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Research Article

Pyroptosis and necroptosis inhibitor necrosulfonamide ameliorates lipopolysaccharide-induced inflammatory hyperalgesia in mice

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Abstract

Objectives: This study aimed to investigate the effect of the gasdermin D (GSDMD) and mixed lineage kinase domain-like pseudokinase (MLKL) inhibitor, necrosulfonamide (NSA), on lipopolysaccharide (LPS)-induced hyperalgesia in mice.

Methods: Reaction time to a thermal stimulus within 30 seconds was measured in male mice injected with saline, LPS, and/or NSA after 6 hours using the hot plate test. Immunoblotting studies were performed to determine changes in caspase-11/GSDMD-mediated pyroptosis, receptor-interacting serine/threonine-protein kinase (RIPK) 1/RIPK3/MLKL necrosome-mediated necroptosis, demyelination, and remyelination in the brains and spinal cords of animals.

Results: NSA demonstrated significant antinociceptive activity compared with LPS-treated mice. In the tissues of LPS-treated mice, NSA decreased expression of caspase-11 p20, p30-GSDMD, interleukin-1β, high-mobility-group-box 1, and semaphorin 3A, and activity of RIPK1, RIPK3, and MLKL. NSA also increased the expression of myelin proteolipid protein.

Conclusion: Therefore, NSA may have therapeutic potential in the treatment of inflammatory painful conditions due to bacterial infections.

Keywords

Inflammatory hyperalgesia, lipopolysaccharide, necroptosis, pyroptosis, necrosulfonamide

Introduction

Several programmed cell death pathways have become known to be associated with innate immunity, including apoptosis, pyroptosis, necroptosis, ferroptosis, and PANoptosis in recent decades (Frank and Vince 2019; Lacey and Miao 2020; Miller et al. 2020; Bertheloot et al. 2021; Yu et al. 2021; Christgen et al. 2022; Gullett et al. 2022; Mázló et al. 2022). Recent studies have provided evidence that two distinct forms of necrotic cell death, particularly caspase-11-mediated pyroptosis and mixed lineage kinase domain-like pseudokinase (MLKL)-mediated necroptosis, triggered by toll-like receptor (TLR) 4 ligands such as lipopolysaccharide (LPS) are involved in the pathogenesis

of several diseases in particular neurodegenerative diseases associated with neuroinflammation (Downs et al. 2020; Burdette et al. 2021; Yu et al. 2021; Jayaraman and Reynolds 2022; Wright et al. 2022).

The non-canonical inflammasome, comprising inflammatory caspases, caspase-11 in rodents (caspase 4 and 5 in human), detects bacterial components including lipid A component of LPS, endotoxin, of Gram-negative bacteria (Abu Khweek and Amer 2020; Lacey and Miao 2020; Burdette et al. 2021; Bezbradica et al. 2022; Wright et al. 2022). Upon activation by cytosolic Gram-negative bacteria or intracellular LPS, caspase-11 as an intracellular LPS receptor undergoes oligomerization and next cleaves its substrate, gasdermin D (GSDMD) into pore-forming peptides (i.e., the catalytic N-terminal domain of GSDMD; p30-GSDMD, GSDMD-N) (Rathkey et al 2018). GSDMD-mediated pore formation leads to pyroptosis, lytic programmed cell death, and the release of pro-inflammatory cytokines including interleukin (IL)-1β and damage-associated molecular patterns (DAMPs) such as high-mobility-group-box (HMGB) 1 thereby promoting inflammatory responses by regulating both the innate and the adaptive immune responses (Heilig et al. 2018).

LPS can also induce necroptosis, a caspase-independent inflammatory form of regulated cell death caused by necrosis (Kang et al. 2014; Gullett et al. 2022; Mázló et al. 2022). This type of necrotic cell death involves the formation of the receptor-interacting kinase (RIPK) 1/RIPK3 necrosome leading to the activation of the MLKL through its phosphorylation (Wang et al. 2014). Following activation of MLKL molecules, they oligomerize and translocate to the cell membrane where they compose cation-selective ion channels. The generated ionic disturbance leads to the swelling of cells and organelles, rupture of the plasma membrane, and release of DAMPs, including HMGB1 (Kolbrink et al. 2020).

Inhibition of GSDMD-mediated pyroptosis and/or MLKL-mediated necroptosis has currently emerged as a therapeutic target in the treatment of various neuroinflammatory neurodegenerative central nervous system (CNS) diseases including Alzheimer's disease (Espinosa-Oliva et al. 2019; Lee et al. 2021; Gullett et al. 2022; Mázló et al. 2022), amyotrophic lateral sclerosis (Mázló et al. 2022; Neel et al. 2022), Huntington's disease (Espinosa-Oliva et al. 2019; Mázló et al. 2022), multiple sclerosis (Mázló et al. 2022), and Parkinson's disease (Espinosa-Oliva et al. 2019; Mázló et al. 2022). The results of a few studies published to date emphasize that GSDMD and MLKL inhibitor, necrosulfonamide (NSA), which can also enter the CNS when administered systemically (Bartzatt et al. 2010; Jiao et al. 2020), may represent a promising therapeutic agent for reducing neuroinflammation and accelerating anti-inflammatory responses through protective mechanisms potentially related to inhibition of pyroptotic and necroptotic signaling pathways as demonstrated in the animal models of systemic inflammation (Rathkey et al. 2018; Wang et al. 2018; Jiao et al. 2020; Motawi et al. 2020; He et al. 2022; Ueda et al. 2022). It has also been reported that the effects of LPS on pyroptosis (Rathkey et al. 2018; Li et al. 2019; Zhang and Wei 2021) and necroptosis (El-Mesery et al. 2015; Saeed et al. 2019; Geng et al. 2022). However, there are no studies focusing on either the role of both types of cell death or the effect of NSA in perceived LPS-induced inflammatory hyperalgesia at the spinal and/or supraspinal level remain unknown.

