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Inhaled hydrogen sulfide prevents neuropathic pain after peripheral nerve injury in mice

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ABSTRACT

Increasing evidence suggests that the pathogenesis of neuropathic pain is mediated through activation of microglia in the spinal cord. Hydrogen sulfide attenuates microglial activation and central nervous system inflammation; however, the role of hydrogen sulfide in neuropathic pain is unclear. In this study, we examined the effects of hydrogen sulfide breathing on neuropathic pain in mice. C57BL/6J mice were subjected to chronic constriction injury (CCI) of the sciatic nerve. After CCI, mice breathed air alone or air mixed with hydrogen sulfide at 40 ppm for 8 h on 7 consecutive days. The expression levels of inflammatory cytokines including interleukin 6 (IL-6) were measured in the spinal cord. Effects of hydrogen sulfide on IL-6-induced activation of microglia were examined in primary rat microglia. Mice that breathed air alone exhibited the neuropathic pain behavior including mechanical allodynia and thermal hyperalgesia and increased mRNA levels of IL-6 and chemokine CC motif ligand 2 (CCL2) after CCI. Inhaled hydrogen sulfide prevented the neuropathic pain behavior and attenuated the upregulation of inflammatory cytokines. Sodium sulfide inhibited IL-6-induced activation of primary microglia. These results suggest that inhaled hydrogen sulfide prevents the development of neuropathic pain in mice possibly via inhibition of the activation of microglia in the spinal cord.

1. Introduction

Chronic pain affects about 30% of the population and is estimated to cost $650 billion a year in health-care costs and lost productivity in the United States [1]. The estimate of the annual cost of chronic pain was greater than the annual costs of heart disease ($309 billion), cancer ($243 billion), and diabetes ($188 billion) [2]. Neuropathic pain is a common chronic pain condition [3] that is caused by peripheral nerve injury and is characterized by long-lasting exaggerated pain behavior such as allodynia and hyperalgesia [4]. Although neuropathic pain is known as a particularly unpleasant type of pain [3], the management of patients with neuropathic pain is challenging because of the multiplicity of mechanisms involved in neuropathic pain conditions [5]. Precise pathophysiological mechanisms of neuropathic pain are still unclear, however, a number of studies have suggested that microglial activation and inflammatory cytokines in the spinal cord play important roles in the development and maintenance of neuropathic pain [6,7]. To date, management of neuropathic pain is aimed only at reducing symptoms; however, current drugs have limited efficacy and dose-limiting toxic effects [8]. Several clinical trials of drugs for neuropathic pain have reported negative results despite encouraging results from preclinical and early clinical studies [9]. Therefore, additional therapeutic strategies are urgently needed.

Hydrogen sulfide is a colorless, flammable and water-soluble gas with the characteristic odor of rotten eggs typically found in sulfur hot spring and sewer [10]. Recently, hydrogen sulfide was rediscovered as an endogenously produced signaling molecule along with nitric oxide and carbon monoxide [11]. An abundance of experimental evidence suggests that hydrogen sulfide plays a prominent role in physiology and pathophysiology [12]. We have previously reported that inhaled hydrogen sulfide prevents neurodegeneration in a mouse model of Parkinson's disease induced by neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [13]. The protective effects of inhaled hydrogen sulfide in the mouse model of Parkinson's disease were associated with inhibition of glial activation and upregulation of antioxidant and detoxification proteins in the brain. Furthermore, we have recently reported that breathing hydrogen sulfide prevents the systemic inflammation induced by lipopolysaccharide (LPS) and improves survival rate in mice [14]. Based on these findings, we hypothesized that breathing hydrogen sulfide prevents the development of neuropathic pain behavior via inhibiting microglial activation and neuroinflammation in the spinal cord. Here we report that inhaled hydrogen sulfide prevents the neuropathic pain behavior induced by chronic constriction injury of the sciatic nerve in mice.

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2. Materials and methods

2.1. Animals

After approval by the Massachusetts General Hospital Subcommittee on Research Animal Care, we studied 2–3 month-old male C57BL/6J wild-type (WT) mice (The Jackson Laboratory, Bar Harbor, ME) and 3–4 month-old male Sprague-Dawley rats (Charles River, Wilmington, MA).

2.2. Surgical procedure

Chronic constriction injury (CCI) of the sciatic nerve was performed in mice as previously described [15,16]. Briefly, after instrumentation under anesthesia, one side of the common sciatic nerve was exposed and two loose ligatures (4-0 chromic gut) were made around the dissected nerve.

2.3. Hydrogen sulfide inhalation

Mice breathed air alone or air mixed with hydrogen sulfide at 40 ppm for 8 h each day for 7 days starting immediately after the CCI operation in custom made chambers, as previously described [13]. We chose the dose of H_2S inhalation based on our recent study in which H_2S inhalation at 40 ppm prevented neurodegeneration in a murine model of Parkinson's disease induced by MPTP [13].

