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Mechanism of the inhibitory effect of melatonin on tumor necrosis factor production in vivo and in vitro

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Abstract

Melatonin is an antioxidant. Since other antioxidants inhibit the production of tumor necrosis factor (TNF) induced by lipopolysaccharide, we investigated its effect on TNF production in vivo and in vitro and on lethality associated with endotoxic shock. Administration of melatonin to mice (5 mg/kg, s.c., 30 min before or simultaneously with lipopolysaccharide) inhibited serum TNF levels by 50–80% and improved survival of mice treated with a lethal dose of lipopolysaccharide. By studying other, structurally related, indolamines (*N*-acetyl-5-hydroxytryptamine, 5-methoxytryptamine and 5-hydroxytryptamine) we found a good correlation between antioxidant activity (for which the 5-methoxy group is essential) and the inhibition of TNF production in vivo and in vitro in mononuclear cells. Melatonin did not increase serum corticosterone and did not modify the elevation of serum corticosterone levels by lipopolysaccharide or by interleukin-1. Furthermore, it exerted its inhibitory effect in adrenalectomized or hypophysectomized mice also, indicating that its effect is independent of the hypothalamus–pituitary–adrenal axis. © 1998 Elsevier Science B.V.

Keywords: TNF-α (tumor necrosis factor-α); Melatonin; Antioxidant; Endotoxin

1. Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine), in addition to its hormonal function, has antioxidant activity and is protective in some in vivo or in vitro models of oxidative damage (Reiter et al., 1997). It inhibits low density lipoprotein oxidation (Pieri et al., 1996) and is protective in a rat model of stroke (Manev et al., 1995). It also protects against paraquat-induced pulmonary damage (Melchiorri et al., 1995), lipopolysaccharide-induced hepatotoxicity (Sewerynek et al., 1995), ethanol-induced gastroduodenal injury (Melchiorri et al., 1997) and viral encephalitis in mice (Ben-Nathan et al., 1995). In vitro studies on human peripheral blood mononuclear cells have shown that melatonin inhibits the production of tumor necrosis factor (TNF) and interferon-gamma (Di Stefano and Paulesu, 1994).

TNF is a pathogenetic mediator of various infective and inflammatory diseases and anti-TNF antibodies are protective in animal models of septic or endotoxic shock (Beutler et al., 1985; Tracey and Cerami, 1993).

The present study was aimed at characterizing the mechanism of the inhibitory effect of melatonin on TNF production and its effect in a murine model of endotoxic shock. We thus studied, in a first series of experiments, the effect of melatonin on the lethality induced by lipopolysaccharide and its effect on TNF and two other cytokines (interleukin-1 and interleukin-6) also implicated in endotoxic shock (Dinarello, 1996). Since melatonin was also reported to be neuroprotective against excitotoxic damage in vivo, a model where TNF was also implicated, we investigated its effect on brain TNF production induced by intracerebroventricular injection of lipopolysaccharide (Faggioni et al., 1995).

A second series of experiments was designed to understand the role of endogenous corticosteroids in this activity. Melatonin is a major regulator of the circadian cycle (Gwinner and Benzinger, 1978) and blood corticosterone, the levels of which vary with the circadian cycle, is a potent inhibitor of TNF production (Fantuzzi and Ghezzi, 1993). Activation of the hypothalamus–pituitary–adrenal

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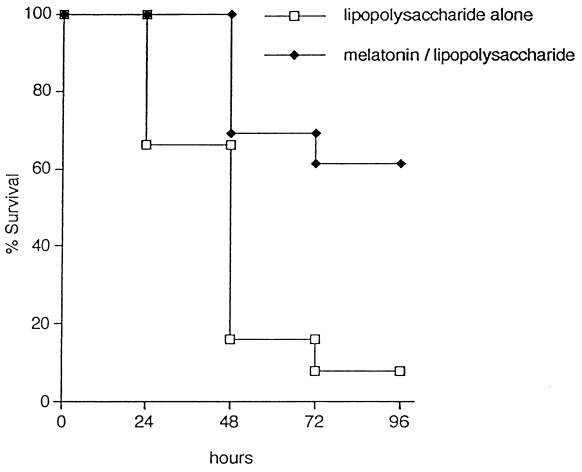

