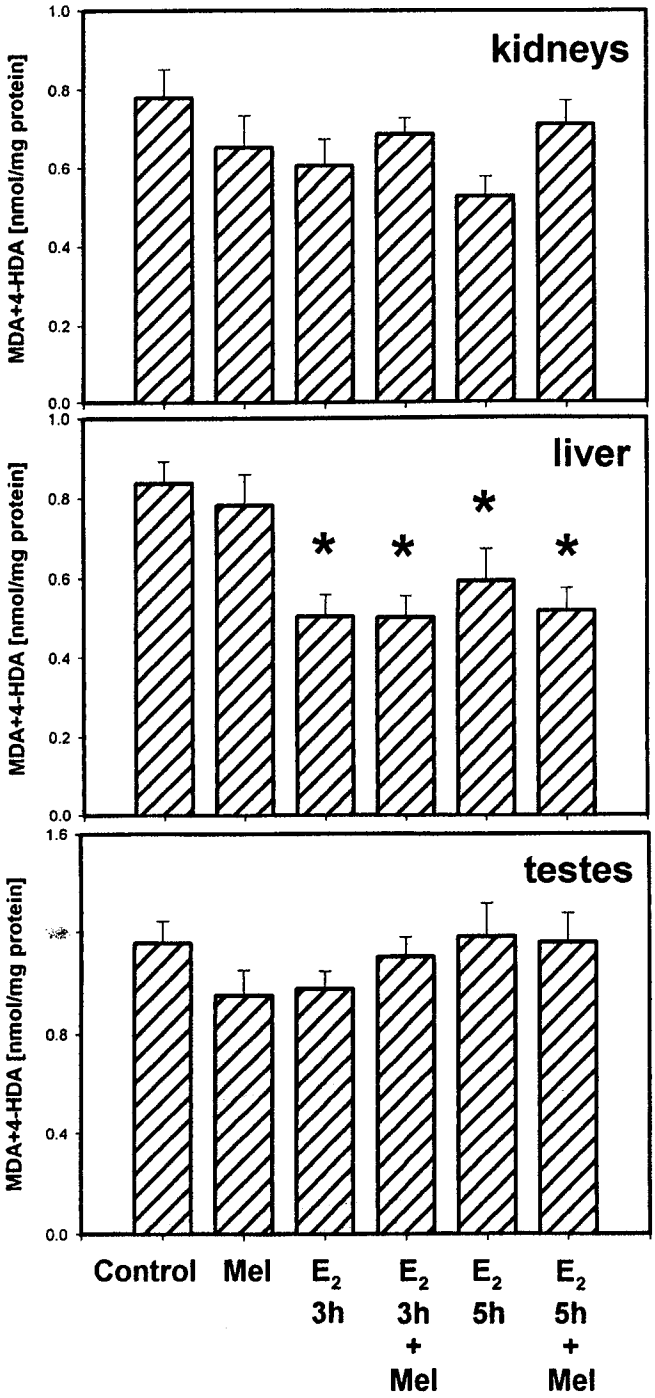

Figure 4. Concentrations of MDA + 4-HDA in kidneys, liver, and testes of hamsters treated only with 17β-estradiol (75 mg/kg body wt) and collected 3 or 5 hr later (E_2 [3 hr] or E_2 [5 hr], respectively), treated only with melatonin (Mel, 15 mg/kg body wt 30 min before and 120 min after the time point of E_2 treatment), or treated with both E_2 and melatonin and collected 3 or 5 hr after E_2 treatment (E_2 [3 hr] + Mel or E_2 [5 hr] + Mel, respectively]. Data are expressed as nanomoles per milligram of protein. Bars represent means ± SE. Eight or nine hamsters per group. *P <0.05 versus Control and versus Mel.

