

**Studies on the expression and role of a transmembrane
receptor CD36 in the mammalian olfactory system**

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GENERAL INTRODUCTION

Chemosensory system is responsible for the detection and recognition of a variety of chemicals in the environment, and thus, plays an important part in the maintenance of life. In mammals, including humans, the chemosensory system consists of gustatory and olfactory ones, serving for the sensing of taste and smell, respectively. The sense of taste is important for distinguishing the nutritive and beneficial compounds from ones that are unnecessary or potentially toxic to the host. On the other hand, the sense of smell is crucial to the rapid judgement of appropriate and inappropriate foods. In recent years, much effort has been directed to identify sensor proteins that are localised in the oral and nasal cavities [1].

Cluster of differentiation 36 (CD36) is a two-transmembrane protein that is found in various tissues and cells of vertebrates. This protein is believed to play diverse functions associated with its ability to recognise a number of amphiphilic molecules such as polar lipids [2, 3]. As a scavenger receptor, CD36 in macrophages recognises oxidised low-density lipoproteins [4]. It can even recognise fatty acids and controls the uptake of the lipid species in small intestine and adipose tissues [5]. Furthermore, its presence in the taste cells [6], a reduced palatability of fatty acids in CD36-knockout mice [7], and a higher response to fatty acids (increase in intracellular calcium concentration) in CD36-positive taste bud cells [8] suggest that this protein contribute to the gustatory detection of fatty acids. To date, a family of membrane proteins called sensory neuron membrane proteins (SNMPs), the insect homologues of CD36, has been demonstrated to exist on the surface of olfactory dendrites and to participate in the detection of volatile compounds, including pheromones [9, 10]. The studies on SNMPs had prompted the author to investigate whether CD36 plays a role in the olfactory system. However, little is known about the relationship between CD36 and olfaction.

This thesis work aimed to provide evidence that CD36 plays a part in the olfactory system of mammals using mice as a model. First, the author investigated the localisation of this protein in the olfactory mucosa. Next, the author assessed whether mice consciously perceive a potent ligand of CD36, 1-(palmitoyl)-2-(5-keto-6-octanedioyl)phosphatidylcholine, and if so, whether CD36 is involved in the sensing of the oxidised phospholipid species. Furthermore, the author investigated if CD36 is involved in the perception (olfactory perception) of an odour-active volatile compound present in

foods, oleic aldehyde [11]. Results obtained through this thesis work indicate that CD36 exists abundantly in the olfactory epithelium and may participate in the detection of specific odorants in the nasal cavity. This work would make a contribution to expanding the knowledge and understanding of not only the diverse biological functions of this receptor but also the mechanism by which mammals recognise individual odorants in the nasal cavity.

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CHAPTER 1

Expression of CD36 in the olfactory receptor cells and its abundance on the epithelial surface in mice

Introduction

CD36 is a transmembrane protein that is expressed in a variety of cells, including monocytes/macrophages, adipocytes, myocytes and taste bud cells [1, 2]. This protein has a variety of physiological functions, which are associated with its ability to recognise a number of amphiphilic molecules such as polar lipids [1–4]. For example, CD36 recognises and binds the oxidised phospholipids that can occur on the surface of oxidised low-density lipoproteins and mediates the uptake of these particles in macrophages [5, 6]. Another important function of CD36 is to detect and regulate the uptake of long-chain fatty acids (LCFAs) [7–9]. CD36-knockout mice exhibit an increase in the level of serum-free LCFAs and a reduced uptake of oleic acid in adipocytes during fasting [8]. Muscle-specific over-expression of CD36 in mice results in enhanced FA oxidation in contracting tissues [9]. Moreover, CD36 appears to play a key role in the gustatory perception of LCFAs, as suggested by its presence in the apical region of rat taste bud cells [10], a reduction of LCFA palatability in CD36-knockout mice [11] and an LCFA-induced increase in the concentration of intracellular free calcium in taste bud cells expressing CD36 [12].

