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RESEARCH ARTICLE

Neuro- and Hepatoprotective Effects of Methylene Blue in Rats Treated with Lipopolysaccharide Endotoxin

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ABSTRACT | The effects of methylene blue (MethyB) on oxidative stress markers in the serum and on the brain and liver tissue damage following lipopolysaccharide (LPS) injection in rats were studied. Rats received intraperitoneal (i.p.) injection of LPS (300 µg/kg) alone or along with MethyB (5 and 10 mg/kg) and euthanized 4 h later. Malondialdehyde (MDA), nitric oxide, paraoxonase-1 (PON-1) activity, cholinesterase activity, and glucose were measured in the serum. The brain and liver histopathology, glial fibrillary acidic protein (GFAP), and caspase-3 immunostaining in the brain were performed. Results indicated that LPS treatment caused significantly raised MDA and nitric oxide concentrations by 52.8% and 47.5%, respectively. Cholinesterase activity was not significantly changed, but PON1 activity fell by 48.1%. Serum glucose increased by 48.7%. When administered to LPS-injected rats, MethyB resulted in significant decreases in serum MDA and nitric oxide concentrations, respectively. PON-1 activity increased by 37.8-89.3%, cholinesterase activity decreased by 33.4-41.2%, and serum glucose decreased by 39.6% upon MethyB treatment. Neurodegeneration, increased capsase-3 staining, decreased GFAP immunostaining in the brain and vacuolar degeneration and inflammatory cell infiltrates in the liver of LPS-treated rats were almost prevented by MethyB. These data suggest that MethyB exerts an antioxidant action and ameliorates brain and liver tissue damage during endotoxemia.

KEYWORDS | Brain injury; Lipid peroxidation; Lipopolysaccharide; Liver injury; Methylene blue

ABBREVIATIONS | AChE, acetylcholinesterase; BChE, butyrylcholinesterase; GFAP, glial fibrillary acidic protein; IL, interleukin; LPS, lipopolysaccharide; MDA, malondialdehyde; MethyB, methylene blue; NOS, nitric oxide synthase; PON-1, paraoxonase-1; TNF-α, tumor necrosis factor-alpha

CONTENTS

1. INTRODUCTION

The redox dye methylthioninium chloride, commonly called methylene blue (MethyB), has long been known as an antidote in the treatment of cyanide poisoning [1] and in methemoglobinemia, causing reduction of methemoglobin to hemoglobin [2]. The dye has also been employed to prevent and/or reverse encephalopathy caused by the chemotherapeutic drug ifosfamide, to treat vasoplegic shock in neonates [3], and refractory hypotension in adults [4], and as an adjunctive agent reducing residual depression and anxiety in subjects with bipolar depression [5]. Studies in experimental animals indicated the ability of the dye to prevent neurodegeneration under different circumstances. In this context, MethyB was shown to prevent neuronal death caused by ischemia/reperfusion injury [6], serum deprivation [7], rotenone [8], traumatic brain injury [9], malathion [10], and toluene [11]. It was also protective in a mouse model of Huntington's disease [12]. MethyB possesses a number of important pharmacological actions that are likely to be of relevance to its neuroprotective effects. The dye is an inhibitor of nitric oxide synthase and guanylyl cyclase [13, 14]. MethyB enhances mitochondrial function due to cycling between its oxidized (leucomethylene blue) and reduced form in the mitochondria, reducing the formation of reactive oxygen metabolites [15]. MethyB exerts: (i) antioxidant activities [10, 11]; (2) anti-inflammatory effects decreasing microglia activation and gene expression of interleukin-1beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) in macrophages and microglia [9], as well as activation of nuclear factor kappa-B [11]; and (iii) antiapoptotic effects inhibiting caspase-3 activation and increasing Bcl2 expression [8].

Lipopolysaccharide (LPS), a constituent of the outer wall of Gram-negative bacteria, is widely used to induce a systemic inflammatory illness [16]. LPS activates the mammalian Toll-like receptor on the surface of the immune cells which is the signal transducing receptor that triggers the inflammatory cascade [17, 18]. When injected peripherally into rodents, LPS activates the immune cells to increase the synthesis and release of pro-inflammatory cytokines,

such as TNF-α, IL-1β, and IL-6, causing both systemic inflammation and neuroinflammation [19–22]. LPS also increases the generation of nitric oxide and reactive oxygen metabolites resulting in oxidative stress, lipid peroxidation, and tissue damage [23–25]. The model is thus suitable to study the influence of peripheral inflammatory signals in the development of neurodegeneration. Accordingly, in this study, MethyB was investigated for its ability to prevent the brain and liver injury during LPS-induced systemic inflammation.

