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Sphingosine-1-phosphate acting via the $S1P_1$ receptor is a downstream signaling pathway in ceramide-induced hyperalgesia

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ABSTRACT

Ceramide is a potent pro-inflammatory sphingolipid recently shown to exert potent hyperalgesic responses in rats. Once generated, ceramide is converted by sphingosine kinase (SphK) 1 and/or 2 to one of its active metabolite sphingosine-1-phosphate (S1P), which in turn signals through G-protein coupled S1P receptors. The objectives of this paper were to define whether ceramide-induced hyperalgesia is driven by S1P. Our results show that intraplantar injection of ceramide in rats led to a time-dependent development of thermal hyperalgesia that was associated with an increase in tumor necrosis factor-α (TNF-α) in paw tissues. The development of hyperalgesia was significantly attenuated by a soluble TNF receptor I. TNF-α is known to activate SphK1, thus S1P production, and our results demonstrate that, the development of hyperalgesia was attenuated in a dose-dependent fashion by a well characterized inhibitor of SphK1 and SphK2 (SK-I) and by a murine monoclonal anti-S1P antibody (LT1002). LT1017, the isotype-matched control monoclonal antibody for LT1002, had no effect. Our results further demonstrate that S1P contributes to the development of hyperalgesia via the S1P receptor 1 subtype ($S1PR_1$), since responses were blocked by a well characterized $S1PR_1$ antagonist, W146, but not by its inactive enantiomer, W140. Collectively, these results provide mechanistic evidence implicating the S1P-to-$S1PR_1$ pathway as a downstream signaling pathway in ceramide-induced hyperalgesia. Targeting S1P may be a novel therapeutic approach in pain management.

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Ceramide, a potent proinflammatory sphingolipid [9], is generated from enzymatic hydrolysis of sphingomyelin by sphingomyelinases (sphingomyelin pathway) and from *de novo* synthesis by serine palmitoyltransferase and ceramide synthase (*de novo* pathway) [5]. Besides its well-established role in inflammation, a potential role of ceramide in peripheral sensitization and hyperalgesia is documented by the observations that intradermal injection of ceramide in rats produces dose-dependent mechanical hyperalgesia and thermal hyperalgesia [7,11] and that tumor necrosis factor-α (TNF-α) mediated peripheral sensitization following intraplantar injection in rats is driven at least in part by *in situ* formation ceramide [11]. Furthermore, and as shown in *in vitro* studies, ceramide increases the excitability of small diameter sensory neurons and is an important mediator in nerve growth factor (NGF)-induced sensitization of sensory neurons [32]. We have recently reported that one pathway engaged by ceramide in promoting hyperalgesia is through a p38 kinase and NF-κB dependent induction of cyclooxygenase 2 and increased levels of prostaglandin E_2 [7]. Once generated, the steady-state availability of ceramide is regulated by several enzymes including ceramidases that convert ceramide to sphingosine and sphingosine kinase 1 and/or 2 [14,27] that convert sphingosine to the biologically active sphingosine-1-phopshate (S1P), which in turn signals through G-protein coupled S1P receptors (S1PRs) [14,26,27]. Five S1PRs subtypes have been identified to date [14,26,27]. A potential role of S1P in peripheral sensitization and hyperalgesia is documented by the observations that S1P directly increases the excitability of rat sensory neurons *in vitro* [31,32] at least in part via activation of $S1PR_1$ [4] and that S1P, derived following bioconversion of ceramide, contributes to NGF-induced excitation of rat sensory neurons [17,33]. Furthermore and as shown in our recent studies, intraplantar injection of S1P in rats led to the development of hyperalgesia; activation of $S1P1R_1$ and subsequent formation of peroxynitrite were found to contribute to S1P-mediated hyperalgesia [6]. Subsequent elegant studies by Mair and co-workers [13] revealed that genetic deletion of $S1PR_1$ receptors in neurons expressing the nociceptor-specific $Na_v1.8$ promoter abrogated hyperalgesia following intraplantar injection of S1P, supporting the role of the S1P to $S1PR_1$ pathway in hypersensitivity. Collectively these observations prompted us

Abbreviations: SphK, sphingosine kinase; S1P, shingosine-1-phosphate; $S1PR_1$, shingosine-1-phosphate receptor subtype 1; S1PR, G-protein coupled S1P receptors; SK-I, 2-(*p*-hydroxyanilino)-4-(*p*-chlorophenyl) thiazole.