A family of membrane proteins in insects called sensory neuron membrane proteins (SNMPs) has structural features similar to those of the class B scavenger receptor family, which includes CD36 [13]. Importantly, some of the SNMPs have been demonstrated to exist on the surface of olfactory dendrites and are involved in the recognition of amphiphilic molecules [14–16]. Despite these studies on SNMPs in insects, few studies have been carried out on the putative function of CD36 in the mammalian olfactory system. The detection of CD36 mRNA in the mouse olfactory epithelium has been reported only to date [17]. In chapter 1, the author aimed to confirm CD36 expression in the olfactory epithelium of mice and to determine the site(s) of production and function within the tissue. CD36 was found to be expressed by the

receptor and supporting cells of the olfactory epithelium, and it is abundant on the epithelial surface containing the olfactory cilia. Furthermore, the author also demonstrated the presence of CD36 on the cilia of nasal respiratory epithelium. These results suggest that CD36 plays role(s) in the olfactory system.

Materials and Methods

Animals

This study was approved by the Kyoto University Animal Experimentation Committee, and experiments were conducted in accordance with both the ethics guidelines of the above-mentioned committee and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. CD36-knockout mice were kindly provided by Mason W. Freeman, MD [18] and maintained on a C57BL6/J background. Wild-type and CD36-knockout littermates were bred from the same cross and were, thus, of identical genetic background. Animals used were 8–12 weeks old, unless otherwise noted. All process was performed under sodium pentobarbital anesthesia, and all efforts were made to minimise suffering and the number of animals that necessary to produce reliable scientific information.

Genotyping

The author extracted genomic DNA from the tails of mice. Genotyping was done using the GoTaq Hot Start green Master Mix (Promega; Madison, WI, USA) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was carried out using a PC-708-02 thermal cycler (ASTEC; Fukuoka, Japan). PCR products were separated on a 2% agarose gel, and visualised using SYBR Gold Nucleic Acid Gel Stain (Invitrogen; Carlsbad, CA, USA). The author used a primer set: 5'-AGCTCCAGCAATGAGCCCA-C-3' (forward for wild-type mice), 5'-TGGAAGGATTGGAGCTACGG-3' (forward for CD36-knockout mice), and 5'-CATACATTGCTGTTTATGCATGA-3' (reverse for wild-type and CD36-knockout mice) [19].

Reverse transcription (RT)-PCR

The author extracted total RNA from the nasal mucosa and liver of mice using the RNeasy Mini Kit (Qiagen; Tokyo, Japan). RT-PCR was performed as described previously [20]. Primer sets used were as follows: 5'-GAGCCATCTTTGAGCCTTCA-3' and 5'-TCAGATCCGAACACAGCGTA-3' (for CD36); 5'-CCCTGTGCTGCTC-ACC-3' and 5'-GCACGATTTCCTCTCAG-3' (for β -actin). The products amplified were analysed as described above.

In situ hybridisation analysis

Two non-overlapping antisense oligonucleotide probes (45 bp in length) were designed for the *in situ* detection of the mRNAs for mouse CD36. The probe was complementary to the sequence of nucleotides 576 to 620 of mouse CD36 mRNA, and was labelled with ^{33}P -dATP using terminal deoxynucleotidyl transferase (Invitrogen). Fresh-frozen sections of 14- μm thickness were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 15 min, and then acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0) for 10 min. Hybridisation was performed at 42°C for 10 h with a hybridisation buffer containing the ^{33}P -labelled oligonucleotide probes (10,000 cpm/ μl). The sections were rinsed in 2 $\times$ SSC (1 $\times$ SSC: 150 mM sodium chloride, 15 mM sodium citrate) containing 0.1% *N*-lauroylsarcosine sodium at room temperature for 30 min, and then rinsed twice in 0.1 $\times$ SSC containing 0.1% *N*-lauroylsarcosine sodium at 55°C for 40 min. The sections were dehydrated through a graded series of ethanol and air-dried. Sections were immersed in an autoradiographic emulsion (NTB-2; Kodak, Rochester, NY, USA) at 4°C for 8–10 weeks. After development, the hybridised sections were counterstained with hematoxylin.

Western blot analysis

Tissue pieces were lysed by sonication in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl and 0.5% (v/v) NP-40 containing protease inhibitor cocktail (P8340, Sigma-Aldrich; St. Louis, MO, USA) and phosphatase inhibitor cocktail (P0044, Sigma-Aldrich). The protein concentrations in the tissue lysates were determined using the bicinchoninic acid protein assay kit (Pierce Chemical; Rockford, IL, USA). The lysates (40 μg of protein) and a molecular weight standard (MagicMark XP, Invitrogen) were subjected to reducing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, on a 15% gel) followed by transfer to a polyvinylidene difluoride membrane. After blocking with Tris-buffered saline (pH 7.4) containing 5% skim milk and 0.1% Tween-20 for 1 h, the blot was incubated overnight at 4°C with a goat polyclonal anti-CD36 antibody (1:2000; AF2519; R&D Systems, Minnesota, MN, USA). After washing the blot, peroxidase-labelled polyclonal rabbit anti-goat IgG secondary antibody (1:1000; P0449; Dako; Tokyo, Japan) was added. Immunolabelled proteins were detected using an ImageQuant LAS-4000 mini chemiluminescent imager (GE Healthcare; Tokyo, Japan).