Fig. 1. Balb/C mice were treated with lipopolysaccharide (160 μg/mouse, i.p.) with or without a simultaneous injection of melatonin (5 mg/kg, s.c.) and survival was assessed daily and expressed as a percentage (No. of mice: lipopolysaccharide alone, $n = 12$; melatonin/lipopolysaccharide, $n = 15$). Animals were followed for up to 7 days and neither were further deaths observed nor did surviving mice look sick in this period. $^{*}P < 0.05$ versus lipopolysaccharide alone by Fisher's exact test (analysis was done on day 4).

axis by stress (Fantuzzi et al., 1995) or other stimuli (Fantuzzi and Ghezzi, 1993) is a potent anti-inflammatory feedback mechanism. It is not clear whether melatonin has an effect on the hypothalamus–pituitary–adrenal axis. While one report indicated that melatonin stimulates the secretory activity of rat and hamster adrenal glands in vitro (Lesniewska et al., 1990), another paper reported a decrease in serum corticosterone in melatonin-treated rats (Yamada, 1990). To investigate the role of endogenous corticosterone in melatonin inhibition of TNF production we studied the effect of melatonin on blood corticosterone levels in naive mice or in mice in which corticosterone was elevated by injection of lipopolysaccharide or interleukin-1. We also studied the effect of melatonin on lipopolysaccharide-induced TNF production in vivo in adrenalectomized or in hypophysectomized mice. Considering its high lipophilicity and its non-toxic nature as well as its ability to readily cross the blood–brain barrier, the neurohormone, melatonin, may prove to be an effective and important molecule in the antioxidative defense system, especially in the central nervous system. (Le Bars et al., 1991).

A third set of experiments was designed to investigate the possibility that inhibition of TNF production is correlated with the antioxidant activity of melatonin. In fact, the production of TNF in vivo and in vitro is redox-sensitive. Various antioxidants were shown to inhibit TNF production (Peristeris et al., 1992; Zhang et al., 1994), while pro-oxidant compounds enhanced it (Chaudri and Clark, 1989). To investigate this possibility, we tested various structurally related molecules differing from the compound under investigation for their antioxidant activity. Along these lines, we compared melatonin (*N*-acetyl-5-methoxytryptamine), its precursors, *N*-acetyl-5-hydroxytryptamine, 5-methoxytryptamine and 5-hydroxytryptamine (5-HT, serotonin), for their ability to inhibit TNF production in vivo in mice or in vitro in human peripheral blood mononuclear cells and for their antioxidant activity.

2. Materials and methods

2.1. Materials

Lipopolysaccharide (phenol-extracted preparation from *E. coli* O55:B5), 5-methoxytryptamine, *N*-acetyl-5-hydroxytryptamine and 5-HT were from Sigma (St. Louis, MO). Melatonin was from Helsinn Chemicals, SA, Switzerland. [^{3}H]Corticosterone was from Amersham (Amersham, UK). Recombinant human interleukin-1β (10^{7} U/mg) was a kind gift from Sclavo, Siena, Italy.

2.2. Animals and treatments

Male CD-1 mice (25 g body weight, Charles River, Calco, Como, Italy) were used, unless otherwise indicated. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies (European Community Council Directive 86/609, OJ L 358, 1, December 12, 1987; Italian Legislative Decree 116/92, Gazzetta Ufficiale della Repubblica Italiana No. 40, February 18, 1992; National Institutes of Health Guide for the Care and Use of Laboratory Animals, National Institutes of Health Publication No. 85-23, 1985).

