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RESEARCH

Targeting the adenosinergic signaling pathway in the inflammatory response in rat lung tissue during moderate and severe chronic hypoxia

Orta ve şiddetli kronik hipoksi sırasında sıçan akciğer dokusunda inflamatuvar yanıtta adenozinerjik sinyal yolunun hedeflenmesi

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Abstract

Purpose: Hypoxia occurs after inflammatory diseases in tissues and is associated with the induction of proinflammatory responses in addition to the breakdown of barriers. Adenosine receptors are critical in the initiation and regulation of this response. The effectiveness of externally applied adenosine receptor agonists/antagonists in such inflammatory diseases is noteworthy. In this study, we aimed to investigate the relationship between hypoxia, adenosine and inflammation, as well as the role of adenosine agonists and antagonists in this situation.

Materials and Methods: For this purpose, two different hypoxia models, moderate and severe, were used. Using a total of 80 male Sprague-Dawley rats for both models, 4 subgroups were designed: control (CON), dimethyl sulfoxide (DMSO), adenosine agonist (AGO; CGS-21680) and adenosine antagonist (ANT; MSX-3). Rats were exposed to moderate groups 13% O_2 and severe groups 10% O_2 in fine-tuned normobaric hypoxia chambers for 7 days. At the end of the 7th day, ventilation measurements were made and the hypoxia model was confirmed. A2AR, CD11c, COX2, NFKB and VEGF antibody expressions were evaluated by immunofluorescence staining method by taking frozen sections from the lung tissues after the experimental stages.

Results: This study showed that the expression of inflammation markers increased in experimental hypoxia models. According to the findings, while the levels of A2AR and VEGF were higher in the agonist group compared to the other groups in both models, the levels of inflammatory markers CD11c, NFKB and COX2 were significantly lower.

Conclusion: Various natural and synthetic drugs are available as treating the inflammation which can be helpful in treating lung disorders. Researchers are still searching for new anti-inflammatory drugs which can produced

Öz

Amaç: Hipoksi, dokulardaki inflamatuar hastalıklardan sonra ortaya çıkar ve proinflamatuar yanıtların indüklenmesiyle ilişkilidir. Adenozin reseptörleri bu yanıtın başlatılması ve düzenlenmesinde kritik öneme sahiptir. Bu tür inflamatuar hastalıklarda haricen uygulanan adenozin reseptör agonistlerinin/antagonistlerinin etkinliği dikkat çekicidir. Bu çalışmada hipoksi, adenozin ve inflamasyon arasındaki ilişkinin yanı sıra adenozin agonist ve antagonistlerinin bu süreçteki rolünü araştırmayı amaçladık.

Gereç ve Yöntem: Bu amaçla, orta ve şiddetli olmak üzere iki farklı hipoksi modeli kullanıldı. Her iki model için toplam 80 erkek Sprague-Dawley sıçanı kullanılarak 4 alt grup tasarlandı: kontrol (CON), dimetil sülfoksit (DMSO), agonist (AGO; CGS-21680) ve antagonist (ANT; MSX-3). Sıçanlar 7 gün boyunca ince ayarlı normobarik hipoksi odalarında orta gruplarda %13 O2 ve ağır gruplarda %10 O2'ye maruz bırakıldı. 7. günün sonunda ventilasyon ölçümleri yapıldı ve hipoksi modeli doğrulandı. Deneysel aşamalardan sonra akciğer dokularından dondurulmuş kesitler alınarak immünofloresan boyama yöntemi ile A2AR, CD11c, COX2, NFKB ve VEGF antikor ekspresyonları değerlendirildi.

Bulgular: Bu çalışma, deneysel hipoksi modellerinde inflamatuar belirteçlerin ekspresyonunun arttığını gösterdi. Bulgulara göre, her iki modelde de agonist grubunda A2AR ve VEGF düzeyleri diğer gruplara göre daha yüksek iken, inflamasyon belirteçleri CD11c, NFKB ve COX2 düzeyleri anlamlı derecede düşüktü.

Sonuç: Enflamasyonu tedavi etmek için akciğer bozukluklarının tedavisinde yardımcı olabilecek çeşitli doğal ve sentetik ilaçlar mevcuttur. Araştırmacılar hala daha hızlı yanıt verebilecek yeni antiinflamatuar ilaçları araştırıyorlar. Bu bulgular hipoksi kaynaklı akciğer

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faster response. These findings highlight the potential benefit of A2A agonists, which can be used in hypoxia-induced lung inflammation.
Keywords: Hypoxia, adenosine, adenosine agonist/antagonist, lung toxicity

inflamasyonunda kullanılabilecek A2A agonistlerinin potansiyel faydasını vurgulamaktadır.

Anahtar kelimeler: Hipoksi, adenozin, adenozin agonist/antagonist, akciğer toksisitesi

INTRODUCTION

The main function of the lung is to exchange gas between the blood and air in the pulmonary capillaries. This event occurs by diffusion at the rate of the partial pressures of the gases in the environment[1]. Thus, the gas pressures in the arterial blood remain constant; during breathing at sea level and at rest, the partial arterial oxygen pressure (PaO_2) is kept between 80 and 100 mmHg, and the partial arterial carbon dioxide pressure ($PaCO_2$) is kept between 35 and 45 mmHg. When PaO_2 falls below 50 mmHg, saturation rapidly decreases. Such a decrease in saturation leads to tissue hypoxia. The lowest PaO_2 that the organism can tolerate is 20 mmHg[2,3]. Historically, the lung was the first organ in which hypoxia damage was studied due to decreased oxygen content in organs and surrounding tissue[4]. At the same time, knowledge of hypoxic effects on lung tissue is poor in the literature. The systemic consequences of alveolar hypoxia have been extensively studied. Experimental hypoxia models and protocols may differ according to the change in the amount of oxygen going into the tissue[4,5].

Adenosine was first isolated from heart muscle and described in 1927 as an "adenine compound" that can alter heart rhythm when injected into guinea pigs[6,7]. Besides its function in heart rhythm, adenosine also modulates inflammatory responses during hypoxic conditions[7,8]. Adenosine exerts its effects through four types of receptors on the cell surface: the adenosine A1 receptor (A1AR), the adenosine A2A receptor (A2AAR), the adenosine A2B receptor (A2BAR), and the adenosine A3 receptor (A3AR)[9,10]. The interaction between hypoxia and adenosine signaling has therapeutic value in lung diseases[7,11]. Especially, the A2A receptor (A2AR) has been identified as an important mediator of inflammatory and immune responses[12]. A2AR is activated by adenosine or its agonists and activates the negative feedback mechanism to suppress the systemic inflammatory response[13,14]. The experimental study of hypoxia models is also clinically important as it mimics the picture in diseases such as chronic obstructive pulmonary disease[15,16]. In this way, solutions may be found to a wide range of clinical problems that may arise in common clinical scenarios where hypoxia and inflammation are combined[17,18].The biggest problem experienced in the COVID-19 outbreak, which has affected many people in recent years, was hypoxia-related lung damage[19]. It is very important to recognize the signaling pathways in hypoxia-related lung damage associated with many diseases clinically and to develop new treatment protocols against this damage. For this reason, in our study, experimental hypoxia models were created as moderate and severe continuous. Thus, the effect of hypoxia, which is known to be associated with inflammation, on the lung tissue was demonstrated by the change in the expressions of the determined proteins. Based on the literature, it is known that adenosine agonists and antagonists have a positive effect on the hypoxia-inflammation pathway[12-14]. For this reason, the therapeutic effects of adenosine agonists and antagonists in rats with a hypoxia model were evaluated by changes in the expressions of A2AR, CD11c, Cyclooxygenase2 (COX2), Nuclear Factor Kappa β (NF-κβ) and Vascular Endothelial Growth Factor (VEGF) proteins, which have an important effect on the hypoxia-inflammation pathway. Thus, we aimed to make a potential contribution to the development of new anti-inflammatory drugs by demonstrating the effects of adenosine agonists and antagonists on lung tissue in hypoxia-induced diseases that are difficult to treat, which have been emphasized recently.