Immunohistochemistry

The mice were anaesthetised with sodium pentobarbital (10 mg/kg body weight) and were perfused *via* the aorta with saline (0.5 ml/g) followed by a fixative (4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4). The heads and tongues were removed and immersed in the same fixative for an additional 2 days. For decalcification, the heads were immersed in 5% ethylenediaminetetraacetic acid for 3 weeks at 4°C. The decalcified heads were immersed in 30% sucrose solution overnight at 4°C, embedded in optimal cutting temperature compound (Sakura Finetek; Tokyo, Japan), and quickly frozen in liquid nitrogen. Frozen sections were mounted on poly-L-lysine-coated glass slides. After pre-treatment with phosphate-buffered saline (PBS) containing 0.3% Triton X-100 and normal donkey serum, the sections were incubated overnight with AF2519 (1:2000). The sections were then incubated with Cy3-labelled donkey polyclonal anti-goat IgG (Jackson ImmunoResearch; 1: 200; 705-165-147; West Grove, PA, USA). The nuclei were counterstained with SYTO 13 (Invitrogen). For double immunofluorescence staining, the sections were incubated overnight with AF2519 and the following antibodies; rabbit polyclonal antibody to olfactory marker protein (OMP) (anti-OMP; 1:1000; O7889; Sigma-Aldrich), rabbit polyclonal antibody to PGP9.5 (anti-PGP9.5; 1:2000; RA95101; UltraClone Ltd, Rossiters, UK) and rabbit polyclonal antibody to S100 protein (anti-S100; RY320: 1:4000; Yanaihara Institute, Fujinomiya, Japan). Then, they were incubated with Cy3-labelled donkey anti-goat IgG and Alexa Fluor 488-labelled donkey anti-rabbit IgG (Invitrogen). The stained sections were mounted in glycerol/PBS and observed under a confocal laser-scanning microscope (FV1000; Olympus; Tokyo, Japan).

Silver-intensified immunogold labelling method for electron microscopy

Frozen sections were incubated overnight with anti-CD36 antibody (1 µg/ml) and, subsequently, with rabbit anti-goat IgG covalently linked with 1-nm gold particles (1:200; Nanoprobes; Yaphank, NY, USA). Following silver enhancement using the HQ SILVER enhancement kit (Nanoprobes), the sections were osmicated, dehydrated and directly embedded in Epon (Nisshin EM; Tokyo, Japan). Ultrathin sections were stained with uranyl acetate and lead citrate, and examined under an electron microscope (H-7100; Hitachi; Tokyo, Japan).

Results

RT-PCR and in situ hybridisation analyses of CD36 in the nasal mucosa of mice

CD36 expression in the murine olfactory mucosa has previously been demonstrated using RT-PCR and *in situ* hybridisation methods [17]. In chapter 1, the author examined RT-PCR detection of CD36 mRNA in the nasal mucosa of wild-type and CD36-knockout mice. The β -actin gene *Actb* was evaluated as a control for RNA degradation. The expression of both genes in the liver was examined as a reference. PCR products for both CD36 (490 bp) and *Actb* (328 bp) were detected in the nasal mucosa and in the liver samples of wild-type mice (Fig. 1A). In the nasal mucosa samples of CD36-knockout mice, amplicons of *Actb*, but not of CD36, were detected (Fig. 1A). In control RT-PCR mixtures prepared without reverse transcriptase, neither CD36 nor *Actb* products were detected (Fig. 1A).

Next, the author aimed to detect CD36 mRNA in the olfactory mucosa by using *in situ* hybridisation with ^{33}P -labelled oligonucleotides. When sections of the olfactory mucosa were probed with an antisense oligonucleotide directed against CD36 mRNA (576–620), aggregated signals were detected within the epithelial layer (Fig. 1B, black arrows in the upper panel). This observation is consistent with previously reported results [17]. However, unlike previous results [17], signals were also detected consistently in the superficial layer of the olfactory epithelium, which is occupied by supporting cells (Fig. 1B, white arrows in the upper panel). In addition, CD36 mRNA expression was detected in the superficial layer throughout the respiratory area (Fig. 1B, a black arrowhead on the lower panel indicates the boundary between olfactory and respiratory areas).