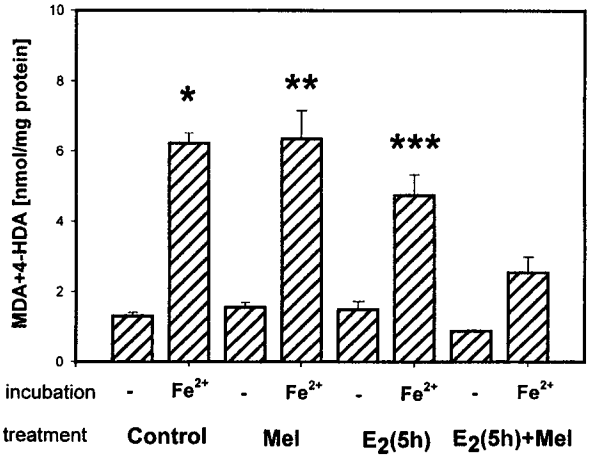

Figure 5. Concentrations of MDA + 4-HDA in liver homogenates from untreated hamsters (Control), animals treated only with 17β-estradiol (75 mg/kg body wt) and collected 5 hr later (E^2 [5 hr]), treated only with melatonin (Mel, 15 mg/kg body wt 30 min before and 120 min after E^2 treatment), or treated with both E^2 and melatonin and collected 5 hr after E^2 treatment (E^2 [5 hr] + Mel), and then exposed *in vitro* to ferrous sulfate (15 μM) (Fe^{2+}) + hydrogen peroxide (H_2O_2, 0.1 mM). Data are expressed as nanomoles per milligram of protein. Bars represent means ± SE. Eight or nine hamsters per group. *P < 0.05 versus Control (without exposure to Fe^{2+}); **P < 0.05 versus Mel (without exposure to Fe^{2+}); ***P < 0.05 versus E_2 (5 hr; without exposure to Fe^{2+}).

hamster kidney and liver in the present study is in agreement with previous findings (20). In contrast, lipid peroxidation not only was not induced within 5 hr after E_2 administration, but rather the steroid decreased MDA + 4-HDA levels in liver. Thus, the dual role of E_2, i.e., both as a pro- and an antioxidant (5), is consistent with previous studies and may relate to the concentration of estrogen in subcellular compartments and the concentration of other reactants. In contrast to the present results, it should be noted that in at least one study, E_2 exposure *in vivo* resulted in a time-dependent increase in lipid peroxidation, the products of which could additionally contribute to DNA damage (21).

The differential actions of estrogens within cells, i.e., either pro-oxidant or antioxidant, depends on their metabolism and the subsequent actions of the metabolites (5). Estrogens are readily converted to catecholestrogens during their oxidation. The catecholestrogens are precursors of quinones; these latter molecules participate in an oxidation reduction reaction, which yields semiquinones and toxic reactants that are capable of damaging not only DNA, but other molecules as well. In contrast, catechol-estrogens also are involved in the redox cycling of iron, an action that contributes to the antioxidant effects of estrogens. The differential actions of estrogens depend on their concentrations in specific subcellular compartments, as well as on those of the other reactants.

The lack of an increase in 8-oxodGuo levels in testes due to E_2 treatment as observed in the present experiment does not preclude the possibility of testicular DNA damage at some other time point after steroid administration. Interestingly, the reduction in the levels of 8-oxodGuo seen after melatonin treatment emphasizes the protective effects of the indoleamine on the testes under physiologically on-going oxidation of DNA bases.

In the hamster kidney, E_2-induced damage to biological macromolecules is believed to be related to the elevated metabolic conversion of E_2 to catechol-estrogens. This same

conversion occurs in other rodent organs (3), as well as in human breast (22) and uterus (23). The catechol-estrogens are capable of the metabolic redox cycling by oxidation to quinones, followed by free radical generation, with subsequent damage to macromolecules, and eventually to carcinogenesis (7). Melatonin is probably protective at several steps of estrogen-related carcinogenesis.

Oxidative damage to DNA (20, present work), lipids (21), and proteins (24) are believed to be involved in estrogen-induced carcinogenesis in animals. In a variety of studies, melatonin has been shown to offer protection against oxidative damage of DNA (11, 16–18), lipids (17, 18, 25, 26), and proteins (27).

