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Original Article

Inhibition of TGF-β-Smad signaling attenuates hyperoxia-induced brain damage in newborn rats

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Abstract: Transforming growth factor-beta (TGF-β) is ubiquitously expressed in various tissues and functions in pathologic processes, including hyperoxia. In the present study, we investigated the expression and functional role of TGF-β in brain tissue during hyperoxia-induced brain damage. Three days old neonatal rats were treated with hyperoxic conditions (80% O_2) for 7 days, followed by TGF-β, Smad, and MAPK detection by western blotting and immunohistochemical staining. The functional role of TGF-β was assessed by treating hyperoxic neonatal rats with neutralizing antibody against TGF-β and caffeine, followed by histological and myelin basic protein (MBP) staining. Our results demonstrated upregulation of TGF-β and activation of the Smad/MAPK signaling pathway in brain tissue of neonatal rats under hyperoxic conditions. Injection of neutralizing antibody against TGF-β efficiently blocked TGF-β expression, accompanied by inactivation of the Smad/MAPK signaling pathway. Further evidence confirmed the attenuation of hyperoxia-induced brain damage by a neutralizing antibody against TGF-β in neonatal rats. Similar attenuation was also observed for caffeine. Collectively, our results indicate that TGF-β is a therapy target for hyperoxia-induced brain damage in neonates.

Keywords: TGF-β, hyperoxia, brain damage, neonate

Introduction

Hypoxia and hyperoxia are antithetic conditions that trigger opposite responses in cells and tissues [1]. Hemorrhage-mediated brain hypoxia is a dangerous feature of stroke and perinatal encephalopathy, and hyperoxic oxygenation is a widely used mandatory therapy for brain survival [2]. However, excess O_2 has also been linked to a worse outcome in patients resuscitated from cardiac arrest and post-ischemic stroke [3, 4]. Studies have also correlated hyperoxia exposure with the development of bronchopulmonary dysplasia, which is a major risk factor for mortality and morbidity in premature infants [5, 6]. Thus, uncovering the underlying mechanisms of hyperoxia in organ damage could provide targets for novel therapies.

Transforming growth factor-beta (TGF-β) belongs to a highly conserved protein family that includes three different isoforms, TGF-β 1, TGF-β 2 and TGF-β 3. Through binding to transmembrane serine/threonine kinase receptors I and II, TGF β proteins are activated and phosphorylate transcription factor SMAD protein subunits 2 and 3 [7]. Transforming growth factor-beta is ubiquitously expressed in various tissues and functions in pathologic processes, including hyperoxia [8-11]. In post-ischemic myocardium, it has been shown that TGF-β expression induces activation of Smad2/3 [12]. Transforming growth factor-beta is expressed in the brains of normal mice, and blocking TGF-β signaling accelerates hypertension progression, through promotion of neuroinflammation [13]. In a mouse model of bronchopulmonary dysplasia, lung TGF-β expression was induced by hyperoxia [14], and blocking TGF-β signaling attenuated bronchopulmonary dysplasia [15]. Significant up-regulation of hepatic TGF-β was also found in hyperoxic rats which

indicates that TGF-β is an accelerating factor for hepatic fibrosis under hyperoxic conditions [16]. However, the expression and function of TGF-β in brain tissue under hyperoxia is still unclear.

In the present study, we aimed to investigate the expression and functional role of TGF-β in brain tissue during hyperoxia-induced brain damage. Our results demonstrated the upregulation of TGF-β and activation of Smad/MAPK signaling in brain tissue of neonatal rats. Inhibition of TGF-β by neutralizing antibody against TGF-β, or caffeine, efficiently attenuated the hyperoxia-induced brain damage in neonatal rats. Our study indicates that TGF-β is a potential therapy target for hyperoxia-induced brain damage.

Materials and methods

Animals and treatment

All animal experiments were carried out in accordance with institutional guidelines concerning animal use and care at West China Medical Center, Sichuan University. Pregnant Sprague-Dawley rats were obtained from the Center of Animal Experiments at Sichuan University. 3 day old pups and nursing mothers were cultured under normoxia (21% O_2), hyperoxia (80% O_2) for days. Nursing mothers were rotated between normoxia and hyperoxia every 24 h. The hyperoxia-exposed groups were placed in an oxygen chamber into which oxygen was continuously delivered (80% percent) at a flow of 30 ml/min. After hyperoxic incubation, all of the pups were cultured at normoxia. The neutralizing antibody against TGF-β was dissolved in normal saline with a concentration of 1 mg/ml and caffeine was dissolved in normal saline with a concentration of 12 mg/ml. The hyperoxia-cultured pups were intraperitoneally injected with neutralizing antibody against TGF-β (100 μl), IgG solution (100 μl), normal saline (100 μl) and caffeine (100 μl) separately for 3 times, 1 times per day. All the pups were killed for brain tissue collection at 5 days post agent injection.