LPS has been shown to enhance pain sensation in response to thermal stimuli at spinal and supraspinal levels, as indicated by the hot plate test, which is considered an integration of supraspinal pathways (Hori et al. 2000; Deuis et al. 2017). In our previous studies, we demonstrated that inflammatory hyperalgesia induced by LPS is associated with decreased expression and/or activity of inducible nitric oxide synthase (NOS), neuronal NOS, caspase-recruitment and activation domain containing nucleotide-binding oligomerization domain-like receptor (NLRC) 3, and peroxisome proliferator-activated receptors α/β/γ in the brain and spinal cord of mice (Tunctan et al. 2006; Buharalioglu et al. 2009; Dolunay et al. 2017; Cagli et al. 2021; Senol et al. 2021). Enhanced activity of canonical NLRC4/apoptosis-associated speck-like protein containing caspase activation and recruitment domain (ASC)/pro-caspase-1, and non-canonical caspase-11 inflammasomes, nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain containing 3/ASC/pro-caspase-1, TLR4/myeloid differentiation factor 88/transforming growth factor-activated kinase 1/nuclear factor κB (NF-κB)/cyclooxygenase-2, and nucleotide-binding oligomerization domain-like receptor X 1/tumor necrosis factor receptor-associated factor 6/inhibitor of κB kinase/inhibitor of κB-α/NF-κB pathways, soluble epoxide hydrolase, and nicotinamide adenine dinucleotide phosphate oxidase, which are associated with the formation of pro-inflammatory cytokines resulted in inflammation, were also observed in the CNS of LPS-treated mice (Tunctan et al. 2006; Buharalioglu et al. 2009; Dolunay et al. 2017; Cagli et al. 2021; Senol et al. 2021; Bahceli et al. 2022). Given the detrimental role of pyroptosis and necroptosis in inflammation, we aimed to investigate whether NSA ameliorates inflammatory hyperalgesia associated with demyelination induced by LPS via inhibiting caspase-11/GSDMD-mediated pyroptosis and RIPK1/RIPK3/MLKL-mediated necroptosis in the CNS of mice. The results of this study have been presented in abstract form (Ozgen et al. 2022).

Materials and methods

Animals

Balb/c mice (male; 20 to 30 g; n = 70) (Research Center of Experimental Animals, Mersin University, Mersin, Türkiye) were used in the experiments. The mice were housed under a 12-hour light/dark cycle and fed on standard chow. The procedures on animals were approved by the Mersin University Experimental Animals Local Ethics Committee (Approval date: June 28, 2021; Protocol number: 2021/31) and performed following the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Inflammatory hyperalgesia model

The inflammatory hyperalgesia model was induced by intraperitoneal injection of LPS as previously reported (Tunctan et al. 2006; Dolunay et al. 2017; Cagli et al. 2021; Senol et al. 2021). Mice were randomly divided into 8 groups, and the dose-response relationship was investigated with different NSA doses to observe the pain behavior (Rathkey et al. 2018; Wang et al. 2018; Jiao et al. 2020; Motawi et al. 2020): (1) Saline (10 mL/kg), (2) LPS (10 mg/kg; 10 mL/kg; intraperitoneal [i.p.]), (3) saline+dimethyl sulfoxide (DMSO) (10 mL/kg; i.p.), (4) saline+NSA (0.01 mg/kg; 10 mL/kg; i.p.), (5) LPS+NSA (0.001 mg/kg; 10 mL/kg; i.p.), (6) LPS+NSA (0.01 mg/kg; mL/kg; i.p.), (7) LPS+NSA (0.1 mg/kg; 10 mL/kg; i.p.), and (8) LPS+NSA (1 mg/kg; 10 mL/kg; i.p.). DMSO (60153; A1584; Applichem GmbH, Darmstadt, Germany) or NSA (dissolved in 6% DMSO; 480073; Sigma Chemical Co., St. Louis, MO, USA) was injected into the mice simultaneously with saline or LPS (dissolved in saline; L4130; *Escherichia coli* LPS, O111:B4; Sigma). Mice treated with saline, LPS, or DMSO were used as control, inflammatory hyperalgesia, and vehicle control groups, respectively. To evaluate pain sensation in response to thermal stimuli at spinal and supraspinal levels, increased sensitivity to the thermal stimulus was measured by the hot plate test at 6 hours after drug administration (Turner 1965; Tunctan et al. 2006; Dolunay et al. 2017; Cagli et al. 2021; Senol et al. 2021). For this procedure, each mouse was placed individually on a plate pre-heated to 55 ± 0.2 °C (AHP 9601, Commat Ltd., Ankara, Türkiye), and the time taken to elicit paw licking was recorded with a cut-off time of 30 seconds to avoid lesions to the animals' paws. According to our previous time-course studies (Tunctan et al. 2006), the time point of 6 hours was chosen for the assessment of LPS-induced hyperalgesia. Mice were euthanized after the test by cervical dislocation and exsanguination, and the brains and spinal cords of the animals were collected for measurement of expression of caspase-11 p20, p30-GSDMD, IL-1β, HMGB1, SEMA3A, and myelin PLP proteins as well as activity of RIPK1, RIPK3, and MLKL in immunoblotting studies.

