Original Article

Olfactory receptor (OR7D4 and OR11I1) expression in stallion testes

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ABSTRACT Olfactory receptors (OR) are primarily responsible for the detection of odorant molecules. We previously demonstrated that OR7D4, an OR for androstenone, is expressed in the vomeronasal organ and olfactory epithelium tissue of stallions. Recently, the expression of OR11I1 in the human testes was reported and the possible roles of OR11I1 in the testicular cells were suggested. The objectives of this study were 1) to explore the expression of OR7D4 and OR11I1 in stallion testes, and 2) to define the specific localization of OR7D4 and OR11I1 in the testicular tissues. Stallion testicular tissue samples were used for this study. Western blot was performed to confirm the cross-reactivity of OR7D4 and OR11I1 antibody with stallion testicular tissue samples. OR7D4 and OR11I1 gene expressions were investigated using reverse transcription-polymerase chain reaction (RT-PCR) in stallion testes. Immunofluorescence was performed to investigate the expression of OR7D4 and OR11I1 in stallion testicular tissues. The protein bands for OR7D4 and OR11I1 from the testes were observed at approximately 38 kDa and 43 kDa, respectively. The mRNA of OR7D4 and OR11I1 were detected in stallion testes. Immunolabeling of OR7D4 and OR11I1 in the cytoplasm of both spermatogonia and Leydig cells was observed. In conclusion, androstenone and another odorant chemical, which is recognized by OR11I1, may play an important role in stallion testes.

Keywords: olfactory receptor, OR11I1, OR7D4, stallion, testis

INTRODUCTION

Olfactory receptors (ORs) are the largest family of G-protein-coupled receptors in the human genome. Almost 1,000 OR genes exist within the entire human genome (Firestein, 2001). Vertebrates can detect a variety of chemicals by smell, which is recognized by ORs expressed in the olfactory epithelium (OE) and pheromone receptors located in the vomeronasal organ (VNO) (Mombaerts, 1999; Fleischer et al., 2009). Although ORs are primarily expressed in chemosensory organs, many ORs have been detected and play a role in non-olfactory tissues. For ex-

ample, the human OR hOR17-4 expressed in the testes mediates the chemical communication between spermatozoa and eggs (Spehr et al., 2003). In mice, MOR23 is expressed in the muscles during myogenesis and muscular regeneration (Griffin et al., 2009). ORs are also expressed in human prostate glands (Neuhaus et al., 2009), mice uterus (Kim et al., 2009), rat cardiac tissues (Drutel et al., 1995), mice autonomic nervous system ganglia (Weber et al., 2002), and rat placenta (Itakura et al., 2006). These findings suggest that the ectopic expression of ORs may play an important role in non-chemosensory tissues.

Twenty genes that code for testicular ORs have been

identified in the testes of various mammalian species, including humans (Vanderhaeghen et al., 1997a). These OR genes are predominantly expressed in male germ cells. In particular, most OR genes become widely distributed during the latter stages of spermatogenesis. Interestingly, these specific ORs were localized in the flagellum of mature sperm in humans and mice. These results suggest that ORs might play a role in chemotaxis during sperm-egg communication in humans and mice (Fukuda et al., 2004; Spehr et al., 2004).

The expression of OR protein in the testes has been reported in several vertebrates, such as humans, dogs, rats, mice, and birds (Vanderhaeghen et al., 1997b; Goto et al., 2001; Fukuda et al., 2004; Steiger et al., 2008; Milardi et al., 2018). A study by Spehr et al. sought to isolate the synthetic agents and antagonists of hOR17-4 to determine their effects on human sperm (Spehr et al., 2003). They demonstrated that human sperm chemotaxis in reaction to specific odorants causes changes in intracellular Ca^{2+} levels through the ORs. Based on that report, ORs may be considered as critical components in male fertility. OR111 was expressed in human testicular tissues (Milardi et al., 2018). The immunolabeling pattern of OR111 in the human testes was also varied. These results indicate that each OR may have distinct localization and function depending on the type of testicular cells.

