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Activation of microglial cells in the trigeminal subnucleus caudalis evoked by inflammatory stimulation of the oral mucosa

By

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Summary: To study the inflammatory hyperalgesia induced by an acute noxious stimulation of oral mucosa with 5% formalin (5%FOR), we performed an immunohistochemical study on the expression of TNFα in the intermolar region of the dorsal lingual eminence (IDLE), and Iba1 and phosphorylated (phospho)- p38 MAPK involved with central nervous system microglial activation in the trigeminal subnucleus caudalis (Vc). The present study observed significantly increased expression of TNFα at either 1h or 24h of 5%FOR nociception, as well as sustained TNFα immunoreactivity in the IDLE. On the other hand, at either 1h or 24h 5%FOR nociception, Iba1- immunoreactive (IR) cells in the Vc were significantly increased after inflammatory stimulation of the IDLE; the increase was more evident at 24h 5%FOR nociception. By using the double-label immunofluorescence technique, the findings in particular demonstrated a significant increase in the number of phospho-p38 MAPK- and Iba1-IR coexpressed cells in the Vc at 24h 5%FOR nociception. The results suggest that 24h persistent microglial activation in subnuclei zonalis and gelatinosus of the Vc is evoked by 5%FOR noxious stimulation of the IDLE oral mucosa, thereby the present study indicates that the MAPK expression plays important roles in microglial activation related with central sensitization and inflammatory hyperalgesia.

Introduction

The mucous membrane surface lining of the oral cavity is constantly exposed to micro-environmental changes elicited by a variety of physical, chemical, microbiological and thermal stimulation. In the oral cavity, there are many kinds of receptors involved in nocifensive (e.g., pain-related) behaviors maintained by mechanisms to escape from noxious events. Because pain sensation deteriorates the quality of life (QOL), the task to elucidate nociceptive mechanisms, which evoke, enhance or hypersensitize pain, is an important assignment.

Some previous studies have reported that hypodermic injection of tumor necrosis factor (TNFα; an inflammatory mediator cytokine) into tissue distributed with C fibers would upregulate IL-1β to evoke inflammatory hyperalgesia[1)–7)]. Additionally, we observed the enhanced expression of TRPV4, serotonin (5-hydroxytryptamine: 5HT) and phosphorylated-extracellular signal-regulated kinase (pERK) —a mitogen activated-protein kinase (MAPK)— in many parts of the brainstem, and confirmed that the expression of TRPV4 and pERK in the trigeminal subnucleus caudalis (Vc) was closely related with central hyperalgesia and descending noradrenergic and serotonergic endogenous pain inhibitory systems evoked by nociceptive stimulation[8)]. The pERK has been recognized as a marker of activation of spinal dorsal horn (DH) neurons following a variety of peripheral noxious stimuli[9, 10)].

There have been many studies reported that the Vc glia were activated to elicit central sensitization, while dental pulp injuries induced plastic neuronal changes of astrocytes in the Vc[11)–14)]. Astrocytes and microglia in the central nervous system (CNS) were activated either by inflammation or peripheral injury, and the activity of spinal glia was deeply involved with activation mechanisms of p38 MAPK to evoke inflammatory pain, hyperalgesia and allodynia[15)–17)]. Other studies also observed that both peripheral inflammation and nerve damage prominently increased the number of microglial markers and phosphorylated(phospho-) p38 MAPK-IR glia in the spinal cord, while application with 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580,

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SB: a p38 MAPK inhibitor) of the spinal cord effectively suppressed allodynia[18)–21)]. In addition, a particular temporal pattern of ERK activation has been found in spinal neuron, microglia and astrocytes, and also in spinal dorsal root ganglia (DRG) neurons and satellite cells. The study indicated that ERK is contributed to neuropathic pain through different mechanisms in neurons and glial cells at different times[22)]. Nevertheless, details concerning the roles of MAPK in neuronal activity of microglial cells and the relationships with the pain crisis of inflammatory hyperalgesia and allodynia in oral mucosa are still unclear.

In the present study, we examined the expression of TNFα related with inflammatory hyperalgesia occurred in 5%FOR nociception of oral mucosa. Furthermore, for the purpose of clarifying the relationship between brainstem microglial activity, central sensitization and hyperalgesia followed by the acute noxious stimulation of the IDLE, we investigated the expression of phospho-p38 MAPK and ionized calcium-binding adapter molecule 1 (Iba1: a marker for microglial activity[23)] in the Vc.

