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Melatonin Administration Prevents Lipopolysaccharide-Induced Oxidative Damage in Phenobarbital-Treated Animals

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Abstract The protective effect of melatonin on lipopolysaccharide (LPS)-induced oxidative damage in phenobarbital-treated rats was measured using the following parameters: changes in total glutathione (tGSH) concentration, levels of oxidized glutathione (GSSG), the activity of the antioxidant enzyme glutathione peroxidase (GSH-PX) in both brain and liver, and the content of cytochrome P450 reductase in liver. Melatonin was injected intraperitoneally (ip, 4mg/kg BW) every hour for 4 h after LPS administration; control animals received 4 injections of diluent. LPS was given (ip, 4 mg/kg) 6 h before the animals were killed. Prior to the LPS injection, animals were pretreated with phenobarbital (PB), a stimulator of cytochrome P450 reductase, at a dose 80 mg/kg BW ip for 3 consecutive days. One group of animals received LPS together with N^{w}-nitro-L-arginine methyl ester (L-NAME), a blocker of nitric oxide synthase (NOS) (for 4 days given in drinking water at a concentration of 50 mM). In liver, PB, in all groups, increased significantly both the concentration of tGSH and the activity of GSH-PX. When the animals were injected with LPS the levels of tGSH and GSSG were significantly higher 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 and enhanced the activity of GSH-PX in LPS-treated animals. Additionally, LPS diminished the content of cytochrome P450 reductase with this effect being largely prevented by L-NAME administration. Melatonin did not change the content of P450 either in PB- or LPS-treated animals. In brain, melatonin and L-NAME increased both tGSH levels and the activity of GSH-PX in LPS-treated animals. The results suggest that melatonin protects against LPS-induced oxidative toxicity in PB-treated animals in both liver and brain, and the findings are consistent with previously published observations related to the antioxidant activity of the pineal hormone.

Key words: melatonin, glutathione, lipopolysaccharide, oxidative damage, oxygen free radicals, antioxidant, phenobarbital, cytochrome P450 reductase

INTRODUCTION

Bacterial lipopolysaccharide (LPS) induces oxidative damage mediated by increased production of reactive oxygen intermediates [Bernard et al., 1984; Ghezzi et al., 1986]. Treatment with LPS is associated with an increase in hepatic lipid peroxidation, one of the toxic consequences of oxidative damage [Peavy and Fairchild, 1986]. LPS also stimulates nitric oxide synthase (NOS) activity, the enzyme which catalyzes the oxidation of L-arginine to citrulline and nitric oxide (NO). At a low concentration of L-arginine, oxygen activation leads to the production of the superoxide anion (0_2^-) and hydrogen peroxide (H_2O_2) with this effect being blocked by the selective blocker of NOS, N^{w}-nitro-L-arginine [Mayer et al., 1992; Sessa, 1994]. Dysfunction of the L-arginine-NO pathway is a common feature in a number of diseases, including artherosclerosis, hypertension, diabetes, sepsis, and cerebral ischemia; the mechanism of action is very important for the understanding of their pathophysiology and treatment [Berdeaux, 1993].

Bacterial LPS, either injected or generated during gram-negative sepsis, initiates lymphocyte transformation, macrophage activation, and

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initiation of the complement, kinin, and blood coagulation pathways [Morrison and Ulevitch, 1978; Peavy et al., 1978; Morrison and Ryan, 1979]. After intravenous administration, LPS accumulates especially in the liver and spleen, organs rich in reticuloendothelial cells [Farrar and Corwin, 1966].

