

Original Article

Promise of 2-methylbenzamide in the prevention of acute kidney injury in rats through inhibition of inflammatory factor expression

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Abstract: The present study aimed to investigate the effect of 2-methylbenzamide on lipopolysaccharide (LPS) induced acute kidney injury rats model. Male Sprague-Dawley rats were assigned to control, untreated, treatment and YOH (yohimbine) groups. Except control group, the other three groups of rats were injected 5 mg/kg LPS. The animals in the treatment group were given 2-methylbenzamide (5 mg/kg) 1 h prior LPS injection whereas those in the YOH group received 1 mg/kg YOH half an hour before 2-methylbenzamide administration. After 12 h of LPS injection, the animals were sacrificed to extract the kidneys for examination of tissue morphology, expression of kidney injury molecule-1 (KIM-1) and high mobility group protein 1 (HMGB-1). Blood samples were collected for the analysis of creatinine, blood urea nitrogen, interleukin 6 (IL-6), IL-18 and tumor necrosis factor α (TNF- α) levels. The results revealed that 2-methylbenzamide treatment caused a significant ($P < 0.005$) reduction in the levels of LPS induced creatinine, blood urea nitrogen, interleukin 6 (IL-6), IL-18 and tumor necrosis factor α (TNF- α) levels. The expression levels of KIM-1 and HMGB-1 enhanced by LPS injection were also reduced markedly by 2-methylbenzamide treatment. In addition, treatment of LPS induced acute kidney injury rats with 2-methylbenzamide prevented the degradation of renal tissues. However, administration of YOH reversed the protective effect of 2-methylbenzamide in rats with acute kidney injury. Thus, 2-methylbenzamide protects against LPS induced acute kidney injury in rats through inhibition of inflammatory factor expression.

Keywords: Lipopolysaccharide, 2-methylbenzamide, yohimbine, acute kidney injury, inflammation

Introduction

Acute kidney injury is the major clinical complication of the kidneys and is associated with high rate of morbidity and mortality [1, 2]. It is reported that the aged patients suffering from diabetes or cardiac disorders are most susceptible to the acute kidney injury. In the patients with acute kidney injury, there is degradation of tissues in the tubular and vascular areas in nephrons induced by the inflammatory reaction [3]. Kidney is considered to be the most frequent target organ for the sepsis caused by various factors including, pathogenic attack, trauma, reperfusion injury, hypoxia, etc. [4]. Among the patients with sepsis, around 60% develop to acute kidney injury with mortality rate of more than 65% [5, 6]. The strategies used for the prevention of acute kidney

injury currently include, supportive care and renal replacement. Despite advancement in the fields of renal replacement and surgery, kidney injury continues to be a serious issue at present. The five-year mortality rate for the patients with acute kidney injury is less than 50% [7]. Therefore, the screening and identification of the molecules which can prevent the acute kidney injury is being constantly performed.

It has been reported that interleukins including, IL-6 and IL-18 are the characteristic and diagnostic markers of acute kidney injury [8]. In the patients with acute kidney injury, IL-18 induces the expression of tumor necrotic factor- α and IL-6 which enhance the progress of inflammatory reactions leading to the tissue injury [9]. Therefore, the inhibition of expression of the inflammatory factors is considered to be prom-

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ising strategy for the prevention of acute kidney injury. The early stage of the acute kidney injury is characterized by the increase in expression of KIM-1 and HMGB-1. Among the two factors, KIM-1 is usually absent in the kidney of normal animals. However, it is reported that the expression of KIM-1 is increased significantly in the animals with kidney injury and its level of expression is indicative of the stage of injury [10-14]. The α -2-adrenergic receptor antagonist, YOH reverses the effect of 2-methylbenzamide in LPS induced acute kidney injury rat model [12]. Thus, it appears that 2-methylbenzamide exhibits its effect through α -2 adrenoceptor by inhibiting the progress by inflammatory processes.

HMGB-1 plays an important role in the process of induction of inflammatory reactions by activating various factors involved in inflammation process.

