N-acetylserotonin reduces lipopolysaccharide-induced lipid peroxidation *in vitro* more effectively than melatonin

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Abstract

OBJECTIVE: Bacterial lipopolysaccharide (LPS) causes lipid peroxidation (LPO). We have found that LPS induces LPO *in vitro*, in tissue homogenates in a concentration-dependent manner, the concentration of 400 μg/ml demonstrating the most efficient lipid damaging effect .
Both melatonin and its precursor, N-acetylserotonin, must possess antioxidant activities, both in vivo or *in vitro*, however, following some claims, N-acetylserotonin is a more effective extra- and intracellular antioxidant than melatonin.
The aim of our study was to compare the effects of melatonin and N-acetylserotonin on the LPS-induced LPO *in vitro*.
METHODS: Malondialdehyde (MDA) plus 4-hydroxyalkenal (4-HDA) concentrations were measured as the indices of induced membrane peroxidative damage in brain, liver and kidney homogenates. Both melatonin and N-acetylserotonin were used at increasing concentrations, starting from 0.01–5 mM, together with LPS at one concentration level of 400 μg/ml.
RESULTS: In all the examined tissues, LPS stimulated LPO, while both melatonin and N-acetylserotonin decreased LPS-stimulated LPO. Furthermore, the capacity of N-acetylserotonin reducing LPO was higher than that of melatonin.
CONCLUSIONS: The results of the reported study clearly indicate that N-acetylserotonin is a much stronger antioxidant *in vitro* than melatonin in terms of reducing oxidative damage to lipid membranes. However, it remains still unclear how the features relate to in vivo circumstances.

ORIGINAL ARTICLE

INTRODUCTION

LPS, a lipopolysaccharide from Gram-negative bacteria and an endotoxin, induces peroxidation of lipids (Kheir-Eldin *et al.* 2001; Portoles *et al.* 1993; Yoshikawa *et al.* 1994) and, consequently, causes oxidative damage in many tissues (Okabe *et al.* 1994; Sewerynek *et al.* 1995a; 1995b; 1995c). For example, exogenous administration of LPS causes lung and liver lipid peroxidation, indirectly manifested by increased levels of malonaldehyde (MDA) and conjugated dienes (Nowak *et al.* 1993; Kouno *et al.* 1994; Sewerynek *et al.* 1995d). In many *in vivo* and *in vitro* animal models, a rela-

tionship has been demonstrated between LPS administration and the resulting overproduction of reactive oxygen species, including superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$) and nitric oxide ($NO^{\bullet}$). In several model systems, free radicals have been postulated to be important mediators of tissue injury (Bautista & Spitzer 1990; Jiang-Shieh *et al.* 2005; Shuter *et al.* 1990; Silva *et al.* 2004; Yoshikawa *et al.* 1994). An administration of antioxidants decreases LPS-induced biochemical and physiological changes.

Melatonin is effective in neutralizing a number of oxygen-based and nitrogen-based toxic agents,, some of which are radicals, while other are related metabolities (Allegra *et al.* 2003; Gitto *et al.* 2009; Peyrot & Ducrocq 2008; Reiter *et al.* 2009; Tengattini *et al.* 2008). Melatonin detoxifies the highly potent $^{\bullet}OH$ (Poeggeler *et al.* 1994; Stasica *et al.* 1998; Tan *et al.* 1998; Tan 1993), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl), $O_2^{\bullet-}$, $NO^{\bullet}$, peroxynitrite anion ($ONOO^-$) and other free radical scavengers (Matuszak *et al.* 2003; Mei *et al.* 2005; Tan *et al.* 2000; Ximenes *et al.* 2005; Zavodnik *et al.* 2004). The pineal hormone is highly lipophilic and quite hydrophilic as well and it readily enters subcellular compartments (Menendez-Pelaez & Reiter 1993). Melatonin *in vivo* also protects against oxygen toxicity induced by paraquat, a toxic herbicide (Melchiorri *et al.* 1996b; Melchiorri *et al.* 1998), carbon tetrachloride (Daniels *et al.* 1995), potassium bromate (Karbownik *et al.* 2006), adriamycin (Dabrowska *et al.* 2008), amyloid beta (Masilamoni *et al.* 2008) and aflatoxin B1 (Gesing & Karbownik-Lewinska 2008). The protective effect of melatonin was also observed after LPS-induced oxidative damage in many tissues (Sewerynek *et al.* 1995b; Sewerynek *et al.* 1995a; Sewerynek *et al.* 1995c; Sewerynek *et al.* 1996; Chen *et al.* 2006; Tamura *et al.* 2009). Additionally, melatonin has been reported to decrease lipid peroxidation in rats in L-thyroxin-induced thyrotoxicosis (Wiktorska *et al.* 2010; Wiktorska *et al.* 2005) and after pharmacological doses of iodine (Sewerynek *et al.* 2006; Swierczynska-Machura *et al.* 2004).

