

Sphingosine 1-Phosphate Mediates Hyperalgesia via a Neutrophil-Dependent Mechanism

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Abstract

Novel classes of pain-relieving molecules are needed to fill the void between non-steroidal anti-inflammatory agents and narcotics. We have recently shown that intraplantar administration of sphingosine 1-phosphate (S1P) in rats causes peripheral sensitization and hyperalgesia through the S1P₁ receptor subtype (S1PR₁): the mechanism(s) involved are largely unknown and were thus explored in the present study. Intraplantar injection of carrageenan in rats led to a time-dependent development of thermal hyperalgesia that was associated with pronounced edema and infiltration of neutrophils in paw tissues. Inhibition of 1) S1P formation with SK-I, a sphingosine kinase inhibitor, 2) S1P bioavailability with the S1P blocking antibody Sphingomab, LT1002 (but not its negative control, LT1017) or 3) S1P actions through S1PR₁ with the selective S1PR₁ antagonist, W146 (but not its inactive enantiomer, W140) blocked thermal hyperalgesia and infiltration of neutrophils. Taken together, these findings identify S1P as an important contributor to inflammatory pain acting through S1PR₁ to elicit hyperalgesia in a neutrophil-dependant manner. In addition and in further support, we demonstrate that the development of thermal hyperalgesia following intraplantar injection of S1P or SEW2871 (an S1PR₁ agonist) was also associated with neutrophilic infiltration in paw tissues as these events were attenuated by fucoidan, an inhibitor of neutrophilic infiltration. Importantly, FTY720, an FDA-approved S1P receptor modulator known to block S1P-S1PR₁ signaling, attenuated carrageenan-induced thermal hyperalgesia and associated neutrophil infiltration. Targeting the S1P/S1PR₁ axis opens a therapeutic strategy for the development of novel non-narcotic anti-hyperalgesic agents.

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Introduction

One-quarter of Americans over the age of 20 suffer from some sort of persistent pain [1]. Current treatment options, such as non-steroidal anti-inflammatory agents and narcotics, result in deleterious side-effects making them unattractive options for persistent use [2]. Therefore, novel classes of pain-relievers are severely needed. In addition to their pro-inflammatory roles [3], sphingolipids including ceramide [4–10] and sphingosine 1-phosphate (S1P) [6,7,10–15] are emerging as important modulators of pain.

S1P derived from the conversion of ceramide to sphingosine by ceramidase, and is a product of the phosphorylation of sphingosine by sphingosine kinase isoenzymes, plays an important role in peripheral and central sensitization. S1P resulting from ceramide bioconversion has been shown to contribute to NGF-induced excitation of rat sensory neurons [11] and is required for the development of ceramide-induced peripheral sensitization following intraplantar injection of ceramide in rats [7]. Furthermore, S1P has the ability to directly increase the excitability of rat sensory neurons *in vitro* [14] and cause thermal hyperalgesia following intraplantar injection in rats [12]. However, apart from

S1P's ability to directly increase nociceptor sensitivity *in vitro* and *in vivo* [13] and our previous reports that S1P exerts its actions at least in part via the upregulation of peroxynitrite [12], S1P's mechanism of action remains largely uninvestigated.

To date, five subtypes of G-protein coupled S1P receptors (S1PR_s) have been identified: S1PR_{1–5} [16]. These receptors are differentially expressed on all cell types and can bind to multiple different heterotrimeric G-proteins [16,17], thereby having varying effects, depending on the signaling cascade they activate. In order to examine the signaling pathways and mechanisms involved in S1P-mediated hyperalgesia it is important to identify the receptor subtype(s) involved. We have focused our studies on S1PR₁ as we have shown this receptor subtype to be of particular importance in S1P-mediated peripheral hyperalgesia [12]. In addition, enhanced excitability in peripheral sensory neurons in response to S1P been shown to occur, at least in part, through the activation of S1PR₁ [18] and S1P hypersensitivity is significantly reduced in mice with a conditional nociceptor-specific deletion of S1PR₁ [13] or those with local knockdown of S1PR₁ in the DRG [19].

