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Research Communication

Inhibitory effect of luteolin on the odorant-induced cAMP level in HEK293 cells expressing the olfactory receptor

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Abstract.

Luteolin is a flavonoid in many fruits and vegetables. Although luteolin has important biological functions, including antioxidant, anti-inflammatory, antimicrobial, and neuroprotective activities, little is known about the functions of luteolin in the olfactory system. Various odorants can be detected and distinguished by using several molecular processes, including the binding of odorants to odorant receptors, activation of adenylyl cyclase (AC), changes of cyclic adenosine monophosphate (cAMP) and Ca^{2+} levels in olfactory sensory neurons, as well as changes in membrane potentials and the transmission of electric signals to the brain. Because AC-cAMP signal transduction plays a pivotal role in the olfactory system, we evaluated the effects of luteolin on the AC-cAMP pathway that had been stimulated by the odorant eugenol. We demonstrated that eugenol caused an upregulation of the cAMP level and the phosphorylation of phosphokinase A (PKA, a downstream target of cAMP) in human embryonic kidney 293 (HEK293) cells expressing the murine eugenol receptor. This upregulation significantly decreased in the presence of luteolin, suggesting that luteolin inhibited the odorant-induced production of cAMP and affected the downstream phosphorylation of PKA.

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1. Introduction

Luteolin, 3,4,5,7-tetrahydroxyflavone (Fig. 1), is a natural poly phenolic compound widely distributed in fruits and vegetables. Luteolin has recently received considerable attention because of its biological activity and potential application for the prevention of cancer [1], cardiovascular disease [2], and diabetes [3], as well as for its role in strengthening the immune system [4–6]. In addition, *in vivo* and *in vitro* studies have shown that luteolin is an important component of various cell-signaling pathways [7]. Although luteolin is involved in the regulation of cyclic adenosine monophosphate (cAMP) level and in downstream signal transduction pathways that are stimulated by different molecules in different cell types [8–10], the functions of luteolin in the odorant-induced signal transduction pathway have not been reported.

Olfactory systems are of great importance for the survival of mammals, and olfactory pathways are involved in mate selection, food uptake, and the stimulation of the fear response to predators [11–13]. Odorant receptors (ORs), such as rhodopsin-like guanine nucleotide-binding protein-coupled receptors, can distinguish between thousands of odorants [14]. Odorant perception is initiated by the binding of odorants to ORs in olfactory sensory neurons (OSNs). The activated olfactory-specific G protein (G_{olf}) by odorant-bound ORs stimulates a type III adenylyl cyclase (AC), resulting in an increased cAMP level in OSNs. This increased cAMP level leads to the accumulation of Ca^{2+} in the cytosol and causes a change in the membrane potential, which converts chemical information into electrical signals that are then transmitted to the brain for odor perception [15–17].

This study was designed to investigate the effects of luteolin in the AC-cAMP pathway that had been induced by the odorant eugenol. The expression of the murine eugenol

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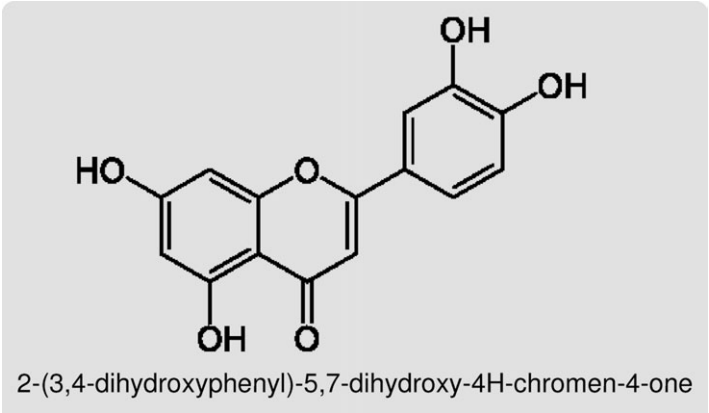


Fig. 1. Chemical structure of luteolin.

receptor (mOR-EG) in human embryonic kidney 293 (HEK293) cells represents an excellent model system for investigating the molecular mechanisms in the mammalian olfactory system [17]. We therefore evaluated the effects of luteolin on the cAMP level and the phosphorylation of phosphokinase A (PKA) in eugenol-stimulated HEK293 cells expressing mOR-EG.

