치의과학석사학위논문

유지놀에 의한 Transient Receptor Potential Ankyrin 1의 활성화

Activation of Transient Receptor Potential Ankyrin 1 by Eugenol

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이 논문을 치의과학석사학위논문으로 제출함

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ABSTRACT

Activation of Transient Receptor Potential Ankyrin 1 by Eugenol

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The structural similarity of eugenol to cinnamaldehyde, an active ligand for transient receptor potential ankyrin 1 (TRPA1), suggests that eugenol might produce its effect via TRPA1 in addition to TRPV1 as we reported previously.

In this study, I investigated effect of eugenol on TRPA1, by Fura-2-based calcium imaging and whole-cell patch clamp recording in trigeminal ganglion neurons and in a heterologous expression system.

As the results, eugenol induced robust calcium responses in rat trigeminal ganglion neurons that responded to a specific TRPA1 agonist, allylisothiocyanate (AITC), but not to capsaicin. Capsazepine, a TRPV1 specific antagonist failed to inhibit eugenol-induced calcium responses in AITC responding neurons. In addition, eugenol response was observed in trigeminal ganglion neurons from TRPV1 knockout

mice, which was inhibited by TRPA1-specific antagonist HC-030031. Lastly, involvement of TRPA1 in action of eugenol was confirmed by calcium imaging and patch clamp experiments using human embryonic kidney 293 cells that stably express TRPA1.

In summary, these results demonstrate that activation of TRPA1 might account for another molecular mechanism underlying pharmacological action of eugenol.

keywords : eugenol, TRPA1, trigeminal ganglion, AITC, pain, analgesia.

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INTRODUCTION

Eugenol, a phytochemical found in several herb plants such as cloves, has been extensively used in dentistry to alleviate pain elicited in various condition including pulpitis and alveolar osteitis. The antinociceptive action of eugenol has been attributed to its ability to inhibit various voltage-gated ion channels (Park et al., 2009; Yeon et al., 2011). However, eugenol shows irritant action in addition to its analgesic effect (Sneddon and Glew, 1973). Since eugenol is a vanilloid compound that shares similar chemical structure with capsaicin, a pungent ingredient found in hot chili peppers, it was proposed that eugenol exerts its irritable action via activation of capsaicin receptor, transient receptor potential vanilloid 1 (TRPV1) (Ohkubo and Kitamura, 1997; Yang et al., 2003). However, TRPV1-specific antagonist, capsazepine induced only a partial inhibition of eugenol-induced cation current (Ohkubo and Shibata, 1997), which suggested that another mechanism might be involved in irritable action of eugenol.

Transient receptor potential ankyrin 1 (TRPA1) is a polymodal nociceptive receptor protein which can be activated by noxious cold temperature (Story et al., 2003), stretch of cell membrane (Corey et al., 2004), and various chemical compounds including mustard oil and cinnamaldehyde (Bandell et al., 2004). Cinnamaldehyde is an intermediate metabolite during biosynthesis of eugenol in plants. Both cinnamaldehyde and eugenol are members of phenylpropanoid compounds, a large family of organic bioactive chemicals that contain

a phenyl ring and a C3 side chain (Chaieb et al., 2007). The structural similarity between eugenol and cinnamaldehyde, and a recent study that described TRPA1-dependent increase of glutamatergic excitatory synaptic currents in spinal substantia gelatinosa neurons in response to eugenol (Inoue et al., 2012) suggests that eugenol might also exerts its irritant effect via activation of TRPA1.

In the present study, to elucidate the TRPV1-independent molecular mechanisms underlying irritant action of eugenol, we investigated whether eugenol could activate trigeminal ganglion neurons in conditions in which activation of TRPV1 was suppressed and whether eugenol could induce calcium response and cationic inward current in a heterologous expression system that stably expresses TRPA1.

MATERIALS AND METHODS

All procedures for animal use were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the College of Dentistry, Seoul National University prior to the experiments.

