수의학 박사학위 논문

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Doctoral Thesis

The role of peripheral P2Y1 receptor-mediated TRPV1 receptor modulation in the development of thermal hyperalgesia

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ABSTRACT

The role of peripheral P2Y1 receptor mediated TRPV1 receptor modulation in the development of thermal hyperalgesia

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BACKGROUND:

During the pathological conditions such as ischemia and inflammation, a huge array of endogenous chemicals are released into the damaged tissue that contribute to peripheral sensitization. Since Transient receptor potential vanilloid 1 receptor (TRPV1R) is known for the endpoint target of sensitizing mediators and receptors in periphery, modulation of TRPV1R is the effective way to control the pain initiation at the damage site. P2Y1 receptor (P2Y1R) is a Gq-coupled receptor located in the peripheral nervous system. Preferred agonists for P2Y1R are ADP and ATP which are released from damaged tissues. Recently, putative involvement of P2Y1R in sensory transduction has been documented and the possibility that P2Y1R could modulate the function of TRPV1R was reported *in vitro* system. However, the underlying

mechanisms of P2Y1R and P2Y1R-TRPV1R interaction in pain hypersensitivity are remain to be addressed.

OBJECTIVES:

The present study was aimed to

1. Examine whether inflammatory insults would increase the expression of peripheral P2Y1R and blockade of peripheral P2Y1R could prevent the development of inflammatory pain. The modulatory effects of P2Y1R on the expression level of TRPV1R during the inflammation was also investigated.

2. Evaluate whether MAPKs activity in dorsal root ganglion (DRG) would increase in response to the inflammatory insults. This study investigated whether the inhibition of peripheral P2Y1R could affect MAPKs activity, and the possible involvement of MAPKs in the P2Y1R induced up-regulation of TRPV1R expression.

3. Examine whether injection of acidic saline into the hind paw causes the development of TRPV1R mediated thermal hyperalgesia under the ischemic state. In addition, the present study investigated whether functional interactions between TRPV1R and P2Y1R would contribute to the development of this ischemic thermal hyperalgesia.

MATERIALS AND METHODS:

All experiments were performed on Sprague-Dawley rats. Inflammation was induced by 2% carrageenan injection to the hind paw, and the ischemic condition was induced by TIIP (thrombus induced ischemic pain) surgery. 20% $FeCl_2$ was applied to the separated femoral artery and the synthesis of thrombus caused the peripheral ischemia in this model. Sensitization to noxious heat stimulation (thermal

hyperalgesia) was examined with Hargreaves apparatus, and sensitization to innocuous mechanical stimulation (mechanical allodynia) was examined using von Frey filaments with forces of a 4g. In the present study, MRS2500 and MRS2179 (P2Y1R antagonist), MRS2365 (a P2Y1R agonist), AMG9810 (a TRPV1R antagonist), chelerythrine (a PKC inhibitor), amiloride (an ASICs blocker) and TNP-ATP (a P2Xs antagonist) were intraplantarly injected. SB203580 (a p38 MAPK inhibitor) was intrathecally injected to inhibit p38 MAPK in DRGs. Immunohistochemistry and western blot assay were performed according to each experiment procedure. The computer-assisted image analysis system (Metamorph) was utilized throughout whole experiments.

RESULTS:

1. The expression of P2Y1R and TRPV1R was significantly increased on day 2 following carrageenan injection. Blockade of peripheral P2Y1R by the P2Y1R antagonist, MRS2500 injection significantly reduced the induction of thermal hyperalgesia, but not mechanical allodynia. Simultaneously, MRS2500 injections suppressed up-regulated TRPV1R expression. In addition, repeated injection of P2Y1R agonist, MRS2365 into the naïve rat's hind paw dose dependently increase the expression level of TRPV1R in naive rats.

2. Following injection of 2% carrageenan into the hind paw, the phosphorylation rates of both p38 MAPK and ERK but not JNK were increased and peaked at day 2 post-injection. Injection of MRS2500 significantly suppressed the ratio of p38 MAPK phosphorylation in DRGs, while p-ERK signaling was not affected. Furthermore, inhibition of p38 MAPK activation in the DRGs by SB203580 (a p38 MAPK inhibitor) prevented the increase of TRPV1R by inflammation.

Furthermore, in naïve rats, repeated stimulation of peripheral P2Y1R dose dependently increased the level of p-p38 MAPK in DRGs.

3. Repeated intraplantar injection of pH 4.0 saline for 3 days following TIIP surgery resulted in the development of thermal hyperalgesia. Moreover, injection of chelerythrine (a PKC inhibitor) and AMG9810 (a TRPV1R antagonist) effectively alleviated the established thermal hyperalgesia. After acidic saline (pH 4.0) injections, there were no changes in the expression of TRPV1R in hind paw skin, whereas a significant increase in TRPV1R phosphorylation was shown in acidic saline injected TIIP animals. Pre-blockade of peripheral P2Y1R significantly prevented the induction of thermal hyperalgesia, and the increase of phosphorylated TRPV1R ratio.

CONCLUSIONS:

This study demonstrated that there was a sequential role for P2Y1R, p38 MAPK and TRPV1R in inflammation-induced thermal hyperalgesia. Peripheral P2Y1R activation modulates p38 MAPK signaling and TRPV1R expression, which ultimately leads to the induction of the inflammatory thermal hyperalgesia. I also have addressed that maintenance of an acidic environment in the ischemic sate resulted in the phosphorylation of TRPV1R by P2Y1R, which leads to the development of thermal hyperalgesia mimicking what occurs in chronic ischemic the patients with severe acidosis. Collectively, these data imply that there is a close relationship between P2Y1R and TRPV1R in the development of thermal hypersensitivity, and this connection could be useful therapeutic targets for alleviating thermal hypersensitivity under the conditions of inflammation or ischemia.

Key words: P2Y1 receptor, TRPV1 receptor, Thermal hyperalgesia, p38 MAPK, inflammation, ischemia

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CONTENTS

LIST OF FIGURES

ABBREVIATIONS

ADP	Adenosine diphosphate
AMG 9810	(2E)-N-(2,3-Dihydro-1,4-benzodioxin-6-yl)-3-[4-(1,1-dimethylethyl)phenyl]-2-propenamide
Amiloride	3,5-Diamino-N-(aminoiminomethyl)-6-chloropyrazinecarboxamide hydrochloride
AMP	Adenosine monophosphate
ASIC	Acid sensing ion channel
AS-TIIP	Acidic saline injected TIIP
ATP	Adenosine triphosphate
CA II	Carbonic anhydrase II
CaMKII	Calmodulin dependent protein kinase II
CFA	Complete Freund's adjuvant
Chelerythrine	1,2-Dimethoxy-12-methyl[1,3]benzodioxolo[5,6-c]phenanthridinium chloride
CPM	C-fiber polymodal afferents
DMSO	Dimethyl sulfoxide
DRG	Dorsal root ganglion
ERK	Extracellular signal-regulated kinase
GFAP	Glial fibrillary acidic protein
HIF-1α	Hypoxia inducible factor-1α
JNK	c-Jun amino-terminal kinase
MAPK	Mitogen-activated protein kinase
MRS2179	2'-Deoxy-N6-methyladenosine 3',5'-bisphosphate tetrasodium salt

MRS2365	[[(1R,2R,3S,4R,5S)-4-[6-Amino-2-(methylthio)-9H-purin-9-yl]-2,3-dihydroxybicyclo[3.1.0]hex-1-yl]methyl] diphosphoric acid mono ester trisodium salt
MRS2500	(1R*,2S*)-4-[2-Iodo-6-(methylamino)-9H-purin-9-yl]-2-(phosphonooxy)bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester tetraammonium salt
PAD	Peripheral arterial disease
PBS	Phosphate-buffered saline
PKA	Cyclic AMP-dependent protein kinase
PKC	Calcium dependent protein kinase
p-p38 MAPK	Phosphorylation of p38 MAPK
pTRPV1R	Phosphorylated TRPV1 receptor
PWF	Paw withdrawal frequency
PWL	Paw withdrawal latency
RTX	Resiniferatoxin
SB	SB203580
SB203580	4-[5-(4-Fluorophenyl)-2-[4-(methylsulfonyl)phenyl]-1*H*-imidazol-4-yl]pyridine
TIIP	Thrombus-induced ischemic pain
TNP-ATP	2',3'-O-(2,4,6-Trinitrophenyl)adenosine-5'-triphosphate tetra(triethylammonium) salt

BACKGROUND

Pain conduction pathway

Although pain is unpleasant sensory and emotional experience, it is the crucial warning mechanism for protection in response to the tissue damages (Cheng and Ji, 2008; Scholz and Woolf, 2002). Free nerve endings function as nociceptors which have high-threshold and specialized for detecting painful mechanical, chemical and heat stimulus (Woolf and Ma, 2007). The cell body of the nociceptors are clustered in the dorsal root ganglion (DRG) located in a posterior root of a spinal nerve (Cheng and Ji, 2008; Obata and Noguchi, 2004). Nociceptors detect noxious stimulus by many channels and receptors and transform the external stimulus into the electrical events, i.e., action potentials in peripheral nerves (Hucho and Levine, 2007; Woolf and Ma, 2007). These neural activity is runs along the DRG neuron and transmitted to the spinal cord. Finally, brain integrates the transmitted signal from the periphery and psychological factors such as emotions and memories, which ultimately causes the painful sensation (Scholz and Woolf, 2002).

Peripheral sensitization

During pathological conditions such as ischemia and inflammation, a huge array of endogenous chemicals are released into the tissue that contribute to abnormal sensory phenomena, i.e., pain hypersensitivity (Scholz and Woolf, 2002). Sustained pathological stimulus results in the neuronal changes occur at the peripheral nervous system, which is called peripheral sensitization (Woolf and Ma, 2007). It lowered the threshold and amplified the responsiveness of nociceptors; peripheral sensitization results in the behavioral consequences characterized by hyperalgesia (increased

sensitivity to a painful stimuli), and allodynia (pain produced in response to a non-nociceptive stimulus) (Sandkühler, 2009; Woolf and Ma, 2007).

TRPV1 receptor in nociceptor

It is well recognized that activation of TRPV1 receptor (TRPV1R) contributes to peripheral sensitization, particularly to heat stimuli (Holzer, 2008; Ma and Quirion, 2007; Wang, 2008). Capsaicin, most potent agonist for TRPV1R, induced hyperalgesia and allodynia are considered to be a model for inflammatory and neuropathic pain (Holzer, 2008; Moulton et al., 2007; Nagy et al., 2004; Planells-Cases et al., 2005). Proton is also an important endogenous agent and a plausible candidate for activating TRPV1R during inflamed and ischemic states accompanying tissue acidosis (Aneiros et al., 2011; Holzer, 2008). Besides directly activated by agonist, TRPV1R has been implicated to be the main signaling pathway stimulated by pro-algesic substances such as ATP, bradykinin, and prostaglandin released in damaged tissues (Ma and Quirion, 2007; Planells-Cases et al., 2005; Wang, 2008).

P2 receptors in nociceptor

ATP is present at low concentrations in the extracellular space in normal tissues, but it is released from swollen cells as a result of tissue damage such as inflammation and ischemia. ATP is a potent extracellular nociceptive molecule through activation of ionotropic P2X and metabotropic P2Y receptors (Shao et al., 2007). Among the 7 subtypes (P2X1–P2X7) of cloned P2X receptors, P2X3 receptor is expressed selectively in small-diameter nociceptive neurons located in dorsal root ganglion (DRG) neurons (Chizh and Illes, 2001). The P2Y receptors are composed of 8 subtypes (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13 and P2Y14) that have

been cloned. Among 8 types of P2Y receptors, P2Y1 and P2Y2 receptors are the most highly-expressed Gq-coupled P2Y receptors in sensory neurons (Malin et al., 2008). Histological analysis suggests that P2Y1 and P2Y2 receptors are likely to be expressed in small nociceptive neurons (Gerevich and Illes, 2004).

OBJECTIVES

This study is aimed to

1. Examine whether: (1) inflammatory insults would increase the expression of peripheral P2Y1 receptor (P2Y1R); (2) blockade of peripheral P2Y1R could prevent the development of inflammatory pain; and finally (3) the expression level of TRPV1 receptor (TRPV1R) would be modulated by inhibition of P2Y1R under the inflammation.

2. Evaluate whether: (1) MAPKs activity in DRGs would increase in response to the inflammatory insults and contribute to pain hypersensitivity; (2) blockade of peripheral P2Y1R result in the decrease of MAPKs activity in DRGs; (3) MAPKs signalling would be involved in the modulatory effects of P2Y1R on the TRPV1R expression.

3. Examine: (1) whether injection of acidic saline into the hind paw causes the development of TRPV1R mediated thermal hyperalgesia under the ischemic state; (2) which proton (ASIC and TRPV1) and ATP (P2X and P2Y1) sensing receptors are involved in this newly developed thermal hyperalgesia; and finally (3) whether there are functional interactions between TRPV1 and P2Y1 receptors.

CHAPTER 1

Modulatory effect of P2Y1 receptor on TRPV1 receptor expression during the inflammation

ABSTRACT

Although previous reports have suggested that P2Y1 receptor (P2Y1R) is involved in cutaneous nociceptive signaling, it remains unclear how P2Y1R contribute to peripheral sensitization. The current study was designed to delineate the role of peripheral P2Y1R in pain and to investigate potential linkages to Transient Receptor Potential Vanilloid 1 receptor (TRPV1R), i.e., endpoint target of peripheral sensitization, in a rodent inflammatory pain model. Following injection of 2% carrageenan into the hind paw, inflammatory thermal hyperalgesia and mechanical allodynia were developed. At day 2 post-injection of carrageenan, the expression of P2Y1R and TRPV1R was increased in peripheral tissues including skin, sciatic nerve and DRGs (dorsal root ganglions). Blockade of peripheral P2Y1R by the P2Y1R antagonist, MRS2500 injection (intraplantar, D0 to D2) significantly reduced the induction of thermal hyperalgesia, but not mechanical allodynia. Simultaneously, MRS2500 injections significantly suppressed the up-regulated TRPV1R expression by inflammatory insults. Lastly, to identify the mechanistic action of P2Y1R, P2Y1R specific agonist, MRS2365, was repeatedly injected into the naïve rat's hind paw. As a result, there was a dose-dependent increase in TRPV1R expression in hind paw skin and DRGs. These data demonstrate the role of P2Y1R in the regulation of TRPV1R expression and inflammatory thermal hyperalgesia; thus, peripheral P2Y1R could be a useful therapeutic target for alleviating thermal hypersensitivity under the inflammation

INTRODUCTION

P2Y1 receptor (P2Y1R) is Gq-coupled receptor located in sensory neurons. ADP (adenosine diphosphate) and ATP (adenosine triphosphate) are released from injured cells under conditions of tissue damage, and they serve as natural ligands for these purinergic receptors (Dussor et al., 2009; Hardy et al., 2005; Nakamura and Strittmatter, 1996; Sacha and Derek, 2010). Recently, the localization of P2Y1R to sensory neurons and their putative involvement in pain transduction have been documented (Gerevich et al., 2004; Jankowski et al., 2012; Sacha and Derek, 2010; Yousuf et al., 2011). Previous reports have concentrated on the role of P2Y1R in heat and cold sensing in C-fiber polymodal (CPM) afferents which had no Transient receptor potential vanilloid 1 receptor (TRPV1R) (Molliver et al., 2011). Moreover, knockdown of P2Y1R prevented the inflammation-induced decrease in CPM heat threshold (Jankowski et al., 2012; Sacha and Derek, 2010). Although these findings support the contribution of peripheral P2Y1R to inflammatory thermal hyperalgesia particularly in CPM fiber, their relationship with TRPV1R and the mechanisms involved in inflammation-induced nociceptor plasticity is poorly understood.

TRPV1R is recognized as a molecular sensor of noxious heat stimuli (Holzer, 2008; Wang, 2008). It is important to note that TRPV1R is not only activated directly by endogenous agonists, i.e., capsaicin, heat and proton, but it also activated indirectly by other proalgesic substances including ATP, bradykinin, and prostaglandin and their related receptors (Ma and Quirion, 2007; Planells-Cases et al., 2005; Wang, 2008). Substantial evidence has indicated that TRPV1R is essential to the development of thermal hyperalgesia under inflammatory conditions, since mice lacking this receptor do not develop thermal hyperalgesia following tissue inflammation (Caterina et al., 2000). Since TRPV1R is the endpoint target of intracellular signaling pathways

triggered by inflammatory mediators (Ma and Quirion, 2007; Planells-Cases et al., 2005), an increase in TRPV1R expression during inflammation is a crucial factor in maintaining a nociceptive phenotype, particularly with respect to thermal hyperalgesia. Therefore, the present study was designed to elucidate possible mechanisms underlying P2Y1R mediated thermal hyperalgesia in a model of inflammation-induced persistent pain. I hypothesized that blockade of peripheral P2Y1R prevented the induction of thermal hyperalgesia via modulation of TRPV1R expression.

MATERIALS AND METHODS

Experimental animals

In all experiments, male Sprague-Dawley rats (200 to 250 g) were used and purchased from the Laboratory Animal Center of Seoul National University (SNU). Animals were housed under standard environmental conditions consisting of a 12 h light/dark cycle, a constant room temperature (maintained between 20-25 °C), and 40-60% humidity. During the experiments animals had free access to standard laboratory food and tap water. The experimental protocols for animal usage were reviewed and approved by the SNU Animal Care and Use Committee and conform to NIH guidelines.

Intraplantar drug administration and procedures

In order to investigate the role of peripheral P2Y1R at the site of inflammation, P2Y1R antagonist intraplantarly injected to the hind paw. Rats were briefly anesthetized with 3% isoflurane in a mixture of N_2O/O_2 gas. For intraplantar injection, each drug was injected into the central sole region of the hind paw using a 27-gauge needle attached to a Hamilton syringe. In order to investigate the role of P2Y1R in persistent inflammatory pain, I used a rat carrageenan inflammatory pain model and the selective P2Y1R antagonist, MRS2500 (50 μl). 2% carrageenan (200 μl) was injected into the plantar surface of the left hind paw, and control animals were injected with the same volume of saline. In naïve rats, the P2Y1R agonist, MRS2365 (30 μl), was injected into the hind paw to investigate the specific effect of peripheral P2Y1R. Each control group received the appropriate vehicle for each drug. Animals were randomly assigned to experimental groups and subsequent drug treatment and

behavioural analyses were performed blindly.

In order to investigate the role of P2Y1R in TRPV1R mediated thermal nociception, the P2Y1R agonist in naïve rats, MRS2365 was injected into the hind paw as either a singular injection or in a repetitive manner (Fig. 1-8). The experimental design for repetitive injections of MRS2365 involved injecting this agonist once per day for 3 consecutive days. Capsaicin was injected intraplantarly 1-hour after MRS2365 treatment. In the repetitive injection group, capsaicin was administrated 1-hour after the last MRS2365 injection on day 3. Capsaicin (Sigma, St. Louis, USA) was initially dissolved to a concentration of 0.1% in 20% alcohol, 7% Tween 80 and saline. Further dilutions were then made in saline giving a 0.1% capsaicin solution.

Assessment of thermal hyperalgesia

To test nociceptive responses to noxious heat stimuli, I measured the paw withdrawal response latency (PWL, sec) using a plantar test apparatus (Series 8, Model 390, IITC Life Science Inc., Woodland Hills, CA, USA) as previously described (Caterina et al., 2000; Seo et al., 2008; Wang, 2008). Before performing the tests, rats were placed in a plastic chamber on an elevated glass plate and were allowed to acclimate for 30 min. A radiant heat source was positioned under the glass floor beneath the hind paw to be tested and the withdrawal latency to the radiant heat was measured by using a photoelectric cell connected to a digital clock. The intensity of the light source was calibrated to produce a withdrawal response within 10 to 15 seconds in normal animals. Room temperature was maintained between 26-28°C

during the entire testing period. The test was duplicated in each hind paw at each time point and the mean withdrawal latency was calculated. A cut-off time of 20 seconds was used to protect the animal from excessive tissue damage. Animals were randomly assigned to experimental groups and subsequent drug treatment and behavioral analyses were performed blindly. To observe behavioral changes during both the acute and persistent pain phases in carrageenan rats, PWL were measured and assessed at 1, 2, 4, 6 hours after carrageenan injection at day 0 (acute phase), and subsequent measurements were performed at days 1, 2, 3, 5, 7 and 10 after inflammation (persistent pain phase). Behavioral testing on days 1 and 2 post-carrageenan injection was performed at least 4 hours after MRS2500 injection in order to avoid potential acute effects of MRS2500.

Assessment of mechanical allodynia

To assess mechanical allodynia of the glabrous skin, the number of paw withdrawal responses to a normally innocuous mechanical stimuli was measured by using a von Frey filament of 4.0 g (North Coast Medical, Morgan Hill, CA). Before performing the tests, rats were placed on a metal mesh grid under a plastic chamber and acclimated for 30 min. The von Frey filaments were applied from underneath the metal mesh flooring to the central sole region of hind paw, applied once every 3±4 s, for 10 trials at approximately 10 s intervals between trails. Baseline withdrawal response frequency (PWF, %) was measured by von Frey filaments prior to carrageenan injection. The data resulting from the mechanical allodynic behavioral testing for each experimental and control group are presented as the percentage of paw withdrawal response frequency (PWF, %). To observe behavioral changes in both the

acute and persistent phases of carrageenan-induced mechanical hyperalgesia, PWF to von Frey stimulation were measured and assessed at 1, 2, 4, 6 hours after carrageenan injection at day 0 (acute phase), and subsequent measurements were obtained at day 1, 2, 3, 5, 7 and 10 days (persistent phase) after inflammation. Behavioral testing on days 1 and 2 post-carrageenan injection was performed at least 4 hours after MRS2500 injection in order to avoid potential acute effects of MRS2500.

Drugs

(1R*,2S*)-4-[2-Iodo-6-(methylamino)-9H-purin-9-yl]-2-(phosphonooxy)bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester tetraammonium salt (MRS2500) and [[(1R,2R,3S,4R,5S)-4-[6-Amino-2-(methylthio)-9H-purin-9-yl]-2,3-dihydroxybicyclo[3.1.0]hex-1-yl]methyl] diphosphoric acid mono ester trisodium salt (MRS2365) were purchased from Tocris (Ellisville, MO, USA). MRS2500 and MRS2365 were dissolved in physiological saline. A 30 μl volume of one of the above drugs was injected to the hind paw.

Western Blot Analysis

Plantar lysates from ipsilateral hind paw, sciatic nerve and DRG were collected from anesthetized rats. The tissue were homogenized in buffer containing 1M Tris (pH 7.5), 1% NP-40, 0.5 M EDTA (pH 7.5), 50 mM EGTA, 1M dithiothreitol, 1M benzanidine and 0.1 M PMSF. The total amount of protein in each sample was determined using the Bradford dye assay prior to loading on polyacrylamide gels. The tissue homogenates (40μg protein) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. After the blots had been washed with

TBST [10mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.05% Tween-20], the membranes were blocked with 5% skim milk for 1-hour. I utilized a rabbit polyclonal P2Y1R antibody (1:2000, alomone labs Ltd, Jerusalem, Israel) for skin, sciatic nerve and DRG samples, and a rabbit polyclonal TRPV1R antibody (1:400, Calbiochem®, EMD Chemicals, Inc., Darmstadt, Germany) for skin and DRG samples. After the secondary antibody reaction, the bands were visualized with enhanced chemiluminescence (Amersham Pharmacia Biotech, England, UK). The positive pixel area of specific bands was measured with a computer-assisted image analysis system (Metamorph®, version 6.3r2, Molecular Devices Corporation, PA). P2Y1R and TRPV1R bands were normalized against the corresponding ß-actin loading control. The mean values of the each positive pixel area in the control or vehicle group were set at 100% and used for comparison with the experimental group.

Immunohistochemistry

Animals were deeply anesthetized with an intra-peritoneal injection of the Zoletil-Rompun-saline mixture (2:1:2) at 2 days after carrageenan injection in rat's hind paw. Animals were perfused transcardially with calcium-free Tyrode's solution, followed by a fixative containing 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). The ipsilateral DRGs (L4-L6) were collected after perfusion, post-fixed in the identical fixative for 4 hours and then placed in 30% sucrose in PBS (pH 7.4) at 4°C overnight. Frozen serial frontal sections (10 μm) were cut through the DRG L4–L6 using a cryostat (Microm, Walldorf, Germany). These serial sections were pre-blocked with 3% normal donkey serum and 0.3% Triton X-100 in PBS for 1 hour at room temperature. Tissue sections were incubated at 4°C with rabbit polyclonal P2Y1R and

goat polyclonal TRPV1R antibody (1:250, Santa Cruz Biotechnology Inc., Santa Cruz, California, USA), and mouse monoclonal Glial fibrillary acidic protein (GFAP) and mouse monoclonal NeuN (Millipore Corp, Bedford, Massachusetts, USA) for 48h and followed by a mixture of AlexaFluor 588 and 555 conjugated secondary antibodies (1:500, invitrogen, Carlsbad, California, USA) for 2 hr at room temperature.

