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Piperine, a component of black pepper, decreases eugenol-induced cAMP and calcium levels in non-chemosensory 3T3-L1 cells



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ABSTRACT

This study investigated the effects of an ethanol extract of black pepper and its constituent, piperine, on odorant-induced signal transduction in non-chemosensory cells. An ethanol extract of black pepper decreased eugenol-induced cAMP and calcium levels in preadipocyte 3T3-L1 cells with no toxicity. Phosphorylation of CREB (cAMP response element-binding protein) was down-regulated by the black pepper extract. The concentration (133.8 mg/g) and retention time (5.5 min) of piperine in the ethanol extract were quantified using UPLC–MS/MS. Pretreatment with piperine decreased eugenol-induced cAMP and calcium levels in 3T3-L1 cells. Piperine also decreased the phosphorylation of CREB, which is up-regulated by eugenol. These results suggest that piperine inhibits the eugenol-induced signal transduction pathway through modulation of cAMP and calcium levels and phosphorylation of CREB in non-chemosensory cells.

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1. Introduction

Black pepper (*Piper nigrum*, PN), of the *Piperaceae* family, is one of the most widely used condiments in the world. In addition to its use as a spice, black pepper has been used in conventional medicines to treat pain, flu, muscle aches, and rheumatism as well as to stimulate appetite [1]. Black pepper contains various active phytochemicals such as alkaloids, terpenes, flavones, and steroids, and many studies have reported their physiological effects in human and animals [1,2]. Although the exact amount of piperine, a main alkaloid in black pepper, varies owing to different methods of extraction and analysis, piperine comprises approximately 2–30% of dried black pepper [3,4]. After piperine (Fig. 2B) was first separated and purified by Oersted in 1820 [5], numerous studies have demonstrated its potential health benefits [1,2]. Piperine is an anti-inflammatory molecule inhibiting the production of prostaglandin E2 and nitric oxide in RAW264.7 cells [6]. It also suppresses stress-induced behavior by increasing serotonin and

Abbreviations: CREB, cAMP response element-binding protein; OST, odorant-induced signal transduction; PNF, *Piper nigrum* fructus

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brain-derived neurotropic factors [7]. In high-fat diet-induced obese mice, piperine was shown to activate AMP-activated protein kinase and PPAR δ and attenuate HFD-induced obesity [8]. Inhibition of ERK1/2 signaling by piperine reduced SREBP-1 and FAS expression in breast cancer cells, suggesting that it could be used as an antitumor agent to prevent or treat human breast cancer [9]. Recently, this possibility was further supported by data showing that piperine induced apoptosis of melanoma cells by decreasing XIAP, Bid, and caspase-3 [10]. However, the physiological roles of black pepper and piperine have not yet been elucidated in the odorant-induced signal transduction (OST) pathway in non-chemosensory cells.

Olfaction is the sense of smell. The perception of odor is important for survival and is required to select food and mates as well as to respond to the fear of predators. However, recent reports demonstrate that OST plays not only a role in olfaction but also other physiological roles in non-chemosensory tissues [11]. Ectopic expressions of olfactory receptors in sperm, kidney, and muscle were involved in the chemotaxis of sperm, glomerular filtration rate, and muscle regeneration, respectively [12–14]. The ectopic expression was also supported by recent reports showing that olfactory receptors were expressed in fat tissue of diet-induced obese mice and eugenol receptor (mOR-EG, Olfr73, MOR174-9) was expressed in 3T3-L1 cells [15,16]. However, their physiological roles and regulations in fat tissue and non-chemosensory cells are