Oxidative damage is defined as a disturbance in the prooxidant and antioxidant balance within tissues [Sies, 1986]. The tripeptide glutathione (GSH) is an important endogenous antioxidant which is found in particularly high concentration in the liver [Meister and Anderson, 1983]. Oxidative stress inactivates superoxide dismutase, catalase and glutathione peroxidase (GSH-PX), the enzymes involved in detoxifying reactive oxygen intermediates such as O_2^- and H_2O_2 [Raes et al., 1987; Pigeolet and Remacle, 1991; Nistico et al., 1992]. GSH-PX plays a key role in the enzymatic defense system against oxygen free radicals generated in the presence of peroxides by reducing H_2O_2 and lipid peroxides with the concomitant oxidation of GSH [Raes et al., 1987]. GSH, present in all tissues, is oxidized to glutathione disulfide (GSSG). An intracellular increase in GSSG levels and an enhanced ratio of GSSG to GSH are used as indices of oxidative stress. GSH may act directly as an antioxidant and, furthermore, it may have a major role in restoring other free radical scavengers and antioxidants such as vitamins E and C to their reduced state [Reed, 1986; Ross, 1988].

It has been found that a precursor of GSH, *N*-acetylcysteine (NAC), protects against LPS toxicity and the inhibitor of GSH synthesis, DL-buthionine-(SR)-sulfoximine (BSO), has the opposite effect and decreases liver GSH levels [Bernard et al., 1984; Ghezzi et al., 1986]. Additionally, GSH-PX protects neurons from oxidative damage [Mirault et al., 1994] and has been demonstrated in the cytoplasm of glial cells in the area of an infarct [Takizawa et al., 1994]. Thus, GSH-PX seems to play either a protective role against lipid peroxidation after cerebral infarction or it may be involved in the healing process after ischemia. Evidence that GSH-PX activity is enhanced by lipid peroxide in peritoneal macrophages [Watanabe and Murakoshi, 1986] and liver cells [Watanabe, 1986] supports the above assumption.

Cytochrome P450 reductase plays a major role in the cytotoxicity of LPS. Cytochrome P450 reductase catalyzes the oxidative metabolism of endogenous and exogenous substances [Guengerich, 1991; Porter and Coon, 1991; Coon et al., 1992]. Specific molecular isoforms of P450 are induced by the exposure of animals to a variety of xenobiotics. Phenobarbital (PB) stimulates the CYP2B1/2 isoform, which is important in metabolism of drugs and environmental substances [Renaud et al., 1993; Karuzina and Archakov, 1994]. The activity of P450 is altered after infection or treatment with cytokines (interleukin-1; interferon-γ; and tumor necrosis factor-α) and immunostimulants such as LPS [Sessa, 1994; Katusic and Cosentino, 1994). LPS has been shown to diminish microsomal P450 activity and content in PB-treated animals [Khatsenko et al., 1993].

The pineal hormone, melatonin is a potent scavenger of free radicals and it may also stimulate other antioxidant activities [Reiter et al., 1994a,b]. Compared to GSH and mannitol, melatonin is more effective as a hydroxyl radical ($^{\cdot}OH$) scavenger and it seems to markedly protect DNA from oxidative damage induced by chemical carcinogens [Tan et al., 1993a]. Melatonin participates in many important physiological functions including the control of seasonal reproduction as well as influences on circadian rhythms and the immune system [Reiter, 1980, 1991]. Melatonin is highly lipophilic so it easily enters cells and gains access to every subcellular compartment. Recently, melatonin was characterized as an anti-aging, life-prolonging molecule [Maestroni et al., 1988; Pierpaoli et al., 1991]. In in vitro studies, melatonin has been shown to be both a $^{\cdot}OH$ [Reiter et al., 1993; Tan et al., 1993b] and a peroxyl radical scavenger [Pieri et al., 1994]. Melatonin has also been shown to stimulate the antioxidant enzyme GSH-PX, which metabolizes H_2O_2 to water. As animals age, they become relatively deficient in melatonin [Reiter, 1992; Reiter et al., 1994c], and the loss of melatonin may contribute to the increased oxidative stress associated with aging [Abe et al., 1994; Reiter et al., 1994b,c].

In this study, we investigated the possible protective role of melatonin on oxidative damage induced by LPS in PB-treated animals. The following parameters were measured: total GSH (tGSH), oxidized GSH (GSSG), and the activity of GSH-PX in brain and liver, and cytochrome P450 reductase content in liver.

