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Original Article

NOX2 is involved in CB2-mediated protection against lung ischemia-reperfusion injury in mice

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Abstract: Lung ischemia-reperfusion injury (LIRI) can occur in many clinical scenarios. Activation of the cannabinoid 2 (CB2) receptor limits tissue injury in some ischemia-reperfusion (I/R) models. However, whether and how CB2 receptor activation alleviates lung injury induced by I/R remain unclear. In this study, we sought to determine whether JWH133, a selective CB2 receptor agonist, could alleviate lung injury induced by I/R and to examine the role of NOX2 in this process. Here, an I/R model was established using male C57BL/6 mice, by blocking the left pulmonary hilum for 1 h, followed by reperfusion for 2 h. Results showed that pretreatment with JWH133 significantly attenuated I/R-induced lung injury (decreased lung injury scores and wet-to-dry weight ratio and increased oxygenation index), alleviated oxidative stress (increased superoxide dismutase (SOD), and decreased Malondialdehyde (MDA) levels). It also significantly increased CB2 receptor mRNA expression and protein levels and significantly reduced NOX2 mRNA and protein expression. Further, the CB2 receptor antagonist AM630 eliminated these effects mediated by JWH133. Pretreatment with the NOX2 inhibitor, gp91 ds-tat, reduced NOX2 expression, but did not affect CB2 receptor expression and failed to alleviate lung injury and oxidative stress after additional JWH133 treatment. Our study suggests that CB2 receptor activation alleviates LIRI by inhibiting oxidative stress and that NOX2 is involved in CB2-mediated protection against LIRI in mice.

Keywords: Lung ischemia-reperfusion injury, cannabinoid 2 receptor, oxidative stress, NOX2 inhibitor

Introduction

Lung ischemia-reperfusion injury (LIRI) can occur in many clinical scenarios such as lung transplantation and can lead to devastating consequences in critically ill patients [1, 2]. Although the mechanisms underlying LIRI have been extensively investigated, they have not been fully elucidated. This has contributed to a lack of effective pharmacotherapies in clinical practice.

Oxidative stress plays a vital role in LIRI, and excess generation of reactive oxygen species (ROS) is considered the primary molecular mechanism underlying lung injury induced by ischemia-reperfusion (I/R) [2, 3]. Following reperfusion, the overproduction of ROS, which exceeds the body's antioxidant compensatory capacity, ultimately leads to cell damage and tissue destruction [4-6]. NADPH-mediated ROS generation is one of the key pathways of ROS formation during LIRI [3]. NADPH oxidase (NOX) is a membrane-related enzyme complex composed of several subunits, including NADPH oxidase 2 (NOX2). Knocking out or inhibiting NOX2 reduces oxidative stress injury in the kidney and heart in I/R mice models [7, 8]. NOX2 might also play an essential role in LIRI; Yang et al. [9] found that p47phox-/- mice or mice treated with then specific NADPH oxidase inhibitor apocynin exhibit significantly alleviated lung injury after I/R.

Increasing evidence indicates that the endocannabinoid system plays an important role in pathophysiologic processes and affects disease severity [10, 11]. This system includes

cannabinoid receptors (CB1 and CB2), the endocannabinoid N-arachidonoylethanolamine (AEA), and 2-arachidonyl glycerol (2-AG), as well as enzymes responsible for the synthesis, transport, and degradation of endocannabinoids [10, 12]. We previously demonstrated that the endogenous cannabinoid-degrading enzyme inhibitor, URB602, alleviated LIRI by increasing endogenous cannabinoid 2-AG levels in mice [13]. Emerging evidence also suggests that the protective effect of the endocannabinoid system on I/R injury might be mediated by activation of CB2 receptors [14-17]. As such, treatment with the CB2 receptor agonist HU-308 protects mice from cisplatin-induced renal injury by reducing the expression of NOX2 and subsequent oxidative stress [18]. However, the effect of CB2 receptor activation on LIRI and whether NOX2 and subsequent oxidative stress are involved in this CB2-mediated protective pathway remain undefined. Therefore, we sought to determine whether JWH133, a selective CB2 receptor agonist, could alleviate lung injury induced by I/R and examine the role of NOX2 in this process. Accordingly, we investigated the mechanisms underlying the lung-protective effect of JWH133.