In the present study, effect of 2-methylbenzamide on the LPS induced acute kidney injury rat model was investigated.

Materials and methods

Reagents and chemicals

2-methylbenzamide, YOH and LPS were purchased from (Sigma-Aldrich, St. Louis, MO, USA). The other common chemicals were obtained from Gibco BRL (Grand Island, NY, USA).

Animals and treatment

Forty male Sprague-Dawley rats (8 week old) were obtained from Shanghai Laboratory Animal Commission (SLAC, Shanghai, China). The animals were assigned randomly into four groups of 10 in each: control, untreated, treatment and YOH group. The animals in the untreated, treatment and YOH groups were injected 5 mg/kg LPS through via the tail vein after 12 h of fasting to induce kidney injury. The rats in the control group received an equal volume of normal saline. The animals in the treatment group were given 2-methylbenzamide (5 mg/kg) 1 h prior to LPS injection. Animals in the YOH group received 1 mg/kg YOH half an h before 2-methylbenzamide administration. After 12 h after LPS injection, the animals were sacrificed to extract the kidneys for investigation. The animal procedures were performed according to the Guidance Suggestions for the Care and Use of Laboratory Animals 2012 administered by the Ministry of Science and

Technology of the People's Republic of China. The study was also approved by the Laboratory Animal Care Committee of Science and Technology of the People's Republic of China.

Analysis of creatinine and blood urea nitrogen levels

The blood samples of the rats were collected from abdominal artery and analyzed for the creatinine and blood urea nitrogen. The levels of creatinine and blood urea nitrogen in the blood of rats was determined by using the automatic biochemistry analyzer (Hitachi 7600-020/7170A; Hitachi High-Technologies Corp., Tokyo, Japan).

Morphological examination of the kidney tissues

From the mice, left kidney was extracted, washed in PBS and fixed in 10% buffered formalin. The samples were the nparaffin embedded, cut into thin 2 μ m sections followed by staining with hematoxylin and eosin (Nanjing Keygen Biotech Co., Ltd., Nanjing, Jiangsu, China). The morphological examination of the tissues was performed by random observation of the five areas under a magnification of \times 500. The quantification of the kidney injury was carried out by Hamar's method.

Measurement of plasma IL-6, IL-18 and TNF- α

The level of inflammatory factors in the serum of control, untreated and treatment groups of rats was determined. For this purpose, the blood samples from the abdominal artery of rats were collected and transferred into the heparinized 2 ml plastic tubes. The tubes were then centrifuged for 20 min at 2,500 \times g to collect the plasma which was stored at -80°C. ELISA assay kits for IL-6, IL-18 and TNF- α (all purchased from Nanjing Keygen Biotech. Co., Ltd) were used for the determination of serum IL-6, IL-18 and TNF- α levels as per the manual protocol.

Western blot analysis

For the purpose of kidney injury molecule-1 (KIM-1) and high mobility group protein 1 (HMGB-1) protein expression determination, the right kidney of the rats was extracted. The tissue samples of the kidney were boiled in sodium dodecyl sulfate (SDS) loading buffer (Bio-Rad, Hercules, CA, USA) for the purpose of solubilisation. The proteins were isolated on

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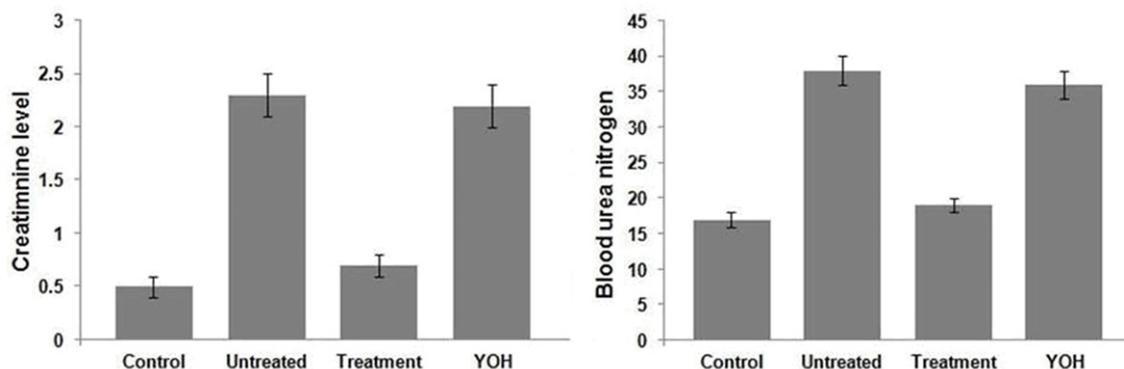