2. MATERIALS AND METHODS

2.1. Animals

Male Sprague-Dawley rats (180–200g in body weight) were used in the study. Rats were obtained from an animal house colony of the National Research Centre (Cairo, Egypt). Standard laboratory food and water were provided ad libitum. All experiments were performed in accordance with animal protocols and guidelines of the Institutional Ethics Committee and that of the Canadian Council on Animal Care (CCAC) guidelines. Equal groups of six rats each were used in experiments.

2.2. Drugs and Chemicals

Lipopolysaccharide (LPS) derived from *Escherichia coli* (Serotype 055:B5, Sigma–Aldrich, St Louis, MO, USA) was used and dissolved in aliquots of sterile saline, and frozen at −20°C. Methylene blue (MethyB, Sigma–Aldrich) was used and dissolved in isotonic saline solution immediately before use. Other chemicals were from Sigma–Aldrich. The doses of LPS and MethyB used were based on those reported in previous studies [26].

2.3. Study Design

Rats were randomly divided into four groups of six rats each. Rats were treated with LPS (300 mg/kg, i.p.) and then received either saline (group 1) or MethyB at doses of 5 or 10 mg/kg (groups 2 and 3). The fourth group received only saline. Rats were euthanized 4 h after LPS or saline injection by decapitation under ether anesthesia; the brains and livers were then removed, washed with ice-cold saline solution (0.9 % NaCl), weighed, and stored at −80°C for biochemical analyses. The tissues were homogenized in 0.1 M phosphate buffer saline at pH 7.4 to give a final concentration of 10 % (w/v) for the biochemical assays.

2.4. Biochemical Analyses

2.4.1. Lipid Peroxidation

Malondialdehyde (MDA), an end product of lipid peroxidation, was determined in the serum according to the method described by Nair and Turne [27]. In this assay, thiobarbituric acid reactive substances (TBA) react with thiobarbituric acid to form TBA-MDA adduct. The latter can be measured at 532 nm spectrophotometrically.

2.4.2. Nitric Oxide

Nitric oxide was measured indirectly in the serum using a colorimetric assay where nitrate is converted to nitrite via nitrate reductase. The Griess reagent then acts to convert nitrite to a deep purple azo compound that can be determined using a spectrophotometer [28].

2.4.3. Paraoxonase-1 (PON-1) Activity

PON-1 arylesterase activity was determined in the serum using phenylacetate as a substrate and the formation of phenol was measured by monitoring the increase in absorbance at 270 nm at 25°C using a spectrophotometer. The enzyme activity is expressed as kilo-international units per liter (kU/l) [29].

2.4.4. Cholinesterase Activity

Butyrylcholinesterase (BChE) activity was measured in the serum using a commercially available kit (Ben Biochemical Enterprise, Milan, Italy), based on the assay principle described before [30].

2.4.5. Glucose Level

The level of glucose was measured in the supernatants of brain homogenates by a standard glucose oxidase-based method as described by Trinder [30[a]], using a commercially available assay kit (Biodiagnostic, Egypt).

2.5. Histopathological Studies

The brain and liver samples of all animals were dissected immediately after decapitation. The specimens were then fixed in 10% neutral-buffered formalin saline for at least 72 h. Specimens were washed in tap water for 30 min and then dehydrated in ascending grades of ethanol, cleared in xylene, and finally embedded in paraffin. Serial sections of 5 μm thickness were cut and stained with hematoxylin and eosin (H&E) for the histopathological investigation. Images were examined and photographed under a digital camera (Microscope Digital Camera DP70, Tokyo, Japan), and processed using Adobe Photoshop version 8.0 (San Jose, CA, USA).

2.6. Immunohistochemistry for Glial Fibrillary Acidic Protein and Caspase-3

The paraffin-embedded brain sections were deparaffinized, and hydrated. Immunohistochemistry was performed with a mouse monoclonal caspase-3 and glial fibrillary acidic protein (GFAP) for the detection of the caspase cleavage and GFAP activity. The paraffin sections were heated in a microwave oven for 25 min (at 720 W) for antigen retrieval and then incubated with either anti-caspase-3 or anti-GFAP antibodies (1:50 dilution) overnight at 4°C. Following washing with phosphate-buffered saline (PBS) and incubation with biotinylated goat-anti-rabbit-immunoglobulin G secondary antibodies (1:200 dilution; Dako, Santa Clara, CA, USA.) and streptavidin/alkaline phosphatase complex (1:200 dilution; Dako) (30 min at room temperature), and the binding sites of antibody were visualized with 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma–Aldrich). The samples were then washed with PBS, counterstained with H&E for 2–3 min, and dehydrated by transferring them through increasing grades of ethanol solutions. Following dehydration, the slices were soaked twice in xylene at room temperature for 5 min, mounted, examined, and evaluated by a high-power light microscope.