The expression of OR family 7 subfamily D member 4 (OR7D4) in the OE and VNO of horses was previously reported. The OR7D4 is a receptor for androstenone (5 α -androst-16-en-3-one), which is the first identified mammalian steroidal pheromone. Androstenone plays a role in the mating habits of gilts (Moe et al., 2008; Robic et al., 2011). As androstenone is synthesized in the Leydig cells of the mature boar (Sinclair et al., 2005), it can be hypothesized that androstenone can function as an autocrine or paracrine factor, impacting testicular function. However, OR7D4 expression in the testes has not been investigated. Based on the results of previous studies, we hypothesized that OR7D4 and OR111 are expressed in stallion testes.

Thus, the main purpose of the present study was to investigate the expression of the OR7D4 and OR111 in stallion testes. The results of the present study may provide clues regarding the roles of OR7D4 and OR111 in stallion testicular function.

MATERIALS AND METHODS

Testicular tissues preparation

No animal welfare statement or animal ethics approval was necessary as the testes used for the study were collected from horse breeding farms. Testicular samples ($n = 3$) were obtained through a field castration service in the Republic of Korea. The age of the castration horses was an adult (4–5 years old) and castration proceeded during the non-breeding season. The testes were kept at 4°C in an icebox and were immediately transferred to the laboratory. Testicular tissues were prepared as previously described (Jung et al., 2014). Testes for fixation were chopped into 1 cm³ pieces and immersed in 4% paraformaldehyde for 24 h. Tissues that were chopped into 0.5 cm³ pieces were snap-frozen in liquid nitrogen and preserved at -80°C prior to conducting Western blot analysis.

Molecular analysis

RT-PCR was conducted using a previously reported procedure with minor modifications (Kim et al., 2015). Briefly, frozen testicular tissues were thawed at room temperature. The tissues were then homogenized for total RNA extraction for 5 min by Polytron PT 1200 CL (Kinematica AG, Switzerland). The extracted RNA was quantified by a Nanodrop spectrophotometer (Thermo Scientific, USA), and diluted to the concentration of 50 ng/ μ L for RT-PCR. The OR7D4 primers, FW. 5'-CATGAACCCACGCCTTTGTG-3' and Rev. 5'-ATGAAGGGGTTTCAGCATGGG-3' (Macrogen, Rep. of Korea), and OR111 primers, FW. 5'-ACCTC-CAAGGGTCCTACTCC-3' and Rev. 5'-AGCATGGCATGACT-CACCAA-3' (Macrogen), were used. RT-PCR was performed by SimpliAmp™ Thermal Cycler (Life Technologies, USA) with the cycling conditions as follow: cDNA synthesis at 50°C for 30 min; activation at 95°C for 10 min; denaturation for 40 cycles at 95°C for 20 sec, annealing at 60°C for 20 sec, and extension at 72°C for 35 sec; and a final extension at 72°C for 5 min. The samples were then subjected to electrophoresis in 2% agarose gel for 30 min. The BioDoc-It™ Imaging System (UVP, USA) was used to capture RT-PCR results.

Western blot analysis

Western blot analysis was performed using a previously reported procedure to verify the specificity of OR7D4 and OR111 in stallion testes (Hwang et al., 2021). In summary,

frozen samples were thawed at room temperature and homogenized in a radioimmunoprecipitation assay buffer for 5 min through Polytron PT 1200 CL (Kinematica AG). Protein sample concentrations were measured by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, USA). The homogenized samples were diluted in 2x Laemmli Sample Buffer (Bio-Rad, USA), and boiled in water for 15 min. After boiling, 15 μ L of boiled protein samples were loaded into a 10% SDS-polyacrylamide gel, and electrophoresis was conducted using a Mini-Protean II system (Bio-Rad). After electrophoresis, proteins were electro-transferred to a nitrocellulose blotting membrane (GE Healthcare, USA) and blocked with a BLOTTO reagent (Becton Dickinson and Company, USA). Then, the membrane was incubated with primary antibodies: OR7D4 (diluted 1:500 in Blotto reagent, rabbit anti-human, ABP59752; Abbkine, China) and OR11I (diluted 1:300 in Blotto reagent, rabbit anti-human, ab174611; abcam, UK), which were diluted in the BLOTTO reagent for 12 h at 4°C. For the negative controls, normal rabbit IgG (2729S, Cell Signaling Technology, USA) was treated with the same concentrations of the primary antibodies onto the membrane. For determining the sequence of the primary antibody, anti-rabbit IgG, which was the HRP-linked Antibody (7074S, 1:10,000, Cell Signaling Technology), was treated as the secondary antibody for 1 h. The iBright™ CL1000 (Thermo Fisher Scientific, USA) kit was used to develop the Western blot film.

