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Melatonin effects on inhibition of thirst and fever induced by lipopolysaccharide in rat

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Abstract

In 24 h water deprived rats we have evaluated the effects of melatonin on the inhibition of thirst and on fever induced by *Escherichia coli* lipopolysaccharide. Intraperitoneal (i.p) injection of lipopolysaccharide (0.32, 0.64 and 0.96 mg/kg) alone induced, a dose-dependent and significant inhibition of water intake as well as fever. In addition, lipopolysaccharide at the same concentrations increased urinary prostaglandins and serum cytokines levels. On the contrary, lipopolysaccharide treatment had no effects on cerebral brain nitric oxide synthase activity. All lipopolysaccharide effects were reverted by a prior, concomitant and subsequent i.p. treatment with melatonin (2, 4 and 6 mg/kg), whereas they were still present when melatonin was injected in combination with the melatonin receptor antagonist luzindole (15, 30 and 60 mg/kg, i.p.). We suggest that melatonin could exert its dipsogenic effects through a reduction of the free radical nitric oxide ($NO^{\cdot}$) whereas it may reduce body temperature by preventing an excessive formation of prostaglandins and cytokines.

Keywords: Melatonin; Lipopolysaccharide; Water intake; Fever; Nitric oxide (NO)

1. Introduction

Bacterial *Escherichia coli* endotoxin (lipopolysaccharide) is regarded as a complex glycolipid localized in the outer membrane of gram negative bacteria (Morrison and Ryan, 1987). Lipopolysaccharide, either injected or generated during the course of gram negative infections participates in several pathophysiological conditions such as fever (Derijk et al., 1993), somnogenic activity (Krueger, 1990), inhibition of water (Calapai et al., 1994; Nava et al., 1996) and food intake (O'Reilly et al., 1988), as well as shock (Tracey et al., 1987) and death (Tracey et al., 1987). Despite extensive studies, the mechanism of action of lipopolysaccharide has yet to be fully elucidated (Sugino et al., 1989; Ulevitch and Tobias, 1995). Several lines of investigations now suggest that lipopolysaccharide primary action in the brain may be mediated by an enhancement of various cytokines and autacoid factors (e.g., nitric oxide (NO)) concentration (Kent et al., 1992; Hopkins and Rothwell, 1995; Rothwell and Hopkins, 1995; Nava et al., 1996). It has also been proposed that lipopolysaccharide stimulates lipid peroxidation and oxidative damage, through an increased production of reactive oxygen intermediates (Sakaguchi et al., 1981; Sugino et al., 1989; Yoshikawa et al., 1994). In particular, many of the tissue injures induced by lipopolysaccharide seem to be mediated by an overproduction of reactive oxygen, such as superoxide anion ($^1O_2^-$), hydroxyl radical ($OH^{\cdot}$), hydrogen peroxide (H_2O_2) and peroxynitrite anion ($ONOO^-$) (Nathan, 1982; Bautista and Spitzer, 1990; Lipton et al., 1993). It has been suggested that lipopolysaccharide may promote brain formation of free radicals, cytokines and NO and, therefore, may play an important role as a mediator of endotoxic injury (Breder et al., 1994; Yirmiya, 1996).

Melatonin, the main hormone of the pineal gland, is cyclically released overnight in response to light–dark environmental alterations (Reiter, 1991). The pineal hormone is synthesized from serotonin by an initial *N*-acetyla-

