Anti-Allodynic Effect of Intracerebroventricularly Administered Antioxidant and Free Radical Scavenger in a Mouse Model of Orofacial Pain

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Aims: *To evaluate possible effects of the intracerebroventricular (icv) injection of either O-Tricyclo [5.2.1.0^{2,6}] dec-9-yl dithiocarbonate potassium salt (D609), a potent antioxidant and inhibitor of phosphatidylcholine specific phospholipase C (PtdCho-PLC) and acid sphingomyelinase (ASMase), or the spin trap/free radical scavenger N-tert-Butyl-α-phenylnitrone (PBN), on mechanical allodynia induced by facial carrageenan injection in mice.* ***Methods:*** *Balb/c mice received icv injection of D609/PBN plus facial carrageenan injection, and the number of face wash strokes to von Frey hair mechanical stimulation of the maxillary skin was quantified. PtdCho-PLC and ASMase activities were also assayed in the brainstem, thalamus, and somatosensory cortex.* ***Results:*** *Mice that received the icv injection of 10 nmol D609 plus facial carrageenan injection showed significantly fewer face wash strokes evoked by von Frey hair stimulation (indicating reduced mechanical allodynia) at 1 and 3 days post-injection, compared to mice that received icv injection of isotonic saline plus facial carrageenan injection. Mice that received icv injection of 1.13 μmol PBN plus facial carrageenan injection likewise showed significantly fewer face wash strokes after facial carrageenan injection, compared to isotonic saline-injected plus carrageenan-injected controls. D609 injection also resulted in significantly reduced ASMase activity in the brainstem, thalamus, and somatosensory cortex 3 days after injection, compared to controls.* ***Conclusion:*** *The icv injections of D609 and PBN were effective in reducing mechanical allodynia after facial carrageenan injection-induced pain. Together, the results point to a possible role of central nervous system sphingolipids and/or free radicals in orofacial pain.* J OROFAC PAIN 2009;23:167–173

Key words: acid sphingomyelinase, free radicals, mechanical allodynia, orofacial pain, phosphatidylcholine specific phospholipase C, reactive oxygen species, ROS

Growing evidence indicates an important role of lipid mediators and free radicals in the central nervous system (CNS) that augment the sensitivity of sensory neurons and enhance pain perception. These lipid mediators can act as second messengers or activate receptors and ion channels in sensory neurons. Intracerebroventricular (icv) injection of phospholipase A_2 (PLA_2) inhibitors attenuates mechanical allodynia after carrageenan-induced inflammation in a mouse model of orofacial pain.[1] In contrast, icv injections of lysophospholipids or platelet activating factor increase allodynic responses.[2] CNS phospholipase C (PLC) may

also be involved in nociceptive transmission. The icv injection of the phosphatidylinositol-specific phospholipase C inhibitor U73122 dose-dependently reduces thermal hyperalgesia induced by morphine.[3] This has been attributed to diacylglycerol (DAG) or inositol 1,4,5-triphosphate ($InsP_3$) produced by the action of PLC.[3–5] DAG is also generated by phosphatidylcholine-specific PLC (PtdCho-PLC). Treatment of LA-N-1 cells with12-O-tetradecanoyl-phorbol-13 acetate and retinoic acid increases DAG levels, indicating the stimulation of PLC activity. This stimulation is blocked by O-Tricyclo [5.2.1.$0^{2,6}$] dec-9-yl dithiocarbonate potassium salt (D609), a potent antioxidant and competitive PtdCho-PLC inhibitor.[6] Besides glycerophospholipids, which are the substrates of PLA_2 or PLC, other lipid components of the neural cell membrane are sphingolipids and cholesterol. The action of sphingomyelinase on sphingomyelin produces ceramide. The latter induces neuropathic pain after intradermal injection to the hind paw of rats.[7] However, little is known about the possible action in the CNS of sphingomyelinase or ceramides on pain perception.