Immunoblotting studies

The immunoblotting method was used according to the protocol as described in detail previously with minor modifications (Dolunay et al. 2017; Cagli et al. 2021; Senol et al. 2021). In brief, tissue homogenates (30 μg of total protein/20 μl) were resolved on nitrocellulose membranes. The membranes were then probed with antibodies in bovine serum albumin (BSA) (1:1.000 in 5% BSA) against (1) caspase-11 p20 (sc-374615; Santa Cruz Biotechnology, Santa Cruz, CA, USA), (2) p30-GSDMD (sc-393656; Santa Cruz), (3) IL-1β (sc-52012; Santa Cruz), (4) HMGB1 (sc-56698; Santa Cruz), (5) RIPK1 (ARG55746; Arigo Biolaboratories, Hsinchu City, Taiwan), (6) phosphorylated RIPK1 (p-RIPK1) (ARG66476; Arigo Biolaboratories), (7) RIPK3 (PA5-19956; Thermo Fisher, Waltham, MA USA), (8) phosphorylated RIPK3 (p-RIPK3) (PA5-105701; Thermo Fisher), (9) MLKL (PA5-102810; Thermo Fisher), (10) phosphorylated MLKL (p-MLKL) (PA5-102810; Thermo Fisher), (11) SEMA3A (sc-74554; Santa Cruz), (12) myelin PLP (bs-11093R-HRP; Bioss Antibodies Inc., Woburn, MA, USA), and (13) β-tubulin (Santa Cruz; sc-5274). Goat anti-rabbit IgG-horseradish peroxidase (Amersham Life Sciences, Cleveland, OH, USA; RPN4301) or sheep anti-mouse (Amersham; RPN4201) in 0.1% BSA (1:1.000) were used as secondary antibodies. Immunoreactive blots were visualized with ECL Prime Western Blotting Detection Reagent (Amersham; RPN2232) using a gel-imaging system (EC3-CHEMI HR imaging system; Ultra-Violet Products, UVP, Cambridge, UK). Band intensities were evaluated using Image J densitometry analysis software (Image J 1.53k, Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). The ratio of each band/β-tubulin was taken into account for the expression level of specific proteins.

Statistical analysis

The sample size determined for each treatment group in each experiment was based on previous studies from our laboratory (Tunctan et al. 2006; Buharalioglu et al. 2009; Dolunay et al. 2017; Cagli et al. 2021; Senol et al. 2021) and complied with power analysis (Festing 2018). The results are expressed as means ± standard error of the mean (SEM). For data normally distributed, parametric statistical analysis was conducted using one-way analysis of variance followed by Student-Newman-Keuls test for multiple comparisons to assess whether there is a significant difference between the mean values of β-tubulin in each group. For data normally or not normally distributed, parametric or nonparametric statistical analysis was performed with Student's *t*-test or Mann-Whitney *U*-test for normally or abnormally distributed data, respectively. Statistical analysis was performed using GraphPad Prism version 7.04 for Windows (GraphPad Software, San Diego California USA; http://www.graphpad.com). $p < 0.05$ was considered to indicate a statistically significant difference.

Results

NSA treatment ameliorated hyperalgesia induced by LPS

To test the effect of NSA on LPS-induced hyperalgesia, NSA was injected into mice alone or in combination with saline or LPS. LPS caused a decrease in the hot plate latency 6 hours after LPS injection compared with control group values (Fig. 1) ($p < 0.05$). NSA at doses of 0.01, 0.1, and 1 mg/kg prevented the reduction in latency compared with LPS-injected mice ($p < 0.05$). NSA at the dose of 0.001 mg/kg was ineffective in preventing the decrease in latency compared with the LPS-injected mice ($p > 0.05$). At a dose of 0.01 mg/kg, treatment with NSA had no effect on hot plate latency in mice treated with saline or DMSO

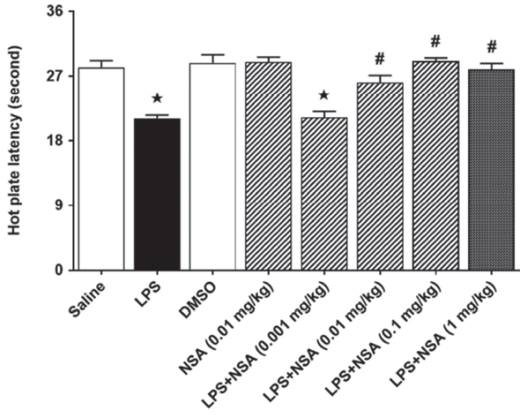

Figure 1. Effect of NSA on LPS-induced hyperalgesia. Data are expressed as means ± SEM from 5–10 animals. *p < 0.05 versus saline-injected group; #p < 0.05 versus LPS-injected group. LPS: lipopolysaccharide; NSA: necrosulfonamide.