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tion followed by methylation of 5-hydroxy moiety by hydroxyindole-*O*-methyltransferase (Reiter, 1991). Serotonin *N*-acetyl-transferase is the first enzyme that catalyses the melatonin synthesis and its activity increases during dark cycle (Borjigin et al., 1995). Melatonin effects are mediated by a family of G protein coupled receptors (Reppert et al., 1996) which are distributed in various areas of the brain, including suprachiasmatic nuclei, mediobasal hypothalamus, preoptic area, thalamus, cerebral cortex and retina (Krause and Dubocovich, 1991; Stankov et al., 1992; Reppert et al., 1996). In addition, non-neuronal sites containing melatonin receptors are present in the hypophysal pars tuberalis and cerebral and caudal arteries (Reppert et al., 1996). Evidence exists in literature pointing out that melatonin plays a modulatory role in physiological functions such as circadian rhythms (Sumova et al., 1995), reproduction (Sumova et al., 1995), sleep (Dijk et al., 1995), mood (Dollins et al., 1994; Thalen et al., 1995), body temperature (Dollins et al., 1994; Thalen et al., 1995), behavioural performance (Dollins et al., 1994) and aging (Pierpaoli and Regelson, 1994; Reiter, 1995a). On the other hand, melatonin has a therapeutic role in anxiety, affective disorders, cancer and endotoxic shock (Reiter, 1991; Reiter, 1995b; Reiter et al., 1995; Maestroni, 1996). Furthermore, melatonin is a potent scavenger of free radicals and stimulates other antioxidant activities by preventing hydroxyl ($OH^{\cdot}$) and peroxyl radical ($ROO^{\cdot}$) formation (Tan et al., 1993; Reiter, 1995a,b; Reiter et al., 1995). An effect of melatonin on lipopolysaccharide induced oxidative damage has also been reported (Sewerynek et al., 1995a,b,c). Melatonin antioxidant properties seem to be due to an increase in cerebral glutathione peroxidase levels (Barlow Walden et al., 1995) and inhibition of brain nitric oxide synthase activity (Pozo et al., 1994; Betthai et al., 1996).

We and others have previously shown that peripheral administration of lipopolysaccharide inhibits water intake and promotes fever by increasing brain release of cytokines, autacoid factors and free radicals (Hesse et al., 1988; Derijk et al., 1993; Calapai et al., 1994; Nava et al., 1996) (Fig. 1). In the present study we have examined the eventual effects of antioxidant properties of melatonin on lipopolysaccharide-induced thirst and fever, in 24 h water deprived rats.

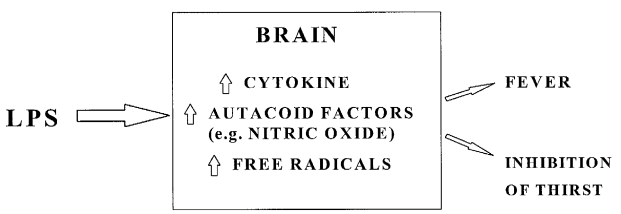


Fig. 1. Peripheral lipopolysaccharide (LPS) administration induces high levels of cytokines, autacoid factors and free radicals in the brain. These and other substances could be responsible of central effects such as inhibition of water intake and fever observed in response to the lipopolysaccharide stimulus.

2. Material and methods

2.1. Animals

Adult male Sprauge-Dawley rats weighing 280–320 g were used. Animals were housed at the constant temperature of 25 ± 1°C, subjected to a 12/12 h light dark cycle (light on at 06.00) and with free access to purina rat chow pellets and tap water. During the light period, the light intensity at the level of the cage was about 250–300 lx. All experiments were carried out in the dark cycle using a red dim light (Kodak, filter A).

2.2. Water intake and rectal temperature evaluation

Thirst was elicited by 24 h water deprivation. During the same period animals had free access to food. At the end of the water deprivation, animals were tested for water intake by measuring the volume of water (ml/rat) drunk over a 60 min period. Water was provided in graduated burettes with drinking spouts allowing direct volumetric measurement of intake to the nearest 0.1 ml.

Rectal temperature was recorded using an Elektrolaboratoriet thermometer type T.E. 3. Temperature was allowed to calibrate for 15–30 s before reading was taken. All measurements were performed at room temperature (25 ± 1°C). Water intake and rectal temperature were measured at the same schedule time after drug injections (see Section 2.6).