Free radicals are also likely to play a role in allodynia-related neurotransmission in the CNS. The number of neurons showing mitochondrial reactive oxygen species (ROS) production is significantly increased in the spinal dorsal horn of rats with neuropathy.[8] Injection of peroxynitrite into the subplantar space of rats enhances carrageenan-induced hyperalgesia.[9] In contrast, icv injections of a peroxynitrite scavenger have an anti-allodynic effect in a mouse model of facial pain induced by carrageen injection.[10] Systemic injection of the spin trap/free radical scavenger N-tert-Butyl-α-phenylnitrone (PBN) relieves neuropathic pain in rats.[11] PBN also reduces the development of thermal and mechanical hyperalgesia 1 and 3 days after chronic constriction injury of the sciatic nerve in mice.[12] In addition, systemic administration or intrathecal injection of PBN or the ROS scavenger 4-hydroxy-2,2,6,6-tetramethylpiperidine-1 oxyl (TEMPOL) alleviates capsaicin-induced hyperalgesia in rats.[13,14]

D609 is a xanthogenate derivative with a variety of biological effects, including antiviral, antitumor, and antiinflammatory activities.[15] It inhibits PtdCho-PLC,[16] sphingomyelin synthase, and acid sphingomyelinase (ASMase) activities.[17–19] Moreover, it is a potent antioxidant, with the ability to scavenge intracellular reduced glutathione and inhibit intracellular ROS accumulation in cells.[15,20,21] The above properties suggest that D609 may have a modulatory effect on pain. This study aimed to evaluate possible effects of the icv injection of either D609 or PBN on mechanical allodynia induced by facial carrageenan injection in mice.

Materials and Methods

Effect of D609 Injection on Mechanical Allodynia Induced by Facial Carrageenan Injection

Twenty-nine adult male Balb/c mice, about 6 to 8 weeks of age and weighing approximately 20 to 30 g each, were purchased from the Laboratory Animals Center, Sembawang, Singapore. They were randomly divided into four groups with seven to eight mice per group. Three of these groups received an icv injection of D609 (Sigma, St Louis, MO) and a facial carrageenan injection, and the fourth group (control) received an icv injection of isotonic saline and a facial carrageenan injection.

The icv injections were carried out as previously described.[1,10] Mice were deeply anesthetized with an intraperitoneal injection of 0.2-0.3 ml of 0.3% pentobarbital sodium salt, followed by exposure of the cranial vault and stereotaxic injection of 5 μL of either 0.02 mM, 0.2 mM, or 2 mM of D609 (equivalent to 0.1 nmol, 1 nmol, and 10 nmol, respectively), or isotonic saline into the right lateral ventricle (coordinates: 0.7 mm caudal to bregma, 1.0 mm lateral to the midline, 3.0 mm from the surface of the cerebral cortex). The median inhibition concentration (IC50) of D609 has been reported to be 50~100 μg/mL *in vitro*.[22,23] The concentrations of D609 injected were thus 1/10, 1, and 10 times of the IC50 (50 μg/mL), although the final brain concentrations would have been lower due to dilution in the cerebrospinal fluid. The needle was withdrawn 10 minutes later and the scalp sutured. Facial carrageenan injection was carried out by injecting 50 μL of lambda carrageenan (40 mg/ml in isotonic saline; Fluka, Buchs, Switzerland) into the subcutaneous tissue of the right maxilla of mice immediately after the D609 injections. All procedures involving animals were in accordance with the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain (IASP), and were approved by the Institutional Animal Care and Use Committee.

The mice were assessed for behavioral responses to von Frey filament (Touch-Test Sensory Evaluator, North Coast Medical, Morgan Hill, CA) stimulation of the right maxillary skin 1 day before injections and at 6 hours, 1 day, and 3 days after injections. The mice were fully awake at 6 hours