2.7. Immunomorphometric Analysis

The morphometric analysis was performed at the Pathology Department, National Research Centre (Cairo, Egypt) using the Leica Qwin 500 Image Analyzer (LEICA Imaging Systems Ltd, Cambridge, England) which consists of a Leica DM-LB microscope with a JVC color video camera attached to a computer system Leica Q 500IW. The morphometric analysis was carried out on GFAP and caspase-3 stained slides. The area is determined as an area per field in micrometer square, area fraction, and area percentage by using the interactive software of the system. The area was measured in 10 fields in each slide**.**

2.8. Statistical Analysis

The results are presented as the mean ± standard error (SE) of the means. Statistical analysis of the data was done using one-way analysis of variance (ANOVA), followed by Duncan's multiple dose range, using the Statistical Package for Social Sciences software (version 16.0; SPSS Inc., Chicago, IL, USA). A probability (p) value of < 0.05 was considered statistically significant.

3. RESULTS

3.1. Biochemical Results

3.1.1. MDA

The level of MDA in the serum of LPS-treated rats increased by 52.8% compared with the saline control group (83.3 ± 3.2 vs. 54.5 ± 2.7 μmol/ml). MDA level fell significantly by 32.3% and 48.9% following treatment with MethyB at 5 and 10 mg/kg, respectively (56.4 ± 3.5 and 42.6 ± 2.1 vs. 83.3 ± 3.2 μmol/ml) (**Figure 1A**).

3.1.2. Nitric Oxide

The serum nitric oxide level increased by 47.5% after endotoxin administration compared with the saline control group (71.7 ± 1.2 vs. 48.6 ± 2.9 μmol/ml). MethyB given at 5 and 10 mg/kg resulted in 35.6% and 72.1% decrements in serum nitric oxide, respectively, compared with the LPS control group (46.2 ± 3.1 and 20 ± 1.5 vs. 71.7 ± 1.2 μmol/ml) (**Figure 1B**).

3.1.3. PON-1 Activity

In the LPS-treated group, the serum PON-1 activity showed a 48.1% decrease as compared to the saline

