Appl Biol Chem (2016) 59(1):103–108
DOI 10.1007/s13765-015-0142-7

Online ISSN 2468-0842
Print ISSN 2468-0834

CrossMark

ARTICLE

Tangeretin synergistically enhances odorant-induced increases in cAMP and calcium levels and CREB phosphorylation in non-neuronal 3T3-L1 cells

Sung-Hee Kim[1] · Yeo Cho Yoon[1] · Jin-Taek Hwang[1] · Mi Jeong Sung[1] · Myung-Sunny Kim[1] · Haeng Jeon Hur[1] · Mee-Ra Rhyu[1] · Jae-Ho Park[1,2]

Received: 19 July 2015 / Accepted: 30 October 2015 / Published online: 19 January 2016
© The Korean Society for Applied Biological Chemistry 2016

Abstract Tangeretin is a natural flavone found mainly in citrus peels. Although emerging evidence suggests the beneficial health effects of tangeretin, little is known about the biological role of tangeretin in the odorant-induced signal transduction pathway. In this study, the effects of tangeretin on odorant-stimulated non-neuronal 3T3-L1 cells were evaluated. Here, we present the first evidence that an olfactory receptor for lyral, an odorant, is expressed in 3T3-L1 cells and that it responds to its ligand. Stimulation with lyral increased the cyclic adenosine monophosphate (cAMP) and Ca^{2+} levels as well as the phosphorylation of cAMP response element binding protein (CREB). Pretreatment with tangeretin synergistically enhanced the effects of stimulation with lyral: up-regulation of cAMP and Ca^{2+} levels and CREB phosphorylation. These data collectively suggest that tangeretin synergistically enhances the odorant-induced signaling pathway through modulation of cAMP and CREB signal transduction in non-chemosensory cells.

Keywords 3T3-L1 · Adenylyl cyclase · Calcium · Cyclic adenosine monophosphate · Olfactory · Tangeretin

S. H. Kim and Y. C. Yoon contributed equally to this work.

✉ Jae-Ho Park
jaehopark@kfri.re.kr

[1] Korea Food Research Institute, 1201-62 Anyangpangyo-ro, Bundang-gu, Seongnam-si 463-746, Gyeonggi, Republic of Korea

[2] Food Biotechnology, University of Science & Technology, 217 Gajeong-ro, Yuseong-su, Daejeon 305-350, Republic of Korea

Introduction

Tangeretin (5,6,7,8-tetramethoxy-2-(4-methoxyphenyl)-4H-1-benzopyran-4-one) is a polymethoxylated flavone found mainly in tangerine and other citrus peels. The five methylated hydroxyl groups in the chemical structure of tangeretin confer high molecular hydrophobicity (Fig. 1) (Murakami et al. 2002), allowing it to be more easily taken up by cells compared to other flavonoids and, thereby, to exert physiological effects. Many reports have demonstrated that tangeretin modulates various physiological processes, including inflammation by inhibiting p38 and JNK phosphorylation (Sung et al. 2013), diabetes by increasing glucose uptake (Onda et al. 2013), atherosclerosis by modulating acetylated low-density lipoproteins (Whitman et al. 2005), carcinogenesis by regulating apoptotic molecules (Dong et al. 2014), and cell cycle by modulating cell cycle regulatory proteins (Pan et al. 2002). However, little is known about the effects of tangeretin on the odorant-induced signal transduction (OST) pathway.

Although OST is crucial to the process of odor perception for food uptake, mate selection, and fear response to predators, emerging data demonstrate that OST also affects different physiological functions including muscle regeneration, cell migration, and sperm motility in non-chemosensory tissues (Fukuda et al. 2004; Griffin et al. 2009; Kang and Koo 2012). In olfactory sensory neurons, binding of odorants to olfactory receptors (ORs) activates olfactory-specific G protein (G_{olf}), which then stimulates type III adenylyl cyclase, leading to an increase in cyclic adenosine monophosphate (cAMP) levels. The increased cAMP levels trigger an increase in Ca^{2+} influx, thereby altering the membrane potential. The chemical information is converted to electrical signals and transmitted to the brain for the perception of odor (Restrepo et al. 1996). In non-chemosensory tissues, the OST