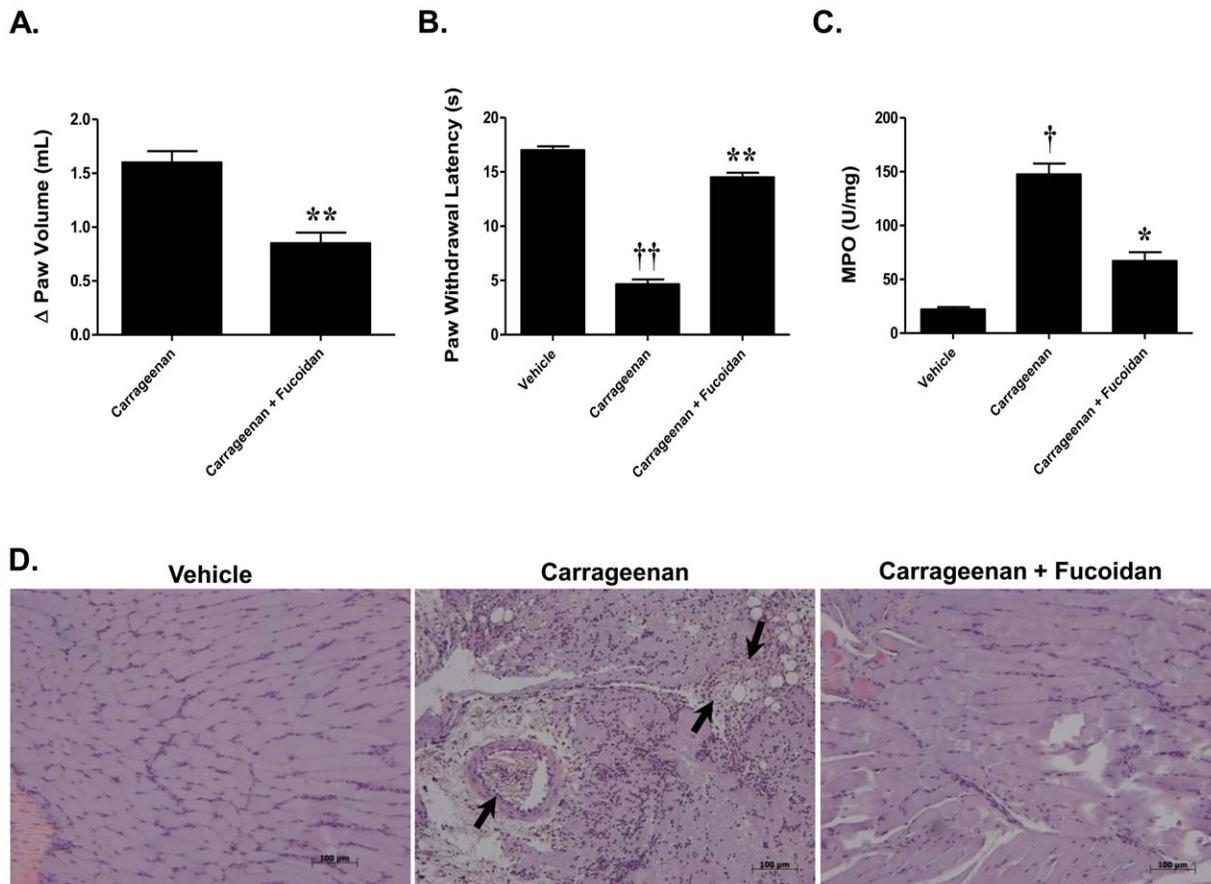


Figure 1. Carrageenan injection leads to an increase in neutrophil infiltration that is attenuated by fucoidan. A–C) Intraplantar injection of carrageenan (1%) led to a time-dependent development of thermal hyperalgesia that was accompanied by an increase in paw volume and an increase in myeloperoxidase activity. All were blocked by fucoidan (40 mg/kg). D) The increased myeloperoxidase activity in response to carrageenan injection correlated with pathological changes as well as a marked increase in neutrophilic infiltration as indicated by H&E staining. Fucoidan (40 mg/kg) attenuated this response. Results are expressed as mean \pm SEM for 6 rats and analyzed by student's unpaired *t* test for paw volume, two-way repeated measures ANOVA with Bonferroni *post hoc* test for behavior and one-way ANOVA with Dunnett's *post hoc* test for MPO, where * $P < 0.01$, ** $P < 0.001$ vs. carrageenan; † $P < 0.01$, †† $P < 0.001$ vs. vehicle. doi:10.1371/journal.pone.0055255.g001

Another important action of S1P is its ability to enhance immune cell migration [20]. Specifically, S1P via activation of S1PR₁ upregulates the expression of the adhesion molecules ICAM-1 and E-selectin on the surface of endothelial cells [21–24] thereby initiating neutrophilic infiltration [25,26]. Many models of inflammation-induced hyperalgesia rely on neutrophilic recruitment [27–30] and this neutrophil-dependent hyperalgesia underlies pain of several etiologies.

Taken together, we hypothesize and demonstrate herein that neutrophils contribute to the development of S1P-induced hyperalgesia acting through the S1PR₁ subtype. Targeting the S1P-to-S1PR₁ pathway may offer a novel approach in the management of pain.

Materials and Methods

Materials

S1P (sphingosine 1-phosphate), W146 (3- amino- 4- (3-hexylphenylamino)- 4- oxobutyl phosphonic acid), W140 (3-amino- 4- (3- hexylphenylamino)- 4- oxobutyl phosphonic acid), JTE-013 (N- (2, 6- dichloro- 4- pyridinyl)- 2- [1, 3- dimethyl- 4- (1-methylethyl)- 1H- pyrazolo[3, 4- b]pyridin- 6- yl]- hydrazinocarboxamide), CAY10444 (2- undecyl- thiazolidine- 4- carboxylic

acid) and FTY720 (2- amino- 2- [2- (4- octylphenyl)ethyl]- 1, 3-propanediol, hydrochloride) were all purchased from Cayman Chemical (Ann Arbor, MI). Carrageenan, fucoidan and o-dianisidine (3,3'-Dimethoxybenzidine dihydrochloride) were purchased from Sigma-Aldrich (St. Louis, MO). The SK inhibitor, SK-I [2-(*p*-hydroxyanilino)-4-(*p*-chlorophenyl) thiazole] was purchased from Calbiochem (La Jolla, CA) and the binchonic acid (BCA) assay was from Thermo-Fisher (Rockford IL). The murine monoclonal anti-S1P antibody, Sphingomab/LT1002 and its isotype mAb control, LT1017, was generated as described previously [31].