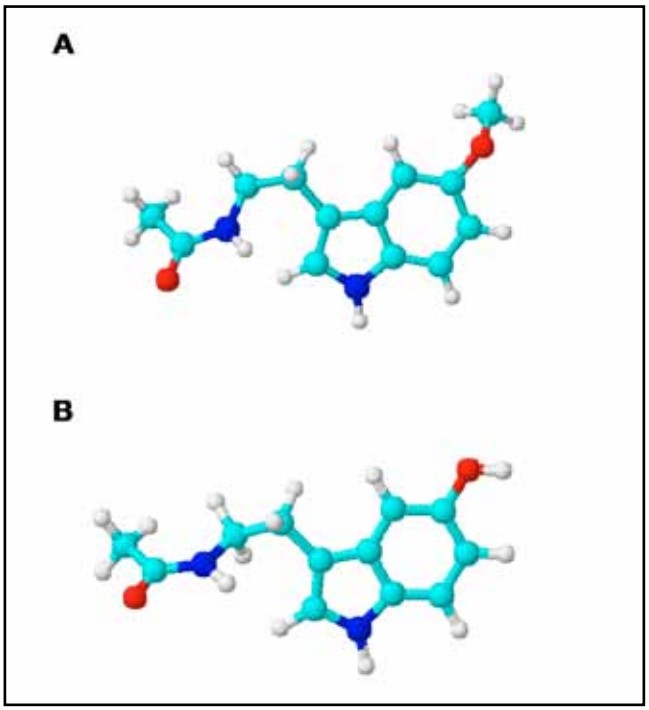

Fig. 1. Biochemical scheme of melatonin (**A**) and N-acetylserotonin (**B**).

Several indole products exhibit antioxidative properties, including N-acetylserotonin, an immediate melatonin precursor (Behrends *et al.* 2007; Poeggeler *et al.* 2002; Qi *et al.* 2000; Keithahn & Lerchl 2005; Gavazza & Catala 2004). N-acetylserotonin, compared to melatonin, has an additional hydroxy group in position 5 of the indole ring (Figure 1). The concentrations of both indoles diminish with age (Miguez *et al.* 1998). Both melatonin and N-acetylserotonin exert protective effects against free radical-mediated damage, induced by xenobiotics (Bachurin *et al.* 1999; Calvo *et al.* 2001). There has been some evidence, suggesting that N-acetylserotonin may be a more effective extra- and intracellular antioxidant than melatonin (Wolfler *et al.* 1999).

The ability of melatonin and N-acetylserotonin to protect against LPS-induced toxicity *in vitro* was tested in the reported study. The concentration of malonaldialdehyde (MDA) plus that of 4 hydroxyalkenals (4-HDA) in liver, kidney and brain homogenates were used as an index of induced oxidative damage to lipid membranes.

MATERIALS AND METHODS

Reagents

Lipopolysaccharide (LPS) (from *Escherichia coli*, serotype 0111:B4), melatonin, N-acetyserotonin and ferrous sulfate were obtained from Sigma-Aldrich, St. Louis, US). A Bioxytech LPO-586 kit, purchased from the Cayman Chemical (Ann Arbor, MI), was used in measurements of lipid peroxidation products. All the other reagents were of the highest, available quality.

Methods

Six groups, of 6 adult Wistar male rats (200 ± 40 g BW) in each, were housed in plexi cages with 3 animals per cage. The animal rooms were windowless with automatic temperature (22 ± 1 °C) and lighting control (light on at 07.00 h and off at 21.00 h; 14 h light/10 h dark). The rats received standard laboratory chow and water *ad libitum*. Melatonin and N-acetylserotonin were dissolved in absolute ethanol (when added to tissue homogenates, the final concentration of alcohol was 1%). LPS was dissolved in 20 mM Tris-HCl buffer, pH 7.4.