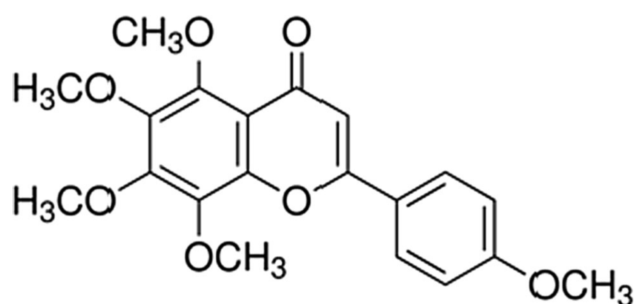

Fig. 1 Chemical structure of tangeretin

pathway reflects the same process in that the odorants bind to ORs, while cAMP and Ca^{2+} act as second messengers to relay the signal and mediate different physiological functions (Busse et al. 2014; Fukuda et al. 2004; Griffin et al. 2009; Pluznick et al. 2009). Although many studies have used heterologous systems to characterize ORs and ligands, ectopic expression of ORs in many different cells presents a model system for investigating the biological significance of OST in non-chemosensory tissues (Kang and Koo 2012; Touhara 2007). In heterologous system and in vivo, one of the well-characterized olfactory receptors is mOlfr16 (Fukuda et al. 2004; Kang and Koo 2012; Touhara 2007). MOlfr16 responds to lyral, a cognate ligand of mOlfr16, in olfactory neurons (Grosmaitre et al. 2006; Lee et al. 2009; Touhara et al. 1999), in heterologous system (Touhara et al. 1999), and in non-neuronal cells (Fukuda et al. 2004; Griffin et al. 2009) and triggers lyral-induced signal transduction pathway. However, the effect of lyral on OST pathway and its regulation in other non-neuronal cells are still unknown.

This is the first report of non-chemosensory 3T3-L1 cells responding to an odorant, lyral. In this study, we also investigated the effects of tangeretin on Ca^{2+} and cAMP levels and the downstream signaling molecules in non-chemosensory 3T3-L1 cells stimulated by lyral.

Pretreatment with tangeretin enhanced the lyral-induced increase in cAMP and Ca^{2+} in 3T3-L1 cells. In addition, tangeretin increased the phosphorylation of CREB, which plays an important role in the OST pathway. Understanding the regulation of the OST pathway in non-chemosensory cells provides a new model to study the physiological roles of ORs and identify their ligands. It also provides a foundation for the development of therapeutic approaches involving modulation of the OST pathway in non-chemosensory tissues.

Materials and methods

Chemicals and reagents

All chemicals used in these experiments were purchased from Sigma (USA). The calcium assay kit was purchased from Molecular Devices (USA), and the cAMP assay kit was obtained from Enzo Life Sciences (USA). The antibody against phospho-CREB was purchased from Cell Signaling Technology (USA) and the LaminB1 antibody was obtained from Abcam (USA). We generated an adenovirus expressing siRNA against mOlfr16 (5′-AAAGGCTTTCTTCTGGCCTT-3′; Griffin et al. 2009) and purchased an adenovirus expressing non-specific siRNA from SignaGen Laboratories (USA).

Cell culture

3T3-L1 cells were purchased from the American Type Culture Collection (USA) and grown in Dulbecco's modified Eagle's medium supplemented with 10 % bovine calf serum (Welgene, Korea) and 1 % antibiotic–antimycotic solution (Welgene) at 37 °C in a 5 % carbon dioxide (CO_2) incubator.

Odorant stimulation and calcium influx assay

Cells were seeded in microplates (96-well, black with clear flat bottoms) and then incubated overnight. Following a 30-min pretreatment with tangeretin (0–1.6 mM), the cells were incubated with the loading buffer (calcium assay reagent dissolved in 1 × Hank's Balanced Salt solution and 20 mM HEPES buffer, pH 7.4) for 30 min at room temperature in the dark and then for 15 min at 37 °C. The assay plate was then transferred directly to the FlexStation3 microplate reader (Molecular Devices, USA), and the cells were stimulated by the addition of lyral (odorant ligand of the mouse olfactory receptor 16) or ionomycin (positive control, 4 μM) (Kim et al. 2015). Intracellular calcium levels were measured using the assay kit according to the manufacturer's instructions, and the results were analyzed using SOFTmax Pro (Molecular Devices).