Preparation of trigeminal ganglion neurons and HEK293 cells stably expressing TRPA1

Trigeminal ganglion neurons from adult Sprague-Dawley rats or TRPV1 knockout mice were prepared as previously described (Yeon et al., 2011). Briefly, trigeminal ganglia prepared in 4°C HBSS (Welgene, Daegu, Korea) were incubated in 2 mL HBSS containing 0.167% trypsin (Invitrogen, Carlsbad, CA) at 37°C for 40 min. The cells were washed in DMEM and triturated with a flame-polished Pasteur pipette to separate cells and remove processes, and subsequently centrifuged (750 RPM, 5 min), resuspended and placed on 0.5 mg/mL poly-L-ornithine (Sigma, St. Louis, MO)-coated glass coverslips (12 mm in diameter). The cells were maintained at 37°C in 95% O2/5% CO_2 incubator.

The HEK293 cell lines stably expressing human TRPA1 were used in some experiments. Cells were grown in DMEM (Invitrogen, Carlsbad, CA, USA) with 5% fetal bovine serum (Invitrogen) plus penicillin G (100 U/mL, Invitrogen), streptomycin sulfate (100 μg/mL, Invitrogen), and geneticin (500 μg/ml, Invitrogen). Cells were replated onto poly-L-ornithine-coated glass coverslips on the day of recording

and were used within 8 hrs.

Intracellular Ca^{2+} Imaging

Ca^{2+} imaging experiments based on fura-2AM (Molecular Probes, Eugene, OR, USA) were performed as previously described (Park et al., 2006a). Briefly, trigeminal ganglion neurons or TRPA1-expressing HEK293 cells were loaded with fura-2AM (2 μM) for 40 min at 37 °C in a Hank's balanced salt solution (HBSS). Cells were then rinsed and incubated in HBSS for additional 30 min, and the coverslips were mounted onto the chamber (Live Cell Instrument, Seoul, Korea). Intracellular calcium concentration ($[Ca^{2+}]_i$) was measured at room temperature by digital video microfluorometry with an intensified CCD camera (Cascade, Roper Scientific, Trenton, NJ, USA) coupled to an inverted microscope (IX70, Olympus, Tokyo, Japan), and a computer with imaging software (MetaMorph, Universal Imaging Corp., West Chester, PA, USA). Cells were illuminated with a 175W xenon arc lamp, and excitation wavelengths (340/380 nm) were selected by a Lambda DG-4 monochromatic wavelength changer (Sutter Instrument, Novato, CA, USA).

Electrophysiologic Recordings

The whole-cell configuration of the patch-clamp technique was performed with an Axopatch 200B amplifier (Axon Instruments, Union City, CA, USA). The resistances of pipette electrodes were 4-8 MN.

The pipette solution was composed of (mM): CsCl 135, $MgCl_2$ 5, Mg-ATP 5, HEPES 10, EGTA 5, Glucose 10, adjusted pH to 7.3 with NaOH. The bath was continuously perfused with extracellular solution containing (mM): NaCl 140, KCl 5, $MgCl_2$ 2.5, $CaCl_2$ 0.5, HEPES 10, glucose 10, adjusted to pH 7.3 with NaOH. Partial series resistance compensation was used, and currents were low-pass-filtered at 2 kHz and sampled at 10 kHz. pClamp8 (Axon Instruments) software was used during the experiments and analysis.

Drugs

All chemicals were purchased from Sigma (St. Louis, MO, USA). Eugenol, capsaicin, allyl isothiocyanate (AITC), HC-030031 were dissolved in dimethylsulfoxide (DMSO) to make a stock solution and were kept at -20°C until the day of experiment. The final concentration of DMSO was less than 0.1% (v/v), which did not affect membrane currents and $[Ca^{2+}]_i$. The drugs were diluted to their final concentration with the extracellular solution, and then applied by gravity through a bath perfusion system. The bath solution was continuously perfused at a rate of 2-3 ml/min.