Materials and methods

Animals and drugs

Healthy male C57BL/6 mice weighing 18-25 g were obtained from the Animal Experimental Center of Sichuan University. The mice were housed in cages at controlled temperature (22±1°C) and humidity (60±10%) with a 12-h light-dark cycle and free access to food and water for 3 days before the experiment. Animal feeding and disposal processes were performed in accordance with the Guiding Principles of the U.S. National Institutes of Health (NIH). The experimental scheme was approved by the Animal Ethics Committee of West China Hospital, Sichuan University (2018118A).

JWH133 (Cayman Chemical, America), AM630 (Sigma, America), and gp91 ds-tat (Absin Bioscience, China) were used in this study. JWH133 and AM630 were dissolved in tween 80:DMSO:saline (1:1:18) at a concentration of 5 mg/mL and 1 mg/mL, respectively. gp91 ds-tat was dissolved in DMSO:ddH_2O (1:9) at a concentration of 1 mg/mL. All drugs were intraperitoneally (i.p.) administered to mice. Doses of JWH133, AM630, and gp91 ds-tat were based on our preliminary experiments using mice.

Establishment of animal models and animal grouping

Based on our previously published studies, an in vivo LIRI mouse model was established [13]. The experiment was conducted in two parts. The first part investigated the effect of CB2 receptor activation on LIRI in mice. Briefly, mice were randomly divided into four groups as follows: (1) sham: the left hilum was separated by thoracotomy without clipping and then ventilated for 3 h; (2) I/R: the left hilum was clamped with a clip to induce ischemia for 1 h and then the clip was removed for reperfusion for 2 h; (3) JWH133+I/R: JWH133 (5 mg/kg) was administered and 5 min later, ischemia was induced for 1 h with subsequent reperfusion for 2 h; (4) AM630+JWH133+I/R: AM630 (2 mg/kg) was injected 30 min before JWH133 injection (5 mg/kg), and then after 5 min, I/R was performed as indicated previously.

The second part of the experiment aimed to determine whether NOX2 is involved in the protective effect of CB2 receptor activation on LIRI. Mice were divided into four groups as follows: (1) sham, (2) I/R, (3) JWH133+I/R, and (4) gp91 ds-tat+JWH133+I/R, where gp91 ds-tat (1.5 mg/kg) was injected 30 min before JWH133 injection (5 mg/kg) and 5 min later, ischemia was performed for 1 h with subsequent reperfusion for 2 h. The mice in groups (1), (2), and (3) were treated as in the first part of the experiment.

During the experiment, gauze was used to cover the thoracic incision to prevent the loss of water via evaporation. Mouse body temperature was maintained within the normal range using a warm blanket. Immediately after the experiment, mice were sacrificed by injecting excess sodium pentobarbital (100 mg/kg, i.p).

Arterial blood oxygenation index

A small volume of peripheral blood extracted for arterial blood gas analysis would probably cause hemorrhagic shock in the C57BL/6 mice due to their small bodies. Therefore, arterial blood in the ventriculus sinister of the mice was collected immediately after I/R for blood gas analysis to determine the partial pressure of

oxygen (PaO_2) value. The oxygenation index was calculated as PaO_2/FiO_2 to reflect physiological lung dysfunction (FiO_2 indicates the fraction of oxygen in the inspired air).

Wet-to-dry weight (W/D) ratio

Immediately after I/R, the left lungs of mice were removed and weighed; this value was recorded as the total wet weight. Subsequently, the wet lung was placed in an oven at 60-70°C until stabilized; this value was taken as the gross dry weight. Then, the W/D ratio was calculated as an estimation of lung tissue edema.

Histopathological examination

Left lung tissue of approximately 0.5 cm^3 was extracted and stained with hematoxylin-eosin. Then, the samples were observed and graded by a pathologist who was blinded to the experimental grouping. The degree of lung injury was evaluated based on a modified acute lung injury score, which included five independent variables as follows: neutrophils in the alveolar space, neutrophils in the interstitial space, hyaline membranes, proteinaceous debris filling the airspaces, and alveolar septal thickening [19].