Animals

Male Sprague Dawley rats (200–220 g) were purchased from Harlan (USA) and housed 3–4 per cage and maintained in a controlled environment (12 h light/dark cycle) 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.

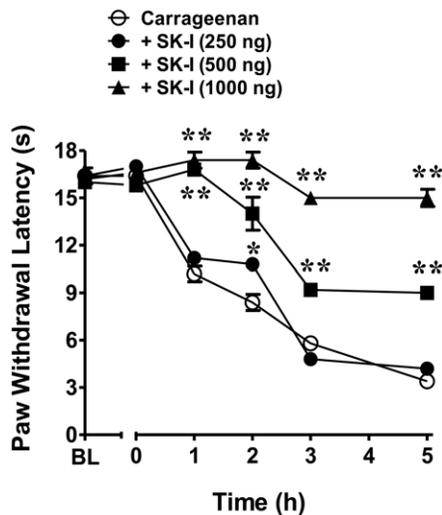


Figure 2. Carrageenan-induced thermal hyperalgesia is blocked by SK-I. Intraplantar injection of carrageenan (1%) led to a time-dependent development of thermal hyperalgesia that was attenuated in a dose-dependent manner by intraplantar injection of SK-I (250 ng, 500 ng, or 1000 ng; $n=6$). Results are expressed as mean \pm SEM and analyzed by two-way repeated measures ANOVA with Bonferroni *post hoc* test where $*P<0.01$, $**P<0.001$ vs. carrageenan. doi:10.1371/journal.pone.0055255.g002

Drug Administration

Male Sprague Dawley rats were lightly anesthetized [CO_2 (80%)/ O_2 (20%)] and given a subplantar injection of S1P (0.3 μg ; using a Hamilton gauge needle 3 $\frac{1}{2}$ ''; 5 μL) or of 1% carrageenan (100 μL) into the left hindpaw. All drugs or their vehicle (6% EtOH in saline for S1P; saline for carrageenan) were given by

intraplantar injection 30 minutes prior to intraplantar S1P or carrageenan injection unless otherwise stated. LT1002 and LT1017 were given in a volume of 40 μL while SK-I, W146, W140, JTE-013 and CAY10444 were given in a volume of 5 μL . Fucoidan was given i.p. in 200 μL saline, 30 minutes prior to S1P injection. FTY720 was given p.o. in 10% DMSO in saline, 30 min prior to carrageenan injection.

Behavioral Analysis

Behavioral testing was done with experimenter blinded to treatment conditions. Hyperalgesic responses to heat were determined by the Hargreaves' Method using a Basile Plantar Test [32] with a cut-off latency of 20 s employed to prevent tissue damage. Rats were individually confined to plexiglass chambers and allowed to habituate. A mobile unit consisting of a high intensity projector bulb 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. Results are expressed as paw-withdrawal latency(s).

Carrageenan-Induced Paw Edema

Changes in paw volume were measured as previously described [33]. Briefly, paw volume was measured with a plethysmometer (Ugo Basile, Comerio, Varese, Italy) immediately prior to the injection of carrageenan and thereafter at hourly intervals for 6 h. Edema was expressed as the increase in paw volume (mL) after carrageenan injection relative to the pre-injection value for each animal. Results are expressed as paw volume change (mL).

Histological Examination

For histopathological examination, biopsies of paws were taken 2 hours following the intraplantar injection of carrageenan, tissue from the pads of the rats hindpaw was removed with a scalpel. The