RESULTS

Eugenol evoked calcium responses in trigeminal ganglion neurons that lack functional TRPV1

Previous studies suggested that eugenol is an active agonist of TRPV1 (Yang et al., 2003; Ohkubo and Kitamura, 1997; Ohkubo and Shibata, 1997). Accordingly, I also observed that eugenol (1 mM) elicited robust calcium transients in trigeminal ganglion neurons of rats (n=62, Fig 1). However, capsaicin responses were observed in only a fraction of the eugenol-responders (n=47/62, Fig 1A, C). When allylisothiocyanate (AITC), a TRPA1-specific agonist, was applied to investigate functional correlation of TRPA1 with eugenol, 28 neurons out of 62 eugenol-responding neurons showed responses to AITC, of which 19 neurons were capsaicin-sensitive (Fig 1A) and 9 neurons were capsaicin-insensitive (Fig 1B). Among 47 neurons that responded to both capsaicin and eugenol, 19 were AITC-sensitive (Fig 1A) and remaining 28 were AITC-insensitive (Fig 1C). Interestingly, a small subpopulation of eugenol responding neurons did not responded to either capsaicin or AITC (n=6/62, data not shown). Summary of responses of eugenol-responding neurons to capsaicin and AITC was illustrated in Fig. 1D and Table 1.

Capsazepine failed to inhibit eugenol response in a subset of neurons

Since capsazepine, a TRPV1-specific antagonist, failed to induce complete blockade of eugenol induced cationic inward currents in previous study (Ohkubo and Shibata, 1997), I investigated the effect of capsazepine in a subset of eugenol-responding neurons that expressed functional TRPA1 but not TRPV1. As the result, capsazepine (10 μM) failed to inhibit eugenol-induced response in neurons that showed response to AITC and capsaicin (n=41, Fig. 2A), or in neurons that showed response to AITC without response to capsaicin (n=21, Fig. 2B). In contrast, capsazepine completely abolished eugenol response in neurons that responded only to capsaicin but not to AITC (n=6, Fig 2C), which was in accordance with the previous report (Yang et al., 2003). Calcium responses induced by eugenol before, during and after application of capsazepine are normalized and summarized in Fig 2D.

Eugenol evoked calcium response in trigeminal ganglion neurons from TRPV1 knockout mice

I next investigated eugenol-evoked calcium response in trigeminal ganglion neurons from TRPV1 knockout mice. Similar to the results obtained from trigeminal ganglion neurons from rats, eugenol-induced calcium transients in the majority of neurons (n=55/69, 80%). 48 out of 55 eugenol responding neurons showed response to AITC (87%, Fig. 3Aa), whereas 7 remaining neurons responded to eugenol but not

to AITC (13%, data not shown). 14 out of 69 neurons that showed responses to high potassium solution did not responded to eugenol or AITC (20%, Fig 3Ab). Absence of functional TRPV1 was confirmed by unresponsiveness to application of capsaicin. Involvement of TRPA1 in eugenol evoked calcium response was further confirmed by blockade of eugenol-responses by TRPA1-specific antagonist, HC-030031 (100 μM) (n=10, Fig. 3B). Results obtained from TRPV1-delete mice are summarized in Fig. 3C.

Eugenol activated TRPA1 in heterologous-expression system

Application of eugenol to HEK293 cells that stably express human TRPA1 induced robust calcium transients in all of the cells tested (n=198, Fig. 4Aa), which was completely blocked by HC-030031 (n=74, Fig. 4Ab). Whole-cell patch clamp recording revealed that eugenol induced inward currents in TRPA1-expressing HEK293 (n=7, Fig. 4Ba), which were also abolished by HC-030031 (n=8, Fig. 4Bb).