Materials and Methods *(see Flowchart)*

Animal preparation

Wistar rats (male, 250 g body weight, n = 20; Japan SLC, Shizuoka, Japan) were used in this study. The protocol was approved by the Animal Research Committee of Osaka Dental University (No. 12-02024) and implemented in accordance with the ethical guidelines for the treatment of animals of the International Association for the Study of Pain[24)].

Control and experimental groups

After anesthesia of the rats with sodium pentobarbital (70 mg/kg, i.p., Nembutal, Dainippon Sumitomo Pharma, Osaka, Japan; n = 20), the intermolar region of the dorsal lingual eminence (IDLE) was stimulated with filter paper discs (2 × 2 mm^2 square) soaked with either 10 μl 0% FOR (normal saline, room temperature: RT, pH = 7.2; n = 10, control:0%FOR) or 5% FOR (RT, pH = 4.0; n = 10, experimental:5%FOR) for 5 minutes[8)]. After 1 hour (h) and 24h of stimulation, the rats of control 0%FOR (1h, n = 5; 24h, n = 5) and experimental 5%FOR (1h, n = 5; 24h, n = 5) groups were euthanized and transcardiac-perfused with 100 ml of normal saline solution followed by 4% chilled (0°C–4°C) paraformaldehyde in 0.1 M phosphate buffer (PB; pH = 7.4) following the conventional methods.

Tissue preparation and immunohistochemical analysis

The tongues and brainstems were dissected, immersed in the same fixative (tongue: 2h; brainstem: 24h), and then transferred to 30% sucrose (W/V) in 0.1 M PB for 48h for cryoprotection. The samples were cut into sections (tongue: 10 μm; brainstem: 30 μm) at –20℃ with a Leica CM3050S cryostat (Leica Microsystem, Wetzlar, Germany), and then serially transferred to multi-well tissue culture plates containing 0.1 M Tris-buffered saline. Frozen sections of each tongue IDLE and every sixth section of each brainstem Vc (between –13.68 mm to –15.66 mm; distance from the bregma) were collected and processed for the present immunohistochemical study[8, 24, 25)]. The IDLE specimens were investigated with anti-TNFα enzyme labeled antibody method, whereas the brainstem Vc specimens were studied with either anti-Iba1 enzyme labeled antibody method or anti-Iba1/anti-phospho-p38 MAPK immunofluoresence (IF) technique[8, 24, 25)]. The present histological study was summarized as following categories (see Flowchart):

(1) The expression of TNFα in the IDLE.
(2) Iba1-IR cells in the Vc.
(3) The coexpression of Iba1 and phospho-p38 MAPK in the Vc.

Subsequently, the specimens of enzyme labeled antibody method were examined and photographed with Olympus BX41 light microscope (Olympus, Tokyo, Japan) mounted with an Olympus FX380 3CCD digital camera system (OS: Windows XP; Microsoft, CA, USA). The specimens of immunofluorescent staining were observed and photographed with a BIOREVO BA-9000 fluorescence microscope (KEYENCE, Osaka, Japan). TNFα-IR cells in 10 areas (area = 20 × 20 $μm^2$ square) of each IDLE of either 1h and 24h 0%FOR (control) specimens or 1h and 24h 5%FOR (1h nociception and 24h nociception; experimental) specimens were counted with a FLvFs software (Flovel Image Filling System, Tokyo, Japan) of the FX380 system. Data of the IR cell counts in the IDLE and Vc (area=800 × 500 $μm^2$) of both the control and experimental groups were recorded, summarized and statistically analyzed by using Student's *t*-test (Excel 2010, Microsoft, USA) by a MS-Windows XP personal computer.

Histological Findings and Results of the Statistical Analysis

The expression of TNFα in the IDLE (Fig. 1)

Distribution of TNFα-IR cells was indistinct in lamina propria of the weakly-stained IDLE mucosa in the 1h (Fig. 1A) and 24h (Fig. 1C) control (0%FOR) specimens. In contrast, TNFα-IR cells were distinctly found in lamina propria of the IDLE mucosa in the 1h (Fig. 1B) and 24h (Fig. 1D) experimental (5%FOR nociception) specimens. Furthermore, the histology indicated that the number of TNFα-IR cells at 24h 5%FOR nociception was significantly increased than at 1h nociception (Figs. 1B & 1D).