We found that luteolin pretreatment decreased the eugenol-induced upregulation of cAMP level and PKA phosphorylation. Because cAMP plays a pivotal role in OSNs, the understanding of its signaling role and its modulation by exogenous molecules such as luteolin provides a foundation for developing a method to erase unpleasant odor sensations while eating and to prevent or treat hyperosmia.

2. Materials and methods

2.1. Reagents

Forskolin was purchased from Calbiochem (San Diego, CA) and was dissolved in dimethylsulfoxide (DMSO). Eugenol and all other chemicals were purchased from Sigma (St. Louis). Antibodies directed against protein kinase B (Akt) and phospho-Akt were purchased from Cell Signaling Technology (Beverly, Danvers, MA). The cAMP assay kit was obtained from Enzo Life Sciences, Farmingdale, NY (Plymouth Meeting). A mammalian expression vector (pCMV6-AC-IRES-GFP) was purchased from Origene (Rockville, MD). Restriction enzymes were obtained from New England BioLabs (Ipswich, MA). Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from WelGENE Inc. (Daegu, Republic of Korea). HEK293 cells were purchased from the American Type Culture Collection (Manassas, VA).

2.2. Vector construction

Murine brain (8 weeks old) and fetal brain (18.5 days post-coitum) were kindly provided by Dr. Y. Lee. Total RNA was extracted from the brains by using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany), and cDNA was synthesized with the SuperScript III First-Strand Synthesis System using the Oligo(dT)$_{20}$ primer according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Polymerase chain reaction (PCR) amplification was performed with the following primer sets (flanked by *Xho*I restriction sites): eugenol receptor (5′-ACG CGGCCGCTCGAGATGACTCTGTCAGATGGAAA-3′ and 5′-GAGTTTC TGCTCG-AGAGAAGAATAGACTTTAGTACCTATT-3′) and G_{olf} (5′-AC GCGGCCGCTCGA-GATGGGCCTATGCTACAGCCT-3′ and 5′-GAGTT TCTGCTCGAGCAAGAGT-TCGTACTGCTTGAGATGC-3′). The amplified products were digested with *Xho*I and then purified with the QIAquick PCR Purification Kit (Qiagen). The purified products and the expression vector (digested with *Xho*I) were ligated using the Quick Ligation Kit (New England BioLabs). The ligation products were transformed into DH5α cells (Invitrogen) and amplified for further studies. The vector expressing mOR-EG and green fluorescent protein (GFP) as well as G_{olf} and GFP were under the control of the same cytomegalovirus (CMV) promoter.

2.3. cAMP assay

HEK293 cells (50–60% confluent) in a poly-D-lysine-coated 12-well plate (BD Biosciences, San Jose, CA) were

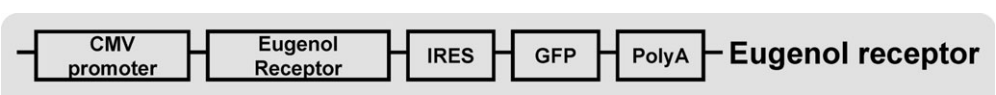


Fig. 2. Construction of a vector expressing the mOR-EG in the pCMV6-AC-IRES-GFP plasmid. The eugenol receptor and GFP were under the control of the same CMV promoter.