Western blotting

Whole brain tissues were collected and lysed on ice for 30 minutes with the RIPA lysis buffer (Merck Millipore, MA, USA) containing 1% protease inhibitor cocktail (Merck Millipore, MA, USA). After centrifugation for 15 minute at 4°C by 12,000 g, the Bradford Protein Assay Kit (Thermo Scientific, MA, USA) was used to determine the protein concentration of the supernatants. Total 20 μg protein was loaded for SDS-PAGE gel electrophoresis, followed by the transfer of proteins onto the PVDF membranes (Merck Millipore). After blocking with 5% milk in TBS/T buffer for 1 hours at room temperature, primary antibodies against TGF-β (Abcam, Cambridge, UK), p-Smad 2/3 (Abcam, Cambridge, UK) Smad 2/3 (Abcam, Cambridge, UK), p-MAPK (Abcam, Cambridge, UK), MAPK (Abcam, Cambridge, UK) and GAPDH (Cell Signaling Technology, MA, USA) were added to incubate at 4°C for overnight. After washing with TBS/T for 3 times at room temperature, the horseradish peroxidase-conjugated secondary antibodies (Zsbio, Beijing, China) was added to incubate at room temperature for 1 h.

Chemiluminescent substrate ECL kit (Merck Millipore, MA, USA) was used to detect the specific bands. The band density was determined by Image J software and the relative expression of specific band was calculated after normalized to GAPDH expression.

Immumohistochemical staining

Brain tissues were fixed in 4% paraformaldehyde at 4°C for 48 hours, then dehydrated, embedded in paraffin, and cut into 5 μm slides. After dewaxing with xylene and hydration, the slides were stained with hematoxylin and eosin (Beyotime, Beijing, China).

Immumohistochemical staining was performed as in a previous study [17]. Briefly, the slides underwent antigen retrieval at high temperature and high pressure for 3 minutes. After blocking with goat serum at room temperature for 15 minutes, the slides were incubated with primary antibody against TGF-β (Abcam, Cambridge, UK), p-Smad 2/3 (Abcam, Cambridge, UK) and p-MAPK (Abcam, Cambridge, UK) at 4°C overnight. The staining kits SP9001 and SP9002 (Zsbio, Beijing, China) were used for linking the specific IgG of primary antibody. The DAB kit (Maixin, Fuzhou, China) was used to detect the positive cells and hematoxylin (Beyotime, Beijing, China) was used to stain the nucleus. Upright metallurgical microscope

(BX51, Olympus, Tokyo, Japan) was used to acquire the immunohistochemical images.

Statistical analysis

All statistical analyses used the statistical software Statistical Package for the Social Sciences (SPSS version 19.0). The Student t test was applied to analyze the difference between two groups. All of the data are presented as the mean ± standard deviation (SD) and a p value < 0.05 was considered significant.

Results

Hyperoxia promotes the activation of TGF-β-Smad brain signaling in neonatal rats

To investigate the expression of TGF-β in brain tissue under hyperoxia, neonatal rats were subjected to hyperoxic conditioning (80% O_2). Seven days later, brain tissues were harvested for TGF-β analysis. As shown in **Figure 1A**, intracephalic TGF-β was significantly upregulated by hyperoxic treatment, whereas low/no expression of TGF-β was observed in brain tissues of neonatal rats under normal conditions. Immunohistochemistry (IHC) also confirmed a dramatic increase of TGF-β positive cells in brain tissues of hyperoxia-treated neonatal rats (**Figure 1B**). Next, we investigated the activity of the Smad-MAPK pathway, which is a downstream target of TGF-β. We found that hyperoxic conditions significantly promoted the phosphorylation of Smad2/3 and MAPK, but expression of total Smad2/3 and MAPK was not significantly affected (**Figure 1C**). Furthermore, more p-Smad2/3 and p-MAPK positive cells were observed in brain tissues from neonatal rats under hyperoxic conditions. Collectively, the results indicate that hyperoxia-induced TGF-β expression in brain tissues activates the Smad/MAPK pathway.