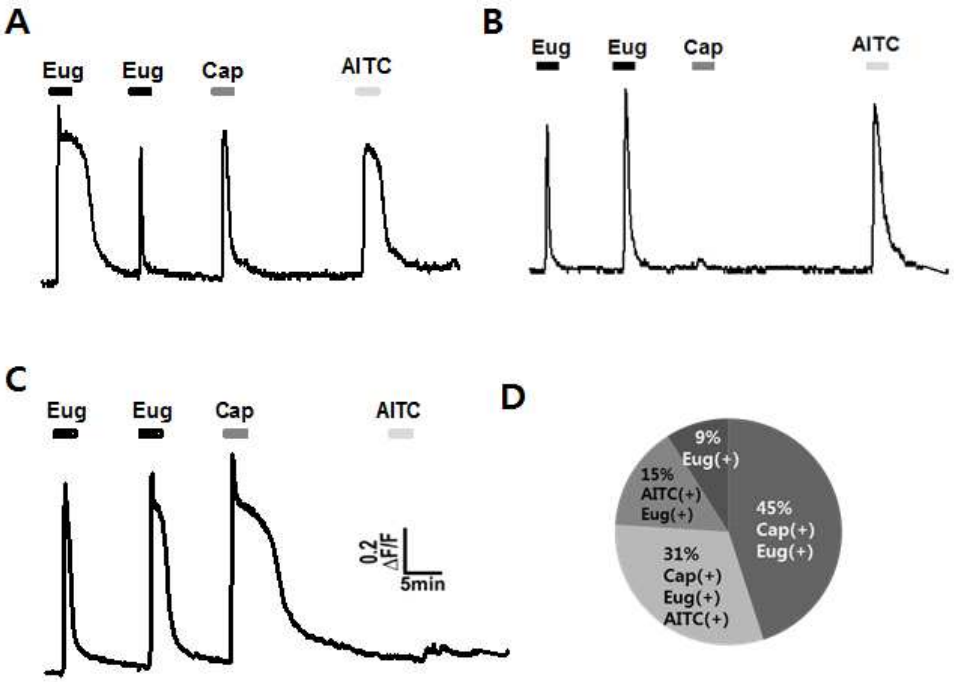

Figure 1 Figure 1. Effect of eugenol on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of rat TG neurons

(A) An example of eugenol-induced calcium response in capsaicin- and AITC-sensitive TG neurons(n=19/61). Drugs were applied at the points indicated by horizontal bars. (B) A representative trace of eugenol-induced calcium response in neurons that were sensitive to AITC but not to capsaicin(n=9/61). (C) An example of calcium response of eugenol in neurons that were sensitive to capsaicin but not to AITC(n=28/61). (D) Population summary of capsaicin- or AITC responders among eugenol-responding neurons. A small fraction of neurons responded only to eugenol. Eug: eugenol, Cap: capsaicin, CZP:capsazepine, AITC: allyl isothiocyanate

Table1. Characterization of eugenol-sensitive TG neurons

Neuron subtype	Eugenol response	Capsaicin response	AITC response	n
Type Ⅰ	+	+	-	28/62(45%)
Type Ⅱ	+	+	+	19/62(31%)
Type Ⅲ	+	-	+	9/62(15%)
Type Ⅳ	+	-	-	6/62(9%)
		47/62(76%)	28/62(46%)	