Cyclic AMP assay

Cells were serum starved overnight and then treated with different concentrations of tangeretin (0–1.6 mM) for 30 min and stimulated with lyral for 7 min. The treated cells were lysed with 0.1 M HCl and the level of cAMP in the supernatant was measured using the assay kit according to the manufacturer's instructions.

Western blot analysis

3T3-L1 cells pretreated with tangeretin for 30 min were stimulated with lyral for 7 min and then lysed with RIPA buffer (Biosesang, Korea) containing protease and phosphatase inhibitors (Roche, Switzerland). Extracted proteins were separated by a sodium dodecyl sulfate polyacrylamide

gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. Phosphorylation and expression of proteins were detected with specific antibodies.

Statistical analysis

Data are expressed as mean ± SD of at least three independent experiments. Values of $p < 0.05$ were considered to be significant. Statistical analyses were conducted using SPSS version 9.0 (SPSS Inc., USA).

Results and discussion

Lyral increased Ca^{2+} and cAMP levels and CREB phosphorylation in 3T3-L1 cells expressing murine olfactory receptor 16

ORs are expressed in both chemosensory and non-chemosensory tissues and mediate the first step in the odorant-induced signal transduction pathway (Kang and Koo 2012). Although numerous studies demonstrate that ORs play various physiological roles in different tissues, Ca^{2+} and CREB are crucial molecules in the odorant-induced signal cascade in non-chemosensory tissues and olfactory perception in olfactory sensory neurons (Moon et al. 1999; Fukuda et al. 2004; Griffin et al. 2009; Pluznick et al. 2009). Recently, it was demonstrated that several olfactory receptors are expressed in 3T3-L1 cells (Choi et al. 2013; von der Heyde et al. 2014). However, there functional roles and their regulations in non-neuronal fat tissues and 3T3-L1 cells have not yet understood. In addition, their putative ligands were not yet characterized. Here we report for the first time, the expression of the murine olfactory receptor 16 (mOlfr16) known as a lyral receptor in 3T3-L1 cells (Fig. 2A). The receptor was significantly reduced by siRNA against mOlfr16, while non-specific siRNA did not affect the expression of mOlfr16. This suggests that mOlfr16 is, at least, one of the expressed olfactory receptors in 3T3-L1 cells and may be involved in physiological roles of 3T3-L1cells.

Investigation of the signal transduction cascade induced by odorant revealed that lyral significantly increased Ca^{2+} levels in a dose-dependent manner (Fig. 2B). To determine the change of cAMP levels in 3T3-L1 cells, lower concentrations of lyral were used because higher concentrations of lyral induced cell death. It may be due to much longer incubation time for cAMP assay (7 min) while short stimulation (less than 12 s) by lyral in calcium assay. Considering experiments with tangeretin, it is important to determine an appropriate concentration of lyral increasing both calcium and cAMP without cell toxicity. After carefully examination of different concentrations of lyral, we found that lyral at 100 μM significantly increased cAMP levels (threefold) without toxicity (Fig. 2C). However, the pattern was not dose dependent. It is may be due to an intrinsic problem of cAMP assay using antibody which is not fully optimized. Compared with Fig. 3A, B, we also found the same results showing that calcium level was changed in dose-dependent manner but not in cAMP. This phenomenon is also supported our previous report showing increase of cAMP by odorant not in dose-dependent manner in AGS cells (Kim et al. 2013). Although assay of cAMP is still controversial, these results at least showed that Ca^{2+} and cAMP are crucial molecules in the odorant-induced signal transduction pathway in non-chemosensory tissues the same as previous reports (Busse et al. 2014; Fukuda et al. 2004; Griffin et al. 2009; Pluznick et al. 2009).

Western blot analysis for investigating the effects of changes in cAMP levels on phosphorylation of CREB revealed that treatment with lyral enhanced CREB phosphorylation in a dose-dependent manner. Since phosphorylation of CREB was also saturated with stimulation with 25 μM lyral, in the following experiments, lower concentration of lyral would be used to investigate the effect of tangeretin. Collectively, these data show the expression of mOlfr16 in non-chemosensory 3T3-L1 cells as well as increase in cAMP and Ca^{2+} levels following treatment with lyral, a ligand of mOlfr16. Phosphorylation of CREB was also increased by lyral. These results suggest that 3T3-L1 cells could serve as a model system to investigate the physiological roles of mOlfr16 and its regulation by exogenous molecules (as described below) in non-chemosensory cells.

