Role for Peroxynitrite in Sphingosine-1-Phosphate Induced Hyperalgesia in Rats

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Abstract

Sphingosine-1-phosphate (S1P) is an important mediator of inflammation recently shown in *in vitro* studies to increase the excitability of small diameter sensory neurons at least in part via activation of the $S1P_1$ receptor subtype. Activation of $S1PR_1$ has been reported to increase the formation of NADPH oxidase-derived superoxide ($O_2^{\bullet-}$) and nitric oxide synthase (NOS)-derived nitric oxide (NO). This process favors the formation of peroxynitrite ($ONOO^-$, PN), a potent mediator of hyperalgesia associated with peripheral and central sensitization. The aims of our study were to determine whether S1P causes peripheral sensitization and thermal hyperalgesia via $S1PR_1$ activation and PN formation. Intraplantar injection of S1P in rats led to a time-dependent development of thermal hyperalgesia that was blocked by the $S1PR_1$ antagonist, W146 but not its inactive enantiomer, W140. The hyperalgesic effects of S1P were mimicked by intraplantar injection of the well characterized $S1PR_1$ agonist, SEW2871. The development of S1P-induced hyperalgesia was blocked by apocynin, a NADPH oxidase inhibitor, L-NAME, a non-selective NOS inhibitor and by the potent PN decomposition catalysts (FeTM-4-PyP^{5+} and MnTE-2-PyP^{5+}). Our findings provide mechanistic insight into the signaling pathways engaged by S1P in the development of hyperalgesia and highlight the contribution of the $S1P_1$ receptor-to-PN signaling in this process.

Keywords

sphingosine-1-phosphate; peroxynitrite; sphingosine kinase; hyperalgesia; superoxide; nitric oxide

Introduction

The bioactive sphingosine-1-phosphate (S1P) is synthesized by phosphorylation of sphingosine by sphingosine kinases (SphK1 and 2) in a wide variety of cell types and in response to many extracellular stimuli including nerve growth factors and cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) [59;64]. Once generated and released extracellularly, S1P binds its cognate receptors (S1PRs) with high affinity and initiates typical G-protein coupled receptor (GPCR) signaling pathways [63;26;64]. Five S1PRs have been identified to date ($S1PR_{1-5}$) [63;26;64] and these are ubiquitously but differentially expressed on all cells [15;64] and couple to different G proteins upon binding to S1P. Whereas $S1PR_1$, $S1PR_4$ and $S1PR_5$ subtypes mainly couple to $G_{i/o}$, $S1PR_2$ and

S1PR$_3$ subtype couple to G$_{i/o}$, G$_q$ and G$_{12/13}$ [40–41]. Over the last decade, S1P has emerged as a potent mediator of inflammation [58;64] with important roles documented in several studies showing that inhibition of SphK (and in particular SphK1) and S1P$_1$ receptor is anti-inflammatory in several animal models [58;57]. Besides its role in inflammation, a potential role of S1P in peripheral sensitization and hyperalgesia is also documented by the observations that S1P directly increases the excitability of rat sensory neurons *in vitro* [69;68] at least in part via activation of S1PR$_1$ [11] and that S1P derived following bioconversion of ceramide, contributes to NGF-induced excitation of rat sensory neurons [70;36].

Interestingly, S1P$_1$ receptor stimulation activates the superoxide ($O_2^{\bullet-}$)-generating enzyme NADPH (nicotinamide adenine dinucleotide phosphate) oxidase [37;9;61] and the nitric oxide (NO)-producing enzyme nitric oxide synthase (NOS) [20;19;42]. The interaction between $O_2^{\bullet-}$ and NO leads to the production of peroxynitrite ($ONOO^-$, PN) [6] that acts as a potent proinflammatory nitroxidative species [44;50;49;62] and a critical signaling molecule in the development of peripheral and central sensitization associated with pain of several etiologies [48;23;47]. In addition, we have recently reported that ceramide contributes to the development of morphine-induced hyperalgesia and antinociceptive tolerance [35] following its bioconversion to S1P in spinal cord which in turn signals via PN [32]. These observations prompt us to consider the potential contribution of PN in S1P-induced peripheral sensitization and hyperalgesia. Our results reveal that the S1P-to-S1P$_1$ receptor pathway contributes to the development of S1P-induced thermal hyperalgesia in a peroxynitrite-dependent manner. These findings, when analyzed collectively with the emerging roles of sphingolipids such as ceramides and nitroxidative species, such as PN in pain, suggest that targeting the ceramide/S1P and nitroxidative pathways may offer novel approaches in pain management.