Figure 1. Effect of 2-methylbenzamide and yohimbine (YOH) on the lipopolysaccharide (LPS)-induced function of renal tubules in rats. The LPS injected rats were given 2-methylbenzamide and yohimbine (YOH) and then the levels of creatinine and blood urea nitrogen were determined. The rats in the control, untreated, treatment and YOH groups were administered saline, 5 $\mu\text{g}/\text{kg}$ LPS, 5 $\mu\text{g}/\text{kg}$ LPS + 5 $\mu\text{g}/\text{kg}$ 2-methylbenzamide, 5 $\mu\text{g}/\text{kg}$ LPS + 1 mg/kg YOH + 5 $\mu\text{g}/\text{kg}$ 2-methylbenzamide, respectively.

10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and then transferred to a nitrocellulose membrane. The membranes were incubated with primary antibodies using blocking buffer and then washed three times with PBS. Following PBS washing, the membranes were incubated for 1 h with secondary antibodies at room temperature. The primary antibodies used were against KIM-1 (cat. no. sc-47495, 1:2,000 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and HMGB-1 (cat. no. sc-26351, 1:2,000 dilution; Santa Cruz Biotechnology, Inc.). The secondary antibodies used for incubation was horseradish peroxidase-conjugated goat anti-rabbit IgG (Wuhan Boster Biological Technology, Ltd.; 1:10,000). The internal loading control used was the β -actin. For the purpose of visualization of protein bands and quantification enhanced chemiluminescent detective system (Amersham Biosciences UK Ltd., Little Chalfont, UK) and Quantity One software package (Bio-Rad), respectively were used.

Statistical analysis

The Statistical Package for Social Sciences (SPSS for Windows, version 17.0; SPSS, Inc., Chicago, IL, USA) was used for the process of the data and monofactorial analysis of variance for the purpose of analysis. The difference between the groups was analyzed by Student's *t*-test. All the data were presented as mean \pm standard deviation. A *P*-value <0.05 is considered to be statistically significant.

Results

Effect of 2-methylbenzamide on blood urea nitrogen and creatinine levels in LPS-induced acute kidney injury rats

Acute kidney injury was induced in the rats by injecting LPS through the vein in tail. It was observed that LPS injection resulted in a marked enhancement in the levels of both creatinine and blood urea nitrogen (Figure 1). The levels of creatinine and blood urea nitrogen were 3 and 1-fold, respectively, higher in the LPS injected rats compared to the control group. However, 2-methylbenzamide treatment suppressed the LPS induced increase in the levels of creatinine and blood urea nitrogen. The levels of creatinine and blood urea nitrogen in the 2-methylbenzamide treatment and control group of rats were found to be similar (Figure 1). Administration of the YOH to 2-methylbenzamide treated rats inhibited the effect of 2-methylbenzamide and caused increase in the levels of creatinine and blood urea nitrogen (Figure 1).

