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Study of the expression of $GABA_A$ receptor in rats during acute lung injury caused by endotoxin

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ABSTRACT. The objective of the present study was to investigate the role of γ-aminobutyric acid type A receptor ($GABA_AR$) in lipopolysaccharide (LPS)-induced acute lung injury (ALI) in rats. Thirty-two male wistar rats were randomly divided into four groups. Rats in the GABA group were pretreated with LPS and GABA, while those in the bicuculline (BIC) group were pretreated with LPS and bicuculline. We assessed the arterial blood gas, dry/wet ratio, and the level of tumor necrosis factor-α (TNF-α), IL-6, malondialdehyde, and superoxide dismutase 6 h after the immunization. Paraffin sections of samples were detected using the steptavidin-peroxidase method. Protein expression was detected using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blotting. PaO_2 in the LPS group was significantly lower than that in the control rats. Activation of GABA-mediated signaling by GABA increased the expression of $GABA_AR$ in airway bronchial and alveolar epithelial cells. Blockade of the $GABA_AR$ by bicuculline limited the expression of this receptor. The GABA group rats had higher levels of tissue TNF-α and IL-6 than in ALI rats and

control rats. The BIC group rats demonstrated an opposite expression level compared to the GABA group rats. Our results suggest that the $GABA_AR$ could aggravate the inflammatory response syndrome and oxidative stress in the lungs and play an essential role in LPS-induced acute lung injury. It provides a novel method to study the incidence and mortality of ALI during the peroperative period.

Key words: Acute lung injury; $GABA_AR$; LPS; Oxidation stress

INTRODUCTION

Lung injury progression usually accompanies acute respiratory distress syndrome (ARDS) and acute lung injury (ALI), caused by trauma, shock and surgical infection (Barbas, 2007; Tsushima et al., 2009). ALI is a common clinical complication associated with excessive inflammatory response (EIR) and oxidative stress (OS)-mediated pathogenesis (Wheeler and Bernard, 2007; Eastwood et al., 2010).

γ-aminobutyric acid (GABA) is one of the important inhibitory neurotransmitters in mammalian central nervous system. In the brain, it activates GABA receptors (GABAR) resulting in mature hyperpolarized neurons that are protected (Jin et al., 2006; Corry and Kheradmand, 2007).

Recent studies reported that GABA and its receptor were expressed in respiratory epithelium and lungs. However, the GABA/GABAR functions in respiratory and central nervous system were entirely different. In GABA signaling pathway, GABAR could be an active mediator in inflammatory response and in lung anti-injury procedure, which are involved in asthma pathogenesis, airway remodeling procedure, and regulation of lung homeostasis (Barbas, 2007; Tsushima et al., 2009; Eastwood et al., 2010).

GABAR has been classified into three types as $GABA_AR$, $GABA_BR$, and $GABA_CR$ (Brosnan, 2011). Among these, $GABA_AR$ mediates the drug signaling pathways initiated by anesthetics and benzodiazepines (Saari et al., 2011). However, there is limited information whether $GABA_AR$ is involved in ALI pathogenesis. Therefore, the aim of the present study was to explore new directions in ALI pathogenesis.

MATERIAL AND METHODS

Reagents

Lipopolysaccharide (Cat. No. L2880) and bicuculline (Cat. No. B103) were purchased from Sigma-Aldrich (Sigma Ltd. China, Shanghai, China); mouse anti-$GABA_A$ receptor primary antibody (Cat. No MAB341), horseradish peroxidase (HRP)-tagged rabbit anti-goat secondary antibody (Cat. No.P8375) and anti- glyceraldehyde-3-phosphate dehydrogenase (GAPDH) IgG (Cat. No ABS16) were purchased from Merck Millipore (Darmstadt, Germany). Interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) kits were purchased from XingboS (Cat. No.FK10211 and MTHE103a, Wuhan, China).

Animals and grouping

Thirty-two male wistar rats were purchased from the Experimental Animal Center of Wuhan University equipped with an animal biosafety level 3 laboratory (Approval No. SCXKe2008-0004).

Rats were randomly divided into the following four groups (N = 8): blank control group, LPS control group, GABA group (pretreated with LPS and GABA), and BIC group (pretreated with LPS and bicuculline).

Immunization program

Normal saline was injected into the tail vein in the blank control group, while 5 mg/kg LPS was injected in the LPS control group. For the GABA group, intraperitoneal injection of 50 mg/kg GABA was administered within 30 min of the tail vein injection of LPS. Similarly, for the BIC group, 10 µmol/kg bicuculline was injected into the tail vein within 30 min of the LPS injection. The rats were fasted (but were allowed drinking water) 12 h prior to the immunization. The arterial blood gas was analyzed 6 h after immunization.