2.4. Behavioral test of neuropathic pain

2.4.1. Mechanical allodynia

Mechanical allodynia was assessed by using the von Frey filament test as previously described [16,17]. To carry out this test, mice were placed in a cage in which the bottom is made of gauze. This set-up allowed an experimenter to touch the mid-plantar surface of mice's hind-paw from the bottom of the cage. An experimenter applied von Frey filaments in ascending order of bending force to the mid-plantar surface of the mice's hind-paw. A von Frey filament was applied perpendicular to the skin and depressed slowly until it bent. A threshold force of response was defined as the first filament in the series that evoked at least one clear paw-withdrawal out of five applications. Each of these five stimuli was applied to slightly different areas of the mid-plantar surface with 2–3 s intervals.

2.4.2. Thermal hyperalgesia

Thermal hyperalgesia to radiant heat was assessed by using a foot-withdrawal test as previously described [16,18]. Mice were placed in plastic boxes on a glass plate. The radiant heat source was applied from a projection bulb placed directly under the planter surface of the mice's hind-paw. The foot-withdrawal latency was defined as the time elapsed from the onset of radiant heat stimulation to withdrawal of mice's hind-paw. The radiant source was adjusted to result in base-line latencies of 12 s and a cut-off time of 20 s was preset to prevent possible tissue damage. Three test trials with 5 min interval were performed and scores from each trial were averaged to yield the mean foot-withdrawal latency.

2.5. Measurements of gene expression in the mice spinal cord after peripheral nerve injury

The lumber parts of spinal cord were obtained from mice at 2 days after sham operation or CCI with or without hydrogen sulfide breathing. RNA was extracted from spinal cord using the RNAspin Mini kit (GE Healthcare, Piscataway, NJ) and cDNA was synthesized using moloney murine leukemia virus reverse transcriptase (M-MLV RT) (Promega, Madison, WI). The mRNA expressions of interleukin 6 (IL-6), tumor necrosis factor α (TNF-α), chemokine CC motif ligand 2 (CCL2), activating transcription factor 3 (ATF3), integrin alpha M (ITGAM), glial fibrillary acidic protein (GFAP), and 18S ribosomal RNA were measured by real-time PCR using Realplex 2 system (Eppendorf North America, Westbury, NY). The primer sequences are listed in Table 1.

2.6. Activation of primary microglia and measurements of gene expression

Primary mixed glial cells were cultured from 0 to 2 days old Sprague Dawley rat pups as previously described [19]. Cerebral cortices were dissected, minced and digested. The cells were seeded in poly-D-lysine-coated 75-cm^2 T flasks with Dulbecco's Modified Eagle's medium containing 20% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cell cultures were confluent in 10–14 days, and then the flasks were shaken for an hour in an orbital shaker at 218 rpm in 36.5 °C to dissociate the microglia. Then, the media from the flasks were extracted. Cells in the extracted media were seeded into poly-D-lysine-coated six-well plates (2×10^5 cells/cm^2). Cells were incubated for 7 days and then serum starved with 0.1% FBS. Vehicle, or sodium sulfide (Na_2S) at

Table 1
List of primer sequences for real-time PCR.

IL-6 for mice	Forward	5′-CCGGAGAGGAGACTTCACAGA-3′
IL-6 for mice	Reverse	5′-GGTCTGGGCCATAGAACTGA-3′
TNF-α for mice	Forward	5′-CAGCCTCTTCTCATTCCTGC-3′
TNF-α for mice	Reverse	5′-GGTCTGGGCCATAGAACTGA-3′
CCL-2 for mice	Forward	5′-ATGCAGGTCCCTGTCATGCTTC-3′
CCL-2 for mice	Reverse	5′-ACTCATTGGGATCATCTTGCTGG-3′
ATF3 for mice	Forward	5′-CAGTTACCGTCAACAACAGACCC-3′
ATF3 for mice	Reverse	5′-CTTTCTGCAGGCACTCTGTCTTC-3′
ITGAM for mice	Forward	5′-CCATGACCTTCCAAGAGAATGC-3′
ITGAM for mice	Reverse	5′-ACCGGCTTGTGCTGTAGTC-3′
GFAP for mice	Forward	5′-ACCAGCTTACGGCCAACAG-3′
GFAP for mice	Reverse	5′-CCAGCGATTCAACCTTTCTCT-3′
18S for mice	Forward	5′-CGGCTACCACATCCAAGGAA-3′
18S for mice	Reverse	5′-GCTGGAATTACCGCGGCT-3′
IL-6 for rat	TaqMan Rn00561420_m1 (Life Technologies, Grand Island, NY)	
TNF-α for rat	TaqMan Rn99999017_m1 (Life Technologies)	
CCL-2 for rat	TaqMan Rn00580555_m1 (Life Technologies)	
18S for rat	TaqMan Hs 99999901_s1 (Life Technologies)	

IL-6, interleukin 6; TNF-α, tumor necrosis factor α; CCL2, chemokine CC motif ligand 2; ATF3, Activating transcription factor 3; ITGAM, integrin alpha M; GFAP, glial fibrillary acidic protein.

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