Endocannabinoid 2-arachidonoylglycerol protects inflammatory insults from sulfur dioxide inhalation via cannabinoid receptors in the brain

Short Title: Endogenous 2-AG protects neuroinflammation from SO₂ inhalation

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Abstract

Sulfur dioxide (SO₂) pollution in the atmospheric environment causes brain inflammatory insult and inflammatory-related microvasculature dysfunction. However, there are currently no effective medications targeting the harmful outcomes from chemical inhalation. Endocannabinoids (eCBs) are involved in neuronal protection against inflammation-induced neuronal injury. The 2-Arachidonoylglycerol (2-AG), the most abundant eCBs and a full agonist for cannabinoid receptors (CB1 and CB2), is also capable of suppressing proinflammatory stimuli and improving microvasculature dysfunction. Here, we indicated that endogenous 2-AG protected against neuroinflammation in response to SO₂ inhalation by inhibiting the activation of microglia and astrocytes and attenuating the overexpression of inflammatory cytokines, including tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, and inducible nitric oxide synthase (iNOS). In addition, endogenous 2-AG prevented cerebral vasculature dysfunction following SO₂ inhalation by inhibiting endothelin 1 (ET-1), vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) expression, elevating endothelial nitric oxide synthase (eNOS) level, and restoring the imbalance between thromboxane A2 (TXA2) and prostaglandin I2 (PGI2). In addition, the action of endogenous 2-AG on the suppression of inflammatory insult and inflammatory-related microvasculature dysfunction appeared to be mainly mediated by CB1 and CB2 receptors. Our results provided a mechanistic basis for the development of new therapeutic approaches for protecting brain injuries from SO₂ inhalation.

Keywords: sulfur dioxide; neuroinflammation; microvasculature dysfunction; 2-arachidonoylglycerol; cannabinoid receptors

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Introduction

Sulfur dioxide (SO₂) is a key factor for air pollution in urban areas, which has been comprehensively studied due to the combustion of coal, power plants, and factories. These facts force us to revisit the adverse health outcomes of SO₂ pollution. Epidemiological investigations reveal that SO₂ pollution not only increase the risk of many respiratory diseases (Guo et al., 2014; Liu et al., 2014) but is also associated with circulatory system diseases (Amancio and Nascimento, 2012), such as heart disease (Li et al., 2015; Dong et al., 2015), congenital malformation (Farhi et al., 2014) and liver disease (Tong et al., 2015). Increasing evidence suggests that SO₂ inhalation is also linked to neurotoxicity and aggravates the risk for hospitalization and mortality of many brain disorders, including ischemic stroke (Amancio and Nascimento, 2014), cerebral hemorrhage (Ye et al., 2009), epilepsy (Cakmak et al., 2010), neurodevelopmental impairment and cognitive deficit (Lin et al., 2014). In the pathophysiology of these neurological diseases, a state of neuroinflammation accompanied by the alteration of the microvasculature function has been shown to be involved (Grammas et al., 2011; Farkas et al., 2000). In particular, our recent results confirm that acute SO₂ treatment causes neuroinflammation and microvasculature dysfunction, including the abnormal expression of inflammatory cytokines (inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and intercellular adhesion molecule 1 (ICAM-1) and endothelial dysfunction related factor (endothelin 1 (ET-1) and endothelial nitric oxide synthase (eNOS)), and exacerbates cerebral ischemic responses (Sang et al., 2010; Yun et al., 2010). Specifically, neuroinflammation following chronic SO₂ inhalation above the environmental standard has been reported to impair neuronal behavior and repress memory-related kinase activation and the gene expression of glutamate receptors (Yao et al., 2015). Taken together, these findings suggest that SO₂ exposure causes brain insults, and inflammation-related responses might be an important molecular modulator during this

process.