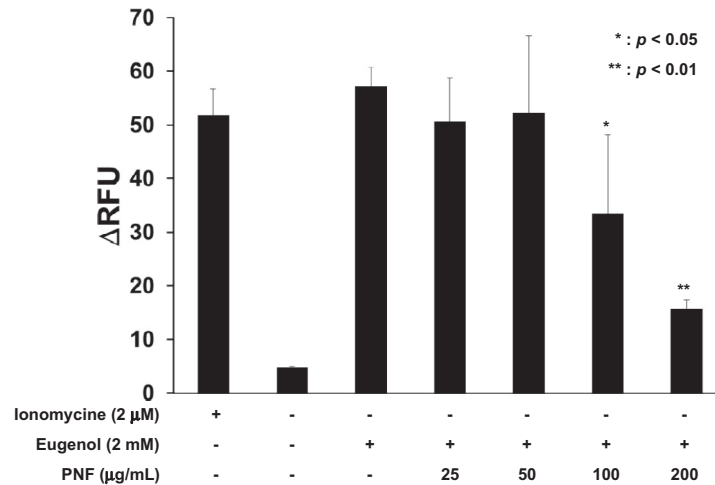


Fig. 1A. Changes in Ca^{2+} level induced by eugenol in 3T3-L1 cells after pretreatment with an ethanol extract of black pepper for 30 min. Ionomycin (2 μM) was used as a positive control and DMSO (1%) was used as a negative control. The data are shown as means \pm SD ($n = 3$ for 25 and 200 $\mu\text{g/mL}$, $n = 5$ for 50 and 100 $\mu\text{g/mL}$). ** $p < 0.01$. ΔRFU , change in relative fluorescence unit.

largely unknown. Interestingly, the OST pathway in these non-chemosensory tissues shares the same mechanism, where odorants stimulate signals by binding to olfactory receptors, and cAMP and Ca^{2+} act as second messengers to relay the signal cascade in order to achieve olfactory perception and other physiological effects in neuronal and non-chemosensory cells, respectively [12–14].

In this study, we investigated the effects of black pepper and its constituent, piperine, on the OST pathway in non-chemosensory 3T3-L1 cells. When an odorant was used to stimulate non-chemosensory cells, we observed inhibitory effects of the ethanol extract of black pepper and piperine through regulation of Ca^{2+} and cAMP levels.

2. Materials and methods

2.1. Plant material

Ethanol extract of *P. nigrum* fructus (PNF) was obtained from the Korea plant extract bank at the Korea Research Institute of Bioscience and Biotechnology (KRIBB, Daejeon, Republic of Korea). The powder was dried at room temperature and then dissolved in 95% ethanol (v/v).

2.2. Reagents and antibodies

Piperine and eugenol were purchased from Sigma (St. Louis, MO, USA). The Ca^{2+} assay kit was obtained from Molecular Devices (Sunnyvale, CA, USA). The cAMP assay kit was purchased from Enzo Life Sciences (Plymouth Meeting, PA, USA). Antibodies against phospho-CREB and lamin B1 were purchased from Cell Signaling Technology (Beverly, MA, USA).

2.3. Cell culture and viability assay

3T3-L1 cells were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM (high glucose), which contained 10% FBS and $1\times$ antibiotic-antimycotic solution (WelGENE Inc., Daegu, Republic of Korea). The cells were incubated at 37 $^{\circ}\text{C}$ in the presence of 5% CO_2 . The viability of cells was determined using the Cell Proliferation Reagent WST-1 (Roche Diagnostics, Mannheim, Germany). Cells at a concentration of 4×10^4 cells/well in 500 μl culture medium were seeded in 24-well plate. Incubated cells for 6 h at 37 $^{\circ}\text{C}$ and 5% CO_2 and washed it with serum-free medium, then added 500 μl serum-free medium containing the extract or piperine. After 24 h, added 20 μl /well Cell Proliferation Reagent WST-1 and incubate the cells for 1 h. Shake