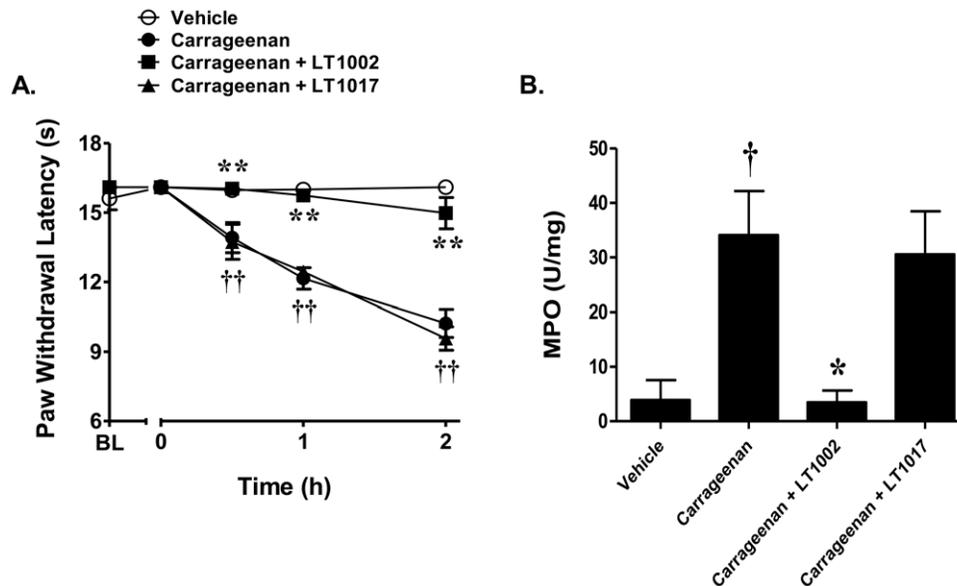


Figure 3. Inhibition of S1P attenuates carrageenan-induced thermal hyperalgesia and the recruitment of neutrophils. A) Intraplantar injection of LT1002 (484 μg , $n=6$) but not of LT1017 (572 μg ; isotype control, $n=6$) attenuated carrageenan-induced thermal hyperalgesia. B) Intraplantar injection of carrageenan led to an increase in neutrophilic recruitment as evidenced by increased levels of MPO activity in paw tissues and this was blocked by LT1002 but not LT1017. Results are expressed as mean \pm SEM and analyzed by two-way repeated measures ANOVA with Bonferroni *post hoc* test for behavior and one-way ANOVA with Dunnett's *post hoc* test for MPO, where $*P<0.01$, $**P<0.001$ vs. carrageenan; † $P<0.05$, †† $P<0.001$ vs. vehicle. doi:10.1371/journal.pone.0055255.g003

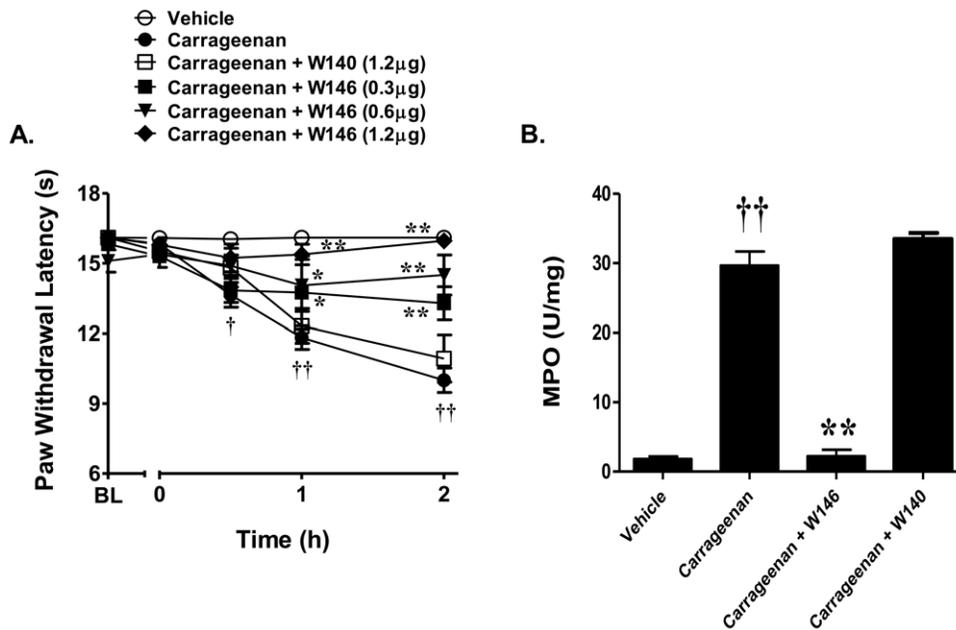


Figure 4. Blockade of S1PR₁ inhibits carrageenan-induced thermal hyperalgesia and the recruitment of neutrophils. A) Intraplantar injection of carrageenan led to a time-dependent development of thermal hyperalgesia that was blocked in a dose-dependent manner by the selective S1PR₁ antagonist, W146 (0.3–1.2 μg, n=6) but not by its inactive S-enantiomer, W140 (1.2 μg, n=6). B) W146 (1.2 μg, n=6) but not W140 (1.2 μg, n=6) attenuated neutrophilic recruitment. Results are expressed as mean ± SEM and analyzed by two-way repeated measures ANOVA with Bonferroni *post hoc* test for behavior and one-way ANOVA with Dunnett's *post hoc* test for MPO, where **P*<0.05, ***P*<0.001 vs. carrageenan; † *P*<0.01, †† *P*<0.001 vs. vehicle. doi:10.1371/journal.pone.0055255.g004

tissue slices were fixed in Dietric solution (14.25% ethanol, 1.85% formaldehyde, 1% acetic acid) for 1 week at room-temperature, dehydrated by graded ethanol and embedded in Paraplast (Sherwood Medical). Section (thickness 7 μm) were deparaffinized with xylene, stained with hematoxylin and eosin and observed in Dialux 22 Leitz microscope.

