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Riboflavin attenuates lipopolysaccharide-induced lung injury in rats

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Abstract
Riboflavin (vitamin B2) is an easily absorbed micronutrient with a key role in maintaining health in humans and animals. It is the central component of the cofactors flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) and is therefore required by all flavoproteins. Riboflavin also works as an antioxidant by scavenging free radicals. The present study was designed to evaluate the effects of riboflavin against acute lung injury induced by the administration of a single intranasal dose (20 μg/rat) of lipopolysaccharides (LPS) in experimental rats. Administration of LPS resulted in marked increase in malondialdehyde (MDA) level (p < 0.01) and MPO activity (p < 0.001), whereas marked decrease in glutathione (GSH) content (p < 0.001), glutathione reductase (GR) (p < 0.001) and glutathione peroxidase (p < 0.01) activity. These changes were significantly (p < 0.001) improved by treatment with riboflavin in a dose-dependent manner (30 and 100 mg/kg, respectively). Riboflavin (100 mg/kg, p.o.) showed similar protective effects as dexamethasone (1 mg/kg, p.o.). Administration of LPS showed marked cellular changes including interstitial edema, hemorrhage, infiltration of PMNs, etc., which were reversed by riboflavin administration. Histopathological examinations showed normal morphological structures of lungs tissue in the control group. These biochemical and histopathological examination were appended with iNOS and CAT gene expression. The iNOS mRNA expression was increased significantly (p < 0.001) and levels of CAT mRNA expression was decreased significantly (p < 0.001) in the animals exposed to LPS, while treatment with riboflavin significantly (p < 0.01) improved expression of both gene. In conclusion, the present study clearly demonstrated that riboflavin caused a protective effect against LPS-induced ALI. These results suggest that riboflavin may be used to protect against toxic effect of LPS in lungs.

Introduction
Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are serious clinical problems with high mortality (Chen et al., 2003). Alveolar edema is an early major change/lesion in the ARDS (Matthay & Wienerkronish, 1990), whereas severe hypoxemia, pulmonary edema and neutrophil accumulation in the lungs occur in ALI (Lee & Downey, 2001; Puneet et al., 2005). Furthermore, airway infiltration and activation of polymorphonuclear neutrophils (PMNs) in response to environmental airborne toxicants, such as ozone, particulate matter and biogenic toxicants, are critical features in ALI (Alexis et al., 2006; Bosson et al., 2008; Dillon et al., 2011). Apoptotic changes in the lungs tissue are considered as the pathogenesis of ALI which may cause delay and abnormal secretion of neutrophil granulocytes, altered the release of cytokines, and imbalance in oxidant/antioxidant factors (Kristof et al., 1998; Rubenfeld et al., 2005).

Mechanistically, infiltrated neutrophils lead to the production of reactive oxygen species (ROS), which play an important role in the pathogenesis of neutrophil-mediated airway injury and disease (Grommes & Soehnlein, 2011; Moraes et al., 2006). Therefore, therapeutic measures that control the airways infiltration or activation of PMNs may be useful in the mitigation of the morbidity (Wagner & Roth, 2000). Interventions, such as glucocorticoids and NADPH oxidase inhibitors, are used to protect from tissue injury mediated by PMNs and PMNs oxidative burst while antioxidant interacts directly with and neutralizes ROS (Condino-Neto et al., 1998; El-Benna et al., 2012). Also micronutrients with antioxidant properties, such as α-tocopherol and ascorbate (vitamin C), have been used to reverse ROS-mediated pulmonary injury associated with a number of acute and chronic airway diseases (Rocksen et al., 2003; Samet et al., 2001).
Riboflavin (vitamin B2) is an easily absorbed micronutrient with a key role in maintaining health in humans and animals. It is the central component of the cofactors flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), and is therefore required by all flavoproteins (Hung et al., 2006). As such, riboflavin is required for a wide variety of cellular processes. Like the other B vitamins, riboflavin plays a key role in energy metabolism and is required for the metabolism of fats, carbohydrates and proteins. In animals, riboflavin deficiency results in lack of growth and failure to thrive.