Efficient blocking of TGF-β activation in the brain of neonatal rats by a neutralizing antibody

To further determine the functional role of TGF-β during hyperoxia-induced brain damage, a neutralizing antibody against TGF-β was used to treat the hyperoxic brain tissue. Immunohistochemistry staining confirmed the efficient blocking of TGF-β expression by anti-TGF-β (**Figure 2A**). Furthermore, p-Smad2/3 positive cells were reduced by TGF-β neutralizing antibody treatment (**Figure 2B**), and accompanied by inactivation of MAPK (**Figure 2C**). These results demonstrate that blocking of TGF-β efficiently decreases the activation of Smad/MAPK signaling in brain tissues of neonatal rats under hyperoxic conditions.

Blocking of TGF-β attenuates hyperoxia-induced brain damage in neonatal rats

Based on the efficient blocking of TGF-β/Smad signaling by anti-TGF-β, brain tissues were also collected for further histologic staining. As shown in **Figure 3A**, cells in the IgG group were disorganized, with vesicular nuclei, whereas well-organized cells were found in the anti-TGF-β group. Brain damage scores were also lower in the TGF-β antibody treatment group (**Figure 3A**). Furthermore, an additional brain damage biomarker, myelin basic protein (MBP), was also detected by IHC staining. These results indicate that inhibition of TGF-β reduces expression of MBP brain tissue (**Figure 3B**). Collectively, the results confirm a protective role of anti-TGF-β during hyperoxia-induced brain damage.

Caffeine inhibits the activation of TGF-β-Smad signaling in neonatal rat brain under hyperoxia

Caffeine has been demonstrated to inhibit TGF-β in various cells [18, 19]. Therefore, we used caffeine to block TGF-β expression in brain tissues of neonatal rats under hyperoxic conditions. As shown in **Figure 4A**, there were fewer TGF-β positive cells in the caffeine group, compared with the normal saline group. Furthermore, activation of Smad2/3 and MAPK were also inhibited by caffeine, evidenced by a reduction of p-Smad2/3 positive (**Figure 4B**) and p-MAPK positive (**Figure 4C**) cells in brain tissues of the caffeine group. These results demonstrate that caffeine efficiently blocks TGF-β expression and inactivates the Smad/MAPK signaling pathway.

Caffeine attenuates hyperoxia-induced brain damage in neonatal rats

Based on the efficient blocking of TGF-β by caffeine, we further investigated the potential protective role of caffeine in hyperoxia-induced