Myeloperoxidase Assay

Myeloperoxidase (MPO; a peroxidase enzyme released by neutrophils and a marker of neutrophilic infiltration [34,35]) activity was assessed by taking tissue at 2 h (time of peak inhibition). Flash-frozen plantar soft tissue was pulverized in liquid nitrogen-chilled mortar and pestle, and then homogenized in 1 mL 0.05% HTAB in 50 mM potassium phosphate buffer and kept on ice. Homogenates were sonicated with an ultrasonicator

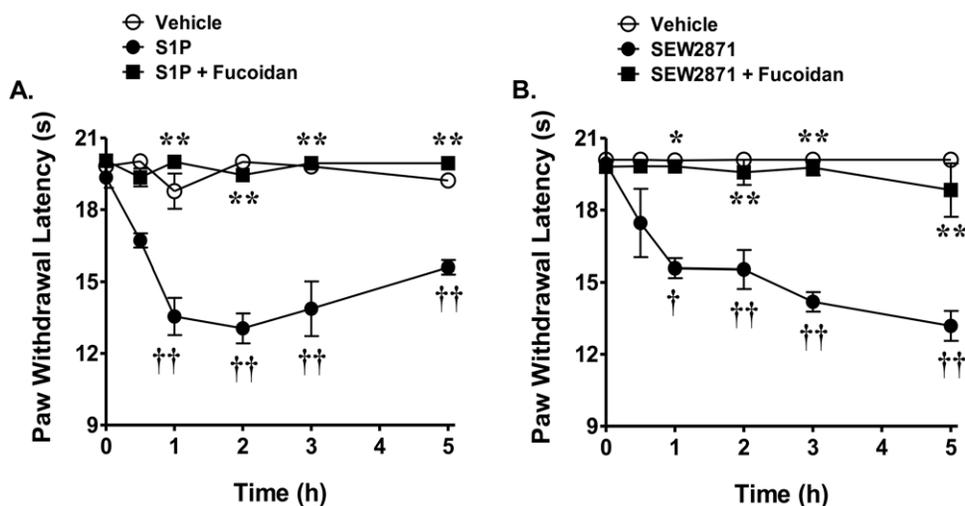


Figure 5. Fucoidan blocks S1P-induced thermal hyperalgesia. Intraplantar injection of A) S1P (0.3 μg) or B) the S1PR₁ agonist, SEW2871 (0.3 μg), led to a time-dependent development of thermal hyperalgesia that was attenuated by pretreatment with fucoidan (40 mg/kg, i.p.). Results are expressed as mean ± SEM for 6 rats and analyzed by two-way repeated measures ANOVA with Bonferroni *post hoc* test where **P*<0.05, ***P*<0.001 vs. carrageenan; † *P*<0.01, †† *P*<0.001 vs. vehicle. doi:10.1371/journal.pone.0055255.g005

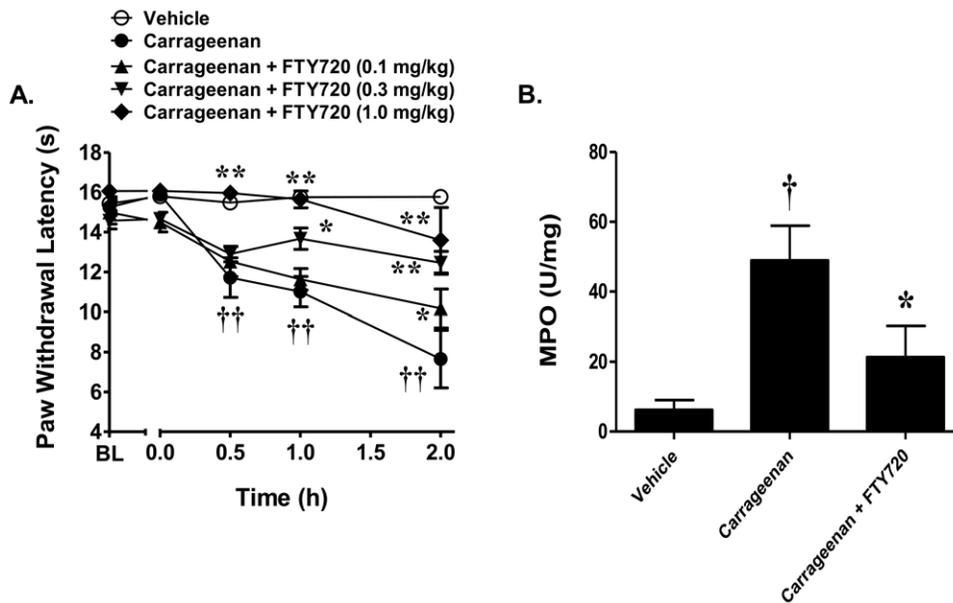


Figure 6. FTY720 attenuates carrageenan-induced thermal hyperalgesia and neutrophilic recruitment. Intraplantar injection of carrageenan (1%) led to a time-dependent development of thermal hyperalgesia. A) Pretreatment with the non-selective functional antagonist, FTY720 (0.1 mg/kg – 1.0 mg/kg, $n=7$) attenuated the development of thermal hyperalgesia in response to carrageenan in a dose-dependent manner. B) FTY720 (1 mg/kg, $n=7$) attenuated carrageenan-induced increases in neutrophilic recruitment. Results are expressed as mean \pm SEM and analyzed by two-way repeated measures ANOVA with Bonferroni *post hoc* test for behavior and one-way ANOVA with Dunnett's *post hoc* test for MPO, where * $P<0.05$, ** $P<0.001$ vs. carrageenan; † $P<0.01$ †† $P<0.001$ vs. vehicle. doi:10.1371/journal.pone.0055255.g006

for 5×10^5 s, centrifuged $40,000 \text{ g}$ @ 4°C for 15 min, then supernatants were pulled off and stored at 4°C . For the assay, $7 \mu\text{L}$ of sample was added to $193 \mu\text{L}$ of 0.167 mg/mL o-dianisidine in 50 mM potassium phosphate buffer with or without 0.0005% H_2O_2 . Absorbance of each sample was read immediately and at 1 min intervals for 3 min at 460 nm . To calculate MPO activity, we plotted absorbance over time to obtain slope and used slope to calculate units of activity per mg (U/mg) using the equation $\text{U/mg} = (\Delta A_{460}/\text{min}) / (11.3 \times \text{mg enzyme/ml reaction mixture})$.