MATERIALS AND METHODS

Experimental animals

Experiments were performed on male Sprague-Dawley rats weighing 250–300 g, housed in a 12:12 light-dark cycle, and fed a standard diet ad libitum. All experimental procedures were approved by the Erciyes University Animal experiments local ethics committee (EÜHADYEK) at the meeting dated 05.05.2020, with decision number 20/081 before investigation. As this study did not involve human participation, informed consent does not require.

All experimental stages of the study were carried out at Erciyes University Genome and Stem Cell Center (GENKÖK). The experiments conformed to national standards for the care and use of experimental animals. At the end of experiments, rats were euthanized with an overdose of sevoflurane (Sleep Away, 5% sevoflurane in 100% O_2) in an anesthetize chamber.

Experimental groups and drug administrations

We used 80 rats and divided them randomly into two main experimental groups: the moderate chronic sustained hypoxia (mCSH) group (n = 40) and the severe chronic sustained hypoxia (sCSH) group (n = 40). Main groups were divided into control (CON), dimethyl sulfoxide (DMSO; Sigma Aldrich, St. Louis, MO, USA), agonist (AGO), AOBIOUS INC, CGS-21680 hydrochloride, CTY 2416, Gloucester MA, USA), and antagonist (ANT), AOBIOUS INC, MSX-3, AOB 5001, Gloucester MA, USA MSX-3. Moderate groups were exposed to 13% O_2, and severe groups were exposed to 10% O_2 in fine-tuned (OxyCycler A84XOV Dynamic O_2 Animal Chamber Controller, BioSpherix, Ltd., RRID: SCR_021185) normobaric hypoxia chambers (Safety Hood for Animal Chambers, BioSpherix, Ltd., RRID: SCR_021206) for 7 days (temperature 21°C and 40% humidity). The chamber was opened for 10 minutes every day for cage cleaning and the replacement of food and water. During this time, drug administrations of 20% DMSO diluted in saline used as a vehicle and CGS21680 and MSX-3 dissolved in 20% DMSO intraperitoneal (ip) (100 µg kg-1) were done for 7 days.

Whole body plethysmography

Ventilatory responses to hypoxia were measured in unrestrained rats using a barometric WBP (TBL3000, FinePointe unrestrained whole-body plethysmography, Buxco Electronics, Wilmington, NC) modified for continuous flow. On the experimental day, before starting the measurement, first the device was automatically calibrated, and then the rats were weighed and sealed into the plethysmograph chambers (model PLY422, Buxco Electronics, Wilmingtan, NC). A constant gas flow (6 L/min) was delivered with a mass flow controller (MFC-4, Sable Systems, Inc., Las Vegas, NV) to a gas mixer connected upstream of the chambers. Gases exited the chamber through a hole connected to the WBP cage to isolate respiration-induced pressure changes in the chamber during steady flow with high input and output impedances. This also allowed us to keep the room pressure close to atmospheric pressure and to make reference pressure measurements in the room relative to the atmosphere. The single chamber plethysmograph uses a barometric analysis technique, which compares the pressure difference between the animal chamber and a reference chamber to measure respiratory physiological parameters. Rats were exposed to 10% O_2 or 13% O_2 with a constant 0.03% CO_2 balance in N_2 for 30 min of acclimation to stabilize the animal movements in the WBP chamber during measurement. Then we started to measure baseline ventilation (V (ml/min * Kg)) at the same O_2 levels for the main groups. All ventilatory parameters were recorded on an analog-digital acquisition system (Buxco FinePointe software, v2.9.0, Buxco Electronics, Wilmington, NC) and analyzed with the same software.

Immunofloresan staining

Frozen sections taken with a cryostat device were kept in an acetone solution at -20°C for 10 minutes for postfixation. Then, by blocking with 10% goat serum, primary antibodies were dropped at the specified rates: anti-adenosine A2AR; 1/500 (mouse monoclonal; Millipore); CD11c; 1/100 (rabbit monoclonal; Abcam); COX-2; 1/100 (rabbit polyclonal; Abcam); NF-κB; 1/200 (mouse monoclonal; Santa Cruz Biotech); and VEGF; 1/200 (mouse monoclonal; Santa Cruz Biotech). The slides were incubated in humidity chambers at +4°C for 24 hours, and the next day, secondary antibodies were added to the antibody-treated slides and left for incubation for 1 hour. After incubation, the slides were treated with DAPI and made ready for examination. Then, tissue slides were covered with a coverslip using mounting medium. Prepared slides were kept at -20 °C, and imaging was performed using a fluorescent microscope (Olympus BX51, Germany).

Statistical analysis

While evaluating the immunofluorescence staining results, the intensity of immunoreactivity of each antibody was calculated using the Image J software program. Measurements were made from 10 different areas randomly selected from each slide, and statistical analysis of the obtained data was done using

the Graphpad prism (Graphpad Software Inc. Version 8.0d, California, United States) package program. After checking the conformity of the normal distribution with the Shapiro Wilk test, results in terms of statistical significance were analyzed using the Student t-test and one-way ANOVA. Comparison of 4 groups (CON, DMSO, AGO, ANT) in 13% moderate and %10 severe groups was made with oneway ANOVA for all markers. In addition, CON, DMSO, AGO and ANT groups were compared with student t test according to 10% and 13% status for all markers. The level of significance was accepted as $p<0.05$, all data presented as mean ±SEM, n=10 for all groups.

RESULTS

Ventilation measurements

After exposure to moderate chronic sustained hypoxia (13%), minute ventilation (V) significantly decreased in the ANT group compared to the DMSO and AGO groups (Figure 1A). After exposure to severe chronic sustained hypoxia (10%), minute ventilation (V) significantly increased in the AGO group compared to the CON, DMSO, and ANT groups (Figure 1B).