Statistical Analysis

Statistical analysis was performed using Prism 5.1 (GraphPad Software, San Diego, CA). Behavioral data were tested using a two-way ANOVA to determine the overall effect of the drugs. For posthoc analysis, the Bonferroni's multiple comparison test was subsequently performed to determine significant differences among groups. One-way analysis of variance (ANOVA) was performed to confirm the change in western blot assay, and Newman-Keuls multiple comparison test was subsequently performed for posthoc analysis. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

1. The localization of P2Y1 receptor in DRG

In order to observe the location of DRG P2Y1R, immunohistochemistry was performed on serial DRG sections. To differentiate the location of P2Y1R in neuron and satellite glial cells, double staining in DRG sections was performed with NeuN (neuronal marker), GFAP (glial marker) and P2Y1R antibody. P2Y1R was mainly co-localized with neurons stained with NeuN, but not with satellite cells that stained with GFAP (Fig. 1-1). These results showed that P2Y1R selectively located in neuronal cells but not in satellite glial cells.

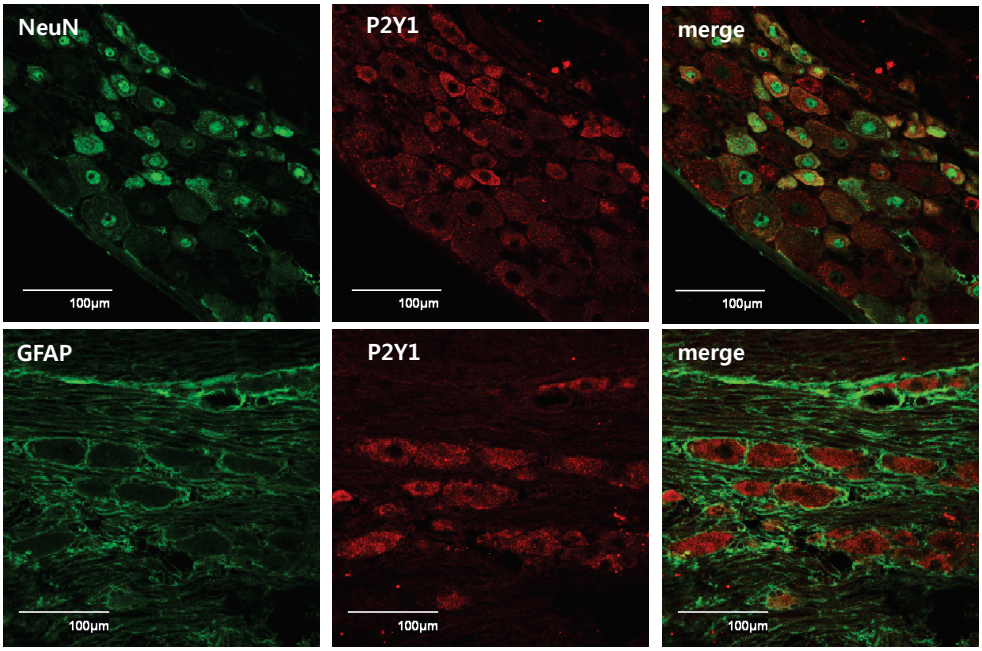


Figure 1-1. Cellular distribution of P2Y1 receptor in DRG.. Thin sections (10μm) of rat DRGs (L4-L6) were stained with antibodies against P2Y1R (red), NeuN (green) and GFAP (green) in control group. Representative double-labelled neurons are stained yellow in the merged panel. P2Y1R were co-localized with NeuN but not GFAP positive cells. Images are shown at 200× magnification. Scale bars represent 100μm.

2. Intraplantar injection of 2% carrageenan induce thermal hyperalgesia and mechanical allodynia in rats

In order to induce peripheral inflammation, carrageenan (2%, 200μl) was intraplantarly injected into the rat's hind paw, and observed the behavioral changes in paw withdrawal latency (PWL, sec) to noxious heat thermal hyperalgesia, and paw withdrawal frequency (PWF, %) to an innocuous mechanical stimulus. After carrageenan injection, rats showed significant pain hypersensitivity to both noxious heat and innocuous mechanical stimulation. With regard to thermal hyperalgesia, rats showed acute thermal hyperalgesia, which peaked at 4h after carrageenan injection, and persistent thermal hypersensitivity, which lasted for at least 10 days post-injection, but started to recover by day 7 post-injection (Fig. 1-2A; $*P < 0.05$ and $***P < 0.001$ as compared to control). Mechanical allodynia also developed immediately after carrageenan injection, and was maintained for more than 10 days (Fig. 1-2B; $**P < 0.01$ and $***P < 0.001$ as compared to control).

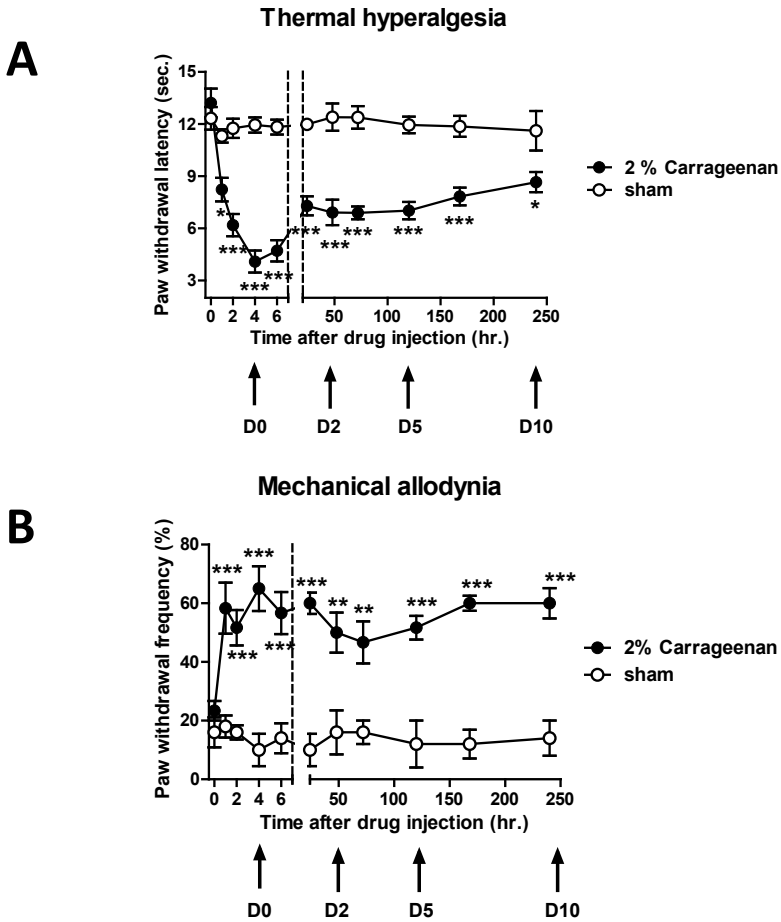


Figure 1-2. The effect of intraplantar 2% carrageenan injection on pain hypersensitivity. 2% carrageenan (200μl) injection into the hind paw significantly decreased paw withdrawal latency (PWL, sec) to the noxious heat (**A**, n=5 in normal group and n=7 in carrageenan group, $*P < 0.05$ and $***P < 0.001$ as compared to control), and also increased paw withdrawal frequency (PWF, %) to innocuous mechanical stimulation (**B**, n=5 in normal group and n=6 in carrageenan group, $**P < 0.01$ and $***P < 0.001$ as compared to control group). Tissue samples are collected 4 time points from day 0 (D0) to day 10 (D10).

3. Up-regulated P2Y1 receptor expression in peripheral tissues during carrageenan-induced inflammation

To elucidate the expression level of P2Y1R in peripheral tissues over time, animals were euthanized at the following 4 time points: 4 hours, 2, 5 and 10 days after inflammation, and then collected hind paw skin, sciatic nerve and DRGs for further processing (Fig. 1-3). I first investigated whether inflammatory insults caused by intraplantar carrageenan injection induced changes in P2Y1R expression in peripheral tissues by using western blot analysis. After inflammation, the expression of P2Y1R in peripheral ipsilateral tissue including skin, sciatic nerve and L4-L6 DRG was increased (Fig. 1-3A to C; * $P < 0.05$, ** $P < 0.01$ as compared to control). Hind paw inflammation induced a gradual increase in P2Y1R expression in immunoblots that peaked at 2 days post-injection. By 10 days after induction of inflammation, P2Y1R expression in peripheral tissue had almost returned to normal values. In addition, to confirm the inflammation induced up-regulation of P2Y1R in DRG, P2Y1R expression appearance was compared between normal and carrageenan injected rats. After inflammation, both staining density and the number of P2Y1R positive cells were increased compared to control group (Fig. 1-3D).

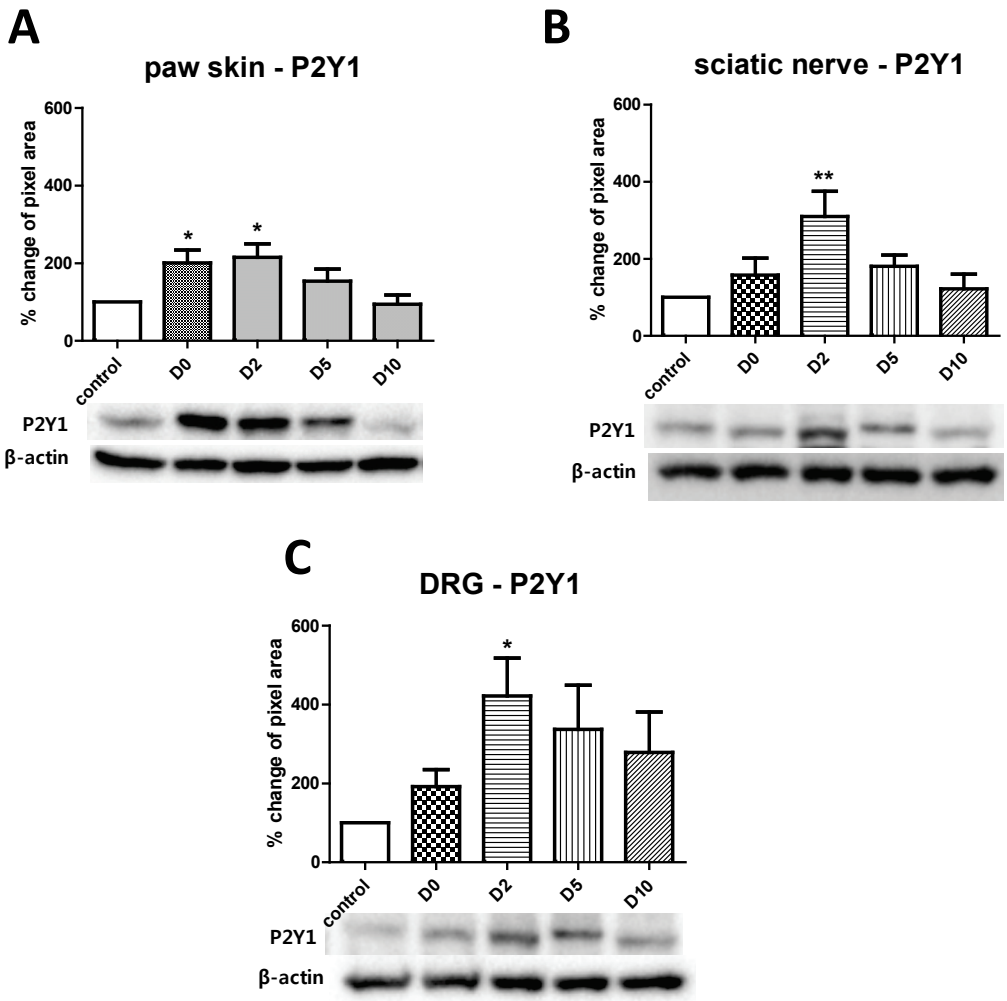

A
paw skin - P2Y1
% change of pixel area
600
400
200
0
*
*
control
D0
D2
D5
D10
P2Y1
β-actin
B
sciatic nerve - P2Y1
% change of pixel area
600
400
200
0
**
control
D0
D2
D5
D10
P2Y1
β-actin
C
DRG - P2Y1
% change of pixel area
600
400
200
0
*
control
D0
D2
D5
D10
P2Y1
β-actin
D
control
carrageenan (D2)
P2Y1
100μm
P2Y1
100μm

Figure 1-3. The effect of intraplantar 2% carrageenan injection on the expression level of P2Y1 receptor. Western blot and graphs illustrate the effect of peripheral inflammation on P2Y1R expression in peripheral tissues including hind paw skin, sciatic nerve and DRG (A to C). The protein expression of P2Y1R in inflammatory skin increased from day 0 (D0, 4 hours after carrageenan injection) to day 2 after inflammation (D2, **A**, n=7 in each group, $*P < 0.05$ as compared to control). P2Y1R expression in the sciatic nerve and DRG significantly increased at 2 days post-carrageenan injection (**B** and **C**, n=7 in each group, $*P < 0.05$ as compared to control group). Representative western blots showing P2Y1 (top) and β-actin (bottom) expression in skin, sciatic nerve and DRG. Data are presented as the percent (%) change relative to the control. Immunofluorescent images of rat DRG neurons (**D**). Thin sections (10μm) of rat DRGs (L4-L6) were stained with antibodies against P2Y1R. The proportion of P2Y1R-immunoreactive neurons is augmented 2 days after inflammation in carrageenan rats compared to control rats. Images are shown at 200× magnification. Scale bars represent 100 μm.

4. Involvement of peripheral P2Y1 receptor on carrageenan-induced inflammatory pain

In attempting to evaluate the role of peripheral P2Y1R in carrageenan induced inflammatory pain, I performed intraplantar injection of P2Y1R antagonist, MRS2500 (1, 3 and 10nmol), and observed the behavioral changes in paw withdrawal latency to noxious heat, and paw withdrawal frequency to innocuous mechanical stimulus (Fig. 1-4). Following a single injection of MRS2500 that was given 10 min before carrageenan injection, there were no significant effects on the carrageenan-induced thermal hyperalgesia and mechanical allodynia (Fig. 1-4A and B). Therefore, MRS2500 (1, 3 and 10nmol, once a day) was repetitively injected from day 0 to day 2 (Fig. 1-4C and D). Following two repeated injections of MRS2500, there was a significant analgesic effect on thermal hyperalgesia, but not mechanical allodynia, during the persistent phase of inflammatory pain (Fig. 1-4C; $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ as compared to vehicle). Behavioral testing on days 1 and 2 post-carrageenan injection was performed at least 4 hours after MRS2500 injection in order to avoid potential acute effects of MRS2500. The anti-thermal hyperalgesic effects of the MRS2500 in the carrageenan model of inflammatory pain were dose related. Cessation of drug treatment resulted in persistence of the anti-hyperalgesia effect, which lasted through day 5 after carrageenan injection (Fig. 1-4C; $**P < 0.01$ as compared to vehicle on day 5, and $***P < 0.001$ as compared to vehicle on day 7).

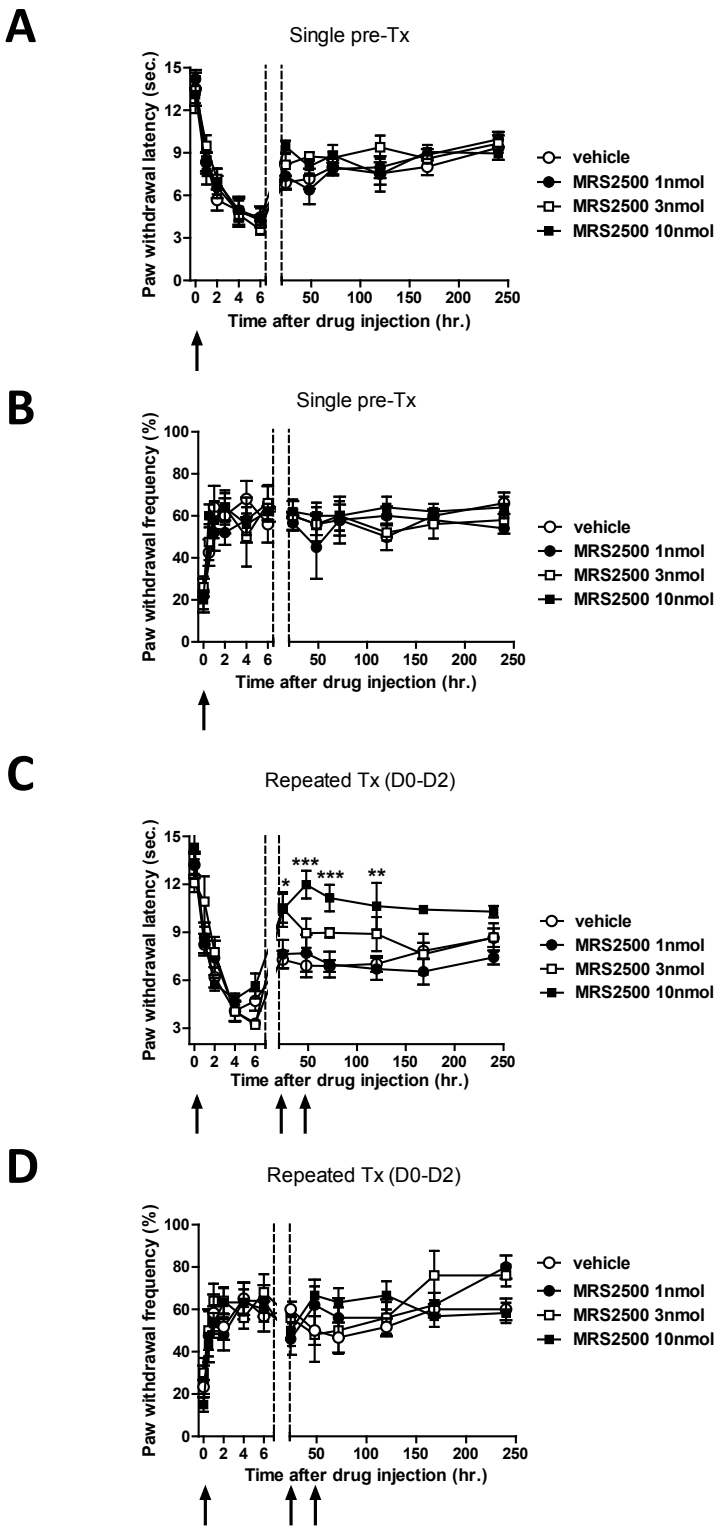

A
Single pre-Tx
Paw withdrawal latency (sec.)
Time after drug injection (hr.)
vehicle
MRS2500 1nmol
MRS2500 3nmol
MRS2500 10nmol
B
Single pre-Tx
Paw withdrawal frequency (%)
Time after drug injection (hr.)
vehicle
MRS2500 1nmol
MRS2500 3nmol
MRS2500 10nmol
C
Repeated Tx (D0-D2)
Paw withdrawal latency (sec.)
Time after drug injection (hr.)
vehicle
MRS2500 1nmol
MRS2500 3nmol
MRS2500 10nmol
D
Repeated Tx (D0-D2)
Paw withdrawal frequency (%)
Time after drug injection (hr.)
vehicle
MRS2500 1nmol
MRS2500 3nmol
MRS2500 10nmol

Figure 1-4. The effects of single and repeated MRS2500 injections on carrageenan-induced thermal hyperalgesia and mechanical allodynia. Single pre-treatment (10 min before carrageenan injection) did not affect carrageenan induced thermal hyperalgesia and mechanical allodynia (**A** and **B**, n=5 in each group). Repeated daily treatment (D0 to D2) with MRS2500 (1, 3 and 10nmol) blocked the persistent thermal hyperalgesia compared to vehicle-treated carrageenan rats from day 1 to day 5 post-carrageenan injection. (**C**, n=7 in vehicle group, n=5 in MRS2500 1 and 3nmol, n=6 in 10nmol group, $*P < 0.05$ and $***P < 0.001$ as compared to carrageenan + vehicle). However, carrageenan-induced mechanical allodynia was unaffected by repeated intraplantar injection of MRS2500 (**D**, n=6 in vehicle group, n=5 in MRS2500 1 and 3nmol, n=6 in 10nmol group).

5. Modulatory effect of peripheral P2Y1 receptor on TRPV1 receptor expression

In order to observe the changes in peripheral TRPV1R expression over time, skin, sciatic nerve and DRG samples were collected from control and carrageenan-injected rats at 0, 2, 5 and 10 days post-injection (Fig. 1-5A to C). Following carrageenan injection, the expression of TRPV1R gradually increased, peaked at day 2 and then slowly decreased back to control levels by day 5 (Fig. 1-5A to C; $*P < 0.05$ as compared to control). Since inhibition of peripheral P2Y1R selectively alleviated inflammatory thermal hypersensitivity, I hypothesized that this inhibitory effect was dependent on TRPV1R expression levels. Therefore, western blot analysis was performed to determine whether TRPV1R expression is regulated by P2Y1R inhibition. After repeated treatment with the P2Y1R antagonist, MRS2500, the animals were euthanized at day 2, the time point at which animals showed a peak anti-thermal hyperalgesic effect in response to MRS2500 treatment. There was a significant increase in TRPV1R expression in the inflammatory skin and DRGs from carrageenan animals compared to the control group (Fig. 1-6A to C; $*P < 0.05$ as compared to control), and MRS2500 treated rats showed a significant decrease in TRPV1R expression compared to vehicle-treated carrageenan rats in both skin and DRG tissues (Fig. 1-6A and C, $\#P < 0.05$ as compared to carrageenan + vehicle). In order to investigate the locational relationship between P2Y1R and TRPV1R, double-staining was performed in DRG sections with P2Y1R and TRPV1R antibodies. As shown in Fig. 1-7, P2Y1R expression overlapped with TRPV1R positive, small-diameter DRG neurons. Compared to control rats, double-stained P2Y1R and TRPV1R positive neurons were markedly increased in rats with inflammation (Fig. 1-7).