(p > 0.05). No mortality was also observed during the study. Accordingly, tissues from mice injected with NSA at the minimum effective dose (0.01 mg/kg) in the LPS-induced hyperalgesia were used for further experiments.

NSA treatment prevented the LPS-induced increase in caspase-11/GSDMD-mediated pyroptosis

To test the effect of NSA on the LPS-induced caspase-11/GSDMD-mediated pyroptosis in the CNS, the brain and spinal cord tissue samples of saline-, LPS-, DMSO-, and/or NSA-treated mice were immunoblotted with specific caspase-11 p20, p30-GSDMD, IL-1β, and HMGB1 antibodies. Increased expression of caspase-11 p20, p30-GSDMD, IL-1β, and HMGB1 was observed in the brain (Fig. 2A–D) and spinal cord (Fig. 2E–H) of LPS-injected

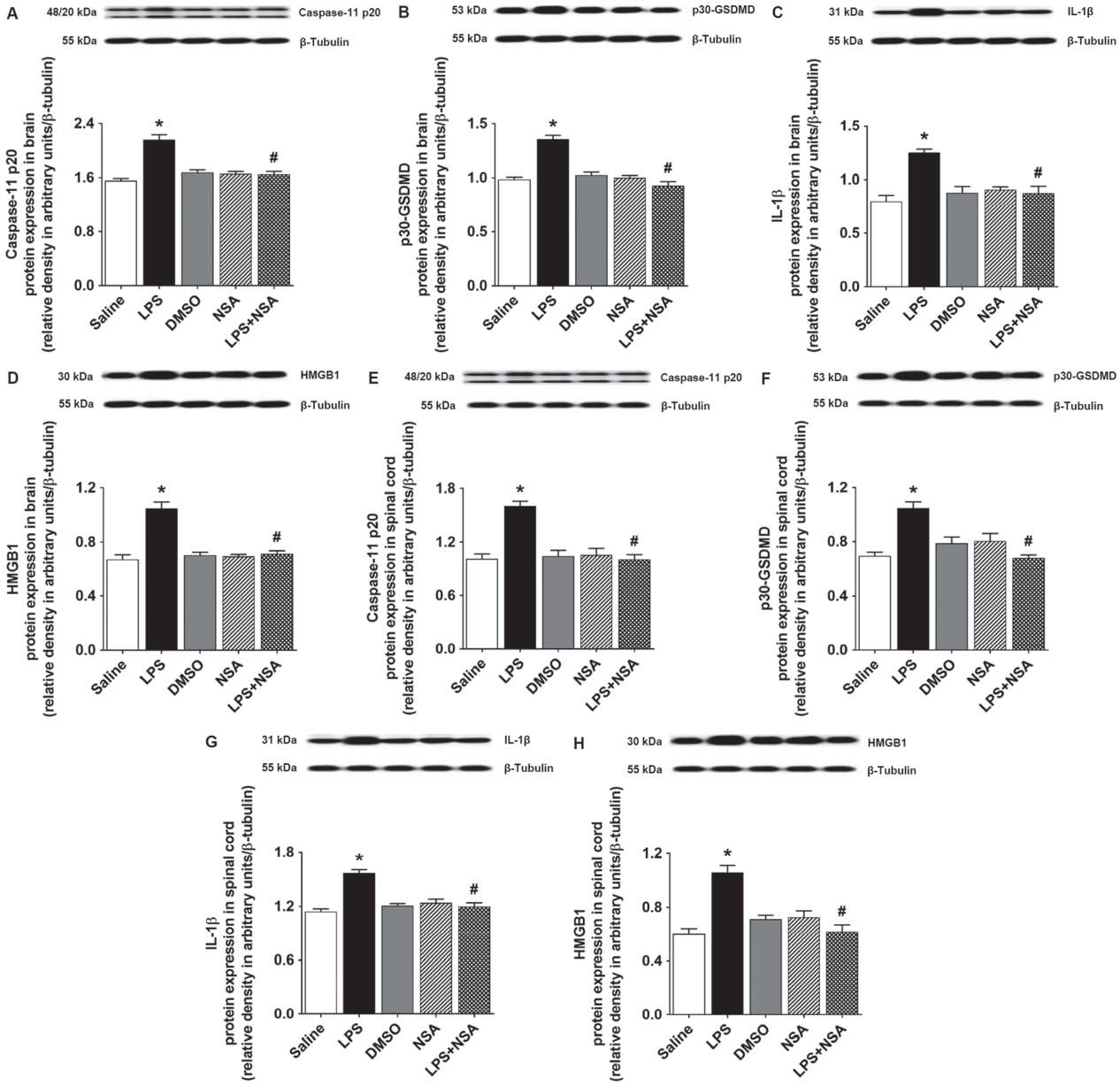

Figure 2. Effect of NSA on LPS-induced increase in caspase-11 p20, p30-GSDMD, IL-1β, and HMGB1 expression in the (**A–D**) brain and (**E–H**) spinal cord. Data are expressed as means ± SEM from 4 animals. *p < 0.05 versus saline-injected group; #p < 0.05 versus LPS-injected group. GSDMD: gasdermin D; HMGB: high-mobility-group-box; IL: interleukin; LPS: lipopolysaccharide; NSA: necrosulfonamide.

mice compared with the levels in the control group values ($p < 0.05$). Treatment with NSA inhibited the LPS-induced increase in caspase-11 p20, p30-GSDMD, IL-1β, and HMGB1 expression in tissues compared with LPS-injected mice ($p < 0.05$). The expression of these proteins in the tissues of NSA-treated mice was not different from the control group ($p > 0.05$).

