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Expression of human olfactory receptor 10J5 in heart aorta, coronary artery, and endothelial cells and its functional role in angiogenesis

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ABSTRACT

ORs are ectopically expressed in non-chemosensory tissues including muscle, kidney, and keratinocytes; however, their physiological roles are largely unknown. We found that human olfactory receptor 10J5 (OR10J5) is expressed in the human aorta, coronary artery, and umbilical vein endothelial cells (HUVEC). Lyral induces Ca^{2+} and phosphorylation of AKT in HUVEC. A knockdown study showed the inhibition of the lyral-induced Ca^{2+} and the phosphorylation AKT and implied that these processes are mediated by OR10J5. In addition, lyral enhanced migration of HUVEC, which were also inhibited by RNAi in a migration assay. In addition, matrigel plug assay showed that lyral enhanced angiogenesis *in vivo*. Together these data demonstrate the physiological role of OR10J5 in angiogenesis and represent roles of ORs in HUVEC cells.

1. Introduction

Olfactory receptors (ORs) are G protein-coupled receptors, normally expressed in olfactory sensory neurons (OSNs). The first step of smell perception involves the binding of odorants to their cognate receptor [1]. Considering that more than 350 and 1,000 putative functional genes encode ORs in humans and in mice, respectively [2,3] and that ORs are combinatorially activated by odorants [4], thousands of possible odorants can be distinguished. The binding of an odorant activates the G protein-coupled receptor, resulting in increasing cAMP levels via the stimulation of a type III adenylyl cyclase (ACIII). Increased cAMP levels lead to the accumulation of Ca^{2+}, thereby causing a change in the membrane potential, which is transmitted to the brain for smell perception [5,6]. In addition, emerging data demonstrate that ORs are expressed in many other tissues [7,8], playing important physiological roles in sperm motility [9,10], modulation of glomerular filtration rate in kidney [11], muscle regeneration [12], and stimulation of keratinocytes [13]. Interestingly, odorant-induced signal transduction (OST) pathways in non-chemosensory tissues recapitulate the same cAMP-Ca^{2+} signaling cascade as that observed in OSNs [10–13]. However, a physiological role for ORs in blood vessels has not yet been described.

To deliver oxygen and nutrients to new growing tissues, the growth of microvessels from parent vessels, a process known as an angiogenesis, is an important step in vascular remodeling, female reproductive cycles, and wound healing in normal life [14]. However, abnormal angiogenesis is implicated in cancer, diabetic retinopathy, and rheumatoid arthritis [15]. Given that angiogenesis plays a central role for both sustained human health as well as the development of abnormal disease states, a detailed understanding of angiogenic regulation is required to effectively treat and prevent vascular diseases. During angiogenesis, endothelial cells play a major role in cellular activation, migration, and tube formation [15]. In addition, Ca^{2+} plays a crucial role in proliferation and invasion of human umbilical vein endothelial cells (HUVEC) during angiogenesis [16]. Since ORs regulate migration of sperm, myocytes, and keratinocytes [9,12,13] and regulate Ca^{2+} levels in non-chemosensory tissues [10,12,13], we hypothesized that ORs may affect angiogenesis through the regulation of Ca^{2+}.

In this study, we demonstrated that OR10J5, homologous to murine olfactory receptor 16 (olfr16), is expressed in human tissues

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including the aorta and coronary artery as well as in HUVEC. In addition, we found that OR10J5 is at least one of the important molecules involved in enhancing angiogenesis in matrigel *in vivo*, and it stimulates cellular migration of HUVEC by altering Ca^{2+} levels *in vitro*.