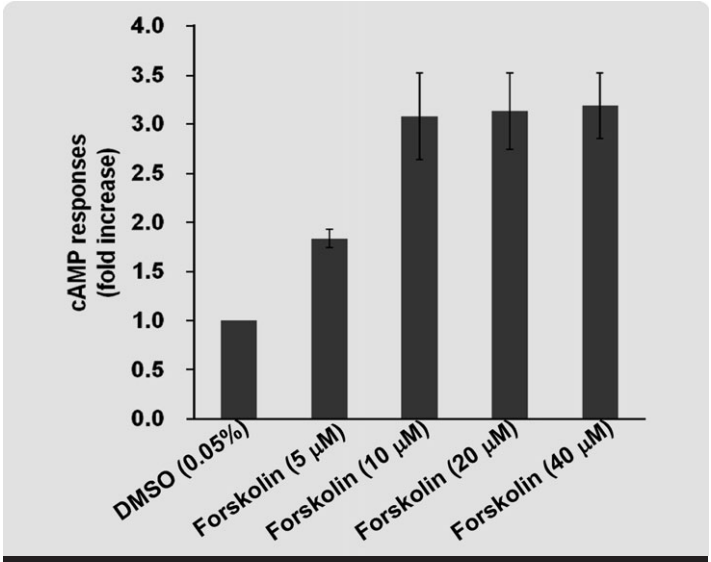
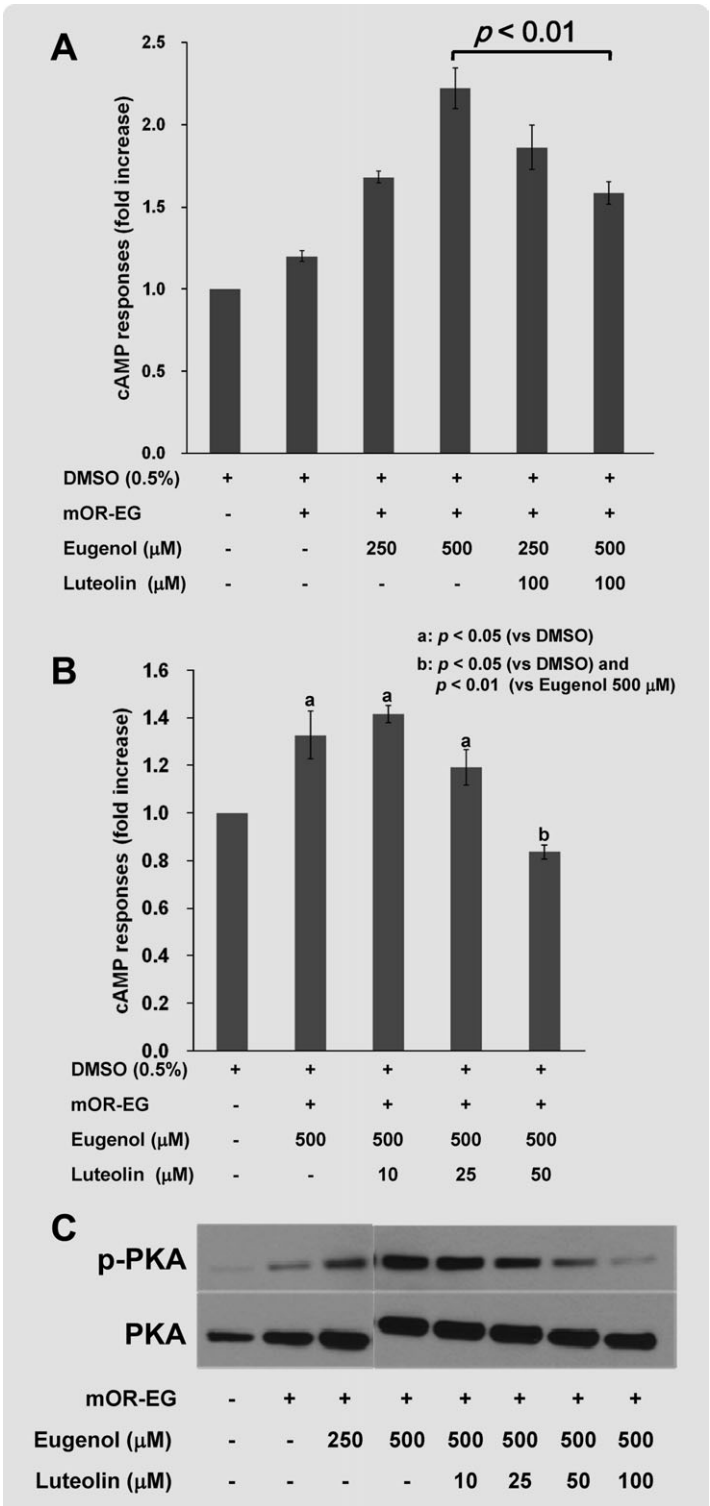