NSA treatment prevented the LPS-induced increase in RIPK1/RIPK3/MLKL necrosome-mediated necroptosis

To investigate the effect of NSA on the LPS-induced RIPK1/RIPK3/MLKL necrosome-mediated necroptosis in the CNS, the brain and spinal cord tissue samples of saline-, LPS-, DMSO-, and/or NSA-treated mice were immunoblotted with specific antibodies for RIPK1, p-RIPK1 (at Ser^{166}), RIPK3, p-RIPK3 (at Ser^{232}), MLKL, and p-MLKL (at Ser^{358}). Increased expression of the p-RIPK1, p-RIPK3, and p-MLKL, but not RIPK1, RIPK3, and MLKL proteins was observed in the brain (Fig. 3A–C) and spinal cord (Fig. 3D–F) of LPS-injected mice compared with levels in the control group values ($p < 0.05$). Treatment with NSA inhibited the LPS-induced increase in the p-RIPK1, p-RIPK3, and p-MLKL expression in tissues compared with LPS-injected mice ($p < 0.05$). The expression of unphosphorylated and phosphorylated proteins in the tissues of NSA-treated mice was not different from the control group ($p > 0.05$).

NSA treatment prevented the LPS-induced increase in SEMA3A expression

To investigate the effect of NSA on the expression of SEMA3A in the CNS, the brain and spinal cord tissue samples of saline-, LPS-, DMSO-, and/or NSA-treated mice were immunoblotted with a specific SEMA3A antibody. Increased expression of SEMA3A was observed in the brain (Fig. 4A) and spinal cord (Fig. 4B) of LPS-injected mice compared with levels in the control group ($p < 0.05$). Treatment with NSA inhibited the LPS-induced increase in SEMA3A expression in tissues compared with LPS-injected mice ($p < 0.05$). The expression of SEMA3A in the tissues of NSA-treated mice was not different from the control group ($p > 0.05$).

NSA treatment prevented the LPS-induced decrease in myelin PLP expression

To investigate the effect of NSA on the expression of myelin PLP in the CNS, the brain and spinal cord tissue samples of saline-, LPS-, DMSO-, and/or NSA-treated mice were immunoblotted with a specific myelin PLP antibody. Decreased expression of myelin PLP was observed in the brain (Fig. 5A) and spinal cord (Fig. 5B) of LPS-injected mice compared with levels in the control group ($p < 0.05$). Treatment with NSA inhibited the LPS-induced decrease in myelin PLP expression in tissues compared with LPS-injected mice ($p < 0.05$). The expression of myelin PLP in the tissues of NSA-treated mice was not different from the control group ($p > 0.05$).