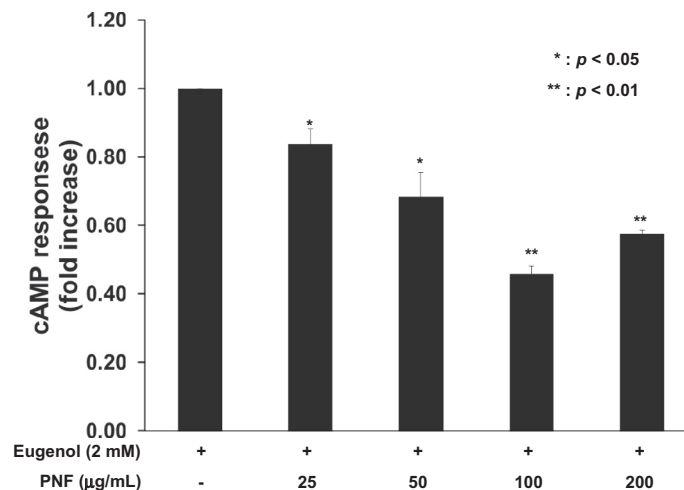


Fig. 1B. Changes in cAMP level induced by eugenol in 3T3L1 cells after pretreatment with an ethanol extract of black pepper for 30 min. The final concentration of DMSO in all samples was 1%. The data are shown as the mean \pm SD ($n = 3$). ΔRFU , change in relative fluorescence unit.

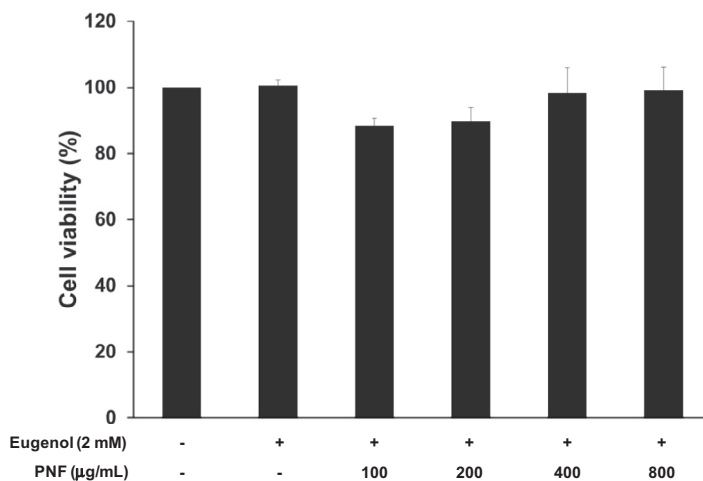


Fig. 1C. Cell viability. 3T3-L1 cells were treated with a series of PNF concentrations for 30 min, cell proliferation was evaluated by WST-1 assay. The data shown as means \pm SD ($n = 3$).

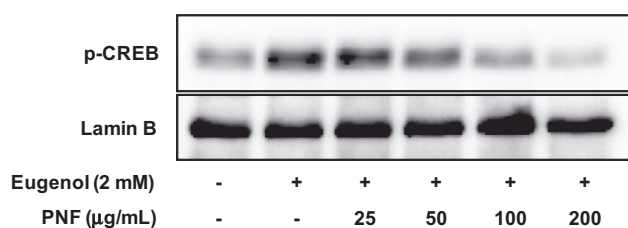


Fig. 1D. Western blot analysis of total and phosphorylated CREB after pretreatment with an ethanol extract of black pepper for 30 min. A total of 40 μ g of protein was separated by SDS-PAGE. The final concentration of DMSO in all samples was 1%.

thoroughly for 1 min on a shaker and measure the absorbance of the samples against a background control as blank using the microplate reader at 440 nm and 690 nm.

2.4. UPLC-MS/MS analysis

The analyses were performed using an Acquity UPLC system (Waters, Milford, MA, USA) with an Acquity UPLC BEH C18 column (2.1 mm \times 100 mm, 1.7 μ m). The eluent was a gradient of 0.1% formic acid aqueous solution (Solvent A) and 0.1% formic acid in methanol (Solvent B) at a flow rate of 0.4 mL/min. The cells were initially treated with 98% A, followed by 98–25% A for 0–5 min, 25% A for 5–6 min, and finally 98% A for 6–7 min, which was held