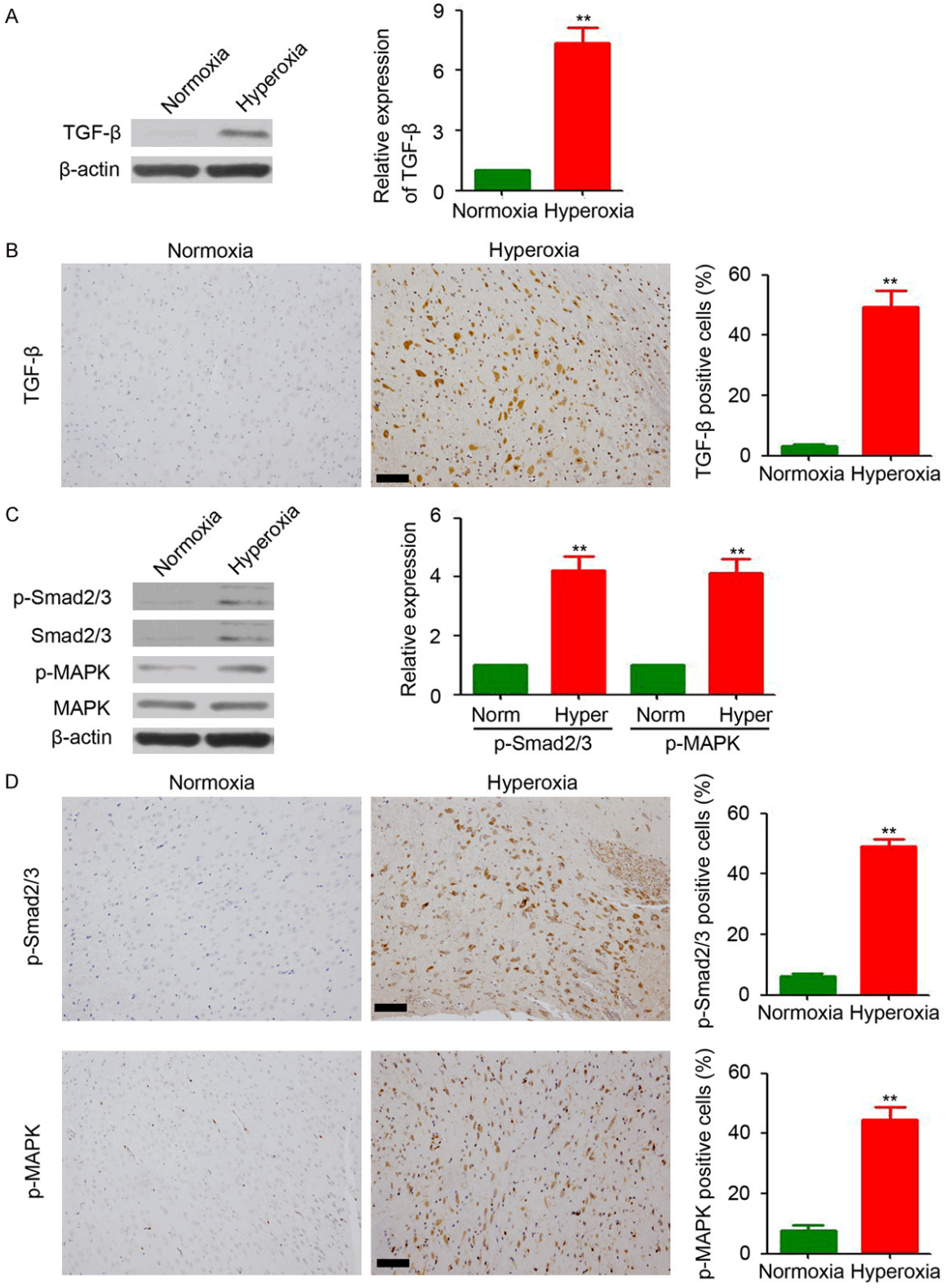

Figure 1. Hyperoxia promotes the activation of TGF-β-Smad signaling in the brain of neonatal rats. A. Western blotting detection of TGF-β expression in the brain tissues of neonatal rats under normoxia and hyperoxic condition. β-actin was used as a loading control. The relative expression of TGF-β was analyzed (n = 4, **, $P < 0.05$). B. IHC staining of TGF-β in the brain tissues of neonatal rats under normoxia and hyperoxic condition. Scale bar = 200 μm.

The percents of TGF-β positive cells were counted and analyzed (n = 4, **, P < 0.05). C. Western blotting detection of p-Smad2/3, Smad2/3, p-MAPK and MAPK expression in the brain tissues of neonatal rats under normoxia and hyperoxic condition. β-actin was used as a loading control. The relative expression of each protein was analyzed (n = 4, **, P < 0.05). D. IHC staining of p-Smad2/3 and p-MAPK in the brain tissues of neonatal rats under normoxia and hyperoxic condition. Scale bar = 200 μm. The percents of p-Smad2/3 and p-MAPK positive cells were counted and analyzed (n = 4, **, P < 0.05).