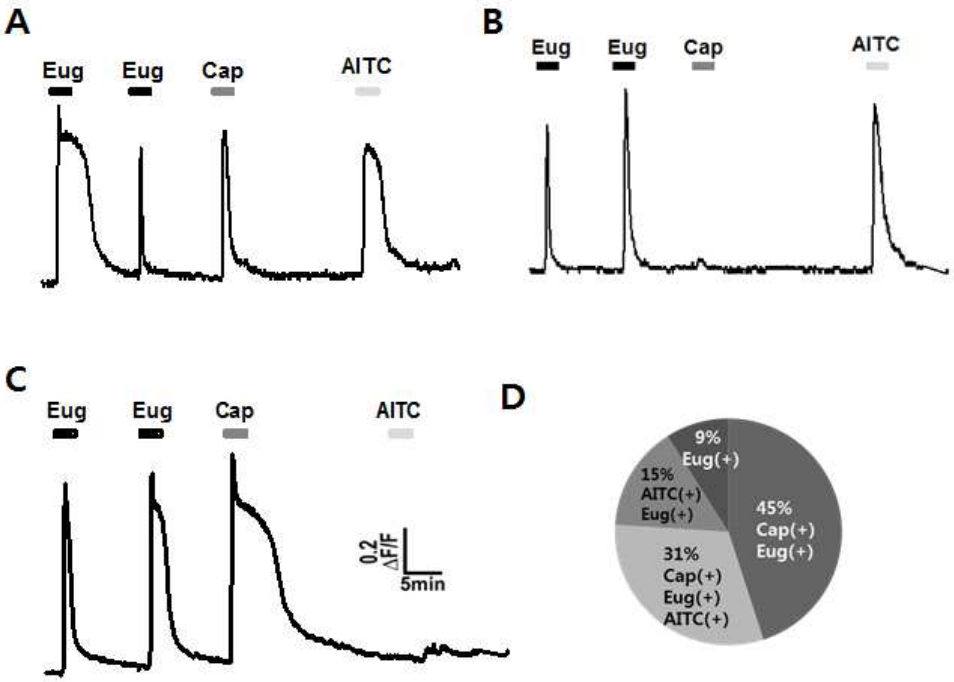

Figure 2 Figure2. Failure of inhibition of eugenol responses by capsazepine in subsets of neurons.

TRPV1-specific antagonist, capsazepine (10 μM) did not block eugenol-induced calcium response in neurons that responded to (A) both capsaicin and AITC (n=41/68), or (B) AITC alone (n=21/68) (C) Eugenol-induced calcium response was successfully inhibited by capsazepine in neurons that responded only to capsaicin but not to AITC (n=6/68). (D) Normalized calcium responses to eugenol before, during and after application of capsazepine. Capsazepine was effective in blocking eugenol-induced calcium responses in neurons that were sensitive to capsaicin, but not to AITC. CTR: Control, CZP: capsazepine, Wash: after washouts.

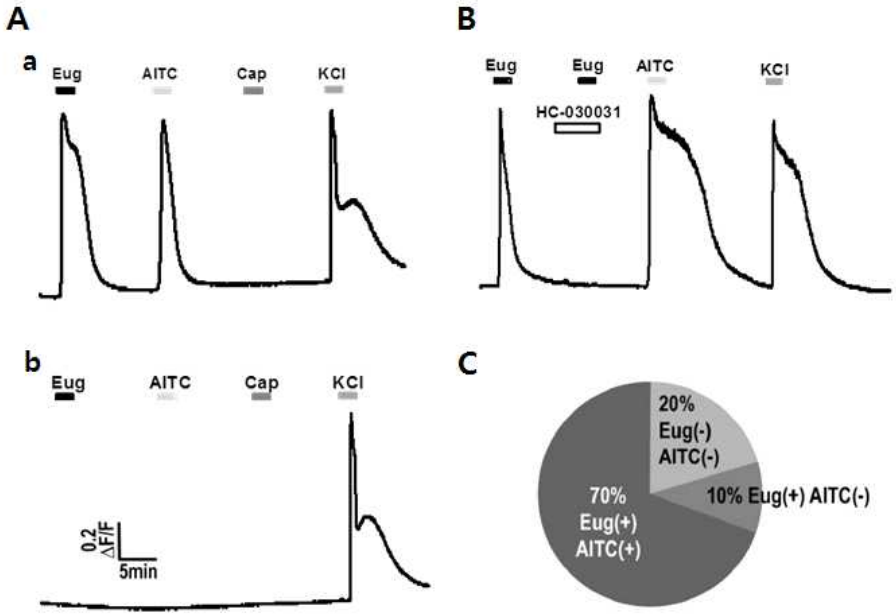

Figure 3. Effect of eugenol on intracellular Ca^{2+} concentration in TG neurons from TRPV1 knock-out mice.