Endocannabinoids (eCBs) have been reported to be involved in a variety of physiological, pharmacological and pathological processes as an endogenous signaling mediator (Marrs et al., 2010; Pertwee et al., 2010) and have the ability to bind to and functionally activate cannabinoid receptors (CB1 and CB2) to exert anti-inflammatory and neuroprotective properties against harmful insults (Mackie, 2006; Chen et al., 2011). As the most abundant eCBs and a full agonist for CB1 and CB2 receptors (Sugiura et al., 2006), 2-arachidonoylglycerol (2-AG) has been shown to play an important role in protecting neurons from inflammatory stimuli (Panikashvili et al., 2001; Kreutz et al., 2007). Subsequent experimental studies demonstrate that indirect elevation of endogenous 2-AG or direct application of 2-AG protected hippocampal neurons from the adverse effects of interleukine-1 beta (IL-1 β) and lipopolysaccharide (LPS), β -amyloid stimuli and other brain harmful insults by weakening the inflammatory reactions (Chen et al., 2011; Nomura et al., 2011). Brain microvascular dysfunction is an important determinant of the inflammatory response to brain injury. The experimental evidence shows that neuroinflammation participates in brain microvascular dysfunction (Wu et al., 2012). Interestingly, 2-AG also affects other physiological processes, such as vasculature dysfunction and platelet function (Kvasnicka, 2010; Bouchard et al., 2003). 2-AG abolishes the toxicity effect of ET-1-induced cerebral microvascular responses via CB1/2 receptors (Mechoulam and Shohami, 2007). Thus, we hypothesized that endocannabinoid 2-AG may play a protective role against inflammatory insults in the brain and following microvascular dysfunction in response to SO₂ inhalation via CB1/2 receptors.

2-AG is mainly produced from diacylglycerol (DAG) by diacylglycerol lipase (DGL) and is hydrolyzed to arachidonic acid (AA) by monoacylglycerol lipase (MAGL) (Nomura et al., 2011). Thus, MAGL inhibition can elevate endogenous 2-AG level. Whereas, JZL184 is a selective inhibitor of MAGL and has been used for disrupting MAGL and improving the endogenous 2-AG content. In the present study, we established sub-acute SO₂ inhalation models (14 mg/m³, 6 hr/day, 7 days) in the absence or presence of pre-intraperitoneal injection of JZL184 and investigated the protection of 2-AG against brain neuroinflammation and proinflammatory cytokines-mediated microvascular dysfunction following SO₂ inhalation.

1 Material and methods

1.1 Experimental animals and exposure treatments

C57BL6 mice, weighing 18-20 g, were purchased from the Center of Experimental Animal of Hebei Province and used according to the guidelines approved by the Institutional Animal Care and Use Committee of Shanxi University. The mice were divided randomly into 5 equal groups, in which each group consist of 8 animals, including vehicle, SO₂, JZL184 (a monoacylglycerol lipase inhibitor, Cayman, USA) +SO₂, SR-1 (SR141716, a CB1 antagonist, Cayman, USA) + JZL184+SO₂, SR-2 (SR144528, a CB2 antagonist, Cayman, USA) + JZL184 + SO₂ groups. Similarly, vehicle and SO₂ groups were intraperitoneally injected with normal saline, and other groups were intraperitoneally injected with JZL184 (12 mg/kg), SR-1 (5 mg/kg) + JZL184, SR-2 (5 mg/kg) + JZL184 for 7 days, respectively. All reagents were injected once every two days for a total of three times in a 7-day timespan. Mice were then treated with SO₂ (14 mg/m³) for 6 hr/day (10:00 a.m. to 16:00 p.m.) for 7 days. To obtain the desired concentration and generate SO₂ gas for distribution among the entire chamber, the SO₂ exposure appliance was prepared as previously described (Yao et al., 2015). In addition, the concentration of SO₂ exposure in the chamber was monitored by applying a real-time SO₂ monitor (FIX660, Wandu, China). An alkali absorption device was used to absorb the gas waste. During the SO₂ exposure process, the mice were allowed free movement under normal circumstances, except for access to food and water. The 1-hr China's Ambient Air Quality Standard Grade II of SO₂ is 0.5 mg/m³ (GB 3095-2012, 2012). The concentration used in this study was 14 mg/m³ (6 hr/day), then the 24-hr average concentration every day was 3.5 mg/m³ and it is approximately 7 times higher than the standard. Although the experiments may be viewed as beyond the normal atmosphere encountered in the human environment, two important points must be taken into account. First, animals were subjected to regular periods of extended exposure, with relief periods between protocols (6 hr/day, for 7 days, with 18 hr between exposures). This finding may provide a corollary to individuals exposed to the gas in an occupational setting. Second, real-time and mean concentrations in the upper and lower airways were higher in humans when compared with rat using a comparative simulation of SO₂ gas transport in airway models (Tsujino et al., 2005).