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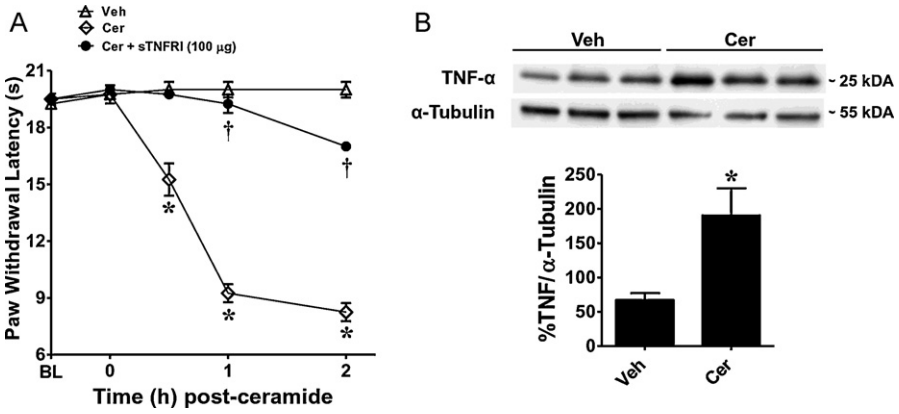

Fig. 1. Role of TNF-α in ceramide-induced hyperalgesia. (A) When compared to rats administered intraplantar sTNFR1vehicle and ceramide vehicle (Veh, △), an intraplantar injection of ceramide (10 μg, ◊) led to a time-dependent development of thermal hyperalgesia that was largely attenuated by 100 μg (●) of sTNFR1. The development of hyperalgesia was associated with increased TNF-α expression in rat paw as shown in 2 h paw lysates (B). Results are expressed as mean ± SEM. For western blot, data from 3 rats are normalized to α-tubulin acquired within the same exposure and analyzed by unpaired *t*-test. Behavioral data from 4 rats were analyzed by ANOVA with Bonferroni *post hoc* test. **P* < 0.001 *vs.* Veh and †*P* < 0.001 *vs.* Cer.

to consider and test whether ceramide induces hyperalgesia via formation of S1P and if so whether the S1P receptor 1 subtype contributes to the ceramide-to-S1P signaling pathway.

Male Sprague Dawley rats (200–220 g) were purchased from Harlan (Indianapolis, IN, USA), housed 3–4 per cage, and maintained in a controlled environment (12 h light/dark cycles) with food and water available *ad libitum*. All experiments were performed in accordance with the International Association for the Study of Pain and the National Institutes of Health guidelines on laboratory animal welfare and the recommendations by Saint Louis University Institutional Animal Care and Use Committee. C_2-ceramide (D-erythro-Sphingosine, N-Acetyl) and SK-I ([2-(*p*-hydroxyanilino)-4-(*p*-chlorophenyl) thiazole] were purchased from Calbiochem (La Jolla, CA, USA). W146 and W140 were purchased from Cayman Chemical (Ann Arbor, MI, USA). Unless otherwise noted, all other chemicals and reagents were from Sigma–Aldrich (St. Louis, MO, USA). SK-I, W146, W140 (or their respective vehicle, 20% DMSO in saline), the soluble TNF receptor I (sTNFR1; Amgen, Thousand Oaks, CA), the murine anti-S1P monoclonal antibody (LT1002) or its isotype-matched control monoclonal antibody (LT1017) or their vehicle (saline) were given by intraplantar injection into the right hindpaw of rats 15 min before intraplantar injections of ceramide or its vehicle, DMSO. All drugs were injected in a 5 μl injection volume using a Hamilton gauge needle (3 1/2″) in lightly anesthetized rats [CO_2 (80%)/O_2 (20%)]. Hyperalgesic responses to heat were determined by the Hargreaves' Method using a Basile Plantar Test (Ugo Basile; Comeria, Italy) [10] with a cut-off latency of 20 s employed to prevent tissue damage. Rats were individually confined to Plexiglas chambers and allowed to habituate/acclimate for 15 min prior to behavioral testing. A mobile infrared generator was positioned to deliver a thermal stimulus directly to an individual hindpaw from beneath the chamber. The withdrawal latency period of injected paws was determined with an electronic clock circuit and thermocouple. Two readings were acquired for each paw to determine a mean latency for each animal. Thermal hyperalgesia results are the mean latency for each group and expressed as Paw Withdrawal Latency (s). All experiments were conducted with the experimenters blinded to treatment conditions. Behavioral testing was done at baseline (defined as BL) in all rats prior to drug/vehicle administration, 15 min post-drug/vehicle administration (defined at time 0) and subsequently at different time points after intraplantar injection of ceramide or vehicle. For western blotting, plantar lysates ($n = 3$) were obtained from 2 h flash-frozen plantar soft tissue and the relative TNF-α expression was determined by chemiluminescent immunoblot analysis as previously described [15,30] using a primary rabbit anti-TNF-α (R & D Systems; Minneapolis, MN, USA) and a secondary goat anti-rabbit-HRP antibody (Thermo Fisher Scientific; Rockford, IL, USA). Images were acquired using Fujifilm LAS-3000 imaging system and Image Reader LAS-3000 v2.2 (Fujifilm, Japan) software at 60 s incremental exposures, standard sensitivity for 13 min. The densities of the TNF-α band for each animal was measured from the original acquired images and normalized to α-tubulin. A multiply mask was applied to the entire western blot image in Adobe Photoshop 6.0 (San Jose, CA, USA) for presentation purposes only. The differences in TNF-α expression were analyzed by one-tailed unpaired *t*-test and the differences in levels of thermal hyperalgesia were assessed by two-tailed, two-way analysis of variance (ANOVA) with Bonferroni *post hoc* comparisons to ceramide-treated animals. Significance was accepted at $P < 0.05$.