(Aa) Eugenol increased $[Ca^{2+}]_i$ in TG neurons from TRPV1 knockout mouse (n=48/69). Absence of functional TRPV1 was confirmed by unresponsiveness to capsaicin. (Ab) Eugenol failed to elevate $[Ca^{2+}]_i$ in neurons that did not respond to AITC nor capsaicin (n=14/69). (B) TRPA1-specific antagonist, HC-030031 (100μM), successfully inhibited eugenol-induced calcium response of AITC-responding neurons (n=10). (C) Population summary of eugenol- or AITC-responders in TG neurons from TRPV1 knockout mice. TG: trigeminal ganglion

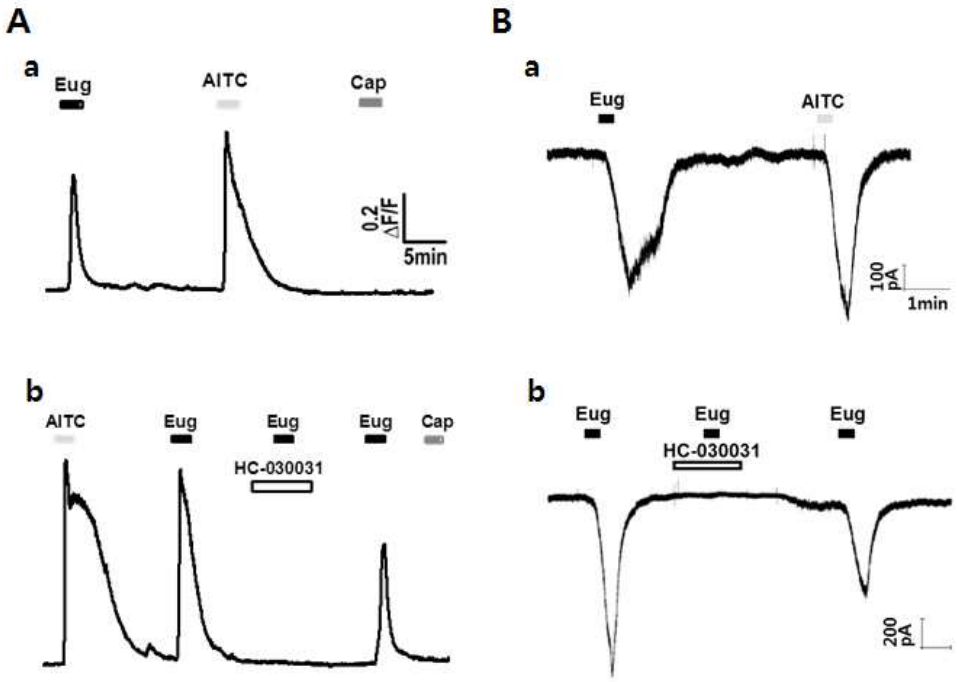

Figure 4. Eugenol-induced calcium response and current in TRPA1-expressing HEK293 cells.

(Aa) Eugenol increased $[Ca^{2+}]_i$ in TRPA1-expressing HEK293 cell (n=198). (Ab) Eugenol-induced calcium response was inhibited by TRPA1-specific antagonist, HC-030031 (n=74). (Ba) Eugenol elicited robust inward current in TRPA1-expressing HEK293 cells (n=7). AITC was applied as a positive control. (Bb) Eugenol-induced inward currents were inhibited by TRPA1-specific antagonist, HC-00030031 (n=8)

DISCUSSION

Irritable pharmacological action and structural similarities shared by eugenol and cinnamaldehyde raised the possibility that eugenol might act on the same receptor as cinnamaldehyde, TRPA1. This study investigated whether eugenol might activate TRPA1 in rodent trigeminal ganglion neurons and in TRPA1-expressing HEK293 cells. Since eugenol has been previously reported to activate TRPV1, neurons that did not show functional expression of TRPV1 were selectively used for this study. As the result, I found that: (1) eugenol-induced increase in $[Ca^{2+}]_i$ might involve TRPV1-independent pathway as well as TRPV1-dependent pathway, (2) the TRPV1-independent calcium response of eugenol might involve activation of TRPA1 as confirmed with TRPV1 knockout mice and and a heterologous expression system; TRPA1-expressing stable cell line, (3) there is a possibility that additional unknown receptors might play a role in neuronal activation of eugenol.