The mice were sacrificed 12 hr after the last exposure, and the cortex was rapidly excised.

A small piece of tissue was removed and fixed with 4% paraformaldehyde for immunofluorescent staining, and the remaining tissue was frozen in liquid nitrogen and stored at -80°C in an ultra-cold storage freezer for immunoblot analysis and enzyme-linked immunosorbent assay.

1.2 Protein isolation and immunoblot analysis

Proteins were extracted from cortical tissues in ice-cold lysis buffer as previously described (Yao et al., 2015). The lysates were centrifuged at 13,000 r/min for 15 min at 4°C , and protein concentrations were determined using the bicinchoninic acid assay. Briefly, 50 μg of total proteins were separated using Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred onto a 0.45- μm aperture nitrocellulose membrane, and the protein bands were cut and blocked with 3% bovine serum albumin (BSA) for 1 hr. After blocking, the membrane was incubated with rabbit polyclonal primary antibodies at 4°C overnight, including polyclonal antibodies ET-1, iNOS, eNOS, VCAM-1, ICAM-1 (volume ratio, 1:200, Bioss, China) and rabbit monoclonal antibodies β -actin (1:1000, CST, USA). The blot was washed with 0.1 mol/L phosphate buffer (PBS) and incubated with a fluorescently labeled secondary antibody (1:5000) (IRDye 800CW goat anti-rabbit IgG (H + L) at room temperature for 1 hr, and the blot was scanned and detected using the LI-COR Odyssey (Odyssey sa, Gene, USA) Infrared Fluorescent System.

1.3 Immunofluorescent staining

The fixed cortical tissues were embedded in paraffin, cut into 4-6 μm slices, and adhered onto glass slides. The sections were heated for 20 min at 60°C prior to deparaffinization with xylene, and antigen retrieval was performed using citric acid buffer (pH 6.0). Next, the sections were blocked with 3% BSA solution for 30 min at room temperature and incubated with anti-CD11b/c antibody (1:500, Abcam, USA) and anti-glial fibrillary acidic protein (GFAP) antibody (1:2000, Abcam, USA), a marker of microglia and astrocyte, respectively, overnight at 4°C ; the sections were washed three times with PBS and incubated with anti-rabbit secondary antibodies for 1 hr at room temperature. After staining with 4', 6-diamidino-2-phenylindole (DAPI), a fluorescent stain which binds strongly to DNA, the

sections were rapidly mounted, coverslipped, and stored in darkness at 4°C until images were obtained. The images were then obtained by applying a fluorescent inverted microscope (IX73, Olympus, Japan), in which the relative fluorescence intensity was calculated using Image-pro Express 6.0 software (Media Cybernetics, USA).

1.4 Measurement of 6-keto-PGF1 α and TXB2 levels

Because both PGI₂ and TXA₂ have a very short half-life and are ready to convert to 6-keto-PGF1 α and thromboxane B₂ (TXB₂), respectively, we measured 6-keto-PGF1 α and TXB₂ levels. Briefly, cortical tissues were homogenized in a buffer containing 1 mmol/L ethylene diamine tetraacetic acid (EDTA), 10 mol/L indomethacin, and 0.1 mol/L PBS. Homogenates were centrifuged at 12000 $\times g$ for 15 min at 4°C. Supernatants were removed and assayed in triplicate using a 6-keto-PGF1 α and TXB₂ enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's guidelines (Cayman, USA).

1.5 Liquid chromatography-tandem mass spectrometry

The 2-AG were quantified in the brain tissues of C57BL/6 J mice using high-performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). The quantification of 2-AG was based on an external standard curve diluted with methanol. All the standards of the analytes were provided by Cayman Chemical (Ann Arbor, MI, USA). The brain tissue was weighed and extracted individually. Frozen samples were placed in extraction solvent containing 2 mL of acetonitrile in a borosilicate glass tube. After rapid homogenization, the sample was centrifuged at 1500 $\times g$ for 10 min. The supernatant was collected and evaporated to dryness under a gentle stream of nitrogen. Then, 200 μ L of methanol were added to the extract, which was dried under N₂ gas. Subsequently, the lipid extract was resuspended using 20 μ L of methanol, and 5 μ L were injected for LC-MS/MS analysis. A Waters UPLC system coupled with a triple quadrupole Quattro Premiere/XE mass spectrometer (Q-tof 2, Waters, USA) was used for the analysis. Liquid chromatography was performed using a Waters XSelect CSH C18 column 2.1 $\times$ 75 mm, 2.5 μ m (Waters, USA), with 0.1% formic acid (A) and 100% acetonitrile (B) as a mobile phase. A flow rate of 400 μ L/min was used for gradient elution, which started at 50% B, linearly