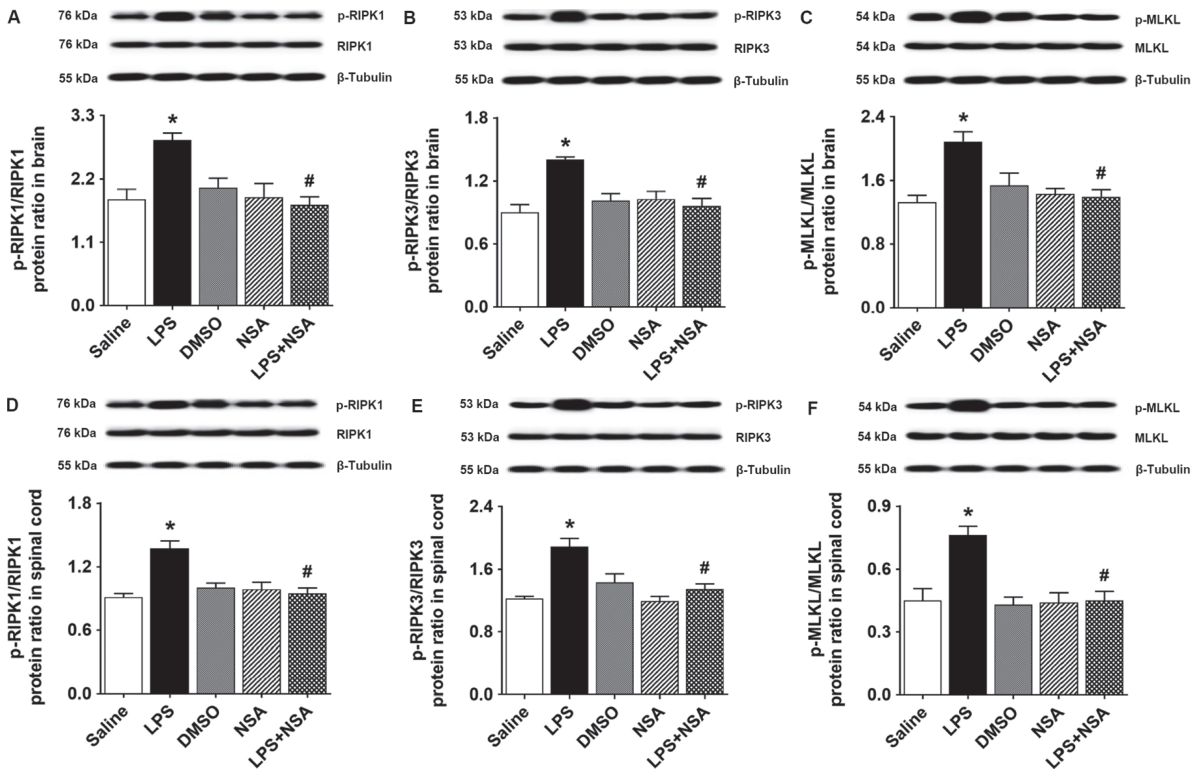

Figure 3. Effect of NSA on LPS-induced increase in RIPK1, RIPK3, and MLKL activity in the (**A–C**) brain and (**D–F**) spinal cord. Data are expressed as means ± SEM from 4 animals. *$p < 0.05$ versus saline-injected group; #$p < 0.05$ versus LPS-injected group. LPS: lipopolysaccharide; MLKL: mixed lineage kinase domain-like pseudokinase; NSA: necrosulfonamide; RIPK: receptor-interacting serine/threonine-protein kinase.

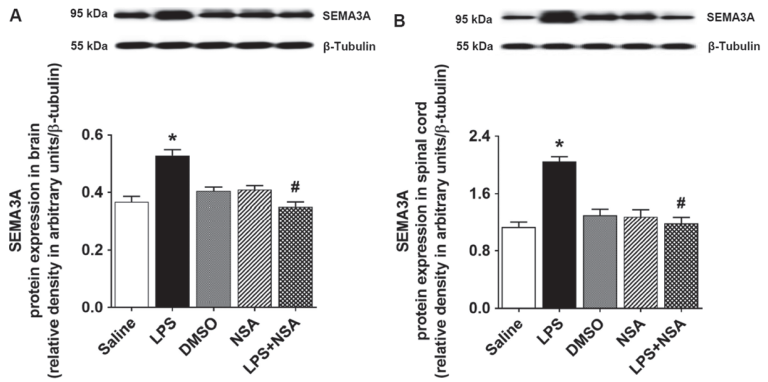

Figure 4. Effect of NSA on LPS-induced increase in SEMA3A expression in the (**A**) brain and (**B**) spinal cord. Data are expressed as means ± SEM from 4 animals. *p < 0.05 versus saline-injected group; #p < 0.05 versus LPS-injected group. LPS: lipopolysaccharide; NSA: necrosulfonamide; SEMA: semaphorin.

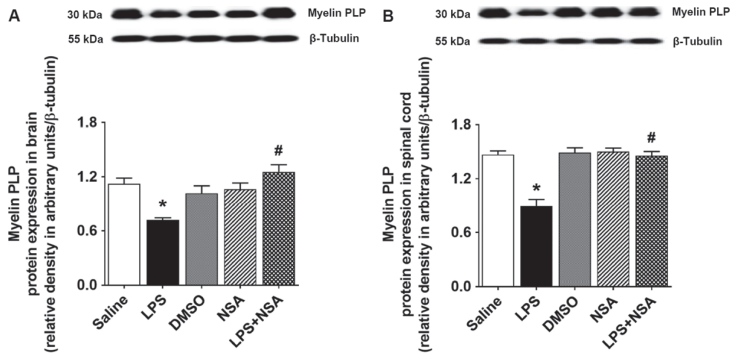

Figure 5. Effect of NSA on LPS-induced decrease in myelin PLP expression in the (**A**) brain and (**B**) spinal cord. Data are expressed as means ± SEM from 4 animals. *p < 0.05 versus saline-injected group; #p < 0.05 versus LPS-injected group. LPS: lipopolysaccharide; NSA: necrosulfonamide; PLP: myelin proteolipid protein.

Discussion

The results of this study suggest, for the first time, that GSDMD and MLKL inhibitor, NSA, ameliorates hyperalgesia by inhibiting increased protein expression of caspase-11 p20, p30-GSDMD, IL-1β, HMGB1, and SEMA3A in addition to RIPK1, RIPK3, and MLKL phosphorylation associated with decreased myelin PLP expression in the CNS of LPS-treated mice. Consistent with our previous findings (Tunctan et al. 2006; Buharalioglu et al. 2009; Dolunay et al. 2017; Cagli et al. 2021; Senol et al. 2021), the results of the present study may provide the first evidence that NSA ameliorates the LPS-induced inflammatory hyperalgesia by not only inhibiting caspase-11/GSDMD-mediated pyroptosis, RIPK1/RIPK3/MLKL necrosome-mediated necroptosis, and demyelination but also promoting remyelination in the CNS of mice (Fig. 6).

