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Melatonin Reduces Fenton Reaction-Induced Lipid Peroxidation in Porcine Thyroid Tissue

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Abstract Free radicals and reactive oxygen species (ROS) participate in physiological and pathological processes in the thyroid gland. Bivalent iron cation (ferrous, Fe^{2+}), which initiates the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$) is frequently used to experimentally induce oxidative damage, including that caused by lipid peroxidation. Lipid peroxidation is involved in DNA damage, thus indirectly participating in the early steps of carcinogenesis. In turn, melatonin is a well-known antioxidant and free radical scavenger. The aim of the study was to estimate the effect of melatonin on basal and iron-induced lipid peroxidation in homogenates of the porcine thyroid gland. In order to determine the effect of melatonin on the auto-oxidation of lipids, thyroid homogenates were incubated in the presence of that indoleamine in concentrations of 0.0, 0.00001, 0.0001, 0.001, 0.01, 0.1, 0.25, 0.5, 1.0, 2.5, or 5.0 mM. To study melatonin effects on iron-induced lipid peroxidation, the homogenates were incubated in the presence of $FeSO_4$ (40 μM) plus H_2O_2 (0.5 mM), and, additionally, in the presence of melatonin in the same concentrations as above. The degree of lipid peroxidation was expressed as the concentration of malondialdehyde + 4-hydroxyalkenals (MDA + 4-HDA) per mg protein. Melatonin, in a concentration-dependent manner, decreased lipid peroxidation induced by Fenton reaction, without affecting the basal MDA + 4-HDA levels. In conclusion, melatonin protects against iron + H_2O_2-induced peroxidation of lipids in the porcine thyroid. Thus, the indoleamine would be expected to prevent pathological processes related to oxidative damage in the thyroid, cancer initiation included. J. Cell. Biochem. 90: 806–811, 2003.

Key words: melatonin; thyroid; iron; lipid peroxidation; cancer

Reactive oxygen species (ROS) and free radicals participate in numerous metabolic processes. Under physiological conditions, there is a balance between the production and detoxification of ROS [Dröge, 2002]. However, any internal or external pathological factor may disrupt this balance, leading to conditions referred to as oxidative stress, playing a significant role in the pathogenesis of several diseases [Dreher and Junod, 1996; Dröge, 2002].

As in other tissues and organs, oxidative reactions occur also in the thyroid gland under physiological conditions. For example, one of ROS, hydrogen peroxide (H_2O_2), is produced in the thyroid gland and it is an essential factor for thyroid hormone biosynthesis, acting as an electron acceptor at each step of this process [Nunez and Pommier, 1982; Sugawara et al., 2002].

The most basic reaction of oxidative stress is Fenton reaction: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$; the most harmful free radical, hydroxyl radical (${}^{\bullet}OH$), is produced during this reaction. Thus, ferrous ion (Fe^{2+}) participates in Fenton reaction. It is known that the increased iron stores in the organism result in cellular toxicity and are associated with increased risks of several diseases, cancer included [Dreher and Junod, 1996; Eaton and Qian, 2002]. At the same time, it is known that lipid peroxidation (which may be induced by ${}^{\bullet}OH$ produced during Fenton reaction) contributes to DNA damage and cancer initiation [Marnett, 1999]. In accordance, there is some evidence that oxidative stress is involved in the pathomechanism

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of several thyroid diseases, also neoplastic ones [Ha et al., 2000; Hasegawa et al., 2002].

On the other hand, iron is a cofactor for many biological reactions and is an essential element for normal metabolic processes in different tissues and organs. In the thyroid gland, iron is present in heme-dependent thyroid peroxidase (TPO), the key enzyme catalyzing thyroid hormone synthesis. Studies on animals and in human subjects have revealed that iron deficiency impairs thyroid hormone biosynthesis, among others by reducing TPO activity, and enhances the consequences of iodine deficiency, while iron supplementation improves the efficacy of iodine supplementation [Zimmermann and Köhrle, 2002].

N-acetyl-5-methoxytryptamine (melatonin), the chief indoleamine produced by the pineal gland, is a well-known protective factor against oxidative damage and carcinogenesis [reviewed in Reiter, 1998, 2000a,b, 2002; Karbownik and Reiter, 2000, 2002; Reiter et al., 2000; Karbownik et al., 2001c; Tan et al., 2003].

As regards the relationship between the thyroid and the pineal gland, the up-to-date experimental evidence, although very abundant, practically resolves itself into the fact that melatonin, in many, but not all, experimental conditions, suppresses thyroid growth and function, and that in majority of studies thyroid hormones are reported to stimulate pineal growth and secretory processes [reviewed in Lewiński et al., 2002; Lewiński and Karbownik, 2002]. Unfortunately, the involvement of oxidative stress in the pineal–thyroid relationships has, up to now, been poorly identified.