Effect of 2-methylbenzamide on the pathological damage of kidneys in LPS-induced acute kidney injury rats

For the purpose of analysis of the effect of 2-methylbenzamide on morphology of the renal tubule, Hamar score method was employed. Examination of the kidney tissues in LPS injected rats showed degradation of the tissues, broad lumen tubules, penetration of the inflam-

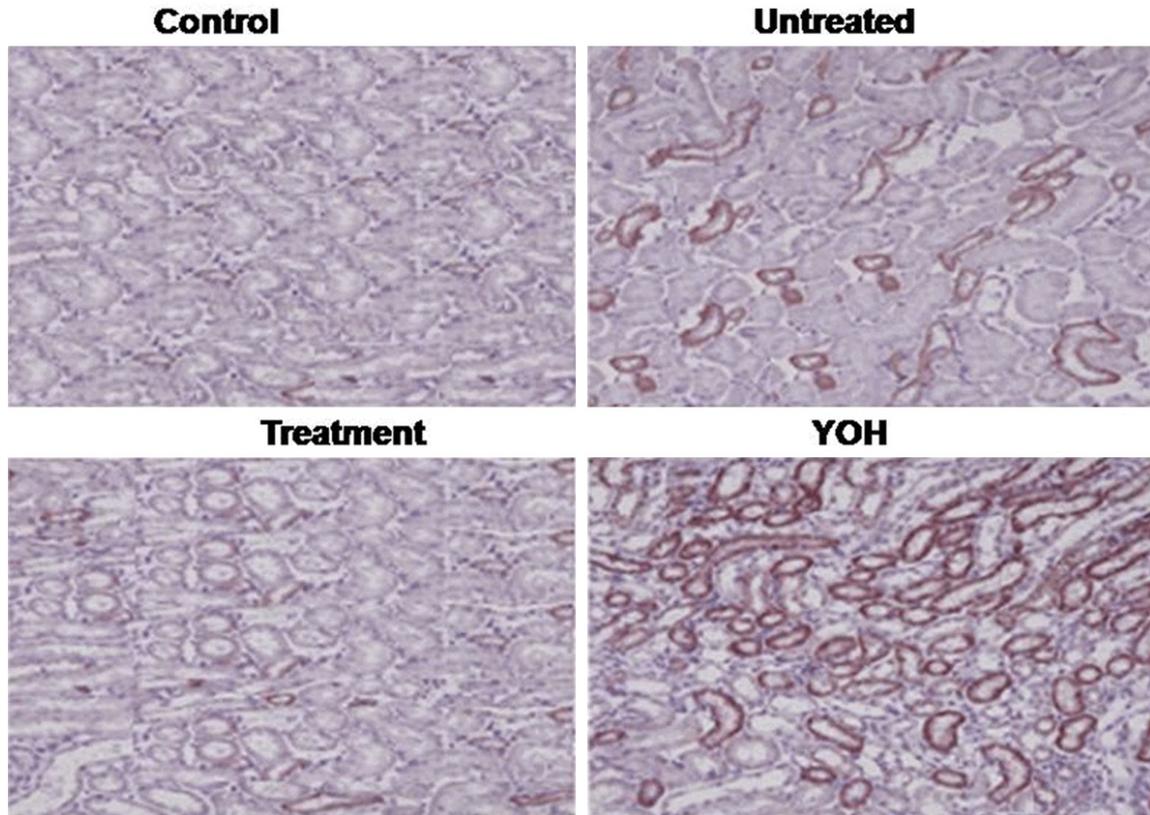


Figure 2. Effect of 2-methylbenzamide and yohimbine (YOH) on lipopolysaccharide (LPS)-induced kidney tissue damage. For the examination of histopathological changes hematoxylin and eosin staining was used followed by original magnification, $\times 200$. The rats in the control, untreated, treatment and YOH groups were administered saline, 5 $\mu\text{g}/\text{kg}$ LPS, 5 $\mu\text{g}/\text{kg}$ LPS + 5 $\mu\text{g}/\text{kg}$ 2-methylbenzamide, 5 $\mu\text{g}/\text{kg}$ LPS + 1 mg/kg YOH + 5 $\mu\text{g}/\text{kg}$ 2-methylbenzamide, respectively.

matory of cells and weakened tissues (**Figure 2**). However, no such alterations in the morphology were observed in the kidney tissues of control group of rats. Treatment of the rats with 2-methylbenzamide inhibited the LPS induced alterations in the morphology of kidney tissues.