Riboflavin also works as an antioxidant by scavenging free radicals and hence it can be evaluated for the prevention of lungs toxicity (Lago & Kaplan, 1981). Riboflavin also plays a key role in the regulation of glutathione reductase (GR), a central enzyme in the maintenance of redox regulation in the cells, by virtue of being a component of the cofactor. Hence, riboflavin may play a direct role in the maintenance of cellular oxidative stress, such as ALI. However, the protective effect of riboflavin against ALI remains unknown. Therefore, the present study was designed to evaluate the possible protective effect of riboflavin on lipopolysaccharide-induced lung injury in rats.

Materials and methods

Materials

All the chemical and reagents used in the study were purchased from commercial sources. Lipopolysaccharide (LPS), dexamethasone and riboflavin were purchased from Sigma Aldrich. DNA primers were obtained from Integrated DNA Technologies (IDT, Coralville, IA). TRIzol reagent was supplied by Invitrogen Co. (San Diego, CA). High Capacity cDNA Reverse Transcription kit and SYBR® Green PCR Master Mix were purchased from Applied Biosystems® (Foster City, CA). All the other chemicals used for experimental work were of analytical grade.

Animals

Male Wistar albino rats weighing 180–200 g (10–12 weeks old) used in the current study were obtained from Experimental Animal Care Center, College of Pharmacy at King Saud University. Animals were housed under ideal laboratory conditions (12 h light/12 h darkness cycle, 45–55% relative humidity and temperature 23–25°C), maintained on standard pellet diet and water ad libitum throughout the experimental period. All experiments were carried out according to the guidelines of the animal care and use committee at King Saud University.

Experimental design

Thirty rats were randomly divided into five groups of six animals each: Group 1, control group, received 50 μl phosphate buffer saline (PBS) intranasally (i.n.) on day 7, under light anesthesia. Group 2, LPS group, LPS 20 μg/rat in 50 μl of PBS was administered i.n. on day 7 after 1 h of scheduled treatment to induce lung injury (Hernandez et al., 2013; Shih et al., 2010; Yingkun et al., 2013). Group 3, treatment group, received riboflavin (30 mg/kg, p.o.) for 7 days followed by LPS similar to group 2. Group 4, treatment group, received riboflavin (100 mg/kg, p.o.) for 7 days followed by LPS similar to group 2. Group 5, positive control, received dexamethasone (1 mg/kg, p.o.) for 7 days followed LPS i.n. on day 7 similar to group 2.

All rats were sacrificed after 6 h of last dose administration by decapitation under ether anesthesia, as per the protocol. The rats lungs were isolated and washed in ice cold PBS and used for the assessment of oxidative stress, histopathology and gene expression.

Histopathology

Among the five different experimental groups, isolated lung tissues of the rats were fixed in 10% buffer formosaline. Paraffin sections of thickness 3–4 μm were prepared and stained with hematoxylin and eosin (H&E) for histopathological examination under light microscopy.

Preparation of tissue homogenate

All the animals were sacrificed under light ether anesthesia. The lung tissues were removed quickly, perfused immediately with ice cold normal saline. A portion of lung sample was homogenized in chilled phosphate buffer (0.1 M, pH 7.4) containing potassium chloride (1.17% w/v), using a Potter Elvehjem homogenizer. The homogenate was centrifuged at 700 g for 10 min at 4°C to separate the nuclear debris. The supernatant obtained was then centrifuged at 9000 g for 20 min at 4°C to get the post-mitochondrial supernatant (PMS).

Determination of lipid peroxidation measured as malondialdehyde

Level of malondialdehyde (MDA), a product of membrane lipids peroxidation, was estimated in lungs tissue by the method of Ohkawa et al. (1979), using the standard calibration curve prepared with tetraethoxy propane. Protein was estimated by the method of Lowry et al. (1951). MDA was expressed as nmole of MDA per mg of protein.

Determination of reduced glutathione

Glutathione (GSH) content was estimated by the method of Sedlak & Lindsay (1968). The absorbance of reaction mixture was read within 5 min of the addition of dithibus-2-nitrobenzoic acid at 412 nm using UV-spectrophotometer, against a reagent blank.