Real-time quantitative PCR (RT-qPCR)

According to the manufacturer's protocols, total messenger RNA (mRNA) was extracted from lung tissues using an RNA extraction kit (RE-03014, FOREGENE Company, China) and complementary DNA (cDNA) was synthesized using a cDNA Synthesia Kit (RT-01023, FOREGENE Company, China). RT-qPCR was performed to evaluate the mRNA expression of genes encoding the CB2 receptor and NOX2 using Real Time PCR EasyTM (QP-01014, FOREGENE Company, China). The primers were designed and synthesized by Shanghai Shenggong Bioengineering Technology Service Co., Ltd. The following PCR primers were used: Beta-actin: upstream: gaagatcaagatcattgctcct, downstream: tactcctgcttgctgatcca; CB2: upstream: gccttctgttccatgctgtgcctt, downstream: agcagttggagcagcctggagttct; NOX2: upstream: ccagtgcgtgttgctcgacaaggatt, downstream: gtggatggcggtgtgctagtgctatc.

The following reaction mixes were prepared (20 μL): 10.0 μL 2× Real PCR EasyTM Mix-SYBR, 0.8 μL Forward Primer (10 μM), 0.8 μL Reverse Primer (10 μM), 2.0 μL DNA template, and 6.4 μL ddH_2O. The PCR cycling conditions were as follows: predenaturation at 95°C for 30 s, then denaturation at 95°C for 5 s, annealing at 55°C for 30 s for 45 cycles, and extension at 72°C for 32 s. The relative mRNA expression level of target genes was calculated using the 2-ΔΔCT method with beta-actin serving as control.

Western blotting

Western blotting was performed to detect the protein expression levels of CB2 receptor and NOX2. RIPA lysis butter was used to extract total cellular protein from the collected tissues, and a BCA protein quantitative kit (Biyuntian, China) was used to determine protein concentration. Primary antibodies used were anti-CB2 (ab3561, 1:500, Abcam, UK) and anti-NOX2 (GTX63960, 1:500, Genetex, China). The gray area value of the protein bands was analyzed with Image-Pro Plus 5.0 software (Media Cybernetics, USA), and relative protein expression levels were normalized to levels of the reference protein, beta-actin.

Enzyme-linked immunosorbent assay (ELISA)

Immediately after treatment, the left lung tissue was removed. Superoxide dismutase (SOD) and Malondialdehyde (MDA) contents were then assessed by ELISA based on the manufacturer's protocols (Nanjing Jiancheng Technology, China).

Statistical analysis

SPSS 20.0 (SPSS Inc., Chicago, IL) was used for statistical analysis. The Kolmogorov-Smirnov test was used to analyze the distribution of quantitative data, and the Levene test was used to test homogeneity of variance. Data were expressed as the mean ± standard deviation and analyzed by one-way ANOVA followed by Tukey-HSD test for multiple comparisons. $P<0.05$ was considered significant.

Results

Activation of the CB2 receptor alleviates acute lung injury induced by I/R

Figure 1A shows the representative histology of the lungs from each experimental group. After I/R, the evident infiltration of neutrophils, aggregation of proteinaceous debris, and