Materials and Methods

Materials

S1P, SEW2871, W146 and W140 were purchased from Cayman Chemical, Ann Arbor MI. Unless otherwise noted, all other chemicals and reagents were from Sigma-Aldrich (St. Louis MO). MnTE-2-PyP^{5+} was synthesized as described previously [4]. Charges on FeTM-4-PyP^{5+} and MnTE-2-PyP^{5+} are omitted for clarity on all Figures.

Experimental animals

Male Sprague Dawley rats (200–220 g) were purchased from Harlan (Indianapolis IN), 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.

Drug administration and induction of thermal hyperalgesia

Apocynin, L-NAME, FeTMPyP, MnTE-2-PyP^{5+} (or their respective vehicle, saline) W146, W140 (or their respective vehicle, 20% DMSO in saline) were given by intraplantar injection into the right hindpaw of rats 15 minutes before intraplantar injections of S1P or SEW2871 (or their respective vehicle, 6% ethanol in saline). All drugs were injected in a 5 µl injection volume using a Hamilton gauge needle (31/2″) 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) [16] with a cut-off latency of 20 s employed to prevent tissue damage. Rats were individually confined to Plexiglas

chambers. 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. Results are expressed as Paw-Withdrawal Latency (s). Experiments were conducted with the experimenters blinded to treatment conditions

Immunoprecipitation and Western Blot analysis of protein nitration

Total paw lysates were obtained from flash-frozen plantar soft tissues harvested at the 5h time point. Frozen tissues were pulverized in liquid nitrogen-chilled mortar and pestles; then homogenized in 0.3 – 1 ml (4 volumes) of homogenization buffer [20 mM Tris–Cl (pH 7.4), 150 mM NaCl, 16.3 mM CHAPS, 0.5% Triton X-100, 0.1% SDS, 2 mM EGTA, 5% glycerol, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM sodium molybdinate, 2 mM PMSF, 1X phosphatase inhibitor cocktail (Sigma, St. Louis, MO, USA) (containing: cantharidin, bromotetramisole, and microcystin LR), 1X protease inhibitor cocktail (Sigma, St. Louis, MO, USA) (final concentration: 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), 15 μM pepstatin A, 14 μM E-64, 40 μM bestatin, 20 μM leupeptin, and 850 nM aprotinin)]. Each lysate was sonicated for 10 min at 4°C; incubated for 1 hr, rotating at 4°C; and clarified by centrifugation at 13,000g, 4°C for 10 min. Total protein concentrations were determined by bicinchoninic acid assay (Pierce, Rockford, IL, USA). Nitrotyrosine-containing proteins in whole paw lysates were immunoprecipitated as previously described [30]. Paw lysate (1 mg total protein) was treated with 12 μg of agarose-conjugated anti-3-nitrotyrosine antibody (Millipore, Billerica, MA, USA) and diluted to 500 μl total volume with PBS pH 7.4 supplemented with 2 mM PMSF and 1X protease inhibitor cocktail. Samples were incubated overnight at 4°C, washed four times in 1X PBS, and denatured with 40 μl of 2X Laemmli Buffer. Samples were boiled 5 min, resolved on 4–20% SDS-PAGE (Bio-Rad, Hercules, CA, USA), and immunoblotted to PVDF membrane in Towbin buffer. The membranes were blocked with 5% low-fat milk in PBS pH 7.4 and 0.05% Tween-20 (PBS-T) for 2 hr at RT before overnight incubation at 4°C with rabbit anti-3-nitrotyrosine affinity purified antibody [14;17] (2 μg/ml) in 2.5% low-fat milk/PBS-T. Membranes were washed 1×10 min and 4×5 min in PBS-T and incubated for 1 hr with horseradish-conjugated goat anti-rabbit IgG (1:1000, Pierce, Rockford, IL, USA). The membranes were washed 1×10 min and 4×5 min in PBS-T and NT-positive bands were visualization by enhanced chemiluminescence detection using a Fujifilm LAS-3000 imaging system with Image Reader LAS-3000 v2.2 (Fujifilm, Japan) software.