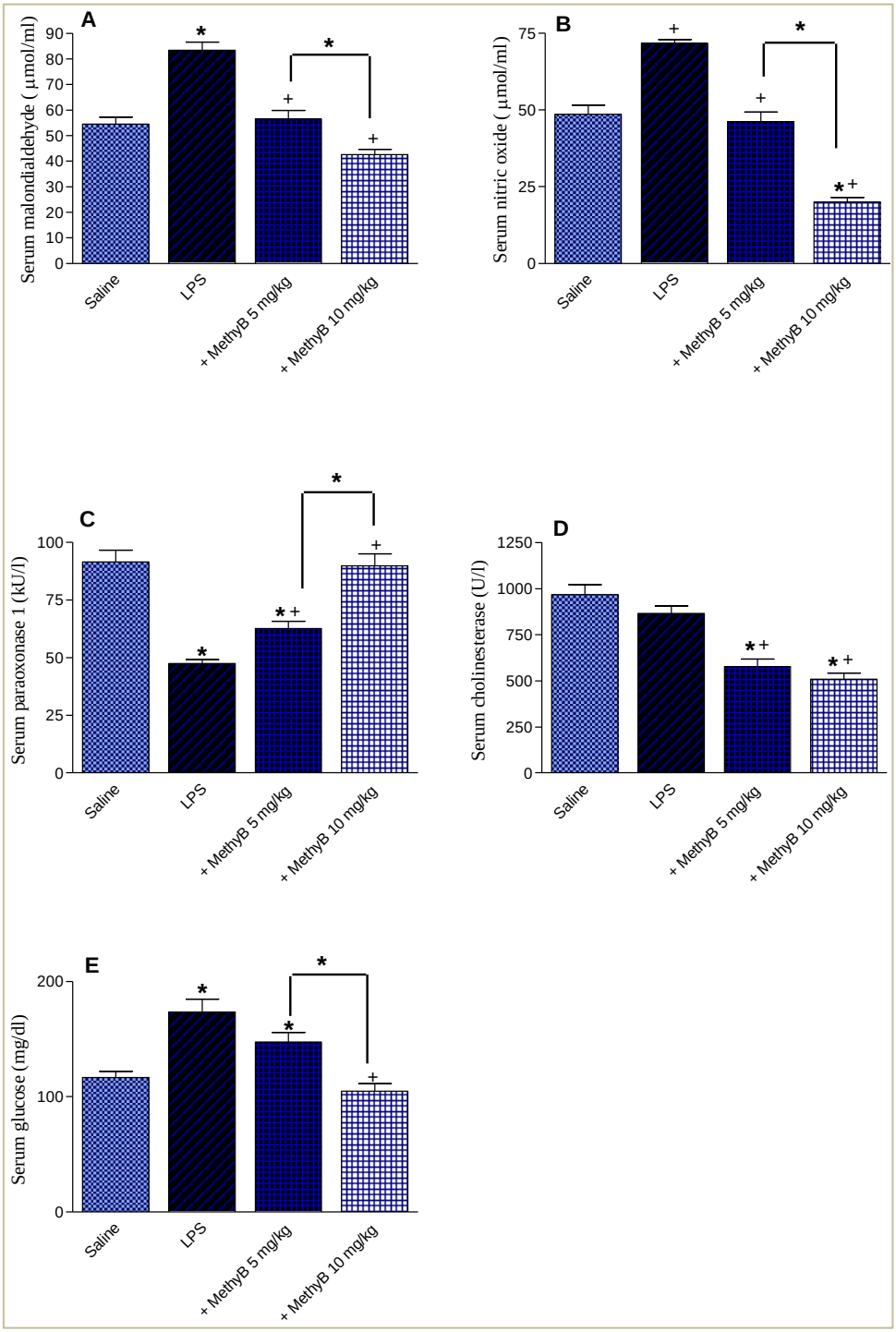

FIGURE 1. The effect of methylene blue (MethyB) on (A) malondialdehyde, (B) nitric oxide, (C) paraoxonase-1 activity, (D) butyrylcholinesterase activity, and (E) glucose in the serum of rats treated with lipopolysaccharide endotoxin (LPS). *, $p < 0.05$ vs. saline and between different groups as indicated in the figure; +, $p < 0.05$ vs. LPS control.

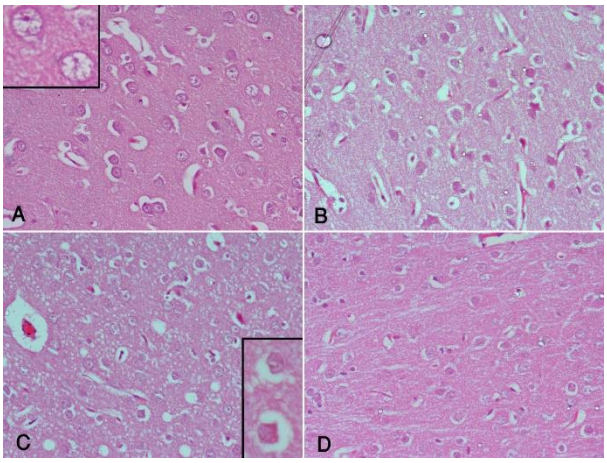

FIGURE 2. Representative photomicrographs of sections of the cerebral cortex from rats treated with various agents. (A) Saline: normal shape of neurons with their large vesicular nuclei (in the upper left part of the figure. (B) LPS only: many neurons with variable degrees of degeneration from slightly dark nuclei to deep dark shrunken neurons. (C) LPS and MethyB at 5 mg/kg: only some dark and/or small neurons are still observed. (D) LPS and MethyB at 10 mg/kg: marked amelioration of signs of damage in the cerebral cortex.

control value (47.5 ± 1.7 vs. 91.6 ± 5.1 kU/l). The serum PON-1 activity increased by 37.8% and 89.3% following treatment with MethyB at 5 or 10 mg/kg, respectively (62.6 ± 3.0 and 89.9 ± 5.2 vs. 47.5 ± 1.7 kU/l) (**Figure 1C**).

3.1.4. Cholinesterase Activity

No significant difference was observed between serum BChE activity in saline and LPS-treated groups (967.4 ± 55.1 vs. 865.3 ± 41.6 U/l). The serum BChE activity significantly decreased by 33.4% and 41.2% after MethyB treatment at 5 and 10 mg/kg, respectively, compared with the LPS control (576.3 ± 41.3 and 508.7 ± 33.4 vs. 865.3 ± 41.6 U/l) (**Figure 1D**).

3.1.5. Glucose

In rats treated with only LPS, the serum glucose level increased by 48.7% compared to the saline group (173.5 ± 11.2 vs. 116.7 ± 5.3 mg/dl). MethyB administered at 10 mg/kg to LPS-treated rats resulted in a significant decrease of 39.6% ($p < 0.05$) in the serum glucose level (104.7 ± 6.6 vs. 173.5 ± 11.2 mg/dl) (**Figure 1E**).