Tangeretin synergistically enhanced lyral-induced increases in intracellular Ca^{2+} and cAMP levels in 3T3-L1 cells

We investigated the effects of tangeretin on the lyral-induced increase in intracellular calcium influx in 3T3-L1 cells. Pretreatment with tangeretin synergistically enhanced the lyral-induced increase in cytosolic Ca^{2+} levels, while tangeretin without lyral stimulation did not (Fig. 3A). Although little change was observed with 0.2 mM tangeretin (data not shown), Ca^{2+} levels were significantly increased by 25, 60, and 80 % following treatment with 0.4, 0.8, and 1.6 mM tangeretin, respectively. Although Takano previously demonstrated that tangeretin regulates Ca^{2+} level and prevents cell death in neuronal cells (Takano et al. 2007), here we report for the first time that tangeretin synergistically enhances the increase in Ca^{2+} levels induced by the odorant

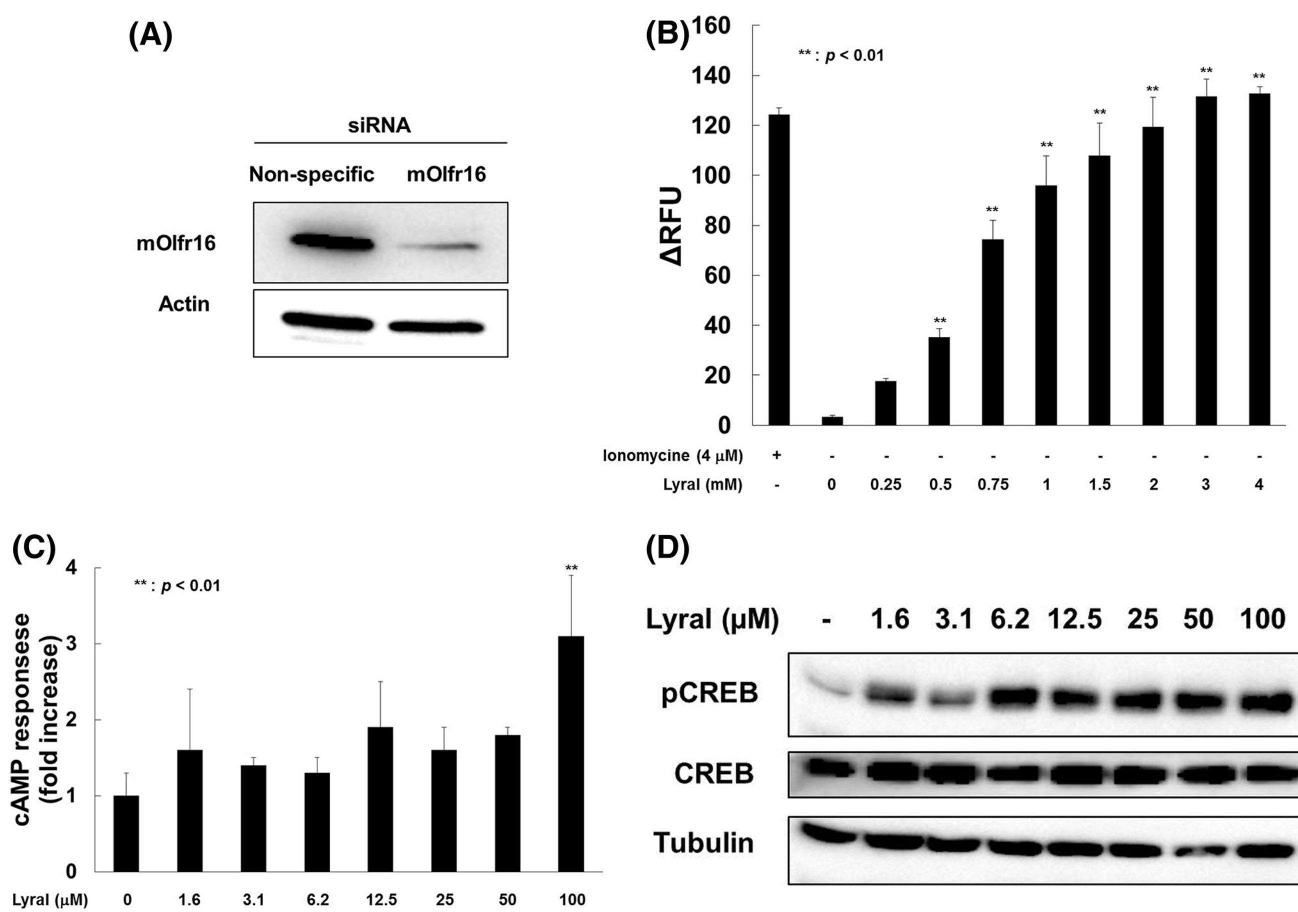

Fig. 2 Stimulation of chemosensory signal transduction pathway by lyral in non-neuronal 3T3-L1 cells. (**A**) Expression of murine lyral receptor (mOlfr16) in 3T3-L1 cells. (**B**) Changes in Ca^{2+} level induced by lyral in 3T3-L1 cells. Cells were stimulated with increasing concentrations of lyral (0–4 mM). Ionomycin (4 μM) was used as a positive control, and DMSO (1 %) was used as a negative control. Data are represented as the mean ± SD, $n = 3$. $**p < 0.01$. ΔRFU, change in relative fluorescence unit. (**C**) Changes in cAMP level induced by lyral in 3T3L1 cells. Cells were stimulated with increasing concentrations of lyral (0–0.1 mM). The final concentration of DMSO in all samples was 1 %. The data are shown as the mean ± SD, $n = 3$. $**p < 0.01$. (**D**). Western blot analysis of phosphorylated CREB by stimulation with 0–0.1 mM lyral for 7 min. Protein (45 μg) was separated by SDS-PAGE. The final concentration of DMSO in all samples was 1 %

lyral in non-neuronal cells. We also examined calcium levels after stimulation with mixture of tangeretin and lyral instead of pretreatment with tangeretin and found that there was no synergistic effect. It suggests that tangeretin could not interact with lyral receptor or lyral and not affect the receptor–ligand interaction. Instead, it is possible that tangeretin in cytosol may affect OST pathway although its underlying mechanism is not yet elucidated. As previously described in the result, lower concentration of lyral was used because of longer stimulation time for cAMP assay than that for calcium assay. It was evident that tangeretin synergistically increased cAMP levels by more than twofold after stimulation with lyral while tangeretin without lyral did not (Fig. 3B). In addition, the pattern of cAMP changes was not the same as that of calcium as previous described in the result.

Tangeretin increased lyral-induced CREB phosphorylation

Next, we investigated the effects of tangeretin on phosphorylation of CREB, which is an important transcription factor that regulates OST pathway genes (Moon et al. 1999). As shown in Fig. 3C, increase in the phosphorylation of CREB by lyral treatment was synergistically enhanced by pretreatment with tangeretin.

In conclusion, in the present study, we demonstrated that tangeretin, which is found in abundance in citrus fruit