Statistical Analysis

All data are expressed as a mean ± SEM. The differences in levels of thermal hyperalgesia were assessed by two-way analysis of variance (ANOVA) with Bonferroni *post hoc* comparisons to S1P-treated animals where significance is defined at $P<0.05$.

Results

Sphingosine-1-phosphate induces thermal hyperalgesia via the S1P1 receptor

When compared to the vehicle group (rats in this group received an intraplantar injection of the vehicle used for W146 or W140 followed by intraplantar injection of the vehicle used for S1P), intraplantar injection of S1P (0.03–0.3 μg, n=4) led to a dose and time-dependent development of thermal hyperalgesia, which peaked by 2h and remained sustained up to the 5h time-point (Fig. 1A). The development of S1P (0.3 μg, n=4) induced thermal-hyperalgesia was blocked in a dose-dependent manner by the well-characterized $S1P_1$ receptor antagonist, W146 (0.1–0.6 μg, n=4, Fig. 1B) [51;40] but not by W140 (1.2 μg, n=4 Fig. 1C) its inactive S-enantiomer with doses chosen from previous studies [51;40]. When tested alone and compared to rats that received an intraplantar injection of the vehicle used

for S1P, W146 (0.6 μg, n = 4) or W140 (1.2 μg, n=4) had no effect on baseline withdrawal latencies (Fig. 1B, C). The role for $S1PR_1$ in the development of thermal hyperalgesia was strengthened by the use of SEW2871, a well-characterized $S1P_1$ receptor agonist [40–41]. When compared to the vehicle group (rats in this group received an intraplantar injection of the vehicle used for W146 followed by intraplantar injection of the vehicle used for SEW2871), intraplantar injection of SEW-2871 (0.3 μg, n=4) elicited a time-dependent development of thermal hyperalgesia that was maximal by 2h and sustained up to the 5h time period (Fig. 2). This effect was blocked as expected by the $S1PR_1$ receptor antagonist, W146 (0.6 μg, n=4, Fig. 2). When tested alone and compared to rats that received an intraplantar injection of the vehicle used for SEW-2871, W146 (0.6 μg, n=4) had no effect on baseline withdrawal latencies (Fig. 2).

Role for superoxide and nitric oxide in S1P induced thermal hyperalgesia

At doses known to block NADPH oxidase activity, intraplantar injection of apocynin (1 μg, n=4), a well characterized inhibitor of superoxide derived from activation of the NADPH oxidase [56;60], attenuated the development of S1P (0.3 μg, n=4)-induced hyperalgesia (Fig. 3A). Likewise, the well characterized and non-selective inhibitor of nitric oxide synthase, N^G-nitro-L-arginine methyl ester (L-NAME, 1.5 μg, n=4) [29], at doses known to block NOS activity mitigated the development of hyperalgesia in response to S1P (Fig. 3B). When tested alone and compared to rats that received an intraplantar injection of the vehicle used for S1P, apocynin or L-NAME had no effect on baseline withdrawal latencies (Fig. 3A, B).