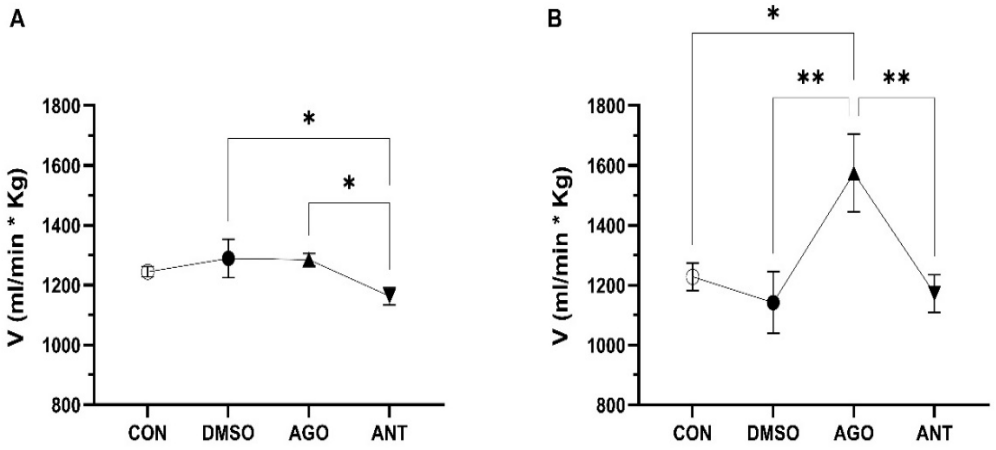

Figure 1. Ventilation (V) measurements after exposure to chronic sustained hypoxia to show baseline ventilation.

After exposure to chronic sustained hypoxia (10% O_2 or 13% O_2) groups (A), Moderate chronic sustained hypoxia group minute ventilation (V) significantly decreased in the ANT group compared to the DMSO and AGO groups. (B) Severe chronic sustained hypoxia group: minute ventilation (V) significantly increased in the AGO group compared to the CON, DMSO, and ANT groups. (Control (CON), dimethyl sulfoxide (DMSO), agonist (AGO), and antagonist (ANT)). After exposure to chronic sustained hypoxia (10% O_2 or 13% O_2) groups, there are no significant differences between 10% O_2 and 13% O_2 levels in CON, DMSO, and ANT groups. There is a significant increase in minute ventilation (V) between AGO groups depending on hypoxic severity (Figure 2).

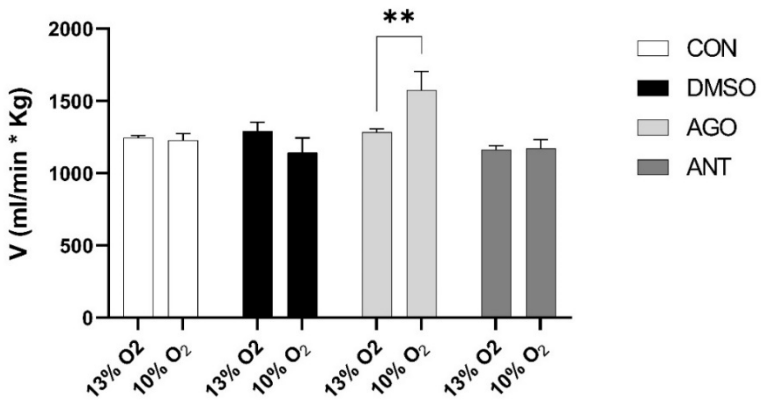

Figure 2. Ventilation (V) measurements after exposure to chronic sustained hypoxia are used in group comparisons to show baseline ventilation changes. Control (CON), dimethyl sulfoxide (DMSO), agonist (AGO), and antagonist (ANT). Moderate sustained hypoxia (13% O_2) and severe sustained hypoxia (10% O_2).

Immunofluorescence staining

A2AR immunofluorescence staining

After exposure to moderate chronic sustained hypoxia, A2AR expression was evaluated between groups by the immunofloresan staining method (Figure 3 and Figure 4). It was determined that there was a statistically significant increase in A2AR expression in the AGO group compared to the other groups ($p<0.001$). A decreased was detected in the ANT group compared to the CON group, but it was not statistically significant ($p>0.05$). On the other hand, the difference between the AGO and ANT groups was significant ($p<0.001$) (Figure 5A).

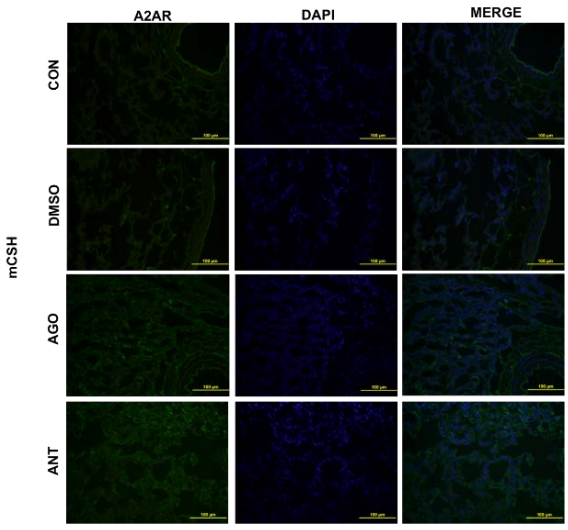

Figure 3. A2AR antibody immunofluorescence staining images in the moderate sustained hypoxia group (mCSH; 13% O_2) (Control (CON), dimethyl sulfoxide (DMSO), agonist (AGO), and antagonist (ANT).

After exposure to severe chronic sustained hypoxia, the results showed strong A2AR staining in the AGO group compared to the other groups ($p<0.001$) (Figure 4). An increase was detected in the ANT group compared to the CON group, but it was not statistically significant ($p >0.05$) (Figure 5A).

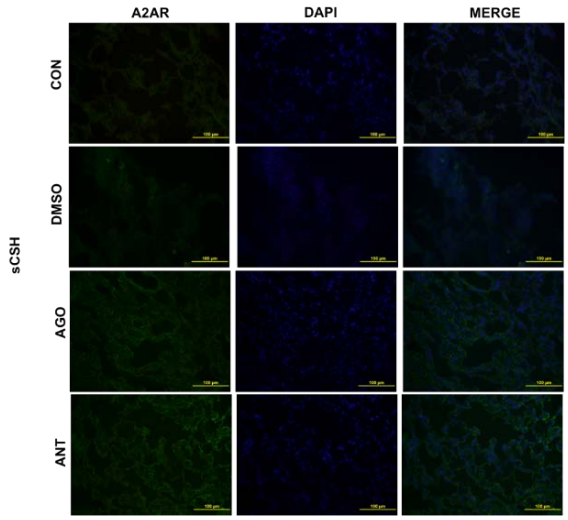

Figure 4. A2AR antibody immunofluorescence staining images in the severe sustained hypoxia group (sCSH; 10% O_2). (Control (CON), dimethyl sulfoxide (DMSO), agonist (AGO), and antagonist (ANT)).

When comparing variables in subgroups (CON, DMSO, AGO, and ANT) of the mCSH and sCSH groups, in the CON and AGO groups of the mCSH group, A2AR expression was higher than that of the sCSH group ($p<0.001$). Differences between other groups were not significant ($p >0.05$) (Figure 5B).