Tissue preparation and assays

The rats were anesthesized with ether and decapitated. Livers, kidneys and brains were removed, frozen and kept at –80 °C until homogenate preparation. On the

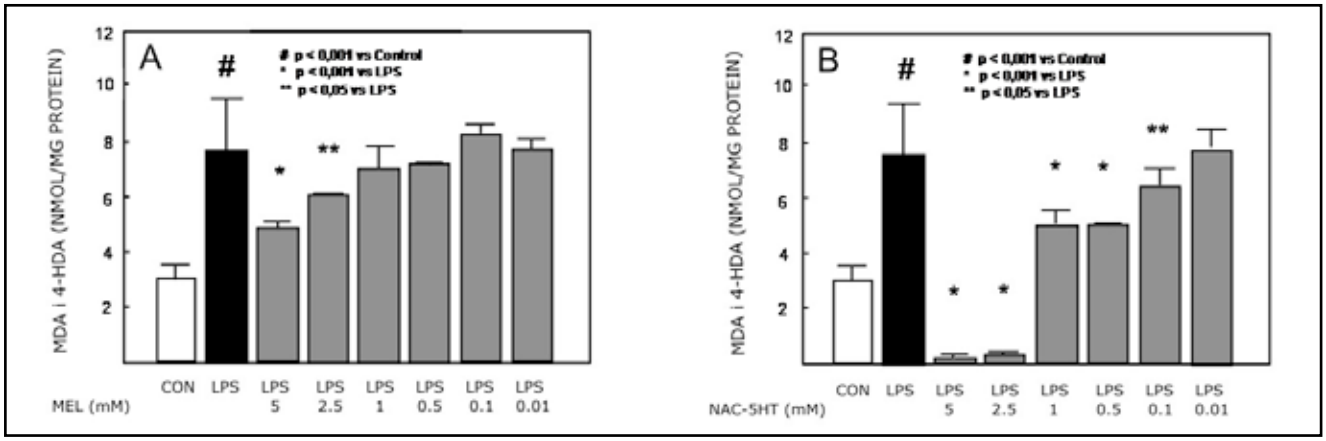

Fig. 2. Effects of different concentrations of melatonin (A) and N-acetylserotonin (B) (0.01–5 mM) on lipid peroxidation, induced by lipopolysaccharide (LPS; 0.4 g/mL) in brain homogenates. The values are means ± SEM. #p<0.001 vs. the control group (without LPS and melatonin); *p<0.001; **p<0.05 vs. the LPS group without melatonin.

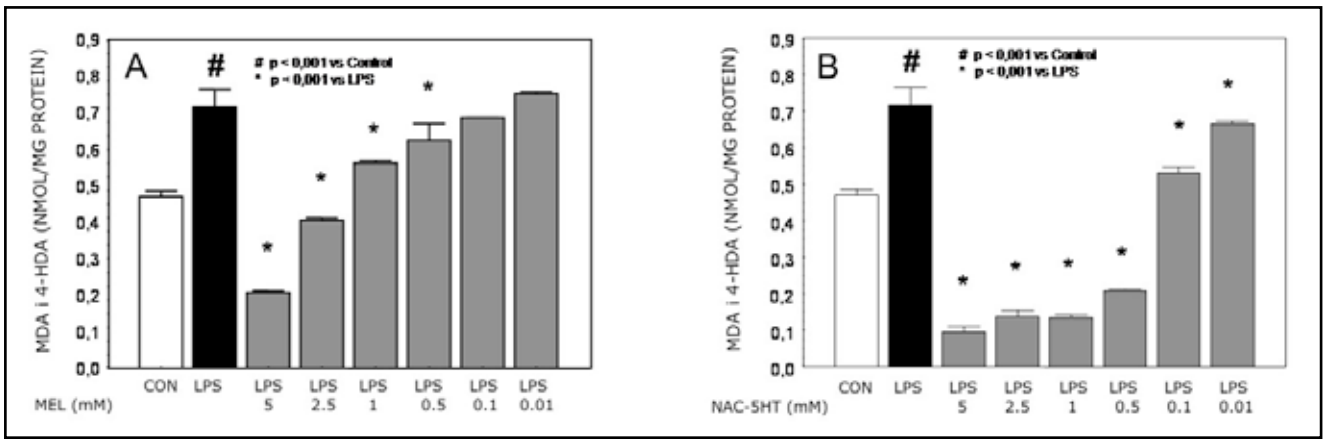

Fig. 3. Effects of different concentrations of melatonin (A) and N-acetylserotonin (B) (0.01 – 5mM) on lipid peroxidation, induced by lipopolysaccharide (LPS; 0.4 g/mL) in kidney homogenates. The values are means ± SEM. #p<0.001 vs. the control group (without LPS and melatonin); *p<0.001 vs. the LPS group without melatonin.