2.3. Nitric oxide synthase activity determination

Nitric oxide synthase activity in the frontal cortex, diencephalon and brainstem was determined. Animals were decapitated under general anaesthesia (ether) and the brain rapidly removed. Brain areas (cortex, diencephalon and brainstem) were rapidly dissected, storaged in aluminium foil and frozen in liquid nitrogen. Thereafter, tissues were homogenized at 4°C in 4 volumes of *N*-[2-hydroxyethyl]piperazine-*N*-[2-hydroxypropanesulfonic acid] (HEPES) buffer 20 mM, pH 7.2. The composition of the buffer fluid was sucrose (320 mM), DL-dithiotreitol (1 mM), soybean trypsin inhibitor (10 μg/ml), aprotinin (2 μg/ml) and leupeptin (10 μg/ml). The homogenates were centrifuged at $100\,000 \times g$ for 30 min at 4°C. The supernatants (i.e., the cytosolic fractions containing nitric oxide synthase activity) were stored at −70°C until use. The protein concentration of the cytosolic fraction was measured spectrophotometrically according to Bradford (1976), using a standard bovine serum albumin (BSA).

As previously shown (Salter et al., 1991) nitric oxide synthase activity was determined by measuring the rate of conversion of L-[U-^{14}C]arginine to [U-^{14}C]citrulline and expressed as nmol/min per g tissue. Aliquots of cytosolic fraction (100 μg of protein) were preincubated for 5 min at 37°C in 50 mM potassium phosphate buffer (pH 7.2)

Table 1
Water intake (ml/rat over a 60 min period) and rectal temperature (°C) after intraperitoneal (i.p.) administration of saline (1 ml/kg) or lipopolysaccharide (LPS) (0.32, 0.64 and 0.96 mg/kg) alone (given 180 min before tests), saline (1 ml/kg) or lipopolysaccharide (0.32, 0.64 and 0.96 mg/kg) (given 180 min before tests) + saline (1 ml/kg) or melatonin (2, 4 and 6 mg/kg) (given 240, 180, 120 and 60 min before tests) and saline (1 ml/kg) or lipopolysaccharide (0.96 mg/kg) + saline (1 ml/kg) or melatonin (2, 4 and 6 mg/kg) (given 240, 180, 120 and 60 min before tests) + saline (1 ml/kg) or luzindole (15, 30 and 60 mg/kg) (given 300, 240, 180 and 120 min before tests)

300, 240, 180, 120 min before testing	180 min before testing	240, 180, 120, 60 min before testing							
		saline (1 ml/kg)		melatonin (2 mg/kg)		melatonin (4 mg/kg)		melatonin (6 mg/kg)	
		water intake	rectal temp.	water intake	rectal temp.	water intake	rectal temp.	water intake	rectal temp.
—	saline	12.04 ± 0.16	36.84 ± 0.11	12.04 ± 0.18	36.66 ± 0.18	12.12 ± 0.27	36.62 ± 0.08	12.06 ± 0.18	36.60 ± 0.11
—	LPS 0.32	1.68 ± 0.13 [a]	38.42 ± 0.08 [a]	2.98 ± 0.31 [a]	38.06 ± 0.11 [a]	4.70 ± 0.23 [a]	37.96 ± 0.11 [a]	5.56 ± 0.15 [a]	37.60 ± 0.06 [a]
—	LPS 0.64	1.06 ± 0.01 [a]	38.72 ± 0.08 [a]	2.58 ± 0.14 [a]	38.44 ± 0.08 [a]	3.70 ± 0.15 [a]	38.32 ± 0.08 [a]	4.34 ± 0.11 [a]	37.90 ± 0.12 [a]
—	LPS 0.96	0.10 ± 0.02 [a]	39.06 ± 0.16 [a]	1.92 ± 0.13 [a]	38.64 ± 0.15 [a]	2.20 ± 0.15 [a]	38.70 ± 0.14 [a]	2.80 ± 0.15 [a]	38.46 ± 0.02 [a]
Saline	saline	12.20 ± 0.15	36.40 ± 0.06	12.18 ± 0.08	36.38 ± 0.08	12.34 ± 0.11	36.36 ± 0.11	12.14 ± 0.11	36.40 ± 0.06
Luzindole 15	LPS 0.96	0.12 + 0.04 [a]	38.98 + 0.16 [a]	0.88 ± 0.16 [a]	38.72 ± 0.08 [a]	1.10 ± 0.01 [a]	38.32 ± 0.08 [a]	2.02 ± 0.14 [a]	38.66 ± 0.08 [a]
Luzindole 30	LPS 0.96	0.10 + 0.0[7]	38.94 + 0.13 [a]	0.14 ± 0.05 [a]	39.06 ± 0.11 [a]	0.10 ± 0.07 [a]	39.00 ± 0.90 [a]	0.06 ± 0.90 [a]	38.94 ± 0.13 [a]
Luzindole 60	LPS 0.96	0.14 + 0.05 [a]	38.99 + 0.11 [a]	0.10 ± 0.07 [a]	39.04 ± 0.08 [a]	0.14 ± 0.05 [a]	38.96 ± 0.11 [a]	0.10 ± 0.07 [a]	38.92 ± 0.16 [a]