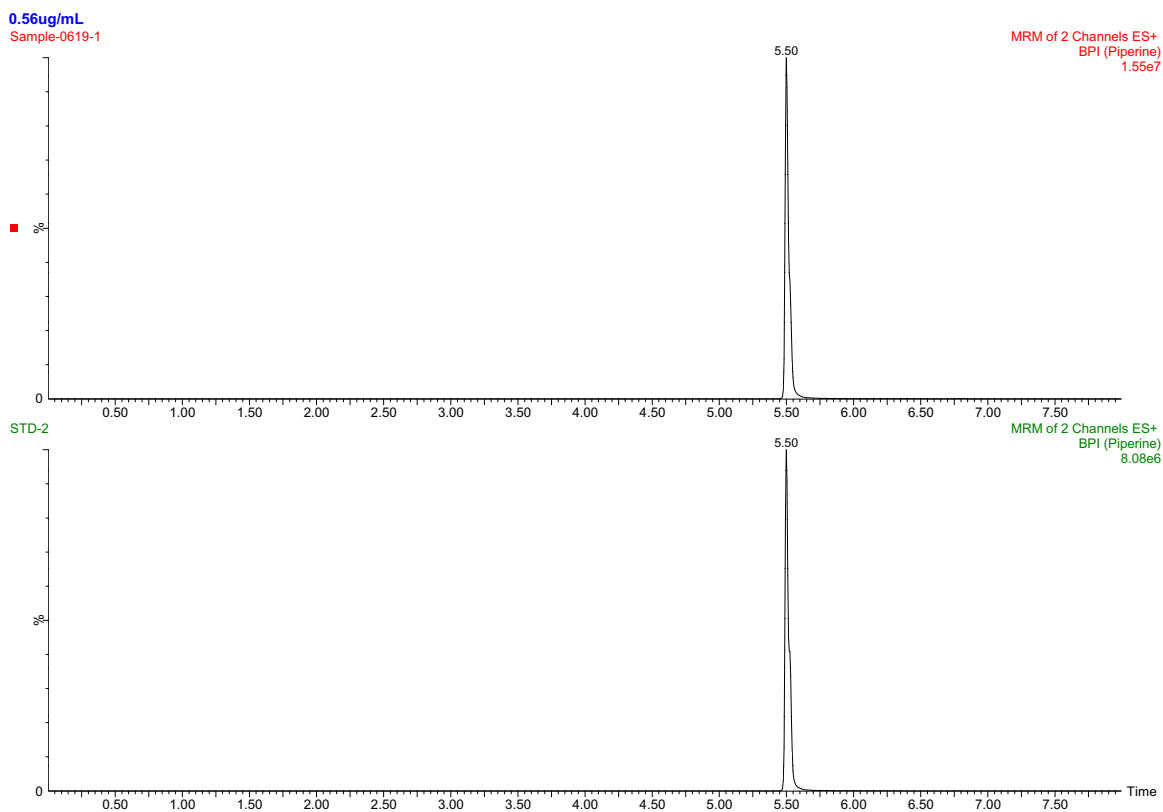
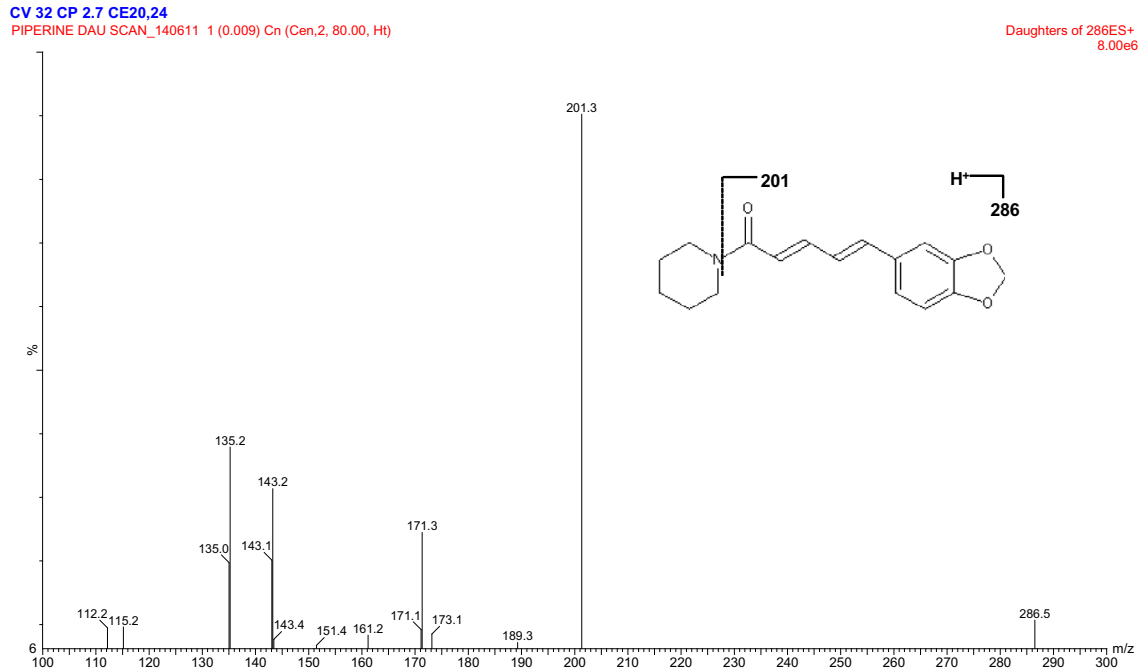


