PROTECTIVE EFFECT OF COX AND NOS INHIBITORS ON LPS INDUCED OXIDATIVE STRESS IN RAT

R Shukla, E Tyagi, R Kumar
Division of Pharmacology, Central Drug Research Institute, Lucknow, India
Corresponding author:
Dr Rakesh Shukla
Division of Pharmacology
Central Drug Research Institute (CDRI)
PO Box 173, Lucknow-226001, India
Email: rakeshshukla_cdri@rediffmail.com
Fax: +915222623405
Tel: +915222612411-18, Ext 4420
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Abstract
The major goal of this study was to examine the ability of Cyclooxygenase (COX) and Nitric Oxide Synthase (NOS) inhibitors namely, indomethacin, a non-selective COX inhibitor, celecoxib, a selective COX-2 inhibitor, N\textsuperscript{o}-nitro-L-arginine methyl ester (L-NAME), a non-selective NOS inhibitor to protect the brain from oxidative stress induced by intraperitoneal injection of lipopolysaccharide (LPS, endotoxin). LPS, a component of the cell wall of gram-negative bacteria, has been recognized as one of the most potent bacterial products in the induction of host inflammatory responses and tissue injury and was used in this study to mimic infections. LPS at a dose of 4 mg/kg resulted in a significant alteration of the brain oxidative status observed as elevation of the level of Malondialdehyde (MDA, index of lipid per oxidation) and reduction of reduced glutathione (GSH), an antioxidant, in all the brain regions. Levels of nitrate/nitrite (NOx), a marker of reactive nitrogen species (RNS), was also found elevated in all the brain regions whereas myeloperoxidase (MPO), a heme-containing enzyme, was significantly increased only in cerebral cortex, hippocampus, thalamus and medulla. Both COX and NOS inhibitors ameliorated the oxidative stress induced by LPS injection by reducing levels of MDA, restoring GSH content and normalizing the NOx and MPO levels in the brain. Results of the study indicate that the COX and NOS inhibitors regulate the increased free radical generation during infections and LPS-induced oxidative stress.

Keywords: Lipopolysaccharide, Oxidative stress, Brain, Celecoxib, Indomethacin, L-NAME

Introduction
Lipopolysaccharide (LPS), among the principal components of all gram-negative bacteria, has been extensively studied as a major factor contributing to the pathogenesis of bacterial infections. LPS induces the production and release of several cytokines, in response of which, several reactive oxygen species (ROS) are produced from cells such as neutrophils, macrophages and other phagocytic cells, creating a status of oxidative stress (1,2). Oxidative stress may induce a rapid alteration in the antioxidant systems by inducing proteins that participate in these systems and/or depleting cellular stores of endogenous antioxidants such as glutathione (3). Thus, this type of stress may hypothetically support the assumption that LPS-induced cell injury would be retarded by modifying free radical metabolism with the aid of antioxidant pretreatment.

In many in vivo and in vitro animal models, a relationship between LPS administration and the resulting overproduction of reactive oxygen species, including free radicals such as superoxide anion (O\textsuperscript{2–}) and hydroxyl radical (OH), has been demonstrated. Reactive oxygen species (ROS), including O\textsuperscript{2–} and the OH as well as other toxic oxygen metabolites, such as hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) and hypochlorous acid, have been postulated to be important mediators in several models of tissue injury (4). Hypochlorous acid, which is a powerful oxidant, is produced by activated neutrophils from hydrogen peroxide by the heme-containing enzyme, Myeloperoxidase (MPO) (5). Nitrite/nitrate (NOx), possessing properties of a free radical, is an important molecule involved in the inflammatory response, is a reactive nitrogen species (RNS). This gaseous free radical is an important biological messenger, highly diffusible, that plays a prominent role in the physiology of the CNS. NO react rapidly with O\textsuperscript{2–} to form peroxynitrite anion (ONOO\textsuperscript{–}) which is the most reactive RNS. ROS and RNS are the cause of oxidative stress in nervous system (6). They are produced in large amounts in pathological conditions, especially NO coming from activated microglia (iNOS) or and from endothelial cells (eNOS). The main sources of ROS in inflammatory process are both damaged mitochondria and activated microglia. Classically oxidative stress is described as an imbalance between generation and elimination of ROS and RNS. These reactive species were originally considered to be exclusively detrimental to cells (7).

COX enzyme has been reported to play a significant role in the pathophysiology of neurodegeneration and brain related disorders and COX inhibition could be a useful drug therapy in neurodegenerative disorders. N\textsuperscript{o}-nitro-L-arginine methyl ester (L-NAME), Indomethacin and Celecoxib exhibited a number of potentially useful clinical effects e.g., neuroprotection for the treatment of conditions such as cerebral stroke, AIDS, dementia and parkinson’s disease (8).