Effect of 2-methylbenzamide on the LPS-induced renal damage in acute kidney injury rats

Injection of LPS caused a significant increase in the expression level of KIM-1 and HMGB-1 in rats compared to control group. However, the expression of KIM-1 and HMGB-1 in the rats of experiment group was reduced and become similar to that of the control group (**Figure 3**). The reduction in the expression levels of KIM-1 and HMGB-1 by 2-methylbenzamide in acute kidney injury rats were also inhibited by administration of YOH (**Figure 3**).

Effect of 2-methylbenzamide on expression of inflammatory factors

The effect of 2-methylbenzamide on the expression level of IL-6, IL-18 and TNF- α (inflammatory factors) in the rats with acute kidney injury was also analysed. LPS treatment increased the expression levels of IL-6, IL-18 and TNF- α in animals. However, 2-methylbenzamide caused a significant reduction in the expression of all the three inflammatory factors in treatment group (**Figure 4**). On the other hand, administration of YOH inhibited the 2-methylbenzamide induced reduction in the expression of IL-6, IL-18 and TNF- α in acute kidney injury rats.

Effect of 2-methylbenzamide on kidney cell apoptosis in rats

The results from western blot analysis revealed that treatment of the LPS induced acute kidney injury rat model with 2-methylbenzamide sig-

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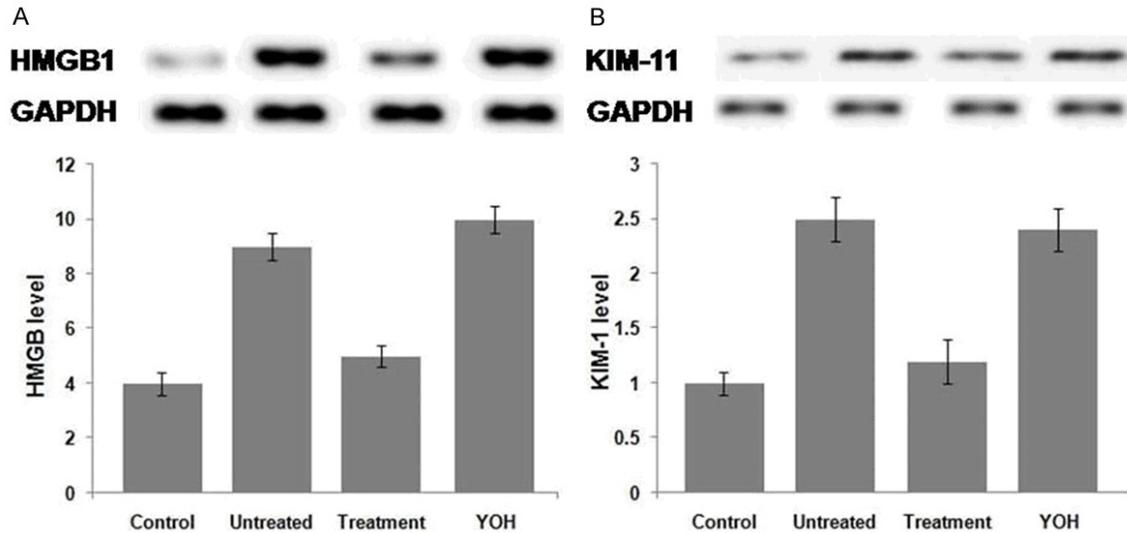


Figure 3. Effect of 2-methylbenzamide and yohimbine (YOH) on lipopolysaccharide (LPS)-induced alterations in expression of high mobility group protein 1 (HMGB-1) and kidney injury molecule-1 (KIM-1) in rats. The rats in the control, untreated, treatment and YOH groups were administered saline, 5 µg/kg LPS, 5 µg/kg LPS + 5 µg/kg 2-methylbenzamide, 5 µg/kg LPS + 1 mg/kg YOH + 5 µg/kg 2-methylbenzamide, respectively.