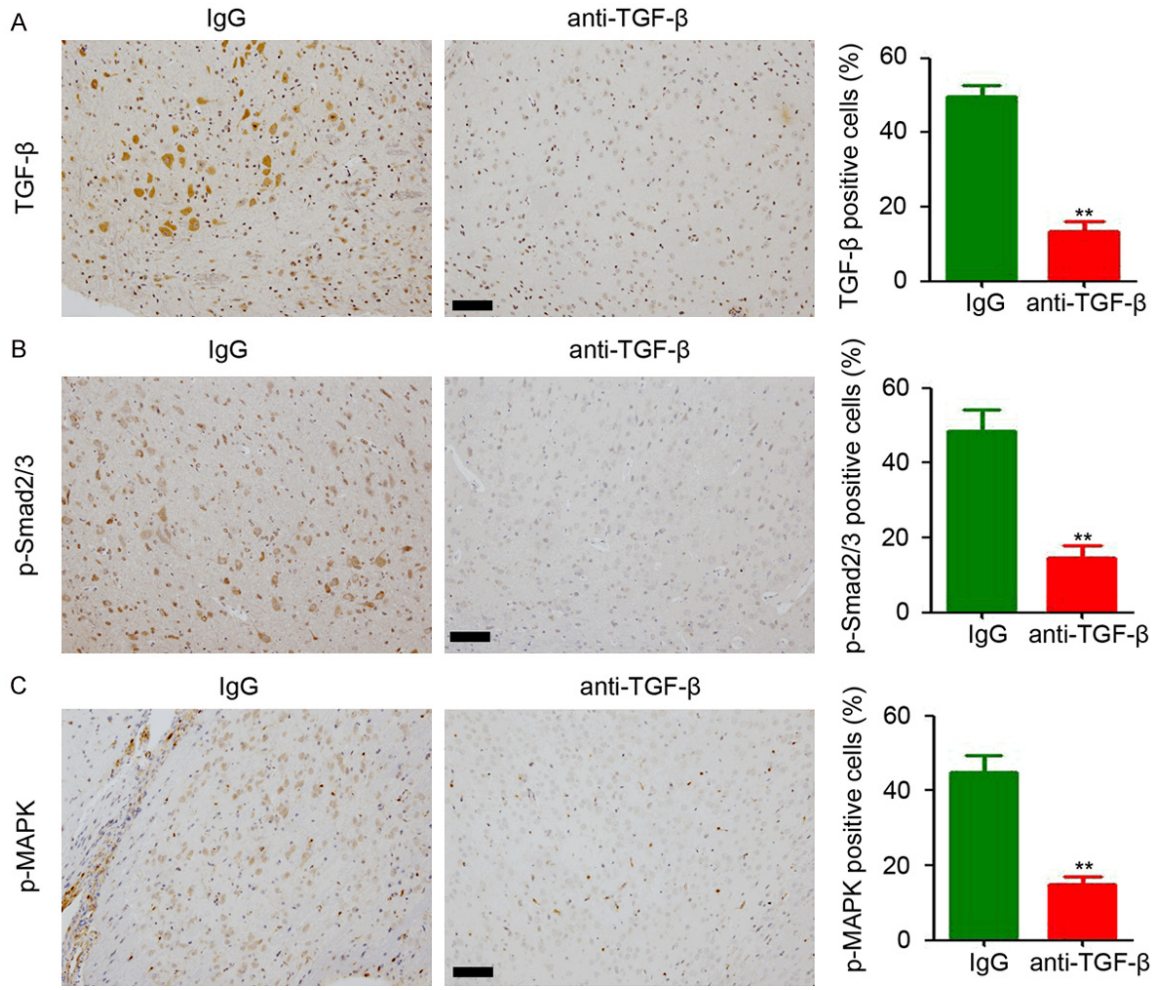

Figure 2. Efficient blocking of TGF-β activation in the brain of neonatal rats by neutralizing antibody. A. IHC staining of TGF-β in the brain tissues of neonatal rats under hyperoxic condition with IgG and neutralizing antibody against TGF-β (anti-TGF-β) treatment. Scale bar = 200 μm. The percents of TGF-β positive cells were counted and analyzed (n = 4, **, P < 0.05). B. IHC staining of p-Smad2/3 in the brain tissues of neonatal rats under hyperoxic condition with IgG and neutralizing antibody against TGF-β (anti-TGF-β) treatment. Scale bar = 200 μm. The percents of p-Smad2/3 positive cells were counted and analyzed (n = 4, **, P < 0.05). C. IHC staining of p-MAPK in the brain tissues of neonatal rats under hyperoxic condition with IgG and neutralizing antibody against TGF-β (anti-TGF-β) treatment. Scale bar = 200 μm. The percents of p-MAPK positive cells were counted and analyzed (n = 4, **, P < 0.05).

brain damage. As shown in **Figure 5A**, fewer damaged brain cells were observed in caffeine-treated rats. Furthermore, MBP staining also indicated fewer MBP positive cells in the caffeine group, compared with the normal saline group (**Figure 5B**). Collectively, the results confirm that caffeine attenuates hyperoxia-induced brain damage in neonatal rats.

Discussion

Under normal conditions, TGF-β is expressed in various cell types and tissues [7]. Various pathologic and physiologic processes upregulate TGF-β expression to accelerate progression of specific processes [12, 15, 20]. Transforming growth factor-beta expression is a driv-