MATERIALS AND METHODS

Materials

LPS (from *Escherichia coli,* serotype 0111: B4), N^{ω}-nitro-L-arginine (L-NAME), saturated

picric acid, NADPH tetrasodium salt, 5-5'-dithiobis(2-nitrobenzoicacid) (DTNB), reduced glutathione (GSH), and glutathione reductase (GSSG-R) were obtained from Sigma; 2-vinyl-piridin monomer was purchased from Fluka (Ronkonkoma, NY). Phenobarbital sodium (130 mg/ml) was purchased from Elkins-Sinn, Inc. Melatonin was obtained from Regis Chemical Company. All other chemicals used were of the highest purity commercially available.

Experimental Procedures

Male Sprague-Dawley rats (250 ± 20 g) were purchased from Harlan (Houston, TX). They were provided laboratory chow and water ad libitum. After 10 days of acclimation to a light: dark (LD) photoperiod (LD14:10, light on 0700–2100 daily), animals were treated with saline (control) or PB (all other groups) (the number of animals per group = 8). Phenobarbital was injected ip in saline, at a dose 80 mg/kg BW, given at 24 h intervals for 3 consecutive days. In 3 groups of PB-treated rats, LPS was administered as a single injection (4 mg/kg, BW) 6 h before the animals were killed; one group of animals also received L-NAME, while 2 other groups received melatonin. Rats received L-NAME in their drinking water at a concentration of 50 mM for 4 days before killing. Melatonin was injected into PB and PB + LPS groups of rats at a dose 4 mg/kg BW, once every hour for 4 hours (the last injection occured 1 h before the animals were killed). The animals were decapitated at 1800 h.

Tissue preparation and assays

Immediately after decapitation, the heart was perfused with phosphate buffer (pH 7.5). Liver and brain were removed and frozen on solid CO_2. All tissues were kept at −80°C until GSH, GSH-PX, and cytochrome P450 analysis. Hepatic microsomes were prepared as reported [Williams et al., 1984] and stored at −80°C until use. Protein concentrations were determined by method of Bradford (1976) using bovine serum albumin (BSA) as standard. Cytochrome P450 reductase content was estimated as described by Omura and Sato (1964) using carbon monoxide difference spectra (changes in absorbance between 450 and 490 μm) of liver microsomes. A portion of brain and liver was homogenized with a Potter-Elvehjem homogenizer (20 mM Tris–HCl buffer, pH 7.4, 1:10 w/v) at 0°C. The resulting liver or brain homogenates were centrifuged in a Beckman microcentrifuge at 100,000*g* for 60 min. The supernatant was collected and frozen to measure GSH-PX activity. GSH-PX was assayed at 30°C using the method of Jaskot et al. [1983]. For GSH analysis, a section of liver and brain was homogenized in a solution of 1% picric acid (1:5 w/v). The homogenate was centrifuged in a Beckman desktop centrifuge at 15,500*g* for 15 min at 0°C. The supernatant was used to measure tGSH and GSSG using the method described by Griffith [1985].

Statistical Analyses

All data were analyzed by one-way analysis of variance (ANOVA). If the F values were significant, the Student–Newman–Keuls test was used. The level of significance was accepted at $P < 0.05$.

RESULTS

Liver

Hepatic tGSH was higher in PB-treated animals compared with that in control rats; when the animals were injected with LPS 6 h before being killed, the level of tGSH was significantly higher compared to all non-LPS-treated rats ($P < 0.002$); both L-NAME ($P < 0.001$) and melatonin ($P < 0.05$) significantly enhanced tGSH levels above those of PB-treated rats given either LPS or melatonin (Fig. 1).