Mice were injected with lipopolysaccharide as indicated in the text. Blood was taken from the retro-orbital plexus

Table 1
Effect of melatonin on lipopolysaccharide-induced serum TNF, interleukin-1 and interleukin-6

Treatment	TNF (ng/ml)	Interleukin-1 (pg/ml)	Interleukin-6 (pg/ml)
Lipopolysaccharide	35.2 ± 7.9	155 ± 87	2818 ± 1450
Melatonin/lipopolysaccharide	7.7 ± 2.1[a]	133 ± 29	3913 ± 63

Mice were treated with lipopolysaccharide (160 μg/mouse, i.p.) with or without melatonin (5 mg/kg, s.c., simultaneously with lipopolysaccharide) and serum cytokines were measured (TNF, 1 h later; interleukin-1 and interleukin-6, 3 h later). Data are means ± S.D. (5 mice/group). No cytokines could be detected (< 15 pg/ml) in mice not treated with lipopolysaccharide (data not shown).
[a] $P < 0.01$ versus lipopolysaccharide alone by ANOVA.

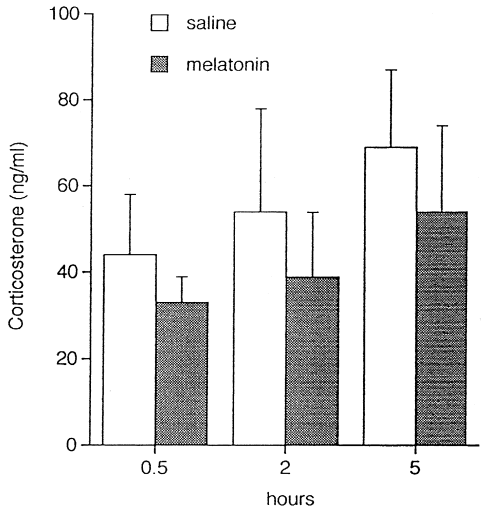

Fig. 2. Effect of melatonin on serum corticosterone levels. Melatonin (5 mg/kg, s.c.) was administered and mice bled 30 min, 2 h and 5 h later. Data are means ± S.D. (5 mice/group).

at the following times after lipopolysaccharide injection: 1 h later for TNF, 1.5 h for corticosterone and 3 h for interleukin-1 and interleukin-6 determinations. Bilateral adrenalectomy was performed under ether anesthesia 7–10 days before the experiments. Adrenalectomized mice were given 0.9% sodium chloride in drinking water as supportive care. Hypophysectomy was performed as previously described (Falconi and Rossi, 1964). Hypophysectomized mice were given 5% glucose in drinking water as supportive care.

For lipopolysaccharide lethality studies, female, adult (18–20 g) Balb/C mice were given lipopolysaccharide as a single dose of 160 μg/mouse i.p., an LD_{100} in our experimental conditions, and survival was assessed daily (Benigni et al., 1995). This mouse strain was chosen for its higher susceptibility to the lethal effect of lipopolysaccharide.

When indicated, lipopolysaccharide was injected intracerebroventricularly (i.c.v.) via a 28-gauge needle into ether anesthetized mice (Haley and McCormick, 1957; Lipton et al., 1991) at the dose of 2.5 μg/mouse in a final volume of 20 μl and TNF was measured in brain homogenate 90 min later as previously described and expressed as ng/g tissue (Faggioni et al., 1995).

2.3. Peripheral blood mononuclear cells preparation

Human peripheral blood mononuclear cells were obtained from the buffy coats of blood donations from normal, healthy volunteers as previously described (De Boer et al., 1981). Cells were suspended in RPMI 1640 medium with 10% fetal calf serum (HyClone, Logan, UT) at the concentration of 2.5×10^6/ml and plated (0.2 ml/well) in 96-well tissue culture plates (Falcon, Lincoln Park, NJ) with 10 ng/ml of lipopolysaccharide in the presence or absence of the test compounds at the indicated concentration. After 4 h culture, TNF was measured in the supernatants.

2.4. Antioxidant activity

The deoxyribose assay for hydroxyl radicals was performed as described earlier (Halliwell et al., 1987). Briefly, the reaction was carried out in a total volume of 1 ml of potassium phosphate buffer (20 mM, pH 7.4) containing deoxyribose (5 mM), $FeCl_3$ (100 μM), EDTA (104 μM), hydrogen peroxide (1 mM), ascorbate (100 μM) and the test compound in various amounts. Reaction mixtures were incubated at 37°C for 1 h. Thiobarbituric acid (1 ml of 1% w/v) was then added, plus 1 ml of 2.8% (w/v) trichloroacetic acid. The tubes were heated for 30 min at 100°C, cooled and absorbance was measured at 532 nm against the appropriate blanks.