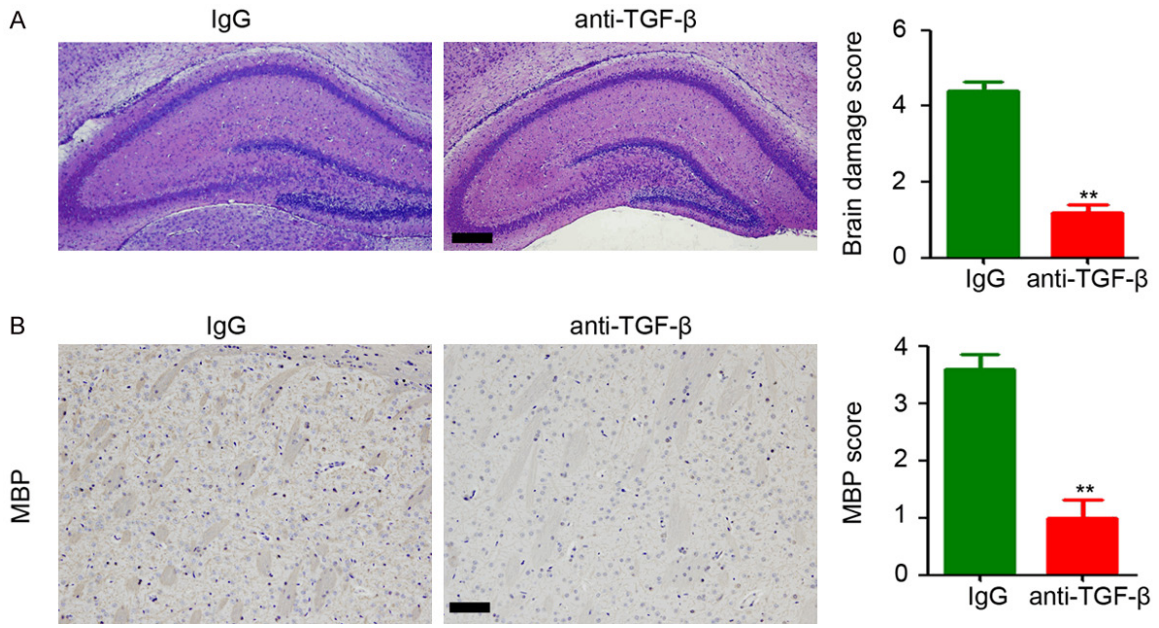

Figure 3. Block of TGF-β attenuates hyperoxia-induced brain damage in neonatal rats. A. Histology analysis of brain tissues from neonatal rats under hyperoxic condition with IgG and neutralizing antibody against TGF-β (anti-TGF-β) treatment. Scale bar = 200 μm. The brain damage score was analyzed (n = 4, **, $P < 0.05$). B. IHC staining of MBP in the brain tissues of neonatal rats under hyperoxic condition with IgG and neutralizing antibody against TGF-β (anti-TGF-β) treatment. Scale bar = 200 μm. The MBP score was analyzed (n = 4, **, $P < 0.05$).

ing factor for tumor cell growth and metastasis in cancer [21, 22]. During IL-1β promoted epithelial-derived alveolar elastogenesis TGF-β is upregulated, and plays a crucial role during this process [23]. In primary lung fibroblasts after exposure to hyperoxia, TGF-β expression increases [14], whereas TGF-β expression decreases in neonatal rats at 7 days post hyperoxic exposure [24]. In the present study, we first demonstrated upregulation of TGF-β in brain tissue of neonatal rats, under hyperoxic conditions. Furthermore, the downstream targets of TGF-β, Smad, and MAPK, were activated by hyperoxic conditions. The results suggest that TGF-β plays a crucial role during hyperoxia-induced brain damage. However, further studies are needed to determine the upstream targets of TGF-β activation during hyperoxia, which may include Anaplastic lymphoma kinase 5 [25, 26].

Transforming growth factor-beta, and downstream targets, play crucial functional roles in the brain. Deficiency in neuronal TGF-β is associated with age-related motor deficits and degeneration of the nigrostriatal system, while increasing TGF-β signaling reduces 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced dopaminergic neurodegeneration and motor deficits [27]. Activation of the astrocytic TGF-β-pathway induces excitatory synaptogenesis that precedes the appearance of seizures [26]. Transforming growth factor-beta is also a regulator of late stages of adult hippocampal neurogenesis, which may have implications for changes in neurogenesis during aging and disease [25]. Aside from the brain, TGF-β also plays a protective or accelerating role in hyperoxia-related bronchopulmonary dysplasia [14], alveologenesis [28], lung injury [15] and fibrosis [20]. Our study used a neutralizing antibody against TGF-β to block TGF-β activation, and demonstrated a decrease in Smad/MAPK signaling in brain tissues of neonatal rats under hyperoxic conditions. Further histologic evidence confirmed the protective role of anti-TGF-β during hyperoxia-induced brain damage.