TRPA1 is a non-selective cation ion channel expressed mostly in a subset of TRPV1-expressing polymodal nociceptors (Story et al., 2003). In this study, I observed that eugenol elicited calcium responses in capsaicin-insensitive neurons which were not blocked by TRPV1-specific inhibitor, capsazepine. I found evidences that support functional expression of TRPA1 in a small population of neurons that responded to eugenol but not to capsaicin. Experiments with neurons from TRPV1 knockout mice verified that eugenol induced calcium responses in neurons that expressed functional TRPA1, which was

inhibited by a TRPA1-specific blocker. Finally, functional correlation of TRPA1 and eugenol was clearly demonstrated by cationic inward current and calcium responses in HEK293 cells that stably express TRPA1.

Chemical activation of TRPA1 elicits noxious burning sensation in human (Bandell et al., 2004). However, activation of TRPA1 in central terminals of dorsal root ganglion neurons was recently shown to depress evoked synaptic excitations from a subgroup of C fibers, thereby contributing to the antinociceptive behavior (Uta et al., 2010), which was in line with observation made with activation of TRPV1 (Jeffry et al., 2009; Dickenson et al., 1990). More recently, it was reported that prolonged activation of nociceptive receptor, TRPA1, led to delayed anti-nociception after initial irritation in vivo, and that activation of TRPA1 resulted in initial depolarization and an extensive and sustained inhibition of voltage-gated sodium and calcium channels in vitro (Andersson et al., 2011). The biphasic action consisting of initial pungent and delayed analgesic action was well documented in activation of TRPV1 by capsaicin (Docherty et al., 1991; Wu et al., 2005; Liu et al., 2001; Onizuka et al., 2011; Su et al., 1999).

It was reported that Various mechanisms for analgesic action of eugenol, such as inhibition of voltage-gated sodium and calcium channels, HCN channels, purinergic receptors (Lee et al., 2005; Park et al., 2006b; Li et al., 2008; Yeon et al., 2011; Park et al., 2009; Chung et al., 2008). Modulation of opioid and glutamatergic system has been recently proposed as another mechanism underlying analgesic effect of eugenol (Dal Bo et al., 2012). In addition, it was

proposed that analgesic action of eugenol might involve activation of TRPV1 (Ohkubo and Kitamura, 1997; Yang et al., 2003), by inducing sustained analgesia after initial irritation of eugenol, in a similar manner to capsaicin (Ohkubo and Shibata, 1997). The observations in the present study suggest that activation of TRPA1 might be another mechanism by which eugenol exerts its biphasic action consisting of sustained analgesia after initial excitation. Since the biphasic action of TRPV1 provided molecular basis for using capsaicin as analgesic agent, it would be interesting to test whether eugenol might produce the similar delayed analgesic effect by activation of TRPA1.

Dental pain can be elicited by direct or indirect transduction of noxious cold temperature. Direct detection of cold temperature involves cellular cold transducer. TRPA1 was shown to play a central role in detection of noxious cold in dental primary afferent neurons (Park et al., 2006a). Indirect transduction of noxious cold involves detection of fluid movement within dentinal tubules (Sessle, 1987). Although controversial, TRPA1 is a plausible candidate of a cellular detector of mechanical force (Kwan et al., 2009). Therefore, pharmacological blockade of TRPA1 might affect both direct and indirect pathway of dental pain transduction. A recent investigation demonstrating the increased expression of TRPA1 in trigeminal ganglion neurons after injury to teeth further support that TRPA1 might play a crucial role in generation of dental pain (Haas et al., 2011). These results suggest that TRPA1 would be more plausible therapeutic target for alleviation of dental pain than TRPV1.