[a] $P < 0.01$ versus controls (ANOVA and Bonferroni *t*-test).

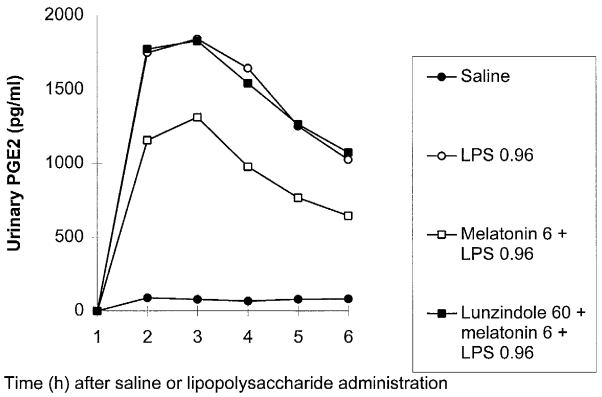


Fig. 2. Effects of intraperitoneal (i.p) administration of saline (1 ml/kg), lipopolysaccharide (LPS) (0.96 mg/kg), melatonin (6 mg/kg) + lipopolysaccharide (0.96 mg/kg) and luzindole (60 mg/kg) + melatonin (6 mg/kg) + lipopolysaccharide (0.96 mg/kg) on urinary prostaglandin E_2 levels.

containing L-valine (60 mM), nicotinamide adenine dinucleotide phosphate (NADPH) (120 μM), L-citrulline (1.2 mM), $MgCl_2$ (1.2 mM) and $CaCl_2$ (0.24 mM) in the presence of drug or vehicle. Samples were then incubated for 10 min at 37°C with L-[U-^{14}C]arginine (150 000 dpm) and L-arginine (0.24 μM). The reaction was stopped by the addition of 1.0 ml of a H_2O/Dowex-50W (200–400, 8% cross-linked Na^+ form) mixture (1:1, v/v). The Na^+ form of Dowex-50W (ion exchange resin) was prepared by washing the H^+-form of the resin four times in 1 M NaOH and several times in bi-distilled water until pH was less than 7.5. The resin was settled by centrifugation (11 000 × *g* for 3 min) in a microfuge (Beckman, Microfuge 11) and a supernatant aliquot was taken for scintillation counting (4 ml Pico-Aqua; Packard 7500). The activity of the brain Ca^{2+}-dependent nitric oxide synthase was determined by measuring the difference between the concentration of L-[U-^{14}C]arginine and [U-^{14}C]citrulline produced in control samples containing 1 mM of ethylene-glycol-*bis*-(β-aminoethyl ether)-tetraacetic acid (EGTA). The activity of the brain Ca^{2+}-independent nitric oxide synthase was determined from the difference between the L-[U-^{14}C]arginine and [U-^{14}C]citrulline produced in samples containing 1 mM EGTA and that produces in samples containing 1 mM EGTA plus 1 mM N^G-monomethyl-L-arginine (L-NMMA). Each experiment was performed in duplicate.