increased to 90% at 2 min and was subsequently reequilibrated at 50% B for a further 2 min after a 4-min hold. The column temperature was 45°C. The samples were kept at 4°C in the autosampler until analysis. The targeted LC-MS/MS analysis was performed in multiple reactions monitoring mode (MRM) using the following transitions: the electrospray ionization (ESI) + MRM transition for 2-AG was 379.4 > 287.3. The amounts of 2-AG were calculated from the ratios of peak areas of compounds to external standards and by comparing the results with standard curves.

1.6 Measurement of TNF- α , IL-1 β

The enzyme-linked immunosorbent assay (ELISA) kit (Xitang, China) was selected to evaluate the concentration of TNF- α and IL-1 β secreted from mouse cortex. Briefly, 0.3-0.5 g of tissue was homogenized in 1 mL of homogenization buffer (0.1 mol/L phosphate, pH 7.4), and the lysates were centrifuged at 1000 $\times$ g for 20 min. The supernatants were collected and assayed in triplicate according to the protocol of the ELISA kit.

1.7 Data analysis

Data are expressed as the mean $\pm$ S.E. and unless stated otherwise, Student's *t*-test and ANOVA with Fisher's least significant difference (LSD) test were used to test for statistical comparison when appropriate, using Origin8.0 Software. Differences were considered significant when *p* < 0.05.

2 Results

2.1 MAGL disruption alters endogenous 2-AG level following SO₂ inhalation

To provide direct evidence for the alteration of endogenous 2-AG levels following MAGL inhibition by JZL184, we first detected the 2-AG contents in the cortex under different treatment conditions. These results showed that the chemical inhalation did not alter the endogenous 2-AG content. However, MAGL inhibition by JZL184 pre-treatment significantly elevated the levels of endogenous 2-AG (**Fig. 1**). This finding suggested that MAGL inhibition by JZL184 increased the content of endogenous 2-AG, which might exert protective effects on neuroinflammation and its related injuries in response to SO₂ inhalation.

2.2 Endogenous 2-AG decreases astroglia activation and neuroinflammation from SO₂ inhalation

For brain inflammation, a major status is astroglia cell activation, and the activation astroglia cells release a broad array of chemokines, cytokines, and toxic factors (Sofroniew and Vinters, 2010). To determine whether endogenous 2-AG protects against neuroinflammation from SO₂ inhalation, we determined the reactive astrocytes and microglia using specific markers in mouse cortex. These results indicated that SO₂ inhalation caused the activation of astrocytes and microglia, and JZL184 subsequently suppressed the elevation (**Fig. 2**). To confirm the preliminary result, we further detected the release of inflammatory cytokines. As shown in **Fig. 3**, SO₂ exposure increased the expression of iNOS, TNF- α and IL-1 β , and JZL184 statistically inhibited the overexpression.

2.3 Endogenous 2-AG protects against inflammatory insults from SO₂ inhalation via CB1/2 receptors

eCBs have been reported to protect against inflammation via binding to and functionally activating cannabinoid receptors. To clarify whether endogenous 2-AG protected inflammatory insults from SO₂ inhalation via CB1/2 receptors, we treated mice with SO₂ (14 mg/m³) in the presence of JZL184, SR-1+JZL184, and SR-2+JZL184, and determined the release of inflammatory cytokines, including iNOS, TNF- α and IL-1 β . As presented in **Fig. 3**, both SR1 (CB1 antagonist) and SR2 (CB2 antagonist) reversed JZL184-induced suppression of iNOS overproduction in response to SO₂ exposure but not TNF- α and IL-1 β . These results suggested that 2-AG protected against brain inflammation from SO₂ inhalation, and the action was partly modulated by CB1/2 receptors.