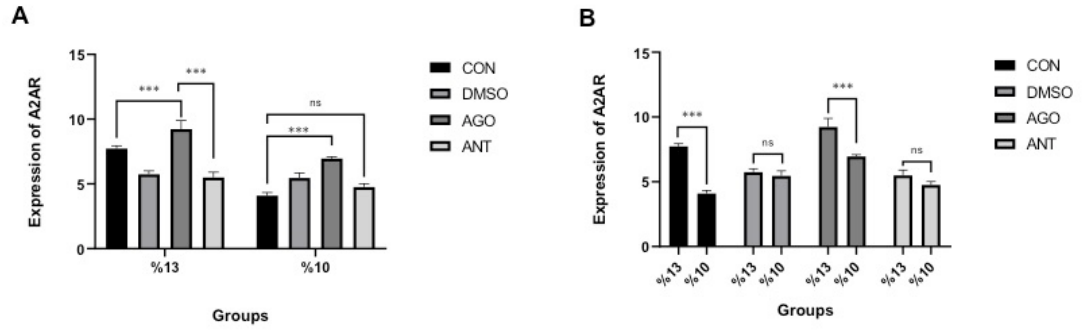

Figure 5. The fluorescence quantification of A2AR. A. Comparison of variables in mCSH (13%) and sCSH (10%) groups B. Comparison of variables in subgroups (CON, DMSO, AGO, and ANT) of mCSH and sCSH groups. *p< 0.05, **p< 0.01, ***p< 0.001, ns: not significant.

CD11c immunofluorescence staining

After exposure to moderate chronic sustained hypoxia, a decrease in CD11C expression was observed in the AGO group compared to the CON group and ANT group, and it was statistically significant ($p<0.05$). The difference between the ANT and CON groups was not statistically significant ($p >0.05$) (Figure 6 and Figure 8A).

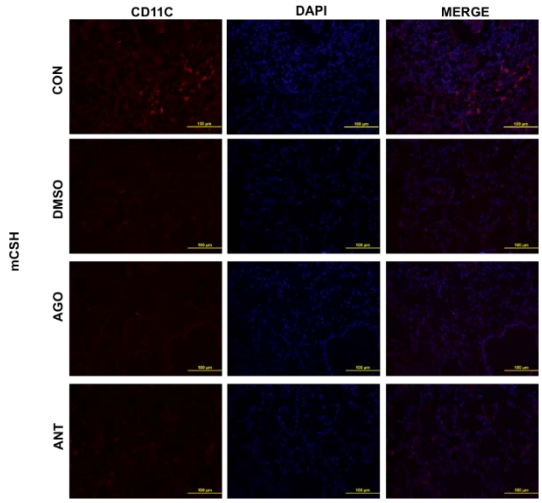

Figure 6. CD11c antibody immunofluorescence staining images in the moderate sustained hypoxia group (mCSH; 13% O_2). (Control (CON), dimethyl sulfoxide (DMSO), agonist (AGO), and antagonist (ANT)).

After exposure to severe chronic sustained hypoxia, AGO and ANT groups were weakly stained with CD11c compared to the CON group ($p<0.05$) (Figure 7). But the difference between the AGO and ANT groups was not statistically significant ($p >0.05$) (Figures 8A).

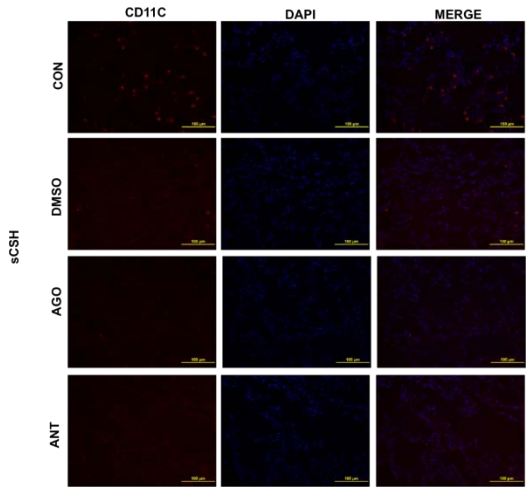

Figure 7. CD11c antibody immunofluorescence staining images of the severe sustained hypoxia group (sCSH; 10% O_2). (Control (CON), dimethyl sulfoxide (DMSO), agonist (AGO), and antagonist (ANT)).

As a result of the comparison of subgroups (CON, DMSO, AGO, and ANT) belonging to the mCSH and sCSH groups, CD11c density was higher in the CON and DMSO group of sCSH than in the mCSH group ($p<0.001$). Differences between other groups were not significant ($p >0.05$) (Figure 8B).

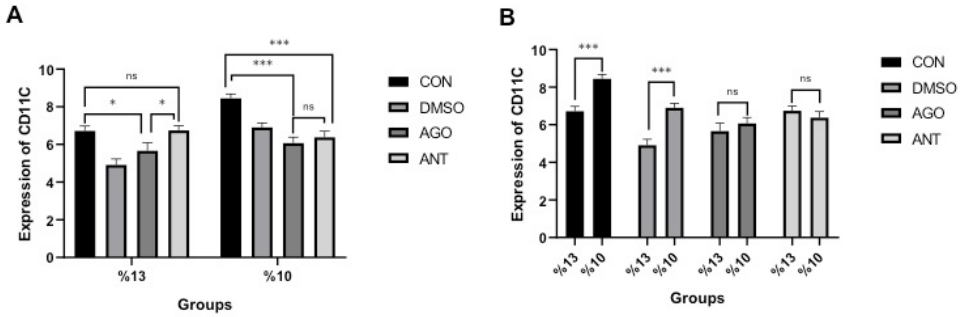

Figure 8. The fluorescence quantification of CD11C. A. Comparison of variables in different subgroups (CON, DMSO, AGO, and ANT) of mCSH (13%) and sCSH (10%). B. Comparison of variables in the mCSH and sCSH groups. *p< 0.05, **p< 0.01, ***p< 0.001, ns: not significant.

COX2 immunofluorescence staining

After exposure to moderate chronic sustained hypoxia, we found that COX2 expression decreased in the AGO and ANT groups compared to the control group ($p<0.01$) (Figure 9 and Figure 11A). The strong staining seen especially in the control group is shown in Figure 9.

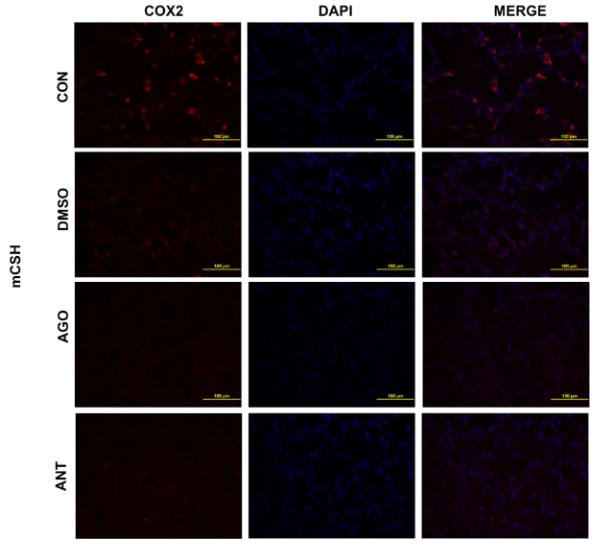

Figure 9. COX2 antibody immunofluorescence staining images in the moderate sustained hypoxia group (mCSH; 13% O_2). (Control (CON), dimethyl sulfoxide (DMSO), agonist (AGO), and antagonist (ANT)).

After exposure to severe chronic sustained hypoxia, in the AGO and ANT groups, COX2 expression was much lower than in the CON and DMSO groups ($p< 0.001$, $p< 0.01$, respectively) (Figure 10). Moreover, there was a significant difference between the AGO and ANT groups ($p< 0.05$) (Figure 11A).