Statistical Analysis

Differences in thermal hyperalgesia were assessed using two-way analysis of variance (ANOVA) with Bonferroni *post hoc* comparisons to S1P or carrageenan-treated animals. Differences in MPO activity levels were assessed by one-way ANOVA followed by Dunnett's *post hoc* comparisons to S1P or carrageenan-treated animals. Differences in paw volume were analyzed by using student's unpaired *t* test. Significant statistical difference was defined when $P\text{-value} < 0.05$.

Results

Carrageenan-induced thermal hyperalgesia is associated with an increase in neutrophilic recruitment which is blocked by fucoidan

The carrageenan model is a well-characterized model of inflammation-induced thermal hyperalgesia which has been suggested to rely on neutrophilic infiltration [28]. The development of edema and thermal hyperalgesia in response to intraplantar injection of carrageenan (1%, $n=6$) seen at peak (6 h) was associated with increased infiltration of neutrophils as shown by an increase in myeloperoxidase activity (MPO; a peroxidase enzyme released by neutrophils and a marker of

neutrophilic infiltration [34,35]) and by histological examination of paw tissues (Figure 1). Administration of fucoidan (40 mg/kg , $n=6$), a well-characterized P- and L-selectin blocker, that is well established in the literature as a potent inhibitor of neutrophil adhesion, rolling and infiltration at inflammatory sites [28,36,37], prevented the edema associated with carrageenan injection (Figure 1A), blocked the thermal hyperalgesia (Figure 1B) and significantly reduced myeloperoxidase activity (Figure 1C). Upon histological examination, the paws revealed pathologic changes that correlated closely with the increases in MPO activity. Paw biopsies showed that after carrageenan administration, marked inflammatory changes were observed including pronounced neutrophil infiltration (Figure 1D, see arrows). Treatment with fucoidan significantly reduced overall pathological changes and neutrophil infiltration in the paw tissues (Figure 1D).

Blocking S1P inhibits carrageenan-induced thermal hyperalgesia

Intraplantar injection of carrageenan led to a time-dependent development of thermal hyperalgesia that peaked at 3 h and was sustained through 5 h (Figure 2). Intraplantar injection of SK-I, a well-characterized, competitive and reversible inhibitor of sphingosine kinase, and thus S1P [38], given 30 minutes before carrageenan, inhibited the development of carrageenan-induced thermal hyperalgesia in a dose-dependent manner (250–1000 ng, $n=6$, Figure 2). Doses were chosen from previous studies [38–41]. Similarly, treatment with LT1002 ($484 \mu\text{g}$, $n=6$), a monoclonal antibody directed against S1P [31], was able to significantly attenuate the carrageenan-induced hyperalgesic response, while its IgG1k isotype control, LT1017 ($572 \mu\text{g}$, $n=6$), had no effect (Figure 3A). These results suggest that S1P contributes to the development of carrageenan-induced thermal hyperalgesia.

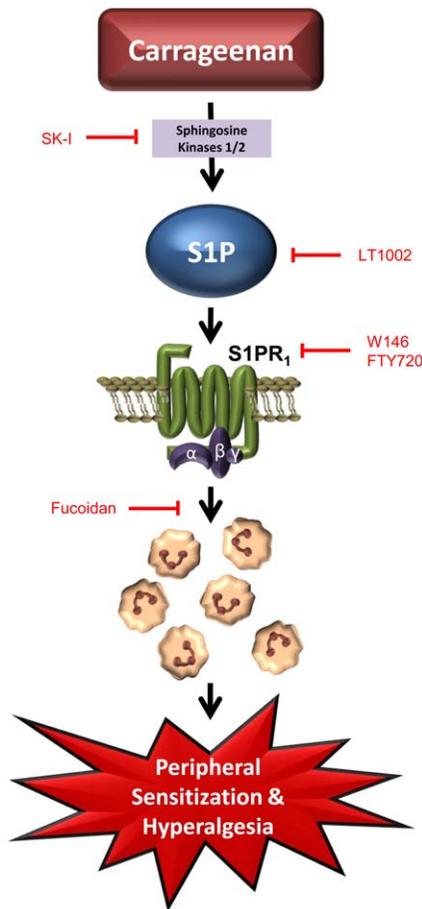


Figure 7. Schematic of proposed mechanisms behind S1P-mediated hyperalgesia. Carrageenan injection leads to the activation of sphingosine kinase enzymes favoring the conversion of sphingosine to bioactive S1P. S1P then goes on to activate S1PR₁, initiating neutrophilic recruitment to the site of injury. Once there, neutrophils release several mediators known to sensitize nociceptors which induce peripheral sensitization and hyperalgesia. doi:10.1371/journal.pone.0055255.g007

Inhibition of S1P blocks the increased neutrophilic recruitment associated with carrageenan-induced thermal hyperalgesia

To determine whether S1P mediates the recruitment of neutrophils in carrageenan-induced thermal hyperalgesia, plantar tissues were taken from animals at 2 h (time of peak inhibition, data not shown) and assayed for MPO activity. As can be seen in Figure 3B, carrageenan injection led to a significant increase in MPO activity that was completely abrogated by pretreatment with LT1002 (484 μ g, $n = 6$), but not by its negative control, LT1017 (572 μ g, $n = 6$).