Sections of whole-head preparations from wild-type and CD36-knockout mice were subjected to *in situ* hybridisation with the probe for CD36 mRNA (576–620). Hybridisation signals were detected in the distinct areas of the section obtained from a wild-type mouse, including those of nasal mucosa, sebaceous gland and tongue, whereas no significant ones could be seen throughout the section from a CD36-knockout mouse (Fig. 2). These results support the validity of *in situ* detection of CD36 mRNA.

Western blot analysis of CD36 expression in the nasal mucosa of mice

The author analysed CD36 protein expression in the nasal mucosa of wild-type mice by SDS-PAGE and western blotting. Lysates of liver and skeletal muscle (tissues for CD36 expression) were subjected to the analysis simultaneously with that of nasal mucosa for a qualitative assessment of the resulting data. Various anti-CD36 antibodies were tested, but only one (AF2519) produced the expected 88-kDa band. The band was detected in the nasal mucosa as well as in the liver and skeletal muscle samples (Fig. 3). It is noted that the level of the signal in the lysate of nasal mucosa was comparable (or even superior) to that in those of the other tissues. Nasal mucosa, liver and skeletal muscle lysates from CD36-knockout mice did not yield apparent protein bands around the 88-kDa position with the anti-CD36 antibody (Fig. 3).

Immunolocalisation of CD36 in the olfactory mucosa of mice

The main purpose was to localise CD36 protein in the olfactory mucosa. The author used AF2519 as the probe for immunohistochemical localisation of CD36. When a section of olfactory mucosa of wild-type mice was probed with the antibody, some but not all cells in the deeper epithelial layer were stained (Fig. 4A). The cells extended a slender process to the surface of the epithelium passing through the supporting cell layer, thus, being identifiable as olfactory receptor cells (ORCs). In addition, the surface layer of the epithelium was found to be intensely stained (Fig. 4A).

The author performed double immunostaining of the olfactory mucosa of wild-type mice with AF2519 and an antibody against OMP (a marker protein for ORCs), and found that CD36-immunoreactive cells within the epithelial layer stained positive with anti-OMP antibody (Fig. 4B), further confirming the production of CD36 by ORCs. However, the CD36 antibody failed to stain the olfactory nerves of various sizes running in the lamina propria (Fig. 4B, white arrows). Olfactory nerve elements, which were stained with anti-OMP antibody, were immunonegative with AF2519 in the olfactory bulb (Fig. 5). When a section of olfactory mucosa from CD36-knockout mice was stained, immunoreactivity with AF2519 was not visible in either the ORCs or the surface layer, while the staining pattern by the anti-OMP antibody was similar to that observed in wild-type mice (Fig. 4C), confirming the validity of the immunolocalisation of CD36 in wild-type mice. Staining of the surface layer in the nasal respiratory area with the antibody was evident in wild-type mice but not in CD36-knockout mice (Fig. 4D and 4E, respectively). In the context of olfactory mucosa of wild-type mice, double

immunostaining with AF2519 and an antibody against a pan-neuronal marker, PGP9.5, gave comparable results to those with AF2519 and anti-OMP antibody (Fig. 6). In double immunostaining of the olfactory mucosa of wild-type mice with AF2519 and an antibody raised against a glial marker S100, the author could not yet detect signals for CD36 protein on the bundles running in the lamina propria where glial (labelled in a green colour) and neuronal elements coexist (Fig. 6).

The author also performed immunostaining with AF2519 of the nasal respiratory mucosa and the site of pheromone detection, vomeronasal organ (VNO), of wild-type and CD36-knockout mice. The staining could not detect apparent signal for CD36 in the sensory epithelium of VNO in either wild-type or CD36-knockout mice (Fig. 7).