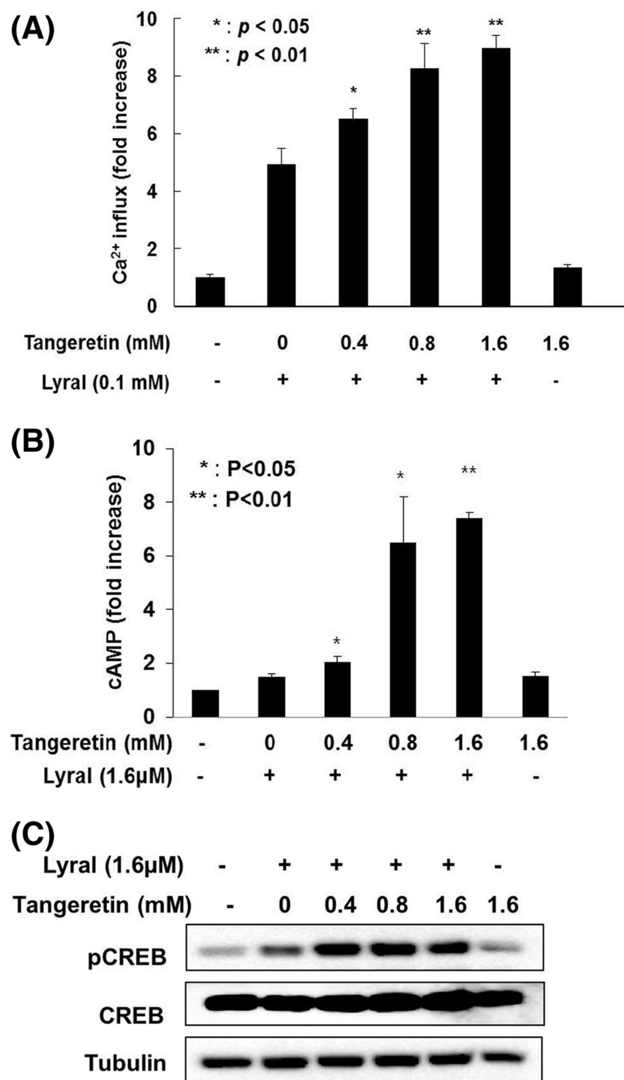

Fig. 3 Effects of tangeretin on lyral-induced signal transduction pathway in 3T3-L1 cells. (**A**) Changes in lyral-induced Ca^{2+} level in 3T3-L1 cells following pretreatment with increasing concentrations of tangeretin (0–1.6 mM) for 30 min and stimulation with 0.1 mM lyral. DMSO (1 %) was used as a negative control. Data are represented as the mean ± SD, $n = 3$. *$p < 0.05$, ** $p < 0.01$. ΔRFU, change in relative fluorescence unit. (**B**) Changes in lyral-induced cAMP level in 3T3-L1 cells following pretreatment with increasing concentrations of tangeretin (0–1.6 mM) for 30 min and stimulation with 1. 6 μM lyral for 7 min. DMSO (1 %) was used as a negative control. Data are represented as the mean ± SD, $n = 3$. *$p < 0.05$, ** $p < 0.01$. (**C**) Western blot analysis of phosphorylated CREB following pretreatment with increasing concentrations of tangeretin (0–1.6 mM) for 30 min and stimulation with 12.5 μM lyral for 7 min. Protein (45 μg) was separated by SDS-PAGE. The final concentration of DMSO in all samples was 1 %

peels, synergistically enhanced the odorant-induced increase in cAMP and Ca^{2+} levels and phosphorylation of CREB in non-chemosensory cells. These findings expand our understanding of physiological roles of ORs and the regulation of the OST pathway in non-neuronal tissues.

Acknowledgments This study was supported by grants from the Korea Food Research Institute (E0111202-05 and E0150301-02). The authors declare that they have no conflicts of interest.

References

Busse D, Kudella P, Gruning NM, Gisselmann G, Stander S, Luger T, Jacobsen F, Seinstraber L, Paus R, Gkogkolou P, Bohm M, Hatt H, Benecke H (2014) A synthetic sandalwood odorant induces would-healing process in human keratinocytes via the olfactory receptor OR2AT4. J Investig Dermatol 134:2823–2832

Choi Y, Hur CG, Par T (2013) Induction of olfaction and cancer-related genes in mice fed a high-fat diet as assessed through the mode-of-action by network identification analysis. PLoS One 8:e56610

Dong Y, Cao A, Shi J, Yin P, Wang L, Ji G, Xie J, Wu D (2014) Tangeretin, a citrus polymethoxyflavonoid, induces apoptosis of human gastric cancer AGS cells through extrinsic and intrinsic signaling pathways. Oncol Rep 31:1788–1794

Fukuda N, Yomogida K, Okabe M, Touhara K (2004) Functional characterization of a mouse testicular olfactory receptor and its role in chemonsensing and regulation of sperm motility. J Cell Sci 117:5835–5845

Griffin CA, Kafadar KA, Pavlath GK (2009) MOR23 promotes muscle regeneration and regulates cell adhesion and migration. Dev Cell 17:649–661