Fig. 2A. Representative UPLC-MS/MS chromatograms of piperine.



for 1 min. The injection volume was 2 μ l, the column temperature was kept at 35 $^{\circ}$ C and the total run time was 8 min. The identification and quantification of piperine for *P. nigrum* fructus was carried out using a Waters Xevo TQ triple-quadrupole mass spectrometer equipped with an electrospray ionization mode. The tandem MS was operated in positive ESI mode, and the data was processed using MassLynx 4.1 (Waters) software and the multiple re-action monitoring mode. The detector was operated at a cone voltage of 32 V and a capillary voltage of 2.7 kV. The source temperature was set at 150 $^{\circ}$ C, while the desolvation flow was set at 800 L/h; the desolvation gas temperature was set at 400 $^{\circ}$ C.

2.5. Ca^{2+} assay

3T3-L1 cells were seeded in 96-well black plates and pretreated with PNF and piperine in a CO_2 incubator for 30 min before adding 100 μ l of component A buffer (Molecular Devices, Sunnyvale, CA, USA). The plate was covered with foil and incubated for 30 min

at room temperature, followed by 15 min at 37 $^{\circ}$ C. Eugenol and ionomycin were added in Flexstation 3 (Molecular Devices). The Ca^{2+} level was measured according to the manufacturer's instructions.

2.6. cAMP assay

Before treatment with PNF and piperine, the 3T3 L1 cells were starved with serum-free DMEM (high glucose) media for 16–18 h. After 30 min of pretreatment with PNF and piperine, the cells were treated with eugenol for 7 min and then lysed with 0.1 M HCl. cAMP levels were measured using the Direct cAMP EIA Kit (Enzo Life Sciences).

2.7. Western blot analysis

Cells were seeded in 6-well plates and after 1 day, they were starved with serum-free DMEM for 16–18 h. The starved cells were