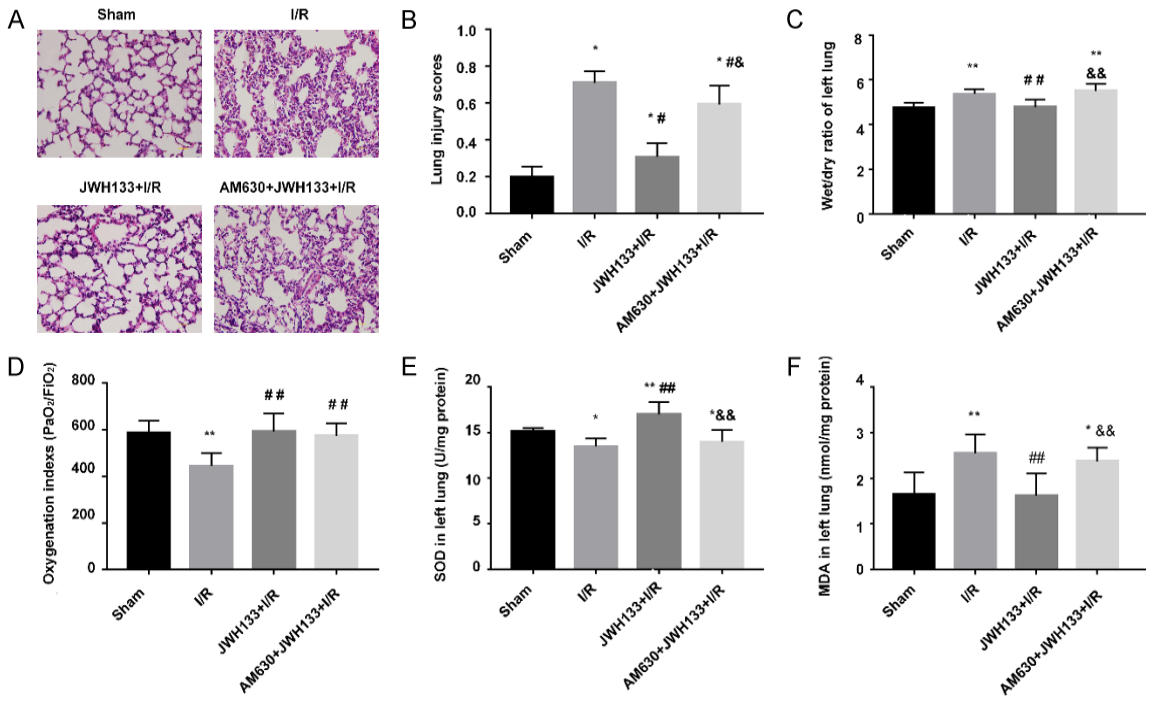

Figure 1. Effects of JWH133 and AM630 on lung ischemia-reperfusion injury (LIRI) and oxidative stress. A. Representative histology of the lungs from each experimental group by H&E staining (original magnification =400×, scale bar =100 μm). B. Lung injury score. C. Wet-to-dry weight (W/D) ratio. D. Oxygenation indexes (PaO_2/FiO_2). E. Superoxide dismutase (SOD) levels. F. Malondialdehyde (MDA) levels. Results are expressed as the mean ± SD (n=6). $^{*}P<0.05$ or $^{**}P<0.01$ versus the sham group; $^{\#\#}P<0.01$ versus the I/R group; $^{\&\&}P<0.01$ versus the JWH133+I/R group.

pulmonary septal edema were observed. Pretreatment with JWH133 significantly alleviated lung damage, and lung injury scores were significantly reduced (**Figure 1B**). Additionally, I/R caused significant increase in the W/D ratio and decrease in the oxygenation index; however, these changes were attenuated by pretreatment with JWH133 (**Figure 1C** and **1D**). To further investigate the effect of the CB2 receptor on LIRI, the CB2 receptor antagonist AM630 was introduced. In the AM630+JWH133+I/R group, histology changes were similar to those in the I/R group, and lung injury scores were significantly higher than those in the JWH133+I/R group (**Figure 1A** and **1B**). Although there was no significant change in oxygenation index, AM630 reversed the effect of JWH133 on the W/D ratio (**Figure 1C** and **1D**).

Activation of the CB2 receptor alleviates I/R-induced oxidative stress

I/R caused a decrease in SOD levels and increase in MDA levels in lung tissue, whereas JWH133 pretreatment reversed such effects. Moreover, AM630 treatment suppressed the effect of JWH133, and no significant differences in SOD and MDA levels were observed relative to those in the I/R group (**Figure 1E** and **1F**).

Activation of the CB2 receptor increases levels of the CB2 receptor and inhibits NOX2 expression

As shown in **Figure 2A**, the mRNA expression level of the gene encoding the CB2 receptor was significantly higher in the JWH133 group than in the sham and I/R groups. However, AM630 was found to antagonize the effect of JWH133. Western blot analysis revealed similar results; specifically, JWH133 significantly increased protein levels of the CB2 receptor, whereas AM630 reversed this effect (**Figure 2B**).