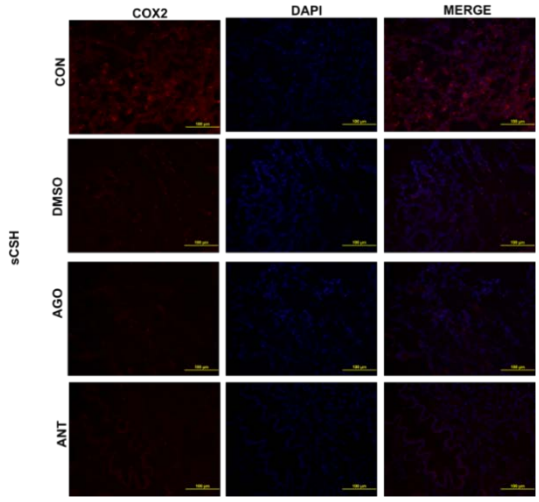

Figure 10. COX2 antibody immunofluorescence staining images in the severe sustained hypoxia group (sCSH; 10% O_2). (Control (CON), dimethyl sulfoxide (DMSO), agonist (AGO), and antagonist (ANT)).

We compared the subgroups (CON, DMSO, AGO, and ANT) with each other to show the difference between the groups (mCSH and sCSH), in the CON and AGO groups of the mCSH group, COX2 expression was higher than that of the sCSH group (*$p< 0.05$, ***$p< 0.001$, respectively). On the other hand, COX2 expression in the DMSO group belonging the mCSH group was lower than in the sCSH group (**$p< 0.01$). The difference in ANT groups was not significant ($p>0.05$) (Figure 11B).

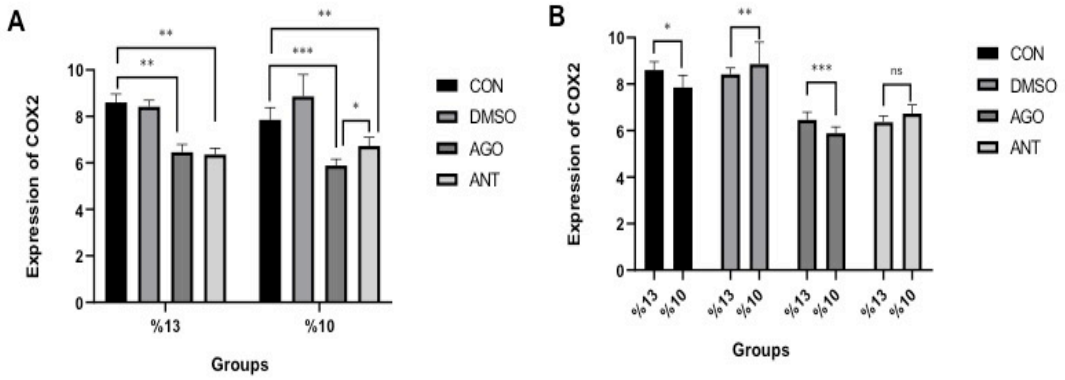

Figure 11. The fluorescence quantification of COX2. A. Comparison of variables in different subgroups (CON, DMSO, AGO, and ANT) of mCSH (13%) and sCSH (10%) B. Comparison of variables in the mCSH and sCSH groups. *$p< 0.05$, **$p< 0.01$, ***$p< 0.001$, ns: not significant.

NF-κβ immunofluorescence staining

Immunofluorescence staining results showed that strong green fluorescence was marked in untreated groups after exposure to moderate chronic sustained hypoxia-induced inflammation, indicating increased NF-κB. After tratment with agonist and antagonist, the green fluorescence intensity weakened (Figure 12). As shown in Figure 14A, compared with the CON group, the staining of NFKB in AGO and ANT groups was weaker (***p<0.001, *p<0.05, respectively) and the difference between the AGO and ANT groups was significant (***p<0.001).

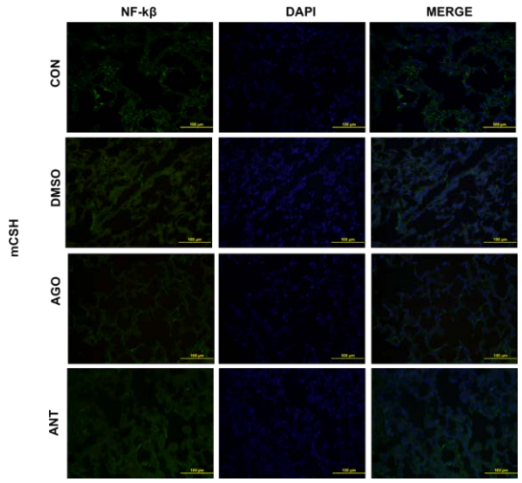

Figure 12. NF-κB antibody immunofluorescence staining images in the moderate sustained hypoxia group (mCSH; 13% O_2). (Control (CON), dimethyl sulfoxide (DMSO), agonist (AGO), and antagonist (ANT)).

As shown is Figure 13, after exposure to severe chronic sustained hypoxia, NF-κB as an indicator of inflammatory stained more strongly in the untreated groups. Expression of NF-κB decreased in the AGO group compared to the CON group (***p< 0.001). However, no significant reduction was seen in the ANT group (p>0.05) (Figure 14A).

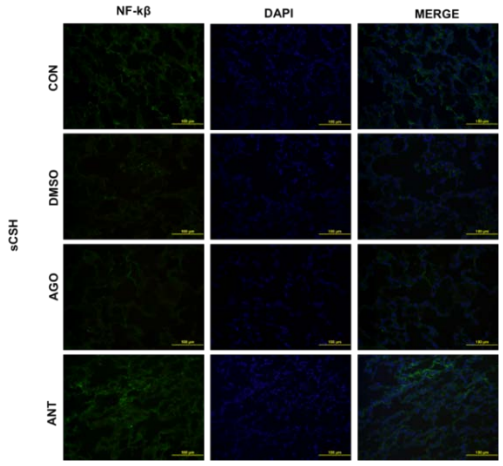

Figure 13. NF-κB antibody immunofluorescence staining images in the severe sustained hypoxia group (sCSH; 10% O_2). (Control (CON), dimethyl sulfoxide (DMSO), agonist (AGO), and antagonist (ANT)).

When comparing variables in subgroups (CON, DMSO, AGO, and ANT) of the mCSH and sCSH groups, in the CON and AGO groups of the mCSH group, NF-ϰB expression was higher than that of the sCSH group (**p< 0.01, ***p< 0.001, respectively). Differences in the DMSO group were not significant (p>0.05). In the ANT group of the sCSH group, NF-ϰB expression was higher than in the mCSH group (***p< 0.001) (Figure 14B).