3.2. Histopathological Results

3.2.1. Brain Tissue

The administration of LPS markedly affected brain tissue as many neurons in the cerebral cortex area lost the characteristic appearance of the large vesicular nuclei with the owl-eye shape of the nucleolus shown in the control group (**Figure 2A** and 2B). Many neurons appeared with dark nuclei, while some neurons showed deeply stained nuclei with dark shrunken body denoting apoptosis (**Figure 2B**). MethyB given at 5 mg/kg resulted in mild improvement of the damaging effects of LPS with some darkly stained neurons being still observed (**Figure 2C**). The higher dose of MethyB nearly restored the normal structure of cerebral cortex tissue with healthy shaped neurons (**Figure 2D**).

Examination of the hippocampal area of the brain tissue of control animals showed the normal structure of this area (**Figure 3A**). LPS caused many of neurons in this area to be smaller in size than normal and darkly stained. The thickness of the area was also decreased (**Figure 3B**). MethyB treatment ameliorated signs of degeneration caused by LPS in a dose-dependent manner. The lower dose decreased the number of darkly stained neurons, although the thickness of the area was still less than normal (**Figure 3C**). The higher dose of MethyB caused the darkly stained neurons to be much decreased in number and the thickness of this area returned to normal (**Figure 3D**).

3.2.2. Liver Tissue

The liver from saline-treated rats showed the normal structure as shown in **Figure 4A**. LPS had damaging effects on the liver tissue in the form of vacuolar degeneration of hepatocytes, dilatation of blood sinusoids, and cellular infiltration around main blood vessels (**Figure 4B**). Treatment with MethyB at 5 mg/kg decreased vacuolar degeneration, but dilatation and congestion of blood vessels were still observed at the periphery of lobules (**Figure 4C**). Rats treated with MethyB at 10 mg/kg showed normal liver tissue (**Figure 4D**).

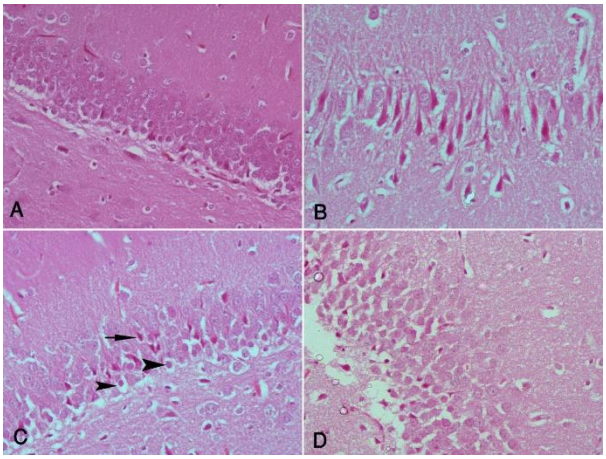

FIGURE 3. Representative photomicrographs of sections of the hippocampal area from rats treated with various agents. (A) Saline: normal thickness of this area and neurons with large vesicular nuclei. (B) LPS only: most of neurons appear elongated, darkly-stained and shrunken. (C) LPS and MethyB at 5 mg/kg: some dark neurons (arrow) and small apoptotic neurons (arrowhead). (D) LPS and MethyB at 10 mg/kg: most of neurons appear normal in shape and size.

3.3. Immunohistochemical Results

Immunohistochemical investigation of the brain tissue stained with caspase-3 antibody confirmed the results obtained by histopathological investigation described above. The saline control group showed negative reaction to the stain (**Figure 5A**), while the LPS-treated group showed high number of positively stained neurons (**Figure 5B**). Treatment with MethyB at 5 mg/kg slightly reduced the number of positively stained neurons (**Figure 5C**), while the high dose of the dye markedly reduced the number of the positively stained neurons (**Figure 5D**). Sections stained with GFAP antibody showed the normal appearance of glial cells in the cerebral cortex of control animals (**Figure 6A**). LPS treatment caused these cells to be decreased markedly (**Figure 6B**). MethyB given at 5 mg/kg mildly ameliorated this damaging effect of LPS (**Figure 6C**). Much better amelioration was observed with MethyB given at 10 mg/kg (**Figure 6D**).