after injection. Our pilot data also showed that the number of responses (face wash strokes) to von Frey filament stimulation after facial carrageenan injection peaked at 3 days after injection; hence, testing of effects of D609 was extended until this time. D609 has a long half-life of 1.5 days.[22] No gross abnormalities in locomotion or other motor behaviors were observed after D609 injection in the mice. A filament delivering ~ 1 g mechanical force (4.08 log units) was used. The mice were tested individually in a deep rectangular stainless-steel tank (60 × 40 × 25 cm). Mice were habituated to the experimental environment for 5 to 10 minutes before testing. Mechanical stimuli were applied only when the mice were in a non-locomotor state, with four paws placed on the ground, neither moving nor freezing but exhibiting sniffing behavior. A new stimulus was applied at least 30 seconds after the previous one.[24] Directed facial grooming, ie, uninterrupted series of face wash strokes by the forelimbs to the stimulated maxillary area, was used as an indicator of unilateral facial pain.[25] The total number of face wash strokes after 20 stimuli was noted for each mouse, and the mean and standard deviation of each group calculated. Twenty stimuli were delivered in each mouse to obtain a sufficient number of responses to reduce variability between individual animals in each group. Possible differences between the means were elucidated using one-way ANOVA with Bonferroni multiple comparison post-hoc test (SPSS 12.0 for Windows software). $P < .05$ was considered significant.

Effect of PBN on Mechanical Allodynia after Facial Carrageenan Injection

A further 14 adult male Balb/c mice were used for this portion of the study. These were randomly divided into two groups of seven mice each. Mice received facial carrageenan injection and, after 3 days, an icv injection of 5 µL of PBN (Sigma, dissolved in isotonic saline at a concentration of 40 mg/mL) or vehicle control (isotonic saline). PBN has a biological half-life of approximately 130 minutes.[26] The injected amount of PBN (0.2 mg or 1.13 µmol) was based on a recent study, which showed an effect of this compound in reducing inflammatory pain 6 hours after intrathecal or icv injection in rats,[27] and took into consideration the difference in brain weight between rats and mice. Mice were assessed for behavioral responses to von Frey filament stimulation on the third post-facial carrageenan injection day, before and at 6 hours after the icv injection. The total number of face wash strokes after 20 stimuli was noted for each mouse, and the mean and standard deviation of each group were calculated. Possible statistical differences between mean were assessed using *t* tests. $P < .05$ was considered significant.

ASMase Activity and PtdCho-PLC Activity Assay after ICV D609 Injection

A further 21 Balb/c mice were used in this portion of the study to examine the enzymatic activity of different portions of the brain after icv D609 injection. The mice were randomly divided into three groups of seven mice each. The first group received an icv injection of 10 nmol D609 and right facial carrageenan injection; the second group received an icv injection of 5 µL of isotonic saline plus right facial carrageenan injection; and the last group did not receive any treatment (control). The dose of D609 used was the same as that found to be effective in reducing mechanical allodynia after icv injection (see below). Mice were deeply anesthetized with an intraperitoneal injection of 0.2 to 0.3 ml of 0.3% Nembutal and sacrificed by decapitation 3 days after injections. The left and right halves of the closed part of medulla oblongata containing the spinal trigeminal nucleus, the left and right ventral posterior nucleus of the thalamus, and the left and right primary somatosensory cortex including the approximate location of the barrel field were dissected out by reference to a rat brain atlas.[28] These sites are the locations of relay neurons along the somatosensory pathway from the orofacial region,[29] and could be potential sites of action of icv delivered D609.

The tissues were homogenized in 0.25% Triton X-100, centrifuged (10,000 g for 5 minutes at 4°C), and protein concentration of the supernatant determined by Bio-Rad protein assay (Bio-Rad, Hercules, CA).[30] ASMase and PtdCho-PLC activity assays were carried out using commercial kits (Molecular Probes, Eugene, OR). The reaction mix for PtdCho-PLC activity assay contained 400 µM Amplex Red reagent, 2 U/mL HRP, 0.2 U/mL choline oxidase, 8 U/mL alkaline phosphatase, and 1 mM phosphatidylcholine. The reaction mix for the ASMase activity assay contained 100 µM Amplex Red reagent, 2 U/mL HRP, 0.2 U/mL choline oxidase, 8 U/mL of alkaline phosphatase, and 0.25 mM sphingomyelin. The reaction mixtures were incubated at 37°C for 30 minutes in the dark, and fluorescence excited at 530 nm and detected at 590 nm using a microplate reader (Tecan, Grödig, Salzburg, Austria). Each assay was performed in duplicate and results normalized to protein concentration. Possible differences between