The aim of the present study was to evaluate potential protective effects of melatonin against Fe^{2+} plus H_2O_2-induced lipid peroxidation in homogenates of porcine thyroid. Our study is the first one showing the direct protective effect of melatonin against oxidative damage in the thyroid gland.

MATERIALS AND METHODS

Chemicals

Melatonin, ferrous sulfate ($FeSO_4$), and H_2O_2 were purchased from Sigma (St. Louis, MO). The LPO-586 kit for lipid peroxidation was obtained from Calbiochem (La Jolla, CA). All the used chemicals were of analytical grade and came from commercial sources.

Animals

The procedures, used in the study, were approved by the Ethical Committee of the Polish Mother's Memorial Hospital-Research Institute at Łódź. Porcine thyroids were collected from 20 animals at a slaughter-house, frozen on solid CO_2 and stored at $-80^{\circ}C$ until assay.

Incubation of Thyroid Homogenates

Thyroid tissue was homogenized in ice cold 50 mM Tris-HCl buffer (pH 7.4) (10%, w/v), and then incubated for 30 min at $37^{\circ}C$ in the presence of examined substances.

In Experiment I, melatonin was used in the following concentrations: 0.0, 0.00001, 0.0001, 0.001, 0.01, 0.1, 0.25, 0.5, 1.0, 2.5, or 5.0 mM. Since in the earlier studies a decrease in the basic level of lipid peroxidation products was observed after 60- or 120-min incubation with melatonin in the monkey [Tan et al., 2000a] and rat [Osuna et al., 2002] brain, the monkey [Cabrera et al., 2001] and rat [Karbownik et al., 2000a; Gitto et al., 2001] liver, and the hamster testis homogenates [Karbownik et al., 2001a], in the present study we have lengthened the incubation time of thyroid homogenates up to 60 and 120 min.

In Experiment II, thyroid homogenates were incubated in the presence of $FeSO_4$, used in different concentrations (7.5, 15, 30, 60, 120, and 240 μM) plus H_2O_2 (0.5 mM). On the basis of the concentration-dependent effect of $FeSO_4$, the concentration of 40 μM was selected for a subsequent study.

Thus, in Experiment III, in order to induce lipid peroxidation, thyroid homogenates were incubated in the presence of $FeSO_4$ (40 μM) plus H_2O_2 (0.5 mM), and, additionally in the presence of melatonin, used in the same concentrations as in Experiment I (to prevent lipid peroxidation).

The reactions were stopped by cooling the samples on ice. Each experiment was run in duplicate and repeated three times.

Measurement of Lipid Peroxidation Products

The concentrations of malondialdehyde + 4-hydroxyalkenals (MDA + 4-HDA), as an index of lipid peroxidation, were measured in thyroid homogenates. The homogenates were centrifuged at 3,000*g* for 10 min at $4^{\circ}C$. The supernatant was mixed with 650 μl of a methanol:acetonitrile (1:3, v/v) solution, containing a

chromogenic reagent, *N*-methyl-2-phenylindole, and vortexed. After adding 150 μl of methanesulfonic acid (15.4 M), the incubation was carried out at 45°C for 40 min. The reaction between MDA + 4-HDA and *N*-methyl-2-phenylindole yields a chromophore, which is spectrophotometrically measured at the absorbance of 586 nm, using a solution of 10 mM 4-hydroxynonenal as the standard. The level of lipid peroxidation is expressed as the amount of MDA + 4-HDA (nmol) per mg protein.

Measurement of Protein

Protein was measured, using the method of Bradford [1976], with bovine albumin as the standard.

Statistical Analyses

Results are expressed as mean ± SE. The data were statistically analyzed, using Student's *t*-test. Statistical significance was determined at the level of $P < 0.05$.

RESULTS

The incubation of thyroid homogenates for 30 min in the presence of melatonin did not cause any changes in the level of lipid peroxidation products (Fig. 1). When we extended the incubation time to 60 and 120 min, still this indoleamine did not affect the basic lipid peroxidation in the rat thyroid gland (data not shown).

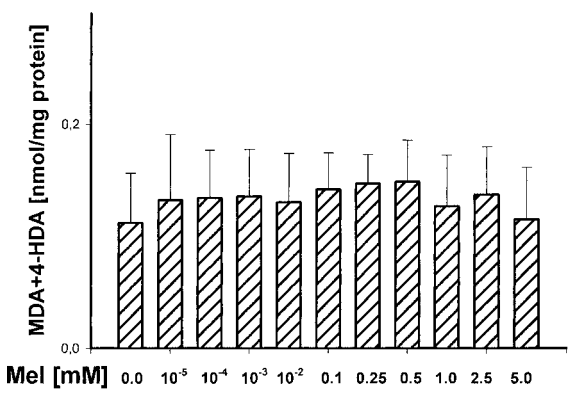

Fig. 1. The concentration of malondialdehyde + 4-hydroxyalkenals (MDA + 4-HDA) in porcine thyroid homogenates, incubated for 30 min in the presence of melatonin (Mel) (0.0, 0.00001, 0.0001, 0.001, 0.01, 0.1, 0.25, 0.5, 1.0, 2.5, or 5.0 mM). Bars represent the mean ± SE of three independent experiments run in duplicates. No significant differences were found.