Systemic administration of NSA has also been shown to be efficacious in several inflammatory rodent models *in vivo* including LPS-induced sepsis in mice (Rathkey et al. 2018), $AlCl_3$-induced Alzheimer's disease in rats (Motawi et al. 2020), ischemia-reperfusion injury in rats (Ueda et al. 2022), spinal cord injury in mice (Jiao et al. 2020), and rats (Wang et al. 2018), and post-resuscitation myocardial dysfunction in mice (He et al. 2022), through protective mechanisms potentially related to inhibition of GSDMD-dependent pyroptotic and MLKL-dependent necroptotic pathways. In only one study conducted to date, Rathkey et al. (2018) demonstrated that NSA (20 mg/kg; i.p.) binds directly to GSDMD and can ameliorate GSDMD-driven mortality associated with increased serum IL-1β and IL-6 levels in murine sepsis induced by LPS (25 mg/kg; i.p.). In a recent study, Wu et al. (2022) also reported that NSA (20 mg/kg; i.p.) alleviates liver injury as demonstrated by histopathological analysis associated with inhibition of pyroptosis pathway, decreased IL-1β and IL-18 levels, and improved survival in acute liver failure model induced by injection of LPS (50 μg kg^{-1}; i.p.)+D-galactosamine (800 mg/kg; i.p.). Nevertheless, to the best of our knowledge, the effect of NSA on the LPS-induced inflammatory hyperalgesia has not been investigated. Increasing evidence also suggests that caspase-11/GSDMD-mediated pyroptosis signaling pathway triggered by LPS plays an important role in the pathogenesis of neuroinflammatory disorders (Lv et al. 2018; Mitchell et al. 2022). Although NSA has been shown to reverse the effects of LPS on pyroptosis both *in vitro* and *in vivo* (Rathkey et al. 2018; Li et al. 2019; Zhang and Wei 2021); its effects on caspase-11/GSDMD-mediated pyroptosis in the CNS during LPS-induced inflammatory hyperalgesia remain unknown. In the present study, we showed that systemic administration of LPS into mice resulted in hyperalgesia as well as increased protein expression of caspase-11 p20, p30-GSDMD, IL-1β, and

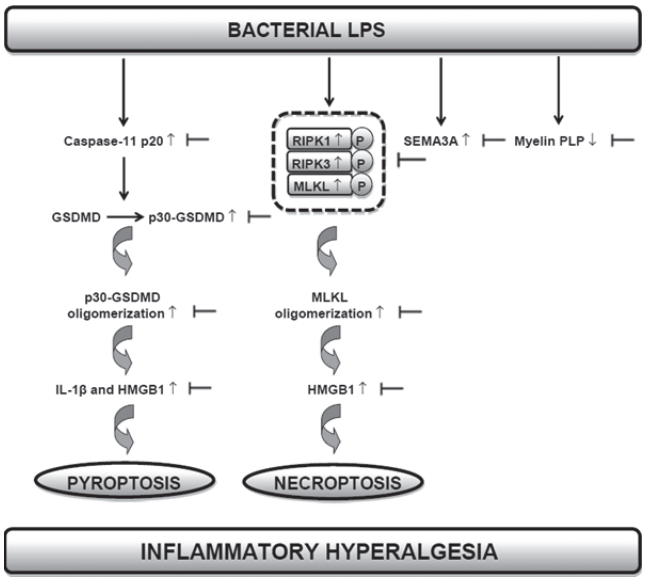

Figure 6. Diagram showing the effect of GSDMD and MLKL inhibitor, NSA on LPS-induced changes in the caspase-11/GSDMD-mediated pyroptosis and RIPK1/RIPK3/MLKL necrosome-mediated necroptosis in the central nervous system of mice during inflammatory hyperalgesia. (↑) Increased by LPS; (↓) decreased by LPS; () prevented by NSA. GSDMD: gasdermin D; LPS: lipopolysaccharide; NSA: necrosulfonamide. LPS: lipopolysaccharide; MLKL: mixed lineage kinase domain-like pseudokinase; NSA: necrosulfonamide; RIPK: receptor-interacting serine/threonine-protein kinase.

HMGB1 in the mouse brain and spinal cord. Therefore, in agreement with the results of previous studies (Rathkey et al. 2018; Wu et al. 2022), and our previous findings (Tunctan et al. 2006; Buharalioglu et al. 2009; Dolunay et al. 2017; Cagli et al. 2021; Senol et al. 2021), this study presents novel data suggesting that NSA ameliorates hyperalgesia through the decreased formation of IL-1β (one of the major pro-inflammatory cytokine) (Lima 2023) and release of HMGB1 (a key mediator of inflammation and inflammatory pain) (Scaffidi et al. 2002; Tanaka et al. 2013; Chen et al. 2022) by pyroptotic cells to promote inflammation as a result of cell rupture and pyroptosis consequently suppressed expression and/or activity of the caspase-11/GSDMD-mediated pyroptotic pathway in the CNS. These findings also suggest that not only binding directly to GSDMD to inhibit p30-GSDMD oligomerization and pyroptosis (Rathkey et al. 2018), but also directly or indirectly decreasing expression of caspase-11 p20, p30-GSDMD, IL-1β, and HMGB1 proteins in the CNS of LPS-treated mice contribute to the protective effect of NSA in the hyperalgesia model. However, additional studies should be done to prove the validity of these hypotheses.