Results of the present statistical analysis revealed that significant differences of the expression of TNFα-IR cells were found between the control and experimental groups (Fig. 1E: Degree of freedom(DF) = 18; 1h: $p < 0.05$, 24h: $p < 0.01$), as well as between the 1h 5%FOR nociception and 24h 5%FOR nociception groups (Figs. 1B & 1D; Fig.

Flowchart of the present study

Materials:

Wistar rats (male, 250g, n=20; Japan SLC, Jpn)
Anesthesia: sodium pentobarbital (70mg/kg, i.p.; Nembutal, Dainippon Sumitomo Pharma, Jpn)

Methods:

Stimulation with medicated filter paper discs on the intermolar region of dorsal lingual eminence (IDLE) of rats (10μl, 5min)

↓

Control group
Normal saline: 0% formalin (0%FOR), pH=7.2
n=10: 1h (n=5), 24h (n=5)

Experimental group
5%FOR, pH=4.0
n=10: 1h (n=5), 24h (n=5)

↓

Immunohistochemical staining

- Transcardiac perfusion (4% paraformaldehyde/0.1M Na-phosphate, pH=7.4, chilled)
- Immerse-fixation (same fixative; tongue:2h, brainstem: 24h)
- Transferred to 30% sucrose (48h) for cryoprotection
- Frozen sections: tongue (10μm), brainstem (30μm; Vc: from -13.68 to -15.66mm from bregma)

↓

- 0.1M phosphate buffer (PB, pH=7.4) containing 0.75% triton X-100 (1h)
- 0.1M PB containing 3% normal goat serum (30min)

↓

Tongue

Enzyme labeled antibody method
1st antibody (performed in 3% normal goat serum)
- rabbit anti-TNFα (1:200, 1h, room temp (RT); Novusbio, CO, USA)

2nd antibody
- biotinylated goat anti-rabbit IgG (1:400, 30min, RT; Vector Labs, CA, USA)
- avidin-biotin-peroxidase complex (30min, RT; Elite ABC Kit, Vector Labs)

Brainstem

1. Enzyme labeled antibody method
1st antibody (performed in 3% normal goat serum)
- rabbit anti-ionized calcium-binding adapter molecule 1 (Iba1) (1:500, 1h, RT; Wako Pure Chemical Industries, Jpn)

2nd antibody
- biotinylated goat anti-rabbit IgG (1:400, 30min, RT)
- avidin-biotin-peroxidase complex (30min, RT)

↓

- distilled water containing 0.05% 3,3'-diaminobenzidine (DAB; 2min, RT; Vector Labs)
- 0.2% nickel sulfate and 0.01% hydrogen peroxide (Elite ABC Kit)

↓

*The IDLE specimens were counterstained with 1% methyl green (5min, RT; MERCK, Germany)

↓

Light microscopy

↓

Light microscopy

2. Immunofluorescent staining
1st antibody (performed in 3% normal goat serum)
- rabbit anti-Iba1(1:500, 1h, RT; Wako)
- mouse anti-MAPK14 (1:250, 1h, RT; Abnova, TW)

2nd antibody
- Alexa Fluor 488 goat anti-rabbit IgG (1:500, 1h, RT; Molecular Probes, OR, USA)
- Alexa Fluor 546 goat anti-mouse IgG (1:500, 1h, RT; Molecular Probes)

↓

Fluorescence microscopy

Observations:

- Light microscopy (LM): BX41(Olympus, Jpn); photography: FX380 (Olympus)
- Data processing (FlvFs; Flovel, Jpn)
- Fluorescence microscopy: BIOREVO BA-9000 (KEYENCE, Jpn)
- Cell counting: TNFα-IR cells in the IDLE, Iba1-IR and Iba1/phospho-p38 MAPK-IR cells in the Vc
- Statistical analysis: student's *t*-test (*$p<0.05$, **$p<0.01$; Excel 2010, Microsoft, CA, USA)