Due to the crucial role of TGF-β in various pathologic and physiologic processes, several intervening strategies have been applied. Curcumin, a lipophilic polyphenol compound that is an active ingredient of the Indian spice turmeric, efficiently blocks TGF-β and protects against hyperoxia-induced neonatal lung injury [29]. Lipoxin A4, a lipid mediator generated dur-

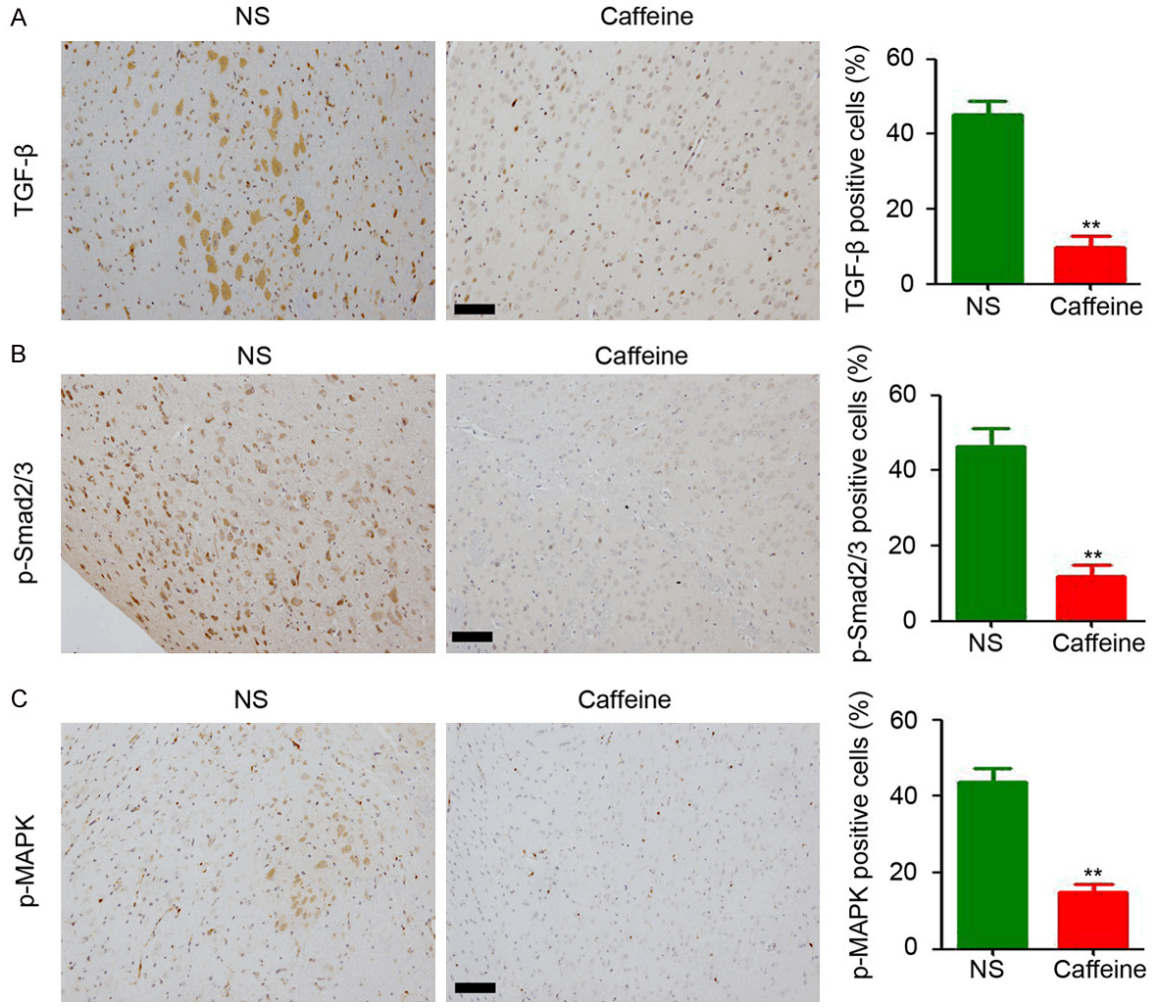

Figure 4. Caffeine attenuates hyperoxia-induced brain damage in neonatal rats. A. Histology analysis of brain tissues from neonatal rats under hyperoxic condition with normal saline (NS) and caffeine treatment. Scale bar = 200 μm. The brain damage score were analyzed (n = 4, **, P < 0.05). B. IHC staining of MBP in the brain tissues of neonatal rats under hyperoxic condition with normal saline (NS) and caffeine treatment. Scale bar = 200 μm. The MBP score was analyzed (n = 4, **, P < 0.05). C. IHC staining of p-MAPK in the brain tissues of neonatal rats under hyperoxic condition with normal saline (NS) and caffeine treatment. Scale bar = 200 μm. The percent of p-MAPK positive was analyzed (n = 4, **, P < 0.05).