Liver GSSG levels were elevated in all groups of rats given LPS, whereas melatonin, when given to PB-treated rats, reduced the level of GSSG in the liver when compared to the control group ($P < 0.001$). L-NAME increased ($P <$

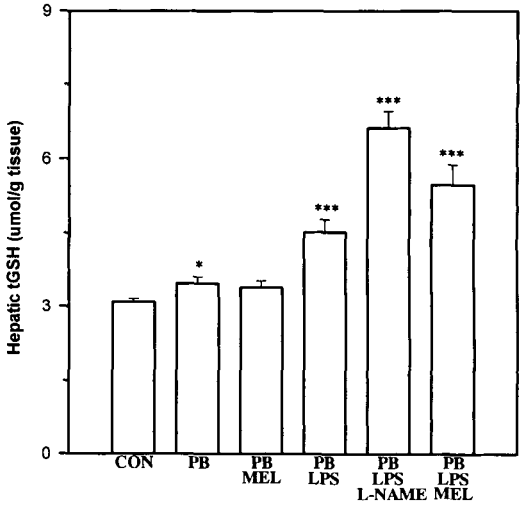

Fig. 1. Effect of melatonin (MEL) and lipopolysaccharide (LPS) on total glutathione levels (tGSH) in liver of phenobarbital (PB)-treated animals. Values are means ± SEM. *$P < 0.05$; ***$P < 0.002$ vs. control (CON).

0.001) GSSG levels compared to those in LPS-only treated rats. Melatonin treatment did not alter GSSG levels in LPS-injected rats (Fig. 2).

The GSSG/GSH ratio, which is an index of oxidative stress, was not significantly changed by treatment with PB alone or in combination with either melatonin or LPS (Fig. 3). However, when PB + LPS rats received either L-NAME or melatonin, the GSSG/GSH ratio was significantly reduced ($P < 0.001$).

GSH-PX is the enzyme which catalyzes the conversion of GSH to GSSG and, in doing so, it uses either H_2O_2 or hydroperoxides. In liver, the activity of GSH-PX was significantly higher in all groups treated with PB. Hepatic GSH-PX activity was significantly stimulated in melatonin-treated animals but only after LPS (PB + LPS compared to PB + LPS + Mel; $P < 0.001$) (Fig. 4).

Treatment of animals for 3 days with PB increased cytochrome P450 content in hepatic microsomes when compared with the control rats. LPS diminished the elevation of cytochrome P450 ($P < 0.002$), with the effect of

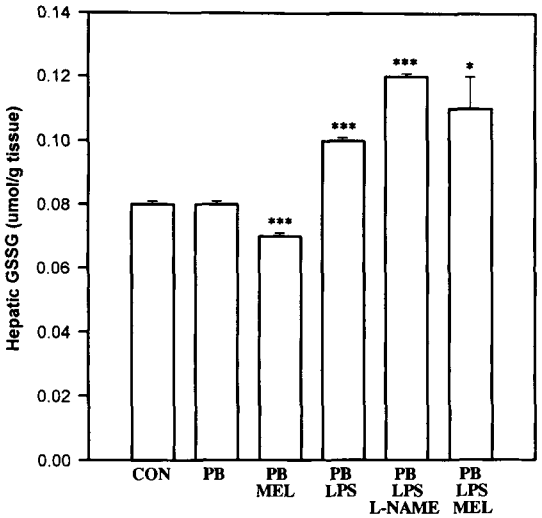

Fig. 2. Effect of melatonin (MEL) and lipopolysaccharide (LPS) on oxidized GSH (GSSG) levels in liver of phenobarbital (PB)-treated animals. Values are means ± SEM *$P < 0.01$; ***$P < 0.001$ vs. control (CON).

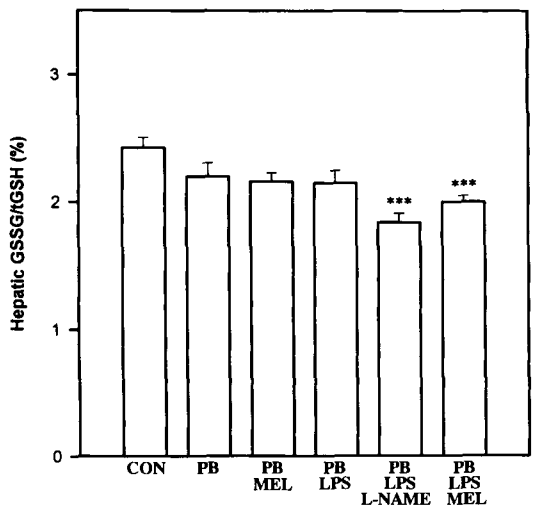

Fig. 3. Effect of melatonin (MEL) and lipopolysaccharide (LPS) on the oxidized glutathione (GSSG)/total glutathione (tGSH) ratio in liver of phenobarbital (PB)-treated rats. Values are means ± SEM. ***$P < 0.001$.