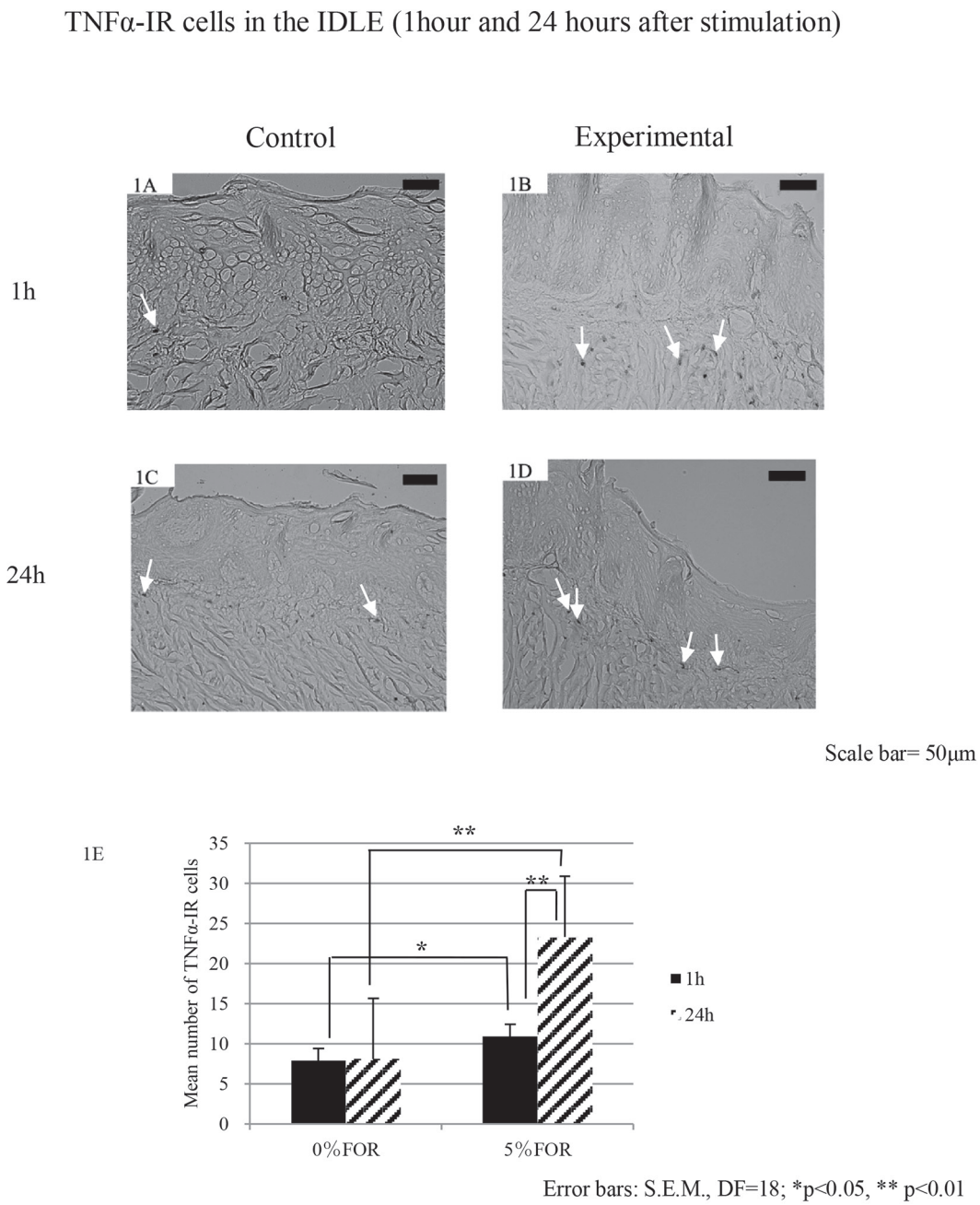


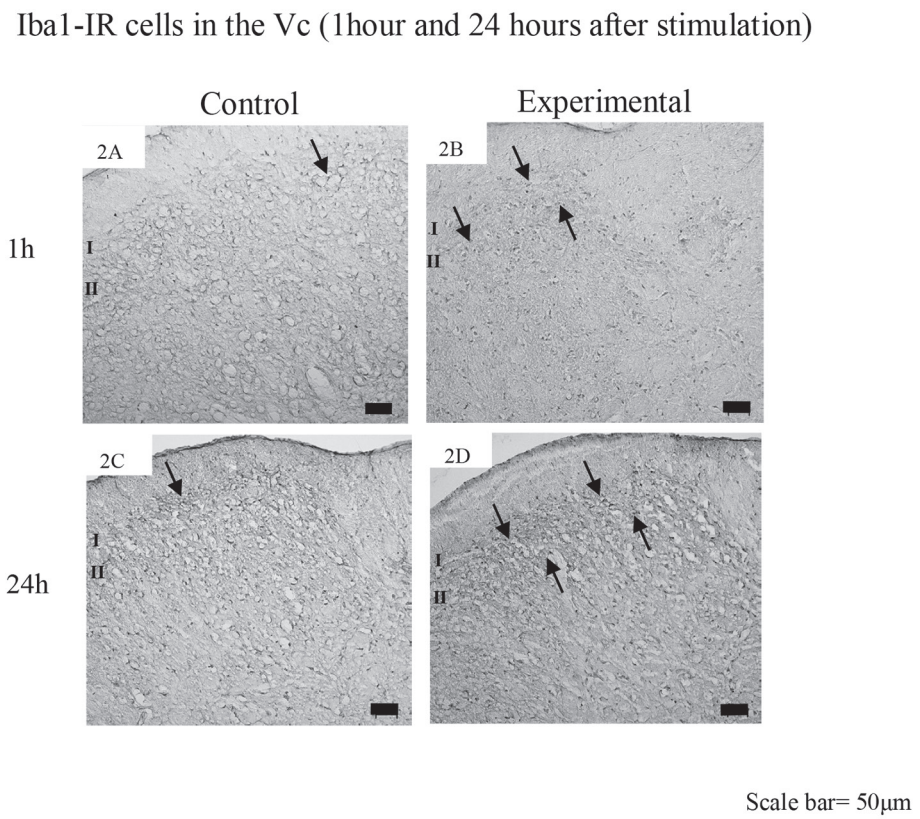
Fig. 1. Figs. 1A and 1C: Light micrograms (LM) of the IDLE mucosa in the control group (0%FOR: normal saline soln. stimulation); Figs. 1B and 1D: LM of the experimental group (5%FOR-induced nociception; 1B: 1h 5%FOR nociception, 1D: 24h 5%FOR nociception)
The histology indicates that TNFα-IR cells (arrows) are localized in lamina propria of the intermolar region of the dorsal lingual eminence (IDLE).
Fig. 1E: Nociception of oral mucosa induces an increase in the number of TNFα-IR cells in the IDLE
The expression of TNFα-IR is found in both the control (0%FOR) and experimental (5%FOR) groups (degree of freedom (DF) =18; 1h: *p<0.05, 24h: **p<0.01). A significant difference is particularly found between at 1h and 24h nociception of the IDLE (5%FOR; DF=18, **p<0.01).

1E: $p < 0.01$).

