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·Report·

Decreased connexin 43 in astrocytes inhibits the neuro-inflammatory reaction in an acute mouse model of neonatal sepsis

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ABSTRACT

Neonatal sepsis is common in neonatal intensive care units, often complicated by injury to the immature brain. Previous studies have shown that the expression of the gap junction protein connexin 43 (Cx43) in the brain decreases when stimulated by neuro-inflammatory drugs such as lipopolysaccharide (LPS). Here we showed that partial deletion of Cx43 in astrocytes resulted in weakened inflammatory responses. The up-regulation of pro-inflammatory cytokines was significantly reduced in mice with partial deletion of Cx43 in astrocytes compared with wild-type littermates after systemic LPS injection. Moreover, microglial activation was inhibited in mice with partial deletion of Cx43. These results showed that Cx43 in astrocytes plays a critical role in neuro-inflammatory responses. This work provides a potential therapeutic target for inhibiting neuro-inflammatory responses in neonatal sepsis.

Keywords: astrocyte; gap junction; Cx43; neonatal sepsis

INTRODUCTION

Neonatal sepsis is a major cause of morbidity and mortality in neonatal intensive care units[1] and is prevalent in premature infants[2]. Many clinical studies have demonstrated that neonatal sepsis directly causes brain damage such as white matter lesions and periventricular leukomalacia, and is closely associated with a high rate of lethality in neonates[3]. Furthermore, survivors of neonatal sepsis often show severe long-term cognitive impairment and adverse neurologic outcomes[4–7].

Microglia are resident immune cells in the brain and play a crucial role in cerebral functions[8]. Mature microglia compose 15–20% of the total cell population of the brain[9]. In the normal adult rodent brain, "resting" microglia are ramified with a small soma, little perinuclear cytoplasm, and a small, dense, and heterochromatic nucleus[10]. When activated by a variety of stimuli such as cell death, excessive aberrant protein, and the presence of viral or bacterial pathogens, microglia transform into cells with larger somata and shorter, coarser cytoplasmic processes[11]. The activation of microglia in the inflammatory response can have deleterious effects on surrounding neurons and glia through the release of inflammatory cytokines such as tumor necrosis factor alpha (TNFα) and interleukin (IL)-1β[9]. It was originally thought that immature microglia are unable to mount a host immune response to injury. However, studies have demonstrated that microglia both prenatally[12] and in adulthood shift to a bushy/ramified morphology, suggesting a similar functional role with stimulation regardless of age.

Astrocytes are the most abundant and widespread cells in the central nervous system and they mediate many functions that are indispensable for brain function. For

example, they play integral roles in synaptic development and disease[14]. Astrocytes supply neurons with vital metabolites such as lactate in response to neuronal activity[15]. Innate immune responses, the most widely-reported function, are also carried out by astrocytes in the brain[16,17]. Astrocytes build a syncytium using gap junctions and hemichannels, which are formed by two apposed connexins or hemichannels that interact and open to form a 'pipeline' for the passage of substances smaller than 1–1.5 kDa[18]. The basic elements of these channels are the transmembrane connexin (Cx) proteins[19]. Of these, Cx43 is the major component of gap junctions in astrocytes[20]. Evidence indicates that pathological stimuli affect Cx43 mRNA and protein levels in astrocytes and thereby regulate intercellular communication through gap junctions[21,22]. However, the role of Cx43 in neuro-inflammation induced by systemic sepsis remains elusive.

The aim of this study was to address this question. We used an acute mouse model of neonatal sepsis by injecting lipopolysaccharide (LPS) and determined whether regulation of Cx43 can protect neonates from neuro-inflammatory injury.

MATERIALS AND METHODS

Animals

The use and care of animals were approved by the Biomedical Research Ethics Committee at the Shanghai Institutes for Biological Science, Chinese Academy of Sciences. $Cx43^{flox/+}$: hGFAP-Cre mice having decreased Cx43 expression in astrocytes were generated by crossing hGFAP-Cre mice (Jackson Laboratory, Sacramento, CA) with Cx43-floxed ($Cx43^{flox/flox}$) mice (Jackson Laboratory). We chose to compare $Cx43^{flox/+}$: hGFAP-Cre mice with hGFAP-Cre controls to define the effect of Cx43 on the neuro-inflammation induced by neonatal LPS injection. Mice were housed under a 12-h light/dark cycle (lights on 07:00–19:00) with *ad libitum* access to water and mouse chow, unless otherwise noted.