I/R increased the mRNA expression of *NOX2* in the lung tissue of mice. JWH133 pretreatment before I/R was found to significantly reduce

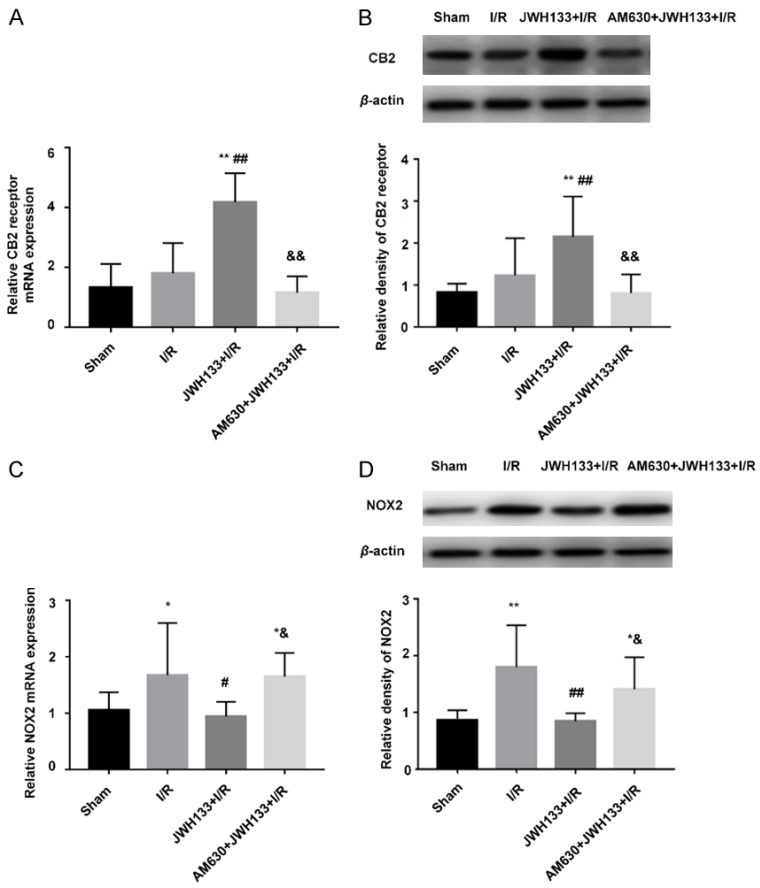

Figure 2. Effect of JWH133 and AM630 on the mRNA expression and protein levels of the CB2 receptor and NOX2. A. mRNA expression of the CB2 receptor. B. Protein levels of the CB2 receptor. C. mRNA expression of *NOX2*. D. Protein levels of NOX2. Results are expressed as the mean ± SD (n=6). *P<0.05 or **P<0.01 versus the sham group; $^{\#}$P<0.05 or $^{\#\#}$P<0.01 versus the ischemia-reperfusion (I/R) group; $^{\&}$P<0.05 or $^{\&\&}$P<0.01 versus the JWH133+I/R group.

NOX2 mRNA expression; however, AM630 could reverse this effect (**Figure 2C**). Western blot analysis revealed similar results for NOX2 at the protein level (**Figure 2D**).

gp91 ds-tat does not alter the protective effect of JWH133 on I/R-induced lung injury

In the second part of the study, we used the NOX2-specific antagonist gp91 ds-tat to determine the role of NOX2 in the CB2 receptor-mediated protective effect on lung injury. Lung tissue histopathology revealed that histologic changes in the gp91 ds-tat+JWH133+I/R group were similar to those in the JWH133+I/R group. Few neutrophils were found to aggregate and infiltrate the alveoli and pulmonary interstitium, and micro-hemorrhage and atelectasis were also observed (**Figure 3A**). I/R also significantly increased lung injury scores. Compared to those in the I/R group, scores in the JWH133+I/R and gp91 ds-tat+JWH133+I/R groups were significantly lower; however, gp91 ds-tat+JWH133 scores did not significantly differ from those with JWH133 alone (**Figure 3B**).

Further, I/R significantly reduced the oxygenation index and increased the W/D ratio of the left lungs of mice. Pretreatment with JWH133 or gp91 ds-tat+JWH133 before I/R increased the oxygenation index and significantly decreased the W/D ratio. However, no significant difference was found in the oxygenation index and W/D ratio between JWH133+I/R and gp91 ds-tat+JWH133+I/R groups (**Figure 3C** and **3D**).

gp91 ds-tat does not alter the effect of JWH133 on SOD and MDA levels

I/R resulted in decrease in SOD levels and increase in MDA levels in the lung tissue. Pretreatment with gp91 ds-tat+JWH133 was found to significantly reverse these changes, causing increase in SOD levels and decrease in MDA levels. However, compared to those with JWH133 alone, SOD and MDA levels did not change significantly in response to gp91 ds-tat+JWH133 (**Figure 3E** and **3F**).