As can be seen in Fig. 1A and when compared to the vehicle group (DMSO), intraplantar injection of C_2-ceramide (10 μg, $n = 4$), given at a dose previously shown to elicit mechanical and thermal hyperalgesia [7,11], led to a time-dependent development of thermal hyperalgesia that peaked by 2 h and was associated with increases levels of TNF-α as detected by western blot ($n = 3$, Fig. 1B). The development of hyperalgesia was attenuated by the sTNFRI (100 μg, $n = 4$, Fig. 1A). When tested alone and compared to rats that received an intraplantar injection of the vehicle used for ceramide, sTNFRI (100 μg, $n = 4$; Fig. 1A) had no effect on baseline withdrawal latencies. These results suggest that TNF-α contributes to the development of ceramide-induced hyperalgesia. TNF-α sensitizes peripheral nociceptors [3,22,23] and interestingly TNF-α mediated peripheral sensitization is blocked by inhibitors of ceramide biosynthesis suggesting a potential contribution of the ceramide metabolic pathway in TNF-α effects [11]. Whether S1P is involved in TNF-α mediated peripheral sensitization is not known but is a likely possibility. TNF-α activates SphK1 and increases the formation of S1P, which has been implicated in several inflammatory disease states [24,26]. Interestingly, TNF-α does not appear to regulate SphK2 [24,26]. The mechanisms whereby SphK1 is regulated by TNF-α are not known for sure, but TNF-α induces the phosphorylation of serine 225 of SphK1 resulting in conformational or electrostatic changes that in turn allow the enzyme to remain at the plasma membrane, thus increasing the identification and thus subsequent metabolism of its substrate, sphingosine [25]. As can be seen in Fig. 2 and when compared to the vehicle group (rats in this group received an intraplantar injection of the vehicle used for SK-I followed by intraplantar injection of ceramide), intraplantar

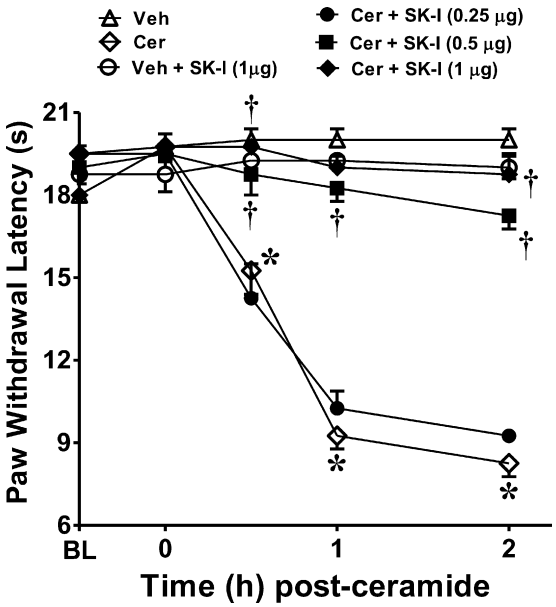

Fig. 2. Role of sphingosine kinases 1 and 2 in ceramide-induced hyperalgesia. When compared to rats administered intraplantar SK-I vehicle and ceramide vehicle (Veh, △), an intraplantar injection of ceramide (10 μg, ◊) led to a time-dependent development of thermal hyperalgesia that was attenuated in a dose-dependent manner by intraplantar SK-I given at 0.25 μg (●), 0.5 μg (■), or 1 μg (♦). Given alone, SK-I (○) had no effect. Results are expressed as mean ± SEM for 4 rats and analyzed by ANOVA with Bonferroni *post hoc* test where $^{*}P < 0.001$ *vs.* Veh and †$P < 0.001$ *vs.* Cer.