Determination of GPx activity

GPx activity was measured according to the modified method of Little et al. (1970) using microplate reader. The conversion of NADPH to NADP is monitored by a continuous recording of the change of absorbance at 340 nm at 1-min interval for 5 min. One unit of GPx was defined as the amount of enzyme causing the oxidation of 1 nmol of NADPH/min/mg protein. The enzyme activity was expressed as nmol NADPH oxidized/min/mg protein.
**Determination of GR activity**

GR activity in PMS was determined as described by Carlberg and Mannervik using microplate reader (Carlberg & Mannervik, 1985). The absorbance was measured at 412 nm at 25 °C. The enzyme activity was expressed as nmol NADPH oxidized/min/mg protein.

**Determination of MPO activity**

MPO activity, which reflects the infiltration of neutrophils into the lung, was measured according to the modified method of Suzuki et al. (1983). The reaction mixture was assayed for MPO activity by measuring the OD at 460 nm.

**RNA extraction, cDNA synthesis and quantification of mRNA expression by real time-polymerase chain reaction (RT-PCR)**

The total cellular RNA was isolated using TRIzol reagent (Invitrogen®) and quantified by measuring the absorbance at 260 nm. RNA quality was determined by measuring the 260/280 ratio (>2.0). High-Capacity cDNA reverse transcription kit (Applied Biosystems®) was utilized to synthesize first strand cDNA, according to the manufacturer’s instructions. Briefly, 1.5 μg of total RNA from each sample was added to a mixture of 2.0 μl of 10× reverse transcriptase buffer, 0.8 μl of 25× dNTP mix (100 mM), 2.0 μl of 10× reverse transcriptase random primers, 1.0 μl of MultiScribe reverse transcriptase and 3.2 μl of nuclease-free water. The final reaction mixture was kept at 25 °C for 10 min, heated to 37 °C for 120 min, heated for 85 °C for 5 min and finally cooled to 4 °C.

Quantitative analysis of specific mRNA expression was performed by RT-PCR by subjecting the resulting cDNA products to PCR amplification using 96-well optical reaction plates in the ABI Prism 7500 System (Applied Biosystems®). The fold change in the level of target mRNA between the control and treated animals was corrected by the level of β-actin. Assay controls were incorporated onto the same plate, namely, no-template controls to test for the contamination of any assay reagents. The RT-PCR data were analyzed using the relative gene expression (i.e. ΔΔ CT) method as described previously (Livak & Schmittgen, 2001).

**Statistical analysis**

Results were expressed as mean ± SEM. (n = 6). One way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test was used to identify significance among groups. Values were considered statistically significant when p < 0.05. Statistical analysis was carried out using Graph pad prism 3.0 (La Jolla, CA).

**Results**

**Effects of riboflavin on lipid peroxidation and GSH in LPS-induced ALI**

In general, it is established that oxidative stress results in damage to the membrane and supplementation of an antioxidant protects from this damage. MDA, a product of lipid peroxidation, is the biomarker for estimating the status of oxidative stress. In this study, administration of LPS to rats caused a significant (p < 0.05) increase in lungs MDA contents approximately by 2-fold as compared to the control (15.65 ± 1.433) versus LPS (29.72 ± 1.251) groups. Treatment with riboflavin 30 and 100 mg/kg showed a dose-dependent reversal in LPS-induced increase in MDA levels (22.40 ± 1.346 versus 29.72 ± 1.251 and 15.06 ± 1.640 versus 29.72 ± 1.251; p < 0.05) compared to the LPS group, respectively. Consequently, administration of LPS to rats caused significant (p < 0.05) decrease in GSH level approximately by 5-fold as compared to the control (1.96 ± 0.139) versus toxic (0.49 ± 0.109) groups. Treatment with riboflavin 30 and 100 mg/kg showed a dose-dependent reversal in LPS-induced decrease in GSH level (0.984 ± 0.114 versus 0.49 ± 0.109 and 1.611 ± 0.117 versus 0.49 ± 0.109; p < 0.05) compared to the LPS group, respectively. These results indicated that riboflavin inhibited LPS-induced lipid peroxidation in a dose-dependent manner. High dose of riboflavin produced similar effect compared to dexamethasone (Figures 1 and 2).