3.4. Immunomorphometric Results

Activated caspase-3 labeling delineates morphologically apoptotic cells, where its expression is localized in the cytoplasm of the apoptotic cells. The least caspase-3 expression was in the normal control group (1.40 ± 0.32%), while the maximum expression was observed in the LPS only group (42.55 ± 2.19%). Caspsae-3 expression decreased by 29.2% and 87.4% after treatment with MethyB at 5 and 10 mg/kg, respectively.

Further quantitative morphometric analysis of the pathological changes was done using GFAP which was detected in the cytoplasm of viable astrocytes. The maximum expression was in the normal control group which received saline only (12.04 ± 0.81%). On the other hand, the lowest GFAP expression was seen in the LPS only group denoting its destructive effect on neuronal tissue (4.03 ± 0.98%). The area% of GFAP increased dose-dependently by 80.0% and 133.0% following MethyB treatment at 5 and10 mg/kg, respectively (**Table 1**).

4. DISCUSSION

In this study, the effect of MethyB on serum oxidative stress and on brain and liver tissue damage following the peripheral injection of the inflammogen LPS was examined. Our aim was to investigate the effect of MethyB in the presence of systemic inflammation. It is shown here that MethyB could decrease oxidative stress in the serum and protect the brain and liver during endotoxemia. When injected peripherally into rodents, LPS causes marked increases in the plasma and tissue levels of cytokines and inflammatory mediators, such as IL-1β, IL-6, TNF-α, and nitic oxide [22, 31–34] and a significant elevation in oxidative stress markers in the brain and other organs, including the liver, kidneys, heart, and lung [23, 24, 35]. The present findings are consistent with the above previous studies and show that treatment with LPS causes a markedly increased serum level of MDA, an end product of lipid peroxidation [36], thereby suggesting the increased release of reactive oxygen metabolites and consequent oxidative attack on polyunsaturated fatty acids. The rise in the serum MDA concentration following LPS injection thus indicates the presence of systemic oxidative stress. Our results also indicate that treatment with

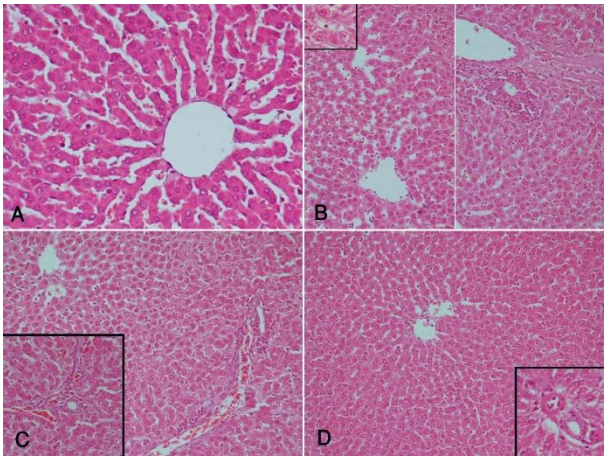

FIGURE 4. Representative photomicrographs of sections of the liver tissue from rats treated with various agents. (A) Saline: normal liver tissue. (B) LPS only: dilation of blood sinusoids with diffuse cellular infiltration, aggregation of inflammatory cells around main blood vessels, the upper left part of figure shows vacuolar degeneration of hepatocytes. (C) LPS and MethyB at 5 mg/kg: reduction of vacuolar degeneration, but mild dilatation and congestion of blood vessels were observed at the portal area and the peripheries of lobules. (D) LPS and MethyB at 10 mg/kg: restoration of normal structure of the liver tissue.

MethyB was able to decrease serum MDA in LPS-treated rats.

Our findings also indicate a significantly raised nitric oxide concentration in the serum of LPS-injected rats. Other studies demonstrated increased nitric acid content as well as increased inducible nitric oxide synthase (iNOS) expression in the brain and liver of LPS-treated rodents [26, 35]. Nitric oxide (nitrogen monoxide) is derived from L-arginine by the action of nitric oxide synthases, which catalyze the oxidation of L-arginine to NO and L-citrulline. The source of nitric oxide during inflammatory conditions is mostly derived from the action of iNOS. This enzyme is capable of generating nitric oxide at greater amounts and for longer duration compared with other two constitutively present isoforms, i.e., the neuronal (nNOS) and endothelial (eNOS) isoforms [37]. This excessive generation of nitric oxide is associated with the so called "nitrosative stress" where nitric oxide results in amine and thiol nitrosation/nitration as well as oxidation reactions. These effects are mediated by nitrogen oxides and other reactive nitrogen metabolites, such as N_2O_3, NO_2, and peroxynitrite derived from the interaction of nitric oxide with molecular oxygen or superoxide [38, 39]. Increased nitric oxide release from inflammatory and immune cells has thus been implicated in tissue damage, brain hypoxia/ischemia, brain trauma, and neurodegenerative disorders [40–42]. Here we show that the administration of MethyB to LPS-treated rats resulted in a significant reduction in serum nitric oxide concentrations. These findings suggest an inhibitory action for the dye on nitric acid synthesis and are in accordance with previously published data showing a reduction in brain nitric oxide by MethyB in rotenone-treated rats [8]. MethyB can inhibit the synthesis of nitric oxide being a guanylate cyclase inhibitor and a direct inhibitor of nitric oxide synthase [13, 14]. In rats treated with LPS, MethyB could reduce iNOS levels in the medial basal hypothalamus [43]. In cerebral ischemia/reperfusion injury, MethyB was shown to decrease cerebral nitric oxide content and the number of iNOS and nNOS activated cortical cells [44]. MethyB co-treatment at 10 mg/kg was also shown to decrease brain nitric oxide and to afford neuroprotection following malathion intoxication in rats [10].