Most of the published studies on this respect referred to the effect of LPS on liver or lung tissue, few studies, however, focused on brain metabolism. Thus, to fulfill the purpose of this aspect, this study has been planned to study the effect of indomethacin, a non-selective COX inhibitor, celecoxib, a selective COX-2 inhibitor and N\textsuperscript{o}-nitro-L-arginine methyl ester (L-NAME), a non-selective NOS inhibitor on malondialdehyde (MDA), a marker of lipid peroxidation, reduced glutathione (GSH), an antioxidant, nitrite/nitrate (NOx), a reactive nitrogen species (RNS) and myeloperoxidase (MPO), a heme containing enzyme in brain induced by intraperitoneal LPS.
Materials and Methods

Animals

The experiments were carried out on adult Sprague-Dawley (SD) rats of wt.175-200 gm. Each group consisted of 5 animals, they were kept in polyacrylic cage and maintained under standard housing conditions (room temperature 25 ± 1°C and humidity 60-65%) with 12 hrs light and dark cycle. The food pellets and water were available ad libitum. The animals were procured from the Laboratory Animal Services Division of Central Drug Research Institute, Lucknow, India. Experiments were performed as per internationally followed ethical standards, after clearance from ethics committee of Central Drug Research Institute and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India on animal experimentation.

Drug administration

The animals were divided into five different groups (n=5). LPS administered intraperitoneally at a dose 4 mg/kg (9) was dissolved in normal saline. Suspensions of celecoxib, indomethacin were prepared with Gum acacia in sterile triple distilled water, while L-NAME was dissolved in sterile triple distilled water and administered orally at a dose of 76 mg, 2 mg and 30 mg per kg body weight (10-11) after 1 hr of LPS treatment respectively. The animals were sacrificed 24 hrs after LPS injection.

Group-1 : Normal saline control. Saline was administered 24 hrs prior to sacrifice.
Group-2 : LPS treated group.
Group-3 : Celecoxib and LPS treated group.
Group-4 : Indomethacin and LPS treated group.
Group-5 : L-NAME and LPS treated group.

Tissue collection

Rats were perfused through heart with ice-cold normal saline after 24 hours of the LPS injection under mild ether anesthesia. The brain was carefully removed and kept over a petri plate after 24 hours of the LPS injection under mild ether anesthesia. The brain was dissected into different regions, viz. hypothalamus (HT), striatum (STR), frontal cortex (FC), cerebral cortex (CC), hippocampus (HP), thalamus (TH), cerebellum (CB) and medulla (MD).

Tissue preparation

Brain regions were homogenized with 10 times (w/v) ice-cold 30 mmol/lit, sodium phosphate buffer, (pH 7) using Ultra-Turrax T25 (USA) homogenizer and aliquots of homogenates were used to estimate MDA and GSH.

After homogenization of tissue, samples were sonicated at 5 sec cycle for 150 sec. using an ultrasonic processor (Heat systems-Ultrasonics, inc). Sonicated samples were then centrifuged for 20 min at 13000 RPM (16600 g) at 4°C using a centrifuge (Biofuge Fresco, Heraeus, Germany). Afterwards, supernatants were collected and stored at −70°C for estimation of NOx and MPO activity.

(a) Estimation of MDA

Malondialdehyde (MDA), a marker of lipid peroxidation, was done in the brain tissue, according to the method of Colado et al (13). After homogenization, tissue homogenate were mixed with 30% TCA, 5 N HCl followed by the addition of 2% TBA in 0.5 N NaOH. The reaction mixture was heated for 15 min at 90°C and centrifuged at 12,000 g for 10 min. The pink colour of the supernatant was measured at 532 nm, using ELISA plate reader (BIOTEK, USA).

(b) Estimation of glutathione

Estimation of reduced glutathione (GSH), an antioxidant marker, was done according to the method of Gupta et al (14). The homogenate was mixed with 10% trichloroacetic acid (TCA) in a 1:1 ratio and then centrifuged for 10 min at 3000 rpm at 4°C to centrifuge out the proteins. To 0.01 ml of this supernatant, 0.2 ml of phosphate buffer (pH 8.4), 0.05 ml of 5, 5-dithiobis (2-nitrobenzoic acid), and 0.04 ml of double distilled water was added. Mixture was vortexed and the absorbance read at 412 nm within 15 min using ELISA plate reader (BIOTEK, USA).