2. Materials and methods

2.1. Materials

4-(4-Hydroxy-4-methylpentyl)-3-cyclo-hexene-1-carboxaldehyde (lyral) and ionomycin were purchased from Sigma (St. Louis, MO, USA). Lyral was prepared by sonication in the appropriate medium or experimental buffer. A Ca^{2+} assay kit was obtained from Molecular Devices (Sunnyvale, CA, USA), and the cell migration assay kit was purchased from Cell Biolabs, Inc. (San Diego, CA, USA). Matrigel was purchased from Millipore Inc. (Darmstadt, Germany). Antibody against the human OR10J5 was obtained from Abcam (Cambridge, UK), and phospho-AKT and phospho-ERK antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). β-actin antibody was obtained from Bethyl Laboratories, Inc. (Montgomery, TX, USA). A set of OR10J5-specific small interfering RNAs (siRNA) and non-targeting siRNA control were obtained from Thermo Scientific, Inc. (Waltham, MA, USA), and the reagent used for their transfection Dharmafect4 was purchased from Dharmacon GE, Inc. (Lafayette, CO, USA).

2.2. Cell culture

HUVC were purchased from American Type Culture Collection (Manassas, VA, USA) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (Welgene, Daegu, South Korea) at 37 °C in a 5% CO_2 incubator.

2.3. OR10J5 siRNA transfection

A combination of four siRNAs (12.5 nM each, total 50 nM) against OR10J5, or non-targeting siRNA control (50 nM), was transfected into HUVEC by using Thermo Scientific Dharmafect4 transfection reagent, according to the manufacturer's protocol. Two days after transfection, HUVEC were used for further analysis.

2.4. Odorant stimulation and Ca^{2+} influx assay

HUVEC plated in microplates (96-well, black with clear flat bottom) were incubated overnight. A loading buffer (Ca^{2+} assay reagent, which was dissolved in 1X Hank's Balanced Salt solution plus 20 mM HEPES buffer, pH 7.4) was added to the cells and incubated for 30 min at room temperature before transferring them to 37 °C for a 15-min incubation. The assay plate was transferred directly to a FlexStation (Molecular Devices, Sunnyvale, CA, USA) and stimulated by the addition of lyral and ionomycin (positive control). Intracellular Ca^{2+} influx levels were measured according to the manufacturer's instructions and analyzed using SoftMax Pro (Molecular Devices, Sunnyvale, CA, USA).

2.5. Cell migration assay

The migratory properties of HUVEC were assayed using The CytoSelect™ Cell Migration Assay Kit containing polycarbonate trans-membrane inserts in a 24-well plate. Medium containing 1% FBS and lyral (0.625, 1.25, and 2.5 μM) or 5% FBS without lyral (positive control for migration) were placed in the lower wells of the migration plate. HUVEC were starved overnight in a medium containing 1% FBS before loading 1.0×10^6 cells into the upper wells. After a 24-h period, migrated cells on the bottom of the polycarbonate membrane were stained and quantified at 560 nm after extraction, according to the manufacturer's instructions.

2.6. Western blot analysis

Extracts of human normal tissue (heart aorta and heart coronary artery) were purchased from Proteus Biosciences, Inc. (Ramona, CA, USA). To determine OR10J5 expression in HUVEC and vascular tissues, the total cellular protein was separated by SDS-PAGE and transferred to a nitrocellulose membrane. Protein expression levels were detected with specific antibodies. HUVEC were stimulated with lyral for 7 min and lysed in RIPA buffer (Biosesang, Seongnam, South Korea) containing protease inhibitor and phosphatase inhibitor (Roche, Basel, Switzerland). As previously described in human tissue samples, extracted proteins were analyzed by western blotting to determine the effects of lyral on signal transduction during angiogenesis.

2.7. Animal experiments

Male C57BL/6J mice (aged 6 wk) were obtained from Nara biotech (Seoul, Korea) and housed under 12 h light/12 h dark cycles in a temperature- and humidity-controlled room (24 ± 1 °C at 50% relative humidity). All mice were handled in accordance with the IACUC guidelines of Korea Food Research Institute (KFRI-M-14029).