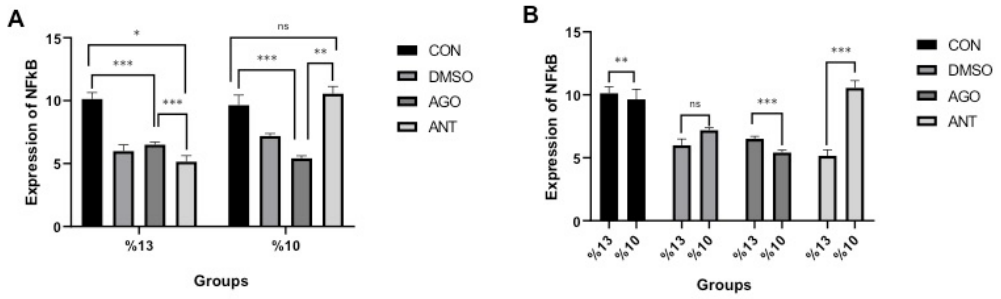

Figure 14. The fluorescence quantification of NF-ϰB. A. Comparison of variables in different subgroups (CON, DMSO, AGO, and ANT) of mCSH (13%) and sCSH (10%). B. Comparison of variables in the mCSH and sCSH groups. *p< 0.05, **p< 0.01, ***p< 0.001, ns: not significant.

VEGF immunofluorescence staining

As seen in figure 15, AGO and ANT groups showed strong green fluorescence after exposure to moderate chronic sustained hypoxia. The results of fluorescence quantification of VEGF showed that it increased in the AGO and ANT groups compared to the CON group (**p<0.01), but the increase in the ANT group was not significant (p>0.05). As shown in Figure 17A, the difference between the AGO and ANT groups was significant (*p<0.05).

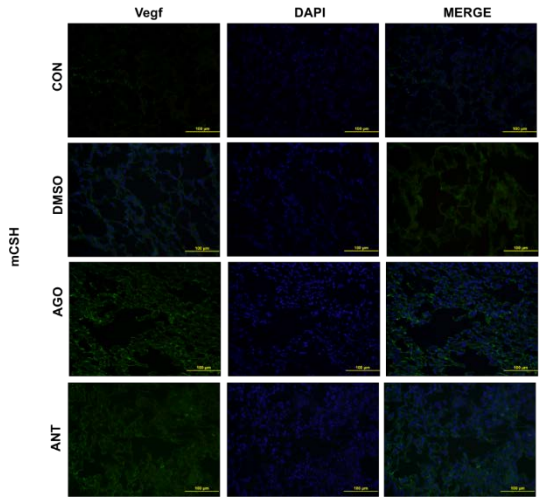

Figure 15. VEGF antibody immunofluorescence staining images in the moderate sustained hypoxia group (mCSH; 13% O_2) (Control (CON), dimethyl sulfoxide (DMSO), agonist (AGO), and antagonist (ANT)).

After exposure to severe chronic sustained hypoxia, VEGF expression increased in the AGO and ANT groups compared to the CON group, but the increase in the ANT group was not significant (**p< 0.01, p>0.05, respectively) (Figure 16). The difference between the AGO and ANT groups was significant (*p<0.01) (Figure 17A).

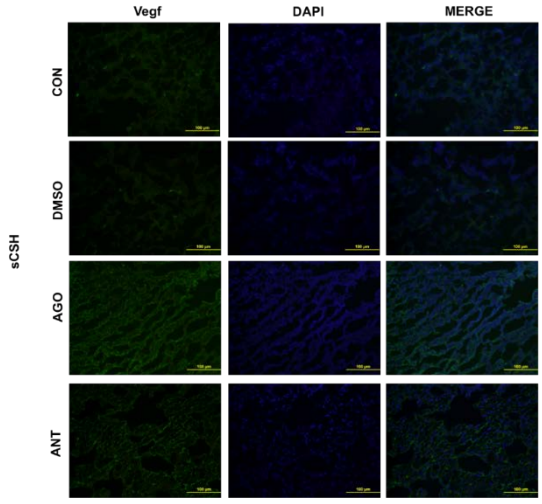

Figure 16. VEGF antibody immunofluorescence staining images in the severe sustained hypoxia group (sCSH; 10% O_2). (Control (CON), dimethyl sulfoxide (DMSO), agonist (AGO), and antagonist (ANT)).

When comparing variables in subgroups (CON, DMSO, AGO, and ANT) of mCSH and sCSH groups, in the CON, AGO, and ANT groups of the mCSH group, VEGF expression was higher than that of the sCSH group (**p<0.01, **p< 0.01, ***p< 0.001, respectively). In the DMSO group of the mCSH group, VEGF expression was higher than in the sCSH group, but it was not significant (p>0.05) (Figure 17B).

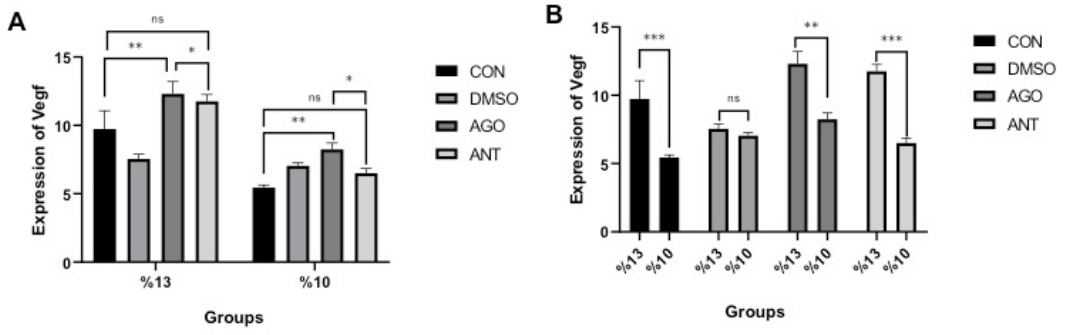

Figure 17. The fluorescence quantification of VEGF A. Comparison of variables in different subgroups (CON, DMSO, AGO, and ANT) of mCSH (13%) and sCSH (10%) B. Comparison of variables in the mCSH and sCSH groups. *p< 0.05, **p< 0.01, ***p< 0.001, ns: not significant.

DISCUSSION

Respiratory plasticity induced by hypoxia: Depending on how often (intermittent or continuous) hypoxia is exposed, at what severity (O_2 percentage), and for how long (depending on time), there are differences in the response of ventilation to these situations[20]. Exposure to intermittent or continuous hypoxia causes the formation of plasticity, which is trying to be defined by different mechanisms within the respiratory control system. Therefore, in this process in which respiratory functions are affected, lung tissue shows protective responses when exposed to chronic changes in oxygenation levels, possibly by changing expression genes that regulate signaling pathway sensitivity[20,21].

It has been reported in the literature that there is a relationship between respiration and adenosine concentrations in a hypoxic environment. Studies indicated that adenosine receptor (AR) agonists and antagonists have a promising future as pharmacological modulators in disease-related inflammation and immune response[22-24]. The first study to support the role of the A2A receptor reported that CGS21680 was the first agonist to activate this receptor [25] and it was supported by subsequent studies[26,27]. There are studies showing the therapeutic effects of A2A antagonists on respiratory plasticity. Johnson et al. examined and reported the positive effects of A2A agonists (CGS21680) and antagonists (MSX-3) on the respiratory plasticity of neonatal rats[28]. For this reason, CGS21680 was preferred as the A2AR agonist and MSX-3 as the antagonist in this study. The main limitation of this study was the lack of comparison with different types of agonists and antagonists. Future studies may compare the efficacy of different Adenosine agonists and antagonists.