LPS Administration

Both $Cx43^{flox/+}$: hGFAP-Cre and hGFAP-Cre mice (3 days old) were randomly assigned to receive LPS or saline injection. They were injected intraperitoneally either with 3 mg/kg LPS (#S1732, Beyotime Biotechnology, Shanghai, China) or with sterile saline. All injections were performed between 09:00 and 11:00 to avoid the effects of circadian variations in corticosterone.

RNA Extraction, Reverse Transcription, and RT-qPCR

Mice were decapitated 3 h after the single LPS or saline injection. The brain was dissected to obtain the cortex for reverse transcription polymerase chain reaction (RT-PCR) assay. Comparative quantitation by real-time RT-PCR was performed for each cytokine using the SYBR-green I fluorescence method. The primers were as follows: GAPDH forward 5′-AGGTCGGTGTGAACGGATTTG-3′, reverse 5′-TGTAGACCATGTAGTTGAGGTCA-3′; TNF-α forward 5′-CCCTCACACTCAGATCATCTTCT-3′, reverse 5′-GCTACGACGTGGGCTACAG-3′; IL-6 forward 5′-TAGTCCTTCCTACCCCAATTTCC-3′, reverse 5′-TTGGTCCTTAGCCACTCCTTC-3′; IL-12β forward 5′-TGGTTTGCCATCGTTTTGCTG-3′, reverse 5′-ACAGGTGAGGTTCACTGTTTCT-3′; IL-1β forward 5′-TCATCTTTGAAGAAGAGCCC-3′, reverse 5′-GTATTTTGTCGTTGCTTGGT-3', IL-9 forward 5′-ATGTTGGTGACATACATCCTTGC-3′, reverse 5′-TGACGGTGGATCATCCTTCAG-3′, IL-10 forward 5′-GCTCTTACTGACTGGCATGAG-3′, reverse 5′-CGCAGCTCTAGGAGCATGTG-3′, IL-11 forward 5′-TGTTCTCCTAACCCGATCCCT-3′, reverse 5′-CAGGAAGCTGCAAAGATCCCA -3′.

Western Blotting

The brain tissues were homogenized on ice in RIPA buffer (50 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 1% TritonX-100, 0.1% SDS, 1% sodium deoxycholate, protease inhibitor cocktail, and phosphatase inhibitor cocktail) followed by sonication, and then centrifuged at 12 000 g for 10 min at 4°C. Twenty-five micrograms of protein were loaded onto 10% SDS-PAGE and run at 120 V. A current of 200 mA was used for transblotting. Blots were probed with anti-IL-1β (9722; Abcam, Cambridge, UK), anti-TNFα (9739; Abcam), anti-Cx43 (4501175, Sigma, St Louis, MO) or anti-GAPDH (8245; Abcam) antibody (1:1 000–10 000) overnight at 4°C. After three washes, blots were incubated

with secondary antibodies (1:5 000) at room temperature for 2 h. Chemiluminescence was used to visualize the protein bands.

Immunohistochemistry

Six hours after LPS or saline injection, the mice were deeply anaesthetized by a single intraperitoneal injection of 5% chloral hydrate (10 μL/g) and transcardially perfused with saline at 4°C followed by 4% paraformaldehyde (PFA) for fixation. The brain was post-fixed overnight in 4% PFA at 4°C, then dehydrated by two successive incubations in 15% and 30% sucrose. Coronal sections were cut at 30 μm and processed for immunohistochemistry. Sections were washed for 10 min in PBS, incubated with primary antibodies (in PBS with 1% BSA and 0.3% TritonX-100) overnight at 4°C, and then incubated with the corresponding secondary antibody (Alexa Fluor-conjugated at 1:1 000; Invitrogen). DAPI was used to label the nuclei and sections were mounted with 75% glycerol. The other antibody used was anti-Iba1 (#019-19741; Wako Pure Chemical Ltd, Chuo-ku, Japan).