2.8. Matrigel plug assay

The *in vivo* angiogenic activity of lyral was determined using modified matrigel plug assay introduced by Passaniti [17]. In total, 0.6 ml of matrigel with or without lyral were injected subcutaneously in the back of male C57BL/6J mice (aged 6 wk). After 2 wk, the mice were anesthetized, and matrigel plugs were removed and analyzed using a stereoscope (Olympus, Tokyo, Japan) and digitally photographed. The blood vessels formed on the matrigel plugs were quantified by measuring the lengths, using Image-Pro Plus V 6.0 (Media Cybernetics, Inc., Bethesda, MD).

2.9. Statistical analysis

Data are expressed as the mean ± SD of at least three independent experiments. Values of $p < 0.05$ and of $p < 0.01$ were considered to be significant. Statistical analyses were conducted using two-tailed Student *t*-test. Dose-response analysis was carried out with GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA).

3. Results and discussion

3.1. Expression of human olfactory receptor 10J5 in non-chemosensory tissues and cells

The human olfactory receptor 10J5 (OR10J5) is an ortholog of mouse olfr16. OR10J5 shares 87% and 85% sequence homology to olfr16 at the protein and DNA levels, respectively. Since olfr16 is ectopically expressed in various tissues [7,8,10–12], we first evaluated the expression of OR10J5 in human tissues and cells. As shown in Fig. 1A, OR10J5 is expressed in both the human aorta and the coronary artery, which are blood vessels consisting of endothelial cells, smooth muscle, and connective tissue. In addition, high expression of OR10J5 was observed in HUVEC. This finding suggests potential functional roles for OR10J5 in endothelial cells. To investigate whether the expression of OR10J5 is functional, Ca^{2+} levels were monitored following stimulation with lyral, a ligand for olr16.

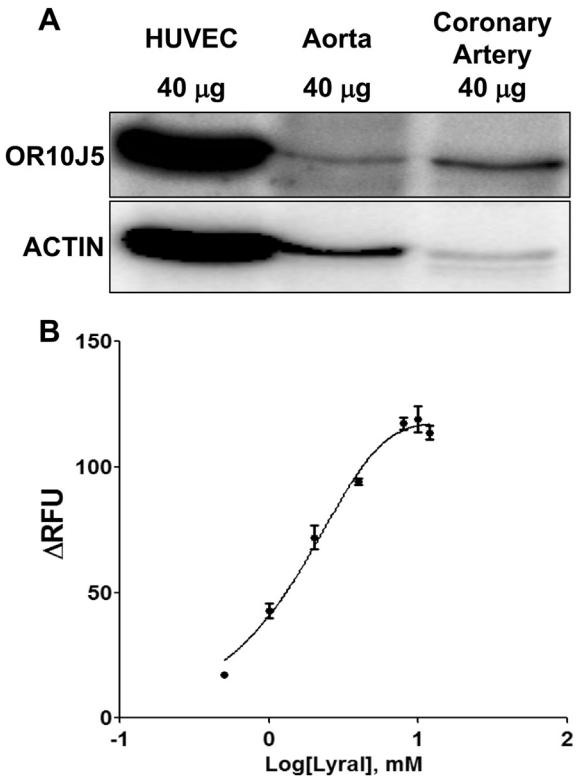

Fig. 1. Olfactory receptor 10J5 (OR10J5) increased Ca^{2+} levels in response to lyral, a ligand for OR10J5. (A) Western blot analysis of OR10J5 protein expression levels in the human aorta, coronary artery, and human umbilical vein endothelial cells (HUVEC). (B) Ca^{2+} levels in HUVEC following treatment with indicated doses of lyral. The data shown as means ± SD (n = 6). *: $p < 0.05$ and **: $p < 0.01$.