2.5. Miscellaneous assays

Corticosterone was measured by radioimmunoassay, using an antiserum obtained from Sigma (C-8784) and following the manufacturer's directions.

Interleukin-1, interleukin-6 and TNF were measured by commercially available enzyme-linked immunosorbent as-

Table 2
Effect of melatonin on lipopolysaccharide- or interleukin-1-induced corticosterone

Experiment 1		Experiment 2	
treatment	corticosterone (ng/ml)	treatment	corticosterone (ng/ml)
Saline	28 ± 19	saline	34 ± 15
Melatonin	42 ± 26	melatonin	64 ± 51
Lipopolysaccharide	273 ± 89[a]	interleukin-1	136 ± 33[a]
Melatonin/lipopolysaccharide	243 ± 29[a]	melatonin/interleukin-1	130 ± 38[a]

Mice were treated with lipopolysaccharide (160 μg/mouse, i.p.) or interleukin-1 (100 ng/mouse, i.v.) and bled at 2 h for corticosterone determination. When indicated, melatonin was given (5 mg/kg, s.c.) 30 min before interleukin-1 or lipopolysaccharide. Data are means ± S.D. (5 mice/group).
[a] $P < 0.01$ versus saline alone by Dunnett's test.

say kits (Genzyme, Cambridge, MA) according to the manufacturer's instructions.

3. Results

3.1. Effect of melatonin on cytokine production and endotoxic shock in mice

As shown in Fig. 1, melatonin, 5 mg/kg, s.c., simultaneously with lipopolysaccharide (160 μg/mouse, i.p.) markedly protected mice from the lethal effect of lipopolysaccharide ($P < 0.05$ by Fisher's exact test).

We then studied the effect of melatonin on serum levels of TNF, interleukin-1 and interleukin-6 in lipopolysaccharide-treated mice, 1 h after lipopolysaccharide for TNF and 3 h for interleukin-1 and interleukin-6, previously reported to be the optimal time-points for these cytokines (Evans et al., 1989). As shown in Table 1 melatonin markedly inhibited serum TNF (by 80% in the experiment shown) but did not affect interleukin-1 and interleukin-6 levels.

3.2. Effect of melatonin on TNF production in the brain

The effect of melatonin (5 mg/kg, s.c., 30 min before lipopolysaccharide) on brain TNF levels induced by i.c.v.-injected lipopolysaccharide (2.5 μg/mouse) was evaluated 90 min after lipopolysaccharide administration. Melatonin significantly inhibited the induction of brain TNF (lipopolysaccharide alone, 35 ± 4.3 ng/g; melatonin/lipopolysaccharide, 22.3 ± 3.7 ng/g; $P < 0.01$ by Tukey's test).

3.3. Effect of melatonin on hypothalamus–pituitary–adrenal axis and its activation by lipopolysaccharide or interleukin-1

Fig. 2 shows the serum corticosterone levels in mice after a subcutaneous (s.c.) injection of saline or 5 mg/kg melatonin. It can be seen that no corticosterone elevation was induced by melatonin at any time point.

We next studied the effect of melatonin on the elevation of serum corticosterone induced 2 h after an injection of lipopolysaccharide (160 μg/mouse, i.p.) or interleukin-1 (100 ng/mouse, i.v.). As shown in Table 2, melatonin did not change the corticosterone response to lipopolysaccharide or interleukin-1. Also, no effect of melatonin was observed with a lower dose of lipopolysaccharide (2.5 μg/mouse, i.p.) or at a different time point (5 h) (data not shown). No significant difference was observed between mice treated with saline or with melatonin alone.