2.4 Endogenous 2-AG inhibits abnormality of neuroinflammation-related microvasculature factors from SO₂ inhalation

Following brain inflammation, neuroinflammation-related microvasculature insults are also associated with many neurological diseases (Amancio and Nascimento, 2014; Ye et al., 2009). During this process, the abnormal metabolite of AA, thromboxane A₂ (TXA₂) and

prostaglandin I₂ (PGI₂), which maintains the state of contraction and relaxation of the microvasculature under normal physiological conditions, occurred (Miller, 2006). Here, we first examined the TXA₂ and PGI₂ expression following SO₂ inhalation in the absence or presence of JZL184 pre-treatment. As shown in **Fig. 4**, SO₂ exposure down-regulated PGI₂ levels, and JZL184 reversed the change in PGI₂. TXA₂ demonstrated no significant changes in the absence or presence of JZL184. Coupled with the imbalance between TXA₂ and PGI₂, the biomarkers of inflammation-related microvasculature dysfunctions, including ET-1, eNOS, ICAM-1, and VCAM-1, caused vascular homeostasis and perturbed vasculature function (Frijns and Kappelle, 2002; Leung et al., 2009). Thus, we further detected the expression of ET-1, eNOS, ICAM-1 and VCAM-1. As shown in **Fig. 5**, SO₂ exposure increased the expression of ET-1, ICAM-1, VCAM-1 and down-regulated the level of eNOS, and JZL184 reversed these alterations. These results suggested that endogenous 2-AG prevents neuroinflammation-related microvasculature dysfunction in response to SO₂ inhalation.

2.5 Endogenous 2-AG reverses neuroinflammation-related microvasculature dysfunction from SO₂ inhalation via CB1/2 receptors

To confirm that endogenous 2-AG protected against inflammation-related microvasculature insults from SO₂ inhalation via CB1/2 receptors, we treated mice with SO₂ in the presence of JZL184, SR-1+JZL184, and SR-2+JZL184 and further detected the expression of ET-1, eNOS ICAM-1 and VCAM-1. As shown in **Fig. 5**, both SR-1 and SR-2 abolished endogenous 2-AG-generated suppression of ET-1, ICAM-1 and VCAM-1 following SO₂ exposure. However, SR-1, but not SR-2, inhibited the endogenous 2-AG-produced restoration of eNOS downregulation. This finding implicated that elevation of endogenous 2-AG by MAGL inhibition protected against neuroinflammation-related injuries from SO₂ inhalation via CB1/2 receptors.

3 Discussion

SO₂ is a widespread pro-inflammatory stimulus to the central nervous system (CNS) and has been largely studied as a risk factor for neurological disease (Shah et al., 2015; Weisskopf et al., 2015; Amancio and Nascimento, 2014; Yun et al., 2013). Although the accurate

mechanisms underlying neurological disease induced by SO₂ exposure are not completely understood, recent studies indicate that oxidative stress, glial activation, neuroinflammation, and the following microvascular dysfunction may be attributed to the primary pathways (sang et al., 2010; yun et al., 2010; Block and Calderón-Garcidueñas, 2009). Moreover, inflammation in the vasculature has been shown to be involved in vasculature dysfunction-related diseases, such as ischemic stroke (Amancio and Nascimento, 2014) and hypertension (Cero et al., 2016). Recent studies have shown that chronic systemic inflammation causes vasculature dysfunction by down-regulating eNOS expression, and up-regulating ICAM-1 expression in blood vessels occurred in the rostral ventrolateral medulla (RVLM) (Wu et al., 2012). Inflammation in the brain, produced by proinflammatory cytokine interleukin (IL)-1 β , accelerates brain microvascular leakage and brain edema (Chakraborty et al., 2010). Thus, the control of inflammation and subsequent vasculature dysfunction are an important target to prevent and treat neurological disorders due to SO₂ inhalation. In recent years, the pleiotropic effects of endogenous 2-AG occurred for the prevention and treatment of inflammation, and vasculature dysfunction began to be investigated and concerned (Zogopoulos et al., 2013; Grabner et al., 2016). Thus, we proposed that strengthening endogenous 2-AG might represent a potential therapeutic target for the treatment of brain injury from SO₂ exposure.