Immunoelectron microscopic analysis of CD36 localisation in the mouse nasal mucosa

The author further analysed the localisation of CD36 in the olfactory and respiratory areas of nasal mucosa by immunoelectron microscopy with AF2519 and a colloidal gold-labelled anti-goat IgG antibody. Representative data for the olfactory epithelium of wild-type mice are presented in Fig. 8A, 8B and 8C. Dotted particles were evident in the soma and dendrites of distinct ORCs within the epithelium (black arrows in A and B). In the surface layer, the particles heavily labelled the olfactory cilia (white arrows in A and B) and also attached on the microvilli of olfactory supporting cells (white arrowheads in B). Electron microscopic images within which no or few dotted particles were visible on ORCs more clearly showed the localisation of signals on the microvilli of supporting cells (white arrowheads in C). Representative data for the respiratory epithelium of wild-type mice are shown in Fig. 8D. Immunogold particles were evident in the cilia of respiratory ciliary cells. On the other hand, very few immunogold particles were present in the somata of supporting cells of olfactory epithelium (Fig. 8A, 8B and 8C) and of ciliary cells of respiratory epithelium (Fig. 8D).

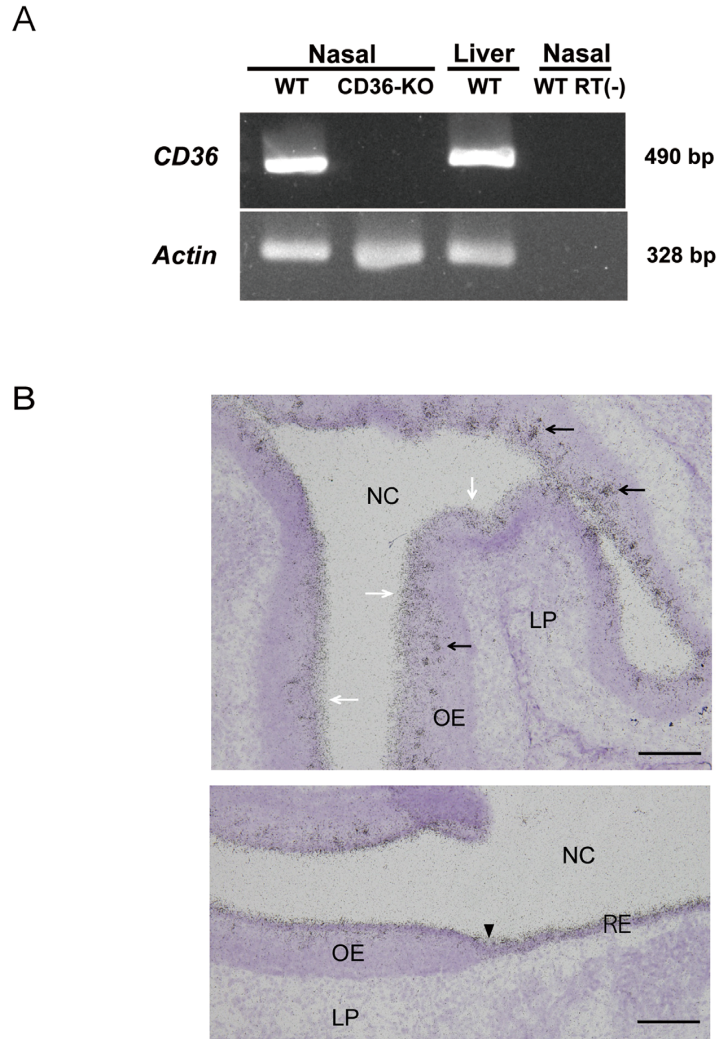


Fig. 1. Detection of CD36 mRNAs in the nasal mucosa of mice. (A) RT-PCR detection. RT-PCR was conducted as described in the Materials and Methods section using nasal samples of wild-type (WT) and CD36-knockout mice (CD36-KO). RT(-) indicates the reaction without reverse transcriptase. (B) *In situ* hybridisation analysis of the nasal mucosa of wild-type mice. The upper and lower panels show the data of olfactory mucosa and the transitional zone to the respiratory area, respectively. Aggregated signals are distributed within the epithelial layer (black arrows in the upper panel). The signals were also detected consistently in the superficial layer of the olfactory epithelium (white arrows in the upper panel). A black arrowhead in the lower panel indicates the boundary between the olfactory (left-hand side) and respiratory (right-hand side) mucosa. LP, lamina propria; NC, nasal cavity; OE, olfactory epithelium; RE, nasal respiratory epithelium. Bar: 100 μ m (upper panel), 50 μ m (lower panel).