day of assay, the tissues were homogenized in ice-cold 20 mM Tris-HCl buffer, pH 7.4, with a Polytron-like stirrer to produce a 1/10 homogenate. The homogenates of each tissue were incubated in a water bath at 37 °C. LPS was used at concentration of 400 µg/ml and after 30-minute incubation (Sewerynek *et al.* 1995c). Melatonin and N-acetylserotonin (0.01–5 mM) were used in combination with LPS (400 µg/ml). Liver homogenates were supplemented with $FeSO_4$ (10 µM) to stimulate lipid peroxidation (Sewerynek *et al.* 1995c). After incubation, the homogenates were centrifuged at 2500 × g for 5 min at 4 °C. The supernatant was collected and immediately assayed for lipid peroxidation products: MDA+4-HDA, which are commonly used lipid peroxidation indices (Esterbauer & Cheeseman 1990). A Bioxytech LPO-586 kit was used for this purpose (Melchiorri *et al.* 1996a). This kit takes advantage of a cromogenic reagent, which reacts with MDA and 4-HDA at 45°C, yielding a stable chromophore with a maximal absorbance at the 586 nm wavelength. The light wavelength and the low temperature of incubation (45 °C), used in the procedure, eliminated interference and undesirable artifacts. Protein concentrations were determined by Branford's method, using bovine serum albumin as standard (Bradford 1976).

Statistical analyses

The data were analyzed, using the one-way analysis of variance (ANOVA) and student's t-test. If F values were significant, the Student-Newman-Keuls t-test was used. All the calculations were performed with the use of the Statistica '99 computer software. The level of significance was accepted at p<0.05.

RESULTS

LPS stimulated lipid peroxidation in all the studied tissue homogenates (Figures 2–4). Both melatonin and N-acetylserotonin were effective in reducing the stimulatory effect of LPS on lipid peroxidation in each tissue (Figures 2–4). Melatonin concentrations, required for significant reduction of induced lipid peroxidation were: 2.5 and 5 mM, regarding brain homogenates, 0.5–5 mM for kidney homogenates, and 0.01–5 mM

Tab. 1. Efficacy of lipid peroxidation inhibition by Melatonin and N-acetylserotonin in brain, kidney and liver homogenates.

Tissue	Concentration	Melatonin (MEL)		N-acetyloserotonin (NAC-5HT)		Comparison
		Mean (%)	SEM	Mean (%)	SEM	
BRAIN	5 mM	41.13	3.41	96.79	0.23	$p<0.001$
	2.5 mM	27.69	4.04	95.27	0.26	$p<0.001$
	1 mM	11.93	5.49	38.03	3.70	$p<0.01$
	0.5 mM	14.42	4.78	39.35	3.39	$p<0.01$
	0.1 mM	–0.64	5.72	19.61	4.83	$p<0.05$
	0.01 mM	6.02	5.37	8.24	8.93	Not significant
LIVER	5 mM	94.05	0.23	96.16	0.14	$p<0.001$
	2.5 mM	91.48	0.41	94.49	0.23	$p<0.001$
	1 mM	80.26	0.44	92.84	0.11	$p<0.001$
	0.5 mM	61.95	0.91	91.26	0.15	$p<0.001$
	0.1 mM	30.75	2.34	65.49	0.48	$p<0.001$
	0.01 mM	14.86	1.89	20.13	1.27	Not significant
KIDNEY	5 mM	72.59	0.39	88.02	0.54	$p<0.001$
	2.5 mM	44.73	0.81	85.32	0.40	$p<0.001$
	1 mM	20.01	1.12	80.08	0.38	$p<0.001$
	0.5 mM	10.42	1.85	69.89	0.42	$p<0.001$
	0.1 mM	0.99	1.37	20.76	1.25	$p<0.001$
	0.01 mM	–3.05	1.43	2.64	1.36	$p<0.05$

for liver homogenates. N-acetylserotonin concentrations, required for significant reduction of induced lipid peroxidation was 0.1–5 mM in brain homogenates, 0.01–5 mM for kidney homogenates and 0.01–5 mM for liver homogenates.