Studies show that extracellular adenosine signaling attenuates inflammation and tissue damage in vivo[29]. Work by Ohta and Sitkovsky also demonstrated that A2AR reduces in vivo inflammation and tissue damage by increasing the level of immunosuppressive cyclin AMP[14]. The observations of Ohta et al. are consistent with studies on inflammation in different animal models[30,31]. Sitkovsky and Thiel assumed that providing oxygen support in diseases that can cause hypoxia may cause serious side effects and patient mortality and stressed the importance of the hypoxia adenosinergic pathway in protecting tissues from damage caused by inflammation in their study[32]. Additional evidence of the therapeutic effects of A2AR agonists showed that treatment with CGS21680 resulted in decreased levels of reactive oxygen metabolites, decreased leukocyte infiltration into the lung, significantly improved lung gas exchange, decreased lung vascular permeability and prevention of oxygenation-induced lung damage[29,32]. The study of Sitkovsky and Thiel was the first to investigate the adenosinergic mechanism of hypoxia, and it was suggested that oxygen therapy in patients with acute respiratory distress syndrome (ARDS) be combined with inhaled A2AR agonists to induce the hypoxia-A2-adenosynergic pathway that protects lung tissue[32]. This study demonstrated that A2AR expression increased in lung tissues exposed to moderate sustained and severe sustained hypoxia due to the administration of the A2A agonist CGS21680 in parallel with the studies. Hoffman et al. showed that the modulation of respiratory plasticity by A2A receptors was explained by cellular mechanisms and contributed to the literature on the basic mechanisms of hypoxia-induced respiratory plasticity. It was also reported that MSX-3 had a unique acute pharmacological intervention at the modulation of respiratory plasticity in the same study[33].

It is known that inflammation due to hypoxia increases the expression of HIF1-α (30), and previous studies have demonstrated the role of HIF-1 on immunity, including its role in T-cells, DCs, epithelial cells, and macrophages. CD11c is a supplemental of the complement receptor 4, which is mainly expressed on DC, but also on activated T-cells, some macrophages and natural kills (NK) cells. Alveolar macrophages (AM) play a key role in regulating immune responses and inflammation within the lung. Previous studies have reported that AMs express high levels of CD11c, unlike other tissue macrophages[34-36]. Draijer et al. reported that alveolar macrophages expressed higher CD11c after the development of allergic lung inflammation in mice [37]. Similarly, we reported that CD11c expression was increased in lung tissues exposed to moderate and severe sustained hypoxia. At the same time, in our study, we showed that the expression of this protein decreased in the groups treated with A2A agonists and antagonists.

There are several causes of lung inflammation, such as hypoxia, and the NF-κB/COX-2/iNOS is a

known important pathway[38,39]. Furthermore, NF-κB is activated the first hours of chronic hypoxia and regulates multiple stages of inflammation and immune modulation. Hypoxia increases the expression of pro-inflammatory genes, including cyclooxygenase-2 (COX-2) by activating NF-κB[39,40]. Lim et al. showed that COX-2 expression increased in the hypoxic environment due to small cell lung cancer[41]. A recently published review targeting novel agents in lung diseases reported that treatment with CGS21680 significantly reduced NF-kB expression in inflamed lungs[42]. Cai et al. reported that Shufeng Jiedu capsule, which they applied for acute lung injury and lung injury with COVID-19, decreased NFKB expression with A2AAR activation [43]. In our study, NF-κB and COX-2 expression levels were evaluated using the immunofluorescent staining method. Our results indicated that moderate and severe sustained hypoxia increased NF-kB and COX-2 expression. After A2A agonist and antagonist administration, we observed decreased expression of NF-kB and COX-2. However, the AG group was more successful than the ANT group. The re-establishment of blood vessels through angiogenesis is important for the healing process, because oxygen and nutrients reach the healing site in this way. VEGF is a key regulator of angiogenesis and vascular permeability[44]. We showed that after exposure to moderate and severe sustained hypoxia, the expression of VEGF decreased. In AG and ANT groups, it was higher than in control groups. The results showed that the A2AR agonist contributed to the healing process after exposure to hypoxia. Impellizzeri et al. investigated the effects of the A2AR agonist CGS21680 on an experimental lung inflammation model. According to the data they obtained, they concluded that CGS21680 is an important anti-inflammatory agent and can be used in the treatment of diseases that cause lung inflammation for different reasons[45].

These and similar data increase interest in A2AR agonists and antagonists as a research topic in chronic lung diseases. Inflammation due to hypoxia increases the expression of HIF1-α, and accordingly, the inflammatory response it creates in the lung tissue and the effect of adenosine agonist/antogonist applications on this response were the subjects of this study. We aimed to make a potential contribution to the development of new anti-inflammatory drugs by showing the effect of adenosine agonists and antagonists on the lung tissue of diseases that are difficult to treat due to respiratory failure or irregularity, such as COVID-19, which has been emphasized a lot recently. The data of the study should be supported by molecular studies and clinical trials should be conducted in the next stage.

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REFERENCES

1. Si XA, Xi J. Pulmonary oxygen exchange in a rhythmically expanding-contracting alveolus-capillary model. J Respir. 2022;2:159-73.
2. Araneda OF, Tuesta M. Lung oxidative damage by hypoxia. Oxid Med Cell Longev. 2012;2012:856918.
3. Cowley NJ, Owen A, Bion JF. Interpreting arterial blood gas results. BMJ. 2013;346:f16.
4. Pamenter ME, Powell FL. Time domains of the hypoxic ventilatory response and their molecular basis. Compr Physiol. 2016;6:1345-85.
5. Pavlacky J, Polak J. Technical feasibility and physiological relevance of hypoxic cell culture models. Front Endocrinol (Lausanne). 2020;11:57.
6. Drury AN, Szent-Gyorgyi A. The physiological activity of adenine compounds with especial reference to their action upon the mammalian heart. J Physiol. 1929;68:213–37.
7. Li X, Berg NK, Mills T, Zhang K, Eltzschig HK, Yuan X. Adenosine at the interphase of hypoxia and inflammation in lung injury. Front Immunol. 2021;11:604944.
8. Boussuges A, Bourenne J, Eloufir F, Fromonot J, Mottola G, Risso JJ et al. Contribution of adenosine in the physiological changes and injuries secondary to exposure to extreme oxygen pressure in healthy subjects. Biomedicines. 2022;10:2059.
9. Sheth S, Brito R, Mukherjea D, Rybak LP, Ramkumar V. Adenosine receptors: expression, function and regulation. Int J Mol Sci. 2014;15:2024-52.
10. Vincenzi F, Pasquini S, Borea PA, Varani K. Targeting adenosine receptors: a potential pharmacological avenue for acute and chronic pain. Int J Mol Sci. 2020;21:8710.
11. Halpin-Veszeleiova K, Hatfield SM. Therapeutic targeting of hypoxia-a2adenosinergic pathway in covid-19 patients. Physiology. 2022;37:46-52.
12. Chen PZ, He WJ, Zhu ZR, Guo-Ji E, Xu G, Chen DW et al. Adenosine a2a receptor involves in neuroinflammation-mediated cognitive decline