Grosmaitre X, Vassalli A, Mombaerts P, Shepherd GM, Ma M (2006) Odorant responses of olfactory sensory neurons expressing the odorant receptor MOR23: a patch clamp analysis in gene-targeted mice. Proc Natl Acad Sci USA 103:1970–1975

Kang N, Koo J (2012) Olfactory receptors in non-chemosensory tissues. BMB Rep 45:612–622

Kim SH, Yoon YC, Kim JI, Hwang JT, Sung MJ, Kim MS, Hur HJ, Rhyu MR, Park MS, Park JH (2013) Equol (4′,7-isoflavandiol) inhibits odorant-induced cAMP and calcium levels in AGS cells expressing the olfactory receptor. Curr Top Nutraceuticals R 11:145–150

Kim SH, Yoon YC, Lee AS, Kang NN, Koo J, Rhyu MR, Park JH (2015) Expression of human olfactory receptor 10J5 in heart aorta, coronary artery, and endothelial cells and its functional role in angiogenesis. Biochem Biophys Res Commun 460:404–408

Lee AC, Tian H, Grosmaitre X, Ma M (2009) Expression patterns of odorant receptors and response properties of olfactory sensory neurons in aged mice. Chem Senses 34:695–703

Moon C, Sung YK, Reddy R, Ronnett GV (1999) Odorants induce the phosphorylation of the cAMP response element binding protein in olfactory receptor neurons. Proc Natl Acad Sci USA 96:14605–14610

Murakami A, Koshimizu K, Ohigashi H, Kuwahara S, Kuki W, Takahashi Y, Hosotani K, Kawahara S, Matsuoka Y (2002) Characteristic rat tissue accumulation of nobiletin, a chemopreventive polymethoxyflavonoid, in comparison with luteolin. BioFactors 16:73–82

Onda K, Horike N, Suzuki T, Hirano T (2013) Polymethoxyflavonoids tangeretin and nobiletin increase glucose uptake in murine adipocytes. Phytother Res 27:312–316

Pan MH, Chen WJ, Lin-Shiau SY, Ho CT, Lin JK (2002) Tnageretin induces cell-cycle G1 arrest through inhibiting cyclin-dependent kinases 2 and 4 activities as well as elevating Cdk inhibitors p21 and p27 in human colorectal carcinoma cells. Carcinogenesis 23:1677–1684

Pluznick JL, Zou DJ, Zhang X, Yan Q, Rodriguez-Gil DJ, Eisner C, Wells E, Greer CA, Wang T, Firestein S, Schnermann J, Caplan

MJ (2009) Functional expression of the olfactory signaling system in the kidney. Proc Natl Acad Sci USA 106:2059–2064
Restrepo D, Teeter JH, Schild D (1996) Second messenger signaling in olfactory transduction. J Neurobiol 30:37–48
Sung MJ, Davaatseren M, Kim SH, Kim MJ, Hwang JT (2013) Boehmeria nivea attenuates LPS-induced inflammatory markers by inhibiting p38 and JNK phosphorylation in RAW264.7 macrophages. Pharm Biol 51:1131–1136
Takano K, Tabata Y, Kitao Y, Murakami R, Suzuki H, Yamada M, Iinum M, Yoneda Y, Ogawa S, Hori O (2007) Methoxyflavones protect cells against endoplasmic reticulum stress and neurotoxi. Am J Physiol Cell Physiol 292:C353–C361
Touhara K (2007) Deorphanizing vertebrate olfactory receptors: recent advances in odorant-response assays. Neurochem Int 51:132–139
Touhara K, Sengoku S, Inaki K, Tsuboi A, Hirono J, Sato T, Sakano H, Haga T (1999) Functional identification and reconstitution of an odorant receptor in single olfactory neurons. Proc Natl Acad Sci USA 96:4040–4045
Von der Heyde S, Fromm-Dornieden C, Salinas-Riester G, Beissbarth T, Baumgartner BG (2014) Dynamics of mRNA and polysomal abundance in early 3T3-L1 adipogenesis. BMC Genom 15:381
Whitman SC, Kurowska EM, Manthey JA, Daugherty A (2005) Nobiletin, a citrus flavonoid isolated from tangerines, selectively inhibits class A scavenger receptor-mediated metabolism of acetylated LDL by mouse macrophages. Atherosclerosis 178:25–32