The results clearly show that the used antioxidants decreased LPS-stimulated lipid peroxidation in all the examined tissues. Furthermore, lipid peroxidation suppression, exerted by N-acetylserotonin in brain and kidney homogenates was higher than that exerted by melatonin (Figures 5–7) (see Table 1).

DISCUSSION

LPS, a Gram-negative bacterial endotoxin, induces a variety of biological responses and diseases (Berdeaux 1993). Some of LPS effects result from anaphylatoxin production, while other ones involve the release of biochemical mediators, including histamine, serotonin, kinins and platelet-activating factors by the reticuloendothelial system. Additionally, LPS directly inhibits both glucose and lipid metabolism, activates protein kinase C, stimulates proinflammatory mediators (cytokines, Nuclear Factor – kappaB), causes lipid peroxidation via the induction of free radical formation and directly induces cellular damage (Berdeaux 1993; Li *et al.* 2005).

Lipid peroxidation plays a significant role in oxidative pathology. Some relationship has been demonstrated between LPS administration and the overproduction of free radicals (Li *et al.* 2005; Yoshikawa *et al.* 1994). Several radical products, including $O_2^{\bullet -}$ and the $^{\bullet}OH$, as well as other toxic oxygen metabolites, including H_2O_2 and HOCl, have been postulated to be important mediators in models of tissue injury (Bautista & Spitzer 1990; Shuter *et al.* 1990). LPS also stimulates nitric oxide synthase (NOS), the enzyme which catalyzes L-arginine oxidation to citrulline and $NO^{\bullet}$ (Jiang-Shieh *et al.* 2005). NO congeners are either neuroprotective or neurodestructive, depending on NO redox states (Lipton *et al.* 1993). $NO^{\bullet}$-mediated neurotoxicity is, in part, a consequence of its reaction with $O_2^{\bullet -}$, leading to $ONOO^-$ formation, i.e., a highly toxic substance. Also, at low concentrations of L-arginine, LPS stimulates the production of $O_2^{\bullet -}$ and H_2O_2, the effect being blocked by N^w-nitro-L-arginine, a selective NOS blocker (Sessa 1994).

There are several enzyme systems in tissues with a number of cellular components that protect them against activated oxygen forms, those protective elements including superoxide dismutase (SOD), catalase, reduced glutathione (GSH) and glutathione peroxidase (GSH-Px), vitamin E and ascorbic acid. Antioxidative enzyme activities have been measured both *in vivo* and *in vitro*, following LPS-administration. When hepatocyte monolayers are treated with LPS (50 μg/ml for 2 h), the endotoxin induces lipid peroxidation (Portoles