Collectively, the results suggested that a single 5%FOR stimulation on the IDLE significantly induced and enhanced TNFα expression in the inflammatory oral mucosa after 24h nociception.

Iba1-IR cells in the Vc (Fig. 2)

The histology of Iba1 expression (an indicator for microglia activity) in the Vc induced by stimulation of the IDLE with 5%FOR (experimental: noxious stimulation, Figs. 2B & 2D) and 0%FOR (control: Figs. 2A & 2C) was



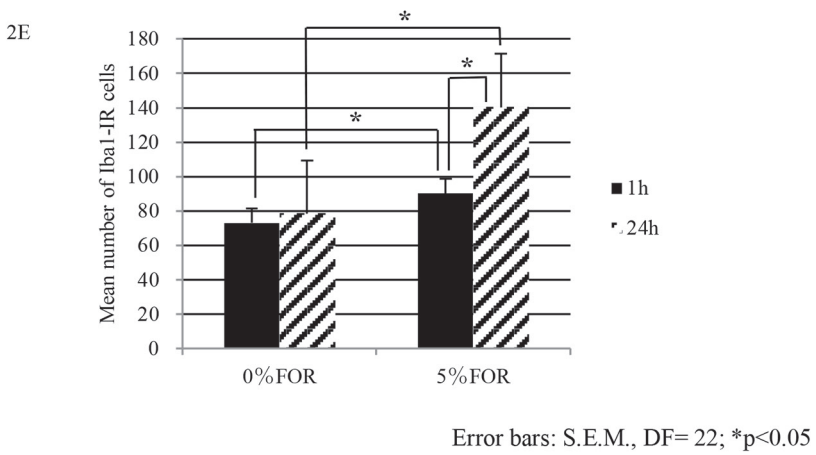


Fig. 2. Figs. 2A & 2C: LM of the control (1h & 24h after 0%FOR stimulation, respectively) group; Figs. 2B & 2D: LM of the experimental (1h & 24h after noxious 5%FOR stimulation, respectively) group (microphotographs of the rat brainstem Vc corresponding to –15.30 mm from bregma)

The histology indicates that Iba1-IR cells (arrows) are particularly localized in subnuclei zonalis (I) and gelatinosus (II) of the trigeminal subnucleus caudalis (Vc).