Fig. 3. cAMP response in HEK293 cells induced by forskolin, a stimulator of AC. Approximately 80–90% confluent HEK293 cells starved with serum-free DMEM for 16 H were treated with different concentrations of forskolin for 30 Min. The increases were calculated based on the cAMP level in HEK293 cells treated with 0.5% DMSO (no forskolin was present). The final concentration of DMSO in all the samples was 0.5%. Data are presented as the mean ± SE (n = 3).

transfected with 4 μg of mOR-EG cDNA containing 5 μL Lipofectamine 2000 (Invitrogen). The transfected cells were incubated for 50–56 H in DMEM medium supplemented with 10% FBS. After the incubation, the cells were washed with phosphate-buffered saline (PBS) and incubated with serum-free DMEM for an additional 16 H. The starved cells, pretreated or not pretreated with luteolin for 30 Min, were then incubated with eugenol for 30 Min, and the cAMP level was measured with the Direct cAMP EIA Kit (Enzo Life Sciences) according to the manufacturer's instructions.



2.4. Western blot analysis

The transfected and chemically treated cells (see the cAMP assay section) were washed with ice-cold PBS and lysed with RIPA buffer (Biosesang, Sungnam, Republic of Korea). The concentration of the extracted proteins was determined with the SMART BCA Protein Assay Kit (iNtRON Biotechnology, Sungnam, Republic of Korea). In total, 50 μg of the protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a nitrocellulose membrane. The membrane was blocked for 1 H at room temperature with 5% non-fat dry milk in 1× tris-buffered saline containing 0.1% Tween-20 (TBST) and then incubated overnight at 4°C with the appropriate primary antibody. The following day, the membrane was washed with TBST and probed with the secondary antibody. Protein bands were detected with Enhanced Chemiluminescence reagents (Amersham, Pittsburgh, PA). Signal intensity was quantified by using ImageQuant (Molecular Devices, Sunnyvale, CA), and the values were statistically analyzed.

2.5. Statistical analysis

All experiments were repeated more than three times, and the data were expressed as the mean ± standard error (SE). Comparisons of the group means were assessed by Student's *t*-test or by one-way analysis of variance using SPSS (SPSS Inc., Armonk, NY).

3. Results and discussion

3.1. Construction of a vector expressing the mOR-EG

To investigate the signal transduction induced by the odorant eugenol, an mOR-EG-expressing vector was constructed (Fig. 2). The eugenol receptor and GFP were coexpressed

Fig. 4. A: cAMP responses induced by different concentrations of eugenol after pretreatment or no pretreatment with 100 μM luteolin for 30 Min. The increases were calculated based on the cAMP level in HEK293 cells treated with 0.5% DMSO (no forskolin was present). The final concentration of DMSO in all the samples was 0.5%. Data are presented as the mean ± SE (n = 6). B: cAMP responses induced by 500 μM eugenol after pretreatment with different concentrations of luteolin for 30 Min. The increases were calculated based on the cAMP levels in HEK293 cells treated with 0.5% DMSO (no eugenol was present). The final concentration of DMSO in all the samples was 0.5%. Data are presented as the mean ± SE (n = 3). C: Western blot analysis of PKA and its phosphorylation. In total, 50 μg of protein was separated by SDS-PAGE. HEK293 cells were pretreated with different concentrations of luteolin for 30 min and then stimulated with eugenol. Lane 1 contained phosphorylated PKA and lane 2 contained total PKA.

because the same promoter controlled the transcription of mOR-EG, the internal ribosomal entry sequence (IRES), and GFP. This strongly implied that the eugenol receptor was expressed when a GPF signal could be detected. Interestingly, GFP signals were observed later with HEK293 cells expressing mOR-EG than with HEK293 cells expressing G_{olf} (unpublished), likely because of the different efficient transcription, translation, or subcellular localization of mOR-EG. The mOR-EG expression system was used in subsequent experiments.