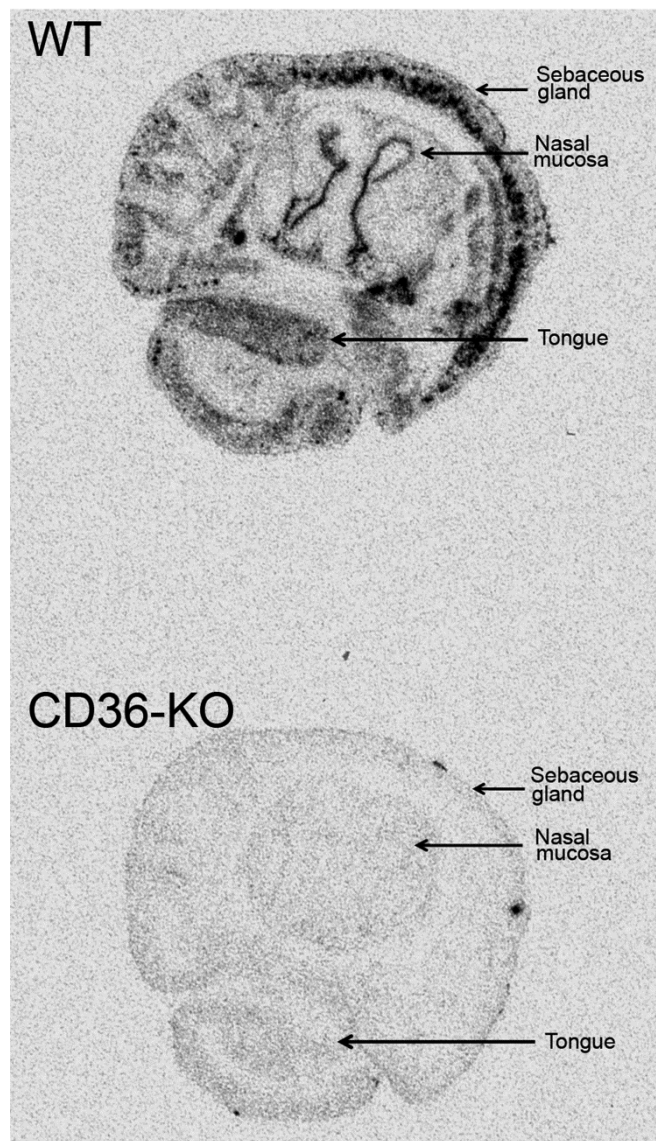


Fig. 2. *In situ* hybridisation analysis of sections of whole-head preparations from wild-type (WT) and CD36-knockout mice (CD36-KO) with ^{33}P -labelled antisense oligonucleotide directed against CD36 mRNA (576–620) as a probe. Sections were obtained using one each of wild-type and CD36-knockout littermates at postnatal day 5. Signals were detected by autoradiography on X-ray film.

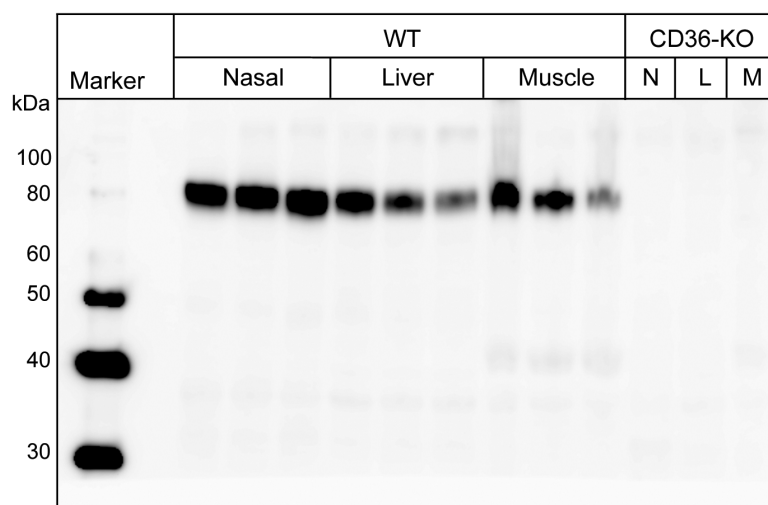


Fig. 3 Western blot analysis of lysates from the nasal mucosa (and other tissues) of mice.

Lysates obtained from the nasal mucosa (Nasal), liver and skeletal muscle (Muscle) of wild-type mice (WT) and those (N; nasal mucosa, L; liver, M; skeletal muscle) of CD36-knockout mice (CD36-KO) were analysed by SDS-PAGE and western blotting using AF2519 as the probe. The molecular sizes of the protein marker (Marker) are indicated on the left in kilodaltons (kDa).