Fig. 2E: Nociception of oral mucosa induces an increase in the number of Ibal-IR cells in the Vc

Iba1-IR cells are significantly increased by 5%FOR nociception than in the control group (1h and 24h after stimulation; *$p < 0.05$). Notice that a significant increase in the number of Iba1-IR cells at 24h than at 1h nociception is observed (5%FOR, *$p < 0.05$).

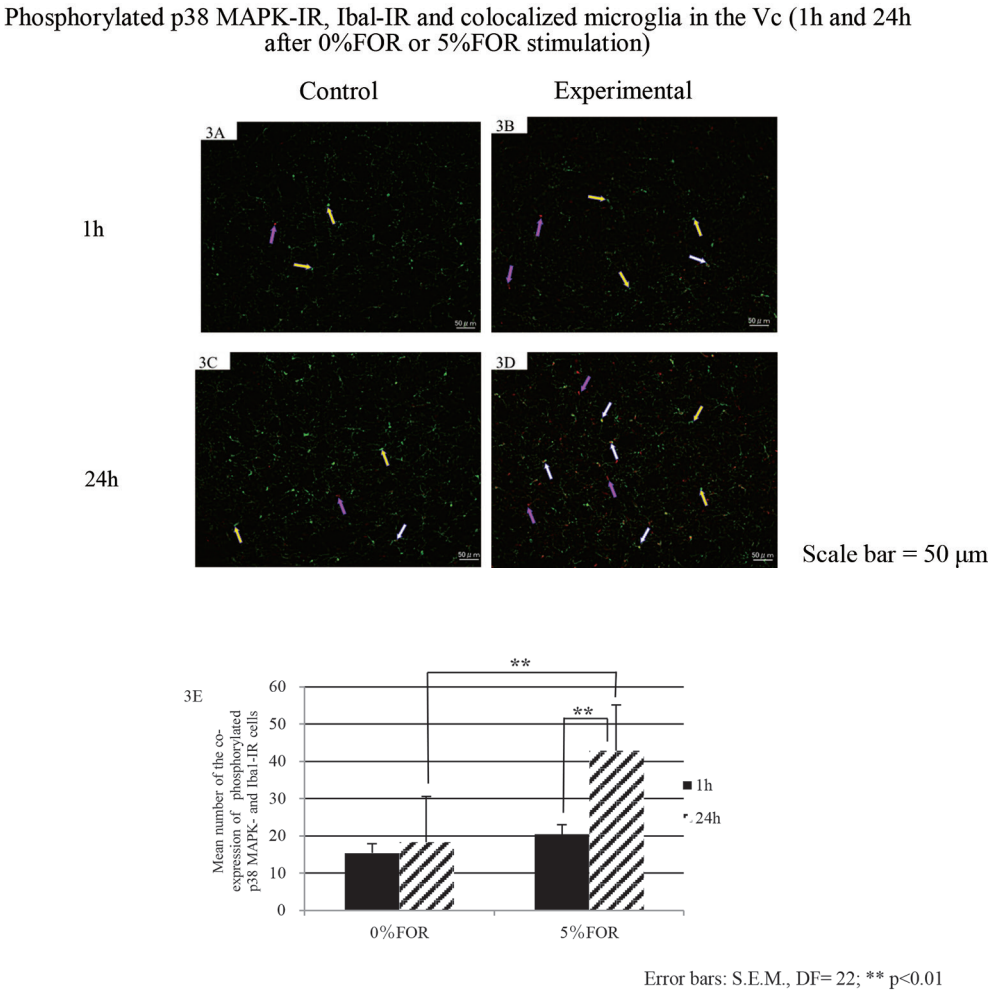


Fig. 3. Double immunohistochemical staining for phosphorylated (phospho-) p38 MAPK-IR (pink arrows), Iba1-IR (yellow arrows) and coexpressed (white arrows) microglial cells in the Vc (–14.58 mm from bregma)

Figs. 3A & 3C: Fluorescent microscopic images of the control group (1h and 24h after 0%FOR stimulation, respectively). Figs. 3B & 3D: Fluorescent microscopic images of the experimental group (1h and 24h 5%FOR nociception, respectively). The phospho-p38 MAPK/Iba1 double-label immunofluorescence staining shows colocalized IR cells (white arrows) in both the control and experimental groups; the coexpression is more distinctly observed at 24h 5%FOR nociception.

