Reprint Article

GRAMINE SUPPRESSES EUGENOL-INDUCED CALCIUM AND CAMP ELEVATION IN NON-NEURONAL 3T3-L1 CELLS

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ABSTRACT: Gramine is an alkaloid found in giant reed and maple. Many reports discuss the health-beneficial effects of gramine in inflammation, angiogenesis, and cell growth. However, the physiological role of gramine in the odorant-induced signal transduction pathway has not yet been investigated. Therefore, in this study, we aimed to evaluate the effects of gramine on odorant-stimulated non-neuronal 3T3-L1 cells endogenously expressing the murine eugenol receptor, olfr73. We showed that gramine inhibited the eugenol-induced calcium influx and cyclic AMP (cAMP) elevation in the 3T3-L1 cells without any toxicity. In addition, gramine decreased the phosphorylation of protein kinase A and cAMP response element binding protein (CREB), which are known as downstream targets of cAMP, in eugenol-stimulated 3T3-L1 cells. These results indicate that gramine suppresses the eugenol-induced signal transduction pathway in non-neuronal 3T3-L1 cells by modulating calcium influx, cAMP levels, and CREB phosphorylation.

KEY WORDS: cAMP, CREB, Gramine, Non-neuronal, Olfactory

INTRODUCTION

Olfactory receptors (ORs) are members of a large family of G protein-coupled receptors embedded in cell membranes and are mainly expressed in olfactory sensory neurons and non-neuronal tissues (Gaillard et al., 2004; Hamadi et al., 2014; Kang and Koo, 2012). During smell perception, ORs play crucial roles in recognizing odorant molecules and stimulating olfactory signaling cascades. Binding of the odorant molecules to their ORs initiates a signal transduction cascade that stimulates adenylyl cyclase III and cAMP production in the cytosol (Restrepi et al., 1996; Touhara, 2007). This increase in cAMP opens cyclic nucleotide-gated (CNG) channels and generates a cation influx, which depolarizes the membrane (Menini, 1999). Thus, an action potential is generated and transmitted to the olfactory bulb and ultimately to the olfactory cortex for the perception of smell.

Recently, many studies have revealed the expression of ORs in different non-neuronal tissues, where they have different physiological roles, including muscle regeneration, kidney function, sperm chemotaxis, and regulation of blood pressure (Griffin et al., 2009; Pluznick et al., 2009; Fukuda et al., 2004; Pluznick et al., 2013). In our previous report, we showed that human olfactory receptor 10J5 (OR10J5) is expressed in the human aorta and coronary artery, where it plays a stimulatory role during angiogenesis (Kim et al., 2015). It was shown that murine olfactory receptor 73 (olfr73), a eugenol receptor, was expressed in non-neuronal 3T3-L1 cells (Yoon et al., 2015). However, the physiological roles of odorant-induced signal transduction (OST) pathway and its regulation in 3T3-L1
cells have not been completely elucidated.

Gramine (N,N-dimethyl-3-aminomethylindole) is an indole alkaloid compound present in several plant families and is the main component of yellow lupin seeds, which are used as protein sources for domestic animal feed in Middle and Eastern Europe (Pastuszewska et al., 2001). Gramine has been shown to play important roles in energy metabolism in the mitochondria (Niemeyer and Roveri, 1984). Pastuszewska et al. demonstrated that gramine affects feed intake and body weight gain in rats and chickens (Pastuszewska et al., 2001). However, the mechanisms underlying the regulation of body weight and food intake by gramine at the cellular and organism level are not well understood.

In this study, we investigated the effects of gramine on odorant (eugenol)-induced signal transduction to understand OST pathway regulation by phytochemicals in non-neuronal 3T3-L1 cells. First, we studied the effects of gramine on eugenol-induced increase in cAMP levels in 3T3-L1 cells. Next, we investigated the effect of this treatment on calcium influx and phosphorylation of cAMP-dependent protein kinase A (PKA) and cAMP-response element binding protein (CREB).

MATERIALS AND METHODS

Materials

Gramine and eugenol were purchased from Sigma (St. Louis, MO, USA) and their solutions in dimethyl sulfoxide (DMSO, Barker, TX, USA) were prepared. Cell Counting Kit-8 and cAMP assay kit were purchased from Enzo Life Sciences Inc. (Farmingdale, NY, USA). The Calcium 4 Assay Kit was obtained from Molecular Devices (Sunnyvale, CA, USA). Antibodies against phospho-CREB and phospho-PKA were obtained from Cell Signaling Technology (Beverly, MA, USA), and the actin antibody was purchased from Bethyl Laboratories, Inc. (Montgomery, TX, USA).