Peroxynitrite formation from nitric oxide and superoxide is a key mediator in S1P induced thermal hyperalgesia

Intraplantar injection of FeTMPyP (1–6 μg, n=4, Fig. 4A) or $MnTE-2-PyP^{5+}$ (1–6 μg, n=4, Fig. 4B) blocked the development of S1P (0.3 μg, n=4)-induced hyperalgesia in a dose and time-dependent manner. When tested alone and compared to rats that received an intraplantar injection of the vehicle used for S1P, $FeTMPyP^{5+}$ (6 μg, n=4) or $MnTE-2-PyP^{5+}$ (6 μg, n=4) had no effect on baseline withdrawal latencies (Fig. 4A, B). We have attempted to measure increased formation of 3-nitrotyrosine (NT), a well characterized and widely used marker of PN [31;62] by immunoprecipitation in paw tissues following intraplantar injection of S1P. Our results yielded inadequate signal to detect changes in NT formation between the groups. These results may have resulted from insufficient sensitivity of the IP method for NT in these tissues as indicated by low signal or may have resulted from a highly localized production of NT in the paw that is capable of participating in the development of hyperalgesia, but whose signal is undetectable in a total paw preparation. Regardless, pharmacological targeting with well characterized PN decomposition catalysts such as $FeTMPyP^{5+}$ and $MnTE-2-PyP^{5+}$ clearly support the contribution of PN in S1P-mediated thermal hyperalgesia.

Discussion

In this study, we demonstrate that S1P is an important mediator of peripheral sensitization and thermal hyperalgesia acting via the $S1PR_1$ –to- PN signaling pathway. To this end, S1P-mediated thermal hyperalgesia was attenuated by the well characterized $S1PR_1$ antagonist W146 but not by its inactive enantiomer W140 [51;40]. In addition, the hyperalgesic effects of S1P were mimicked by the well known $S1PR_1$ agonist SEW2871 [51;40] and SEW-2871 induced hyperalgesia was attenuated by W146. A literature survey of $S1PR_1$ activation in research unrelated to pain mechanisms revealed that activation of $S1PR_1$ subtype by S1P can activate in some cell types, the NADPH oxidase and to activate and/or induce the NO-generating enzyme, NOS [37;39;65–66;9;61]. The multicomponent membrane-associated NADPH oxidase system has been shown over the years to be a central source for $O_2^{\bullet-}$

generation during several pathological conditions [34]. This $O_2^{\bullet-}$ generating enzyme is dormant in resting cells and produces $O_2^{\bullet-}$ only upon activation. The principal regulation of NADPH oxidase is post-translational and depends on the assembly of several membrane-bound and cytosolic components to form an active enzyme complex [2]. In resting cells, the enzyme consists of two membrane-bound components, gp91*phox* and p22*phox*, and several cytosolic components, including p47*phox*, p40*phox*, p67*phox*, and Rac1/2 [2]. Gp91*phox* is a flavocytochrome and the catalytic core of the enzyme. Upon activation, the cytosolic components translocate to the membrane and associate with membrane components to form an assembled, activated, and $O_2^{\bullet-}$ producing enzyme complex [2]. Importantly, $O_2^{\bullet-}$ auto-augments its formation by up-regulating the expression of the Rac1 and gp91*phox* subunits of the holoenzyme creating a self-perpetuating cascade [38;33]. The mechanisms whereby S1P activates this enzyme are not fully understood but include activation of Rac small GTPase [66] and PKCζ [39;65]. In our studies, the critical role of the NADPH oxidase as a source of $O_2^{\bullet-}$ in PN formation was highlighted by the findings that local administration of apocynin blocked the development of S1P induced hyperalgesia. Apocynin prevents serine phosphorylation of p47*phox* and blocks its association with gp91phox, thus blunting NADPH oxidase activation [56;60]. The important role of NOS as a source of NO-derived PN was highlighted in our studies by the findings that local administration of L-NAME, a well characterized non-selective inhibitor of NOS [29], blocked the development of S1P-induced hyperalgesia. Because NO is known to react with $O_2^{\bullet-}$ at a diffusion-limited rate to form PN [6] results obtained with apocynin and L-NAME indirectly suggest that PN from these reactive oxygen and nitrogen species is the common denominator in the molecular and biochemical pathways leading to S1P-induced thermal hyperalgesia. The role of PN was confirmed by the use two well characterized PN decomposition catalysts, namely, FeTMPyP^{5+} and MnTE-2-PyP^{5+} [27;44;50;3]. These results establish that PN formed in response to S1P activation of S1PR$_1$ is a key signaling molecule in the development of S1P-induced thermal hyperalgesia. It is also likely that S1P via activation of S1PR$_1$ directly sensitizes peripheral sensory afferents as shown *in vitro* studies [11] thus contributing to peripheral sensitization and hyperalgesia.