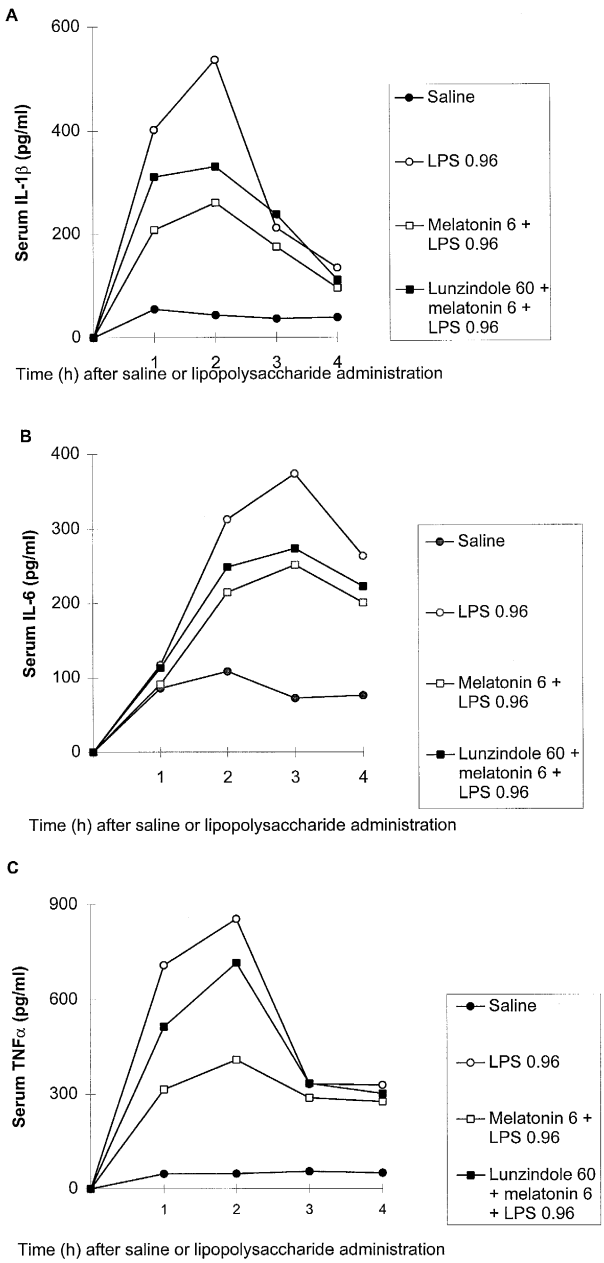
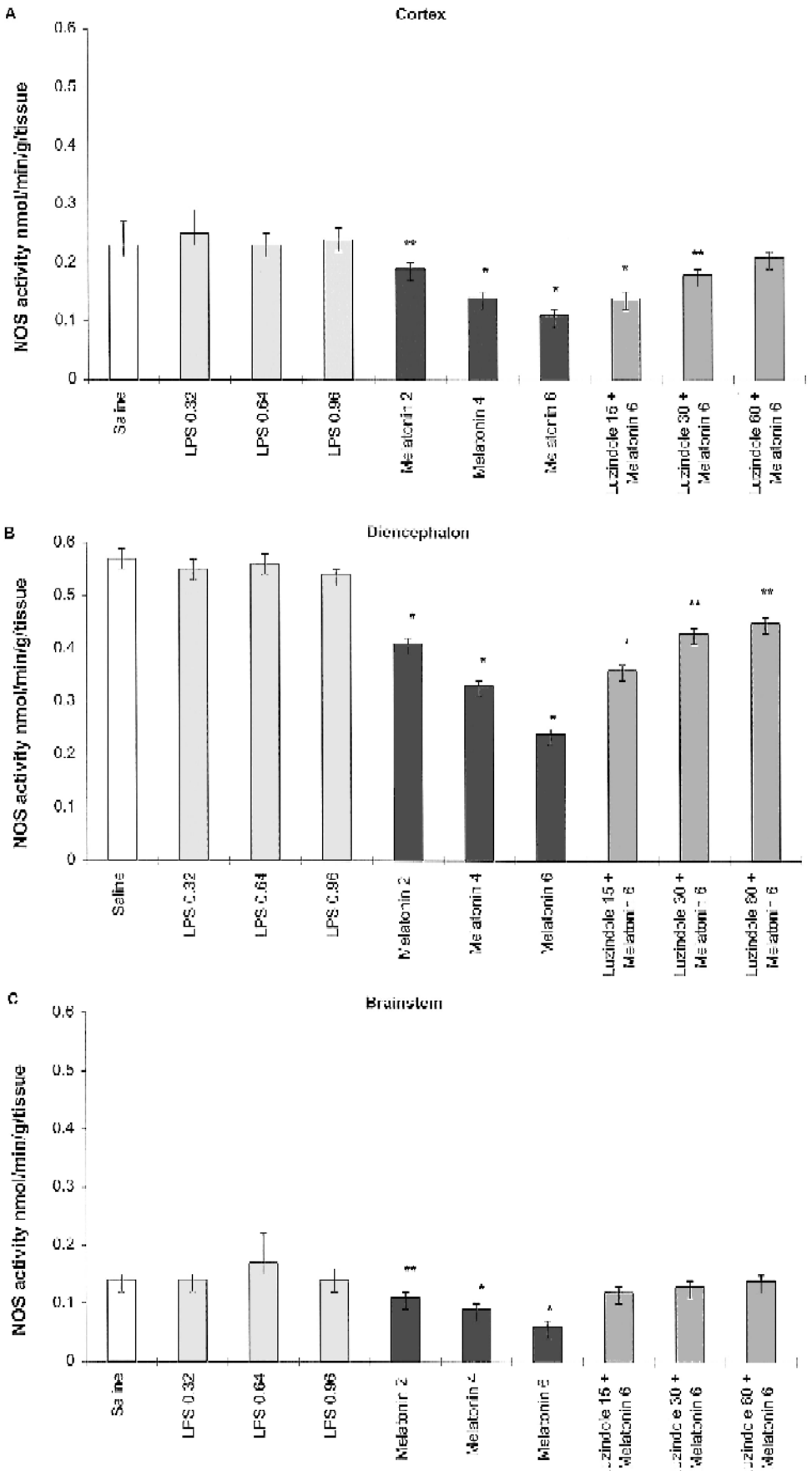