Blocking the S1P-S1PR₁ axis attenuates carrageenan-induced thermal hyperalgesia and neutrophilic recruitment

In order to define whether S1P recruits neutrophils through the S1PR₁ receptor, we used the well-characterized, selective S1PR₁ antagonist, W146 [42]. As can be seen in Figure 3, intraplantar injection of W146 (0.3–1.2 μ g, $n = 6$) [43], but not its inactive S-enantiomer, W140 (1.2 μ g, $n = 6$) [43], dose-dependently abrogated the carrageenan-induced hyperalgesic response (Figure 4A).

When tested at the highest dose, W146 but not W140 (1.2 μ g, $n = 6$) attenuated neutrophilic recruitment in response to carrageenan (Figure 4B). Doses of W146 and W140 were chosen from previous studies [42].

S1P and SEW2871-mediated thermal hyperalgesia is attenuated by fucoidan

To further strengthen the relationship between S1PR₁ and neutrophil infiltration we investigated whether the development of thermal hyperalgesia in response to exogenous intraplantar injection of S1P or the S1PR₁ agonist, SEW2871 [43,44], was driven by neutrophils. As previously reported by our group [7,12], intraplantar injection of S1P (0.3 μ g, $n = 6$) or SEW2871 (0.3 μ g, $n = 6$) led to a time-dependent development of thermal hyperalgesia (Figure 5) which was blocked by i.p. injection of fucoidan (40 mg/kg, $n = 6$, Figure 5) given 30 min prior to S1P or SEW2871. S1P and SEW2871 were used at doses previously shown by our group to provide maximal hyperalgesia [7,12] and were chosen from previous studies [45]. We attempted to measure increased formation of MPO in paw tissues following intraplantar injection of S1P but our results yielded inadequate signal to detect changes in MPO formation between the groups. This may be due to insufficient sensitivity of the assay in these tissues or may have resulted from a highly localized infiltration of neutrophils at sites of damage that is capable of participating in the development of hyperalgesia, but whose signal is undetectable in a total paw preparation. Nevertheless, pharmacological targeting with a well-characterized anti-neutrophil agent [28,36,37] clearly supports the contribution of neutrophils in S1P-mediated thermal hyperalgesia.

FTY720 inhibits carrageenan-induced thermal hyperalgesia and neutrophilic recruitment

To assess the therapeutic potential of targeting S1P-S1PR₁ signaling in the inflammatory pain setting, we examined the ability of the orally active S1PR modulator, FTY720 (fingolimod), to block carrageenan-induced thermal hyperalgesia and neutrophilic recruitment. FTY720 has been recently FDA-approved for the treatment of multiple sclerosis and is postulated to exert its actions, at least in part, through the binding, internalization, and subsequent blockade of S1PR₁ signaling [46,47]. Inhibition of S1PR signaling using FTY720 (0.1 mg/kg–1.0 mg/kg, $n = 7$), with doses chosen from previous studies [46], attenuated the carrageenan-induced hyperalgesia and associated neutrophilic infiltration (Figure 6).

Discussion

In the present study we demonstrate that S1P acting through the S1PR₁ receptor subtype plays an important role in the development of thermal hyperalgesia associated with inflammation. In addition we present evidence that S1PR₁-triggered neutrophil infiltration is a central component in this setting. Inhibition of sphingosine kinases 1 and 2 with SK-I, which prevents the phosphorylation of sphingosine to form S1P [38], inhibits the development of thermal hyperalgesia in the carrageenan model, a well-characterized model of inflammation-induced hyperalgesia. Similarly, neutralizing S1P with the anti-S1P blocking antibody, LT1002, prevents the development of the carrageenan hyperalgesic response.

Our present work focuses on the role of S1PR₁ as it is emerging as an important subtype in the mediation of peripheral sensitization and hyperalgesia. As we have previously reported, blockade of S1PR₁ with W146 attenuates S1P-induced thermal hyperalgesia [12] and the enhanced excitability in peripheral sensory neurons

in response to S1P has been shown to occur at least in part through the activation of S1PR₁ [18]. It has also been demonstrated that a S1PR₁ agonist injected intracutaneously induces heat hypersensitivity *in vivo* and that mice lacking S1PR₁ in Na_v1.8 expressing nociceptors or in the DRG exhibit reduced S1P-induced hypersensitivity, suggesting that nociceptor sensitization by S1P predominantly occurs through activation of S1PR₁ [13,19]. Our results support these previous findings and extend them to also implicate the role for this receptor subtype in inflammatory pain. Indeed, the selective S1PR₁ antagonist, W146 [43], blocked carrageenan-induced thermal hyperalgesia.