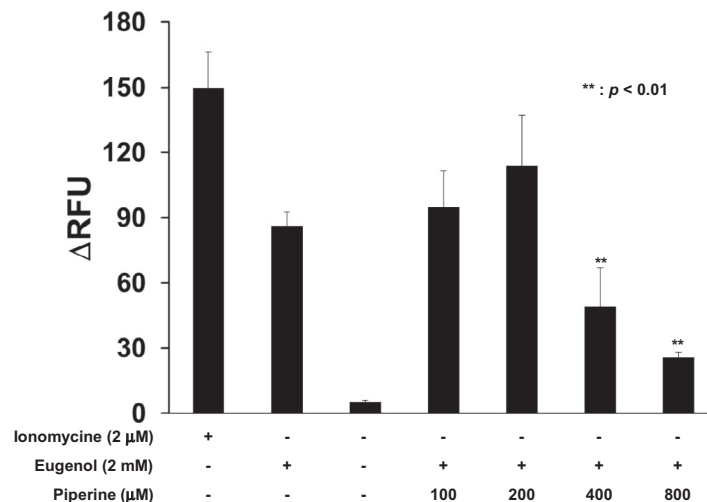


Fig. 3A. Changes in Ca^{2+} levels induced by eugenol in 3T3-L1 cells after pretreatment with piperine for 30 min. Ionomycin (2 μ M) was used as a positive control and DMSO (1%) was used as a negative control. The data are shown as means \pm SD ($n = 3$). ** $p < 0.01$. Δ RFU, change in relative fluorescence unit.

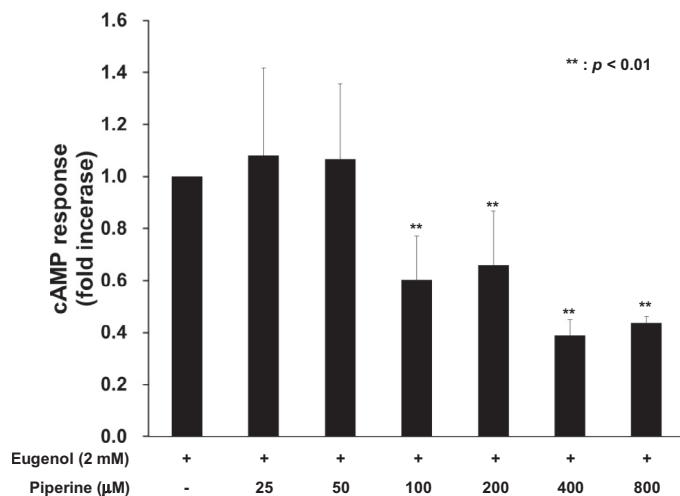


Fig. 3B. Changes in cAMP levels induced by eugenol in 3T3-L1 cells after pretreatment with piperine for 30 min. The final concentration of DMSO in all samples was 1%. The data are shown as the mean \pm SD ($n = 6$ for 100 and 200 μM , $n = 3$ for 25, 50, 400, and 800 μM). ΔRFU , change in relative fluorescence unit.

pretreated with PNF and piperine for 30 min and treated with eugenol for 7 min. The cells were lysed with RIPA buffer (Bioseang, Seongnam-si, Republic of Korea), which contained a protease inhibitor cocktail (Roche, Basel, Switzerland) and a phosphatase inhibitor cocktail (Roche). The lysate was collected using cell scrapers and centrifuged at 12,000 rpm for 30 min at 4 °C. To measure protein concentration, the SMART BCA Protein Assay Kit (iNTRON Biotechnology, Seongnam-si, Republic of Korea) was used. A total of 40 μg of the protein was electrophoresed by SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were incubated overnight with the suitable primary antibody at 4 °C. The membranes were probed with HRP-conjugated secondary antibody at room temperature for 1 h, and then they were incubated with enhanced chemiluminescence reagents (Amersham, Piscataway, NJ, USA).

2.8. Statistical analysis

All experiments were repeated at least three times, and the data are expressed as the mean \pm standard deviation (SD). Group means

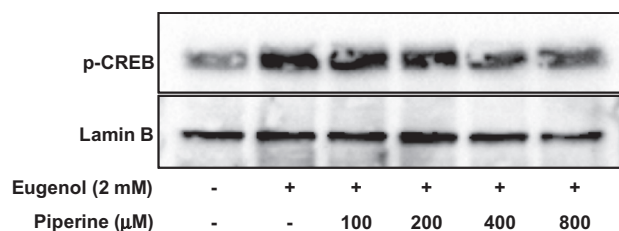


Fig. 3D. Western blot analysis of total and phosphorylated CREB after pretreatment with piperine for 30 min. A total of 45 μg of protein was separated using SDS-PAGE. The final concentration of DMSO in all samples was 1%.

were compared using the non-parametric Kruskal–Wallis and Mann–Whitney analysis using SPSS (SPSS Inc., Armonk, NY).