Cell culture

3T3-L1 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), and antibiotics were obtained from WELGENE Inc. (Daegu, Republic of Korea). Cells were grown in DMEM supplemented with 10% FBS and 1% antibiotics at 37°C, in a 5% CO₂ incubator. To avoid differentiation of 3T3-L1 cells without stimulation, all cells were split whenever the cells were ~70% confluent. All experiments were carried out with the cells having passage 4 to 8 after purchasing them from ATCC. After determining cell number using a cell counter (Scepter, Millipore corporation, Billerica, MA, USA), 2 × 10⁴ and 3 × 10⁴ cells were incubated for 20 hr in 6-well and 96-well plate, respectively for this study.

Cell cytotoxicity assay

To determine gramine cytotoxicity, cells were grown in a 96-well plate and treated with gramine for 30 min. Cell viability was determined by quantitating viable cells using Cell Counting Kit-8.

cAMP assay

3T3-L1 cells in 6-well plate were starved by incubating in serum-free media at 37°C overnight and treated with different concentrations of gramine for 30 min, followed by stimulation with eugenol for 7 min. To investigate the specificity of gramine, the cells were treated with different concentrations of gramine for 30 min followed by stimulation with forskolin for 7 min. The treated cells were lysed with 0.1 M HCl, and the cAMP levels in the supernatants were measured, according to the manufacturer’s instructions.

Calcium influx assay

Intracellular calcium level was assayed using the Calcium 4 Assay Kit. 3T3-L1 cells were cultured in black, clear, flat-bottom 96-well plates. The cells were pretreated with gramine for 30 min and then the loading buffer was added. The cells were incubated at 25°C for 30 min without light and further incubated for 15 min at 37°C. After incubation, the assay plate was directly transferred to a FlexStation microplate reader (Molecular Devices) and stimulated by addition of eugenol. Intracellular calcium influx level was measured according to the manufacturer’s instructions and analyzed using SoftMax Pro (Molecular Devices).

Western blot analysis

Cells in 6-well plate were treated with gramine for 30 min and then stimulated with eugenol for 7 min. The treated cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed with RIPA buffer (Biosesang, Seongnam, Republic of Korea) containing protease and phosphatase inhibitors (Roche, Basel, Switzerland). The extracted proteins were separated on the basis of their molecular weight and electrophoretically transferred to a nitrocellulose membrane. Phosphorylation and expression levels of proteins were detected using specific antibodies.

Statistical analyses

Statistical analyses were performed using SPSS version 9.0 (SPSS Inc., Chicago, IL, USA). Statistical differences were evaluated by one-way analysis of variance (ANOVA) followed by the Duncan’s test. Differences with p < 0.05 were considered to be significant. Results of cAMP and calcium were normalized with cell number based on the cell viability. Data are expressed as means ± standard deviation (SD) of at least three independent experiments.

RESULTS AND DISCUSSION

Suppression of eugenol-induced increase in cAMP by gramine in 3T3-L1 cells

Odorant binding to ORs stimulates adenylyl cyclase III,
leading to an increase in the levels of the secondary messengers, cAMP and calcium (Maritan et al., 2009). Previously, we found that eugenol increased cAMP and calcium levels in 3T3-L1 cells expressing the eugenol receptor olfr73. To understand the regulation of the OST pathway by gramine, we evaluated the effect of gramine on eugenol-induced cAMP signaling. Eugenol-induced upregulation of cAMP was significantly suppressed by gramine (Fig. 1). Eugenol treatment increased cAMP level by 2.5-fold, whereas gramine treatment (0.1–0.8 mM) decreased the cAMP levels to normal. To investigate the specificity of gramine, we determined the level of forskolin-induced cAMP after gramine treatment. Gramine did not affect the levels of forskolin-induced cAMP (Fig. 2). This shows that gramine inhibits the eugenol-induced increase in cAMP levels via a forskolin-independent cAMP pathway. Consistent with a recent report that adenylyl cyclase 3, a crucial molecule regulating cAMP in OST pathway, modulated obesity and insulin sensitivity in mice (Tong et al., 2016), it strongly suggested that changes of cAMP level may be involved in adipogenesis of 3T3-L1 cells. In addition, regulation of cAMP with exogenous molecules such as gramine will be a therapeutic target to treat and prevent obesity.

**Toxicity of gramine in 3T3-L1 cells**

To further investigate whether this significant decrease in cAMP was due to the cytotoxic effects of gramine, viability of gramine-treated 3T3-L1 cells was assessed. No cytotoxicity was detected at all gramine concentrations (0–0.8 mM; FIGURE 3. Gramine cytotoxicity in 3T3-L1 cells. Cell viability was determined after treatment with various concentrations of gramine (0, 0.1, 0.2, 0.4, and 0.8 mM) for 30 min. Data are shown as mean ± SD (n = 4).

![Graph showing the effect of gramine on forskolin-induced cAMP levels in 3T3-L1 cells.](image)

**FIGURE 2. Effect of gramine on forskolin-induced cAMP levels in 3T3-L1 cells.** After pretreatment with gramine for 30 min, 3T3-L1 cells were stimulated with 0.05 mM forskolin for 7 min. Data are expressed as mean ± SD (n = 4). Significant differences were not detected between the gramine-treated and untreated groups.