Statistical Analyses

Data are expressed as mean ± SEM. Analyses were performed using GraphPad Prism version 6.03 (GraphPad Software, La Jolla, CA). The unpaired *t*-test (between two groups) and two-way ANOVA followed by Tukey's test (among multiple groups) were used to evaluate statistical significance. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

To determine whether the level of Cx43 protein was altered by the genomic deletion, we performed western blotting in cortical tissues from $Cx43^{flox/+}$: hGFAP-Cre and hGFAP-Cre mice. The Cx43 protein level was significantly decreased in the $Cx43^{flox/+}$: hGFAP-Cre mice (Fig. 1A).

To test whether the decrease of Cx43 in astrocytes impacts the secretion of inflammatory factors in cortex, we chose IL-1β and TNFα as indicators of inflammatory activation, and found that the mRNA levels of both factors were most pronounced at 3 h following LPS injection (Fig. 1B). Therefore, we chose 3 h as the time point to assess the difference in inflammatory activation after LPS stimulation between hGFAP-Cre and $Cx43^{flox/+}$: hGFAP-Cre mice.

Pro-inflammatory cytokine expression in the cortex was higher in hGFAP-Cre mice than in $Cx43^{flox/+}$: hGFAP-Cre mice after LPS stimulation. We measured the mRNA levels of pro-inflammatory (IL-1β, IL-6, IL-12β, and TNFα) and anti-inflammatory (IL-9, IL-10, and IL-11) cytokines in the cortex at 3 h following LPS injection. There was a substantial increase in IL-1β, IL-6, TNFα, and IL-12β mRNA expression (Fig. 1C) after LPS stimulation in hGFAP-Cre mice. In contrast, there was little difference between saline and LPS injection in $Cx43^{flox/+}$: hGFAP-Cre mice (Fig. 1C). The levels of anti-inflammatory factors did not differ between saline and LPS injection in hGFAP-Cre or $Cx43^{flox/+}$: hGFAP-Cre mice (Fig. 1D). Further, western blots showed that TNFα protein was elevated in hGFAP-Cre mice injected with LPS while this effect was not significant in $Cx43^{flox/+}$: hGFAP-Cre mice (Fig. 1E).

We also examined the microglial response in the cortex at 6 h after LPS injection. The number of Iba1-positive ($Iba1^+$) cells per section was significantly higher than in the saline-injection group in hGFAP-Cre mice, but not in $Cx43^{flox/+}$: hGFAP-Cre mice (Fig. 1F). Moreover, the percentage of round/amoeboid $Iba1^+$ cells in the LPS-injection group was increased, suggesting the activation of microglia (Fig. 1F). Taken together, these experiments showed a defect in the ability to induce a neuro-inflammatory reaction in $Cx43^{flox/+}$: hGFAP-Cre mice.

Despite recent advances in neonatal intensive care, sepsis remains an important cause of morbidity and mortality, particularly among preterm infants[1,2]. Neonatal sepsis causes poor neurodevelopmental outcomes in preterm infants[3–7]. Thus, timely and precise means of protecting preterm infants from sepsis is imperative to reduce the risk of adverse nervous system outcomes. Systemic LPS injection induces inflammatory responses in mice, and subsequently, inflammatory mediators lead to inflammation in the nervous system[23]. Therefore, acute LPS administration in mice simulates the clinical features of neonatal sepsis, and is a good model for studying its mechanisms.

Astrocytes make up the major cell population in the brain and express the highest amount of gap junction proteins. Cx43 is the main connexin protein in