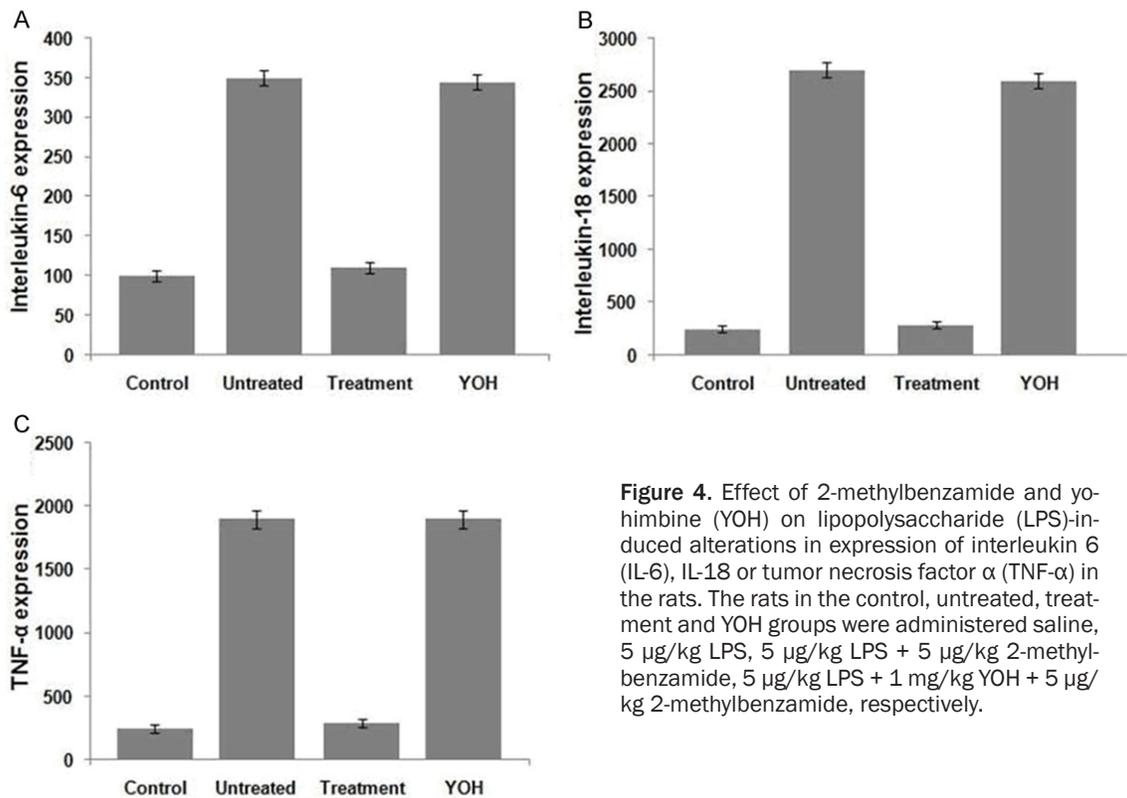


Figure 4. Effect of 2-methylbenzamide and yohimbine (YOH) on lipopolysaccharide (LPS)-induced alterations in expression of interleukin 6 (IL-6), IL-18 or tumor necrosis factor α (TNF-α) in the rats. The rats in the control, untreated, treatment and YOH groups were administered saline, 5 µg/kg LPS, 5 µg/kg LPS + 5 µg/kg 2-methylbenzamide, 5 µg/kg LPS + 1 mg/kg YOH + 5 µg/kg 2-methylbenzamide, respectively.

nificantly ($P < 0.005$) decreased the expression of caspase-3 compared to LPS injected rats (Figure 5). However, administration of YOH reversed the inhibitory effect of 2-methylbenza-

mide on the expression of caspase-3. Thus, 2-methylbenzamide treatment protects the acute kidney injury in rats through the reduction in caspase-3 expression.