ing inflammation, attenuates development of bronchopulmonary dysplasia through inhibition of TGF-β signaling [30]. Caffeine, a commonly used food additive found naturally in many products, has been demonstrated as an antifibrotic agent in the lung, through inhibition of TGF-β [19]. In a mouse model of bronchopulmonary dysplasia, caffeine normalized body mass under hyperoxic conditions, through normalizing TNF-β expression and Smad2 phosphorylation in the lung [31]. In the present study, we also used caffeine to treat neonatal rats under hyperoxic conditions. Transforming growth factor-beta expression and Smad/MAPK signaling was efficiently blocked by caffeine, followed by attenuation of hyperoxia-induced brain damage. The findings suggest that caffeine could be also used as an anti-hyperoxia-induced damage agent in the brain, as well as the lung [18, 32].

In summary, our results demonstrate the role of upregulated TGF-β in hyperoxia-induced brain damage. Furthermore, TGF-β is indicated as a potential therapeutic target for hyperoxia-induced brain damage in neonates.

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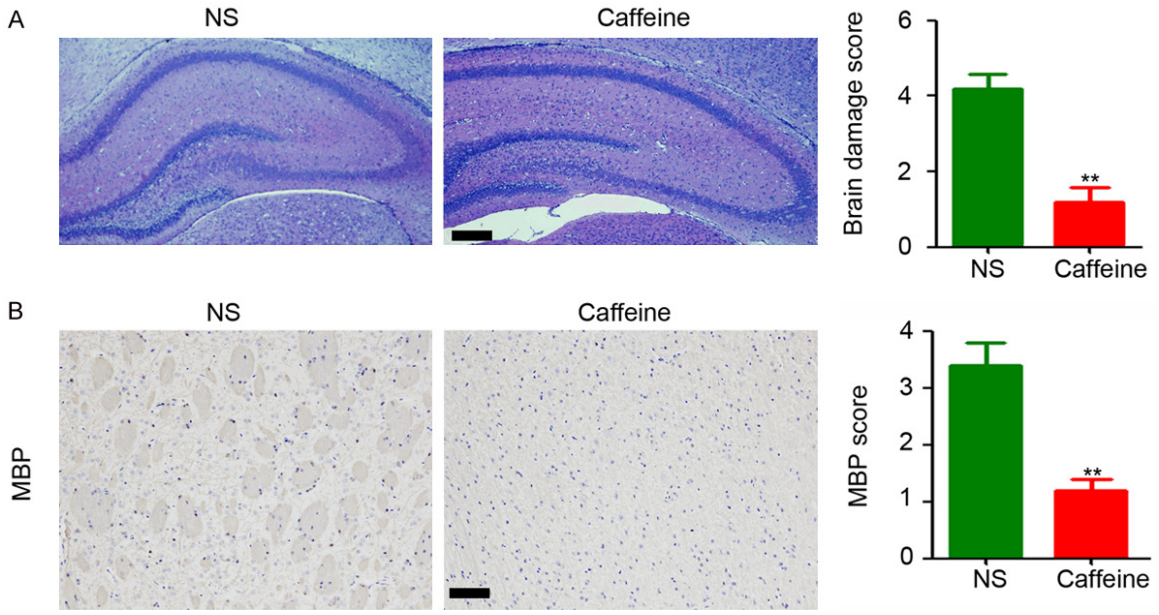

Figure 5. Caffeine inhibits the activation of TGF-β-Smad signaling in the brain of neonatal rats under hyperoxia. A. IHC staining of TGF-β in the brain tissues of neonatal rats under hyperoxic condition with normal saline (NS) and caffeine treatment. Scale bar = 200 μm. The percents of TGF-β positive cells were counted and analyzed (n = 4, **, $P < 0.05$). B. IHC staining of p-Smad2/3 in the brain tissues of neonatal rats under hyperoxic condition with normal saline (NS) and caffeine treatment. Scale bar = 200 μm. MBP score were analyzed (n = 4, **, $P < 0.05$).

Disclosure of conflict of interest

None.

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