In addition, the present study demonstrates significant inhibition of serum PON-1 activity following LPS injection which is in accordance with previously published observations showing decreased PON-1 activity in the serum, liver, and brain of rodents treated with LPS [35, 45]. This enzyme is synthesized and released by the liver cells into the plasma being found in association with high-density lipoproteins [46] and protects high-density lipoproteins and low-density lipoproteins from oxidation [47] and inhibits the release of TNF-α, IL-6, and reactive oxygen metabolites from macrophages [48], while its deficiency results in increased oxidative stress in the serum and macrophages [49]. PON-1 possesses arylesterase and lactonase activities and has also an important role in the hydrolysis of a number of organophosphate insecticides and nerve agents, and in xenobiotic metabolism [46, 50]. Variation in the catalytic efficiency of the enzyme appears to determine the susceptibility of those exposed to organophosphates to develop Parkinson's disease [51, 52]. The activity of PON-1 is also decreased in the se-

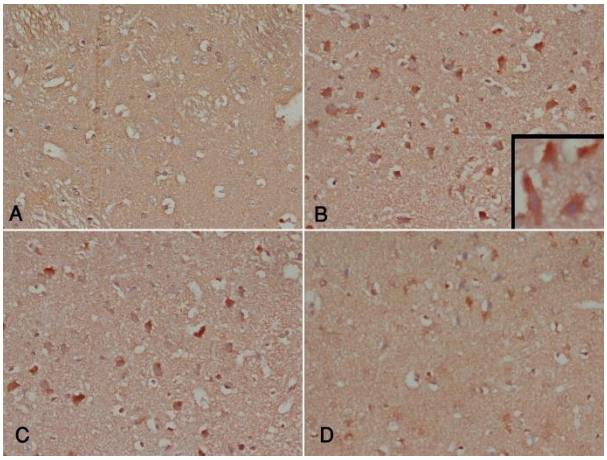

FIGURE 5. Representative photomicrographs of sections of the cerebral cortex tissue stained immunohistochemically with caspase-3 antibody from rats treated with various agents. (A) Saline: negative reaction to the stain. (B) LPS only: many neurons with positive reaction. (C) LPS and MethyB at 5 mg/kg: slight reduction of positively stained neurons number. (D) LPS and MethyB at 10 mg/kg: only a few neurons with positive reaction to the stain.

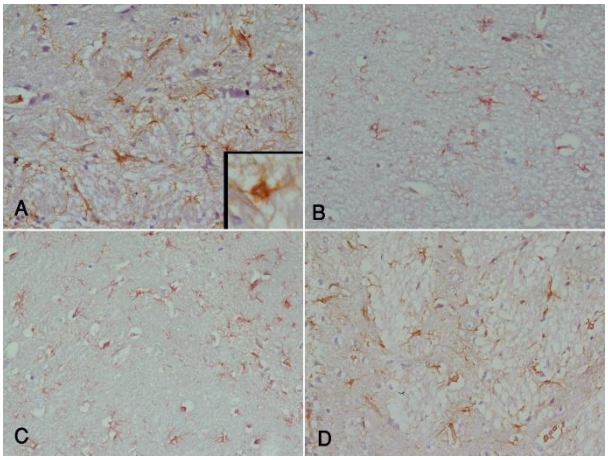

FIGURE 6. Representative photomicrographs of sections of the cerebral cortex tissue stained immunohistochemically with GFAP antibody from rats treated with various agents. (A) Saline: normal appearance of glial cells in this tissue. (B) LPS only: marked decrease in positively stained glial cells. (C) LPS and MethyB at 5 mg/kg: increase in the number of positively stained cells. (D) LPS and MethyB at 10 mg/kg: marked increase in the number of positively stained cells.