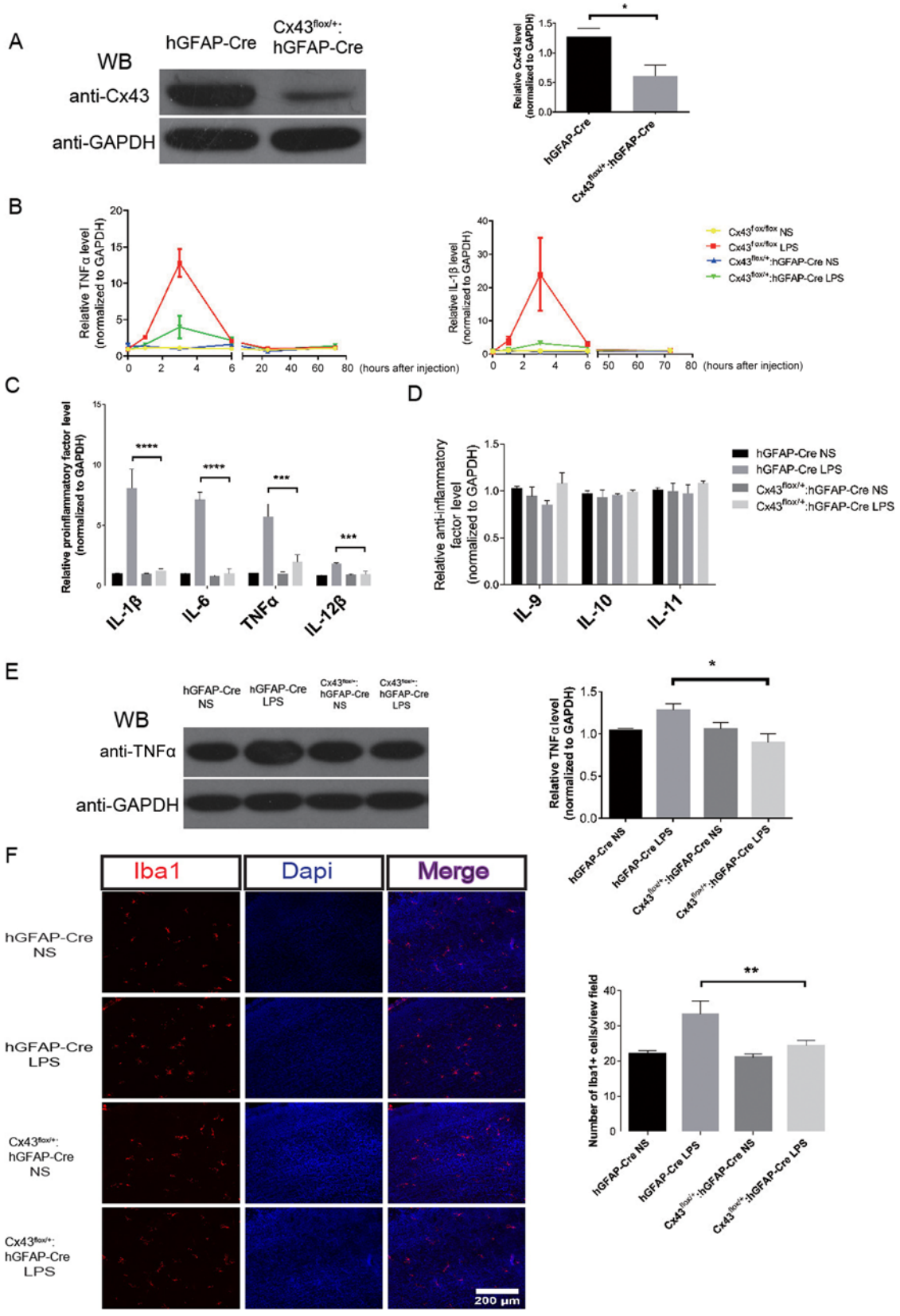
A
WB
hGFAP-Cre
Cx43flox/+:hGFAP-Cre
anti-Cx43
anti-GAPDH
Relative Cx43 level (normalized to GAPDH)
hGFAP-Cre
Cx43flox/+:hGFAP-Cre
B
Relative TNFα level (normalized to GAPDH)
Relative IL-1β level (normalized to GAPDH)
(hours after injection)
Cx43flox/flox NS
Cx43flox/flox LPS
Cx43flox/+:hGFAP-Cre NS
Cx43flox/+:hGFAP-Cre LPS
C
Relative proinflammatory factor level (normalized to GAPDH)
IL-1β
IL-6
TNFα
IL-12β
D
Relative anti-inflammatory factor level (normalized to GAPDH)
IL-9
IL-10
IL-11
hGFAP-Cre NS
hGFAP-Cre LPS
Cx43flox/+:hGFAP-Cre NS
Cx43flox/+:hGFAP-Cre LPS
E
WB
anti-TNFα
anti-GAPDH
Relative TNFα level (normalized to GAPDH)
F
Iba1
Dapi
Merge
hGFAP-Cre NS
hGFAP-Cre LPS
Cx43flox/+:hGFAP-Cre NS
Cx43flox/+:hGFAP-Cre LPS
200 μm
Number of Iba1+ cells/view field