Although our results have identified potential enzymatic sources in the formation of PN (NADPH oxidase and NOS), our results have not identified the cellular sources of PN. To this end and since NADPH oxidase and NOS enzymes are expressed not only in resident cells of the paw (i.e. endothelial cell and resident tissue neutrophils/macrophages and mast cells) [1;25;13;21] but also in peripheral nociceptors [7–8], it is possible and likely that activation of S1P$_1$ receptors in both contribute to PN formation. The mechanisms whereby PN contribute to S1P-induced hyperalgesia must be multiple since PN contributes to peripheral and central sensitization by acting at multiple sites and through several biomolecular signaling pathways [see [48;23;47] for updated review articles]. For example, and in the context of the results presented in this paper, it is possible that PN contributes to the development of S1P-induced peripheral sensitization and hyperalgesia by favoring the *in situ* production of mediators such as PGE$_2$ or TNF-α and IL-1β that, in addition to their pro-inflammatory effects, are also known to sensitize peripheral nociceptors [52;10;53]. These possibilities are supported by the fact that PN activates/induces COX-1 and COX-2 to increase a variety of prostaglandins including PGE$_2$ [see [45;28] for review articles] and that PN can also activate redox-sensitive transcription factors and MAPK kinases to increase the production of various cytokines [see [48;47] for review articles]. Additionally, PN may contribute to the development of S1P-induced hyperalgesia by altering the sensitivity of TRPV1 on peripheral nociceptors highlighted by evidence that NADPH oxidase augments capsaicin-induced TRPV1 activity in DRG neurons through PKC activation and translocation [18]; nitro-oleic acid, a fatty acid nitrated by NO or PN [5], evokes TRPV1-mediated Ca^{2+} currents in DRG neurons [54]; and S-nitrosylation of cysteine residues of TRPV1 elicit Ca^{2+} influx into transfected HEK cells [67]. In light of its potent chemotactic

activity [44;50;46], PN also may contribute to the development of S1P-induced hyperalgesia by favoring neutrophil infiltration that in turn perpetuates the cycle by providing mediators able to sensitize peripheral nociceptors [22;24] or directly activate them [43]. These possibilities highlighted in the schematic shown in Fig. 5 are currently being investigated in our laboratories. It should be recognized that S1P has also been reported to exert antinociceptive effects [12;55]. However, our studies reveal a potential multifaceted role of S1P in nociceptive processing that could depend on the site of S1P biosynthesis and the relative distribution of the S1P receptors involved in signaling. Although in our study we focused on the involvement of $S1P_1$ receptors, we are not excluding the contribution of other S1P receptors such as S1PR2that will be investigated in future studies. In summary, our results have identified S1P acting via the S1P1 receptor as a potent inducer of peripheral sensitization and hyperalgesia signaling via peroxynitrite.