Fig. 3E: Statistical results for specimens processed with the double staining to colocalize phospho-p38 MAPK-IR and Ibal-IR microglia in the Vc

Phospho-p38 MAPK/Iba1-IR cells are significantly increased by 5%FOR nociception than in the control group (24h after stimulation; **p < 0.01). The present analysis indicates a significant increase in the number of phospho-p38 MAPK/Ibal-IR coexpressed cells at 24h 5%FOR nociception than at 1h 5%FOR nociception (**p < 0.01).

analyzed at 1h and 24h (1h: Figs. 2A & 2B; 24h: Figs. 2C & 2D). The subsequent statistical analysis indicated significant increase in Iba1-IR cells in the experimental group compared to the control group (Fig. 2E: DF = 22, $p < 0.05$). At 24h stimulation, the increase of Iba1-IR expression was particularly identified in the subnucleus zonalis (Fig. 2, layer I) and subnucleus gelatinosus (Fig. 2, layer II) of the Vc (Figs. 2C & 2D). Furthermore, a significant increase in the number of Iba1-IR cells was observed at 24h nociception than at 1h nociception (Fig. 2E: $p < 0.05$).

The coexpression of Iba1 and phospho-p38 MAPK in the Vc (Fig. 3)

The present double-label immunofluorescence histology and statistical analysis did not observe coexpression of Iba1 and phospho-p38 MAPK at 1h stimulation in both the control 0%FOR and experimental 5%FOR specimens (Figs. 3A & 3B). However, the histological and analytical results demonstrated and indicated that the number of Iba1/phospho-p38 MAPK coexpressed cells was increased due to 5%FOR nociceptive stimulation (Figs. 3B & 3D; Fig.

3E: DF= 22, $p < 0.01$). Moreover, a significant increase in the number of Iba1/phospho-p38 MAPK coexpressed cells was observed in the subnucleus zonalis and subnucleus gelatinosus of the Vc at 24h 5%FOR nociception (Fig. 3D; Fig. 3E: $p < 0.01$).

Discussion

Peripheral inflammation induced by nociception of the IDLE

The TNF-family refers to a group of inflammatory mediator and pain modulator cytotoxins synthesized and released by monocytes, keratinocytes and fibroblasts that elicits hyperalgesia during inflammation or after injury[26, 27]. There are several studies that have elucidated mediators of pain transmission (e.g., 5HT, cytokines) might sensitize TRPV4-dependent release of nociceptive peptides, which invoke peripheral hypersensitivity[8, 29, 30]. Nevertheless, a previous study has reported that activated ERK took part in increasing TRPV1 by induction of TNFα expression in the spinal DRG; the pERK expression is utilized as an indicator for leveling neuronal activity in the spinal DH following peripheral noxious stimuli[6, 9, 10]. Another study has elucidated that Schwann cells of myelinated nociceptive nerve fibers are peripheral nervous system (PNS) macroglia having histological functions similar to CNS oligodendrocytes in that they phagocytose and clear cellular debris; they also produce TNF after injury[31].

A previous study in the literature has reported that TNFα-induced nocifensive responses were observed as early as 30 min, reached a plateau between 2-3 h, declined at 6 h and recovered to pre-injection values at 24 h after intraplantar injection of the rat hindpaw[32]. Another study has reported that complete Freund's adjuvant (CFA) injection also resulted in a significant elevation in the TNFα expression of the inflamed hindpaw; the elevation was detected at 3h, peaked at 24h, and remained elevated at day5 of nociception[3]. Some studies have focused on the inflammatory mediators and reported that polymodal receptors of non-myelinated C fibers were activated by administration of TNFα, a pain-producing substance (PPS), to evoke nociception related with central hyperalgesia[1, 2, 5, 6, 7]. Besides, TNFα in particular sensitized DRG and spinal cord TRPV1 receptors to response with the endogenous agonist N-oleoyldopamine[7].