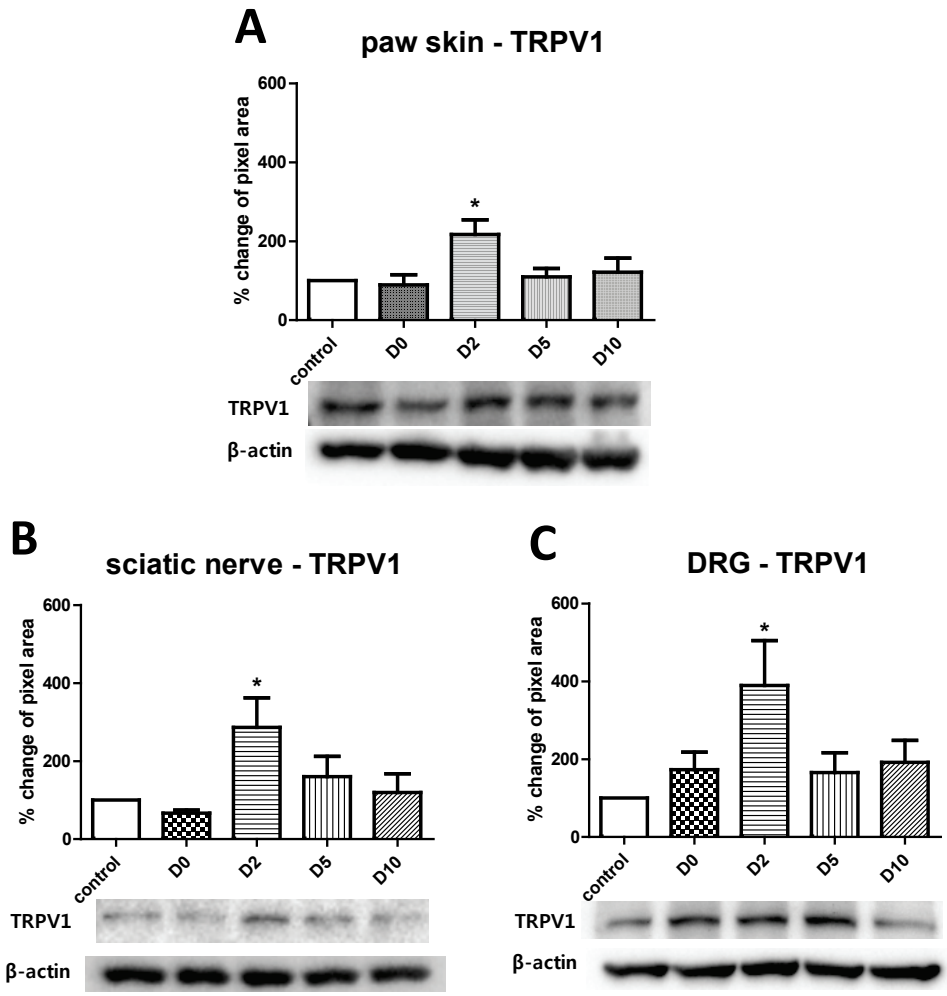
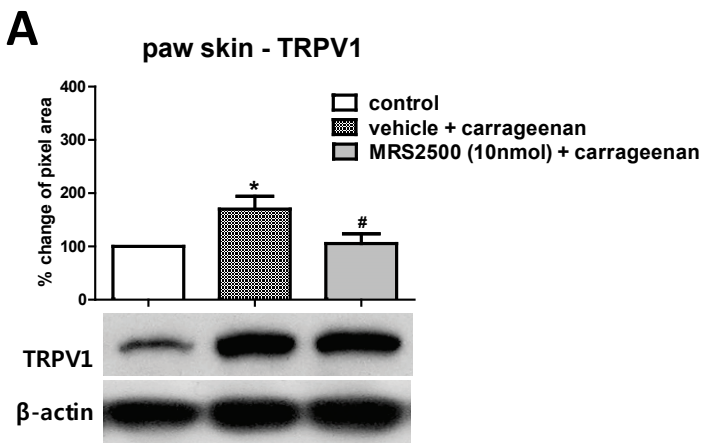
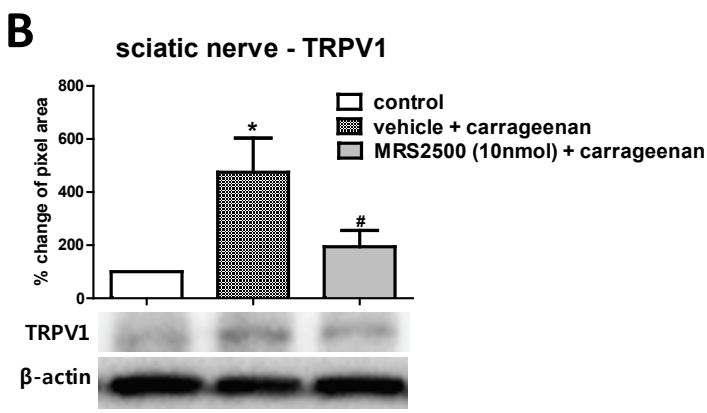


Figure 1-5. The effect of intraplantar 2% carrageenan injection on the expression level of TRPV1 receptor. Western blot and graphs illustrate the changes of TRPV1R expression over time in peripheral tissues including hind paw skin, sciatic nerve and DRG (**A** to **C**). The protein expression of TRPV1R in both skin (**A**, n=5 in each group, $*P < 0.05$ as compared to control), sciatic nerve (**B**, n=6 in each group, $*P < 0.05$ as compared to control) and DRG (**C**, n=6 in each group, $*P < 0.05$ as compared to control) significantly increased at day 2 (D2) after inflammation.


A
paw skin - TRPV1
% change of pixel area
400
300
200
100
0
control
vehicle + carrageenan
MRS2500 (10nmol) + carrageenan
*
#
TRPV1
β-actin


B
sciatic nerve - TRPV1
% change of pixel area
800
600
400
200
0
control
vehicle + carrageenan
MRS2500 (10nmol) + carrageenan
*
#
TRPV1
β-actin

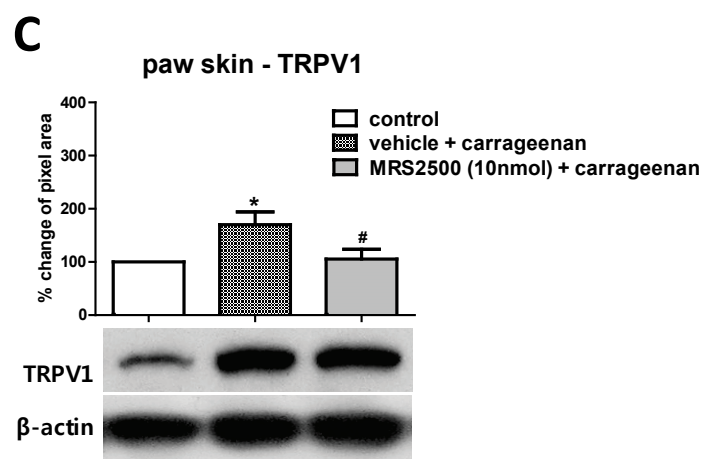

C
paw skin - TRPV1
% change of pixel area
400
300
200
100
0
control
vehicle + carrageenan
MRS2500 (10nmol) + carrageenan
*
#
TRPV1
β-actin

Figure 1-6. The effect of MRS2500 on TRPV1 receptor up-regulation in carrageenan-induced inflammatory tissues. Western blot and graphs depicting the effect of MRS2500 (a P2Y1R antagonist, 10nmol) on carrageenan-induced TRPV1R expression in skin (**A**, n=5 in each group, $*P < 0.05$ as compared to control), sciatic nerve (**B**, n=6 in each group, $*P < 0.05$ as compared to control) and DRGs (**C**, n=4 in each group, $*P < 0.05$ as compared to control). Repeated MRS2500 injection caused a significant decrease in TRPV1R expression in skin, sciatic nerve and DRG lysates (**A** to **C**, $\#P < 0.05$ as compared to carrageenan + vehicle). Representative western blots showing TRPV1R (top) and β-actin (bottom) expression levels in skin, sciatic nerve and DRGs. Data are presented as the percent (%) change relative to the control.

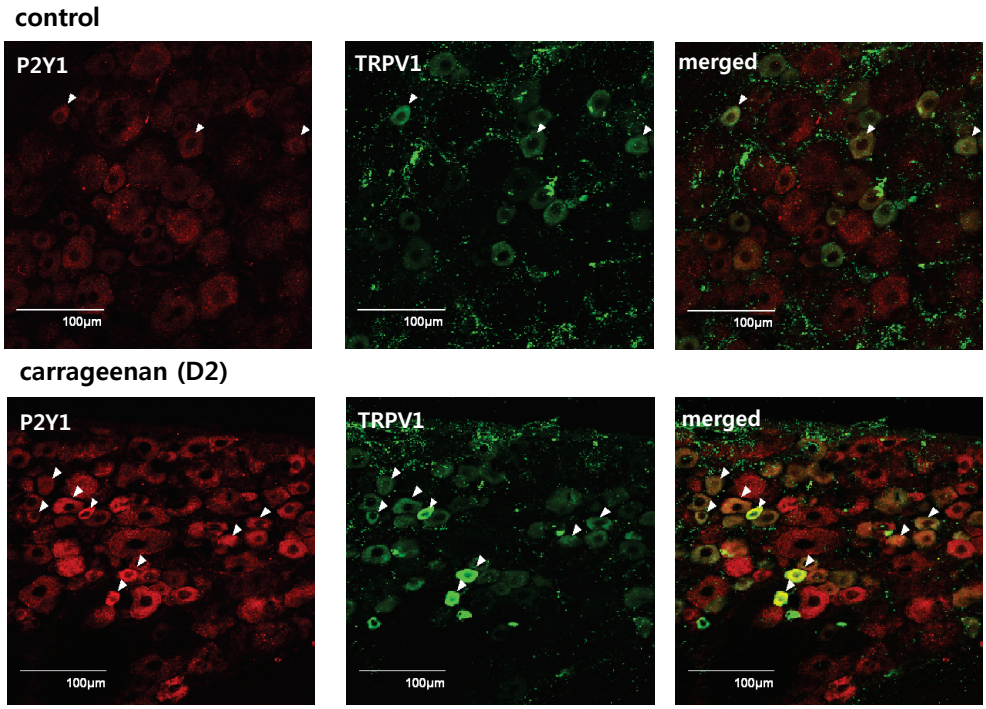
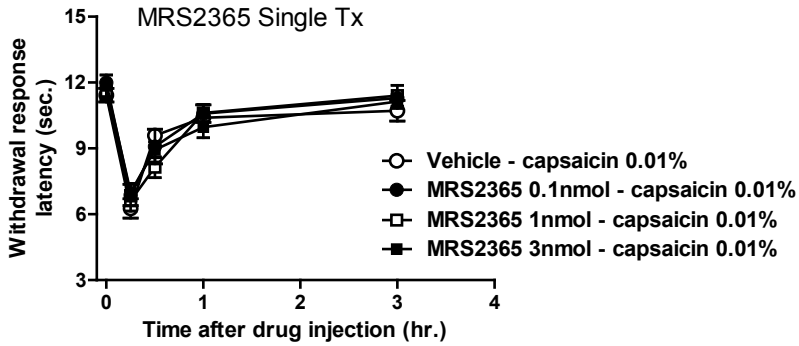


Figure 1-7. Co-localization of P2Y1 and TRPV1 receptors in DRG. Immunofluorescent images of rat DRG neurons. Thin sections (10μm) of rat DRGs (L4-L6) were stained with antibodies against P2Y1R (red), TRPV1R (green) in control and 2 days (D2) after inflammation. Representative double-labelled neurons (arrow-head) are stained yellow in the merged panel. Immunoreactivity of both TRPV1R and P2Y1R was augmented after inflammation and the proportion of double stained immunoreactive neurons was increased after inflammation. Images are shown at 200× magnification. Scale bars represent 100 μm.

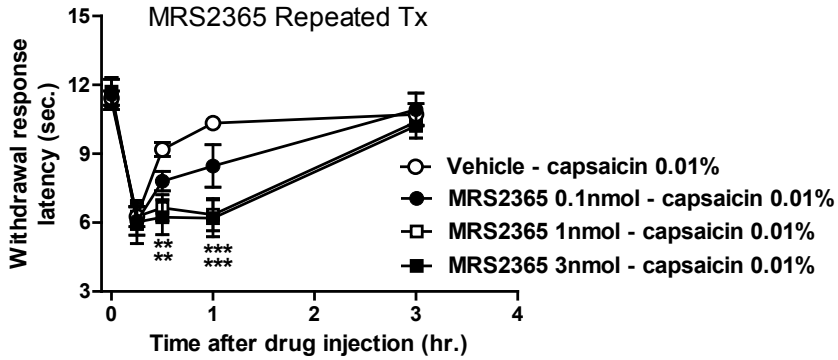
6. Modulatory effect of peripheral P2Y1 receptor on TRPV1 receptor mediated thermal hyperalgesia in naïve rats

To evaluate the potential relationship between P2Y1R and TRPV1R in naïve rats, the TRPV1R agonist, capsaicin (0.01%) was injected 1h after either a single or following repetitive injections of MRS2365 (0.1, 1, and 3nmol). The single injection of MRS2365 had no effect on capsaicin-induce thermal hyperalgesia as compared to saline treated rats (Fig. 1-8A). On the other hand, repeated daily injection of the P2Y1R agonist produced a significant increase in capsaicin-induced thermal hyperalgesia, which was dependent on the dose of MRS2365 that was administered (Fig. 1-8B). In the vehicle-capsaicin (0.01%) treated group, the capsaicin-induced decrease in withdrawal latency returned to baseline levels prior to the 1-hour post-injection measurement time point. By contrast, the 1nmol MRS2365 - 0.01% capsaicin treatment group showed prolonged and significant thermal hyperalgesia that was evident at the 30 min post-injection time point and was still significantly different at the 1-hour post-injection time point. In order to confirm that the P2Y1R-induced facilitatory effect on thermal hyperalgesia is indeed mediated by TRPV1R, pre-treatment with AMG-9810, a potent TRPV1R antagonist, was performed 30 min before capsaicin injection (Fig. 1-8C). I found that AMG9810 (1, 3, and 10nmol) dose-dependently blocked the P2Y1R-induced enhancement of thermal hyperalgesia produced by capsaicin (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ as compared to those in the 1nmol MRS2365-vehicle injected group).

A


MRS2365 Single Tx
Withdrawal response latency (sec.)
Time after drug injection (hr.)
Vehicle - capsaicin 0.01%
MRS2365 0.1nmol - capsaicin 0.01%
MRS2365 1nmol - capsaicin 0.01%
MRS2365 3nmol - capsaicin 0.01%

B


MRS2365 Repeated Tx
Withdrawal response latency (sec.)
Time after drug injection (hr.)
Vehicle - capsaicin 0.01%
MRS2365 0.1nmol - capsaicin 0.01%
MRS2365 1nmol - capsaicin 0.01%
MRS2365 3nmol - capsaicin 0.01%

C

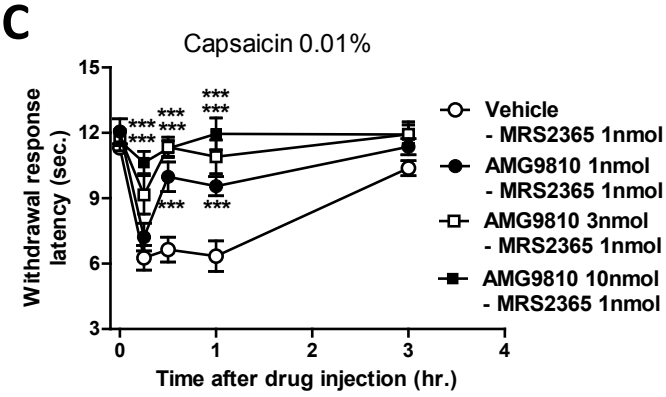
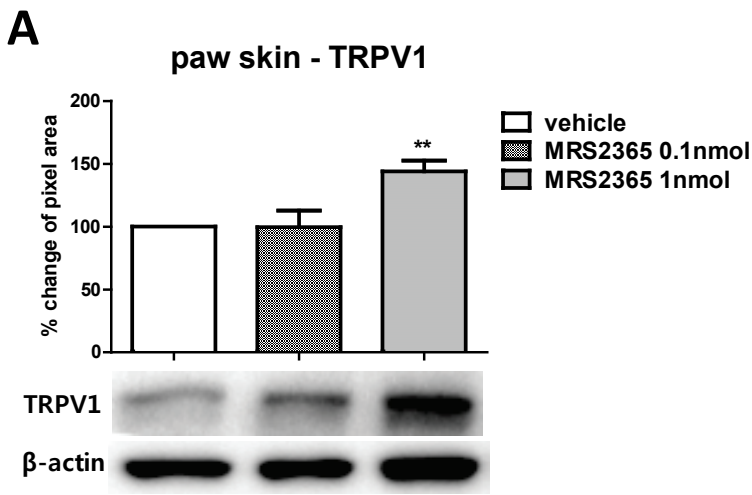

Capsaicin 0.01%
Withdrawal response latency (sec.)
Time after drug injection (hr.)
Vehicle
- MRS2365 1nmol
AMG9810 1nmol
- MRS2365 1nmol
AMG9810 3nmol
- MRS2365 1nmol
AMG9810 10nmol
- MRS2365 1nmol

Figure 1-8. The effect of MRS2365 injections on capsaicin induced thermal hyperalgesia. 1h after MRS2365 (a P2Y1R agonist, 0.1, 1 or 3nmol) injection, a sub-threshold dose of capsaicin 0.01% (**A** to **C**, n=6 in each group) was injected into the hind paw. A single injection of the P2Y1R agonist, MRS2365 (at doses of 0.1, 1, and 3nmol) did not significantly affect 0.01% capsaicin-evoked thermal hyperalgesia (**A**). Repetitive injections of MRS2365 significantly potentiated the 0.01% capsaicin-evoked thermal nociception at the 30 min to 1h time points following injection (**B**, $^{**}P < 0.01$ and $^{***}P < 0.001$ as compared to those in animals treated with vehicle-capsaicin 0.01%), and the maximal facilitatory effect was produced by 1nmol MRS2365. In addition, TRPV1R antagonist, AMG9810 was pre-treated 30 min before the injection 0.01% capsaicin (**C**). Intraplantar pre-treatment with AMG9810 (1, 3 or 10nmol) dose dependently reduced capsaicin-induced thermal hyperalgesia ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ as compared to those in animals treated with 1nmol MRS2365 - vehicle).

7. Modulatory effect of peripheral P2Y1 receptor on TRPV1 receptor expression in naïve rats

To confirm the specific modulatory role of P2Y1R on TRPV1R expression, experiments were performed in naïve rats. To determine the effect of chronic repetitive stimulation on peripheral P2Y1R, I injected the selective P2Y1R agonist, MRS2365 (0.1 and 1nmol), daily for 3 consecutive days (Fig. 1-9). Following these three sequential injections, animals were euthanized and the potential changes TRPV1R expression were investigated in DRG and hind paw lysates using western blot analysis. Repetitive administration of MRS2365 significantly increased the amount of TRPV1R expression, and this was increased in a dose dependent fashion (Fig. 1-9A and B; $^{**}P < 0.01$ as compared to vehicle) in both skin and DRG.


A
paw skin - TRPV1
% change of pixel area
200
150
100
50
0
**
vehicle
MRS2365 0.1nmol
MRS2365 1nmol
TRPV1
β-actin

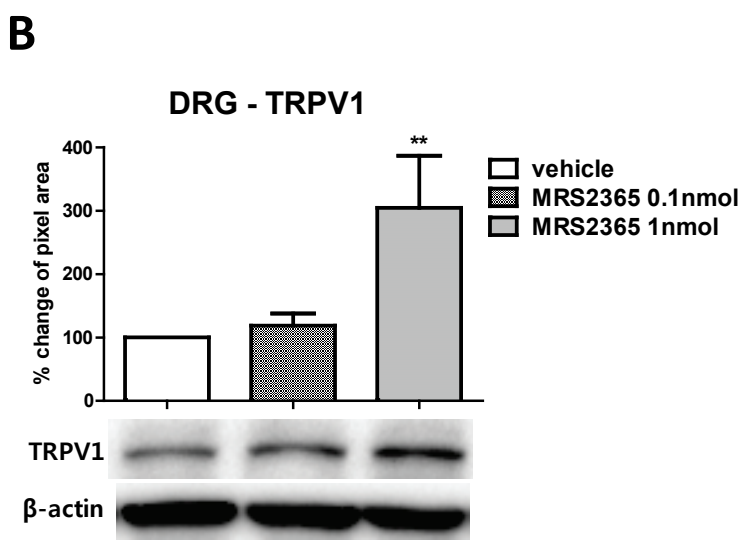

B
DRG - TRPV1
% change of pixel area
400
300
200
100
0
**
vehicle
MRS2365 0.1nmol
MRS2365 1nmol
TRPV1
β-actin

Figure 1-9. The effects of intraplantar injection of MRS2365 on the expression of TRPV1 receptor in naïve rats. Quantitative western blot analysis was performed to determine the expression level of TRPV1R in hind paw skin in the DRG lysates in vehicle, MRS2365 0.1, and 1nmol treated animals (A and B, n=6 in skin, n=8 in DRG). Repeated injection of MRS2365 over 3 consecutive days dose dependently increased TRPV1R expression in hind paw lysates and DRG (A and B, $^{**}P < 0.01$ as compared to vehicle). Representative western blots bands showing TRPV1R (top) and β-actin (bottom) in skin and DRG. Data are presented as the % change relative to the vehicle control.

DISCUSSION

During the process of inflammation, leukocyte-induced tissue damage results in the release of various sensitizers into the skin including ATP. The elevated concentrations of ATP present at sites of tissue damage contribute to the activation of nociceptive sensory afferents and contribute to pain hypersensitivity. In addition to ATP, ADP is also prevalent in damaged tissues (Dussor et al., 2009; Pearson et al., 1980), but its role in the inflammation-induced nociception is not as well understood. In this regard, the P2Y1R is a Gq-coupled receptor whose preferred agonists are ADP and ATP (Dussor et al., 2009; Hardy et al., 2005; Nakamura and Strittmatter, 1996; Sacha and Derek, 2010). Recently, the localization of this receptor in sensory neurons and their putative involvement in pain transduction has been reported (Gerevich et al., 2004; Jankowski et al., 2012; Sacha and Derek, 2010; Yousuf et al., 2011). The current study, for the first time, demonstrates that peripheral P2Y1R-mediated signals regulate inflammation-induced phenotypic changes in TRPV1R expression. This study also provide data showing that P2Y1R play an important role in the maintaining inflammatory thermal hyperalgesia, but not mechanical allodynia.

In the initial experiments, I performed a western blot analysis of tissue lysates from the skin, sciatic nerve and DRG to determine the changes in peripheral P2Y1R expression over time in an established rodent model of inflammatory pain. The expression of P2Y1R in skin, sciatic nerve and DRGs gradually increased following carrageenan injection, and it was increased on day 2 post-injection and then decreased again by day 5 post-injection (Fig. 1-3A to C). These results were in line with previous study reported by Jankowski *et al.* (2012) that CFA injection into the mouse hairy hind paw skin increased P2Y1R expression in L2-3 DRG lysates. Increased

DRG P2Y1R could be transported to both peripheral and central terminal located in spinal dorsal horn. However, in the current study, I focused on the peripheral P2Y1R located in inflammatory tissue, and utilized intraplantar injection route to inhibit of peripheral P2Y1R. In an attempt to delineate the specific role of peripheral P2Y1R, specific P2Y1R antagonist, MRS2500, was injected to the hind paw and subsequently investigated its effect on carrageenan-induced thermal and mechanical hypersensitivity. A single injection of MRS2500 failed to prevent the induction of thermal hyperalgesia and mechanical allodynia (Fig. 1-4A and B). However, repetitive treatment with MRS2500 on days 1 and 2 post-carrageenan injection effectively blocked the persistent thermal hyperalgesia without affecting mechanical allodynia (Fig. 1-4C and D). These results implicated that peripheral inhibition of P2Y1R contributed to long-term plastic changes particularly in inflammation induced thermal hypersensitivity.

A putative relationship between P2Y1R and TRPV1R has been suggested in a few previous studies. Thus, Tominaga *et al*. (2001) suggested an interaction between P2Y1R and TRPV1R based on the work performed in HEK 293 cells. They demonstrated that extracellular ATP lowered the temperature threshold and increased capsaicin- or proton-evoked TRPV1R currents, and this augmentative action of ATP was mediated by P2Y1R. Seo *et al.* (2011) previously proposed the possible inter-relationship of these two receptors *in vivo*; there was a facilitatory interaction of ATP and protons on TRPV1R-mediated thermal hyperalgesia in experiments in which αβmeATP was administered under acidic pH conditions. Although these results implicated a potential relationship between P2Y1R and TRPV1R in the development of thermal hyperalgesia, the experimental design only allowed us to evaluate a short-

term effect. Furthermore, Malin *et al*. (2008) addressed that this acute TRPV1R modulation by extracellular ATP was mainly mediated by P2Y2R but not P2Y1R. Since short-term modulation of P2Y1R on TRPV1R has been reported to be controversial, this study focused on the role of peripheral P2Y1R for TRPV1R expression in terms of long-term nociceptor plasticity. The role of peripheral P2Y1R were examined in inflammation-induced phenotypic increases of TRPV1R expression. Repetitive blocking of peripheral P2Y1R by administration of the P2Y1R antagonist, MRS2500, resulted in a significant decrease in TRPV1R expression and inhibition of the development carrageenan-induced thermal hyperalgesia (Fig. 1-6A to C). In addition, repeated injection of the P2Y1R agonist, MRS2365 into the hind paw of naïve rats dose dependently increased expression of TRPV1R in peripheral tissues (Fig. 1-9A and B). These results indicate that peripheral P2Y1R contributed to the maintenance of a thermal hypersensitive nociceptive phenotype by up-regulation of TRPV1R expression.

Immunohistochemistry data showed that each P2Y1R and TRPV1R positive neurons and co-localized neurons were also increased in carrageenan rats compared to control (Fig. 1-7). Earlier studies observed that P2Y1R is largely expressed in small diameter neurons in the DRG (Ruan and Burnstock, 2003), and P2Y1R and TRPV1R are also reported to be located on the same population of DRG neurons (Gerevich et al., 2004; Jankowski et al., 2012). Therefore, it is plausible to consider that an activity-dependent stimulus from peripheral P2Y1R directly modulates TRPV1R expression in the same neuron. However, there is a still another possibility that TRPV1R expression can be indirectly activated by secondary substances released through P2Y1R activation. There are several studies reporting the P2Y receptor-mediated

release of PGE and CGRP at both the cellular and whole animal levels (Brambilla et al., 1999; Brambilla et al., 2002; Sanada et al., 2002; Zimmermann et al., 2002). In this regard, further *in vivo* and *in vitro* investigations are needed to address whether P2Y1R directly or indirectly modulate TRPV1R expression in the peripheral level.

Based on the present study, the increases in TRPV1R expression require a prolonged stimulation period rather than acute activation of P2Y1R. I found that while a single injection of the P2Y1R agonist, MRS2365, did not enhance TRPV1R mediated thermal hyperalgesia, repetitive injections of MRS2365 significantly potentiated thermal hyperalgesia (Fig. 1-8). Although it is unclear why a single injection of MRS2365 failed to modulate TRPV1R, the present results suggest that the facilitatory action of P2Y1R on TRPV1R might be dependent on the chronic activation of P2Y1R, which would mimic what actually occurs during a variety of pathological states including ischemic injury, inflammation and nerve injury.