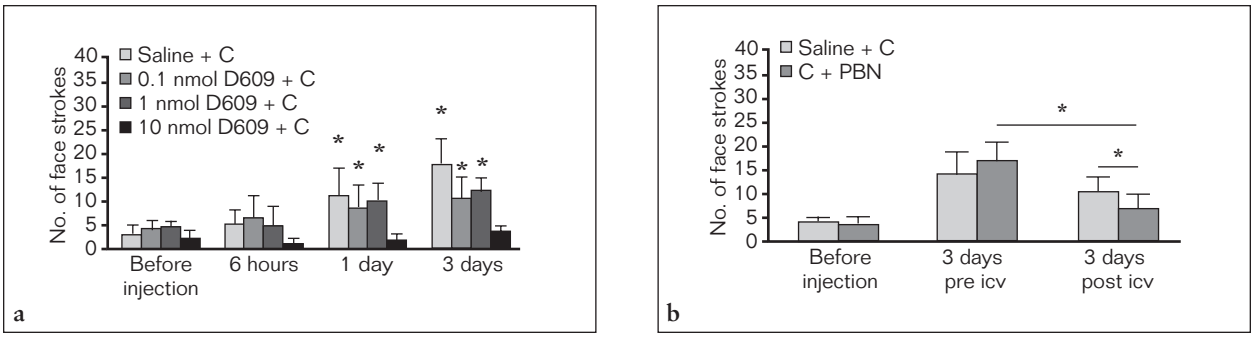

Fig 1 *(a)* Number of face wash strokes after von Frey filament stimulation to the face, before injection, and at 6 hours, 1 day, and 3 days after icv D609/isotonic saline injection plus facial carrageenan injection. The mean and standard deviation are indicated. Significant reduction in responses was observed at 1 day and 3 days after injection of 10 nmol D609, compared to isotonic saline-injected mice, or mice injected with lower doses of D609. *Indicates significant difference by one-way ANOVA test with Bonferroni multiple comparison post-hoc test ($P < .05$). *(b)* Responses to von Frey filament stimulation on the third post-facial carrageenan injection day: before icv injections and 6 hours after icv PBN/isotonic saline injections. The mean and standard deviation of responses are indicated. Significantly fewer responses were observed after injection of 1.13 μmol PBN, compared to before PBN injection. Mice injected with PBN also showed significantly fewer responses, compared to mice injected with isotonic saline. *Indicates significant difference by t-test ($P < .05$). *C* = carrageenan injection.

the means were elucidated using one-way ANOVA with Bonferroni multiple comparison post-hoc test (SPSS 12.0 for Windows software). $P < .05$ was considered significant.

Results

Effect of D609 Injection on Mechanical Allodynia Induced by Facial Carrageenan Injection

Mice injected with isotonic saline plus carrageenan showed significantly increased responses at 1 day (11.2 ± 5.4 responses) and 3 days (17.5 ± 5.3 responses) post-injection, compared to the same mice before injections (3.0 ± 2.0 responses), indicating mechanical allodynia after facial carrageenan injection (Fig 1a).

Mice injected with 10 nmol D609 plus carrageenan showed significantly fewer responses compared to mice injected with isotonic saline plus carrageenan at 1 day (1.6 ± 1.6 responses compared to 11.2 ± 5.4 responses, respectively) and 3 days (3.4 ± 1.4 responses compared to 17.5 ± 5.3 responses) post-injection. No significant difference in responses was observed between mice injected with the two lower doses of D609 (ie, 1 nmol and 0.1 nmol), and isotonic saline-injected controls (Fig 1a).