3.4. Effect of melatonin on TNF production in adrenalectomized or hypophysectomized mice

In the experiments shown in Table 3, the effect of melatonin (5 mg/kg, s.c., 30 min before lipopolysaccharide) was studied on lipopolysaccharide-induced serum TNF production in normal, adrenalectomized or hypophysectomized mice. In adrenalectomized or hypophysectomized mice a sublethal dose (80 μg/mouse, i.p.) of lipopolysaccharide was used. In all cases, melatonin significantly reduced serum levels, despite the high levels induced by lipopolysaccharide in adrenalectomized or hypophysectomized mice.

3.5. Antioxidant activity of melatonin and its precursors and their effect on TNF production in vivo and vitro

The antioxidant activity of melatonin, *N*-acetyl-5-hydroxytryptamine, 5-methoxytryptamine and 5-HT was tested as the scavenging of hydroxyl radicals generated by a mixture of ascorbic acid, water and ferric-EDTA (Halliwell et al., 1987). The sugar deoxyribose is degraded on exposure to hydroxyl radicals, setting off a series of reactions that eventually result in the formation of malondialdehyde detected as a pink chromogen. Fig. 3 shows the antioxidant capability of the various compounds, measured as inhibition of deoxyribose degradation (the antioxidant, dimethylsulfoxide, 10% v/v, was included as a positive control). As expected, 5-HT and *N*-acetyl-5-hydroxytryptamine were at least 10-fold less active than melatonin or 5-methoxytryptamine.

In the next series of experiments, these compounds were tested for their ability to inhibit serum TNF production in lipopolysaccharide-treated mice. As shown in Fig.

Table 3
Effect of melatonin on lipopolysaccharide-induced serum TNF in normal, adrenalectomized or hypophysectomized mice

Treatment	TNF (ng/ml)		
	normal mice	adrenalectomized mice	hypophysectomized mice
Lipopolysaccharide	32.5 ± 9.1	489 ± 16	1269 ± 672
Melatonin/lipopolysaccharide	12.9 ± 5.8^{b}	112 ± 34^{a}	362 ± 273^{b}

Mice were treated with lipopolysaccharide (normal mice: 160 μg/mouse, i.p.; adrenalectomized or hypophysectomized mice, 80 μg/mouse, i.p.) and bled 90 min later for serum TNF determination. When indicated, melatonin was given (5 mg/kg, s.c.) 30 min before lipopolysaccharide.
Data are means ± S.D. (5 mice/group). No TNF was detectable (< 15 pg/ml) in mice treated with saline alone or with melatonin alone.
[a] $P < 0.05$, [b] $P < 0.01$ versus lipopolysaccharide alone by Student's *t*-test.

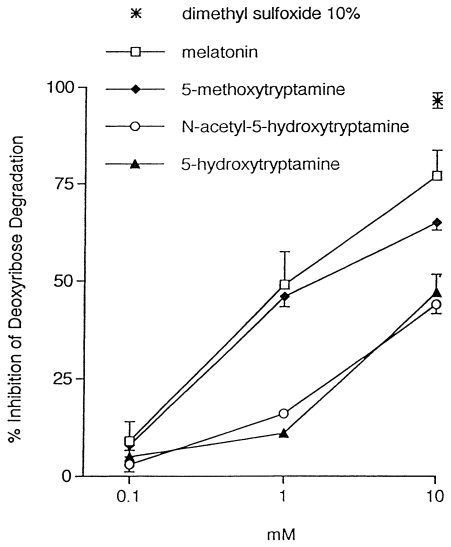

Fig. 3. Antioxidant activity of melatonin and other indolamines in vitro. The antioxidant activity was assayed by measuring the release of thiobarbituric acid-reactivity (A_{532}) from deoxyribose. Data are expressed as the percent inhibition of control activity (no drugs, 100% degradation) and are the means ± S.D. of triplicate samples.