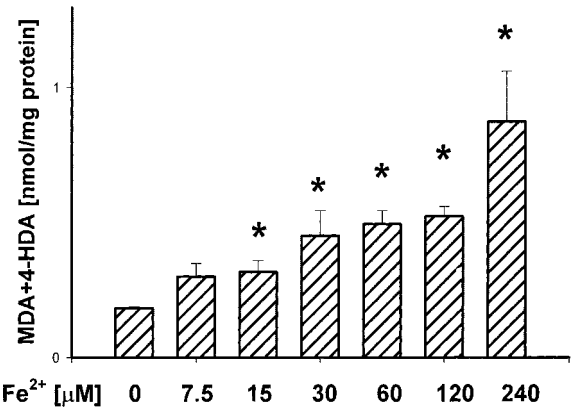

Fig. 2. The concentration of MDA + 4-HDA in porcine thyroid homogenates, incubated for 30 min in the presence of $FeSO_4$ (Fe^{2+}) (7.5, 15, 30, 60, 120, and 240 μM) plus H_2O_2 (0.5 mM), used to induce lipid peroxidation. Bars represent the mean ± SE of three independent experiments run in duplicates. $^*P < 0.05$ versus control (in the absence of Fe^{2+}).

The incubation of thyroid homogenates in the presence of $FeSO_4$ used in different concentrations (7.5, 15, 30, 60, 120, and 240 μM) plus H_2O_2 (0.5 mM), resulted in a concentration-dependent increase in lipid peroxidation; an increase in the level of MDA + 4-HDA was observed for $FeSO_4$ added to the incubation medium in the concentrations of 15, 30, 60, 120, and 240 μM (Fig. 2). For a subsequent study, the concentration of 40 μM of $FeSO_4$ was selected.

Melatonin, added to the incubation medium together with $FeSO_4$ and H_2O_2 (0.5 mM), decreased in a concentration-dependent manner lipid peroxidation induced by Fenton reaction; melatonin was effective in the concentrations of 0.25, 0.5, 1.0, 2.5, and 5.0 mM (Fig. 3).

DISCUSSION

As we mentioned before, melatonin was found to reduce the basal level of lipid peroxidation in numerous tissues. However, no reports exist concerning that process in the thyroid gland. Our study is the first attempt devoted to the subject in question; we have shown that melatonin does not affect the basal level of lipid peroxidation in the thyroid gland, unlike as in many other organs, in which the indoleamine has been found to reduce the value of this parameter of oxidative stress. However, the observation that melatonin did not reveal any ability to reduce auto-oxidation in porcine thyroid, does not rule out its potential to prevent oxidative changes, due to the action of any pro-oxidant

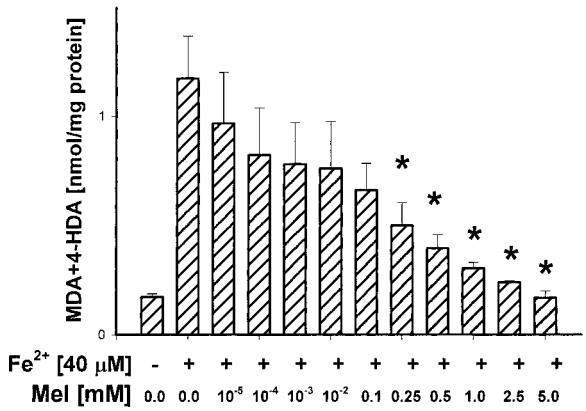

Fig. 3. The concentration of MDA + 4-HDA in porcine thyroid homogenates, incubated for 30 min in the presence of $FeSO_4$ (Fe^{2+}) (40 μM) plus H_2O_2 (0.5 mM), used to induce lipid peroxidation, and, additionally, in the presence of melatonin (Mel) (0.0, 0.00001, 0.0001, 0.001, 0.01, 0.1, 0.25, 0.5, 1.0, 2.5, or 5.0 mM). Bars represent the mean ± SE of three independent experiments run in duplicates. $^*P < 0.05$ versus control (in the presence of Fe^{2+} and in the absence of melatonin).

agent. Indeed, in our study melatonin effectively protected against oxidative damage to lipids caused by $Fe^{2+} + H_2O_2$ in the thyroid. Additionally, it is well-known that oxidative processes occur in biological structures with certain intensity, resulting in formation of several products of oxidative damage to lipids, DNA, protein, etc. Thus, it is probably not required and, in consequence, not recommended to reduce auto-oxidation under physiological conditions.