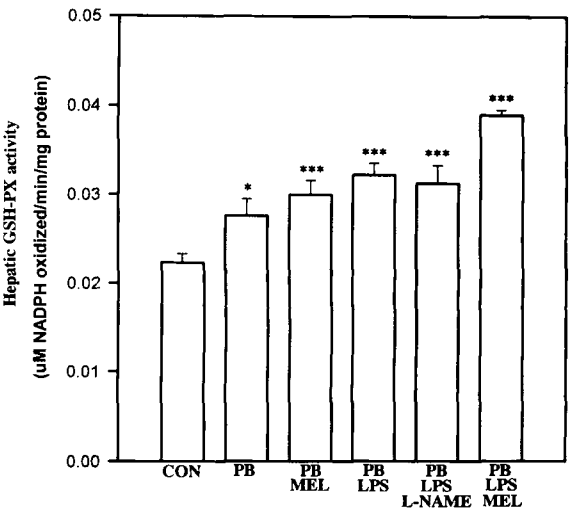

Fig. 4. Effect of melatonin (MEL) and lipopolysaccharide (LPS) on liver glutathione peroxidase activity (GSH-PX) in phenobarbital (PB)-treated animals. Values are means ± SEM. *$P < 0.05$; ***$P < 0.001$.

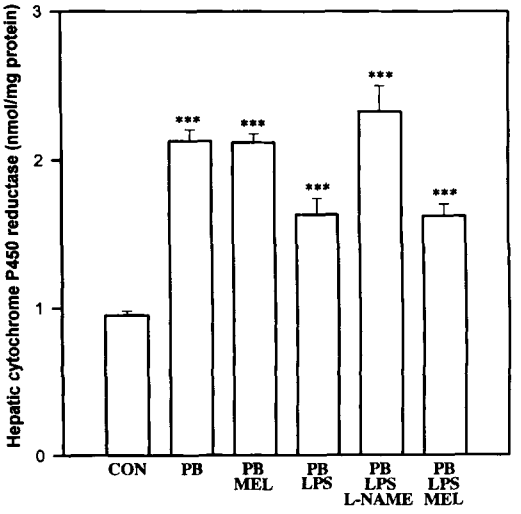

Fig. 5. Effect of melatonin (MEL) and lipopolysaccharide (LPS) on induction of microsomal cytochrome P450 reductase in phenobarbital (PB)-treated animals. Values are means ± SEM. ***$P < 0.001$.

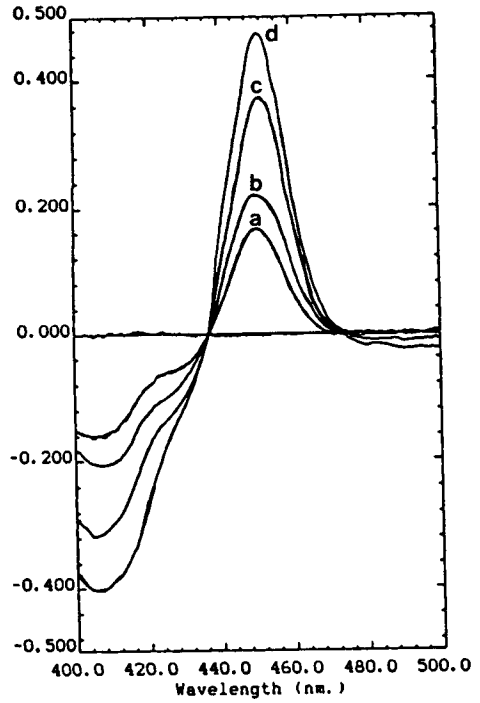

Fig. 6. Absorption spectra of microsomes from phenobarbital (PB)-treated rats after exposure to carbon monoxide (CO). Curves represent the prototypic absoption spectrum of hepatic microsomes with a 450-nm peak after reduction by sodium dithionite and bubbling with CO; **A**, control (CON); **B**, PB + LPS (lipopolysaccharide) and PB + LPS + MEL (melatonin); **C**, PB and PB + MEL; **D**, PB + LPS + L-NAME.