3. Results and discussion

3.1. PNF decreased Ca^{2+} , cAMP levels in 3T3-L1 cells

In our previous findings, Ca^{2+} and cAMP levels were shown to increase with eugenol treatment in 3T3-L1 cells [16]. Our current investigation explores the effects of PNF on the eugenol-induced signal transduction pathway in 3T3-L1 cells. As shown in Fig. 1A, lower concentrations of PNF (25–50 $\mu\text{g}/\text{mL}$) had little effect on Ca^{2+} levels. However, Ca^{2+} levels were significantly decreased up to 40% with 100 $\mu\text{g}/\text{mL}$ of PNF and more than 70% with 200 $\mu\text{g}/\text{mL}$ of PNF. Since cAMP is an important molecule that affects Ca^{2+} levels in odorant signaling transduction (OST), we determined cAMP levels after treatment with PNF. cAMP levels were gradually reduced from an initial concentration of 25 $\mu\text{g}/\text{mL}$ to nearly 50% of that after treatment with 100 $\mu\text{g}/\text{mL}$ of PNF (Fig. 1B). We observed a difference in eugenol-responsiveness as determined with cAMP and Ca^{2+} assay, which may due to cell type and receptor-coupled G proteins as previously described by Touhara [17]. Responsiveness of Ca^{2+} and cAMP have different patterns with the stimulation of the same odorants in HKE293 cells [18] and HeLa/Olf cells [19]. These suggest that change of Ca^{2+} does not fully reflect change of cAMP. Instead, changes of Ca^{2+} and cAMP mutually represent activation or inhibition of OST. Our data suggest that PNF inhibits the OST pathway through regulation of Ca^{2+} and cAMP levels induced by eugenol in 3T3-L1 cells. To remove the possibility of toxic effect of the extract on the change

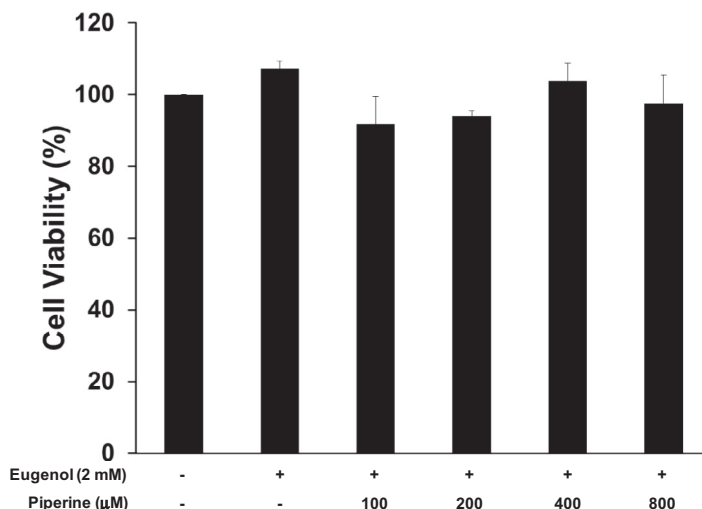


Fig. 3C. Cell viability. 3T3-L1 cells were treated with a series of piperine concentrations for 30 min, cell proliferation was evaluated by WST-1 assay. The data shown as means \pm SD ($n = 3$).

of Ca^{2+} and cAMP, the cell viability was measured by WST-1 assay. As shown in Fig. 1C, up to 800 $\mu\text{g}/\text{mL}$ treatment with PNF for 30 min did not inhibit 3T3-L1 proliferation. It suggests that the change of cAMP and Ca^{2+} is not caused by toxicity.