These results demonstrate that P2Y1R located in inflammatory site modulates long term changes of primary afferent fiber's activity, i.e., nociceptor plasticity especially in heat hypersensitivity. This study suggests that peripheral P2Y1R could be useful targets for alleviating thermal hypersensitivity under the conditions of chronic inflammation.

CHAPTER 2

Causal relations among P2Y1 receptor, p38 MAPK in DRG, and TRPV1 receptor during the inflammation

ABSTRACT

Although previous reports have suggested that P2Y1 receptor (P2Y1R) is involved in cutaneous nociceptive signaling, it remains unclear how P2Y1R contribute to peripheral sensitization. The current study was designed to delineate the role of peripheral P2Y1R in pain and to investigate potential linkages to mitogen-activated protein kinase (MAPK) in DRGs and Transient Receptor Potential Vanilloid 1 receptor (TRPV1R) expression in a rodent inflammatory pain model. Following injection of 2% carrageenan into the hind paw, expressions of P2Y1R and TRPV1R and the phosphorylation rates of both p38 MAPK and ERK but not JNK were increased and peaked at day 2 post-injection. Blockade of peripheral P2Y1R by the specific antagonist, MRS2500 injection (intraplantar, D0 to D2) significantly reduced the induction of thermal hyperalgesia, but not mechanical allodynia. Simultaneously, MRS2500 injections suppressed up-regulated TRPV1R expression and DRG p38 MAPK phosphorylation, while p-ERK signaling was not affected. Furthermore, inhibition of p38 MAPK activation in the DRGs by SB203580 (a p38 MAPK inhibitor, intrathecal, D0 to D2) prevented the up-regulation of TRPV1R and a single intrathecal injection of SB203580 reversed the established thermal hyperalgesia, but not mechanical allodynia. Lastly, to identify the mechanism of action of P2Y1R, the P2Y1R agonist, MRS2365 was repeatedly injected into the naïve rat's hind paw and observed a dose-dependent increase in TRPV1R expression and p38 MAPK phosphorylation. These data demonstrate a sequential role of P2Y1R, p38 MAPK and TRPV1R in inflammation-induced thermal hyperalgesia; thus, peripheral P2Y1R activation modulates p38 MAPK signaling and TRPV1R expression, which ultimately leads to the induction of thermal hyperalgesia.

INTRODUCTION

Mitogen-activated protein kinases (MAPKs) are a family of kinases that mediate many of the cellular responses to a variety of external stimuli (Cheng and Ji, 2008; Ji et al., 2009; Obata and Noguchi, 2004). Although the investigations of MAPKs in pain hypersensitivity is rather restricted in the spinal cord level, there are some reports demonstrating the increased activity of MAPKs in sensory neurons regarding to the peripheral noxious stimulus (Ji et al., 2009). Among these MAPK members, p38 MAPK and ERK are reported to be crucial signaling cascades related to pain hypersensitivity in primary afferent fibers (Dai et al., 2002; Dai et al., 2004; Ji et al., 2002; Mizushima et al., 2005; Obata and Noguchi, 2004). In addition, a few studies also reported the involvement of DRG JNK pathway in sensitized primary afferent fiber (Doya et al., 2005; Kenney and Kocsis, 1998). These findings suggest that DRG p38 MAPK, ERK and JNK cascades could be key factors in maintaining nociceptor plasticity and persistent pain.

Previous studies have suggested that MAPKs signaling in DRGs regulate the expression of TRPV1R (Bron et al., 2003; Cui et al., 2008; Ji et al., 2009; Zhuang et al., 2004). It has also been shown that repeated morphine treatment increases the expression of TRPV1R produced tolerance-associated thermal hyperalgesia, and this is mediated by DRG MAPK phosphorylation (Cui et al., 2008). Nerve growth factor also leads to an increase in TRPV1R levels in DRG neurons and inflamed skin through the activation of p38 mitogen-activated protein kinase (MAPK) (Ji et al., 2002). Since the modulatory effects of P2Y1R on TRPV1R expression were examined in chapter 1, there is a possibility that MAPKs signaling in DRGs contributed to the P2Y1R mediated regulation of TRPV1R expression. Therefore, I examined whether 1) MAPKs activity in DRGs would increase in response to the

inflammatory insults and contribute to pain hypersensitivity; 2) blockade of peripheral P2Y1R result in the decrease of MAPKs activity in DRGs; 3) MAPKs signaling would be involved in the modulatory effects of P2Y1R on the TRPV1R expression.

MATERIALS AND METHODS

Experimental animals

Experimental animal and maintaining condition is identical with that of chapter 1.

Intraplantar and intrathecal drug administration

Methods for the intraplantar administration was described in chapter 1. In order to inhibit DRG p38 MAPK, p38 MAPK inhibitor, SB203580 (10μl) was intrathecally injected. For intrathecal administration, rats were briefly anesthetized with 3% isoflurane in a mixture of N_2O/O_2 gas to prevent any handling-induced stress. Modified method of direct transcutaneous intrathecal injection (Mestre et al., 1994) was used in this study. Intrathecal injections were delivered into the subarachnoid space through the L5-L6 intervertebral space of animals using a 50μl Hamilton syringe connected to 26-gauge needle. The flick of the tail was considered indicative of a successful intrathecal administration. Each control group received the appropriate vehicle for each drug. Animals were randomly assigned to experimental groups and subsequent drug treatment and behavioural analyses were performed blindly.

Behavioral assessments

Experimental methods for pain behaviors (thermal hyperalgesia and mechanical allodynia) were identical with those of chapter 1.

Drugs

All drugs used in this study were previously described in chapter 1 except (1*R**,2*S**)-4-[2-Iodo-64-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl)-

1H-imidazole (SB203580; SB). SB was purchased from Sigma–Aldrich (St. Louis, MO)). SB was dissolved in 1% DMSO in saline, and 10μl was injected intrathecally for individual experiments.

Western Blot Analysis

Experimental procedures are identical with those of chapter 1. Rabbit polyclonal p38 MAPK, rabbit polyclonal p-p38 MAPK, rabbit polyclonal phospho-p44/42 MAPK (1:1000, Cell signaling technology, Beverly, Massachusetts, USA), rabbit polyclonal ERK, mouse monoclonal JNK and mouse monoclonal p-JNK (1:1000, Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) antibodies were used for analysis of DRG samples. After the secondary antibody reaction, the bands were visualized with enhanced chemiluminescence (Amersham Pharmacia Biotech, England, UK). P-p38 and p-ERK levels are normalized against corresponding total p38 MAPK and ERK level. The mean values of the each positive pixel area in the control or vehicle group were set at 100% and used for comparison with the experimental group.

Immunohistochemistry

Experimental procedures are identical with those of chapter 1. Rabbit polyclonal p-p38 MAPK was used 1:100, Cell signaling technology, Beverly, Massachusetts, USA). Incubation for 48h and followed by a mixture of AlexaFluor 555 conjugated secondary antibodies (1:500, invitrogen, Carlsbad, California, USA) for 2 hr at room temperature.

Statistical Analysis

Experimental procedures are identical with those of chapter 1.

RESULTS

1. Carrageenan induced inflammation activate activated MAPKs in DRG

Initially, a change in carrageenan-induced MAPKs activity were observed in the DRG over time using western blot analysis (Fig. 2-1). The activities of p38 MAPK, ERK and JNK cascades were examined in this study. The inflammation slowly increased the percentage of both the p38 MAPK and ERK phosphorylation rate in the DRG, reaching significance at day 2, and then decreasing back down to basal level by day 10 (Fig. 2-1A and B; $*P < 0.05$ as compared to control). However, percentage of JNK phosphorylation rate was not significantly changed during the inflammation (Fig. 2-1C).

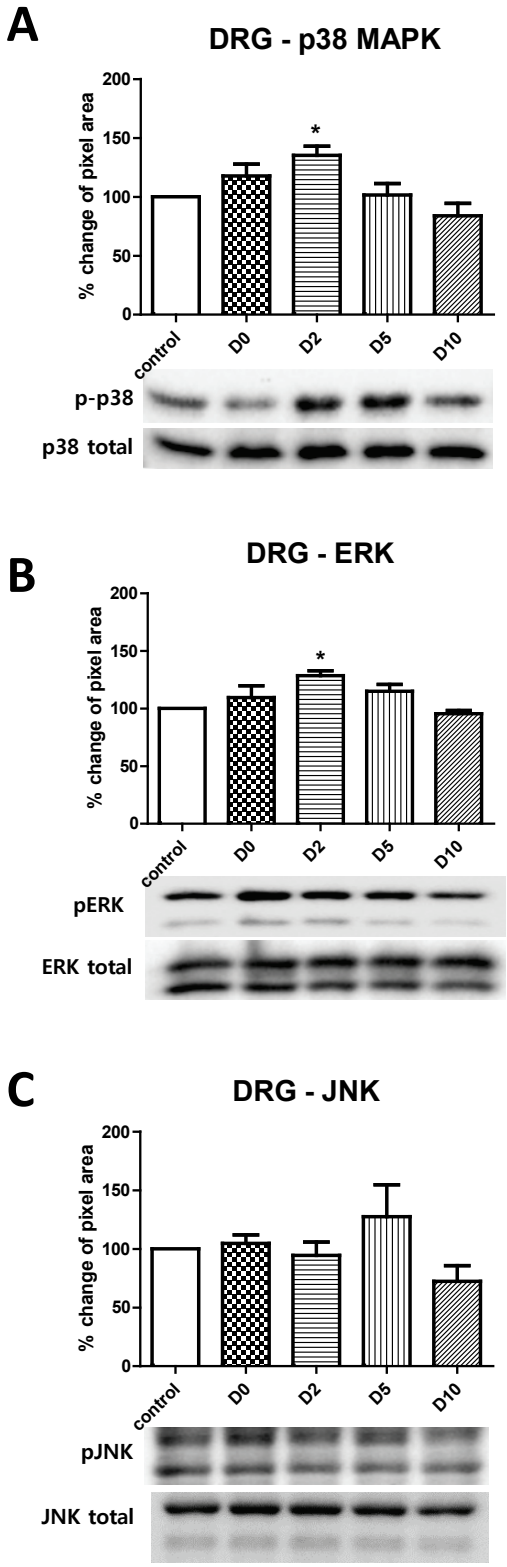
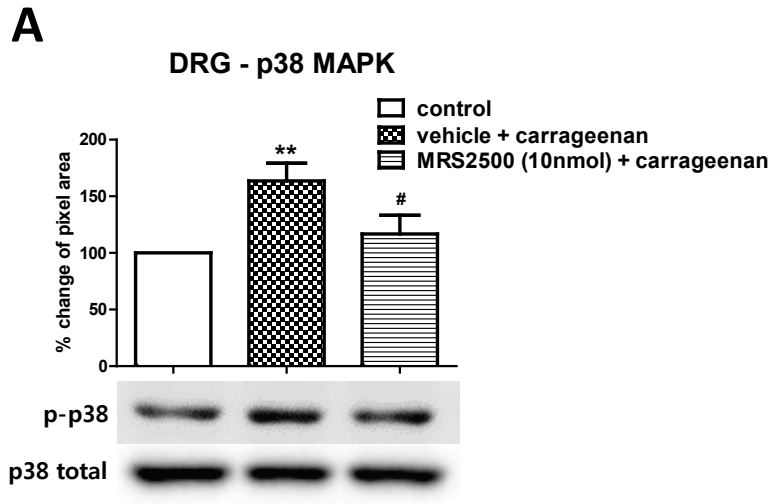

A
DRG - p38 MAPK
% change of pixel area
200
150
100
50
0
*
control
D0
D2
D5
D10
p-p38
p38 total
B
DRG - ERK
% change of pixel area
200
150
100
50
0
*
control
D0
D2
D5
D10
pERK
ERK total
C
DRG - JNK
% change of pixel area
200
150
100
50
0
control
D0
D2
D5
D10
pJNK
JNK total

Figure 2-1. The effect of intraplantar 2% carrageenan injection on the MAPKs activity in DRG. Western blot and graphs illustrate the changes in DRG MAPK activity over time including p38 MAPK, ERK and JNK signalings (**A** to **C**). Quantification of p38 MAPK and ERK phosphorylation rate in DRGs illustrates a significant increase by 2 days (D2) after carrageenan-induced inflammation compared to controls (**A** and **B**, n=5 in the skin lysates group, and n=6 DRG lysates group $*P < 0.05$ as compared to control); however, JNK phosphorylation rate do not significantly changed during the inflammation (**C**, n=6 in each group). Representative western blots show the expression of p-p38 MAPK, p-ERK and p-JNK (top) and p38 MAPK, ERK and JNK total (bottom).

2. Modulatory effect of peripheral P2Y1 receptor on DRG MAPKs activity

Since the activities of p38 MAPK and ERK but not JNK significantly increased at day 2, I investigated whether inhibition of peripheral P2Y1R regulated elevated p38 MAPK and ERK signaling in DRG (Fig. 2-2A and B). After repeated treatments of vehicle and MRS2500 (D0-D2), rats were euthanized on day 2 following carrageenan injections. Carrageenan induced a significant increase in the phosphorylation rate of both p38 MAPK and ERK in the DRG as compared with the control group (Fig. 2-2A and B; $*P < 0.05$, $**P < 0.01$ as compared to control). Intraplantar administration of the P2Y1R antagonist, MRS2500 significantly decreased the level of carrageenan-induced p-p38 MAPK expression as compared with vehicle-treated carrageenan rats (Fig. 2-2A; $\#P < 0.05$ as compared to carrageenan + vehicle). However, there was no significant change in p-ERK expression in the MRS2500 treated rats compared to vehicle-treated carrageenan rats at this same time point (Fig. 2-2B).


A
DRG - p38 MAPK
control
vehicle + carrageenan
MRS2500 (10nmol) + carrageenan
% change of pixel area
200
150
100
50
0
**
#
p-p38
p38 total

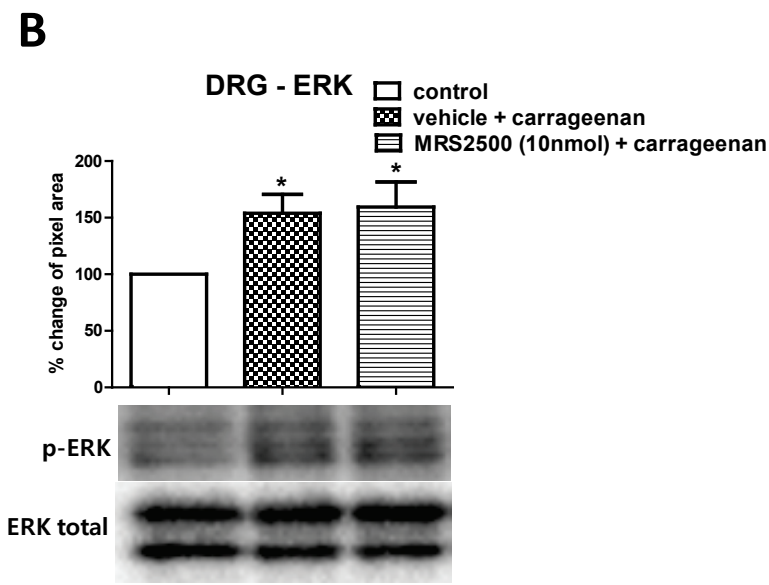
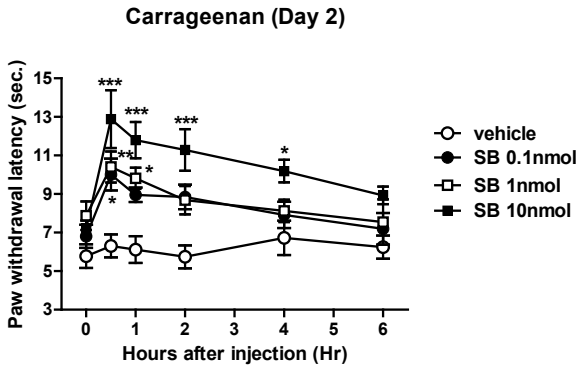

B
DRG - ERK
control
vehicle + carrageenan
MRS2500 (10nmol) + carrageenan
% change of pixel area
200
150
100
50
0
*
*
p-ERK
ERK total

Figure 2-2. The effect of MRS2500 treatment on up-regulated p-p38 MAPK and p-ERK in DRGs. Western blot analysis depicts the effects of intraplantar administration of the MRS2500 on p38 MAPK and ERK activity (**A** and **B**, n=7 in each group $*P < 0.05$ and $**P < 0.01$ as compared to control). The increase in p38 MAPK phosphorylation rate induced by inflammation was significantly blocked by repeated treatment with MRS2500 (**A**, a P2Y1R antagonist, 10nmol, $\#P < 0.05$ as compared to vehicle + carrageenan). There was no change in ERK phosphorylation rate in MRS2500 treated rats (**B**, n=7 in each group). Data are presented as the percent (%) change relative to the control.

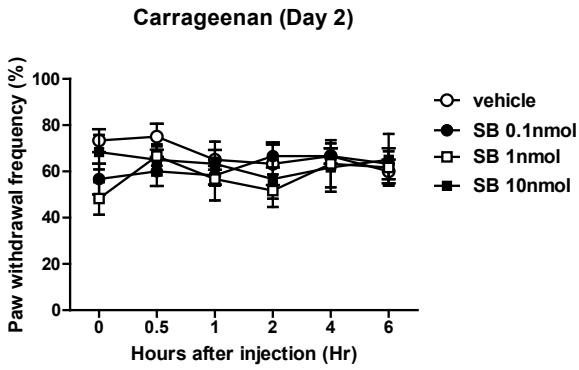
3. Involvement of p38 MAPK in carrageenan-induced inflammatory pain

To evaluate the role of DRG p38 MAPK in persistent inflammatory pain, pharmacological blockade of p38 MAPK in DRG was performed by using intrathecal injection route (Fig. 2-3A and B). Since direct injection to the DRG (L4-6) is difficult to perform, intrathecal injection has been used as an alternative method for modulating DRG (Ji et al., 2002; Mizushima et al., 2005). I intrathecally injected p38 MAPK inhibitor, SB203580, at day 2 after carrageenan injection. To confirm the intrathecal treatment of SB203580 inhibit DRG p38 MAPK activity, animals were euthanized 2 hours after injection and the level of p-p38 MAPK activity was evaluated. Western blot analysis represented the inhibitory effects of single intrathecal administration of the SB203580 (10nmol) on DRG p38 MAPK activity (Fig. 2-4A; $*P < 0.05$ vs control, $\#P < 0.05$ as compared to carrageenan + vehicle). Intrathecal treatment with SB203580 (0.1, 1 and 10nmol) dramatically reduced the carrageenan induced decrease in PWL (sec) to noxious heat stimulus, as compared with vehicle-treated carrageenan rats (Fig. 2-3A; $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ as compared to vehicle). SB203580 dose dependently reversed the established thermal hyperalgesia induced by carrageenan. On the other hand, the carrageenan-induced increase in PWF (%) to innocuous mechanical stimulus was not influenced by intrathecal treatment with SB203580. Since intrathecal injection primarily affect spinal cord, I investigated the p-p38 MAPK level changes during the inflammation (Fig. 2-4B). There were no significant up-regulation of phosphorylation rate of p38 MAPK; therefore, behavioral effects of intrathecally injected SB203580 were induced by down regulation p-p38 MAPK in DRG.

A


Carrageenan (Day 2)
Paw withdrawal latency (sec.)
Hours after injection (Hr)
vehicle
SB 0.1nmol
SB 1nmol
SB 10nmol

B


Carrageenan (Day 2)
Paw withdrawal frequency (%)
Hours after injection (Hr)
vehicle
SB 0.1nmol
SB 1nmol
SB 10nmol

C

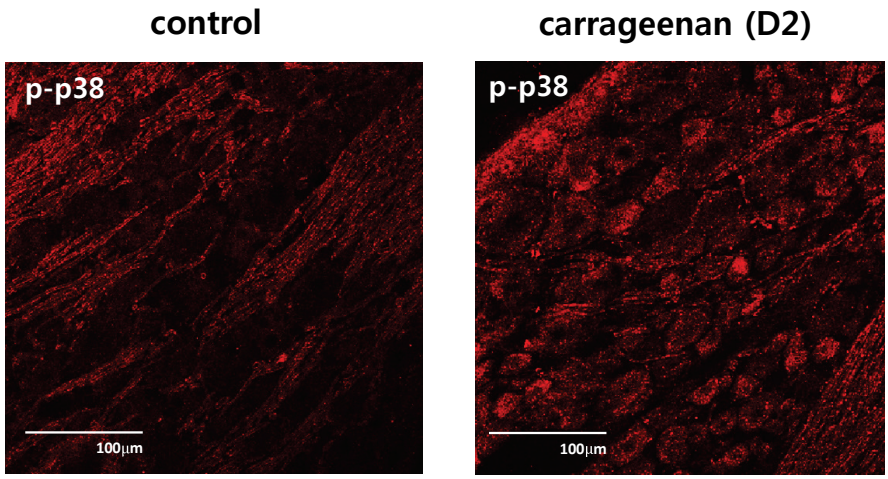

control
carrageenan (D2)
p-p38
100㎛
p-p38
100㎛

Figure 2-3. The effect of SB203580 treatment on established inflammatory thermal hyperalgesia and mechanical allodynia. Graphs illustrate the effect of intrathecal injection of p38 MAPK inhibitor, SB203580 (1, 3, 10nmol, administered at days 2 after inflammation), on persistent thermal hyperalgesia and mechanical allodynia in carrageenan rats (**A** and **B**). Thermal hypersensitivity was effectively alleviated by intrathecal injection of SB203580 (**A**, n=5 in vehicle and SB 0.1nmol treated rats, and n=6 in SB 1nmol and 10nmol treated rats, $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ as compared to vehicle). Mechanical allodynia was not affected by injection of SB203580 (**B**). Thin sections (10μm) of rat DRGs (L4-L6) were stained with antibodies against p-p38 MAPK (**C**). The proportion of p-p38 MAPK-immunoreactive neurons was augmented 2 days (D2) after inflammation in carrageenan rats compared to control rats. Images are shown at 200× magnification. Scale bars represent 100 μm.

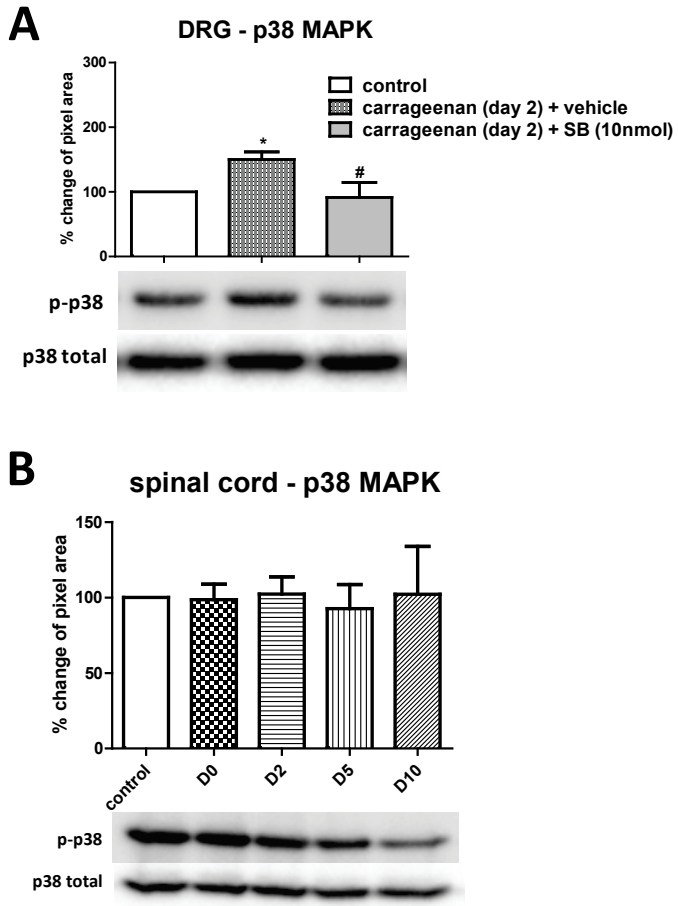
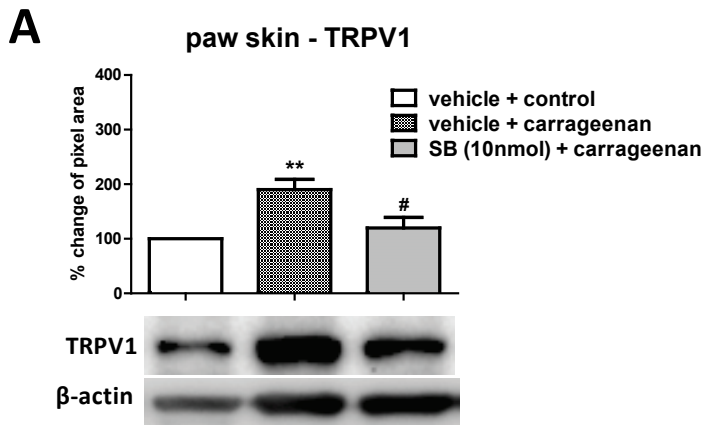
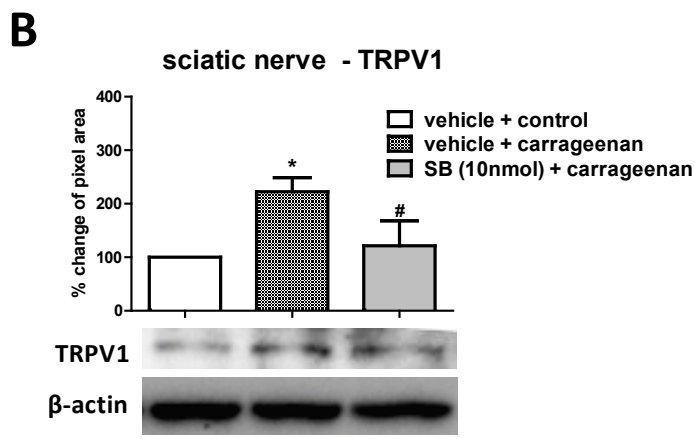


Figure 2-4. The effect of intrathecal injection of SB203580 on p38 MAPK activity in DRG. Representative western blots showing the expression of p-p38 MAPK (top) and p38 MAPK total (bottom). Western blot analysis depicts the effects of intrathecal administration of the SB203580 (10nmol) on DRG p38 MAPK activity (**A**, n=5 in each group $*P < 0.05$ vs control, $\#P < 0.05$ as compared to carrageenan + vehicle). Western blot and graphs illustrate the changes in spinal p38 MAPK activity over time during the inflammation **(B)**. Quantification of p38 MAPK phosphorylation rate in spinal cord did not show significant increase after carrageenan-induced inflammation compared to controls (**B**, n=6 in each group). Data are presented as the percent (%) change relative to the control.