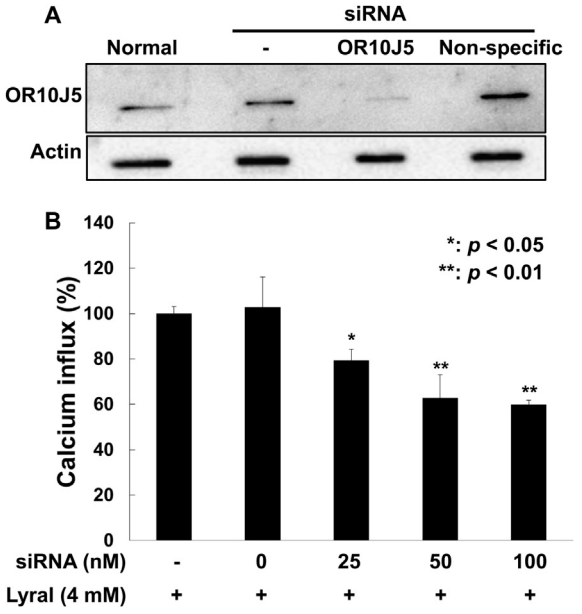

Fig. 2. OR10J5 knockdown suppressed lyral-induced elevation in Ca^{2+} levels and phosphorylation of AKT and ERK in HUVEC. (A) HUVEC transfected with a set of OR10J5 siRNAs, or control siRNA and incubated for 2 d. Levels of OR10J5 were determined by western blotting. β-actin was used as the loading control. (B) Ca^{2+} levels in transfected HUVEC following exposure to lyral. The data shown as means ± SD (n = 4). *: $p < 0.05$ and **: $p < 0.01$.

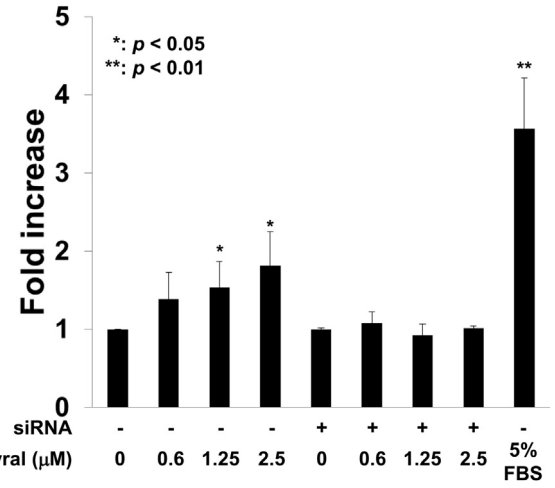

Fig. 3. OR10J5 knockdown inhibited lyral-induced migration of HUVEC. Migration of HUVEC was stimulated by lyral. Transfected and non-transfected HUVEC (1.0×10^6 cells) were placed on the upper wells and incubated with DMEM containing 1% FBS and different concentrations of lyral (0–2.5 μM) for 24 h. The cells at the bottom of the membrane were quantified. The data shown as means ± SD (n = 5 for non-transfected HUVEC, n = 4 for transfected HUVEC). *: $p < 0.05$ and **: $p < 0.01$.

Lyral increased Ca^{2+} levels in a dose-dependent manner (Fig. 1B). These data demonstrate that OR10J5 is at least one of ORs functionally responding to lyral in HUVEC.

3.2. Lyral-induced Ca^{2+} increase was decreased by OR10J5 knockdown in HUVEC

Since multiple olfactory receptors are activated by the same odorant with different efficacies [4,18], we investigated whether the lyral-induced increase in Ca^{2+} levels was mediated by OR10J5 activation by using a combination of four small interfering RNAs (siRNAs). As shown in Fig. 2A, siRNAs against OR10J5 effectively downregulated OR10J5 expression by 90% in HUVEC, while non-specific siRNA did not. Using a Ca^{2+} influx assay, HUVEC were transfected with siRNAs and stimulated with lyral to measure the