Fig. 3. Effects of intraperitoneal (i.p.) administration of saline (1 ml/kg), lipopolysaccharide (LPS) (0.96 mg/kg), melatonin (6 mg/kg) + lipopolysaccharide (0.96 mg/kg) and lunzindole (60 mg/kg) + melatonin (6 mg/kg) + lipopolysaccharide (0.96 mg/kg) on serum interleukin-1β (A), interleukin-6 (B) and tumor necrosis factor-α (C) levels. Each point represents the mean ± S.D. of five animals. S.D. were always smaller than symbol size. Experiments were performed in duplicate 1, 2, 3 and 4 h after saline or lipopolysaccharide administration. $P < 0.01$ versus controls (1–4 h) (ANOVA and Bonferroni *t*-test).

Fig. 4. Effects of intraperitoneal (i.p.) administration of saline (1 ml/kg), lipopolysaccharide (LPS) (0.32, 0.64 and 0.96 mg/kg), melatonin (2, 4 and 6 mg/kg) and luzindole (15, 30 and 60 mg/kg) + melatonin (6 mg/kg) on the activity of nitric oxide synthase measured in cortex (A), diencephalon (B) and brainstem (C). Each column represents the mean ± S.D. of five animals. The experiments were performed in duplicate. For schedule time of drug injections see text. * $P < 0.01$ versus controls; ** $P < 0.05$ versus controls (ANOVA and Bonferroni *t*-test).


A
Cortex
NOS activity nmol/min/g/tissue
0.6
0.5
0.4
0.3
0.2
0.1
0
Saline
LPS 0.32
LPS 0.64
LPS 0.96
Melatonin 2
Melatonin 4
Melatonin 6
Luzindole 15 + Melatonin 6
Luzindole 30 + Melatonin 6
Luzindole 60 + Melatonin 6
B
Diencephalon
NOS activity nmol/min/g/tissue
0.6
0.5
0.4
0.3
0.2
0.1
0
Saline
LPS 0.32
LPS 0.64
LPS 0.96
Melatonin 2
Melatonin 4
Melatonin 6
Luzindole 15 + Melatonin 6
Luzindole 30 + Melatonin 6
Luzindole 60 + Melatonin 6
C
Brainstem
NOS activity nmol/min/g/tissue
0.6
0.5
0.4
0.3
0.2
0.1
0
Saline
LPS 0.32
LPS 0.64
LPS 0.96
Melatonin 2
Melatonin 4
Melatonin 6
Luzindole 15 + Melatonin 6
Luzindole 30 + Melatonin 6
Luzindole 60 + Melatonin 6

2.4. Prostaglandin E_2 determination

Urinary levels of prostaglandin E_2 were determined by means of a specific enzyme immuno assay (EIA) method (Amersham Italia, Milan, Italy). Each experiment was performed in duplicate.