Effect of PBN on Mechanical Allodynia Induced by Facial Carrageenan Injection

Mice injected with carrageenan showed significantly increased responses on the third day after injection. As shown in Fig 1b, the number of responses decreased significantly at 6 hours after icv injection of PBN (6.6 ± 3.1 responses, compared to 16.7 ± 3.9 responses before PBN injection). In contrast, no significant decrease was found after icv injection of isotonic saline (10.1 ± 3.0 responses, compared to 14.1 ± 4.6 responses before isotonic saline injection). There was also a significant difference between PBN-injected mice and isotonic-saline injected mice at 6 hours after icv injection (Fig 1b).

ASMase Activity and PtdCho-PLC Activity Assay After ICV D609 Injection

No significant differences in PtdCho-PLC activity were observed between D609 plus carrageenan-injected mice, isotonic saline plus carrageenan-injected mice, and untreated mice (Fig 2a). Significantly decreased ASMase activity was observed in all investigated parts of the brain, ie, brainstem, thalamus, and cortex in the D609 plus carrageenan-injected mice, compared to the untreated mice and vehicle-injected mice (Fig 2b).

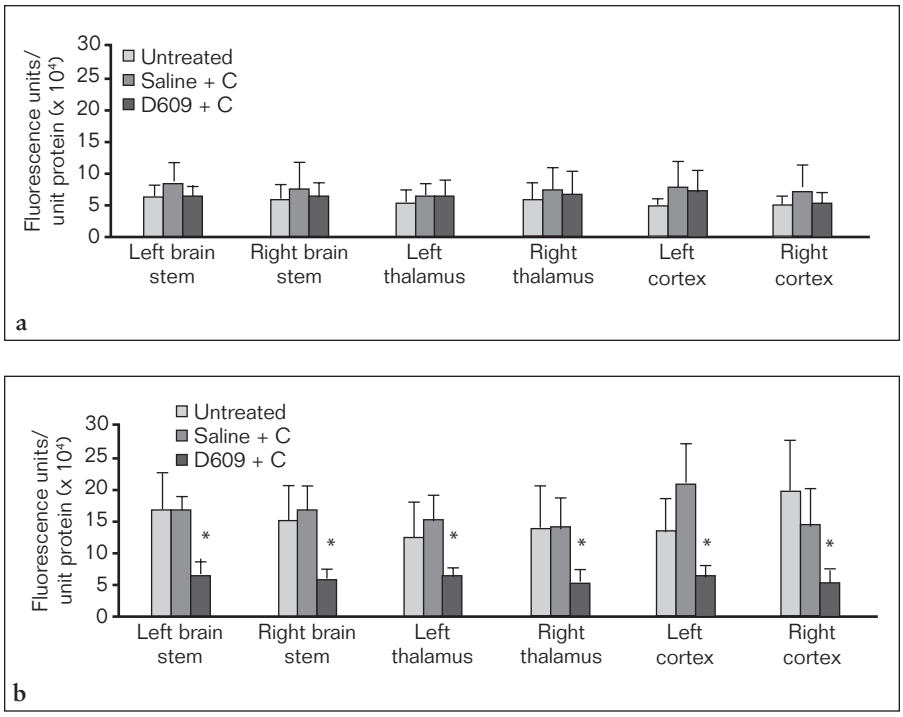

Fig 2 *(a)* PtdCho-PLC activity in different parts of the brain. No significant difference in PtdCho-PLC activity was found between D609 plus carrageenan-injected mice, isotonic saline plus carrageenan injected, and untreated mice. *(b)* ASMase activity assay in different parts of the brain. The icv injection of 10 nmol D609 resulted in significant reduction in fluorescence readings in all brain areas examined. *Indicates significant difference by one-way ANOVA test with Bonferroni multiple comparison post-hoc test ($P < .05$). C = carrageenan injection.