LPS being prevented by the selective inhibitor of NOS, L-NAME ($P < 0.004$) (Figs. 5,6). Melatonin did not change the level of P450 either in PB- or LPS-treated animals.

Brain

Brain tGSH was significantly higher both after melatonin and after L-NAME in LPS-treated animals ($P < 0.02$) (Fig. 7), but no significant differences in either GSSG levels or the GSSG/tGSH ratio were observed (Figs. 8,9).

The activity of brain GSH-PX was enhanced in PB-treated rats compared to levels in controls. GSH-PX activity was significantly higher in melatonin-treated animals in PB- or LPS + PB-treated rats ($P < 0.001$) (Fig. 10).

DISCUSSION

Bacterial LPS is both an endogenous and exogenous toxin which induces oxidative damage by stimulating the production of the reactive oxygen intermediates [Bernard et al., 1984; Ghezzii et al., 1986]. One of the toxic consequences of its administration is an increase in lipid peroxidation with the peroxidation of membrane lipids

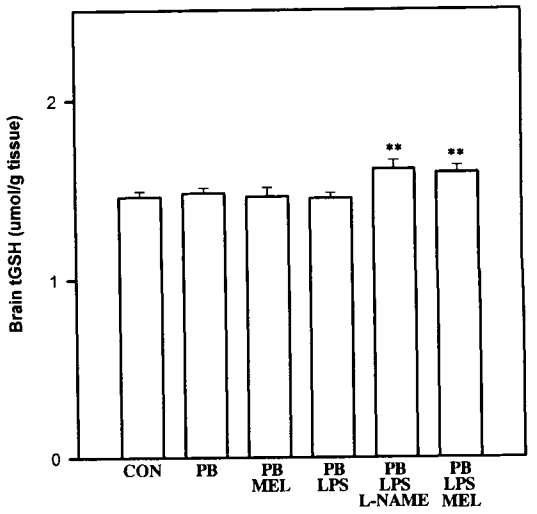

Fig. 7. Effect of melatonin (MEL) and lipopolysaccharide (LPS) on total glutathione levels (tGSH) in brain of phenobarbital (PB)-treated animals. Values are means ± SEM. $^{**}P < 0.02$.

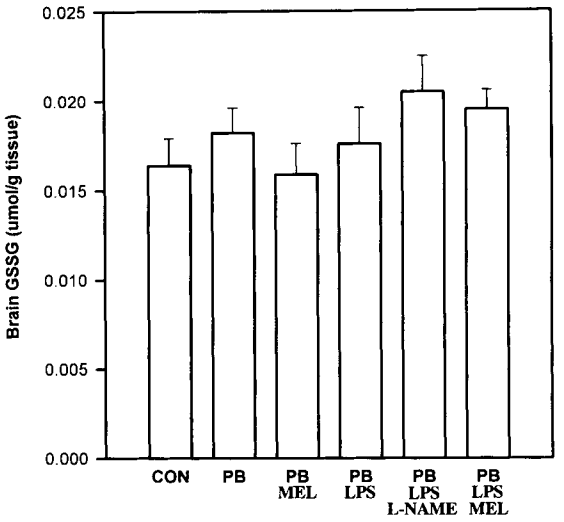

Fig. 8. Effect of melatonin (MEL) and lipopolysaccharide (LPS) on oxidized GSH (GSSG) levels in brain in phenobarbital (PB)-treated animals. Values are means ± SEM.

generally being assumed to be one of the mechanisms by which oxygen radicals exert their damage [Stein et al., 1991]. Under usual circumstances, tissues appear to have a functional anti-oxidative reserve that must be depleted before oxidative injury occurs. Oxidative stress inactivates superoxide dismutase, catalase, and glutathione peroxidase, the enzymes involved in detoxifying O_2^-. and H_2O_2 [Raes et al., 1987; Rigeolet and Remacle, 1991; Nistico et al., 1992]. A reduction in the content of the antioxidant tripeptide GSH normally occurs during drug- and alcohol-induced hepatocellular injury [Meis-