Immunofluorescence

Immunofluorescence was conducted according to a previously reported procedure with modifications (Park et al., 2017). Paraffin was removed from testicular tissue sections ($n = 3$) by washing with xylene and gradually rehydrated with 100% to 25% ethanol. Sodium citrate buffer was used for antigen retrieval in tissues at 97.5°C for about 30 min and then cooled down at room temperature for another 30 min. The tissues were then blocked with 5% donkey serum (Jackson Immuno Research, USA) diluted in PBS. The slides were incubated with OR7D4 antibody (diluted 1:200 in blocking buffer, Abbkine) and OR11I antibody (1:100, abcam) as the primary antibodies for 1.5 hours in a humid container. For negative controls, the slides were incubated with normal rabbit IgG (Cell Signaling Technology) using the same concentrations as those of the primary antibody. After washing the slides three times, the slides were incubated with 1:1,000 donkey

anti-rabbit IgG Alexa Fluor 488 (Life Technologies) as a secondary antibody for 45 min. The antibody-stained tissues were mounted with Vectashield antifade mounting medium (H-1200, Vector Laboratories, USA).

Imaging

The expression of OR7D4 and OR11I of testicular tissues were examined under LEICA DM 2500 microscope (Leica, Germany). The images of testicular tissues were captured using Leica DFC 450 C camera. Cells from OR7D4 and OR11I exhibiting green fluorescence were considered positive whereas those that did not stain with green were considered negative cells.

RESULTS

Cross-reactivity of OR7D4 and OR11I antibodies in stallion testes

Western blotting was performed to assess the cross-reactivity between testicular tissues of adult stallions ($n = 3$) and OR7D4 and OR11I. The protein band that reacted with OR7D4 appeared at an approximate weight of 38 kDa. Then, the OR11I band was identified at an approximate weight of 43 kDa (Fig. 1). The lane for negative con-

Fig. 1. Cross-reactivity of OR7D4 and OR11I in testicular tissues of the horse. The protein bands of OR7D4 and OR11I were observed at an approximate weight of 38 kDa and 43 kDa, respectively. Protein band was not detected in the negative control treated with normal rabbit IgG.

trol, which was treated with the same IgG concentration of the antibodies, did not exhibit any protein band (Fig. 1). These results suggested that the OR antibodies cross-reacted to the OR proteins in stallion testes.

The expression of OR7D4 and OR11I1 mRNA in stallion testes

RT-PCR was performed to detect the expression of OR7D4 and OR11I1 genes in stallion testes. RT-PCR results revealed that the mRNA bands of OR7D4 and OR11I1 were expressed at expected sizes: 462 bp and 655 bp, respectively (Fig. 2). No band was observed in the negative

Fig. 2. The mRNA expression of OR7D4 and OR11I1 in stallion testes. The mRNA bands of OR7D4 and OR11I1 were observed at approximately 462 bp and 655 bp, respectively. The bands were not observed in the negative control lane.

control-treated without primers (Fig. 2).

The expression of OR7D4 and OR11I1 in stallion testes

Immunofluorescence was performed to observe the localization of OR in adult stallion testicular tissues. OR7D4 immunolabeling was observed in the cytoplasm of spermatogonia only among germ cells (Fig. 3A-C). OR7D4 was also concentrated in the cytoplasm of Leydig cells (Fig. 3A-C). OR11I1 expression was concentrated in the cytoplasm of spermatogonia (Fig. 3E-G) and the cytoplasm of Leydig cells (Fig. 3E-G). No immunolabeling of OR7D4 and OR11I1 was observed in the Sertoli cells. The negative control treated with normal rabbit IgG instead of antibody showed no immunolabeling of OR7D4 (Fig. 3D) and OR11I1 (Fig. 3H), respectively.