Melatonin probably protects against E_2-induced DNA damage via a variety of mechanisms. The indoleamine easily enters all cellular compartments due to its small size, high lipophilicity, and modest hydrophilic nature (8–10, 28). Melatonin directly or indirectly neutralizes a variety of free radicals and reactive species. The indoleamine directly detoxifies hydrogen peroxide (H_2O_2) (15, 29) and secondarily the superoxide anion radical (O_{2-}) (9), and it is especially effective in reducing damage caused by the highly toxic hydroxyl radical ($^{\cdot}OH$) (13, 14). Each of these toxic species is generated during the oxidation of catechol-estrogens to quinones (3, 7). It is worth emphasizing that melatonin also scavenges nitric oxide ($NO^{\cdot}$) (30) and the peroxynitrite anion ($ONOO^-$) (12, 31), as well as inhibiting the activity of nitric oxide (NO) synthase (10, 12), which determines the amount of NO produced. This is important since NO has been shown to contribute to estrogen-induced DNA damage. Thus, during the oxidation of catechol-estrogens to quinones, $ONOO^-$ is formed when NO couples with $O_2^{-\cdot}$ (32). Additionally, melatonin is known to quench the reactive species singlet oxygen ($^1O_2$) (10). The effectiveness of melatonin in protecting against lipid breakdown results from a complex process that relates to its ability to scavenge the initiating agents, e.g., $^{\cdot}OH$, $ONOO^-$, etc., and to the localization of melatonin in a superficial position of the lipid bilayers near the polar heads of membrane phospholipids (33). This action of melatonin may permit membrane lipids to more easily resist oxidative destruction. Whether melatonin scavenges the peroxyl radical ($LOO^{\cdot}$) (34) is controversial (35), but if it does, this action would also reduce the accumulation of MDA + 4-HDA.

Melatonin favorably influences the redox cycling of glutathione (GSH), an important intracellular antioxidant, by stimulating the activity of glutathione peroxidase (GSH-Px) (10, 11), which utilizes H_2O_2 and other hydroperoxides as cofactors, thereby reducing the intracellular concentrations of these damaging agents. During this process, GSH is oxidized to its disulfide (GSSG), which is quickly reduced to GSH by the activity of glutathione reductase (GSH-Rd), another antioxidative enzyme shown to be stimulated by melatonin (10, 11). Furthermore, melatonin promotes glucose-6-phosphate dehydrogenase activity, which enzymatically induces the formation of NADPH, an important co-factor for GSH-Rd (10). Additionally, melatonin stimulates γ-glutamylcysteine synthetase (36), the rate-limiting enzyme in the synthesis of GSH. Finally, treatment of cells *in vitro* with melatonin stimulates mRNA levels for superoxide dismutase (SOD), which dismutases $O_2^{-\cdot}$ to H_2O_2 and stimulates the activity of catalase, thereby further reducing H_2O_2 levels and $^{\cdot}OH$ generation (10). By stimulating the activities of a number of antioxidant enzymes, melatonin can offer protection during early steps of estrogen-induced oxidative damage. Interestingly, the activities of several of these enzymes, i.e., GSH-Px, GSH-Rd, SOD, and catalase have been found to be reduced in E_2-induced hamster kidney tumors and in the surrounding tissue (37).

Dietary iron enhances the incidence and severity of estrogen-induced tumors (38), but at the same time, estrogens, including catechol-estrogens, protect against iron-induced *in vitro* lipid peroxidation (39). Because of this and since iron-induced lipid peroxidation is a well-established phenomenon (25, 26), we examined the changes in the latter process in homogenates of liver collected from untreated or E_2-treated hamsters. Although *in vivo* E_2 treatment appeared to be protective against iron-induced lipid peroxidation at the early time point (3 hr) after treatment with the steroid, it was not effective as an antioxidant in tissue collected at the later time point (5 hr). However, in the latter case, cotreatment with melatonin resulted in effective protection against iron-induced lipid peroxidation. It has been shown previously that melatonin enhances the protective actions of other agents against oxidative abuse, e.g., melatonin in combination with the antiestrogen tamoxifen is more effective in reducing iron-induced lipid peroxidation than is treatment with either agent alone (26).

Estrogens, at physiological concentrations, can directly induce primary epithelial cell proliferation in hamster kidney, supporting its potential role in neoplastic transformation (40). The role of proliferation in the pathogenesis of estrogen-related human cancer is well known (41). On the other hand, melatonin is known to inhibit estrogen-related cell proliferation (42, 43). In fact, melatonin is a well-known oncostatic agent (44). The concentrations of melatonin are reduced in patients with endometrial cancer (45) and generally after menopause (46). Therefore, providing melatonin supplements to patients with a risk of estrogen-related cancer may be a consideration.

In summary, the present results confirm the ambiguity of estrogens with respect to oxidative processes and illustrate that estrogens, while offering protection against lipid peroxidation, may also directly cause oxidative damage to nuclear DNA, a process preceding carcinogenesis. Evidence is provided that melatonin is exclusively antioxidative in the presence of E_2, i.e., it prevents E_2-induced DNA damage and it acts synergistically with E_2 in protection against lipid peroxidation.

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