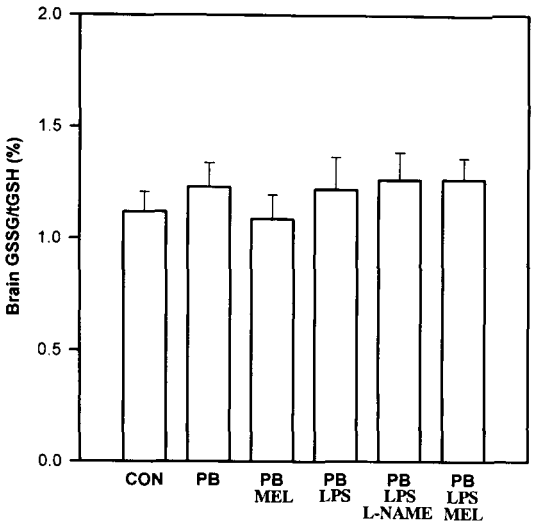

Fig. 9. Effect of melatonin (MEL) and lipopolysaccharide (LPS) on the oxidized glutathione (GSSG)/total glutathione (tGSH) ratio in brain of phenobarbital (PB)-treated animals. Values are means ± SEM.

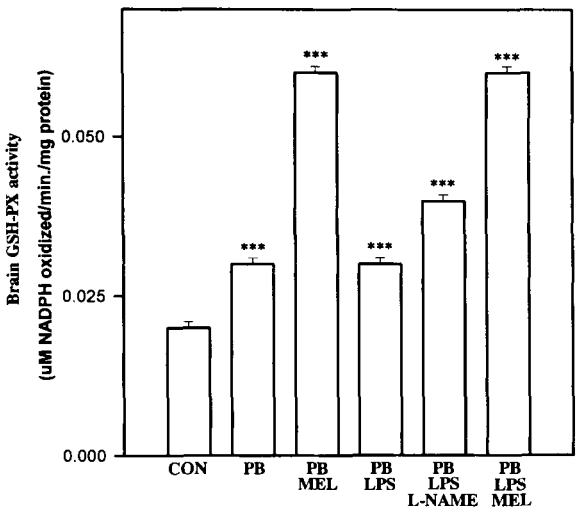

Fig. 10. Effect of melatonin (MEL) and lipopolysaccharide (LPS) on brain glutathione peroxidase activity (GSH-PX) in phenobarbital (PB)-treated animals. Values are means ± SEM. ***$P < 0.001$.

ter, 1988; Kaplowitz et al., 1985]. A depression in liver GSH is observed also during inflammatory processes, indicating that LPS actually induces oxidative stress during which endogenous GSH is used [Peristeris et al., 1992]. GSH depletion is also observed in pathological conditions, e.g., in viral infections [Akaike et al., 1990], possibly due to induction of the superoxide generating enzyme, xanthine oxidase (XO), which is induced after the administration of interferons and LPS and during ischemia/reperfusion [Ghezzi et al., 1985; McCord, 1985]. Depletion of GSH and GSH-PX has been reported in Parkinson's disease [Youdim and Riederer, 1993] and in other neurodegenerative processes in the aged [Nistico et al., 1992]. The release of oxygen-derived free radicals and consumption of GSH has been implicated as a mediator of the reperfusion injury in a variety of organs including the brain, liver, heart, kidney and interstinal mucosa [Curello et al., 1984; Adkinson et al., 1986; Linas et al., 1987; Stein et al., 1990]. Finally, LPS stimulates the production of reactive oxygen intermediates by macrophages either directly or through LPS-induced interleukin-1 (IL-1) [Klempner et al., 1979] or tumor necrosis factor (TNF) release [Peristeris et al., 1992; Lloyd et al., 1993].