**Effects of riboflavin on GPx and GR activities in LPS-induced ALI**

We measured the effect of riboflavin on GPx and GR activities to determine the involvement of antioxidant markers in LPS-induced lung injury. We found that treatment of rats with LPS resulted 78% and 75% decrease in GPx and GR activities, respectively as compared to the control. Treatment with riboflavin 30 and 100 mg/kg ameliorates LPS-induced decrease in GPx and GR activities (Figures 3 and 4). High dose of riboflavin produced significant improvement in the GPx and GR activities and their effect was comparable to dexamethasone standard treatment. Treatment of rats with dexamethasone significantly reversed LPS-induced decrease in GPx (74.84%) and GR (77.66%) activity (Figures 3 and 4).

**Effects of riboflavin on MPO activity in LPS-induced ALI**

MPO activity in lungs was determined to assess the effects of riboflavin on neutrophil accumulation. LPS challenge caused an increase in lung MPO activity (15.81 ± 1.953 versus 32.864 ± 0.480; p < 0.05) compared with the control. Dose-dependent improvements in MPO activity were seen with riboflavin treatment (Figure 5). This infers that riboflavin treatment reversed LPS-induced increase neutrophils accumulation reflected by MPO activity in lungs.

**Effects of riboflavin on histopathological changes in LPS-induced ALI**

Normal morphological structures of lungs tissue were observed in the control group (Figure 6A).
However, treatment with LPS showed marked inflammatory response characterized by presence of interstitial edema, hemorrhage, infiltration of inflammatory PMNs, leucocytes and destruction of lung parenchyma (Figure 6B). These changes were reversed in a dose-dependent manner by the treatment with riboflavin (Figure 6C and D). High dose of riboflavin ameliorates LPS-induced inflammation in the lung similar to Dex (Figure 6E).

Effects of riboflavin on iNOS and CAT gene expression in LPS-induced ALI

iNOS and catalase activity is considered to be a reliable marker of lung inflammation. Therefore, we measured its expression in different experimental groups. Figures 7 and 8 show that treatment of rats with LPS significantly induced iNOS and suppressed CAT mRNA expression by approximately 40-fold and 65%, respectively as compared to the control. However, riboflavin treatment significantly decreased the LPS-induced iNOS and improved CAT expression by approximately 50% and 74%, respectively. These results showed that riboflavin is equally potent as Dex in attenuating LPS-induced iNOS and CAT gene expression (Figures 7 and 8).

Discussion

The present study provides the first evidence that riboflavin protects against LPS-induced ALI. This is supported by the oxidant/antioxidant enzyme system, histopathology and iNOS and CAT gene expression.

LPS is a prototypic pathogen-associated molecular pattern moiety that activates airway monocytes and macrophages. Inhalation challenge studies of human volunteers demonstrate that LPS induces increased PMNs, proinflammatory cytokine...
Figure 6. Effects of riboflavin on lung histopathology of different experimental groups. (A) Control, (B) LPS, (C) LPS + R(30), (D) LPS + R(100) and (E) LPS + Dex (n = 6 per group; magnification = 20×). Arrow, arrow head and double arrow heads indicate interstitial edema, hemorrhage and destruction of lung parenchyma, respectively.

Figure 7. Effects of riboflavin on iNOS gene expression of lung tissue in different experimental groups. Data were presented as mean ± SEM (n = 6). *p < 0.05 compared to the control; #p < 0.05 compared to the LPS.

Figure 8. Effects of riboflavin on CAT gene expression of lung tissue in different experimental groups. Data were presented as mean ± SEM (n = 6). *p < 0.05 compared to the control; #p < 0.05 compared to the LPS.
levels and oxidative stress, thus serving as a model of ALI (Alexis et al., 2005, 2008; Hernandez et al., 2010, 2012).

Riboflavin (vitamin B2) is a central component of the cofactors FAD and FMN and hence is required for a wide variety of cellular processes (Hung et al., 2006; Lago & Kaplan, 1981). Recently, some studies have reported that riboflavin produced a dose-related antiinociceptive and antiinflammatory effects (Granados-Soto et al., 2004), as well as neuro-protective effects through reduction of ischemic brain injury in focal cerebral ischemia (Tripathi et al., 2014). In addition, it has been shown that high dose of riboflavin decreased the LPS-induced mortality through increased expression of heat shock protein 25 (Shih et al., 2010). A clinical study reported that riboflavin is effective and low-cost prophylactic treatment in children and adolescents suffering from migraine (Condo et al., 2009). Yet the protective effect of riboflavin on LPS-induced lung injury in rats was not investigated.