gp91 ds-tat does not alter the effect of JWH133 on CB2 receptor expression in lung tissue

As shown in **Figure 4A** and **4B**, JWH133 pretreatment significantly increased the mRNA expression and protein levels of the CB2 receptor in lung tissue after I/R. However, these did not change after the addition of gp91 ds-tat, as compared to levels with JWH133 alone. Similarly, JWH133 pretreatment significantly

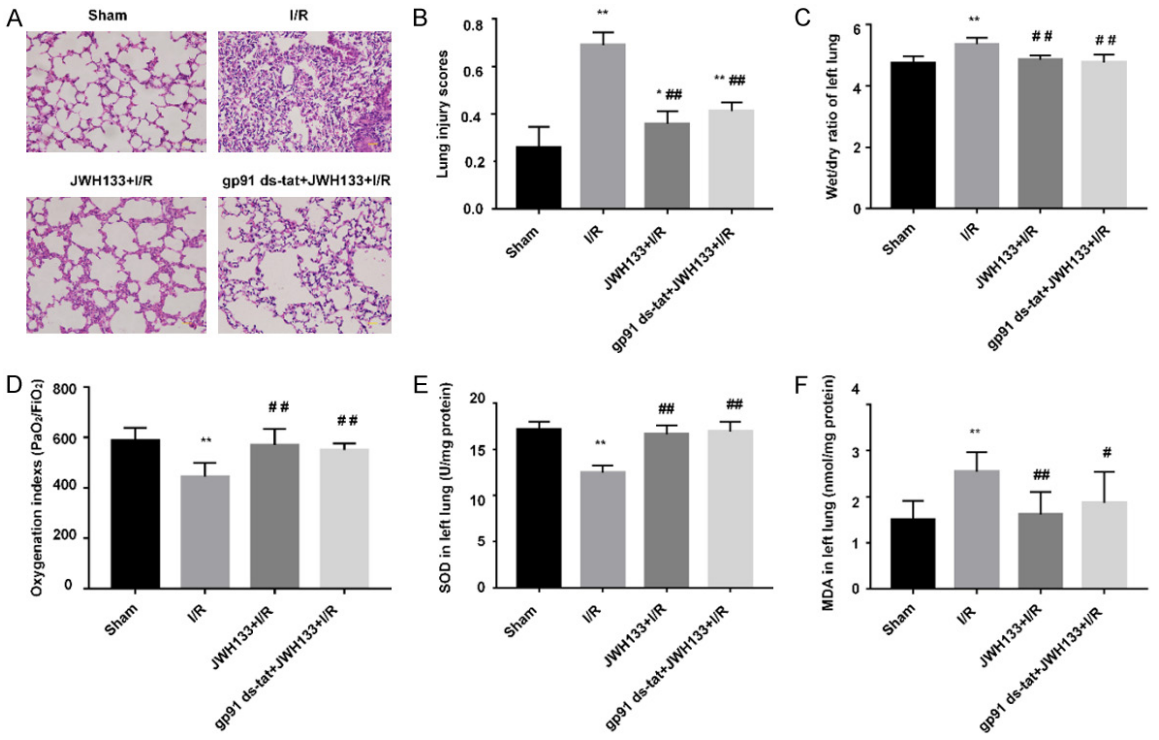

Figure 3. gp91 ds-tat does not alter the protective effect of JWH133 on ischemia-reperfusion (I/R)-induced lung injury and further alleviates oxidative stress reactions. A. Representative histology of the lungs from each experimental group by HE staining (original magnification =400×, scale bar =100 μm). B. Lung injury scores. C. Lung wet-to-dry weight (W/D) ratio. D. Oxygenation indexes (PaO_2/FiO_2). E. SOD levels. F. MDA levels. Results are expressed as the mean ± SD (n=6). *P<0.05 or **P<0.01 versus the sham group; $^{\#}$P<0.05 or $^{\#\#}$P<0.01 versus the I/R group.

decreased the mRNA expression and protein levels of NOX2 in lung tissue compared to levels in the I/R group. After addition of gp91 ds-tat, NOX2 mRNA and protein levels in the gp91 ds-tat+JWH133 group were lower than those in the JWH133 group, although only protein levels were significantly different (**Figure 4C** and **4D**).