Fig. 1. Decreased Cx43 in astrocytes inhibits the inflammatory response in cortex. A: Left, protein levels of Cx43 from hGFAP-Cre and Cx43$^{flox/+}$: hGFAP-Cre mice. Right, quantitation of immunoblots. Error bars indicate SEM; *P <0.05, t-test. B: Dynamic changes of IL-1β and TNFα mRNA levels in hGFAP-Cre and Cx43$^{flox/+}$: hGFAP-Cre mice treated with LPS. Cortical tissues were collected at 1, 3, 6, 24, and 72 h after the systemic injection of LPS. C: mRNA expression levels of pro-inflammatory factors IL-1β, TNFα, IL-6, and IL-12β, in hGFAP-Cre and Cx43$^{flox/+}$: hGFAP-Cre mice after systemic injection of LPS. D: mRNA levels of anti-inflammatory factors in the hGFAP-Cre and Cx43$^{flox/+}$: hGFAP-Cre groups after systemic injection of LPS. E: Left, protein levels of TNFα in hGFAP-Cre and Cx43$^{flox/+}$: hGFAP-Cre mice. Right, quantitation of immunoblots. F: Left, immunostaining for Iba1 and the nuclear marker DAPI in sections of brains from hGFAP-Cre and Cx43$^{flox/+}$: hGFAP-Cre mice. Scale bar, 200 μm. Right, quantitation of immunostaining. n = 3–4 per bar. Data are mean ± SEM; n = 3–4 animals per group; **P <0.005; two-way ANOVA.

astrocytes. As previously reported, intraperitoneal injection of LPS decreases Cx43 expression levels, which leads to the hypothesis that the gap junctional signaling pathway impacts the regulation of the inflammatory response[22]. In this study, we showed that a decrease of Cx43 in astrocytes correlated with neuro-inflammation induced by systemic sepsis in preterm mice. Our experiment demonstrated that Cx43 deficiency in astrocytes attenuated the activation of microglia. Based on immunohistochemical analyses, we found that reduction of Cx43 prevented the change of microglial morphology to the activated form, as indicated by increased cell size, abnormal shape, and thickened and shortened processes. Iba-1 is highly and specifically expressed in microglia and macrophages. In this study, we detected fewer Iba-1$^+$ cells in Cx43$^{flox/+}$: hGFAP-Cre mice than in hGFAP-Cre. Meanwhile, the pro-inflammatory factors secreted by microglia were also reduced in the Cx43$^{flox/+}$: hGFAP-Cre mice. We demonstrated that activation of microglia is highly dependent on the presence of Cx43.

Previous studies have shown that the ATP released by astrocytes through Cx43 hemichannels is a critical trigger for microglial responses[24]. In the situation of trauma, the astrocytes are the first to sense injury, and the activation of microglia is triggered by astrocytic ATP release. Lack of Cx43 hemichannels causes reduced leakage of ATP and thus inhibits the activation of microglia[25–27]. The release of TNF-α and IL-1β from LPS-activated microglia targets astrocytes mainly through Cx43 hemichannels[28,29]. We suggest that the deletion of Cx43 inhibits the activation of astrocytes by IL-1β and TNFα, and meanwhile, the activated astrocytes cannot activate microglia through the release of ATP. In the future we will focus on exploring the underlying mechanisms.

In conclusion, we have demonstrated that a decrease in astroglial Cx43 profoundly weakens the neuroinflammatory response to systemic inflammatory stimulation. Our results suggest that astroglial Cx43 is a promising target to consider when planning immunotherapies for neonatal sepsis.

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