Given that S1P plays a prominent role in the inflammatory process through its ability to recruit neutrophils, which are also implicated in pain [27–30], we hypothesized that S1P-induced peripheral sensitization and hyperalgesia may be triggered by neutrophils. In support, we show that carrageenan-induced neutrophil infiltration is dependent upon S1P and subsequent activation of S1PR₁ as both neutralization of S1P with the anti-S1P mAb, LT1002, and blockade of S1PR₁ activation with W146 was able to inhibit carrageenan-induced neutrophil infiltration. This evidence, taken with the ability of fucoidan to abrogate the development of thermal hyperalgesia in response to S1P alone, supports our hypothesis that S1P-mediated peripheral sensitization and hyperalgesia occurs via a neutrophil-dependent mechanism.

How neutrophils are recruited at sites of inflammation following activation of the S1P-to-S1PR₁ pathway remains to be investigated and was not the focus of the present study. However, scientific literature allows us to speculate as to how this might occur. S1PR₁ activation has been shown to increase the production of the adhesion molecules ICAM-1 and E-selectin in response to inflammatory stimuli, making this a promising candidate for a potential mechanism in our neutrophil-dependent induction of hyperalgesia [22,48]. Several studies have implicated S1PR₁ in the activation of the inflammatory transcription factor NFκB and p38 MAP kinase as well [23,49]. Activation of both NFκB and p38 leads to the increased production of many pro-inflammatory cytokines and chemokines such as TNF-α, IL-1β, IL-6 and CINC-1, the rat homolog of human IL-8 [50–52]. These cytokines are known to enhance the migration of neutrophils through their ability to upregulate the expression of adhesion molecules such as ICAM-1 and E-selectin on resident endothelial cells [53] while the chemokine CINC-1 is a potent neutrophil attractant through a mechanism independent of adhesion molecule expression [54]. Interestingly, the potent proinflammatory and pronociceptive nitroxidative species, peroxynitrite [55–57], has been shown to play a prominent role in the recruitment of neutrophils in inflammatory conditions, including those induced by carrageenan [58–60]. In addition, previous work suggests that peroxynitrite may play a role in the upregulation of the adhesion molecules ICAM-1 and P-selectin as well as the increased production of proinflammatory cytokines such as TNF-α and IL-1β [59,60]. Taken together with previous work showing that S1P via S1PR₁ exerts its actions at least in part through the upregulation of peroxynitrite [12], the activation of these signaling pathways elucidates a possible mechanism by which S1PR₁ may recruit neutrophils to the site of injury.

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Whereas this study has clearly demonstrated the role of the S1PR₁, we are not excluding the potential contribution of other receptor subtypes in S1P's roles; however, this was not the focus of our work. Noteworthy, the tools that are available to examine other receptor subtypes are limited by off-target effects and selectivity issues. For example, the selective S1PR₂ antagonist, JTE-013, has been shown to actually sensitize sensory neurons independently of S1PR₂ activation [61]. The S1PR₃ antagonist, CAY10444 has only been shown to be selective *in vivo* at very low dosages which may not be enough to sufficiently block due to low affinity of the compound for the receptor [62]. Also, CAY10444 has been shown to inhibit [Ca²⁺]_i increases via purinergic P₂ receptor or α_{1A}-adrenoceptor stimulation and α_{1A}-adrenoceptor-mediated contraction, while not affecting the S1P₃-mediated decrease of forskolin-induced cAMP accumulation [63]. Inhibitors are not presently available for S1PR₄ and S1PR₅.

In the present study, FTY720 serves to demonstrate the potential clinical significance of targeting S1PR₁ receptor activation in the inflammatory pain setting. It has been reported that blockade of the S1P-to-S1PR₁ signaling pathway accounts for the observed beneficial effect of FTY720 in MS [64]. In support of this, recently developed S1PR₁ antagonists, such as NIBR-0213, have been shown to have comparable therapeutic efficacy to FTY720 in models of MS [65]. As we show in this study, FTY720, like the S1PR₁ antagonist W146, blocked carrageenan-induced thermal hyperalgesia and neutrophilic recruitment. In addition, FTY720 has been shown to be efficacious in the treatment of rheumatoid arthritis [66] and similar effects are observed with the S1PR₁ antagonist, TASP0277308 [67]. Our work suggests that FTY720's clinical efficacy may extend into the chronic inflammatory pain setting, as in for the treatment of arthritis-induced pain.

While current and emerging therapeutics like NSAIDs and TRPV1 antagonists have been shown to have potent antinociceptive actions in the inflammatory pain setting, in part through their ability to block neutrophilic recruitment, adverse side effect profiles limit their viability as a long-term solution to chronic pain. Novel classes of drugs, such as those targeting S1P, whether used in combination with current analgesics or as a stand-alone treatment, may represent a novel approach in effectively treating chronic pain while avoiding unattractive side effects.

In summary our findings show that S1P, through the activation of S1PR₁, and the subsequent recruitment of neutrophils, plays a key role in inflammatory pain (summarized in Figure 7). Elucidating the mechanisms behind S1P's involvement in inflammatory pain can serve to identify targets for new therapeutic agents that may fill the void between NSAIDs and narcotics in the management of pain.

Author Contributions

Conceived and designed the experiments: AF DS. Performed the experiments: AF ZC EE SC. Analyzed the data: AF EE SC. Contributed reagents/materials/analysis tools: RS. Wrote the paper: AF DS.

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