2.5. Interleukin-1β, interleukin-6 and tumor necrosis factor-α evaluation

The serum concentration of interleukin-1β, interleukin-6 and tumor necrosis factor-α were measured by using an enzyme-linked immunosorbent assay (ELISA) (Genzyme Cinisello Balsamo, Milan, Italy). Each experiment was performed in duplicate.

2.6. Pharmacological treatments

Different groups of rats were injected i.p. as follows:

(1) Saline (1 ml/kg) or lipopolysaccharide (0.32, 0.64 and 0.96 mg/kg) alone: 180 min before tests (i.e., water intake, rectal temperature and nitric oxide synthase activity determinations).

(2) Saline (1 ml/kg) or lipopolysaccharide (0.32, 0.64 and 0.96 mg/kg) (180 min before tests) in combination with saline (1 ml/kg) or melatonin (2, 4 and 6 mg/kg) (240, 180, 120, 60 min before tests).

(3) Saline (1 ml/kg) or lipopolysaccharide (0.32, 0.64 and 0.96 mg/kg) (180 min before tests) in combination with saline (1 ml/kg) or melatonin (2, 4 and 6 mg/kg) (240, 180, 120, 60 min before tests) and saline (1 ml/kg) or luzindole (15, 30 and 60 mg/kg) (300, 240, 180, 120 min before tests).

In different groups of rats (treated as above) urinary prostaglandin E_2 and serum interleukin-1β, interleukin-6 and tumor necrosis factor-α concentrations were measured every hour (up to 6 h) after saline or lipopolysaccharide treatment.

2.7. Drugs

Escherichia coli lipopolysaccharide (055: B5 phenol extract) and melatonin were purchased from Sigma (St. Louis, MO, USA), whereas luzindole was obtained from Tocris Cookson (London, UK).

Lipopolysaccharide was dissolved in 0.9% NaCl and warmed to 37°C, immediately before use. Melatonin was pre-dissolved in absolute ethanol and subsequently diluted in 0.9% NaCl (the final concentration of ethanol was 1%), immediately before use. Luzindole was dissolved in an amount of ethanol (95%) and additional dilutions were done in 0.9% NaCl, immediately before use.

L-[U-^{14}C]arginine (specific activity 304 mCi/μmol) was obtained from Amersham (Amersham, Amersham, UK).

2.8. Statistical analysis

Data are expressed as $\pm$ standard deviation (S.D.) of the mean. Statistical analysis of data was performed by one way analysis of variance (ANOVA) followed by Bonferroni *t*-test.

3. Results

3.1. Effects on water intake, rectal temperature, urinary prostaglandin E_2 and serum interleukin-1β, interleukin-6 and tumor necrosis factor-α levels

After 24 h water deprivation, an injection of lipopolysaccharide (0.32, 0.64 and 0.96 mg/kg; i.p.; $n = 5$ for each group of treated rats), administered 180 min before tests, significantly ($P < 0.01$ and 0.05) and in a dose dependent way, induced inhibition of water intake (measured as ml/rat over a 60 min period) (Table 1) and increased body temperature (°C) (Table 1), as well as growth urinary prostaglandin E_2 (Fig. 2) and serum interleukin-1β, interleukin-6 and tumor necrosis factor-α levels (Fig. 3).

Melatonin (2, 4 and 6 mg/kg; i.p.; $n = 5$ for each group of treated rats), administered 240, 180, 120 and 60 min before tests, inhibited all the lipopolysaccharide-induced effects, in a significant ($P < 0.01$) and dose dependent manner (Table 1; Figs. 1 and 2). The protective effects of melatonin disappeared following a pre-treatment with the melatonin receptor antagonist luzindole, administered i.p. (15, 30 and 60 mg/kg) 60 min prior of each melatonin injection (Table 1; Figs. 2 and 3).