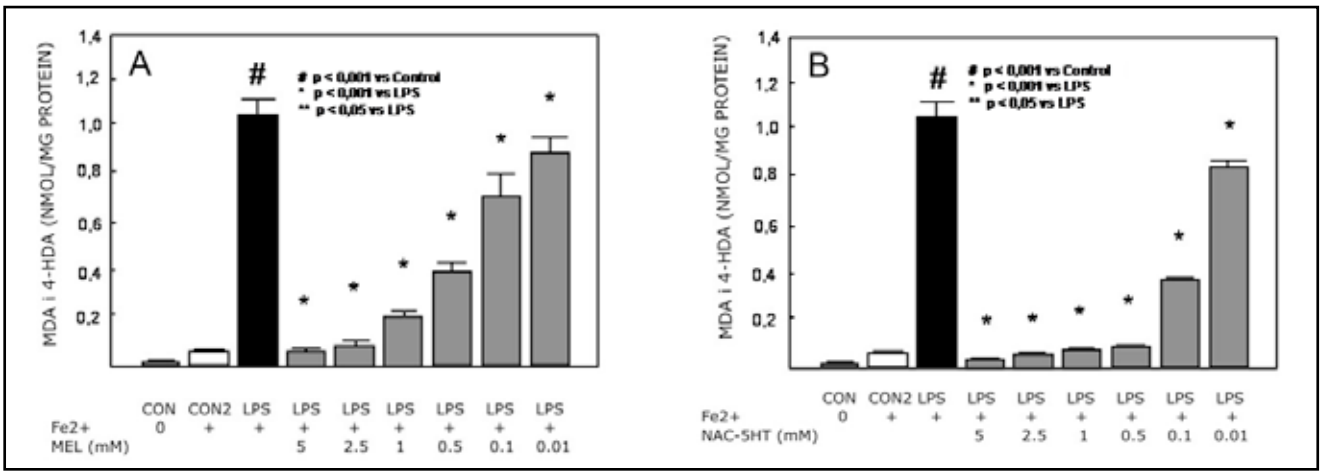

Fig. 4. Effects of different concentrations of melatonin (A) and N-acetylserotonin (B) (0.01–5 mM) on lipid peroxidation, induced by lipopolysaccharide (LPS; 0.4 g/mL) liver homogenates. The values are means SEM. #p<0.001 vs. the control group (without LPS and melatonin); *p<0.001;**p<0.05 vs. the LPS group without melatonin.

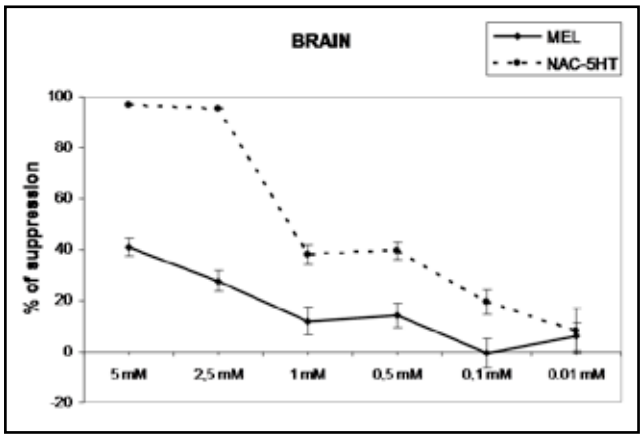

Fig. 5. Percentages of lipopolysaccharide (LPS)-induced lipid peroxidation suppression by melatonin and N-acetylserotonin in brain homogenates. The values are means (%) ± SEM.

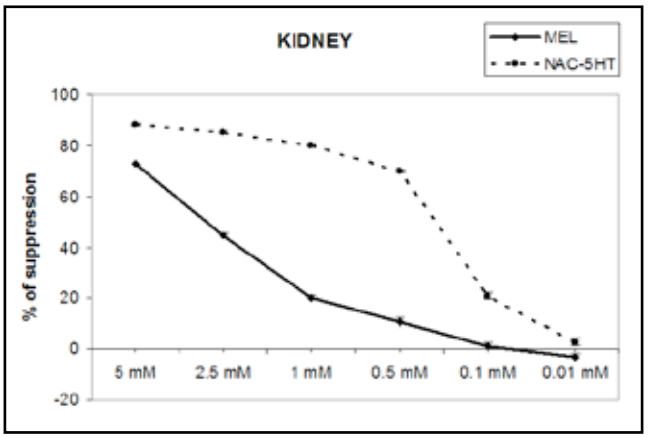

Fig. 6. Percentages of lipopolysaccharide (LPS)-induced lipid peroxidation suppression by melatonin and N-acetylserotonin in kidney homogenates. The values are means (%) ± SEM.

et al. 1993). At the same time, the authors reported an increased SOD activity, demonstrating a potentially protective role of this enzyme against the toxic effects of $O_2^{\bullet -}$. Additionally, a similar stimulatory action of SOD was found in astrocytes, cultured with LPS (1 μg/ml for 3 days) (Mokuno *et al.* 1994). In our previous paper, melatonin also enhanced GSH-Px in LPS-treated animals, GSH-Px being another important antioxidative enzyme (Sewerynek *et al.* 1995b). Additionally, LPS diminished cytochrome P450 reductase content, while that effect was largely prevented by nitric oxide synthase inhibitor: N-nitro-L-arginine methyl ester (L-NAME) administration. Melatonin did not change P450 content, either in phenobarbital- or LPS-treated animals. Thus, LPS induced, at least, two antioxidative enzymes, SOD and GSH-Px, which could possibly help protect tissues from LPS-induced oxidative stress.