Discussion

It is well known that endocannabinoids regulate cellular function through cannabinoid receptors [20]. Among the two major cannabinoid receptors, the CB1 receptor is mainly expressed in various cells of the central nervous system, whereas the CB2 receptor is widely distributed in peripheral immune tissues and cells [10, 11]. In this study, we observed expression of the CB2 receptor in healthy lung tissues, which was consistent with the findings of Liu [21]. We also proved that JWH133, as a highly selective CB2 agonist, significantly upregulated expression of the CB2 receptor in lung tissue. Additionally, pretreatment with JWH133 increased oxygenation index and decreased the W/D ratio after I/R. The corresponding lung injury scores were also significantly decreased. Such findings suggest that JWH133 can improve lung ventilation function, reduce pulmonary edema and neutrophil infiltration, and ultimately, alleviate lung injury. JWH133 was also found to reduce tissue damage in other I/R models. Li et al. [22] found that CB2 receptor activation by JWH133 protects the myocardium from I/R injury. Feizi [23] and Presly [24] reported similar findings in renal I/R models using this compound. These findings suggested that the administration of CB2 receptor agonists are a potential pharmacological approach for LIRI. In contrast, blockade of the CB2 receptor by pretreating with a selective CB2 receptor antagonist, AM630, eliminated the effects of JWH133 on CB2 receptor expression, as well as the protective effects on lung injury, by reversing the effect on the W/D ratio and lung injury scores. Therefore, we conclude that the lung-protective effect of JWH133 is achieved by activation of the CB2 receptor and

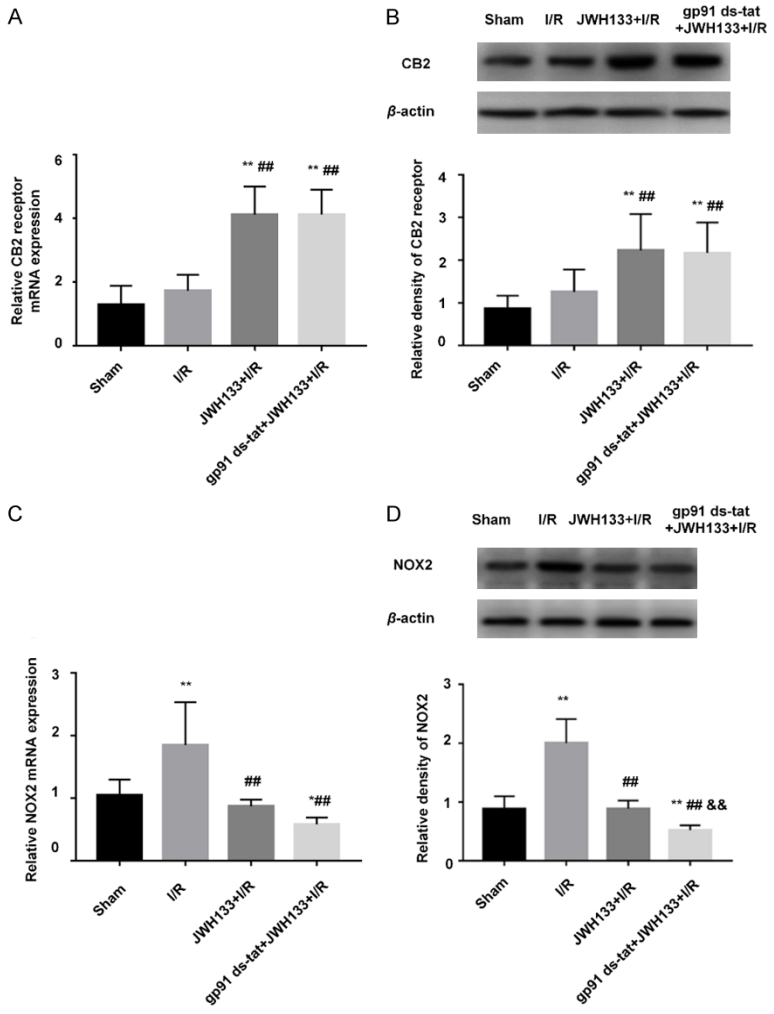

Figure 4. Effects of gp91 ds-tat on mRNA expression and protein levels of the CB2 receptor and NOX2. A. mRNA expression of the CB2 receptor. B. Protein levels of the CB2 receptor. C. mRNA expression of *NOX2*. D. Protein levels of NOX2. Results are expressed as the mean ± SD (n=6). *P<0.05 or **P<0.01 versus the sham group; $^{\#\#}$P<0.01 versus the ischemia-reperfusion (I/R) group; $^{\&\&}$P<0.01 versus the JWH133+I/R group.