In the present study, hepatic GSH levels and GSH-PX activity increased after PB treatment. This is in agreement with previous studies in which mRNA for canalicular GSH transporter, a unique characteristic of GSH secretion, increased after a single dose of PB [Yi et al., 1994]. Also in that study, animals that were injected with LPS exhibited increased levels of GSH and GSH-PX. These results are similar to the findings reported herein. In our experiment GSH levels and GSH-PX activity were measured at 6 h after LPS administration. This relatively short interval allows the detoxification system to increase the production and release of GSH; however, during longer exposure to the toxin the protective system becomes exhausted, GSH is consumed and finally GSH levels decrease. We also found that melatonin enhanced the levels of GSH and activity of GSH-PX in LPS-treated animals both in liver and brain. This is consistent with earlier observations of Kothari and Subramanian [1992] and suggests that melatonin may play an important role in regulating GSH synthesis and release, thereby helping maintain the intracellular redox state and reduce oxidative stress.

GSH is a major antioxidant and it is found in particularly high concentrations in the liver [Meister and Anderson, 1983]. Rapid efflux of GSH is known to occur in response to physiological stimuli such as hormonal signals and oxidative stress [Lu et al., 1990]. Catabolism of GSH by γ-glutamyl transpeptidase in the presence of transition metals leads to oxidative damage [Stark et al., 1994]. Glutathione in all tissues is oxidized during the metabolism of H_2O_2 or hydroperoxides, with the resultant production of GSSG. GSSG is released from liver, brain and other tissues during oxidative damage [Sies,

1986; Jaeschke et al., 1994]. In the present study, hepatic GSSG levels were increased after LPS injection. Melatonin alone decreased the level of GSSG suggesting it protected against oxidative stress. In the current report we observed a strong inhibitory effect of L-NAME on the GSSG/GSH ratio in liver and a significant stimulatory effect of L-NAME on the concentration of GSH in liver and brain. The observation that LPS stimulates oxidative processes in liver is in agreement with previous papers.

The cytochrome P450-dependent monooxygenase system is one of the major producers of active oxygen species in the liver cell. It is known that partially reduced oxygen species, such as O_2^-, H_2O_2, and $^{\cdot}OH$ are formed in cytochrome P450-catalyzed reactions [Karuzina and Archakov, 1994]. It is known that LPS increases liver XO activity [Ghezzi et al., 1984]. This enzyme is also well known to produce reactive oxygen intermediates and cytochrome P450 is sensitive to oxidative damage. XO generates particularly 0_2^- and H_2O_2 as reduction products of molecular oxygen [McCord and Fridovich, 1968; Fridovich, 1970], and an increase of XO activity might play a role in LPS-mediated depression of liver drug-metabolizing enzymes.

In the present study, animals were treated with PB which is a strong inducer of P450 in rat liver microsomes [Puntarulo and Cederbaum, 1992; Khatsenko et al., 1993]. LPS diminished microsomal P450 content in PB-treated rats. Additionally, L-NAME significantly reduced the inhibition in P450 content by LPS. Our results are in agreement with previous reports [Khatsenko et al., 1993]. A preliminary report by Kothari and Subramanian [1992] found that melatonin had an inhibitory effect on the content of cytochrome P450; we could not confirm this observation and found instead that melatonin did not change P450 activity either in PB- or LPS-injected animals. These results suggest that the antioxidative effect of melatonin may not be via cytochrome P450, but might involve the production and release of other antioxidants, e.g., GSH. A previous paper, Bartsch et al. [1992] suggested that PB may influence the metabolism of melatonin in the liver. They found that the 6-hydroxylation of melatonin is carried out by PB-type enzymes. Thus PB could change the local concentration of melatonin in liver. Alternatively, the stimulatory effect of PB could overwhelm the inhibitory effect of melatonin.

Considering melatonin's apparently potent role as an antioxidant and free radical scavenger, it is possible that this indole may find clinical value. There are a variety of diseases that may be, at least in part, a consequence of cellular damage induced by free radicals. Melatonin easily enters subcellular compartments where it could provide local protection against oxidative stress; thus, melatonin could play a significant protective role against both initiation and progression of disease states that involve free radicals.

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