DISCUSSION

This study was performed to detect the presence of OR7D4 and OR11I1 in stallion testes. The expression of OR7D4 and OR11I1 in the horse testes were verified by using RT-PCR procedure and Western blot. As a result of RT-PCR, the band of mRNA for both OR7D4 and OR11I1 appeared at expected molecular sizes. These results of

Fig. 3. Localization of OR7D4 and OR11I1 in horse testes. OR7D4 and OR11I1 were expressed in the cytoplasm of spermatogonia and Leydig cells (A-C, E-G). The expressions of OR7D4 and OR11I1 were not detected in the negative control treated with normal rabbit IgG (D, H). The arrowhead indicates the following by color: Red = spermatogonia; White = Leydig cell. Scale bar = 25 μ m.

RT-PCR indicate that mRNA of OR7D4 and that of OR11I are expressed in the horse testes. Subsequently, Western blot bands for OR7D4 and OR11I were observed at appropriate locations, 38 kDa and 43 kDa, respectively. The present of the Western blot bands indicate that the antibodies cross-react with stallion testicular tissues. The OR7D4 antibody used in this study was previously validated for horse tissues (Choi and Yoon, 2021). However, the bands at 50 kDa, which is not expected, appeared in both Western blot bands for OR7D4 and OR11I. Previously, no band at 50 kDa appeared with VNO and OE tissue of horses when Western blot was performed with the same antibody used in the present study (Choi and Yoon, 2021). Western blot procedures with several different running conditions were conducted to get rid of this band, but the 50 kDa band still appeared. Normally, vast of proteins are expressed in the testicular tissues (Djureinovic et al., 2014). It is considered that protein with size of 50 kDa expressed in the testes was non-specifically reacted with both antibodies during Western blot procedure. As the result of immunofluorescence, there were no same pattern of immunofluorescent staining for both antibodies. If both antibodies truly recognize the protein located at 50 kDa, at least one of the immunofluorescent staining on the testes for both antibodies must be matched. Therefore, the band that appeared at 50 kDa is the result of false staining. Based on the results of this study, it is concluded that mRNA and protein for OR7D4 and OR11I are expressed in the horse testes.

The result of immunofluorescence revealed that OR7D4 was localized in the cytoplasm of spermatogonia and Leydig cells. However, there was no immunolabeling of OR7D4 in Sertoli cells. OR7D4 is the receptor for androstenone, which is produced in Leydig cells (Sinclair et al., 2005). The production of androstenone is controlled by the neuroendocrine system, mainly through the effects of luteinizing hormone (LH) (Zamaratskaia and Squires, 2009). Thus, the presence of OR7D4 in spermatogonia and Leydig cells suggests that androstenone plays a role as a paracrine and autocrine factor, respectively. In addition, the secretion of LH is driven by photoperiod and less secretion in autumn than in spring (Clay et al., 1988; Aurich et al., 1994; Dhakal et al., 2011). Thus, the androstenone-OR7D4 system in the testes could vary between the breeding and non-breeding season. The difference in seasonal OR7D4 expression in horse testes can be a clue

to identify the functional roles of androstenone-OR7D4 system in spermatogonia and Leydig cells.

In this study, OR11I was localized in the cytoplasm of the spermatogonia of stallions and Leydig cells. Interestingly, the localization pattern of OR11I in stallion testes was different from that in human testes. In humans, OR11I expression was localized in spermatocytes and spermatids, but not in spermatogonia and Leydig cells (Milardi et al., 2018). These results indicate that OR11I expression in the testes varies depending on the species. Although the presence of OR11I in the testes of humans and stallions was explored, the odorant molecule that reacts with OR11I has remained to identify. Therefore, in order to establish the functional role of OR11I in the horse testes, it is necessary to establish a specific odorant molecule targeted by OR11I.

CONCLUSION

The expression of OR7D4 and OR11I in testicular cells suggests that the odorant molecules for these receptors may have various functions within the testes as paracrine and autocrine factors. In the future study, it should be identified the functional roles of OR7D4 and OR11I in the horse testis.

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