3.2. The phosphorylation of CREB was decreased by PNF in 3T3-L1 cells

To test whether PNF also decreases signal molecules as a result of decreasing Ca^{2+} and cAMP levels in 3T3-L1 cells, we investigated the phosphorylation of CREB, known to be regulated by cAMP levels in the OST signaling pathway [20]. Phosphorylation of CREB by eugenol was decreased in a dose-dependent manner and remarkably decreased after 30 min of pre-treatment with 200 $\mu\text{g}/\text{mL}$ of PNF (Fig. 1D). This result suggests that cAMP levels decreased by PNF affect the phosphorylation of CREB.

3.3. Determination of piperine in the ethanol extract of black pepper using UPLC-MS/MS

Since piperine is a major active alkaloid in black pepper [21], UPLC-MS/MS was used to evaluate its concentration in an extract of black pepper. Under the chromatographic conditions described above, the retention time of piperine was 5.5 min (Fig. 2A) and its molecular mass was 285 Da. To further characterize the mass peak, the peak was analyzed using a mass spectrometer. The parent fragmentation ions were m/z 286.5 $[\text{M}+\text{H}]^+$ for piperine. The parent-to-daughter ion transitions were monitored at m/z 286.5 \rightarrow 201.3, 135.2 (Fig. 2B). The piperine content was 133.8 ± 17.6 mg/g (in the ethanol extract).

3.4. Reduction of odorant-induced Ca^{2+} and cAMP levels by piperine in 3T3-L1 cells

Because PNF inhibited the OST pathway and piperine is one of its components in the ethanol extract, we examined the effects of piperine on the OST pathway in 3T3-L1 cells. First, it was found that increased Ca^{2+} levels in response to eugenol began to decrease with pretreatment of 400 μM piperine for 30 min and was further decreased by 75% with 800 μM (Fig. 3A). In addition, cAMP levels also decreased up to 50% with 30 min of pretreatment with piperine (100, 200, 400, and 800 μM) (Fig. 3B). These results showed that piperine inhibits the OST pathway by regulating Ca^{2+} and cAMP levels in 3T3-L1 cells as PNF did. Again, we observed the difference in eugenol-responsiveness by piperine in cAMP and Ca^{2+} assay as previously described in Fig. 1. However, decreases of both cAMP and Ca^{2+} level by piperine represent the inhibition of eugenol-induced signal transduction in 3T3-L1 cells. In addition, it strongly suggests that in the ethanol extract of black pepper, piperine is at least one of the active components to regulate eugenol-induced signal transduction. To whether the change of cAMP and Ca^{2+} was due to toxicity of piperine, the cell viability was determined. As shown in Fig. 3C, piperine (100–800 μM) did not inhibit cell proliferation. It suggests that the change of cAMP and Ca^{2+} is not caused by toxic effect of piperine on 3T3-L1 cells.

3.5. Piperine suppressed phosphorylation of CREB in 3T3-L1 cells

Next, we investigated whether the upregulated phosphorylation of CREB by eugenol was decreased by piperine as it was with PNF. As shown in Fig. 3D, piperine significantly decreased the eugenol-induced CREB phosphorylation in 3T3-L1 cells. This is consistent with the previously observed effects of piperine and PNF on the OST pathway. It further strengthens the previous conclusion that piperine is one of the active molecules inhibiting eugenol-induced signal transduction in the ethanol extract of black pepper.

In summary, our study showed that pretreatment with PNF and piperine modulated Ca^{2+} influx, cAMP levels, and phosphorylation of CREB in response to eugenol in non-chemosensory 3T3-L1 cells. These findings also suggest that mOR-EG is at least one of mediators responding to eugenol in 3T3-L1 cells. However, further study will be required to investigate the physiological roles of eugenol receptor in 3T3-L1 cells.

Acknowledgments

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Y.C.Y., S.H.K., M.J.K., and H.J.Y. performed the experiments and analyzed the data. M.R.R. and J.H.P. contributed conceptual insights and designed the studies. Y.C.Y., S.H.K. and J.H.P. wrote the manuscript.

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