3.2. cAMP assay in HEK293 cells

The cAMP level was determined in HEK293 cells after pretreatment with the known AC stimulator forskolin. As shown in Fig. 3, forskolin (5 and 10 μM) increased the cAMP level in HEK293 cells in a dose-dependent manner; however, a higher concentration of forskolin (20 or 40 μM) did not further increase the cAMP level (the level was the same as that observed with 10 μM forskolin). This suggested that the maximum increase in the cAMP level in these HEK293 cells was up to threefold.

3.3. Luteolin inhibited the eugenol-induced cAMP increase in HEK293 cells expressing mOR-EG

In HEK293 cells expressing mOR-EG, the cAMP level was increased up to 1.7- and 2.2-fold when the eugenol concentrations were 250 and 500 μM, respectively (Fig. 4A). The increase of cAMP by eugenol in a dose-dependent manner can be explained by that over-expression of eugenol receptors are not saturated by 250 and 500 μM eugenol. Because a high concentration of eugenol (1 mM) caused cells death after the 30-Min treatment, we excluded the data (unpublished). Other groups have reported that 1 and 3 mM eugenol stimulate the cAMP pathway in HEK293 T-cells and dorsal root ganglion neurons [18,19]. However, Baud et al. treated Hana 3A cells with a range of eugenol concentrations (up to 100 μM) and demonstrated that the half maximal effective concentration of eugenol for its receptor is 11 μM [20]. Although these different results can be attributed to the different cell types used in the experiments, the effects of starvation processes for the synchronization of cells and different solutions to dissolve eugenol and the toxic effects of eugenol should also be investigated further.

The 2.2-fold upregulated cAMP level induced by 500 μM eugenol decreased significantly in the presence of 100 μM luteolin (Fig. 4A). Interestingly, the 1.7-fold increased cAMP level induced by 250 μM eugenol was unaffected by 100 μM luteolin. Although these results are not easily explained, it is possible that a high concentration of luteolin may affect cell survival and therefore affect the cAMP assay results. This explanation is supported by the toxic effect of 100 μM luteolin on HepG2 cells (>30% cell death), as reported by Liu et al. [21]. Thus, we investigated the effects of different concentrations of luteolin on the eugenol-induced cAMP level.

As shown in Fig. 4B, the cAMP level increased in the presence of eugenol in HEK293 cells expressing mOR-EG; this finding was consistent with that of previous studies. However, the upregulated cAMP level was significantly decreased when 50 μM luteolin was added. At low concentrations of luteolin (10 and 25 μM), the cAMP levels were unchanged. A higher concentration of luteolin (100 μM) caused death of many cells. This implied that 100 μM luteolin affected cell survival and hence influenced the cAMP assay results (Fig. 4A). Consequently, 50 μM luteolin is enough to decrease the eugenol-induced upregulation of the cAMP level in HEK293 cells.

To investigate the effects of luteolin on downstream signal transduction, the amount of phosphorylation of PKA was determined. As shown in Fig. 4C, the phosphorylation of PKA increased in the presence of luteolin as a result of the increased cAMP level. However, the upregulated phosphorylation of PKA decreased in a dose-dependent manner because of luteolin treatment.

In summary, we have shown for the first time that pretreatment with luteolin prevented the upregulation of the eugenol-induced cAMP level and PKA phosphorylation in HEK293 cells. This finding enhances our understanding of the olfactory system and will help prevent or treat hyperosmia.

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