Recent evidence also indicates that RIPK1/RIPK3/MLKL necrosome-mediated necroptosis plays a crucial role in the pathogenesis of acute/ischemic brain injury and LPS-induced models of neuroinflammation-related CNS disorders (Suda et al. 2016; Cruz et al. 2018; Liu et al. 2020; Kondo et al. 2021; Wang et al. 2021; Zhang et al. 2022). There are only a few studies in the literature showing that NSA reverses the effects of LPS on the changes in the expression and/or activity of key components of necroptosis, RIPK1, RIPK3, and MLKL, *in vitro* (El-Mesery et al. 2015; Saeed et al. 2019; Geng et al. 2022). On the other hand, it is unknown whether NSA prevents the LPS-induced RIPK1/RIPK3/MLKL necrosome-mediated necroptosis in the CNS during inflammatory hyperalgesia. In the current study, we demonstrated that systemic administration of LPS into mice also resulted in not only increased phosphorylation of RIPK1, RIPK3, and MLKL proteins but also HMGB1 protein expression in the brain and spinal cord tissues. These data suggest that HMGB1 released by necrotic cells to promote inflammation as a result of cell rupture and necrosis may be due to increased activity of the RIPK1/RIPK3/MLKL necrosome-mediated necroptotic pathway in the CNS of mice seems to be involved in inflammatory hyperalgesia induced by LPS. Our findings also suggest that not only binding directly to MLKL to inhibit MLKL oligomerization and necroptosis, but also directly or indirectly, decreasing phosphorylation of RIPK1, RIPK3, and MLKL proteins in the CNS of LPS-treated mice contributes to the protective effect of NSA in the hyperalgesia model. However, additional studies should be done to prove the validity of these hypotheses.

SEMA3A, one of the remyelination inhibitors, is reported to be increased in the mouse brain in response to intracerebroventricular injection of LPS (Ito et al. 2014). In recent years, loss of myelin proteins such as PLP has also received major attention in the neuroinflammatory changes in the brain induced by systemic or intrauterine injection of LPS into rodents (Chang et al. 2011; Huang et al. 2020a, 2020b). On the other hand, whether NSA prevents the LPS-induced changes in the protein expression of SEMA3A and myelin PLP have not been investigated until recently. Consistent with the findings of previous studies (Huang et al. 2020a, 2020b), we obtained additional evidence confirming that systemic administration of LPS into mice results in increased protein expression of SEMA3A associated with

decreased myelin PLP expression in the mouse brain and spinal cords. Moreover, LPS-induced changes in the expression of SEMA3A and myelin PLP were reversed by NSA. These results suggest that NSA not only prevents demyelination but also promotes remyelination in the LPS-induced inflammatory hyperalgesia model in mice.

Collectively, in line with the above-mentioned studies and our previous findings, directly or indirectly decreased formation of pro-inflammatory mediators as a result of inhibition of caspase-11/GSDMD-mediated pyroptotic and RIPK1/RIPK3/MLKL necrosome-mediated necroptotic pathways at transcriptional and/or post-transcriptional level in the CNS seems to contribute to the ameliorating effect of NSA against LPS-induced enhanced pain sensitivity to thermal stimuli associated with demyelination. Furthermore, NSA may also exert its beneficial effects due to its synergistic effect on the signaling pathways that play a role in the pathogenesis of inflammatory hyperalgesia caused by LPS. In addition, it is not clear from our results whether the amelioration of LPS-induced hyperalgesia by NSA is due to inhibition of caspase-11/GSDMD-mediated pyroptosis and RIPK1/RIPK3/MLKL necrosome-mediated necroptosis in the brain and spinal cord. Therefore, further histopathological and immunohistochemistry studies are needed to verify our findings. Whether NSA protects not only the brain and spinal cord, but also vital organs such as the heart, lung, kidney, and liver from LPS-induced systemic inflammation and tissue injury associated with increased pro-inflammatory cytokine formation, and improves survival also needs further investigation. Hence, further investigations are also necessary to clarify the potential mechanisms through both *in vivo* and *ex vivo* studies. Therefore, exploring molecular mechanisms of the effects of NSA on the LPS-induced inflammatory hyperalgesia associated with demyelination in the CNS will ensure the framework for the extension of this research into comprehension of the involvement of caspase-11/GSDMD-mediated pyroptosis and RIPK1/RIPK3/MLKL necrosome-mediated necroptosis. Consequently, GSDMD and MLKL inhibitors such as NSA may be effective and safe therapeutic agents for the treatment of hyperalgesia-related demyelinating inflammatory diseases.

Conclusion

In this study, we demonstrated for the first time that the GSDMD and MLKL inhibitor, NSA, can ameliorate LPS-induced inflammatory hyperalgesia by preventing caspase-11/GSDMD-mediated pyroptosis, RIPK1/RIPK3/MLKL necrosome-mediated necroptosis, and demyelination, and also promotes remyelination in the CNS of mice. Thus, we suggest that GSDMD and MLKL inhibitors such as NSA which can be applied systemically and able to cross the blood-brain barrier, may be useful as analgesic/anti-inflammatory drugs in the prevention and treatment of acute and chronic painful conditions in which inflammation plays a role in the pathophysiology that may result from bacterial infections.

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