It is well known that microglia are the most important immune defense in the CNS, and they have been reported to produce a variety of pro-inflammatory cytokines, such as tumor necrosis factor (TNF), nitric oxide, interleukin and other neurotoxic substances in response to environmental toxins (Block and Hong, 2005). Although the experimental study showed that astrocytes are not as effective as microglia in pro-inflammation function (Lindsay, 1994), it can be obviously activated and produce a variety of neuronal survival-related factors in response to inflammation stimulates or brain injuries (Aloisi, 1999; Tacconi, 1998). Thus, we first confirmed that SO₂ exposure enhanced the activation of astroglia and the expression of iNOS, TNF- α and IL-1 β , and the processes were observably inhibited by endogenous 2-AG. These results indicated that endogenous 2-AG pose the ability to protect against neuroinflammation from SO₂ inhalation.

Vasoactive mediators produced by inflammatory cells may trigger acute cerebrovascular

diseases. TXA₂/PGI₂ and ET-1/eNOS are two groups of important vasoactive substances. PGI₂ and eNOS exert vasodilatation effects that counterbalance the harmful vasoconstriction effects of TXA₂ and ET-1, respectively (Miller, 2006). Microvasculature dysfunction has increasingly been implicated as a crucial player in the pathogenesis of a wide variety of neuroinflammation-induced diseases upon exposure to air pollutants (Grammas et al., 2002). The clinical and animal experiments demonstrated that the two groups of substances are involved in a variety of cerebrovascular diseases (Kunos et al., 2000). Acute and chronic exposures to vehicular source pollutants have also been associated with vasculature dysfunction by altering brain ET-1 and eNOS gene expression (Thomson et al., 2007). Chronic exposure to cigarette smoke causes an imbalance of PGI₂ and TXA₂ production (Sherratt et al., 1988). Moreover, there are many inflammatory adhesion molecules that contribute to vascular endothelial functional properties, including ICAM-1, VCAM-1 (Frijns and Kappelle, 2002). Thus, we examined the expression of TXA₂, PGI₂, ET-1, eNOS, ICAM-1, and VCAM-1 and found that SO₂ inhalation caused microvasculature dysfunction by elevating ET-1, ICAM-1, and VCAM-1 expression, down-regulating eNOS levels and disrupting the balance between TXA₂ and PGI₂. However, these inflammation-related microvasculature insults were obviously reversed from SO₂ inhalation by elevating endogenous 2-AG via MAGL inhibition, suggesting that endogenous 2-AG contributed to restoration of inflammation-related microvasculature dysfunction caused by SO₂ exposure.

eCBs have been reported to exert anti-inflammation and protect microvascular function via the activation of their cannabinoid receptors (Chen et al., 2011; Eljaschewitsch et al., 2006; Mechoulam and Shohami, 2007). In the present study, endogenous 2-AG resulting in downregulation of iNOS, ET-1, ICAM-1 and VCAM-1 in response to SO₂ inhalation was reversed by both SR-1 and SR-2 (antagonist of CB1 and CB2), while a change in eNOS was only targeted by SR-1. Interestingly, iNOS, but not TNF- α and IL-1 β , inhibition was significantly restored in the presence of SR-1 and SR-2. These findings implicated that the CB1/2-dependent signaling pathway was the primary mechanism mediating the protective effects of 2-AG from inflammation and inflammation-related microvasculature function, but other mechanisms could not be excluded. Recent evidence suggest that eCBs bind to peroxisome proliferator-activated receptors (PPARs) or vanilloid receptors to exert the effect

of anti-inflammatory and neuroprotection (Paterniti et al, 2013; Pini et al., 2012; Rockwell et al, 2006), suggesting that other receptors mediate endogenous 2-AG protection against brain inflammatory insult and inflammation-related microvasculature dysfunction from SO₂ inhalation.

4 Conclusions

In summary, endogenous 2-AG prevented neuroinflammation and its related microvasculature dysfunction from SO₂ inhalation, and the action was mediated mainly via CB1/2 receptors. This study provides a mechanistic basis for the development of new therapeutic approaches for protecting against brain inflammation from SO₂ inhalation.

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Figure legend

Fig. 1 JZL184 improves the 2-AG level in mice brain after SO₂ exposure. The level of 2-AG in the brain of mice under different treatment conditions ($n=8$). ^a $p < 0.05$ compared with vehicle controls.