through activating microglia under acute hypobaric hypoxia. Behav Brain Res. 2018;347:99-107.
13. Andres RM, Terencio MC, Arasa J, Paya M, Valcuende-Cavero F, Navalon P et al. Adenosine a2a and a2b receptors differentially modulate keratinocyte proliferation: possible deregulation in psoriatic epidermis. J Invest Dermatol. 2017;137:123–31.
14. Ohta A, Sitkovsky M. Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. Nature. 2001;414:916–20.
15. Groneberg DA, Chung KF. Models of chronic obstructive pulmonary disease. Respir Res. 2004;5:18.
16. Shukla SD, Walters EH, Simpson JL, Keely S, Wark PAB, O'Toole RF et al. Hypoxia-inducible factor and bacterial infections in chronic obstructive pulmonary disease. Respirology. 2020;25:53-63.
17. Herrmann J, Mori V, Bates JHT, Suki B. Modeling lung perfusion abnormalities to explain early covid-19 hypoxemia. Nat Commun. 2020;11:4883.
18. Xu Z, Shi L, Wang Y, Zhang J, Huang L, Zhang C et al. Pathological findings of Covid-19 associated with acute respiratory distress syndrome. Lancet Respir Med. 2020;8:420-2.
19. Kashani KB. Hypoxia in Covid-19: sign of severity or cause for poor outcomes. Mayo Clin Proc. 2020;95:1094-6.
20. Puri S, Panza G and Mateika JH. A comprehensive review of respiratory, autonomic and cardiovascular responses to intermittent hypoxia in humans. Exp Neurol. 2021;341:113749.
21. Powell FL, Milsom WK, Mitchell GS. Time domains of the hypoxic ventilatory response. Respir Physiol. 1998;112:123–34.
22. Jacobson KA, Gao ZG. Adenosine receptors as therapeutic targets. Nat Rev Drug Discov. 2006;5:247–64.
23. Ramlackhansingh AF, Bose SK, Ahmed I, Turkheimer FE, Pavese N, Brooks DJ. Adenosine 2a receptor availability in dyskinetic and nondyskinetic patients with parkinson disease. Neurology. 2011;76:1811–6.
24. Mills JH, Thompson LF, Mueller C, Waickman AT, Jalkanen S, Niemela J et al. CD73 is required for efficient entry of lymphocytes into the central nervous system during experimental autoimmune encephalomyelitis. Pro Natl Acad Sci USA. 2008;105:9325–30.
25. Hutchinson AJ, Webb RL, Oei HH, Ghai GR, Zimmerman MB, Williams M. CGS21680, an a_2-selective adenosine receptor agonist with preferential hypotensive activity. J Pharmacol Exp Ther. 1989;251:47–55.
26. Dong Z, Huang B, Jiang C, Chen J, Lin H, Lian Q et al. The adenosine a2a receptor activation in nucleus accumbens suppress cue-induced reinstatement of propofol self-administration in rats. Neurochem Res. 2021;46:1081-91.
27. Friedman B, Corciulo C, Castro CM, Cronstein BM. Adenosine a2a receptor signaling promotes foxo associated autophagy in chondrocytes. Sci Rep. 2021;11:968.
28. Johnson SM, Vasdev RMS, Miller MM, Baker TL, Watters JJ. Adenosine A2A receptors modulate trkb receptor-dependent respiratory plasticity in neonatal rats. Respir Physiol Neurobiol. 2021;294:103743.
29. Halpin-Veszeleiova K, Hatfield SM. Therapeutic targeting of hypoxia-a2-adenosinergic pathway in covid-19 patients. Physiology. 2022;37:46-52.
30. Thiel M, Caldwell CC, Sitkovsky MV. The critical role of adenosine a2a receptors in downregulation of inflammation and immunity in the pathogenesis of infectious diseases. Microbes Infect. 2003;5:515–26.
31. Kojima H, Gu H, Nomura S, Caldwell CC, Kobata T, Carmeliet P et al. Abnormal b lymphocyte development and autoimmunity in hypoxia-inducible factor 1 alpha deficient chimeric mice. Proc Natl Acad Sci USA. 2002;99:2170-4.
32. Thiel M, Chouker A, Ohta A, Jackson E, Caldwell C, Smith P et al. Oxygenation inhibits the physiological tissue-protecting mechanism and thereby exacerbates acute inflammatory lung injury. PLoS Biol. 2005;3:e174.
33. Hoffman MS, Golder FJ, Mahamed S, Mitchell GS. Spinal adenosine a2(a) receptor inhibition enhances phrenic long-term facilitation following acute intermittent hypoxia. J Physiol. 2010;588:255-66.
34. Gonzalez-Juarrero M, Shim TS, Kipnis A, Junqueira-Kipnis AP, Orme IM. Dynamics of macrophage cell populations during murine pulmonary tuberculosis. J Immunol. 2003;171:3128–35.
35. Grundy M, Sentman CL. GFP Transgenic mice show dynamics of lung macrophages. Exp Cell Res. 2005;310:409–16.
36. Van Rijt LS, Jung S, Kleinjan A, Vos N, Willart M, Duez C et al. In vivo depletion of lung cd11c+ dendritic cells during allergen challenge abrogates the characteristic features of asthma. J Exp Med. 2005;201:981–91.
37. Draijer C, Florez-Sampedro L, Reker-Smit C, Post E, van Dijk F, Melgert BN. Explaining the polarized macrophage pool during murine allergic lung inflammation. Front Immunol. 2022;13:1056477.
38. Surh YJ, Chun KS, Cha HH, Han SS, Keum YS, Park KK et al. Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of cox-2 and inos through suppression of nf-kappa b activation. Mutat Res. 2001;480-482:243-68.
39. Bulgonda RK, Kumar KA, Gangappa D, Beeda H, Philip HG, Rao DM et al. Mangiferin from pueraria tuberosa reduces inflammation via inactivation of nlrp3 inflammasome. Sci Rep. 2017;7:42683.
40. Lee SY, Cho SS, Li YC, Bae CS, Park KM, Park DH. Anti-inflammatory effect of curcuma longa and allium

hookeri co-treatment via nf-κb and cox-2 pathways. Sci Rep. 2020;10:5718.
41. Lim W. and Kang C. Avenanthramide c suppresses hypoxia-induced cyclooxygenase-2 expression through sirtuin1 activation in non-small-cell lung cancer cells. Anim Cells Syst. 2020;24:79-83.
42. Pardeshi CV, Pardeshi SR, Naik JB. Strategies for enhanced drug targeting to inflamed lungs: novel perspectives. In Advanced Drug Delivery Strategies For Targeting Chronic Inflammatory Lung Diseases. (Eds DK Chellappan, K Pabreja, M Faiyazuddin):219-258. Singapore, Springer. 2022.
43. Cai J, Wang YL, Sheng XD, Zhang L, Lv X. Shufeng Jiedu capsule inhibits inflammation and apoptosis by activating a2aar and inhibiting nf-κb to alleviate LPS-induced ALI. J Ethnopharmacol. 2022;298:115661.
44. Laddha AP, Kulkarni YA. VEGF and FGF-2: Promising targets for the treatment of respiratory disorders. Respir Med. 2019;156:33-46.
45. Impellizzeri D, Paola RD, Esposito E, Mazzon E, Paterniti I, Melani A et al CGS 21680, an agonist of the adenosine (a_{2a}) receptor, decreases acute lung inflammation. Rheumatology. 2011;38:2119-29.