Acknowledgments

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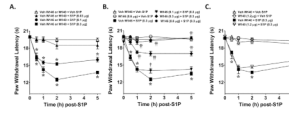
Fig. 1. S1P induced thermal hyperalgesia is blocked by a S1P receptor antagonist
When compared to rats administered intraplantar W146/W140 vehicle and S1P vehicle
(Veh, △), an intraplantar injection of S1P given at 0.03 μg (▲), 0.1 μg (●), or 0.3 μg (■) led
to a time and dose-dependent development of thermal hyperalgesia (**A**). The development of
S1P-mediated (0.3 μg, ■) thermal hyperalgesia was attenuated in a dose-dependent manner
by the active $S1PR_1$ antagonist, W146, given at 0.1 μg (▼), 0.3 μg (◆), or 0.6 μg (●) (**B**)
but not of its inactive enantiomer W140 (1.2 μg, ▽, **C**). When tested alone W146 (0.6 μg, ○,
B) or W140 (1.2 μg, ◇, **C**) and compared to animals receiving S1P vehicle W146 or W140
(△), had no effect on baseline withdrawal latency. Results are expressed as mean ± SEM for
4 rats. *$P < 0.001$ versus Veh, and † † $P < 0.01$ or ††$P < 0.001$ versus S1P by ANOVA with
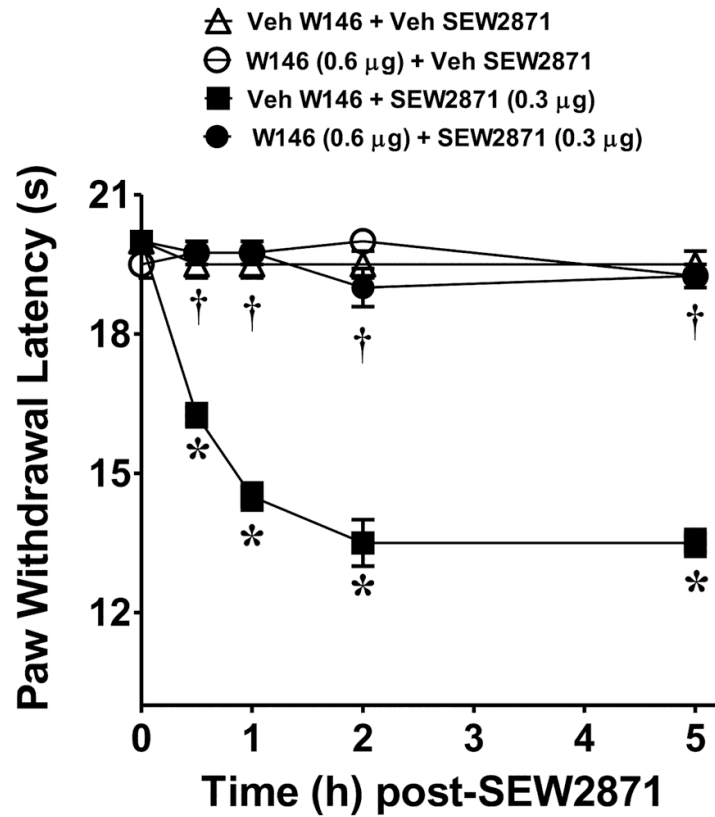Bonferroni *post hoc* test.