injection of SK-I, a well characterized, potent, competitive, and reversible specific inhibitor of SphK 1 and 2 activity and thus of S1P formation [8], attenuated in a dose-dependent fashion (0.25–1 μg, $n = 4$) ceramide-induced thermal hyperalgesia (Fig. 1). Doses of SK-I were chosen from previous studies [5,8,12,29]. When tested alone and compared to rats that received an intraplantar injection of the vehicle used for ceramide, SK-I (1 μg, $n = 4$, Fig. 1) had no effect on baseline withdrawal latencies. These results implicate the contribution of the SphK-mediated S1P pathway in the development of ceramide-induced hyperalgesia.

With full recognition that our results do not demonstrate a direct role of TNF-α in the activation of SphK1; based upon the known link between ceramide, TNF-α, and SphK1 activation, we hypothesize that ceramide-derived TNF-α formation provides a stimulus in SphK1 activation and thus S1P formation. The role of S1P was confirmed by the use of a well characterized murine monoclonal anti-S1P antibody, LT1002 [18]. LT1002 has high affinity and specificity for S1P and does not cross react with structurally related lipids [18] thus offering a unique pharmacological tool to dissect the roles of S1P in pathophysiological settings. As shown in Fig. 3A, formation of S1P contributes to the development of ceramide-induced hyperalgesia since LT1002 (242 μg, $n = 3$) but not the isotype-matched control monoclonal antibody LT1017 (286 μg, $n = 3$) blocked the hyperalgesic responses to ceramide. When tested alone and compared to rats that received an intraplantar injection of the vehicle used for ceramide, LT1002 or LT1017 (242 μg and 286 μg, respectively, $n = 3$, Fig. 3A) had no effect on baseline withdrawal latencies. The potential contribution of the S1P-to-$S1PR_1$ signaling pathway was underscored by the findings that the development of ceramide (10 μg, $n = 4$) induced thermal-hyperalgesia was blocked in a dose-dependent manner by the well-characterized $S1PR_1$ antagonist, W146 (0.1–0.6 μg, $n = 4$, Fig. 3B) [19,21] but not by W140 (1.2 μg, $n = 4$ Fig. 3C), its inactive S-enantiomer with doses chosen from previous studies [19,21].

Collectively, our results support a role S1P/$S1PR_1$ pathway in signaling pathways engaged by ceramide in peripheral sensitization and hyperalgesia (Fig. 4); we are not excluding the potential contribution(s) of other S1PRs subtypes. This will be the focus of future investigations. Ceramide and S1P are emerging as important signaling molecule in the development of central sensitization. Indeed, ceramide has been implicated in the development of central sensitization observed in a model of carrageenan-induced orofacial nociception [28] and as recently reported by our group in the development of central sensitization associated with the induction of morphine-induced hyperalgesia and antinociceptive tolerance [2,16] following bioconversion to S1P [15]. In turn, S1P itself has shown to be a potent inducer of hypersensitivity [4,6,13,17,31,33].

Understanding the relative and preferential contribution(s) of the kinases (SphK1 *vs.* SphK2) involved in the formation of S1P from ceramide, as well as unraveling the contribution(s) of S1PRs