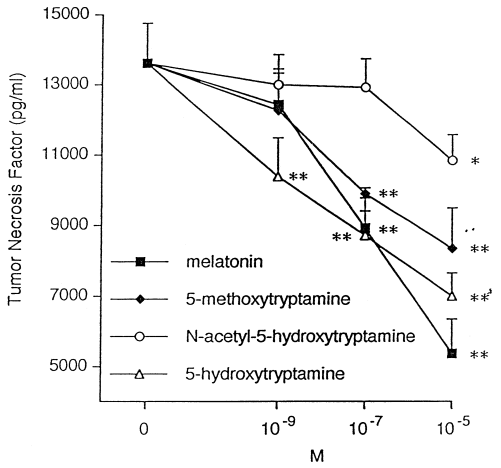

Fig. 5. Effect of melatonin and other indolamines on lipopolysaccharide-induced TNF production in vitro. Human peripheral blood mononuclear cells from one donor were cultured for 4 h with lipopolysaccharide (1 μg/ml) and the indicated concentration of the test compound. At the end of incubation, TNF produced was measured in the supernatant. Data are means ± S.D. from triplicate samples. $^{**}P < 0.01$ versus lipopolysaccharide alone by Tukey's test.

4, all the compounds except *N*-acetyl-5-hydroxytryptamine inhibited TNF production. It should be noted that 5-HT has been reported to inhibit TNF production via 5-HT-1 and 5-HT-2 receptors (Artz et al., 1991). Similar results were obtained when the different compounds were tested in an in vitro model of TNF production, using human peripheral blood mononuclear cells. Fig. 5 shows the result obtained with human peripheral blood mononuclear cells from one blood donor and identical data were obtained for a second donor.

We then chose two of these compounds, *N*-acetyl-5-hydroxytryptamine (which is not an antioxidant and does not inhibit TNF production) and 5-methoxytryptamine (which is an antioxidant and inhibits TNF) and tested them in the endotoxic shock model in vivo described in Fig. 1. As shown in Fig. 6, 5-methoxytryptamine, but not *N*-acetyl-

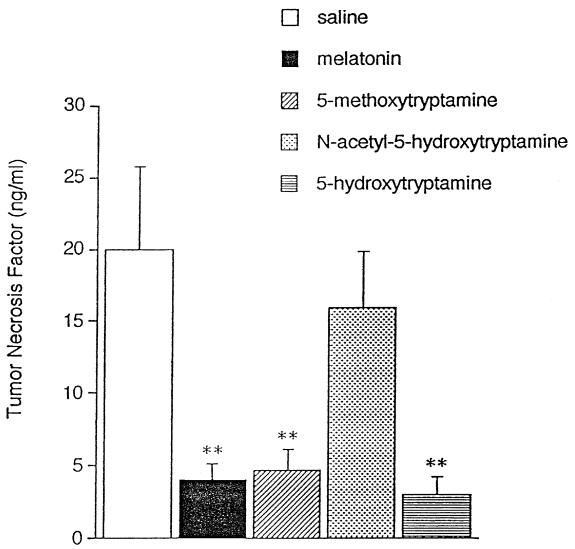

Fig. 4. Effect of melatonin and other indolamines on lipopolysaccharide-induced TNF production in vivo. Mice were treated with lipopolysaccharide (160 μg/mouse, i.p.) and bled 1 h later for serum TNF determination. When indicated, melatonin or the other indolamines were given s.c. (0.02 nmol/kg, corresponding to 5 mg melatonin/kg) 30 min before lipopolysaccharide. Data are means ± S.D. (5 mice/group). No TNF was detectable (< 15 pg/ml) in mice treated with saline alone or with melatonin alone. $^{**}P < 0.01$ versus lipopolysaccharide alone by Tukey's test.