Fig. 2. S1PR$_1$ agonist, SEW2871, induces thermal hyperalgesia
When compared to rats administered intraplantar W146/W140 vehicle and SEW2871
vehicle (Veh, △), an intraplantar injection of the S1PR$_1$-specific agonist, SEW2871, given
at 0.3 μg (■) induced time-dependant thermal hyperalgesia that was attenuated by
intraplantar W146 (1.2 μg, ●). When tested alone and compared to animals receiving
SEW2871 vehicle (△), intraplantar injection of W146 (1.2 μg, ○) had no effect on baseline
withdrawal latency. Results are expressed as mean ± SEM for 4 rats. *P < 0.001 versus Veh,
and †P < 0.001 versus SEW2871 by ANOVA with Bonferroni *post hoc* test.
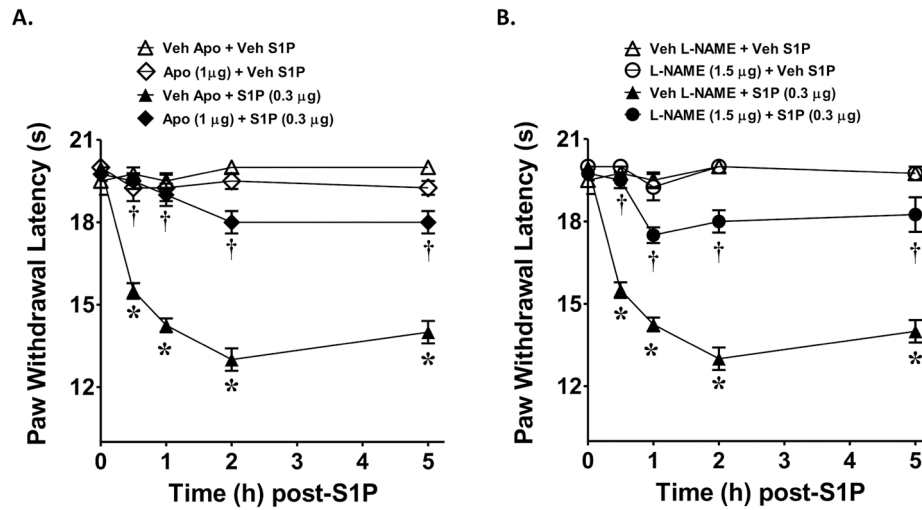
Fig. 3. Role of superoxide and nitric oxide in S1P induced thermal hyperalgesia
When compared to rats administered intraplantar apocynin/L-NAME vehicle and S1P
vehicle (Veh, △), an intraplantar injection of S1P (0.3 μg, ▲, **A** and **B**) led to a time-
dependent development of thermal hyperalgesia that was attenuated by intraplantar
administration of apocynin (1 μg, ◆, **A**) or L-NAME (1.5 μg, ●, **B**). When tested alone and
compared to animals receiving S1P vehicle (Veh, △), intraplantar injection of apocynin (1
μg, ◇, A) or L-NAME (1.5 μg, ○, **B**) had no effect on baseline withdrawal latency. Results
are expressed as mean ± SEM for 4 rats. *$P < 0.001$ versus Veh, and †$P < 0.001$ versus S1P
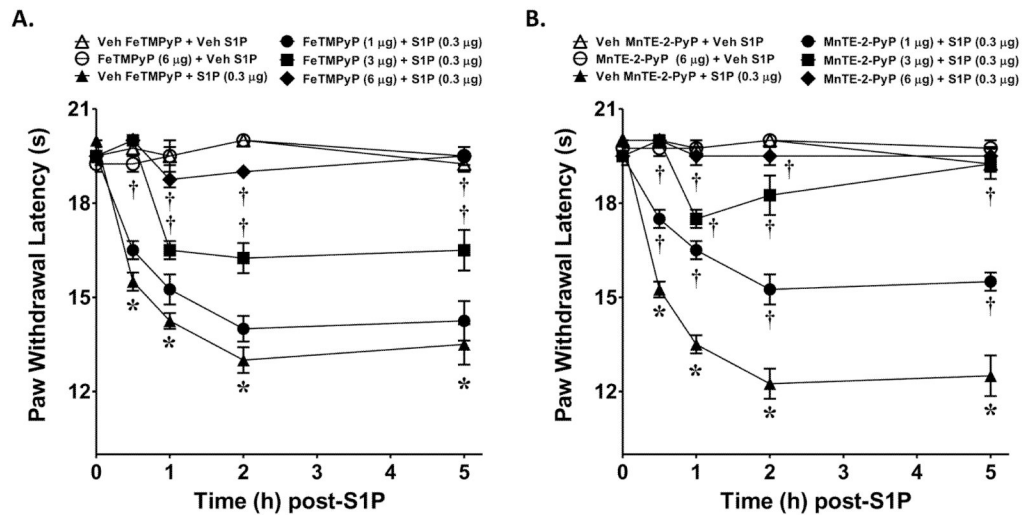by ANOVA with Bonferroni *post hoc* test.