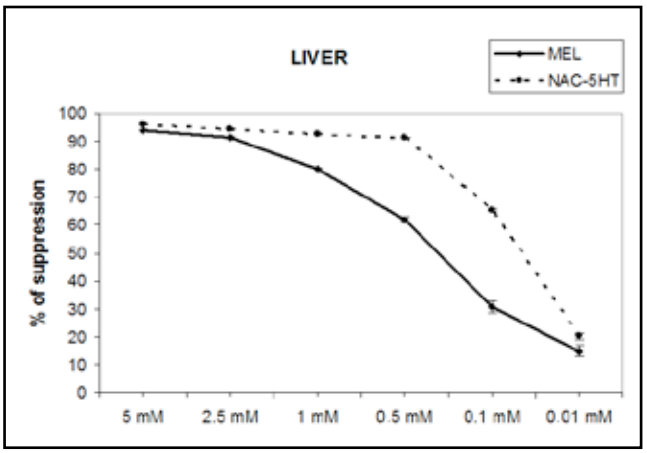

Fig. 7. Percentages of lipopolysaccharide (LPS)-induced lipid peroxidation suppression by melatonin and N-acetylserotonin in liver homogenates. The values are means (%) ± SEM.

In the present study, LPS was used in dose of 400 μg/ml and stimulated LPO production in all the examined tissues, what is conformable with our previous results (Sewerynek *et al.* 1995c). That concentration was used to study the capacity of melatonin and N-acetylserotonin to influence LPS-induced oxidative damage. The strongest LPO stimulation was observed in liver homogenates, when iron was added, what influenced •OH production (Sewerynek *et al.* 1995c).

Melatonin is reported to be a potent •OH radical scavenger (Poeggeler *et al.* 1994; Poeggeler *et al.* 2002; Tan 1993; Tan *et al.* 2005). Furthermore, being highly lipophilic and hydrophilic, it can potentially reach all body fluids, tissues and subcellular compartments. Melatonin, as a scavenger of free radicals, is supported by numerous studies in various models (Peyrot & Ducrocq 2008; Reiter *et al.* 2009; Vijayalaxmi *et al.* 1995; Vijayalaxmi *et al.* 2004). Moreover, N-acetylserotonin, the immediate melatonin precursor, exhibits antioxidative properties as well (Gavazza & Catala 2004; Keithahn & Lerchl 2005; Pless *et al.* 1999; Poeggeler *et al.* 2002; Qi *et al.* 2000). N-acetylserotonin, compared to melatonin, has a hydroxy group in position 5 of the indole ring.

The results of the reported study demonstrate that both melatonin and N-acetylserotonin effectively protect against LPS-induced toxicity. Bacterial LPS is both an endo- and exogenous toxin and induces oxidative damage via generation of free radicals (Kheir-Eldin *et al.* 2001; Portoles *et al.* 1993; Yoshikawa *et al.* 1994). One of the commonly measured consequences of its administration is an increased lipid peroxidation, as indicated by the levels of MDA or conjugated dienes (Cadenas *et al.* 1998; Kheir-Eldin *et al.* 2001).

In the present studies, melatonin and N-acetylserotonin depressed lipid peroxidation levels after LPS treatment of liver, kidney and brain homogenates in a concentration-dependent manner. In kidney and brain homogenates, the inhibitory effect of N-acetylserotonin in LPS-induced lipid peroxidation was higher than that of melatonin. When $FeSO_4$ was added to liver homogenates, a stronger lipid peroxidation followed; in that case, MDA+4HDA formation was markedly suppressed by the same concentrations of melatonin and N-acetylserotonin (0.01–5 mM). $FeSO_4$ is involved in •OH generation by the Fenton reaction (Muiras *et al.* 1993; Sewerynek *et al.* 1995d), what may be suggestive that the reduction of lipid peroxidation products, as observed in the reported study could, at least partially, have been due to the •OH scavenging capacity of both indoles.

In normal conditions, tissues have a functional antioxidative system, depleted during oxidative stress. Reduced glutathione is one of the most important endogenous antioxidants. In a previous paper (Sewerynek *et al.* 1995b), we reported that melatonin protected against LPS-induced toxicity in both the liver and the brain in phenobarbital-treated animals, as shown by reduced oxidized glutathione levels (GSSG) and stimulation of GSH-Px activity. When the animals were injected with LPS, the levels of total glutathione (tGSH) and GSSG were significantly higher, when compared with other groups, while melatonin and L-NAME significantly enhanced tGSH, when compared with that in the LPS-treated rats. Melatonin alone reduced GSSG levels.

In conclusion, the results of the reported study indicate that N-acetylserotonin is more effective from melatonin in reducing lipid peroxidation *in vitro*.

ACKNOWLEDGEMENTS

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