4. Role of p38 MAPK on TRPV1 receptor expression in carrageenan-induced inflammation

To determine if the P2Y1R-induced up-regulation of TRPV1R expression is mediated by a p38 MAPK-dependent pathway, I treated rats intrathecally with the p38 MAPK inhibitor, SB203580 (10nmol), daily for 3 consecutive days (D0 to D2) in carrageenan rats (Fig. 2-5). Repetitive vehicle (intrathecal) injected carrageenan rats demonstrated a significant increase in TRPV1R expression compared to vehicle (intrathecal) treated control rats (Fig. 2-5A to C; $*P < 0.05$ and $**P < 0.01$ as compared to control + vehicle), while inhibition of DRG p-p38 MAPK by SB203580 (10nmol, intrathecal) resulted in a significant decrease in carrageenan-induced TRPV1R expression (Fig. 2-5A to C; $\#P < 0.05$; as compared to carrageenan + vehicle).


A
paw skin - TRPV1
% change of pixel area
400
300
200
100
0
**
#
vehicle + control
vehicle + carrageenan
SB (10nmol) + carrageenan
TRPV1
β-actin


B
sciatic nerve - TRPV1
% change of pixel area
400
300
200
100
0
*
#
vehicle + control
vehicle + carrageenan
SB (10nmol) + carrageenan
TRPV1
β-actin

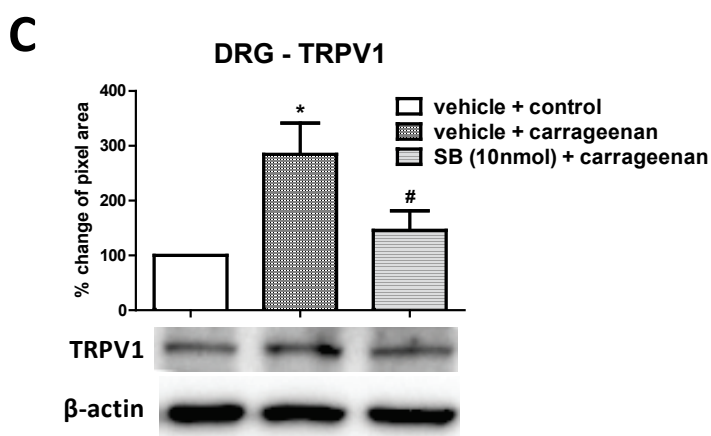

C
DRG - TRPV1
% change of pixel area
400
300
200
100
0
*
#
vehicle + control
vehicle + carrageenan
SB (10nmol) + carrageenan
TRPV1
β-actin

Figure 2-5. The effect of intrathecal injection of SB203580 treatment on TRPV1 receptor expression in carrageenan-induced inflammation. Quantitative western blot analysis of the expression of TRPV1R was performed in hind paw skin (**A**, n=4 in each group, $^{**}P < 0.01$ as compared to control + vehicle), sciatic nerve (**B**, n=4 in each group, $^{*}P < 0.05$ as compared to control + vehicle) and DRG (**C**, n=6 in each group, $^{*}P < 0.05$ as compared to control + vehicle). Repeated daily treatment (D0 to D2) with SB203580 (10nmol, intrathecal) caused a significant decrease in TRPV1R expression in skin, sciatic nerve and DRG lysates (**A** to **C**, $\#P < 0.05$ as compared to carrageenan + vehicle). Representative western blots showing TRPV1R (top) and β-actin (bottom) expression levels in skin, sciatic nerve and DRGs. Data are presented as the percent (%) change relative to the control.

5. Modulatory effect of peripheral P2Y1 receptor on DRG MAPK activity and TRPV1 receptor expression in naïve rats

To confirm the specific modulatory role of P2Y1R on p38 MAPK activity, additional experiments were carried out in naïve rats (Fig. 2-6 and 2-7). In order to determine the effect of chronic repetitive stimulation on peripheral P2Y1R, selective P2Y1R agonist, MRS2365 (0.1 and 1nmol), was daily injected for 3 consecutive days (Fig. 2-6 and 2-7). Following these three sequential injections, the animals were euthanized and tissue samples were collected to investigate the potential changes in MAPK in DRG using western blot analysis. Repetitive administration of MRS2365 significantly increased the expression of p-p38 MAPK in MRS2365 treated rats; however, ERK and JNK phosphorylation rates were not affected by P2Y1R agonist administration (Fig. 2-6A to C; $*P < 0.05$ as compared to vehicle). To observe the modulatory effect of p38 MAPK on TRPV1R expression, Rats were intrathecally treated with the p38 MAPK inhibitor, SB203580 (10nmol), daily for 3 consecutive days in MRS2365 injected group. As a results, SB203580 (10nmol, intrathecal) + MRS2365 (1nmol, intraplantar) injected rats showed effective blockade of P2Y1R stimulation induced TRPV1R up-regulation in both skin and DRG (Fig. 2-7A and B; $\#P < 0.05$ as compared to vehicle + MRS2365).

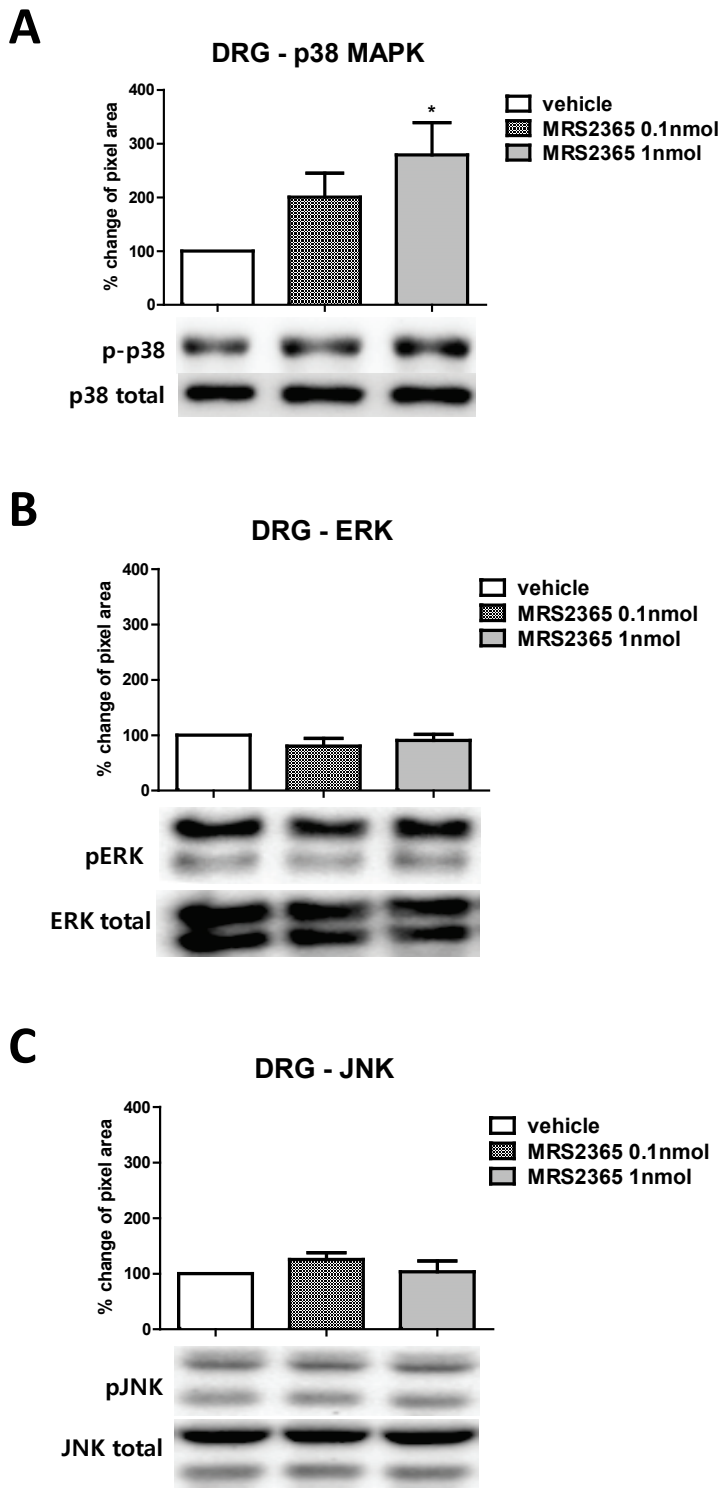
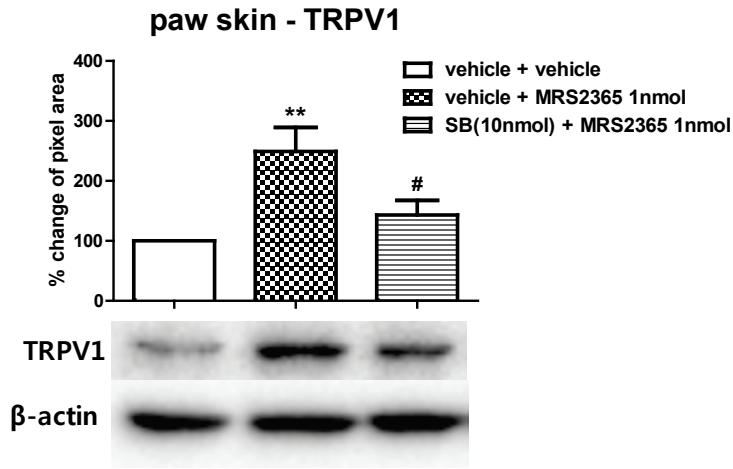

A
DRG - p38 MAPK
% change of pixel area
400
300
200
100
0
*
vehicle
MRS2365 0.1nmol
MRS2365 1nmol
p-p38
p38 total
B
DRG - ERK
% change of pixel area
400
300
200
100
0
vehicle
MRS2365 0.1nmol
MRS2365 1nmol
pERK
ERK total
C
DRG - JNK
% change of pixel area
400
300
200
100
0
vehicle
MRS2365 0.1nmol
MRS2365 1nmol
pJNK
JNK total

Figure 2-6. The effect of repeated intraplantar injections of MRS2365 on DRG MAPK activity in naïve rats. Quantitative western blot analysis of the expression of DRG MAPK in the DRG lysates in vehicle, MRS2365 0.1, and 1nmol treated animals (**A** to **C**, n=7 in p38 MAPK in DRG, n=6 in ERK in DRG, n=6 in JNK in DRG). Injection of MRS2365 dose dependently increased p38 MAPK phosphorylation rate, but did not affect ERK and JNK phosphorylation in DRGs (**A**, $*P < 0.05$ vs vehicle). Representative western blots showing the expression of p-p38 MAPK, p-ERK and p-JNK (top) and p38 MAPK, ERK and JNK total (bottom).

A


paw skin - TRPV1
% change of pixel area
400
300
200
100
0
**
#
vehicle + vehicle
vehicle + MRS2365 1nmol
SB(10nmol) + MRS2365 1nmol
TRPV1
β-actin

B

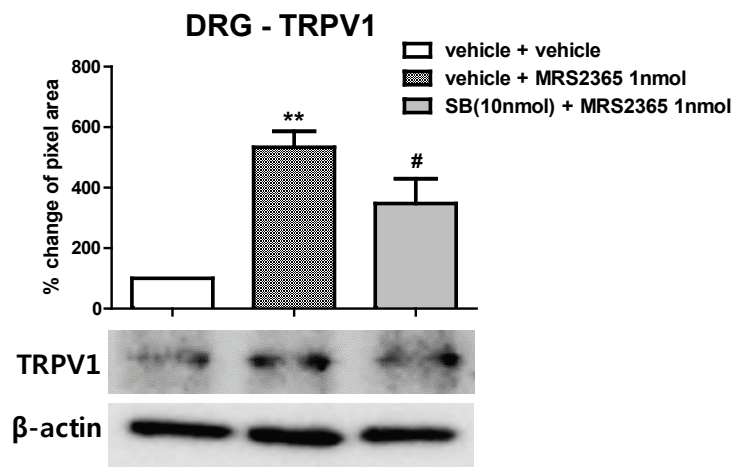

DRG - TRPV1
% change of pixel area
800
600
400
200
0
**
#
vehicle + vehicle
vehicle + MRS2365 1nmol
SB(10nmol) + MRS2365 1nmol
TRPV1
β-actin

Figure 2-7. The effect of the intrathecal injection of SB203580 on the MRS2365 induced TRPV1 receptor up-regulation. Quantitative western blot analysis of the expression of TRPV1R in the hind paw and DRG in vehicle (intrathecal) + vehicle (intraplantar), vehicle (intrathecal) + MRS2365 (1nmol, intraplantar) and SB203580 (10nmol, intrathecal) + MRS2365 (1nmol, intraplantar) treated animals. (**A** and **B**, n=8 TRPV1R in skin, n=5 TRPV1R in DRG). I injected rats intrathecally with the SB203580 (10nmol) and intraplantarly with the MRS2365 daily for 3 consecutive days in rats. SB203580 effectively inhibited MRS2365-induced increases in TRPV1R expression in both skin and DRGs (**$P < 0.01$ as compared to vehicle (intrathecal) + vehicle, #$P < 0.05$ as compared to vehicle (intrathecal) + MRS2365 1nmol).

DISCUSSION

In this part, the potential mechanisms of P2Y1R induced long-term changes in DRG MAPKs activities were addressed. P2Y1R selectively modulate p38 MAPK activity which ultimately control the thermal hypersensitivity and expression of TRPV1R during the inflammation in the hind paw. Intraplantar injection of 2% carrageenan up-regulated the phosphorylation of p38 MAPK and ERK but not JNK signaling in DRGs (Fig. 2-1). There are several reports demonstrating that noxious peripheral stimulation resulted in the acute elevation of ERK and p38 MAPK signaling in DRGs (Dai et al., 2002; Mizushima et al., 2005; Obata and Noguchi, 2004). In addition, Ji *et al.* (2002) reported that phosphorylation of p38 MAPK in DRG was increased in CFA induced chronic inflammation. Otherwise, there is a little information concerning DRG JNK activity and their contribution to pain. In the current study, JNK phosphorylation rate during the carrageenan induced inflammation did not increase during the time points that I observed (Fig. 2-1C). There was a report that DRG JNK activity was increased within 30 min after CFA injection, and the pharmacological inhibition of JNK pathway attenuated thermal hypersensitivity (Doya et al., 2005). Since I did not observe the early time points after inflammation, there was a possibility that JNK activation contributed to thermal hyperalgesia in early phase in this carrageenan model.

In terms of changes in pain sensitivity in different modality, ERK signaling in primary afferent fibers has been reported that it is important for both mechanical and thermal hypersensitivity. Intrathecal injection of the mitogen-activated protein kinase (MAPK) kinase 1/2 inhibitor, U0126 has been shown to inhibit mechanical and thermal hypersensitivity induced by inflammation in a variety of peripheral tissues

including the hind paw, knee joint, nerve root and DRG (Dai et al., 2002; Obata et al., 2003; Obata et al., 2004a; Seino et al., 2006). Although there is far less information regarding the role of p38 MAPK compared to ERK signaling in primary afferent fibers, the specific role of p38 MAPK in thermal hyperalgesia have been suggested in a number of previous reports (Ji et al., 2002; Mizushima et al., 2005). Mizushima *et al*. (2005) reported that capsaicin-induced thermal hyperalgesia, but not mechanical allodynia, was reversed by intrathecal injection of the p38 MAPK inhibitor, FR167653. Furthermore, in a rat CFA model, inhibition of DRG p38 MAPK resulted in a reversal of established thermal hypersensitivity without affecting mechanical allodynia (Ji et al., 2002). In accordance with these previous reports, here, I documented the specific role of DRG p38 activation in persistent inflammatory thermal hyperalgesia in experiments using intrathecal injection of the p38 MAPK inhibitor, SB203580. Intrathecal administration of SB203580 resulted in specific alleviation of thermal hyperalgesia, but not mechanical allodynia (Fig. 2-3A and B). Although prime target of intrathecal injection is spinal cord, many studies have used this method to modulate DRG pharmacologically (Ji et al., 2002; Mizushima et al., 2005; Obata et al., 2003; Obata et al., 2004b). Moreover, since p38 MAPK signaling was not significantly activated in the spinal cord at 2 days post-carrageenan injection (Fig. 2-4B), I would hypothesize that the effect of SB203580 is primarily on DRGs rather than on spinal cord targets. In this regard, this study confirmed the modality specific, distinct role of p38 MAPKs pathways on inflammatory thermal hyperalgesia.

In carrageenan rats, pharmacological blockade of peripheral P2Y1R down-regulated the phosphorylation rate of p38 MAPK without affecting ERK activity in DRGs (Fig. 2-2). To confirm this specific modulatory role of peripheral P2Y1R on DRG MAPK, additional experiments performed in naïve rats. In order to mimic a

peripheral chronic inflammatory condition, P2Y1R agonist was repeatedly injected to naïve rats. Interestingly, there was a significant increase in p38 MAPK phosphorylation, but no effect on ERK and JNK signalling in DRGs (Fig. 2-6). These results implicated that activation of peripheral P2Y1R selectively up-regulated the activity of p38 MAPK in DRG. Previously, there was a report that P2Y1R regulated ERK phosphorylation in a time and concentration dependent manner in skeletal muscle cells (May et al., 2006). In addition, in astrocyte culture system, P2Y receptors are involved in the intracellular MAPK signaling which is critical for astrocyte activity (Franke et al., 2012). Although this study did not delineate the underlying mechanisms involved, this is the first report that activation of P2Y1R facilitated the phosphorylation rate of MAPK in primary sensory neurons. In this regard, further diversified investigations in both vivo and vitro level will be needed to address the relationship between P2Y1R and p38 MAPK in the peripheral sensitization.

Since MAPKs work at the level of transcription and translation, increased activity of MAPKs results in plastic changes in sensory neurons (Ji et al., 2002), I hypothesized that P2Y1R induced up-regulated p38 MAPK ultimately control the expression level of TRPV1R. In this study, experiments were performed to identify the relationship between up-regulated p38 MAPK and TRPV1R expression. Repeated inhibition of DRG p38 MAPK by SB203580 (D0 to D2) prevented the elevation of TRPV1R expression in both carrageenan rats (Fig. 2-5) and naïve rats treated with MRS2365 (Fig. 2-7). Overall, these results demonstrated that stimulation of peripheral P2Y1R contributed to the maintenance of a thermal hypersensitive nociceptive phenotype by p38 MAPK phosphorylation, and the activation of p38 MAPK resulted in TRPV1R up-regulation. These consequences are addressing that sequential relationship of P2Y1R, p38 MAPK and TRPV1R could be useful targets for

alleviating thermal hypersensitivity under conditions of chronic inflammation.

CHAPTER 3

Modulatory effect of P2Y1 receptor on TRPV1 receptor phosphorylation during the ischemia

ABSTRACT

Thrombus-induced ischemic pain (TIIP) animal model was characterized by chronic mechanical allodynia without thermal hyperalgesia. On the other hand, it has been shown that intraplantar injection of acidic saline facilitates ATP-induced pain, which does result in the induction of thermal hyperalgesia in normal rats. Because acidic pH and increased ATP are closely associated with ischemic conditions, this study was designed to: (1) examine whether acidic saline injection into the hind paw causes the development of thermal hyperalgesia in TIIP, but not control, animals; and (2) determine which peripheral mechanisms are involved in the development of this thermal hyperalgesia. Repeated intraplantar injection of pH 4.0 saline, but not pH 5.5 and 7.0 saline, for 3 days following TIIP surgery resulted in the development of thermal hyperalgesia. After pH 4.0 saline injections, protein levels of carbonic anhydrase II (CA II) and hypoxia inducible factor-1α (HIF-1α) were elevated in the plantar muscle indicating that acidic stimulation intensified ischemic insults with decreased tissue acidity. At the same time point, there were no changes in the expression of TRPV1R in hind paw skin, whereas a significant increase in TRPV1R phosphorylation was shown in acidic saline (pH 4.0) injected TIIP (AS-TIIP) animals. Moreover, intraplantar injection of chelerythrine (a PKC inhibitor) and AMG9810 (a TRPV1R antagonist) effectively alleviated the established thermal hyperalgesia. In order to investigate which proton- or ATP-sensing receptors contributed to the development of thermal hyperalgesia, amiloride (an ASICs blocker), AMG9810, TNP-ATP (a P2Xs antagonist) or MRS2179 (a P2Y1R antagonist) were pre-injected before the pH 4.0 saline. Only MRS2179 significantly prevented the induction of thermal hyperalgesia, and the increased pTRPV1 ratio was also blocked in MRS2179 injected animals. Collectively, these data show that maintenance of an acidic

environment in the ischemic hind paw of TIIP rats results in the phosphorylation of TRPV1R via a PKC-dependent pathway, which leads to the development of thermal hyperalgesia mimicking what occurs in chronic ischemic patients with severe acidosis. More importantly, peripheral P2Y1R plays a pivotal role in this process, suggesting a novel peripheral mechanism underlying the development of thermal hyperalgesia in these patients.

INTRODUCTION

Ischemic pain typically results from a shortage of blood supply to the tissues and is associated with a number of pathological states including atherosclerosis and intermittent claudication resulting from peripheral arterial disease (PAD) (Birdsong et al., 2010; Meru et al., 2006). Although patients with PAD demonstrate a range of ischemic pain severity, with abnormal response to mechanical and heat stimulus (Lang et al., 2006; McDermott et al., 2001; Meru et al., 2006), mechanistic differences in pain intensity and modality have been poorly explored. Since there were no proper animal models that mimic the mechanisms of peripheral ischemia observed in PAD patients, Seo *et al.* (2008) previously developed and described a new animal model of thrombus-induced ischemic pain (TIIP) in the rat and investigated the peripheral mechanisms underlying this ischemia induced pain (Seo et al., 2010). TIIP rats show persistent bilateral mechanical allodynia and peripheral acid-sensing ion channels (ASICs) and P2X receptors are involved in the maintenance of this thrombus-induced ischemic pain (Seo et al., 2010). These results indicated that protons associated with low ischemic pH and an increased concentration of ATP at the ischemic site play crucial roles in the development of ischemia-induced peripheral sensitization.

Thermal hyperalgesia has been shown to develop in chronic ischemic patients with severe acidosis and thus I would anticipate that it should be present in the TIIP model. While ischemia-induced conditions are closely associated with TRPV1R activation (Pan and Chen, 2004; Premkumar and Bishnoi, 2011; Takemura et al., 2008; Xing et al., 2008; Zahner et al., 2003), and TRPV1R is the endpoint target of a variety of sensitizing substances including protons and ATP (Holzer, 2008; Huang et al., 2006; Planells-Cases et al., 2005), TIIP rats show no hypersensitivity to heat stimuli, i.e. thermal hyperalgesia, and changes in the pharmacological activity of

peripheral TRPV1R was not detected in this model. These findings suggest that the lack of thermal hyperalgesia development in TIIP animals might be caused by the internal ischemic environment, such that the acidity and the ATP concentration are inadequate to activate TRPV1R.