(c) Nitrite/nitrate (NOx) estimation

Nitrite/nitrate (NOx) was estimated in brain samples by Griess reagent (15). Briefly 100 μl of sodium nitrite or samples were seeded in each well (in duplicates) of a 96-microtitre plate except for the blank well. Subsequently 100 μl of freshly prepared Griess reagent (1:1 ratio, mixture of 0.1% of N-1-naphthyl-ethylenediamine in water and 1% of Sulfanilamide in 5% phosphoric acid) was added to each well and the plate was incubated in dark, at room temperature for 20 min. The intensity of the colour developed, was read at 540 nm using ELISA plate reader.

(d) Myeloperoxidase (MPO) activity

Myeloperoxidation activity was determined by modified technique of Bird et al (16). Briefly 10 μl of samples were seeded in each well (in duplicates) of a 96 well microtitre plate and 230 μl of sodium phosphate buffer (30 mmol/L, pH 7.0) was added. Subsequently 50 μl of 0.2% tetramethyl benzidine (in 10% acetic acid) and 10 μl of H2O2 (30%) was added to initiate the reaction. Absorbance was measured at 492 nm at an interval of 30 sec for 3 min using ELISA plate reader and change in optical density/minute was calculated.

(e) Protein estimation

Protein was estimated in brain samples by the method of Lowry et al (17). Bovine serum albumin (BSA) 0.01–0.1 mg/ml was used as standard.
Statistical Analysis

The data was analyzed by one-way analysis of variance (ANOVA) followed by post hoc Newman Keuls test to determine the significance of difference. A probability level of p<0.05 was accepted as statistically significant.

Results

Effect of drugs on LPS induced Malondialdehyde (MDA) in different brain regions:

Intraperitoneal administration of LPS (4 mg/kg, ip) produced significant increase in malondialdehyde (MDA) level in all the brain regions as compared with their respective control, Which were taken as 100 %. Treatment with all the drugs celecoxib (76 mg/kg, po), indomethacin (2 mg/kg, po) and L-NAME (30 mg/kg, po) showed significant decrease in MDA level in all the brain regions as compared to LPS treated group (Fig 1).

Effect of drugs on LPS induced reduced Glutathione (GSH) in different brain regions:

In all the brain regions, LPS (4 mg/kg, ip) produced significant decreases in GSH level in all the brain regions as compared with their respective control, which were taken as 100 %. Treatment with celecoxib (76 mg/kg, po) showed significant increase in GSH level only in hypothalamus and cerebral cortex as compared to LPS treated group. Indomethacin (2 mg/kg, po) did not show any significant change in GSH level in all the brain regions whereas L-NAME (30 mg/kg, po) showed significant increase in all the brain regions as compared to LPS treated group (Fig 2).

Effect of drugs on LPS induced Nitric Oxide (NOx) in different brain regions:

LPS administration (4 mg/kg, ip) significantly increased NOx level in all the brain regions as compared to their respective control, which were taken as 100 %. Treatment with celecoxib (76 mg/kg, po), indomethacin (2 mg/kg, po) and L-NAME (30 mg/kg, po) showed significant decrease in NOx level in thalamus, cerebellum and medulla as compared to LPS treated group. Indomethacin (2 mg/kg, po) treatment showed significant increase in NOx level in hippocampus, thalamus, cerebellum and medulla whereas L-NAME (30 mg/kg, po) showed significant decrease in all the brain regions as compared to LPS treated group (Fig 3).
Lipopolysaccharide (LPS, an endotoxin) from gram negative bacteria induces lipid peroxidation (18) and consequently oxidative damage in many tissues (19). Lipid peroxidation plays a significant role in oxidative pathology. A relationship between LPS administration and the overproduction of reactive oxygen species has been demonstrated. LPS also stimulates nitric oxide synthase (NOS) activity, the enzyme which catalyzes the oxidation of L-arginine to citrulline and nitric oxide (NO). Congeners of NO are neuroprotective or neurodestructive depending on the redox status of NO. NO-mediated neurotoxicity is in part a consequence of its reaction with $\cdot OH$ - leading to formation of the peroxynitrite anion (ONOO$^-\cdot$), which is highly toxic. At low concentrations of L-arginine, LPS stimulates the production of $\cdot OH$ and $H_2O_2$, with this effect being blocked by the non-selective blocker of NOS, L-NAME.