subsequent upregulation of its expression. Interestingly, AM630 could not suppress the effect of JWH133 on the oxygenation index. We speculate that the reason for this might be that lung injury caused by I/R is still within the compensatory range of the body; similar phenomena were previously observed after unilateral lung transplantation [25].

Oxidative stress plays a vital role in cell function and survival. The accumulation of oxygen radicals or the excessive consumption of antioxidants can disrupt cell metabolism and even lead to cell death [4, 5]. In this study, MDA and SOD were used to reflect oxidation and antioxidant levels in the lung. We found that I/R significantly reduced SOD and increased MDA levels in lung tissue, suggesting that I/R alters oxidative stress levels in lung tissue cells. Pretreatment with JWH133 significantly increased SOD and decreased MDA levels, which was reversed by AM630, suggesting that CB2 receptor activation alleviates oxidative stress by restoring intracellular oxidation and antioxidant balances.

Our study also found that CB2 receptor activation reduced NOX2 expression and that CB2 receptor antagonism reversed this effect. NOX2 is a vital subtype of NADPH oxidase. The main function of NADPH oxidase is to convert oxygen received by the membrane to oxygen radicals; this is an essential source of oxygen radicals in vivo. Knocking out or inhibiting NOX2 was previously shown to help reduce oxidative stress-related injury [7, 8]. Recent evidence further suggests that NOX2 is closely related to LIRI [26-28]. Based on in vitro and in vivo results, Weissmann et al. [27] found that in the LIRI mouse model, global deletion of the NOX2 catalytic subunit (gp91 phox) prevents lung injury. In our study, NOX2 inhibition and lung-protective effects were achieved by pretreating with JWH133. Therefore, we speculate that NOX2 might be downstream of the CB2 receptor and that JWH133 could alleviate oxidative stress reactions during LIRI by regulating NOX2. We then used gp91 ds-tat, a selective NOX2 inhibitor, for further verification. Gp91 ds-tat inactivates NOX2 by simulating the binding of NOX2 and p47phox, thereby reducing the production of oxygen radicals [29]. One previous study showed that gp91 ds-tat decreased the expression of NOX2 in the spinal cord and alleviated

oxidative stress and spinal cord injury in a rat spinal cord injury model [30]. In the current study, compared to the group treated with JWH133 alone, there were no significant changes in the arterial oxygenation index, lung W/D ratio, lung injury score, and SOD and MDA levels in the gp91 ds-tat+JWH133 group. This indicated that gp91 ds-tat could not enhance the pulmonary-protective effect of JWH133 or further inhibit oxidative stress. Furthermore, pretreatment with gp91 ds-tat did not change CB2 expression. These findings suggest that there is no synergistic effect between JWH133 and gp91 ds-tat. The lung-protective effect of CB2 receptor activation might be achieved by inhibiting NOX2 to alleviate oxidative stress.

Some limitations of this study must be noted. The primary purpose of this study was to determine whether CB2 receptor activation has a protective effect on LIRI and to delineate the possible underlying mechanism. Therefore, we determined the dose of JWH133 according to previous reports and based on preliminary experiments and did not include different groups based on alternate doses. Additionally, only specific receptor agonists and antagonists were used for the preliminary validation of the protective effect of CB2 receptors on LIRI, and as such, gene knockout might be informative in further studies.

In conclusion, we confirmed that CB2 activation could alleviate I/R-induced lung injury and that NOX2 might be involved in the CB2-mediated protective effects against LIRI. Although the pathologic mechanism of LIRI has not been fully elucidated, our research indicates that activating the CB2 receptor pharmacologically could be a strategy to treat LIRI.

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Disclosure of conflict of interest

None.

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