It has been demonstrated that there appears to be a close interaction between protons and ATP in their effect on sensory neurons, but to date this relationship has been examined primarily in *in vitro* experiments (King et al., 1996; Li et al., 1996; Li et al., 1997; Naves and McCleskey, 2005; Tominaga et al., 2001; Wildman et al., 2003). Recently, there was a report regarding to the facilitatory effects of acidic pH on ATP-induced hypersensitivity in rat's hind paw (Seo et al., 2011). Although acidic pH itself failed to activate TRPV1R and produce thermal hyperalgesia, an acidic pH in the presence of ATP, did induce a transient thermal hyperalgesia via the activation of peripheral P2Y1R and TRPV1R (Seo et al., 2011). Since decreased pH and increased ATP concentration are the pivotal features of ischemia, this previous results implicated that peripheral P2Y1R and TRPV1R would contribute to the ischemic thermal hyperalgesia. Therefore, in the present study, TIIP animal model was used to investigate the peripheral induction mechanisms of thermal hyperalgesia shown in chronic ischemic patients with severe acidosis. In this regard, I examined: (1) whether injection of acidic saline into the hind paw caused the development of TRPV1R mediated thermal hyperalgesia in TIIP rats; (2) proton (ASIC and TRPV1) and ATP (P2X and P2Y1) sensing receptors were involved in this newly developed thermal hyperalgesia; and finally (3) whether there were functional interactions between TRPV1 and P2Y1 receptors.

MATERIALS AND METHODS

Experimental animals

Sprague-Dawley rats (weighing 350 to 400 g) were obtained from the Laboratory Animal Center of Seoul National University (SNU). Maintaining condition is identical with that of chapter 1.

TIIP surgery

TIIP surgery was performed according to the method described by Seo et al (Seo et al., 2008). Briefly, rats were first anesthetized intraperitoneally with a Zoletil 50® (Virbac laboratories, 06516 Carros) and Rompun® (Bayer Korea Ltd, Ansan) mixture (combining 2.5 mg of Zoletil 50® with 0.47 mg of Rompun® in saline), and then a small incision was made in the femoral triangle of the left hind limb. The femoral artery was separated from the femoral vein and nerve by a piece of moisture-resistant film (Parafilm®, Chicago, IL, USA, 1 X 2.5 cm) to prevent possible ferrous chloride damage to these structures. A filter paper disc (0.5 X 0.5 cm; No. 2, Toyo Roshi Kaisha, Ltd., Japan) soaked with 20% $FeCl_2$ ($FeCl_2 \cdot 4H_2O$, Sigma, St. Louis, MO, USA) solution was placed on the femoral artery for 20 minutes. After the incision was surgically closed and covered by surgical dressing, animals were kept in a warming chamber (28 °C) until they completely recovered from the anesthesia.

Intraplantar drug administration procedures

Methods for intraplantar administration was described in chapter 1. 30μl of each drug or 50μl of acidic saline was injected into the central sole region of the hind paw. The pH was adjusted with 1N HCl solution (Seo et al., 2011; Sluka et al., 2001). In

order to provide a more acidic environment to the ischemic hind paw, pH 4.0 saline was injected intraplantarly once daily from day 0 to 3 following TIIP surgery (acidic saline injected TIIP, AS-TIIP).

The first injection of pH 4.0 saline was performed immediately after the surgery (day 0), and nociceptive behavioral testing was started beginning on day 1 post-surgery. Acid-induced thermal hyperalgesia was measured in two parts. The long-term effects of acid-induced thermal hyperalgesia were evaluated at days 1, 2, 3, 5, 7 and 12 after TIIP surgery. In order to avoid possible acute effects of acidic stimulation, during our examination of the long-term effects of acidic saline, I first examined thermal hyperalgesia beginning 2 hours after pH 4.0 saline injections (Fig. 3-1A). In attempt to examine the possible short-term effects of acidic saline, withdrawal response latency (sec) to heat was measured over a 12-hour period following pH 4.0 injection on days 1 and 3 post-surgery. In this set of experiments, behavioural changes were observed at 30 min, 1, 2, 4, 8 and 12 hours following the injection of pH 4.0 saline.

AMG9810 (a TRPV1R antagonist), chelerythrine (a PKC inhibitor), amiloride (an ASICs blocker), TNP-ATP (a P2X receptor antagonist), or MRS2179 (a P2Y1R antagonist) was injected into the hind paw in order to investigate the mechanisms underlying the development of thermal hyperalgesia. The potential involvement of TRPV1R and PKC dependent pathways during the maintenance phase of thermal hyperalgesia was evaluated by a single injection of AMG9810 and chelerythrine at day 3 post-surgery. To further explore the intrinsic mechanisms underlying the development of thermal hyperalgesia, amiloride, AMG9810, TNP-ATP and/or MRS2179 were repeatedly injected once a day (D0-D3 after surgery) 30 min before the acidic saline injection. Each control group received an intraplantar injection of the

appropriate vehicle for each drug. Animals were randomly assigned to experimental groups and subsequent drug treatment and behavioural analyses were performed blindly.

Resiniferatoxin treatment

In order to examine the possible role of TRPV1R containing peripheral nerve fibers, animals were treated with the potent capsaicin analog, Resiniferatoxin (RTX, 0.3mg/kg; Sigma, St. Louis, MO) dissolved in a mixture of 10% Tween 80, 10% ethanol, 80% normal saline (Roh et al., 2008). After rats were anesthetized with 3% isoflurane in a mixture of N_2O/O_2 gas, either RTX or vehicle was injected subcutaneously in a volume of 0.2 ml into the scruff of the neck. The eye wipe reflex test was subsequently performed to check whether RTX treatment destroyed capsaicin sensitive primary afferent fibers at day 2 post-RTX injection. A diluted capsaicin solution (0.01%, dissolved in saline) was dropped onto cornea, and then the number and the duration of eye wipes were counted for 1 min. Following the eye wipe reflex test, I performed sham and TIIP surgery in vehicle-treated animals and in RTX-treated animals that showed no response to diluted capsaicin solution.

Behavioral assessments

Experimental methods for pain behaviors (thermal hyperalgesia and mechanical allodynia) were identical with those of chapter 1.

Western Blot Analysis

Experimental procedures are identical with those of chapter 1. The muscle samples were incubated with primary antibodies for carbonic anhydrase II (CA II, 1:1000) or hypoxia inducible factor-1α (HIF-1α, 1:1000), which were purchased from the Santa Cruz Biotechnology (Delaware, CA). A TRPV1R polyclonal antibody (1:400, Calbiochem®, EMD Chemicals, Inc., Darmstadt, Germany) and a pTRPV1 (phosphor S800) polyclonal antibody (1:1000, Abnova corporation, Taipei City, Taiwan) were used for skin samples. β-actin antibody was used as a loading control (Sigma, St. Louis, MO). After the secondary antibody reaction, the bands were visualized with enhanced chemiluminescence (Amersham Pharmacia Biotech, England, UK). The positive pixel area of specific bands was measured with a computer-assisted image analysis system (Metamorph®, version 6.3r2, Molecular Devices Corporation, PA) and normalized against the corresponding β-actin loading control bands. Then the ratio of pTRPV1/TRPV1 expression was calculated. The mean values of the TRPV1 and pTRPV1/TRPV1 positive pixel area in the sham group was set at 100% and used for comparison with the experimental group.

Immunohistochemistry

Experimental procedures are identical with those of chapter 1. Frozen serial frontal sections (10μm) were cut through the DRG L4–L5 using a cryostat (Microm, Walldorf, Germany). These serial sections were pre-blocked with 3% normal donkey serum and 0.3% Triton X-100 in PBS for 1 hour at room temperature. Tissue sections were incubated at 4°C with goat polyclonal TRPV1R antibody (1:250, Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) for 48h and followed by AlexaFluor

488 conjugated secondary antibodies (1:500, invitrogen, Carlsbad, California, USA) for 2 hr at room temperature.

Drugs

3,5-Diamino-N-(aminoiminomethyl)-6-chloropyrazinecarboxamide hydrochloride (Amiloride), (2E)-N-(2,3-Dihydro-1,4-benzodioxin-6-yl)-3-[4-(1,1-dimethylethyl)phenyl]-2-propenamide (AMG9810), Chelerythrine, 2′,3′-O-(2,4,6-Trinitrophenyl)adenosine-5′-triphosphate triethylammonium salt (TNP-ATP), 2′-Deoxy-N6-methyladenosine 3′,5′-bisphosphate tetrasodium salt (MRS2179) and [[(1R,2R,3S,4R,5S)-4-[6-Amino-2-(methylthio)-9H-purin-9-yl]-2,3-dihydroxybicyclo[3.1.0]hex-1-yl]methyl] diphosphoric acid mono ester trisodium salt (MRS2365) were purchased from Tocris (Ellisville, MO, USA). Amiloride, TNP-ATP, MRS2179 were dissolved in saline, respectively. AMG9810 was dissolved in 10% ethyl alcohol. Chelerythrine was dissolved in 5% DMSO. A 30μl volume of one of the above drugs was injected intraplantarly for individual experiments.

Statistical Analysis

Experimental procedures are identical with those of chapter 1.

RESULTS

1. Long-term effects of intraplantar acidic saline injection on thermal hyperalgesia in TIIP rats

pH adjusted saline solution (pH 4.0, 5.5, or 7.0) was injected into the hind paw of TIIP rats once per day from days 0 to 3 after TIIP surgery to determine the potential effect of a more acidic, ischemic environment on thermal nociception. Repeated injection of pH 4.0 saline (Days 0 to 3 after surgery) caused a significant reduction in paw withdrawal latency to thermal stimuli in TIIP rats (Fig. 3-1A). In order to investigate long-term effects of acid induced changes, thermal hyperalgesia was measured at day 1, 2, 3, 5, 7 and 12 after the surgery. The threshold for heat stimulation was evaluated at 2 hours post-injection in order to avoid potential acute effects of the pH 4.0 saline solution. Thermal hyperalgesia developed on day 1 post-injection following TIIP surgery and was maintained throughout the entire 3-day injection period (Fig. 3-1A; $^{**}P < 0.01$, $^{***}P < 0.001$ vs sham + pH 7.0 saline), and gradually recovered over a period of several days after the injection period ended. By contrast, the thermal threshold was unchanged in TIIP rats injected intraplantarly with pH 7.0 saline in the ipsilateral paw and thus, thermal hyperalgesia never developed. Importantly the two control groups (sham rats injected daily with pH 4.0 or pH 7.0 saline solutions) did not develop thermal hyperalgesia (Fig. 3-1A) indicating that the ischemic condition was necessary for this acidic-induced thermal hyperalgesia to develop. Furthermore, one group of TIIP rats was injected into the ischemic hind paw with pH 5.5 saline on 3 consecutive days, but the injection of the pH 5.5 saline solution did not significantly decrease the threshold for the thermal stimulus (Fig. 3-

1A). Since only pH 4.0 saline induced the behavioral changes in thermal hypersensitivity in TIIP rats, I injected pH 4.0 saline into the ischemic hind paw in the remaining experiments as outlined below.

2. Short-term effects of intraplantar acidic saline injection on thermal hyperalgesia in TIIP rats

The changes of behavioral responses by a single injection of acidic saline were observed at postoperative days 1 and 3. After pH 4.0 injection, the changes in the withdrawal response latency (sec) was evaluated at 30 min, 1, 2, 4, 8 and 12 hours post-injection (Fig. 3-1B and C). On day 1, TIIP rats showed no significant thermal hyperalgesia prior to pH 4.0 injection compared to sham + pH 4.0 rats (Fig. 3-1B). Following pH 4.0 saline injections, TIIP rats developed a significant thermal hyperalgesia which was observed from 30 min to 8 hours post-injection. This heat hypersensitivity slowly decreased beginning at 8 hours post-injection and the animals showed normal thermal responses by 12 hours after injection (Fig. 3-1B; $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ vs sham + pH 4.0 saline). At day 3 post-surgery, significant thermal hyperalgesia was already present in acidic saline injected TIIP (AS-TIIP) rats before pH 4.0 saline injection (Fig. 3-1C; $** P < 0.01$ vs sham + pH 4.0 saline). Moreover, after pH 4.0 injections, AS-TIIP rats showed persistent thermal hyperalgesia and did not recover until 12 hours post-injection (Fig. 3-1C; $**P < 0.01$ and $***P < 0.001$ vs sham + pH 4.0 saline). Collectively, the thermal hyperalgesia induced by a single acidic saline injection was transient at day 1; however, following 2 more acidic saline injections, the TIIP rats developed persistent thermal hypersensitivity which was maintained for 2 days following the cessation of acidic

saline injection (until day 5 post-surgery).

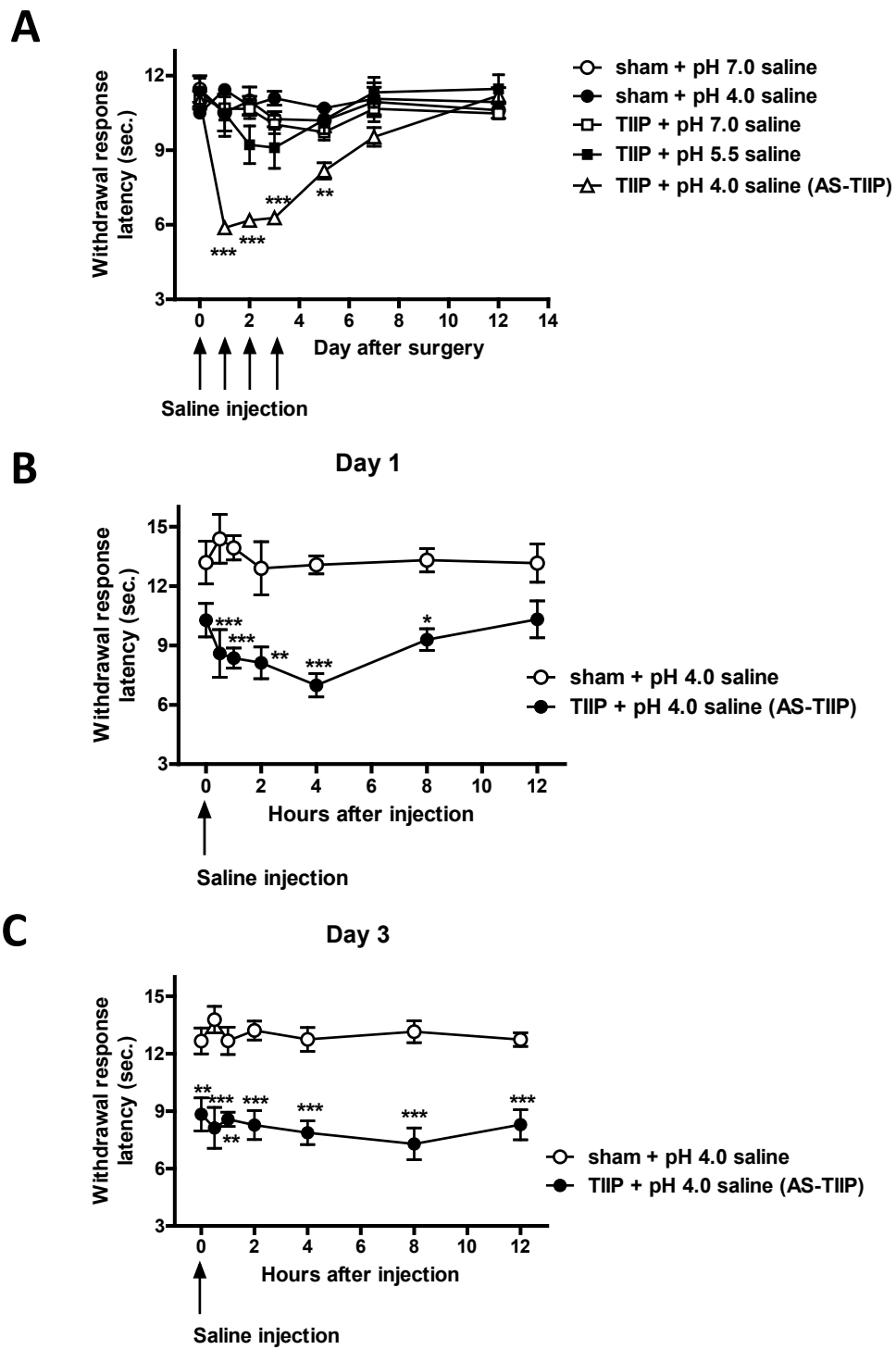

A
Withdrawal response latency (sec.)
Day after surgery
Saline injection
sham + pH 7.0 saline
sham + pH 4.0 saline
TIIP + pH 7.0 saline
TIIP + pH 5.5 saline
TIIP + pH 4.0 saline (AS-TIIP)
B
Day 1
Withdrawal response latency (sec.)
Hours after injection
Saline injection
sham + pH 4.0 saline
TIIP + pH 4.0 saline (AS-TIIP)
C
Day 3
Withdrawal response latency (sec.)
Hours after injection
Saline injection
sham + pH 4.0 saline
TIIP + pH 4.0 saline (AS-TIIP)

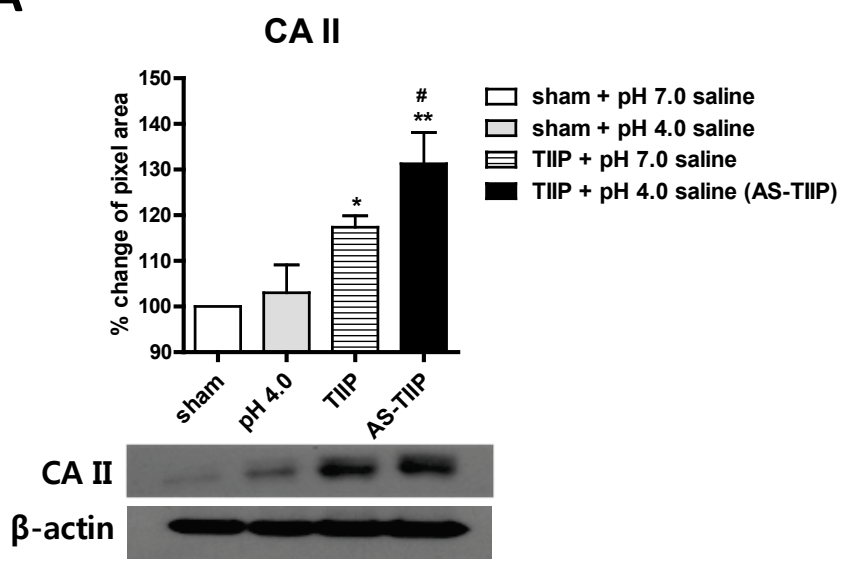
Figure 3-1. The effect of single and repeated acidic saline injection on the heat sensitivity in the ischemic hind paw. Graph showing the changes in thermal hyperalgesia over time caused by injection of acidic saline into the hind paw of thrombus-induced ischemic pain (TIIP) rats. pH adjusted saline (at pH 7.0, 5.5, or 4.0) was injected daily into the hind paw beginning on day 0 through day 3 after the induction of ischemic pain in TIIP rats or into sham control rats. Long-term effects of acid induced thermal hyperalgesia in TIIP rats (**A**). Thermal hyperalgesia was evaluated in sham control rats (pH 7.0 and 4.0, n=7 and 5 in each group) and TIIP rats (pH 7.0, 5.5 and 4.0, n=5, 5 and 9 in each group) at day 1, 2, 3, 5, 7 and 12 after the surgery. Thermal hyperalgesia was measured 2 hours after saline injection to avoid its acute effects. Only in the TIIP + pH 4.0 saline (acidic saline injected TIIP, AS-TIIP) group, did significant thermal hyperalgesia develop beginning on day 1 after the injection of pH 4.0 saline and continuing for the duration of the injection period. As a control, either pH 7.0 or pH 4.0 saline was repeatedly injected into sham rats, and there were no significant change in thermal nociception (**A**, $^{**}P < 0.01$, $^{***}P < 0.001$ vs sham + pH 7.0 saline). Short-term effects of acid-induced thermal hyperalgesia in TIIP rats at day 1 post-surgery (**B**). Thermal hyperalgesia was evaluated in sham and TIIP rats following injection of pH 4.0 saline (n=6 in each group) at 30 min, 1, 2, 4, 8 and 12 hours after pH 4.0 saline injection. Sham + pH 4.0 and AS-TIIP rats did not exhibit thermal hyperalgesia prior to pH 4.0 injection. After pH 4.0 saline injection, AS-TIIP rats showed prominent thermal hyperalgesia compared to sham rats; thermal hyperalgesia was observed from 30 min to 8 hours post-injection. This thermal hyperalgesia in AS-TIIP rats slowly decreased beginning at 8 hours post-injection and was no different than sham + pH 4.0 rats by 12 hours after injection (**B**, $^{*}P < 0.05$,

$P < 0.01$ and ***$P < 0.001$ vs sham + pH 4.0 saline). Short-term effects of acid-induced thermal hyperalgesia in TIIP rats at day 3 post-surgery (C**). Thermal hyperalgesia was evaluated in sham and TIIP rats following injection of pH 4.0 (n=6 in each group) at 30 min, 1, 2, 4, 8 and 12 hours after pH 4.0 saline injection. Significant thermal hyperalgesia was present in AS-TIIP rats prior to pH 4.0 saline injections. Following pH 4.0 saline injections, AS-TIIP rats showed sustained thermal hyperalgesia, which did not recover until 12 hours post-injection. (**C**, **$P < 0.01$, ***$P < 0.001$ vs sham + pH 4.0 saline).

3. Injection of acidic saline results in an increase in acidity and hypoxic status the ischemic hind paw

In order to evaluate whether repeated pH 4.0 saline injection induced changes in hypoxic status and acidity of peripheral hind paw tissue, hind paw muscle samples were collected at 3 days post-surgery following three days of acidic saline injection. To avoid potential acute effects of pH 4.0 and 7.0 saline injection, the animals were euthanized at least 2 hours post-injection. Since carbonic anhydrase II (CA II) has been reported to be a major indicator of pH imbalance and hypoxia inducible factor-1α (HIF-1α) is a well-known index of hypoxia (Heyman et al., 2011; Ho et al., 2006; Li et al., 2002; Mekhail et al., 2004; Potter and Harris, 2004; Riley et al., 1982; Švastová et al., 2004), the changes in these two factors were examined by western blot analysis (Fig. 3-2). The protein concentration of CA II was also significantly increased in both the pH 7.0-treated TIIP and acidic saline (pH 4.0) injected TIIP animals compared to the pH 7.0-treated sham group (Fig. 3-2A). The additional acidic saline injection resulted in significant increase in CA II expression in the AS-TIIP group compared to the pH 7.0-treated TIIP group (Fig. 3-2A; $*P < 0.05$, $**P < 0.01$ vs sham + pH 7.0 saline, and $\#P < 0.05$ vs TIIP + pH 7.0 saline). The protein concentration of HIF-1α was significantly increased in pH 7.0-treated TIIP rats, but there was a larger increase in acidic saline (pH 4.0) injected TIIP (AS-TIIP) rats (Fig. 3-2B; $*P < 0.05$, $**P < 0.01$ vs sham + pH 7.0 saline). As a control group, I tested sham rats repeatedly injected with pH 4.0 saline, and there were no significant changes in either CA II or HIF-1α expression.