![Graph showing the effect of gramine on eugenol-induced calcium influx in 3T3-L1 cells.](image)

**FIGURE 4. Effect of gramine on eugenol-induced calcium influx in 3T3-L1 cells.** Cells were pretreated with gramine for 30 min and stimulated with 2 mM eugenol for 15 s. Calcium influx is expressed as a relative fluorescence unit (ΔRFU). The values are presented as mean ± SD (n = 3). Significant differences between the gramine+eugenol-treated group and the eugenol-treated group are expressed as *p < 0.05.
Fig. 3). This suggested that the inhibitory effect of gramine on eugenol-induced cAMP elevation was not caused by the cytotoxicity of gramine but was a result of its effect on the cAMP signal cascade. This is consistent with a previous report that gramine acts as an antagonist against octopamine-induced cAMP elevation in neurosecretory cells (Orchard et al., 1983).

**Gramine suppressed eugenol-induced increase in calcium levels in 3T3-L1 cells**

As cAMP is known to stimulate calcium influx in olfactory neuronal cells, we next examined the effects of gramine on eugenol-induced increase in intracellular calcium in non-neuronal 3T3-L1 cells. Gramine treatment gradually suppressed the eugenol-induced upregulation of calcium influx (Fig. 4). Approximately 30% of the calcium signal decreased after treatment with 0.8 mM gramine. The inhibitory effects of gramine on calcium influx were supported by a study showing that gramine analogs inhibited calcium entry and led to the inhibition of smooth muscle contraction in rat aorta (Iwata et al., 2001). However, treatment with lower concentrations of gramine (0.1 – 0.4 mM) did not affect the calcium level, although they decreased cAMP levels in 3T3-L1 cells. This difference in the responses observed may be attributed to the difference in eugenol stimulation time, 7 min and less than 15 s for cAMP and calcium, respectively. Because different assay systems were used for detecting calcium and cAMP levels, the same stimulation time could not be used when measuring calcium and cAMP levels. As previously described, cAMP and calcium play pivotal roles in the OST pathway. Our data suggest that gramine suppresses the OST pathway by downregulating the crucial molecules, cAMP and calcium, and consequently alleviates adipogenesis of 3T3-L1 cells as well as obesity in mice.

**FIGURE 5. Effect of gramine on eugenol-induced phosphorylation of CREB and PKA in 3T3-L1 cells.** Cells were pretreated with different concentrations of gramine (0–0.8 mM) for 30 min and then stimulated with 2 mM eugenol for 7 min. Expression of pCREB and pPKA was measured by western blot analysis.

<table>
<thead>
<tr>
<th>Gramine (µM)</th>
<th>0</th>
<th>100</th>
<th>200</th>
<th>400</th>
<th>800</th>
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<tr>
<td>Eugenol (2 mM)</td>
<td>-</td>
<td>+</td>
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**Decrease in phosphorylation of PKA and CREB by gramine in 3T3-L1 cells**

Odorant-induced cAMP also activates PKA and CREB, the well-known modulators of gene expression related not only to olfactory signal transduction but also to various other physiological signaling pathways (Moon et al., 1999; Wetzl et al., 2001). PKA is involved in long-term memory formation associated with olfactory learning and phosphorylates CREB, a protein implicated in the learning and memory development processes. In this study, we investigated whether gramine regulates odorant-induced PKA and CREB phosphorylation in non-neuronal 3T3-L1 cells. We found that gramine gradually attenuated eugenol-induced CREB phosphorylation (Fig. 5). Although CREB phosphorylation increased in response to eugenol, treatment with 0.1 – 0.8 mM gramine decreased the eugenol-induced phosphorylation of CREB in a dose-dependent manner while phosphorylation of PKA decreased after treatment with 0.8 mM gramine. These results are consistent with the inhibitory effects of gramine on eugenol-induced cAMP and calcium levels in 3T3-L1 cells, as CREB is one of the known downstream targets of cAMP. In addition, PKA pathway induced by olfactory receptor 10J5 plays a significant role in hepatic steatosis in mice (Tong et al., 2017). Together, it suggests that regulation of PKA and CREB induced by olfactory receptors may affect lipid metabolism in non-neuronal tissues and be a target to treat and prevent hepatic steatosis.

**CONCLUSIONS**

Our results show that gramine decreased eugenol-induced increase in calcium influx, cAMP level, and CREB phosphorylation in 3T3-L1 cells via a forskolin-independent cAMP pathway. In addition, gramine regulates the OST pathway in non-neuronal 3T3-L1 cells. Future studies should focus on determining whether the effects of gramine on eugenol-induced cAMP and calcium levels are direct or indirect. These findings expand our understanding of the OST pathway and its regulation in non-neuronal cells and identify potential physiological roles of ectopically expressed olfactory receptors during lipid accumulation in hepatic steatosis and diet-induced obesity. In addition, this study will give insights on treatment or prevention of lipid accumulation in liver and obesity.

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**REFERENCES**