It should be noted that there were a small subset of neurons that were responsive to eugenol but not to AITC or capsaicin. The

calcium responses elicited by eugenol in these neurons might be mediated by receptor other than TRPA1 or TRPV1. The molecular identity of the additional eugenol receptor expressed in trigeminal ganglion neurons needs to be addressed in the future study. In addition, only 68% of AITC-sensitive trigeminal ganglion neurons were shown to be sensitive to capsaicin in this study, which was lower than previously reported result from in situ hybridization experiment on dorsal root ganglion (97%) (Story et al., 2003). The reason for the discrepancy could be due to the difference between trigeminal ganglion and dorsal root ganglion or due to the difference in the experimental methods, that I did functional analysis with primary cultured neurons whereas Story et al. did anatomical analysis on the whole ganglion tissue. On the other hand, 40% of capsaicin-sensitive neurons were sensitive to AITC, which was similar to 30% from the in situ results from dorsal root ganglion.

In summary, these results demonstrate that eugenol activates TRPA1 expressed in trigeminal ganglion neurons, which could account for the irritating effect of eugenol. However, since TRPA1 produces biphasic action consisting of sustained analgesia after initial excitement, activation of TRPA1 by eugenol might provide another molecular mechanism underlying the analgesic action of eugenol. Since TRPA1 might play crucial roles in detection of noxious cold temperature or movement of dentinal fluids, elucidation of prolonged analgesia provided by activation of TRPA1 by eugenol might provide additional molecular mechanism explaining the usage of eugenol in treatment of dental pain.

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국 문 초 록

유지놀에 의한 Transient Receptor Potential Ankyrin 1(TRPA1)의 활성화

유지놀은 치과에서 마취성분으로 사용되는 식물추출물로써 역설적인 기전을 나타냅니다. 이 특이한 기전을 설명하기 위해, 유지놀이 캡사이신, 열 그리고 산성에 의해 활성화 되는 transient receptor potential vanilloid 1 (TRPV1)을 활성화 시킴을 연구하였다. 그러나 유지놀이 TRPV1이 녹아웃된 생쥐에서 배양한 삼차 신경절 세포에서 내부전류를 일으킨다는 사실을 관찰하였다. 이에 그 새로운 기전을 확인하기 위해 transient receptor potential ankyrin 1 (TRPA1)의 리간드로 작용하는 신남알데히드의 구조와 유사한 유지놀이기에 TRPA1의 활성을 유발 할 것이다 라는 가설을 제시하고 연구하였다.

이 연구는 TRPA1에 대한 유지놀의 영향을 연구했으며, 삼차 신경절 세포와 이형 발현 시스템에서 fura-2 기본으로한 칼슘이미징과 홀셀 패치클램프 기록을 통해 연구되었다.

결과적으로, 유지놀은 TRPA1 작용제인 AITC에만 특이적으로 반응하는쥐의 삼차 신경절 세포에서 강력한 칼슘 반응을 유도했으며 반면, 캡사이신은 칼슘 반응을 유도하지 못하였다. 또한 TRPV1 길항제인 capsazepine은 AITC에 반응한 신경세포에 대한 칼슘반응을 억제 하지 못하였다. 추가적으로 유지놀은 TRPV1 녹아웃 생쥐로 배양한 삼차 신경절 세포에서 칼슘반응이 관찰됐으며, TRPA1 길항제인 HC-030031에

의해서는 칼슘반응이 억제 되었다. 마지막으로, 유지놀의 활성을 TRPA1이 발현되어있는 인간배아신장293세포에서 칼슘이미징과 홀셀 패치클램프로 최종 점검하였다. 요약하면, 이 결과들은 유지놀의 새로운 약리기전 작용으로 TRPV1 뿐 아니라 TRPA1 또한 활성화시킴을 증명하고 있다.

주요어 : 유지놀, TRPA1, 삼차신경절, AITC, 통증, 마취.

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