3.2. Effects on nitric oxide synthase activity in brain

In 24 h water deprived rats lipopolysaccharide (0.32, 0.64 and 0.96 mg/kg; i.p.), administered 180 min before test, did not modify the concentrations of Ca^{2+}-dependent nitric oxide synthase activity in all the brain areas tested (i.e., cortex, diencephalon and brainstem) (Fig. 4). By contrast, melatonin (2, 4 and 6 mg/kg; i.p.), administered 240, 180, 120 and 60 min before test, significantly and dose dependently, inhibited nitric oxide synthase activity (Fig. 4). This effect was prevented by luzindole (15, 30 and 60 mg/kg; i.p.) administered 60 min prior of each melatonin injection (Fig. 4).

In all groups studied brain Ca^{2+}-independent nitric oxide synthase activity was not detectable.

4. Discussion

We believe that melatonin effects on lipopolysaccharide-induced inhibition of thirst may be due to a reduction of $NO^{\cdot}$ levels in diencephalon and also in the central areas which exerts a controlling role on thirst (e.g., preoptic area). Our findings also reinforce previous investigations showing that $NO^{\cdot}$ acts in the central nervous system as an antidipsogenic agent (Calapai et al., 1992; Calapai et al., 1994). Since there is evidence that prostaglandins and pyrogenic cytokines are not implicated in the inhibition of

thirst induced by lipopolysaccharide (Masotto et al., 1992; Calapai et al., 1994; Nava et al., 1996), it is possible that $NO^{\cdot}$ may directly mediate the antidipsogenic actions of lipopolysaccharide. This is supported by our previous data indicating that N^{G}-nitro-L-arginine methyl ester (L-NAME), a selective NOS inhibitor and methylene blue, an inhibitor of guanylate cyclase activity, given into the preoptic area, antagonize in a specific manner the lipopolysaccharide-induced inhibition of drinking (Calapai et al., 1994). We also believe that melatonin could contribute to reduce the inhibition of thirst induced by lipopolysaccharide through its antioxidant and free radical scavenging properties (Reiter, 1995a,b; Gilad et al., 1997).

The results showing that melatonin may reduce fever induced by lipopolysaccharide are in agreement with previous investigations (Deacon and Arendt, 1995). In particular, it has been suggested that melatonin is involved in thermoregulatory mechanisms by acting on the tone of the caudal arteries and those arteries that form the circle of Willis at the base of brain (Viswanathan et al., 1990). In view of our present results, we also support the idea that melatonin may affect body temperature by preventing an excessive formation of urinary prostaglandins and serum cytokines levels (Deacon and Arendt, 1995). In fact, in the present study melatonin was found to control the increase in urinary prostaglandin E_2 and serum interleukin-1β, interleukin-6 and tumor necrosis factor-α levels which were observed after lipopolysaccharide treatment. However, since it has been demonstrated that lipopolysaccharide may induce fever by enhancing the release of pyrogenic cytokines and a subsequent (cytokines-mediated) increase in prostaglandins (prostaglandin E_2 and prostaglandin $F_{2\alpha}$) concentrations (Kluger, 1991; Coceani et al., 1992; Klir et al., 1993), it is not unlikely to speculate that melatonin could prevent lipopolysaccharide-induced fever through, primarily, an inhibition of cytokines, rather than prostaglandins release.

In conclusion, we believe that melatonin effects on inhibition of lipopolysaccharide thirst could be due to a reduced production of the free radical and potent antidipsogenic factor $NO^{\cdot}$, whereas melatonin antifebrile effects may be mediated by a decrease of urinary prostaglandins and serum cytokines levels. However, based on our observations and also considering that large concentrations of melatonin were used in the present study, we are unable to conclude whether additional mechanisms are involved in such melatonin-induced effects. Nevertheless, we can exclude a direct relationship between melatonin-induced antifebrile and dipsogenic actions because our previous studies have demonstrated that fever is not a real impediment in drinking behaviour (Nava et al., 1996). Finally, we further support the idea that melatonin may exert neuroprotective actions (Melchiorri et al., 1995; Uz et al., 1996) and suggest that it could play a significant protective role in a variety of diseases in which the cellular damage arises from the production of free radicals.

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