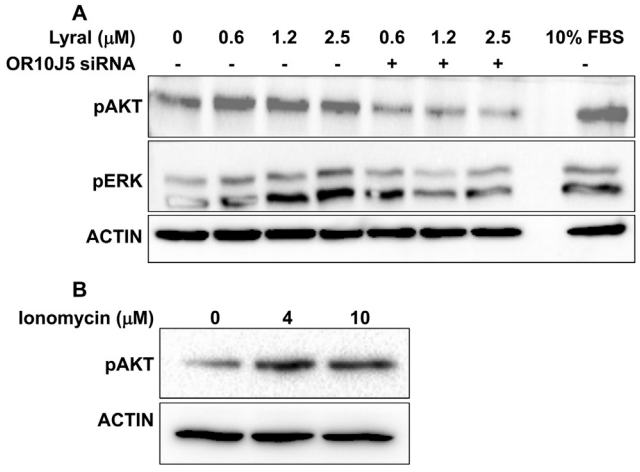

Fig. 4. Lyral-induced Ca^{2+}-dependent phosphorylation of AKT was significantly inhibited by OR10J5 knockdown in HUVEC. (A) Transfected HUVEC were incubated for 2 d before being stimulated with lyral for 7 min. Subsequently, phosphorylation of AKT and ERK was determined by western blotting. (B) HUVEC were treated with ionomycin known as a calcium ionophore (4 and 10 μM) for 10 min. Phosphorylation of AKT was determined by western blotting.

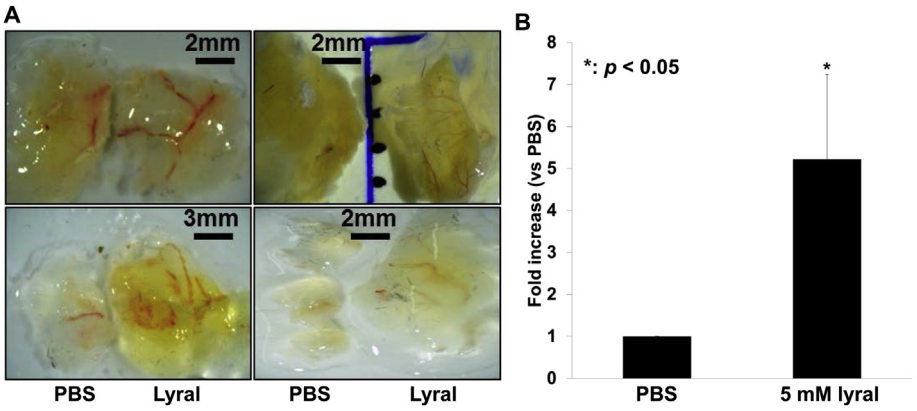

Fig. 5. Lyral enhanced angiogenesis *in vivo*. Matrigels containing 5 mM lyral were injected into the right back of the mouse, while matrigel with PBS was injected into the left back of the same mouse. After 2 wk, matrigels were removed from the mice, washed, and the total length of the blood vessels that had formed within the matrigel was quantified and the blood vessels were photographed. (A) New blood vessel growth in the indicated matrigel. (B) Fold increase in new blood vessel growth in lyral-containing matrigels, relative to that observed in the PBS-containing matrigels. The data are shown as means ± SE (n = 22). *: $p < 0.05$.

effect of OR10J5 on the changing Ca^{2+} levels. OR10J5 knockdown significantly reduced the lyral-induced Ca^{2+} increase (Fig. 2B). In addition, the reduction of Ca^{2+} was dose-dependent, with 25, 50, and 100 nM siRNAs decreasing Ca^{2+} levels by 20%, 42%, and 50%, respectively. This demonstrates that OR10J5 is at least one of the major receptors responsible for increasing Ca^{2+} levels in response to lyral in HUVEC. However, we could not exclude the possibility that lyral activates other olfactory receptors contributing to the observed changing Ca^{2+} levels because 90% suppression of OR10J5 could not fully inhibit the increase of Ca^{2+} levels.