COX and NOS are part of a family of primary inflammatory response genes, whereby COX and NOS expression are coordinately modulated by LPS, bacterial endotoxins, and various cytokines. The most prominent among this evidence was the increase in free radical generation, as reflected by an increase in brain MDA and reduction in GSH. Such elevation could be explained on the basis that LPS induces the production of proinflammatory cytokines in the pituitary, hypothalamus, and hippocampus. The enhanced production of brain MDA observed in our study by LPS injection is in agreement with the in vivo study of Mostafa et al. (20) and in vitro study of Sewerynek et al. (4). Several mechanisms were postulated to explain this phenomenon. One depends on the enhanced release of cytokines that promote the formation and release of reactive oxygen species (ROS) and nitric oxide from microglial cells (21). Another mechanism is based on the release of excitatory amino acids, aspartate and glutamate, that induce free radical formation during their physiological action (22). A third mechanism is related to the LPS-induced mobilization of mitochondrial calcium, which in turn, activates the arachidonic acid cascade that produces ROS (23). The fall in brain GSH content following LPS injection is compatible with another similar study (20). The increased oxidative stress depletes cellular stores of brain antioxidants such as GSH (3). Regarding the effect of COX and NOS inhibitors pretreatment, the most prominent accomplishment was the normalization of brain MDA and GSH. In conclusion, the studied antioxidant pretreatments were effective in reducing LPS-induced brain oxidative stress, as evidenced by elevation of the antioxidant GSH.

Another consideration to the cross talk between iNOS and COX-2 would be required from the angle that COX-2 and its products, including PGE$_2$, may modulate the iNOS pathway. Several studies focused on this point have been performed by the application of either COX inhibitors or PGE$_2$ to in vivo or in vitro investigations. These studies showed that the COX enzyme widely contributes to iNOS gene induction and resultant NO production (24).

There is ample evidence that free radicals are actively involved in the physiological processes during oxidative stress induced by LPS. Among all free radicals the hydroxyl free radicals (OH$^*$) are considered to be the most reactive and hazardous. COX isomers also lead to the formation of hydroxyl free radicals and form peroxynitrite free radicals due to peroxidase activity. Therefore, inhibition of COX isomers could prevent the LPS induced oxidative stress.

In the present study, there was an increase in MDA levels (indicator of the lipid peroxidation due to free radicals) in all brain regions and thus formation of free radicals by LPS administration and furthermore indomethacin (non-selective COX inhibitor), Celecoxib (selective COX-2 inhibitor) and L-NAME (non-selective NOS inhibitor) reversed this increase in lipid peroxidation levels. Celecoxib and L-NAME also showed respective increase in the reduced GSH levels in hypothalamus & cerebral cortex, and all brain regions and hypothalamus, cerebral cortex & hippocampus respectively, suggesting the neuroprotective action of COX-2 as well as NOS inhibitors. L-NAME attenuated the increased nitrite levels in all brain regions where as celecoxib showed reduction in nitrite levels in hippocampus, thalamus, cerebellum and medulla regions of brain. However, indomethacin significantly reduced NO level only in thalamus, cerebellum and medulla regions of brain. Inflammatory events leading to increased myeloperoxidase activity, is indicative of polymorphonuclear leukocytes recruitment in the brain. Both Celecoxib and L-NAME reversed the increase in myeloperoxidase activity in cerebral cortex, hippocampus and thalamus regions of the brain. Whereby, indomethacin reduced the increase in myeloperoxidase activity, only in cerebral cortex and hippocampus regions.

One source of OH$^*$ radicals is the peroxynitrite (ONOO$^-\cdot$), which is generated by the spontaneous reaction of O$_2$ and NO. When NO synthesis is enhanced, the formation of OH$^*$ is favored strongly thus initiating the process of lipid peroxidation and formation of protein adducts, which induces cell damage. COX
isoenzyme leads to an increase in the peroxynitrite levels in rat brain. Both enzymatic sources of oxidative/ nitrosamine mediators are up-regulated in the brain of stressed animals. Once COX and NOS are expressed, the formation of large amounts of oxygen and nitrogen reactive species may account for the oxidation of cellular components found after LPS induced neuroinflammation (oxidative stress) in the rat brain. Moreover, it has been reported that peroxynitrite (ONOO\(^-\)), a product of NO and superoxide, both activates and inhibits the COX-1 and COX-2 activities, depending on (ONOO\(^-\)) concentration.

Celecoxib, Indomethacin and L-NAME attenuated the oxidative stress in brain regions by inhibiting COX. Alternatively, COX inhibitors could also scavenge NO and hydrogen peroxide and prevent excitotoxicity and subsequent oxidative stress. In fact, various COX and NOS inhibitors have also shown neuroprotection in CNS disorders of different etiologies.

In conclusion, the results of the present study suggest that COX and NOS inhibitors are protective against LPS-induced oxidative stress in some of the brain regions of rat.

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