A


CA II
% change of pixel area
150
140
130
120
110
100
90
#
**
*
sham + pH 7.0 saline
sham + pH 4.0 saline
TIIP + pH 7.0 saline
TIIP + pH 4.0 saline (AS-TIIP)
sham
pH 4.0
TIIP
AS-TIIP
CA II
β-actin

B

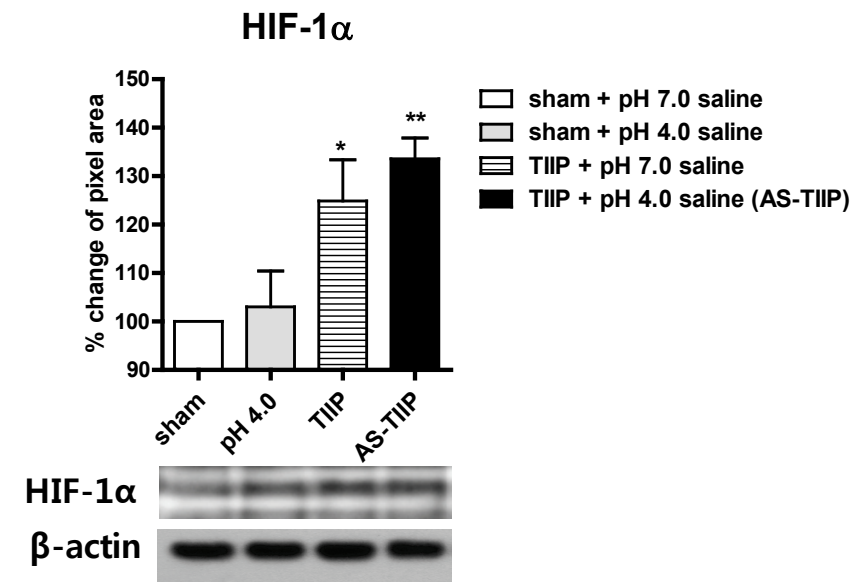

HIF-1α
% change of pixel area
150
140
130
120
110
100
90
*
**
sham + pH 7.0 saline
sham + pH 4.0 saline
TIIP + pH 7.0 saline
TIIP + pH 4.0 saline (AS-TIIP)
sham
pH 4.0
TIIP
AS-TIIP
HIF-1α
β-actin

Figure 3-2. Western blot analysis of carbonic anhydrase II (CA II, **A**) hypoxia inducible factor-1α (HIF-1α, **B**) in the hind paw. The protein level of CA II was significantly increased in pH 7.0-treated TIIP and AS-TIIP groups compared to the pH 7.0-treated sham group. pH 4.0 saline injection induced an additional increase in CA II expression in the AS-TIIP group compared to the pH 7.0-treated TIIP group. (**A**, **P* < 0.05, ***P* < 0.01 vs sham + pH 7.0 saline and #*P* < 0.05 vs TIIP + pH 7.0 saline). The protein concentration of HIF-1α was also significantly increased in the pH 7.0-treated thrombus induced ischemic pain (TIIP) group, and the protein concentration of HIF-1α was increased to an even greater extent in the pH 4.0 saline injected TIIP group (AS-TIIP); (**B**, **P* < 0.05, ***P* < 0.01 vs sham + pH 7.0 saline). CA II (n=4 in each group, day 3 post pH 4.0 saline injection) and HIF-1α (n=5 in each group, day 3 post pH 4.0 saline injection) were quantitatively evaluated in sham and ischemic hind paw muscle lysates by western blotting. Data are presented as percentage of change (%) relative to sham control. β-actin was used as a loading control.

4. TRPV1 and phosphorylated TRPV1 receptor expression of hind paw lysates in acidic saline injected TIIP (AS-TIIP) rats

Since peripheral TRPV1R is considered to be pivotal thermal sensors that contribute to the development of thermal hypersensitivity, I investigated possible changes in TRPV1R expression and phosphorylation rate in skin from the ischemic hind paw (Fig. 3-3). To address this, western blot analysis was performed in both TRPV1R and phosphorylated TRPV1R (pTRPV1R) to determine whether peripheral ischemia alone or coupled with increased tissue acidity (induced by pH 4.0 saline injection) caused a change in TRPV1R expression and phosphorylation in paw lysates (Fig. 3-3) at postoperative day 3 following three days of acidic saline injection. Expression of peripheral TRPV1R was not significantly changed in pH 7.0-treated TIIP rats or in the acidic saline injected TIIP (AS-TIIP) group (Fig. 3-3A). No statistical differences were detected among any of the 4 conditions at this time point. Quantitative analysis of TRPV1R expression was normalized against corresponding β-actin. TRPV1R has two phosphorylation sites for PKC-mediated phosphorylation: S502 and S800 (Planells-Cases et al., 2005). The pTRPV1R antibody used in this study reacted with pTRPV1 at S800. Injection of acidic saline significantly increased the ratio of pTRPV1R and this was positively correlated with the induction of thermal hyperalgesia (Fig. 3-3B; $^{**}P < 0.01$ vs sham + pH 7.0 saline). Quantitative analysis of pTRPV1R expression was normalized against corresponding total TRPV1R expression.

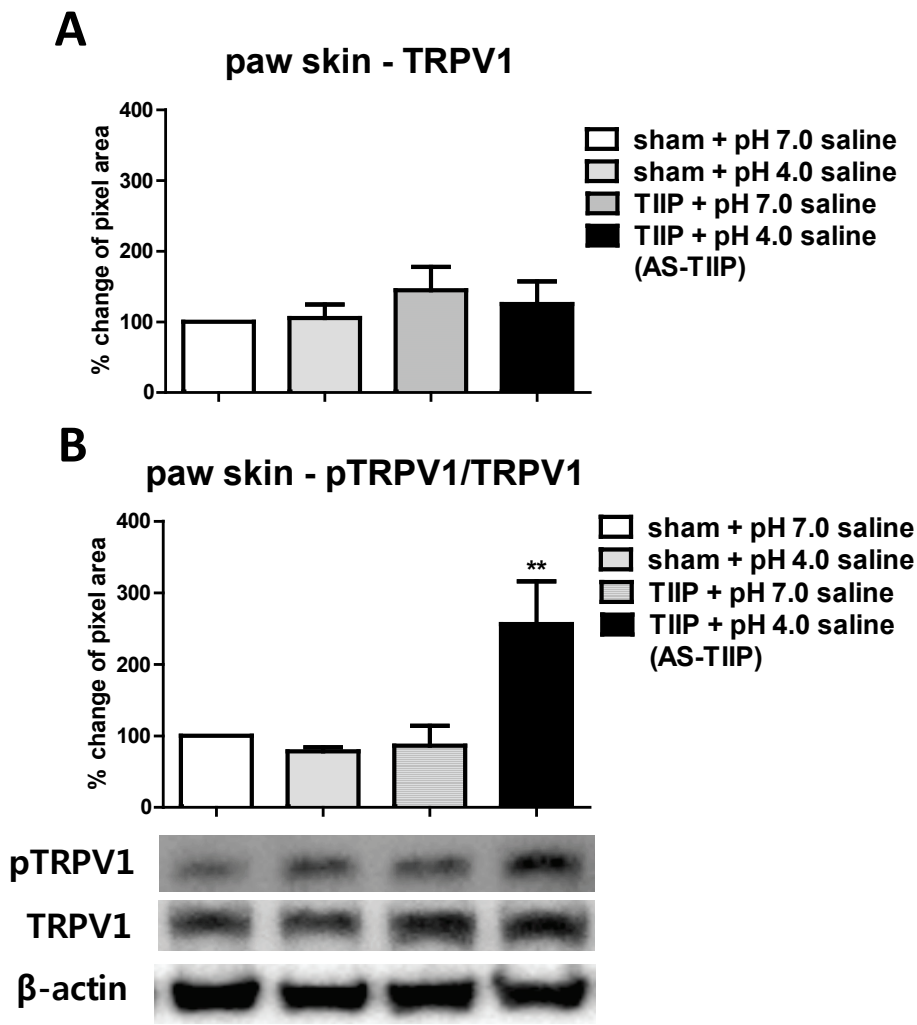

A
paw skin - TRPV1
% change of pixel area
400
300
200
100
0
sham + pH 7.0 saline
sham + pH 4.0 saline
TIIP + pH 7.0 saline
TIIP + pH 4.0 saline
(AS-TIIP)
B
paw skin - pTRPV1/TRPV1
% change of pixel area
400
300
200
100
0
**
sham + pH 7.0 saline
sham + pH 4.0 saline
TIIP + pH 7.0 saline
TIIP + pH 4.0 saline
(AS-TIIP)
pTRPV1
TRPV1
β-actin

Figure 3-3. Western blot analysis of TRPV1 and phosphorylated TRPV1 (pTRPV1) receptor expression in hind paw. TRPV1R expression in hind paw skin lysates was quantitatively evaluated in sham + pH 7.0 saline, sham + pH 4.0, thrombus-induced ischemic pain (TIIP) + pH 7.0 saline, acidic saline (pH 4.0) injected TIIP (AS-TIIP) by western blotting (**A**). The protein expression of TRPV1R did not change in pH 7.0-treated TIIP and AS-TIIP rat groups compared to 7.0-treated sham groups (A, n=4 in each group, 3 days after surgery and pH 4.0 injection). Each band was normalized against the corresponding β-actin band used as loading control. The expression of PKC dependent pTRPV1 in the hind paw skin of sham + pH 7.0 saline, sham + pH 4.0 saline, TIIP + pH 7.0 saline and AS-TIIP (**B**, n=4 in each group, 3 days after surgery and pH 4.0 saline injections). The expression of pTRPV1 levels significantly increased in AS-TIIP rats (**B**, $^{**}P < 0.01$ vs sham + pH 7.0 saline) but not in pH 4.0 treated sham and pH 7.0-treated TIIP rats. The pTRPV1 level was normalized against corresponding total TRPV1R. Data are presented here as a percentage change (%) compared to the sham control.

5. TRPV1 and phosphorylated TRPV1 receptor expression of hind paw lysates in acidic saline injected TIIP (AS-TIIP) rats with Resiniferatoxin (RTX) treatment

Since TRPV1R is highly expressed in epidermal nerve fibers as well as in keratinocytes distributed in the epidermis of the skin (Dussor et al., 2009; Gopinath et al., 2005; Li et al., 2007; Mandadi et al., 2009), additional set of experiments were performed to determine whether ischemic conditions result in the phosphorylation of TRPV1R located in nerve terminals and/or epidermal keratinocytes (Fig. 3-4). I treated separate groups of rats with the potent capsaicin analog Resiniferatoxin (RTX) two days before TIIP surgery to abolish capsaicin sensitive sensory neurons. Following RTX treatment, AS-TIIP rats did not show significant thermal hypersensitivity at day 3 (Fig. 3-4B), and there were no detectable TRPV1R positive cells in lumbosacral DRGs indicating that TRPV1R positive neurons were completely abolished in these ganglia (Fig. 3-4A). However, after RTX treatment, TRPV1 and pTRPV1 receptors were still detected with western blotting; indicating that a significant portion of TRPV1 is found in non-neuronal sources compared to that in nerve endings (Fig. 3-4C and D). Interestingly, although a large amount of TRPV1R still existed in RTX treated rats, there were no significant changes in pTRPV1/TRPV1 levels in AS-TIIP rats (Fig. 3-4D). These results suggest that TRPV1R from non-neuronal cells does not contribute substantially to thermal hyperalgesia and that the up-regulation in pTRPV1 occurs predominantly in peripheral nerve fibers rather than keratinocytes.

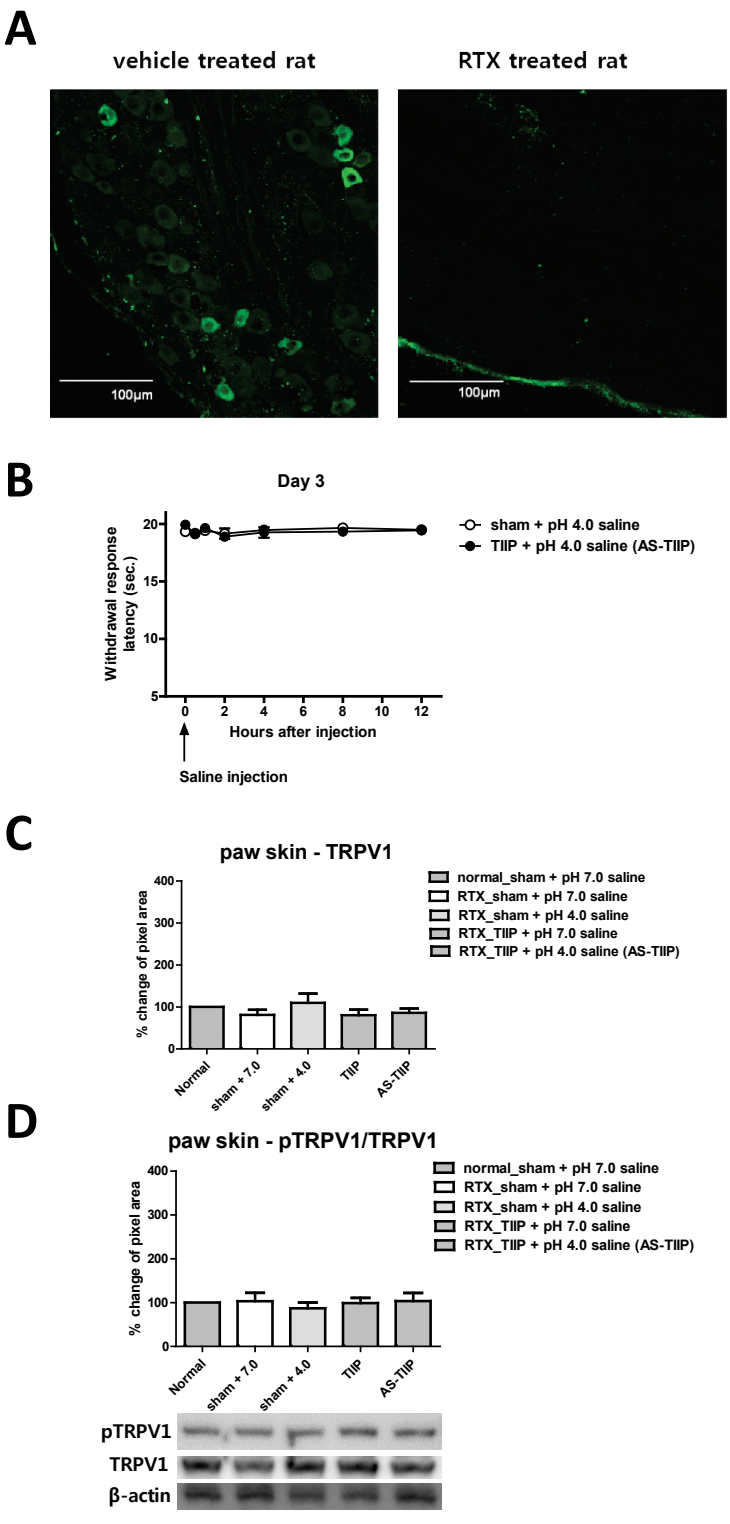

A
vehicle treated rat
RTX treated rat
100μm
100μm
B
Day 3
Withdrawal response latency (sec.)
20
15
10
5
0
2
4
6
8
10
12
Hours after injection
Saline injection
sham + pH 4.0 saline
TIIP + pH 4.0 saline (AS-TIIP)
C
paw skin - TRPV1
% change of pixel area
400
300
200
100
0
Normal
sham + 7.0
sham + 4.0
TIIP
AS-TIIP
normal_sham + pH 7.0 saline
RTX_sham + pH 7.0 saline
RTX_sham + pH 4.0 saline
RTX_TIIP + pH 7.0 saline
RTX_TIIP + pH 4.0 saline (AS-TIIP)
D
paw skin - pTRPV1/TRPV1
% change of pixel area
400
300
200
100
0
Normal
sham + 7.0
sham + 4.0
TIIP
AS-TIIP
normal_sham + pH 7.0 saline
RTX_sham + pH 7.0 saline
RTX_sham + pH 4.0 saline
RTX_TIIP + pH 7.0 saline
RTX_TIIP + pH 4.0 saline (AS-TIIP)
pTRPV1
TRPV1
β-actin

Figure 3-4. The effect of acidic saline in thermal hyperalgesia, expression of TRPV1 and phosphorylated TRPV1 (pTRPV1) receptor in Resiniferatoxin (RTX) treated rat. Immunofluorescent images of rat DRG neurons. Thin sections (10μm) of rat DRGs (L4-L5) were stained with antibodies against TRPV1R in the vehicle and RTX treated groups (**A**). The TRPV1R-immunostaining in DRG neurons is almost completely abolished in RTX treated rats. Images are shown at 200× magnification. Scale bars represent 100μm. Short-term effects of acid induced thermal hyperalgesia in RTX treated TIIP rats at day 3 (**B**). Thermal hyperalgesia was measured in RTX treated sham + pH 4.0 and acidic saline (pH 4.0) injected TIIP (AS-TIIP) rats (**B**, n=4 in each group) at 30 min, 1, 2, 4, 8 and 12 hours after saline injection. RTX treated rats did not show thermal hyperalgesia following TIIP surgery and pH 4.0 injection. TRPV1R expression in hind paw skin lysates was quantitatively evaluated in RTX treated groups including sham + pH 7.0 saline, sham + pH 4.0 saline, TIIP + pH 7.0 saline and AS-TIIP by western blotting (**C**). The protein expression of TRPV1R did not change in any of the RTX treated groups compared to 7.0-treated sham groups (**C**, n=6 in each group, 3 days after surgery and pH 4.0 injection). Each band was normalized against the corresponding β-actin band used as loading control. pTRPV1R expression in hind paw skin lysates was quantitatively evaluated in RTX treated group including sham + pH 7.0 saline, sham + pH 4.0 saline, TIIP + pH 7.0 saline and AS-TIIP by western blotting (**D**). The protein expression of pTRPV1 did not change in any of the RTX treated groups compared to 7.0-treated sham groups (**D**, n=6 in each group, 3 days after surgery and pH 4.0 injection). The pTRPV1R level was normalized against corresponding total TRPV1R. Data are presented here as a percentage change (%) compared to the sham control.

6. The involvement of TRPV1 receptor and PKC dependent pathways in the maintenance of thermal hyperalgesia in acidic saline injected TIIP (AS-TIIP) rats

To investigate the involvement of TRPV1R and PKC dependent signaling in this acid induced thermal hyperalgesia, the selective TRPV1R antagonist (AMG9810) or the PKC inhibitor (chelerythrine) was injected into the hind paw of AS-TIIP rats at day 3 post-surgery following daily pH 4.0 saline injection. (Fig. 3-5). Following the injection of AMG9810 (Fig. 3-5A) or chelerythrine (Fig. 3-5B) into the hind paw that had established thermal hyperalgesia, I examined possible behavioral changes in thermal hypersensitivity for 3 hours post-injection. Both AMG9810 (3 and 10nmol) and Chelerythrine (10 and 30nmol) dose-dependently reversed the established thermal hyperalgesia induced by acidic saline injection in TIIP rats. AMG9810 significantly reversed thermal hyperalgesia at 30 min and 1h after injection, and chelerythrine inhibited thermal hyperalgesia at 1h and 3h after injection (Figure 3-5; $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ vs vehicle).

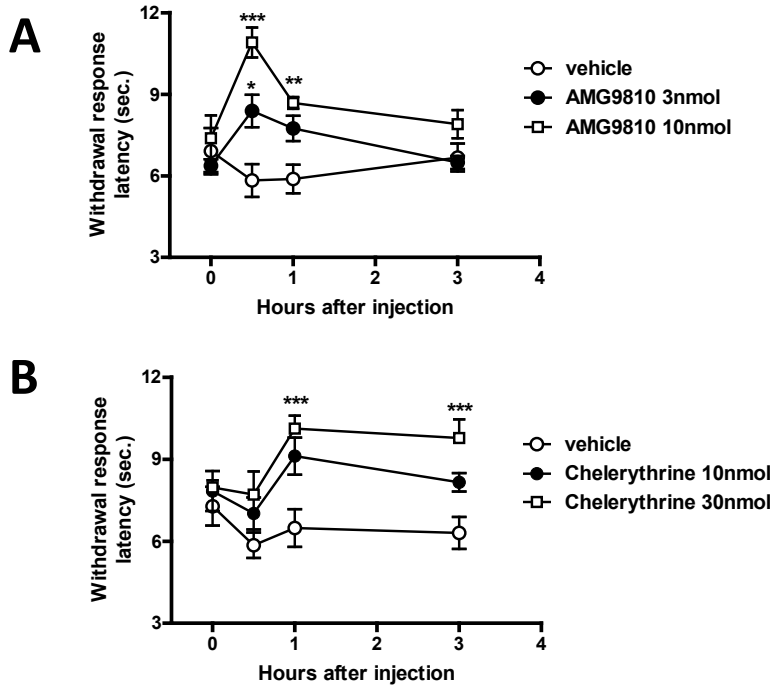
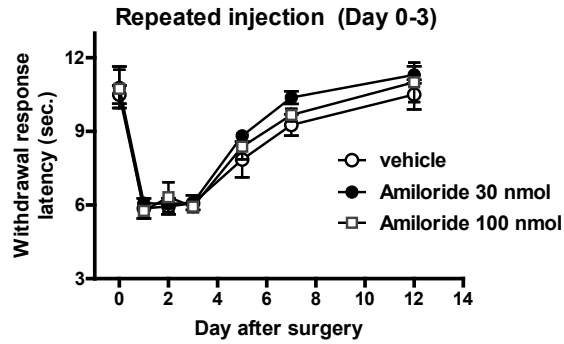


Figure 3-5. The effect of AMG9810 and chelerythrine on established thermal hyperalgesia in acidic saline injected TIIP (AS-TIIP) rats. The effects of AMG9810 (a TRPV1R antagonist) and chelerythrine (a PKC inhibitor) in established thermal hyperalgesia was evaluated at day 3 post-surgery and pH 4.0 injection. Intraplantar injection of AMG9810 (3 and 10nmol) was effective in reversing the established thermal hyperalgesia in a dose dependent manner (A, n=4, 4, 5 in each group). The analgesic effect of AMG9810 was significant at 30 min and 1 h post-injection (**A**, **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 vs vehicle). Intraplantar injection of chelerythrine (10 and 30nmol) dose dependently reversed thermal hyperalgesia at the 1 and 3 h time point after injection in established thermal hyperalgesia rats (**B**, n=4, 6, 6 in each group, ****P* < 0.001 vs vehicle).

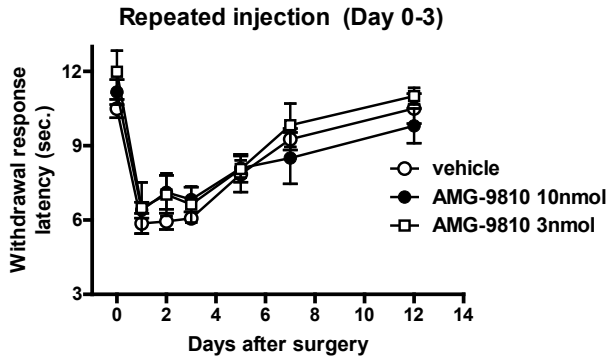
7. The involvement of proton sensing ion channels and P2 receptors in the induction of thermal hyperalgesia in acidic saline injected TIIP (AS-TIIP) rats.

To examine the potential role(s) of proton sensing ion channels and P2 receptors in the development of thermal hyperalgesia in the ischemic hind paw, I investigated the pharmacological effects of ASIC blockers and TRPV1, P2X and P2Y1 receptors antagonists on thermal hyperalgesia in AS-TIIP rats. The potential role of proton-sensing receptors in the development of thermal hyperalgesia was evaluated first. To do this, rats were initially injected intraplantarly with either amiloride or AMG9810 daily 30 min before the pH 4.0 saline injections (Fig. 3-6A and B, from days 0 to 3 after surgery). Both amiloride and AMG9810 were ineffective in preventing the development of thermal hyperalgesia in AS-TIIP rats (Fig. 3-6A and B). It is interesting to note that the TRPV1R antagonist, AMG9810 failed to block the development of thermal hyperalgesia even though TRPV1R appear to be involved in maintaining the lowered threshold for a noxious heat stimulus (Fig. 3-5A). I next evaluated whether P2 receptors are involved in the development of thermal hyperalgesia caused by acidic saline injection under ischemic conditions (Fig. 3-6C and D). To elucidate which P2 receptor subtype (P2X or P2Y1) contributes to induce thermal hyperalgesia, TIIP rats receiving pH 4.0 saline were pre-treated with a potent P2X antagonist, TNP-ATP (100 and 300mol) or a selective P2Y1R antagonist, MRS2179 (100 and 300nmol). Animals were pre-treated with TNP-ATP or MRS2179 daily 30 min before the 4.0 saline injection (Fig. 3-6C and D, from day 0 to 3). MRS2179 (100 and 300nmol) dose-dependently blocked the development of thermal hyperalgesia, while TNP-ATP did not affect the onset of thermal hyperalgesia (Fig. 3-6D; $*P < 0.05$, $** P < 0.01$ vs vehicle).