Pathological detection

Section staining

Lung tissues of rats were cut and fixed in 4% paraformaldehyde solution. Paraffin sections (4 µm thick) of the samples from each of the four groups were analyzed using the streptavidin peroxidase enzyme (SP) conjugate. Sample preparation was done by two processes. In the first process, the paraffin sections were dewaxed in xylene after 2 h baking at 60°C, hydrated in graded ethanol, and then blocked with 3% hydrogen peroxide for 5 min at 25°C. In the second process of preparation, dewaxing was performed under high temperature (140°C) for 2 min in citrate buffer. Both the processes were followed by 3 washes of 2 min each with phosphate buffer saline (PBS) at 25°C.

The $GABA_AR$ expression was detected using mouse anti-$GABA_AR$ primary antibody, biotin-labeled goat anti-rabbit IgG secondary antibody, and horseradish peroxidase (HRP)-tagged streptavidin. Chromogenic peroxidase substrate, 3, 3'-diaminobenzidine (DAB) was used for the detection of peroxidase activity.

Counterstaining with hematoxylin against a transparent background created by xylene was done to make the stained sections clearer and identifiable. PBS was used as a negative control. The specific brown granules in the tissues and cells were observed using an optical microscope (Olympus, Beijing, China).

Protein detection by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

Fresh lung tissues were washed in PBS, ground using a tissue grinder and the homogenate was centrifuged at 1000 *g* for 10 min (Thermo Scientific, USA). The supernatant was discarded, and the precipitate was resuspended in TNE buffer (40 mM Tris, 100 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, pH 7.4). The extract was frozen at -80°C and frozen-thawed four times. It was then centrifuged at 10000 *g* for 30 min at 4°C. The supernatant containing protein $GABA_AR$ IIs was collected and analyzed using 12% SDS-PAGE.

The protein bands were then transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% skimmed milk for 2 h and incubated with mouse anti- $GABA_AR$ primary antibody and HRP-tagged rabbit anti-goat IgG secondary antibody. GAPDH was used as an internal control. The hybridized protein bands were detected using an enhanced

chemiluminescence (ECL) system (Bestbio, China).Intensity of the visualized protein bands was compared with that of GAPDH.

Ratio of the tissue weight moisture: dry/wet ratio of lung

Right middle-upper lobe of fresh lung was weighed. It was then dried in an oven at 76°C and weighed after 48 h to calculate the ratio of tissue weight moisture.

Enzyme linked immunosorbent assay (ELISA)

Lung tissue lysate and standard samples of TNF-a and IL-6 were added to separate wells of the ELISA plate and allowed to incubate at 37°C for 1 hour. Thereafter, biotinylated primary antibody working solution, enzyme-conjugated working solution, and chromogenic agent (100 µL/well) were added sequentially and incubated. The color reaction was terminated by addition of 2 M H_2SO_4. Optical density (OD) at 450 nm was measured using an automated plate reader within 5 min of stopping the reaction.

Malondialdehyde (MDA) (ML255, shanghai, china) and superoxide dismutase (SOD) ELISA kits (GD-S0387, shanghai, china) were used to test the MDA and SOD levels in the lung tissue lysate, according to the manufacturer instructions. Optical density (OD) at 550 nm was measured using a spectrophotometer within 5 min of terminating the color reaction.

Statistical analysis

Data were expressed as mean ± SD (X ± s) and statistically analyzed using SPSS 17.0 software. Comparisons between the groups were performed using a one-way ANOVA (LSD-t), and correlation analyses were performed using Spearman's correlation method. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Pathological findings

Lung tissue structure in the control group was clear under the optical microscope. The alveolar epithelial cells were integral, the nucleus was basophilic, and no infiltration of inflammatory cells was observed. In the LPS group, the structure of the lung tissue was destroyed, cellular arrangement was disordered, infiltration with inflammatory cells was observed, and some alveolar septa were fractured. Destruction of the lung tissue structure in the GABA group was more severe than that in the LPS group with infiltration of inflammatory cells, especially neutrophils. In the BIC group, the destruction was lesser compared to the GABA and LPS group, but infiltration of the lung tissue with neutrophils was detected. The representative pictures of the histological sections are presented in Figure1.