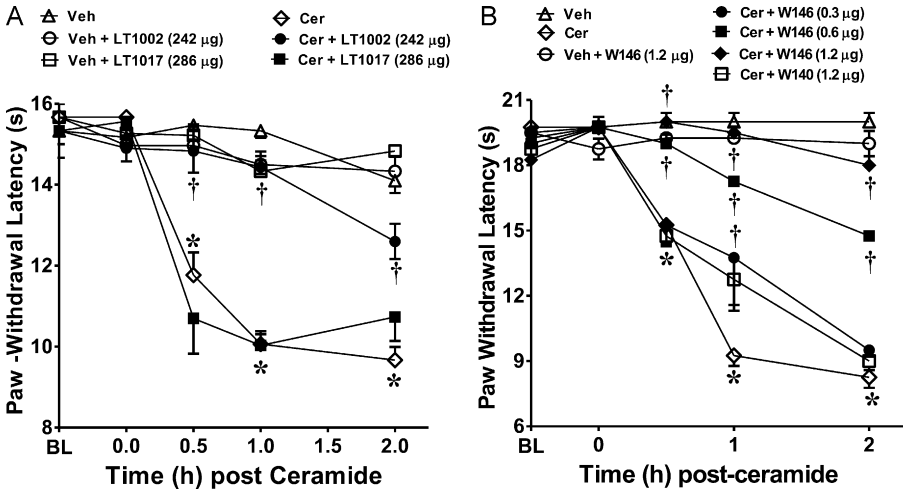

Fig. 3. Role of the S1P to $S1P_1$ receptor pathway in ceramide-induced hyperalgesia. (A) When compared to rats administered intraplantar LT1002 or LT1017 vehicle and ceramide vehicle (Veh, △, $n = 3$), an intraplantar injection of ceramide (10 μg, ◊, $n = 3$) led to a time-dependent development of thermal hyperalgesia that was attenuated by the anti-S1P antibody LT1002 (242 μg, ●, $n = 3$), but not by LT1017 (286 μg, ■, $n = 3$). Given alone, LT1002 (○, $n = 3$) and LT1017 (□, $n = 3$) had no effect. (B) When compared to rats administered intraplantar W146 or W140 vehicle and ceramide vehicle (Veh, △, $n = 4$), an intraplantar injection of ceramide (10 μg, ◊, $n = 4$) led to a time-dependent development of thermal hyperalgesia that was inhibited by the $S1P_1$ receptor antagonist, W146, at 0.3 μg (●, $n = 4$), 0.6 μg (■, $n = 4$), or 1.2 μg (♦, $n = 4$), but not by W140 (1.2 μg, □, $n = 4$). Given alone, W146 (○, $n = 4$) had no effect. Results are expressed as mean ± SEM for (n) rats and analyzed by ANOVA with Bonferroni *post hoc* test where $^{*}P < 0.001$ *vs.* Veh and †$P < 0.001$ *vs.* Cer.

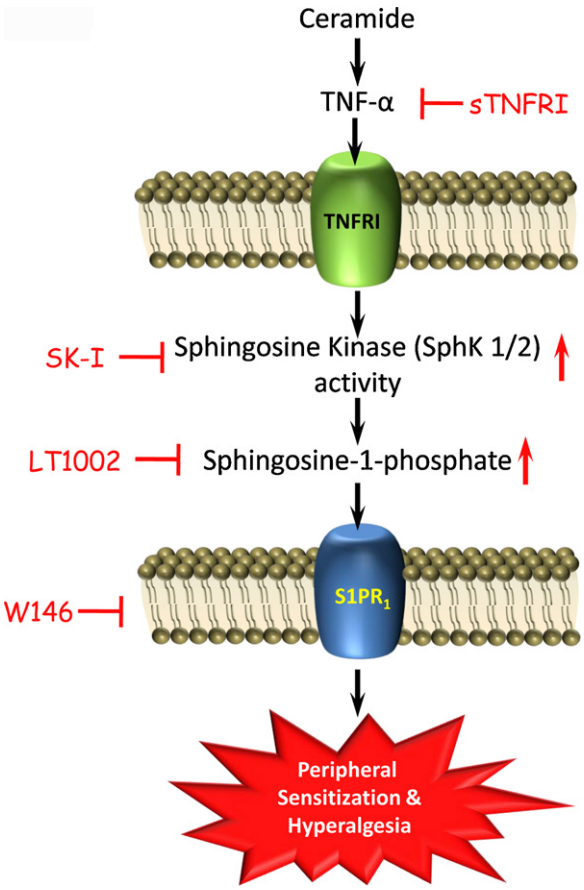

Fig. 4. S1P contributes to hyperalgesic responses to ceramide. Intraplantar injection of ceramide induces formation of TNF-α, which in turn, activates sphingosine kinase 1 and/or 2 leading to S1P formation and subsequent activation of $S1PR_1$.

subtypes involved in pain of several etiologies, will undoubtedly provide the potential for a multilevel therapeutic approach in the development of novel analgesics. The relative advantage of one approach *vs.* the other will ultimately be dictated by the side effect profile of each approach. In this setting, anti-S1P antibodies such as Sphingomab, currently in Phase 2 clinical trials for cancer [20] or novel agents targeting the S1PRs such as Fingolimod, recently approved by FDA for multiple sclerosis [1] may offer promise.

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Conflicts of interest: The authors declare no conflicts of interest.

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