The thyroid gland, as other organs, may be exposed to excessive amounts of either Fe^{2+} or H_2O_2, or both. Under such conditions, Fenton reaction occurs, producing highly toxic $^{\bullet}OH$. While iron is essential for thyroid hormone synthesis and for the normal course of other metabolic processes in the thyroid gland [Zimmermann and Köhrle, 2002], this metal, deriving, for example, from heme-linked histidine residue of TPO and being in excess, produces free radicals which inactivate TPO [Sugawara et al., 2002], and definitely induces other pathological processes.

There is some evidence that thyrotropin (TSH), the main secretory and growth stimulatory hormone for the thyroid, is involved in the production of H_2O_2 in that gland. Using dog thyroid cells, it has been found that TSH stimulates H_2O_2 generation through the cyclic adenosine 3′,5′-monophosphate cascade [Raspe and Dumont, 1995]. The major meaning of this observation is that an increased production of H_2O_2 with a subsequently enhanced formation of free radicals (especially $^{\bullet}OH$) takes place in any conditions accompanied by the increased blood TSH concentration. TSH stimulation results in goiter formation and, under certain conditions, in thyroid cancer initiation.

The protective effects of melatonin against $Fe^{2+} + H_2O_2$-induced lipid peroxidation in the porcine thyroid suggest that the indoleamine could prevent against certain processes in the thyroid, such as disturbances in hormone synthesis, goiter formation, cancer, and probably some others.

The protective effects of melatonin against lipid peroxidation, induced by Fenton reaction, were observed before in the monkey [Tan et al., 2000a] and rat [Osuna et al., 2002] brain, the monkey [Cabrera et al., 2001] and rat [Karbownik et al., 2000a; Gitto et al., 2001] liver, and the hamster testis [Karbownik et al., 2001a] homogenates. Also, in in vivo studies, melatonin was documented to reveal protective effects against lipid peroxidation due to numerous pro-oxidants, carcinogens included [Karbownik et al., 2000b,c, 2001b; Calvo et al., 2001]. Compounds chemically related to melatonin were found to prevent oxidative damage caused by iron as well [Karbownik et al., 2001d].

The mechanisms by which melatonin protects against lipid peroxidation most likely involve direct or indirect antioxidant and free radical scavenging activities of this indoleamine. Among them, the most important seems to be the ability of melatonin to scavenge such toxic species, like $^{\bullet}OH$ and peroxynitrite anion ($ONOO^-$), which are sufficiently reactive to initiate the breakdown of lipids [Reiter, 2000a]. Melatonin is one of the most effective neutralizers of the highly toxic $^{\bullet}OH$, scavenging this radical with a rate constant roughly on the order of $10^{10}\ M^{-1}\ s^{-1}$ [Tan et al., 1993].

Melatonin has also been shown to detoxify $ONOO^-$, one of the most toxic ROS destructive products of the interaction between the $O_2^{-\bullet}$ and $NO^{\bullet}$, and to directly neutralize $NO^{\bullet}$, as well as to indirectly detoxify the superoxide anion ($O_2^{-\bullet}$) [Reiter, 1998, 2000a, 2000b]. Recently, melatonin has been found to directly detoxify H_2O_2 [Tan et al., 2000b]. Furthermore, melatonin quenches singlet oxygen ($^1O_2$), a high-energy form of O_2, which exhibits high toxicity at the molecular level [Reiter et al., 2000].

Melatonin, which is highly lipid soluble, is believed to be widely distributed in cellular membranes where it may intercalate between the polar heads of fatty acids [Reiter et al., 2000]. The last property facilitates melatonin to reduce peroxidative damage to lipids.

Additionally, in the conditions in vivo, it appears important that melatonin can stimulate the activities of antioxidative enzymes (γ-glutamylcysteine synthetase, glutathione peroxidase, glutathione reductase, glucose-6-phosphate dehydrogenase, superoxide dismutase, and catalase), and inhibit the activity of nitric oxide synthase, i.e., a pro-oxidative enzyme [Reiter, 1998; Reiter et al., 2000].

Since melatonin is known to inhibit growth processes in the thyroid [Lewiński and Karbownik, 2002; Lewiński et al., 2002], and—as it results from the present study—melatonin can protect against oxidative damage in the thyroid, the indoleamine may be believed to protect against thyroid cancer.

In conclusion, melatonin protects against iron + H_2O_2-induced peroxidation of lipids in the porcine thyroid tissue. Thus, the indoleamine would be expected to prevent against pathological processes related to oxidative damage in the thyroid, cancer initiation included.

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