Arterial blood gas

Table 1 shows the results of arterial blood gas analysis after 6 h of immunization. The arterial partial pressure of oxygen (PaO_2) in the three experimental groups, except that in the

control group, was less than 80 mm Hg, which indicated that the experimental group rats were under hypoxia but respiratory failure was not evident. No differences in $PaCO_2$ and HCO_3^- were observed between the four groups.

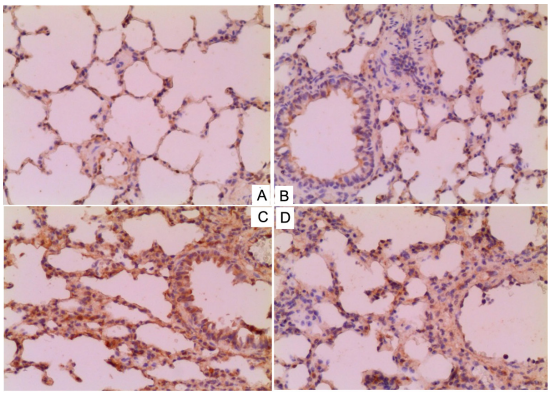

Figure 1. Immunohistochemical evaluation of GABAAR expression in the rat lungs of different treatment groups as observed under a microscope. Paraffin-embedded sections from the four groups were detected using the method of enzymatic streptavidin peroxidation (SP). **A.** Blank control group; **B.** LPS control group; **C.** GABA group (pretreated with LPS and GABA); **D.** BIC group (pretreated with LPS and bicuculline).

Table 1 shows the dry/wet ratio of lung. The ratio was significantly higher in the three experimental groups in comparison to the control group ($P < 0.05$). It was highest (7.5:1) in the GABA group. A similar trend was observed in the estimated levels of TNF-α, IL-6, and malondialdehyde (MDA), particularly the IL-6 level in the GABA group was almost twice that of the other groups (Table 1). In contrast, the SOD levels displayed an opposite trend: the control group had the highest, while the GABA group had the lowest SOD levels.

Table 1. Variety of monitoring experimental data.

Monitoring Data	Control group	LPS group	GABA group	BIC group
P_aO_2 (mmHg)	110.3 ± 2.3	77.9 ± 1.9	75.4 ± 0.6	79.6 ± 2.1
P_aCO_2 (mmHg)	32.07 ±10.1	35.53 ± 6.7	36.08 ± 5.2	32.94 ± 11.4
HCO_3^-	25.13 ± 2.5	21.43 ± 2.3	22.88 ± 5.4	23.88 ± 1.9
W/D	4.4	6.7	7.5	4.8
TNF-α	1042.5	1368.2	1690.1	1158.6
IL-6	221.8	271.3	400.2	252.9
MDA	1.68	3.13	4.13	2.08
SOD	26.3	20.6	16.2	21.0
Gray value of $GABA_AR$	0.796	1.219	1.456	1.166

Western blotting

Western blotting (WB) results were consistent with the identification of $GABA_AR$. The given three experimental group proteins (LPS, GABA, and BIC) increased in the lung tissues of rats after endotoxin exposure (Figure 2).

After calculation of the WB protein bands with GADPH as the internal standard, the maximum variables, protein $GABA_AR$, declined 1.83-fold in the GABA group, and the minimum variables declined 1.46-fold in the BIC group ($P < 0.01$).

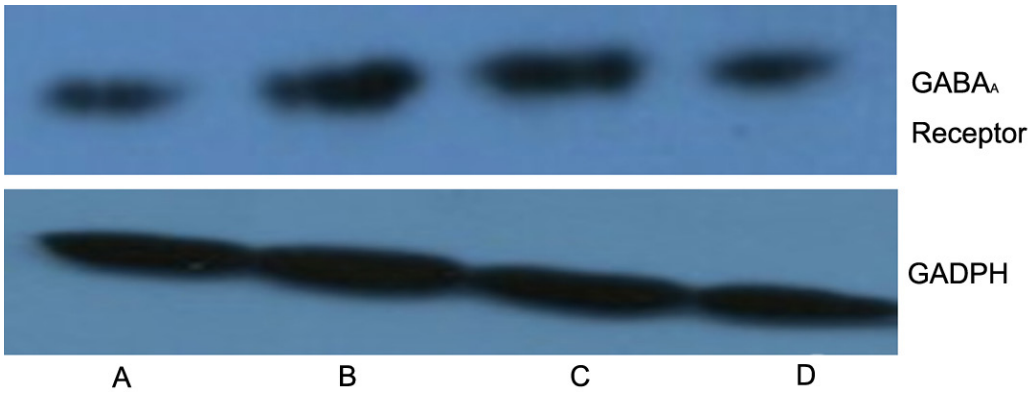

Figure 2. Results of western blotting showing the expression of GABAAR in the four groups with GAPDH as the internal standard. **A.** Blank control group; **B.** LPS control group; **C.** GABA group (pretreated with LPS and GABA); **D.** BIC group (pretreated with LPS and bicuculline).