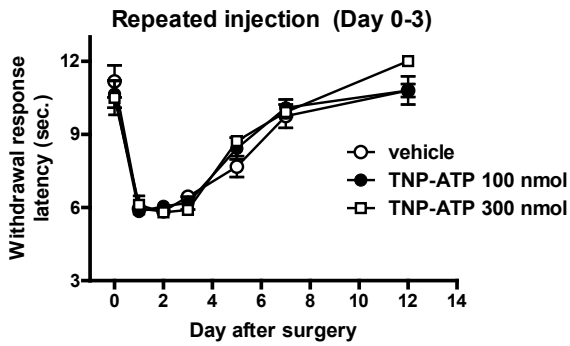
A


Repeated injection (Day 0-3)
Withdrawal response latency (sec.)
Day after surgery
vehicle
Amiloride 30 nmol
Amiloride 100 nmol

B


Repeated injection (Day 0-3)
Withdrawal response latency (sec.)
Days after surgery
vehicle
AMG-9810 10nmol
AMG-9810 3nmol

C


Repeated injection (Day 0-3)
Withdrawal response latency (sec.)
Day after surgery
vehicle
TNP-ATP 100 nmol
TNP-ATP 300 nmol

D

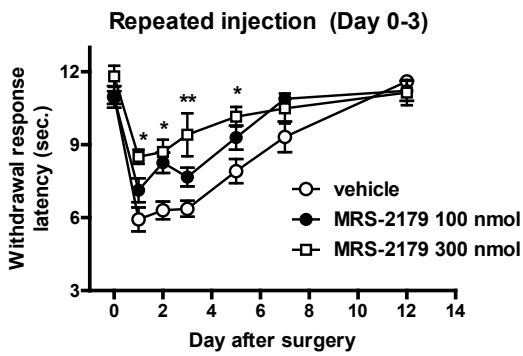

Repeated injection (Day 0-3)
Withdrawal response latency (sec.)
Day after surgery
vehicle
MRS-2179 100 nmol
MRS-2179 300 nmol

Figure 3-6. The Effect of proton sensing ion channels and P2 receptors antagonists in the induction of thermal hyperalgesia in acidic saline injected TIIP (AS-TIIP) rats. Graphs showing the effects of amiloride (a ASICs blocker), AMG9810 (a TRPV1R antagonist), TNP-ATP (a P2X antagonist) and MRS2179 (a P2Y1R antagonist) on acidic injection-induced thermal hyperalgesia in TIIP rats. Each drug was repetitively injected to the hind paw 30 min before pH 4.0 saline injections (D0-3 post-surgery). amiloride (**A**, 30 and 100nmol), AMG9810 (**B**, 3 and 10nmol) and TNP-ATP (**C**, 100 and 300nmol) given before pH 4.0 saline injection did not prevent the development of thermal hyperalgesia (n=7 in each group). Conversely, repetitive treatment with MRS2179 (**D**, 100 and 300nmol, n=4, 6, 5 in each group) effectively prevented the development of thermal hyperalgesia in the AS-TIIP group (*$P < 0.05$, **$P < 0.01$ vs vehicle).

8. Peripheral P2Y1 receptor modulate TRPV1 receptor activity by PKC-dependent phosphorylation in acidic saline injected TIIP (AS-TIIP) rats

Since P2Y1R appears to play a crucial role in acidic saline induced thermal hyperalgesia in TIIP rats (Fig. 3-6D), an additional experiment was performed to test the hypothesis that P2Y1R might modulate TRPV1R activity under ischemic conditions. To address this, I performed a western blot analysis of phosphorylated TRPV1R (pTRPV1R) in the hind paw skin of sham + vehicle, AS-TIIP + vehicle and AS-TIIP + MRS2179 groups at postoperative day 3 following daily injections of pH 4.0 saline (Fig. 3-7). Vehicle and MRS2179 were repetitively injected into the hind paw 30 min before pH 4.0 saline injection. Acidic saline injection into the hind paw of TIIP rats increased the expression of pTRPV1 (Fig. 3-7; $*P < 0.05$ sham + vehicle). More importantly, repeated intraplantar treatment with MRS2179 prior to the pH 4.0 saline injection returned the expression of pTRPV1 to baseline levels (Fig. 3-7; $\#P < 0.05$ vs AS-TIIP + vehicle). Quantitative analysis of pTRPV1 expression was normalized against corresponding total TRPV1R expression and calculated as a percent (%) change with respect to the sham group.

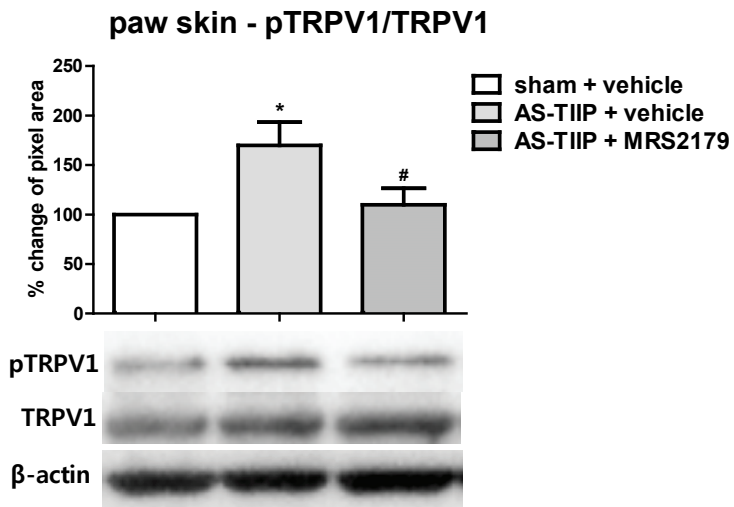


Figure 3-7. The effect of MRS2179 on phosphorylated TRPV1 receptor (pTRPV1R) expression in acidic saline injected TIIP (AS-TIIP) rats. TRPV1R and pTRPV1R were detected by using western blots in the hind paw skin of sham + vehicle, AS-TIIP + vehicle and AS-TIIP + MRS2179 (n=6 in each group, 3 days after surgery and pH 4.0 saline injections). Vehicle and MRS2179 were repetitively injected into the hind paw 30 min before pH 4.0 injection. The level of pTRPV1 expression significantly increased in AS-TIIP rats, and repeated MRS2179 treatment decreased the ratio of pTRPV1 back to the sham control level (*$P < 0.05$ vs sham + vehicle and #$P < 0.05$ vs AS-TIIP + vehicle). The level of pTRPV1 expression was normalized against the corresponding total expression of TRPV1R. Quantitative analysis of pTRPV1 was calculated as the percent (%) change with respect to the sham group.

DISCUSSION

It has been reported that tissue acidity corresponds to the severity of hypoxic status, and the tissue acidic environment is a major factor for triggering ischemia related pathological consequences (Li and Siesjö, 1997; Mekhail et al., 2004; Siesjö, 1988). In terms of pain hypersensitivity, although low tissue pH at the ischemic site has been considered a crucial factor in the development of ischemic pain (Birdsong et al., 2010; Naves and McCleskey, 2005; Seo et al., 2010), the contribution of the acidic environment to ischemia-induced thermal hypersensitivity has not been delineated. The present study is the first to demonstrate that under peripheral ischemic conditions, an increase in tissue acidity results in the development of thermal hyperalgesia. Here, I injected pH 4.0 saline into the ischemic hind paw to evaluate how an acidic tissue environment affects both thermal hypersensitivity and TRPV1R activity under peripheral ischemic condition. Since carbonic anhydrase II (CA II) has been reported to be a major indicator of pH imbalance and hypoxia inducible factor-1α (HIF-1α) is a well-known indicator of hypoxia (Heyman et al., 2011; Ho et al., 2006; Li et al., 2002; Mekhail et al., 2004; Potter and Harris, 2004; Riley et al., 1982; Švastová et al., 2004), these two factors were examined in order to evaluate the degree of hypoxic injury and tissue acidity. Repetitive acidic saline injection into the ischemic hind paw increased protein levels of both carbonic anhydrase II (CA II) and hypoxia inducible factor-1α (HIF-1α) in hind paw muscle (Fig. 3-2). These results indicate that the presence of an acidic tissue environment intensified the ischemia-associated insults to the hind paw muscle tissue. Therefore, I postulated that injection of acidic saline into TIIP (AS-TIIP) rats represented a model of the ischemic condition with severe tissue acidosis.

Although pH 4.0 is quite an extreme pH not often seen even in pathophysiological conditions, several studies have examined the actual pH level in the plantar tissue, and they demonstrated that the measured tissue pH was considerably higher than solution pH level before injection (Ikeuchi et al., 2008; Sluka et al., 2001). For example, Sluka *et al*. (2001) reported that the repeated injection of pH 4.0 saline into gastrocnemius muscle lowered the muscle pH averaged 6.5 with decreases in individual animals to pH 6. Therefore, I have assumed that a single injection of pH 4.0 saline would not extremely decrease the tissue pH, and further considered that temporary acidic pH tissue condition induced by pH 4.0 saline could mimic the pathophysiological state shown in peripheral ischemic condition. Collectively, the acidic environment is easily buffered by physiological buffering systems (Hamamoto et al., 1998; Pan and Chen, 2004; Sluka et al., 2001), it may be difficult to activate TRPV1R directly, since they only respond under relatively low tissue pH (pH < 5-6) (Aneiros et al., 2011; Gavva, 2004; Neelands et al., 2005). Therefore, there is the strong possibility that the interaction of the acidic environment and ATP indirectly activate TRPV1R via P2Y1R which up-regulates intracellular signaling cascades, but this only occurs in an ischemic environment in which tissue acidosis is present.

Previously, I demonstrated that inflammatory insults up-regulated TRPV1R expression and contributed to the inflammatory thermal hyperalgesia (in chapter 1 and 2). On the other hand, there was no significant changes in TRPV1R expression level when ischemic thermal hyperalgesia developed; otherwise, phosphorylated pTRPV1/TRPV1 ratio was increased in AS-TIIP rats (Fig. 3-3). It is well recognized that TRPV1R is activated by pro-algesic substances such as ATP, bradykinin and

prostaglandins sensitize nociceptors by up-regulating intracellular signaling cascades. These signaling cascades include calcium dependent protein kinase (PKC), cyclic AMP-dependent protein kinase (PKA), and calcium-calmodulin dependent protein kinase (CaMKII) dependent phosphorylation (Planells-Cases et al., 2005). Since PKC dependent modulation of TRPV1R has been reported to be the main pathway stimulated by inflammatory mediators (Ma and Quirion, 2007; Wang, 2008), an analysis of phosphorylated TRPV1R that targeted S800 was performed in the present study. Sham (pH 7.0 and 4.0), TIIP and AS-TIIP rats did not show any alterations in TRPV1R expression (Fig. 3-3A), whereas the ratio of pTRPV1/TRPV1 was significantly elevated in AS-TIIP rats (Fig. 3-3B). Although it has been reported that PKC dependent TRPV1R phosphorylation is critical in the TRPV1R response to its agonist *in vitro* (Mandadi et al., 2004; Mandadi et al., 2006; Tominaga et al., 2001), this is the first evidence that increased phosphorylation of TRPV1R in peripheral tissues directly correlates with behavioral changes *in vivo*. Since TRPV1R is not only located in peripheral nerve fibers, but also in the keratinocytes distributed in the epidermis of the skin, our initial western blot data contained TRPV1R from both neuronal and non-neuronal origins. Therefore, in attempt to evaluate the contribution of non-neuronal TRPV1R to the up-regulation of the pTRPV1/TRPV1 ratio observed in AS-TIIP rats, resiniferatoxin (RTX) was given to several groups of rats to destroy TRPV1R containing nerve fibers and subsequently examined the changes in the ratio of TRPV1R and pTRPV1 expression in paw lysates from sham (pH 7.0 and 4.0) and TIIP (pH 7.0 and 4.0) rats (Fig. 3-4). Although TRPV1R was still present in hind paw lysates from RTX treated rats, there were no significant changes in the ratio of pTRPV1/TRPV1 receptor in AS-TIIP rats (Fig. 3-4D). These results indicate that

TRPV1R associated with keratinocytes or other non-neuronal sources did not contribute to the increase in pTRPV1/TRPV1 receptor ratios that I observed in AS-TIIP animals. Collectively, these findings suggest that the transitional rate of PKC dependent phosphorylation of TRPV1R in peripheral nerve fibers is an important factor in the development of heat hypersensitivity in chronic ischemic conditions.

In this chapter, I addressed that activation of P2Y1R modulated the up-regulated pTRPV1 level which result in the development of heat hypersensitivity during the ischemic condition. There are some reports suggesting that locational and functional relationship between P2Y1R and TRPV1R (Gerevich et al., 2004; Ruan and Burnstock, 2003; Tominaga et al., 2001; Yousuf et al., 2011). P2Y1R is located primarily in small diameter sensory neurons, and thus it is perhaps not surprising that over 80% of CGRP neuronal profiles in DRGs contain P2Y1R (Ruan and Burnstock, 2003). Interestingly Gerevich *et al.* demonstrated that P2X3, TRPV1, and P2Y1 receptors are co-expressed in ~80% of small diameter DRG cells (Gerevich et al., 2004). Previously, Tominaga *et al*. (Tominaga et al., 2001) demonstrated in *in vitro* experiments that extracellular ATP potentiated proton- or capsaicin- induced TRPV1R currents, and lowered the heat threshold through interactions with P2Y1R in HEK 293 cells. They suggested that the augmentative action of ATP was mediated by P2Y1R, and they activate TRPV1R by PKC dependent pathway at the cellular level (Tominaga et al., 2001). On the other hand, the relationship and interaction between P2Y1R and phosphorylation of TRPV1R have not been examined *in vivo*. In the current part of the study, I examined the potential interaction between P2Y1R and TRPV1R and demonstrated that this interaction results in the development of a thermal hyperalgesia response in ischemic rats. As indicated in Fig. 3-7, creation of a

more acidic environment in the ischemic hind paw resulted in the up-regulation of PKC-dependent pTRPV1R expression. Moreover, pre-injection of the P2Y1R antagonist, MRS2179 significantly reduced this increase in pTRPV1R expression. Based on these results, I conclude that activation of P2Y1R in an acidic environment modulates TRPV1R activity (i.e. phosphorylation at the S800 site) and ultimately causes the development of thermal hyperalgesia under peripheral ischemic conditions.

One of the questions resulting from the current study relates to explaining the mechanism by which acidic tissue conditions such as that associated with ischemia affects P2Y1R. One possibility is that the lower pH found in ischemic tissue changes the agonist affinity of the P2Y1R. Many reports have demonstrated that extracellular protonation modulates the affinity of the ATP binding site and enhances the agonist potency of P2 receptors. For example, Li *et al*. (1997) demonstrated that extracellular protons regulate the function of P2X receptors by modulating the affinity of the ATP binding site. Furthermore, extracellular protons have been shown to significantly potentiate the agonist potency of recombinant P2Y4 receptors, indicating the functional potentiation of P2Y receptors by protons (Wildman et al., 2003). Furthermore, I should also consider the possibility that the concentration of P2Y receptor agonists at the tissue site might be changed by lower tissue pH. In this regard, Dulla *et al*. reported that ATP hydrolysis to ADP and adenosine (by ectonucleotidases) is closely link to the pCO_2 level and pH in hippocampal slices, and changes in nucleotides levels ultimately modulate neuronal excitability in the forebrain (Dulla et al., 2005). In addition, Sowa *et al*. (2009) demonstrated that prostatic acid phosphatase (PAP), which is expressed in nociceptive neurons and functions as an ectonucleotidase, has pH-dependent ectonucleotidase activity. At

neutral pH, mPAP primarily dephosphorylates AMP; however, under acidic extracellular conditions, mPAP can dephosphorylate all purine nucleotides (AMP, ADP, ATP). Collectively, these reports suggest that the acidic condition of the tissue can change nucleotidase activity leading to increased de-phosphorylation of ATP, which would result in an increase in ADP concentration and increased activation of P2Y1R

In conclusion, the present study clearly demonstrates that the presence of chronic ischemia in combination with increased tissue acidity can alter TRPV1R sensitivity, not by a direct action of protons on the TRPV1R, but rather by an indirect action of ATP on TRPV1R that is mediated via a Gq-coupled P2Y1R. Furthermore, this activation of P2Y1R by an increase acidic tissue environment results in a PKC-dependent TRPV1R phosphorylation, which ultimately contributes to the development of thermal hyperalgesia. Thus, this study suggests a novel peripheral mechanism that may underlie the development of thermal hyperalgesia in chronic ischemic patients with severe acidosis.

SUMMARY

The P2Y1 receptor (P2Y1R) is a Gq-coupled receptor, which is widely distributed in various organs including the nervous system. Recently, there is emerging evidence that P2Y1R could affect the function of Transient Receptor Potential Vanilloid 1 receptor (TRPV1R) which is considered as an endpoint target of peripheral sensitization. However, the precise mechanisms regarding to the role of P2Y1R and their relationship with TRPV1R in pain hypersensitivity remain to be addressed. The current study was designed to delineate the potential linkages of P2Y1R and TRPV1R and their role in the rodent inflammatory and ischemic pain models.

In the first study, the role of peripheral P2Y1R in carrageenan induced inflammation was investigated. Following injection of 2% carrageenan into the hind paw, expression of P2Y1R and TRPV1R was significantly increased and peaked at day 2 post-carrageenan injection. Blockade P2Y1R by MRS2500 selectively prevented the induction of inflammatory thermal hyperalgesia but not mechanical allodynia. Simultaneously, MRS2500 injections suppressed up-regulated TRPV1R expression in peripheral tissues. These results implicate that P2Y1R modulates TRPV1R expression, and this interaction is the crucial components in the development of inflammatory thermal hyperalgesia.

In the second study, I examined whether MAPKs activity in DRGs would contribute to P2Y1R induced modulation of TRPV1R under the inflammatory condition. Phosphorylation rates of both p38 MAPK and ERK but not JNK were increased and peaked at day 2 post-carrageenan injection. Blockade of peripheral P2Y1R significantly suppressed the ratio of p38 MAPK phosphorylation in DRGs,

while p-ERK signaling was not affected. Furthermore, inhibition of p38 MAPK in the DRGs prevented the inflammation induced up-regulation of TRPV1R. These data demonstrate that peripheral P2Y1R activates p38 MAPK signaling in DRGs which ultimately results in the up-regulation of TRPV1R during the inflammation.

In the third study, the role of P2Y1R and TRPV1R in the ischemic thermal hyperalgesia was investigated. Seo *et al.* (2008) previously developed a thrombus-induced ischemic pain (TIIP) animal model, which was characterized by chronic mechanical allodynia without thermal hyperalgesia. Repeated intraplantar injection of pH 4.0 saline for 3 days following TIIP surgery resulted in the development of thermal hyperalgesia. After acidic saline (pH 4.0) injections, there was a significant increase in pTRPV1/TRPV1 ratio in acidic saline injected TIIP animals. Inhibition of peripheral P2Y1R significantly prevented the induction of thermal hyperalgesia, and it also prevented the increase of pTRPV1 ratio in acidic saline injected TIIP animals. These results suggest that maintenance of an acidic environment is critical factors in the ischemic thermal hyperalgesia, and P2Y1R induced TRPV1R phosphorylation plays a pivotal role in this process.

Collectively, these findings clearly demonstrate that there is a close relationship between P2Y1R and TRPV1R in the development of thermal hyperalgesia, and this connection could be useful therapeutic targets for alleviating thermal hypersensitivity under the conditions of inflammation and/or ischemia.

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국 문 초 록

말초 P2Y1 수용체 매개 TRPV1 수용체 조절이 열성 통각과민의 형성에 미치는 영향

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실험배경 및 연구목적

염증 및 허혈성 손상으로 인한 병적 상태에서 내인성 통증 유발 물질들이 손상된 조직으로 방출됨으로써 말초감작현상이 나타난다. TRPV1 수용체는 말초감작현상을 일으키는 주요 인자로서 자극유발물질들이 (sensitizing mediators) 세포 내 신호전달을 통해 TRPV1 수용체를 활성화 시킨다. P2Y1 수용체는 말초 신경에 존재하는 Gq-단백질과 연결된 수용체로서 조직 손상 시 분비되는 ATP 및 ADP가 효현제로 작용한다. 최근, P2Y1 수용체가 통증과 연관성이 있다는 것과 *in vitro* 시스템에서 P2Y1 수용체가 TRPV1 수용체의 기능을 조절할 수 있다는 가능성이 발표된 바 있다. 그러나 P2Y1 수용체의 통증에서의 역할 및 TRPV1 수용체와의 관계가 통증에 미치는 영향에 대한 특정 조절 기전은 밝혀진

바 없다.

따라서 본 연구는 (1) 염증성 자극이 말초의 P2Y1 수용체의 발현 정도에 미치는 영향, 말초 P2Y1 수용체의 억제가 염증성 통증을 조절하는지 여부 및 TRPV1 수용체의 발현 조절에 대하여 조사하였다. (2) 염증성 자극 후 DRG (dorsal root ganglion)의 MAPKs (ERK, p-38 MAPK 및 JNK) 활성 변화, 말초 P2Y1 수용체의 억제가 MAPKs의 활성에 미치는 영향 그리고 최종적으로 활성화된 MAPKs 억제 시 TRPV1 수용체의 발현 변화를 평가하였다. (3) 마지막으로 허혈성 통증 모델에서 P2Y1 수용체에 의한 TRPV1 수용체의 기능 조절이 열성 통각과민의 형성에 기여하는지 여부를 평가하고자 한다.

실험방법

실험동물로 수컷 Sprague-Dawley 랫드를 사용하였다. 염증성 통증은 2% carrageenan을 발바닥 내로 투여하여 유도하였고, 허혈성 통증은 혈전유래 허혈성 통증 (thrombus induced ischemic pain, TIIP) 모델을 사용하였다. 이 모델은 대퇴동맥 노출 후 $FeCl_2$를 점적함으로써 혈전을 생성시켜 말초 허혈을 유도한다. 열성 통각과민증은 Hargreaves 검사를 이용하여 열 자극에 대한 회피반응의 시간을 측정함으로써 평가되었고 기계적 이질통은 von-Frey를 이용하여 무해한 물리적 자극(4g)을 주고 자극에 대한 반응빈도로 측정하였다. 약물은 각각의 실험목적에 따라 처치되었다. MRS2500과 MRS2179 (P2Y1 길항제), MRS2365 (P2Y1 효현제), AMG9810 (TRPV1 길항제), chelerythrine (PKC 억제제), amiloride (ASICs 억제제) 및 TNP-ATP (P2Xs 길항제)는 발바닥 내로 투여했다. SB203580 (p38 MAPK 억제제)는 척수 내 투여를 통해 DRG 내 p38 MAPK 활성을 억제하였다. 면역조직화학법 및 Western blot assay를 실시하였으며, 컴퓨터와

연계된 image analysis program인 Metamorph를 이용하여 단백질의 발현 정도를 분석하였다.

실험결과

1. 발바닥에 2% carrageenan 투여 후 2일째 P2Y1과 TRPV1 수용체의 발현이 유의하게 증가되었다. 말초의 P2Y1 수용체를 억제하면 열성통각과민 형성이 억제되지만 기계적이질통의 형성에는 영향을 미치지 못했다. 또한 말초 P2Y1 수용체 억제 시 염증으로 인해 증가된 TRPV1 수용체의 발현이 감소되는 양상을 규명하였다. 정상 랫드에서 P2Y1 수용체의 효현제를 반복적으로 투여하면 농도 의존적으로 TRPV1 수용체의 발현이 증가되었다.

2. Carrageenan으로 염증 유발 후 DRGs의 p38 MAPK와 ERK의 인산화는 증가하였고 JNK의 인산화 비율에는 변화가 없었다. 말초 P2Y1 수용체를 억제하면 특이적으로 p38 MAPK의 인산화가 감소되는 반면 ERK의 인산화에는 변화가 없었다. 그리고 DRGs의 p38 MAPK 억제 시 염증으로 인해 증가되는 TRPV1 수용체의 발현이 억제됨을 관찰하였다. 또한, 정상 랫드에서 P2Y1 수용체의 효현제를 반복적으로 투여하면 농도 의존적으로 DRG 내 p38 MAPK의 인산화 비율이 증가하였다.

3. TIIP 수술 후 허혈이 유발된 발바닥에 pH 4.0의 산성 식염수를 수술 후 3일간 투여한 결과 열성통각과민 현상이 관찰되었다. 이 열성 통각과민은 PKC 억제제 및 TRPV1 수용체 길항제로 인하여 완화되었다. 산성 식염수를 투여하면 발바닥 피부의 TRPV1 수용체 발현에는 변화가 없었지만 TRPV1 수용체의 인산화 비율은 증가하게 되었다. 말초 P2Y1 수용체 억제 시 열성 통각과민이 완화됨과 동시에 TRPV1 수용체의

인산화 비율을 감소시키는 현상을 관찰하였다.

결론

본 연구에서는 염증 및 열성 통각과민에서 P2Y1 수용체, p38 MAPK 그리고 TRPV1 수용체의 단계적 역할을 규명하였다. 염증상태에서 말초 P2Y1 수용체의 활성은 DRG의 p38 MAPK 신호전달을 활성화시키고 이는 최종적으로 TRPV1 수용체의 발현을 조절함으로써 염증성 열성 통각과민을 일으키게 된다. 또한 허혈성 통증에서는 말초 조직의 산증이 열성 통각과민의 주요 인자라는 점을 밝혔고, 이 열성 통각과민은 말초 P2Y1 수용체가 TRPV1 수용체를 인산화 시킴으로써 형성됨을 규명하였다. 이러한 연구결과들은 열성 통각과민의 형성에 기여하는 말초에서의 세부기전을 제시함으로써 P2Y1 및 TRPV1 수용체가 향후 치료 인자로서 고려될 수 있음을 명확히 제시하고 있다.

주요어: P2Y1 수용체, TRPV1 수용체, 열성 통각과민, p38 MAPK, 염증, 허혈

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