rum/plasma in a variety of neurologic and neurodegenerative disorders, suggesting a neuroprotective role for the enzyme [53, 54]. PON-1 has also an important role in maintaining liver integrity via its antioxidant and anti-inflammatory actions [55, 56]. Mice deficient in the enzyme showed marked steatosis when fed high cholesterol diet compared with their wild-type counterparts [57]. In this study, treatment with MethyB was shown to increase PON1 activity. The recovery of enzyme activity by MethyB has been demonstrated previously in neurotoxicity caused by rotenone or malathion and could probably due to a decrease in oxidative stress [8, 11]. The latter has been shown to result in inactivation of PON-1 [58].

Studies have shown decreased brain acetylcholinesterase (AChE) and BChE activities in rats challenged with a single intraperitoneal dose of LPS (300 μg/kg) [26]. In the present study, serum BChE activity was decreased, though not significantly, by treatment with LPS. On the other hand, we found an inhibitory action of MethyB on serum BChE activity in rats treated with LPS. An interaction between MethyB and the cholinergic system was suggested by Pfaendorf et al. [59] who observed a significant inhibition of both AChE and BChE by MethyB in vitro. Moreover, azure B, a metabolite of MethyB, acts as a reversible competitive inhibitor of AChE and BChE [60]. This action is likely to underlie the enhancement of memory processing by the dye [61, 62]. MethyB also appears to compete with acetylcholine at the muscarinic binding sites [59] which could explain the neuroprotective effect of the dye in rats intoxicated with the organophosphate compound malathion [10].

The neuro- and hepatoprotective actions of MethyB were demonstrated by histopathologic examination of the brain and liver tissue combined with caspase-3 and GFAP immunohistochemistry. It was shown that treatment with LPS resulted in neurodegeneration in the form of darkly stained, shrunken, and apoptotic neurons in both the cerebral cortex and hippocampus. These pathological changes were ameliorated by MethyB in a dose-dependent manner. In

TABLE 1. Immunomorphometric measurements of caspase-3 and GFAP in the brain tissue of rats treated with various agents

Group	Caspase-3 area% (mean ± SE)	GFAP area% (mean ± SE)
Normal control (saline)	1.40 ± 0.32	12.04 ± 0.81
LPS only	42.55 ± 2.19*	4.03 ± 0.98*
LPS + MethyB (5 mg/kg)	30.14 ± 3.3*+	7.27± 0.55*+
LPS + MethyB (10 mg/kg)	5.36 ± 1.05*+	9.39± 1.18+

Note: *, $p < 0.05$ vs. saline; +, $p < 0.05$ vs. LPS control.

addition, the pathological changes induced by LPS in the liver were ameliorated to a great extent, by treatment with MethyB.

Caspases, a family of cysteine proteases, are mediators of programmed cell death or apoptosis. Caspase-3 is the most important effector caspase that, when activated, acts to disassemble the cell [63]. In the present study, increased caspase-3 immunoreactivity was shown in sections from the cerebral cortex of LPS-treated rats, indicating increased apoptosis. In contrast, caspase-3 immunoreactivity decreased dose-dependently after treatment with MethyB, thereby indicating inhibition of caspase-3 activation. Moreover, in rats treated with toluene or rotenone, MethyB was shown to inhibit caspase-3 activation [8, 11]. MethyB also increased the expression of the antiapoptotic protein Bcl2 in the striatum of rotenone-treated rats [8].

Astrocytes are the most abundant glial cells population in the brain and provide structural support to neurons. They also perform important metabolic actions, including removal of neurotransmitters after their release by active neurons. In addition, these cells can release neuroactive substances such as adenosine triphosphate (ATP), glutamate, and serine [64]. GFAP constitutes an intermediate filament protein and a major component of glial of the cytoskeleton in astrocytes. In this study, sections from the cerebral cortex stained with GFAP antibody showed marked decrease in the number of positively stained glial cells, suggesting astrocyte cell death. This was prevented dose-dependently by MethyB. Other studies showed that MethyB rescued astrocytic cell death caused by toluene [11].

In conclusion, the present study indicated that treatment with MethyB reduced LPS-induced increase in lipid peroxidation and nitric oxide in the serum, protected against the decline in serum PON-1 activity, decreased brain caspase-3 activation, and provided neuro- and hepatoprotection. It is likely that the inhibitory effect on nitric oxide synthesis largely mediated the observed neuro- and hepatoprotective effects. These findings lend further support to the potential benefit of MethyB in the treatment of neurodegenerative diseases.

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