Previously, we have elucidated an enhanced TRPV4 expression in the IDLE led to peripheral hyperalgesia, as well as demonstrated a similar distribution of TRPV4- and pERK-IR cells in the Vc that might be related with central hyperalgesia caused by an acute inflammatory stimulus[8]. In the present study, we investigated that the TNFα expression in the IDLE was also significantly increased by 5%FOR stimulation of the IDLE mucosa. Additionally, we identified that the level of the TNFα expression was enhanced with significant differences based on elapsed time; nociception of the IDLE persisted and sustained after 24h of stimulation. The present study demonstrated a significant elapse-time dependent increase of TNFα receptors expression and thereby the results could be regarded as a general explanation for hyperalgesia of the irritated IDLE.

The relationship between microglial activity and the phospho-p38 MAPK expression in the Vc induced by nociception of the IDLE

Some immunohistochemical studies have observed neuronal plastic changes occurring in glia of the brainstem modulatory system to elicit central sensitization caused by dental pulpal injuries[12, 14]. Other studies have reported that a prominent increase in phospho-p38 MAPK-IR spinal microglia was induced by peripheral inflammation or nerve tissue damage, and the glia was involved with certain expression mechanisms to evoke inflammatory pain, hyperalgesia and allodynia[15, 16, 17, 33]. Meanwhile, capsaicin- or FOR-induced nociception elicited an increase in neuronal transmitters (e.g., substance P: SP, calcitonin gene-related peptide: CGRP) secretion that subsequently activated protuberant phosphorylation of ERK signaling pathway molecules in the Vc and upper cervical (C1/C2) spinal DH neurons within 10 min following the peripheral noxious stimulation; the number of pERK-positive neurons progressively increases as stimulus intensity is increased[13, 22, 30, 34, 35, 36]. Furthermore, the expression of TRPV4 in I and II_0 layers of the spinal DH has been elucidated to be coexpressed with afferent nerves containing protease-activated receptor 2 (PAR2), CGRP and SP. The same study consequently concluded that the activation of PAR2 and TRPV4 induced secretion of CGRP and SP into the synaptic cleft to initiate certain neuronal activity in the DH[30]. We have observed an increase in TRPV4-, pERK- and 5HT-IR neurons in the Vc, and summarized that the expression might activate the descending pain modulatory system in analgesia evoked by FOR stimulation of the IDLE[8]. In the present study, we distinctly demonstrated phospho-p38 MAPK/Iba1 coexpressed microglia in the zonal subnucleus and gelatinous subnucleus of the Vc, which is histologically similar and continuous with the DH.

There have been several studies on responses of the brainstem glial induced by peripheral inflammation. The studies found the genesis of enhanced nociceptive behavior with the presence of a sustained and prolonged microglial activity in the Vc; the activity did not correspond to phagocytosis of activated microglia[37, 38]. In the present study, by using the enzyme-label antibody method and double-label immunofluorescence techniques, we observed that the phospho-p38 MAPK expression coincided with a significant increase in the Iba1 (a marker for microglial activity) expression depending upon the elapsed time after 5%FOR nociception of the IDLE. The present results were indicative that the MAPK pathway was signaled to implicate activated microglia in the Vc

to evoke central sesitization in response to FOR-induced nociception of the IDLE[22, 34, 35, 36]. However, one study on peripheral inflammation has reported that stimulation with carrageenan induced an increase in the phospho-p38 MAPK and c-*Jun* N-terminal kinase (JNK) expression, whereas CFA did not evoke an increase in the phosphorylation of p38 MAPK and ERK5 in spinal microglia and primary sensory neurons[39].

Further studies to clarify the histology of the activated microglia and subsequent neuronal activities affecting primary afferent terminals and related nocicpetive specific neurons in the Vc will be the endeavor of our future investigations.

The following conclusions can be made from this study: 1) Acute 5%FOR inflammatory stimulation of the IDLE significantly induced increased and sustained tissue expression of TNFα. 2) Enhanced and prolonged microglial activity was observed in the zonal subnucleus and gelatinous subnucleus of the Vc at 24h nociception. 3) Coexpression of phospho-p38 MAPK and Iba1 was coincidental with the microglial activity in the genesis of enhanced nociceptive behavior. 4) The increase in phospho-p38 MAPK/Iba1-IR coexpressed microglia suggests that the MAPK pathway regulating neuronal activity to activate microglia in the Vc may contribute to sustaining the IDLE nociception.

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