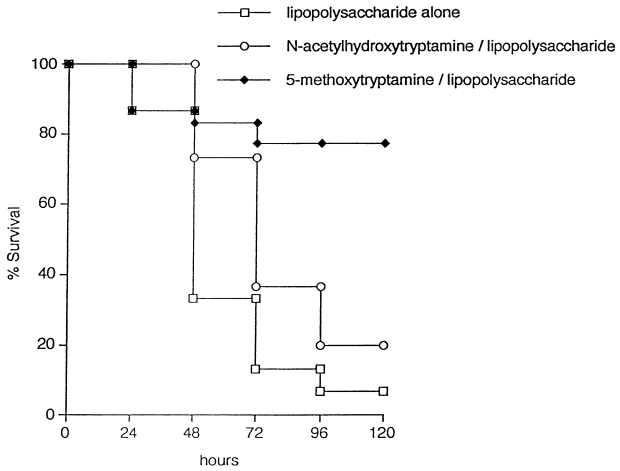

Fig. 6. Balb/C mice were treated with lipopolysaccharide (160 μg/mouse, i.p.) with or without a simultaneous injection of 5-methoxytryptamine or *N*-acetyl-5-hydroxytryptamine (at the doses of 4.9 and 4.7 mg/kg, s.c., respectively), survival was assessed daily and expressed as a percentage (15 mice/group). Animals were followed for up to 7 days and neither were further deaths observed nor did surviving mice look sick in this period. $^{*}P < 0.05$ versus lipopolysaccharide alone by Fisher's exact test (analysis was done on day 4).

5-hydroxytryptamine (at the doses of 4.9 and 4.7 mg/kg, s.c., respectively, equimolar to 5 mg/kg of melatonin), shared the protective effect of melatonin, thus paralleling the results for the effects on TNF production and antioxidant activity.

4. Discussion

The present study showed that melatonin has a protective effect on endotoxic shock, in agreement with previous studies (Maestroni, 1996). We found that this effect was associated with inhibition of TNF production. This might well explain the protective activity of melatonin, since anti-TNF antibodies and TNF inhibitors are fully protective in this experimental model (Beutler et al., 1985; Gadina et al., 1991). The effect of melatonin appears to be specific for TNF since two other inflammatory cytokines, interleukin-1 and interleukin-6, were not affected.

The effect of melatonin is independent of the hypothalamus–pituitary–adrenal axis. In fact, melatonin neither increases corticosterone levels nor potentiates the increase induced by lipopolysaccharide or interleukin-1. More important, adrenalectomized or hypophysectomized mice are equally susceptible to melatonin's inhibitory effect on TNF production.

Our results support the hypothesis that melatonin inhibits TNF production through its antioxidant activity.

In fact, various antioxidants were reported to inhibit the synthesis of TNF, both in vivo and in vitro (Chaudri and Clark, 1989; Peristeris et al., 1992; Eugui et al., 1994). We previously observed that the antioxidant, *N*-acetylcysteine, inhibits TNF production in lipopolysaccharide-treated mice, without affecting the production of interleukin-1 or interleukin-6 (Peristeris et al., 1992), thus with the same pattern as melatonin. Interestingly, melatonin also inhibits the transcription factor, nuclear factor-κB (NF-κB) (Chuang et al., 1996), as do other antioxidants (Schreck et al., 1992).

While it is difficult to definitively prove that melatonin inhibits TNF production through its antioxidant activity, the experiments with structurally related precursors of melatonin biosynthesis further support this theory. In fact, the ability of the various compounds to inhibit TNF production, both in vivo and in vitro, correlated with their antioxidant activity. The different antioxidant potency of these compounds reported here is in agreement with a previous report identifying the 5-methoxy group as essential for the antioxidant activity (Tan et al., 1993).

The only exception to the correlation observed between inhibition of TNF production and antioxidant activity was represented by 5-HT that, although having low antioxidant activity, was a potent inhibitor of TNF production. However, 5-HT was already reported to inhibit TNF production through the 5-HT receptors, as its inhibitory effect was antagonized by methysergide and ketanserin (Artz et al., 1991). Thus we cannot exclude that the 5-HT receptors might mediate the inhibition of TNF production for other, structurally related molecules also. However, this may not be the case for melatonin as various authors have shown that it does not bind to 5-HT receptors (Dubocovich, 1995; Popova and Dubocovich, 1995; Brzezinski, 1997).

The relatively high dose of melatonin required to inhibit TNF production in vivo, as well as its ability to directly inhibit TNF production in an in vitro system further support the possibility that melatonin does not act through the hypothalamus–pituitary–adrenal axis, suggesting that its antioxidant activity might play a primary role in the effect observed.

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