Discussion

The present study was carried out to elucidate the possible antiallodynic effect of D609 and PBN in a mouse model of orofacial pain. Carrageenan was injected into the subcutaneous tissue over the right maxilla, which is assumed to result in inflammation and also activation of neural elements in the maxillary nerve, the right spinal trigeminal nucleus, the left ventral posterior nucleus of thalamus, and the left primary somatosensory cortex.[29] A previous study has shown that partial trigeminal injury in mice produces persistent pain behaviors, activation of astrocytes and microglia in the ipsilateral caudal medulla and persistent satellite cell reaction in the ganglion.[31] In the present study, significantly increased responses indicating mechanical allodynia to von Frey filament stimulation of the maxillary skin was detected in mice after facial carrageenan injection. Mice treated with icv injection of 10 nmol D609 plus facial carrageenan injection showed significantly fewer responses to von Frey filament stimulation compared to controls treated with icv isotonic saline plus facial carrageenan injection at 1 day and 3 days after injection, indicating reduced mechanical allodynia.

One possibility is that D609 exerts its anti-allodynic effect due to inhibition of PtdCho-PLC. The latter hydrolyzes phosphatidylcholine to produce DAG, which is an important regulator of protein kinase C (PKC).[32] PKC has been shown to affect nociceptive transmission. For example, intrathecal injection of PKC inhibitors significantly reduces formalin-induced central sensitization[33] or substance P-mediated hyperalgesia[34] in rats. The possibility that D609 exerts its actions by inhibiting PtdCho-PLC is, however, not supported by our results, which showed no change in PtdCho-PLC activity in different parts of the brain after icv D609 injection.

A second possibility is that D609 reduces mechanical allodynia by inhibiting ASMase activity. This enzyme catalyzes the hydrolysis of sphingomyelin and generates phosphocholine and ceramide. It is proposed that ceramide modulates pain-related behavior via a proinflammatory cytokine tumor necrosis factor α-mediated mechanism in small-fiber sensory neuropathies.[7] In HeLa cells, levels of mitochondrial ceramide are increased as early as 2 hours after ultraviolet (UV) irradiation and remain elevated after 6 hours,[35] and this increase is inhibited by D609. Inhibition of ceramide generation correlates with protection of the mitochondrial transmembrane potential and prevention of cytochrome C release and generation of ROS following UV irradiation.[35] Ceramide and its metabolites including sphingosine 1-phosphate, or ceramide 1-phosphate, are important signaling molecules that not only facilitate neurotransmission[36,37] but also modulate PLA_2 and cyclooxygenase activities, which regulate the production of

free radicals and ROS levels[37] and may contribute to the pathogenesis of neuropathic and inflammatory pain in rats.[7,38] In addition, sphingomyelinase itself can induce nitric oxide release/inducible nitric oxide synthase mRNA expression in cultured rat brain microglia[39] and could have a pro-nociceptive effect. This possibility is supported by the present finding of significantly reduced ASMase activity in all the brain regions sampled after D609 injection. It is to be noted, however, that the regions sampled (medulla, thalamus, somatosensory cortex) process non-nociceptive information as well as possibly nociceptive signals.

A third possibility is that D609 modulates allodynia due to its antioxidant properties. D609 is one of the derivatives of xanthates which scavenge hydroxyl radicals and hydrogen peroxide and reacts with electrophilic products of lipid oxidation in a manner similar to glutathione,[15,20] and has been shown to reduce free radical-induced changes in synaptosomal lipid peroxidation, protein oxidation, intracellular ROS accumulation, and apoptosis.[20,21,40] The notion that CNS free radicals are important in inducing mechanical allodynia is supported by the present observation that injection of the spin trap/free radical scavenger PBN significantly reduced mechanical allodynia. Intrathecal or icv injection of PBN has also been shown to reduce secondary hyperalgesia caused by intradermal injection of capsaicin into the rat paw.[27] A recent study showed that icv injection of a peroxynitrite scavenger reduces mechanical allodynia in a mouse model of facial pain induced by carrageenan injection.[10] Other studies have shown reduction in inflammatory pain after intrathecal injection of the superoxide dismutase mimetic M 40403[41] or TEMPOL.[13,14]

In conclusion, icv injections of D609 and PBN were effective in reducing mechanical allodynia after facial carrageenan injection-induced pain. Together, the results point to a role of CNS sphingolipids and/or free radicals in orofacial pain.

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