Fig. 4. Role of peroxynitrite in S1P induced thermal hyperalgesia
When compared to rats administered intraplantar FeTMPyP or MnTE-2-PyP^{5+} vehicle and S1P vehicle (Veh, $\triangle$), an intraplantar injection of S1P given at 0.3 μg ($\blacktriangle$, **A** and **B**) led to a time-dependent development of thermal hyperalgesia that was attenuated in a dose-dependent manner by intraplantar FeTMPyP (**A**) or MnTE-2-PyP^{5+} (**B**) given at 1 μg ($\bullet$), 3 μg ($\blacksquare$) or 6 μg ($\blacklozenge$). When tested alone and compared to animals receiving S1P vehicle (Veh, $\triangle$), intraplantar administration (6 μg) of FeTMPyP ($\circ$, **A**) or MnTE-2-PyP^{5+} ($\circ$, **B**) had no effect on baseline withdrawal latency. Results are expressed as mean ± SEM for 4 rats. *$P < 0.001$ versus Veh, and †$P < 0.001$ versus S1P by ANOVA with Bonferroni correction
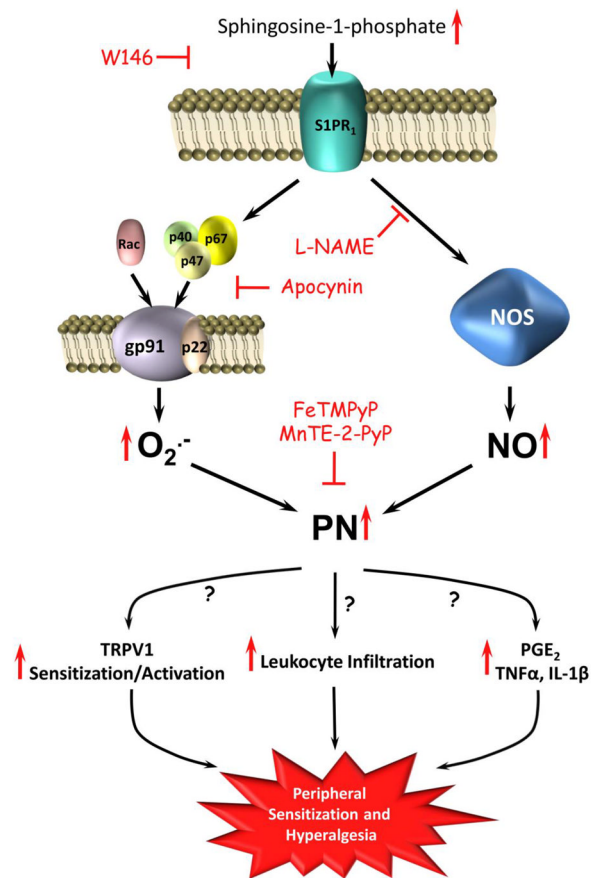
Fig. 5. Illustration of proposed S1PR$_1$-to-PN signaling in S1P-induced hyperalgesia
Activation of S1PR$_1$ with its S1P ligand induces the apocynin-sensitive translocation of
cytosolic subunits of NADPH oxidase to membrane-bound gp91*phox* and p22*phox* forming
the active superoxide producing NADPH oxidase holoenzyme. NADPH oxidase-derived
superoxide, together with nitric oxide produced from NOS activation, form PN leading to
the development of peripheral sensitization and hyperalgesia. Thus, peroxynitrite
decomposition with PN decomposition catalysts (i.e. FeTMPyP or MnTE-2-PyP^{5+}) inhibit
hyperalgesia. Possible mechanisms by which PN facilitates peripheral sensitization and
hyperalgesia include increased TRPV1 sensitization and activation, leukocyte infiltration,
and increased formation of prostaglandins (i.e. PGE$_2$ synthesis) or cytokines (i.e. TNF-α and
IL-1β).