The formation of MDA, a lipid peroxidation biomarker, leads to the activation and generation of free radicals by the pulmonary endothelium and neutrophils, up-regulation of adhesion molecules and production of cytokines and chemokines promoting the recruitment of macrophage and neutrophils within the pulmonary microvasculature (Chow et al., 2003; Ivanova et al., 2006). The exaggeration of these events ultimately leads to air space epithelial injury and respiratory failure as a result of oxidant/antioxidant imbalance (Chow et al., 2003; Valenca et al., 2008). In this study, treatment with riboflavin showed a significant improvement in LPS-induced lipid peroxidation in a dose-dependent manner. In general, it is established that oxidative stress results in damage to the membrane and supplementation of an antioxidant protects from this damage.

The pathogenesis of ALI involves oxidative stress, up-regulation of adhesion molecules and increased production of inflammatory cytokines (Bhargava & Wendt, 2012; Kovach & Standiford, 2011; Kuo et al., 2011). Reactive oxygen species (ROS) decisively contribute to cellular signaling, affecting almost all aspects of cellular function including gene expression, proliferation, migration and cell death (Brandes & Kreuzer, 2005). The cellular damaging effects of ROS (including nitric oxide and peroxynitrite) entail the lipid peroxidation. In the present study, administration of LPS to generates lipid peroxidation of membrane phospholipids and culminates in the formation of MDA (Balkan et al., 2005).

Tissue GSH depletion is one of the primary factors which permit lipid peroxidation (Konukoglu et al., 1998). In the present study, administration of LPS decreased GSH level, GPx and GR activities as compared to the control group, while treatment with riboflavin reversed LPS-induced decrease in lungs GSH content, GPx and GR activities in a dose-dependent manner. Increased intracellular GSH content, GPx and GR activities might be due to up-regulation of enzymatic/nonenzymatic antioxidants or decreased ROS production in the riboflavin-treated group.

Primary granules of neutrophils mainly contain MPO enzyme and thus adhesion and margination of neutrophils in the lungs parenchyma are predicted by increased MPO activity (Chen et al., 2013). To quantify the magnitude of pulmonary permeability, the MPO activity in the lungs tissue was evaluated to assess the effects of riboflavin on neutrophil accumulation. LPS challenge caused significant increases in lung MPO activity compared with the control group. However, treatment with riboflavin or dexamethasone significantly lowered the MPO compared with the LPS-treated group. These findings indicated that riboflavin significantly decreases the high lung permeability and protective effects on LPS-induced ALI.

Nitric oxides (NO) play a role in the occurrence and development of systemic inflammatory responses (Su et al., 2006; Wang et al., 2009). Since they have been found to be the potent mediators of potentially damaging tissue responses, several mechanisms exist to ensure that the effects of these cytokines are restricted (Gando et al., 2003; Galani et al., 2010). In the present study, treatment of rats with LPS significantly induced iNOS mRNA expression by approximately 40-fold, whereas treatment with riboflavin and dexamethasone significantly decreased iNOS levels as compared to the LPS-treated groups, showing that riboflavin attenuates LPS-induced inflammation in the lung. These observation are in agreement with the previous studies showing that selective inhibition of iNOS and its biosynthetic products NO has been shown to suppress inflammation in a variety of inflammation states (de las Heras et al., 2001). At the histopathology level, treatment with LPS showed marked inflammatory response characterized by the presence of interstitial edema, hemorrhage, infiltration of inflammatory PMNs, leucocytes and destruction of lung parenchyma. However, treatment with riboflavin or dexamethasone significantly improved interstitial edema, hemorrhage, infiltration of inflammatory PMNs and leucocytes, in a manner similar to dexamethasone treatment. These changes were reversed in a dose-dependent manner by the treatment with riboflavin.

In conclusion, we found that riboflavin caused a protective effect against LPS-induced ALI due to its antioxidant and anti-inflammatory effects. These findings were confirmed by biochemical markers, histopathological examination and RT-PCR. Therefore, we suggest that riboflavin may be used to protect against toxic effect of LPS and other chemical agents in lungs injury.

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Declaration of interest

The authors declare that there are no conflicts of interest.

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