DISCUSSION

GABA is the most important inhibitory neurotransmitter in the mammalian central nervous system and plays a central role in the mechanism of general anesthesia (Barbas, 2007; Tsushima et al., 2009; Eastwood et al., 2010). The effects of GABA signal transduction pathway activation on the central nervous system and the physiological and pathophysiological mechanisms has been focused in the anesthesia research. Since 2007, studies have reported that GABA expresses in the airway epithelium and lung tissue, and as an excitatory neurotransmitter, it has been implicated in diseases of the respiratory system (Chen et al., 2004; Xiang et al., 2007; O'Dea et al., 2011). In clinical anesthesia, the incidence of ALI is high and effective measures for its control are lacking. Thus, prevention of ALI and elucidation of the effects of anesthesia drugs on ALI have always been crucial topics of research on perioperative organ protection. Understanding the effects of the signal transduction pathway of GABA on the pathogenesis of acute lung damage holds great significance in clinical anesthesia for prevention and treatment of acute lung injury.

ALI is pathophysiological and a result of other injuries in the body. The inflammatory response and oxidative stress damage due to various causes are considered as the main pathological basis of ALI (Liu et al., 2006; Kobuchi et al., 2011; O'Dea et al., 2011). In this study, a model of ALI in rats was established by tail vein injection of LPS. Compared to the rats in the control group, those in the ALI model groups showed pathological changes typical of the ALI. Wet to dry weight ratio of lungs increased significantly, and arterial blood gas analysis showed significant decrease in the PaO2 and $HCO3^-$. The levels of inflammatory factor, TNF- α and IL-6 increased significantly suggesting an increase in the oxidative stress injury in the lung tissue. These results validated the success of ALI animal model prepared in this study.

Immunohistochemistry results revealed that GABA$_A$R expressed both in the cytoplasm of alveolar epithelial cells and in the bronchial epithelial cells. The intensity of the color was low, suggesting low expression of GABA$_A$R under normal conditions, especially in the bronchial epithelial cells. This observation is consistent with the findings of Chen et al. (2004). In the LPS group, the alveolar structure was destroyed which correlated with the significantly higher expression of GABA$_A$R in the alveolar and bronchial epithelial cells as evident by dark yellow-brown color in the IHC. In two experimental groups, GABA and BIC were used to pre-treat the rats before the tail vein

injection of LPS. In the GABA group, the expression of $GABA_AR$ increased significantly due to the induction by GABA while in the BIC group, the expression was significantly inhibited due to the effect of BIC, a $GABA_AR$ specific inhibitor. It could, therefore, be speculated that $GABA_AR$ might be a key signaling protein in LPS-induced ALI.

The western blot results further proved the increased expression of $GABA_AR$ after the injection of LPS in the lung tissue, which also demonstrated pathological changes typical of the ALI. The wet to dry weight ratio of lung tissue in such rats increased significantly compared to that in the control group, and the inflammatory factor (TNF- α and IL-6) and oxidative stress indicators also increased significantly. Upon the induction of $GABA_AR$ protein expression by GABA, ALI specific changes increased significantly in the GABA group. In the BIC group, ALI injury decreased significantly after the $GABA_AR$ protein expression was inhibited by the $GABA_AR$ specific inhibitor BIC. These results suggest that the increased $GABA_AR$ could lead to an increase in inflammatory cytokines and oxidative stress indicators in lung tissue along with an increase in lung water content and the appearance of ALI. However, this effect could be inhibited by the $GABA_AR$ specific inhibitor BIC. Therefore, $GABA_AR$ was identified to play a very important role in the pathogenesis and development of ALI.

In summary, the results of this study indicate that $GABA_AR$ can express in rat bronchial epithelium and alveolar epithelial cells in small amounts under normal conditions. The expression increases significantly through the induction by LPS and ALI symptoms appeared in the rats. It is, therefore, suggested that the increased expression of $GABA_AR$ might be play the central role in the pathogenesis of ALI and the $GABA_AR$ signaling pathway might be the junction signal between anesthesia drugs and ALI. However, the validation of these mechanisms warrants further research.

Conflicts of interest

The authors declare no conflict of interest.

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