3.3. Lyral-induced migration of HUVEC was decreased by OR10J5 knockdown

Since endothelial cellular migration is a key characteristic of angiogenesis [14], migration of the cells was carefully examined using a trans-well containing polycarbonate membrane inserts. As shown in Fig. 3, lyral stimulated migration of HUVEC in a dose-dependent manner. However, OR10J5 knockdown completely inhibited lyral-induced migration. This strongly suggests that migration of HUVEC is predominantly mediated by OR10J5/lyral interaction.

3.4. Lyral-induced phosphorylation of AKT and ERK was inhibited by OR10J5 knockdown in HUVEC

To investigate the effect of lyral on the signaling transduction pathway activated during angiogenesis, we examined the phosphorylation of AKT and ERK, which are both important signaling molecules for angiogenic migration [19,20]. Phosphorylation of AKT and ERK was increased by stimulation with lyral, whereas OR10J5 knockdown inhibited the lyral-induced phosphorylation in HUVEC (Fig. 4A). To investigate whether the phosphorylation of AKT is Ca^{2+}-dependent in HUVEC, we further determined the phosphorylation of AKT after treatment with ionomycin known as a calcium ionophore. As shown in Fig. 4B, the phosphorylation of AKT was Ca^{2+}-dependent in HUVEC, which is consistent with the previous report showing that AKT phosphorylation is Ca^{2+}-dependent in response to VEGF stimulus in endothelial cells [21]. It suggests that the observed lyral-induced HUVEC migration (Fig. 3) is regulated by the Ca^{2+}-dependent AKT signaling cascade. Collectively, these data strongly suggest that OR10J5 is a crucial receptor for modulating cell migration and phosphorylation of important signaling molecules during angiogenesis.

3.5. Lyral enhanced angiogenesis in the murine matrigel plug assay

To investigate the effects of lyral on angiogenesis *in vivo*, angiogenic activity was assessed using the matrigel plug assay. Matrigel containing PBS or 5 mM lyral was subcutaneously injected into the left and right backs, respectively, of the same male mouse. Two weeks after injection, the matrigels were photographed and quantified. Additional new blood vessels were evident in the lyral-treated matrigel (Fig. 5A). Among 30 mice, inflammation was observed at the injection sites of two mice. Hemolysis was examined in two mice, and blood vessels were not detected in four mice. In other 22 mice, the matrigel containing lyral was more turbid and yellow in color in many of the cases. Since the number and length of new blood vessels varied among individual mice, fold increases of lyral-treated matrigel over PBS matrigel were quantified. As shown in Fig. 5B, the total length of blood vessels in matrigels containing lyral was significantly increased by more than 5-fold as compared to that in the controls (n = 22, $p < 0.05$). Blood vessels were increased in length by 9-fold in twelve mice treated with lyral, whereas blood vessels decreased in length in six other mice. In four mice, no difference in blood vessel length was observed. Although future studies will need to assess additional environmental factors potentially influencing the *in vivo* responses, we demonstrated here that lyral is at least one of the important factors enhancing angiogenesis *in vivo*.

In summary, we have demonstrated that OR10J5 is a crucial regulator of angiogenesis. OR10J5 stimulates migration of HUVEC by activating the Ca^{2+}-dependent AKT signal transduction pathway. Additionally, in mice, enhanced angiogenesis is stimulated by lyral, a ligand of OR10J5, clearly showing the significance of our findings *in vivo*. Our study expands the current knowledge on the physiological functions of ORs in non-chemosensory tissues. Future investigations seeking to identify endogenous OR ligands, as well as the mechanisms regulating their production, will be crucial for understanding OR-mediated signaling in non-chemosensory tissues.

Conflict of interest

The authors declare that they have no conflict of interest.

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Author contributions

SHK, YCY, ASL, and NNK performed the experiments and analyzed the data. JHK, MRR, and JHP contributed conceptual insights and designed the studies. SHK, YCY, and JHP wrote the manuscript.

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