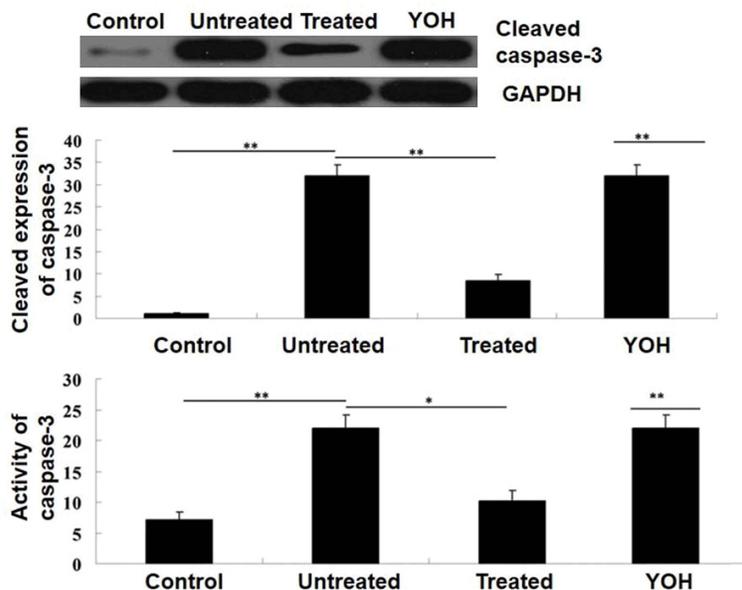


Figure 5. Effect of 2-methylbenzamide and yohimbine (YOH) on lipopolysaccharide (LPS)-induced changes in expression of caspase-3 in the rats. The rats in the control, untreated, treatment and YOH groups were administered saline, 5 $\mu\text{g}/\text{kg}$ LPS, 5 $\mu\text{g}/\text{kg}$ LPS + 5 $\mu\text{g}/\text{kg}$ 2-methylbenzamide, 5 $\mu\text{g}/\text{kg}$ LPS + 1 mg/kg YOH + 5 $\mu\text{g}/\text{kg}$ 2-methylbenzamide, respectively.

Discussion

The present study demonstrates the effect of 2-methylbenzamide on LPS induced acute kidney injury in rats. The results revealed that treatment of rats with 2-methylbenzamide inhibited the LPS induced increase in creatinine and blood urea nitrogen levels, prevented renal tissue degradation, reduced the expression of KIM-1, HMGB-1, IL-6, IL-18 and TNF- α . On the other hand, α -2-adrenergic receptor antagonist, YOH administration reversed the effect of 2-methylbenzamide in LPS induced acute kidney injury rat model. Thus, it appears that 2-methylbenzamide exhibits its effect through α -2 adrenoceptor by inhibiting the progress by inflammatory processes.

The early stage of the acute kidney injury is characterized by the increase in expression of KIM-1 and HMGB-1. Among the two factors, KIM-1 is usually absent in the kidney of normal animals. However, it is reported that the expression of KIM-1 is increased significantly in the animals with kidney injury and its level of expression is indicative of the stage of injury [10-14]. HMGB-1 plays an important role in the process of induction of inflammatory reactions

by activating various factors involved in inflammation process. The results from the current study revealed that LPS injection in the rats increased expression of KIM-1 and HMGB-1. However, LPS induced increase in the expression of KIM-1 and HMGB-1 was inhibited by the treatment of rats with 2-methylbenzamide.

The proteins, KIM-1 and HMGB-1 are involved in the process of release of proinflammatory cytokines and inflammatory factors. It is well known that acute kidney injury involves several types of inflammatory reactions which are regulated by TNF- α and IL-6 genes [15, 16]. In the present study, LPS injection increased the expression of

inflammatory factors including, IL-6, IL-18 and TNF- α in rats. Treatment of the LPS injected rats with 2-methylbenzamide caused a significant reduction in the expression of IL-6, IL-18 and TNF- α . Administration of YOH to the 2-methylbenzamide treated rats inhibited the effect of 2-methylbenzamide on expression of IL-6, IL-18 and TNF- α . Thus our results demonstrated that 2-methylbenzamide inhibited the acute kidney injury in LPS induced rat model by reducing the expression of inflammatory factors.

Therefore, 2-methylbenzamide exhibits inhibitory effect on the acute kidney injury in rats induced by LPS injection through activation of α -2-adrenergic receptor and reduction in expression of inflammatory factors.

Disclosure of conflict of interest

None.

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