Fig. 2 Endogenous 2-AG suppresses SO₂ inhalation caused activation of microglia and astrocytes in mice brain. (A1) Activated microglial cells (CD11b/c, a microglial marker, red) in the brain of mice in vehicle control, SO₂ exposure and JZL184+SO₂ groups; (A2) Quantifications of CD11b/c positive staining cells under different conditions; (B1) Activated astrocytes (GFAP, an astrocytic marker, red) in the brain of mice in vehicle control, SO₂ exposure and JZL184+SO₂ groups; (B2) Quantifications of GFAP positive staining cells under different conditions. ^{aa} $p < 0.01$ compared with vehicle controls; ^b $p < 0.05$ and ^{bb} $p < 0.01$ compared with SO₂ groups. Data are averaged from 3 to 4 sections in three groups of mice, $n=8$ mice per group.

Fig. 3 Endogenous 2-AG inhibits neuroinflammation following SO₂ inhalation through CB1 and CB2 receptors. (A) Quantifications of TNF- α contents in cortex of mice exposed to SO₂ in the absent or present of JZL184, SR-1+JZL184 and SR-2+JZL184 ($n=8$); (B) Quantifications of IL-1 β contents in cortex of mice exposed to SO₂ in the absent or present of JZL184, SR-1+JZL184 and SR-2+JZL184 ($n=8$); (C1) Immunoblot analysis of iNOS in cortex of mice exposed to SO₂ in the absent or present of JZL184, SR-1+JZL184 and SR-2+JZL184; (C2) Quantifications of iNOS expression under different conditions ($n=8$). +: with; -: without. ^{aa} $p < 0.01$ and ^{aaa} $p < 0.001$ compared with vehicle controls; ^b $p < 0.05$ and ^{bb} $p < 0.01$ compared with SO₂ group; ^c $p < 0.05$ compared with SO₂ group in the presence of JZL184.

Fig. 4 Endogenous 2-AG reverses PGI₂ down-regulation in response to SO₂ inhalation. Levels of TXA₂ (A), PGI₂ (B) and TXA₂/PGI₂ ratio (C) in the cortex of mice under different conditions. ^a $p < 0.01$ compared with vehicle controls; ^b $p < 0.05$ and ^{bb} $p < 0.01$ compared with

SO₂. Data are averaged from 3 to 4 sections in three groups of mice, *n*=8 mice per group.

Fig. 5 Endogenous 2-AG abolishes ET-1, VCAM-1 and ICAM-1 elevation and eNOS attenuation by CB1/2 receptors. (A1) Immunoblot analysis of ET-1 in cortex of mice exposed to SO₂ in the absent or present of JZL184, SR-1+JZL184 and SR-2+JZL184; (A2) Quantifications of ET-1 expression under different conditions (*n*=8); (B1) Immunoblot analysis of eNOS in cortex of mice exposed to SO₂ in the absent or present of JZL184, SR-1+JZL184 and SR-2+JZL184. (B2) Quantifications of eNOS expression under different conditions (*n*=8). (C1) Immunoblot analysis of ICAM-1 in the cortex of mice exposed to SO₂ in the absent or present of JZL184, SR-1+JZL184 and SR-2+JZL184. (C2) Quantifications of ICAM-1 expression under different conditions (*n*=8). (D1) Immunoblot analysis of VCAM-1 in the cortex of mice exposed to SO₂ in the absent or present of JZL184, SR-1+JZL184 and SR-2+JZL184. (D2) Quantifications of VCAM-1 expression under different conditions (*n*=8). Abbreviations: +, with; -, without. ^{aa}*p* < 0.01 compared with vehicle controls; ^b*p* < 0.05 compared with SO₂ group; ^c*p* < 0.05 compared with SO₂ group in the presence of JZL184.

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Fig. 4 Endogenous 2-AG reverses PGI2 down-regulation in response to SO_2 inhalation. Levels of TXA2 (A), PGI2 (B) and TXA2/PGI2 ratio (C) in the cortex of mice under different conditions. ^a $p < 0.01$ compared with vehicle controls; ^b $p < 0.05$ and ^{bb} $p < 0.01$ compared with SO_2 . Data are averaged from 3 to 4 sections in three groups of mice, $n=8$ mice per group.

Fig. 5 Endogenous 2-AG abolishes ET-1, VCAM-1 and ICAM-1 elevation and eNOS attenuation by CB1/2 receptors. (A1) Immunoblot analysis of ET-1 in cortex of mice

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