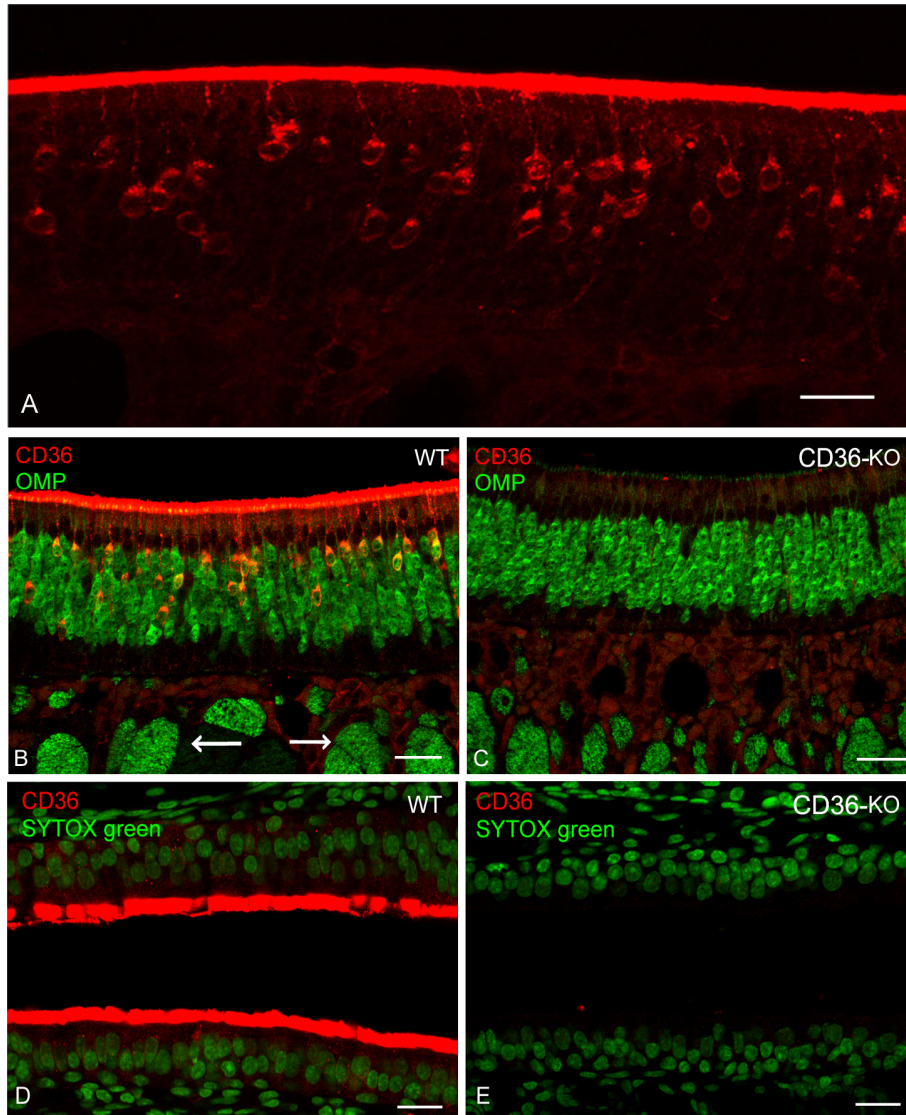


Fig. 4. Immunolocalisation of CD36 in the mouse nasal mucosa. (A) Immunostaining of the olfactory mucosa of wild-type mice with AF2519. Positive immunoreactivity for CD36 is seen in cells with thin dendrites and in the surface layer. (B, C) Double immunostaining of olfactory mucosa with AF2519 (red) and an anti-OMP antibody (green) of olfactory mucosa in wild-type (WT) and CD36-knockout (CD36-KO) mice. In (B), bundles of nerve fibres in the lamina propria are indicated by white arrows. (D, E) Immunostaining of respiratory mucosa of wild-type (WT) and CD36-knockout mice (CD36-KO) with AF2519. The red-coloured immunoreactivity for CD36 in the surface layer was not evident in CD36-knockout mice. In (D) and (E), nuclei are labelled with SYTOX (green). Bar: 100 μ m (A), 50 μ m (B, C, D and E).

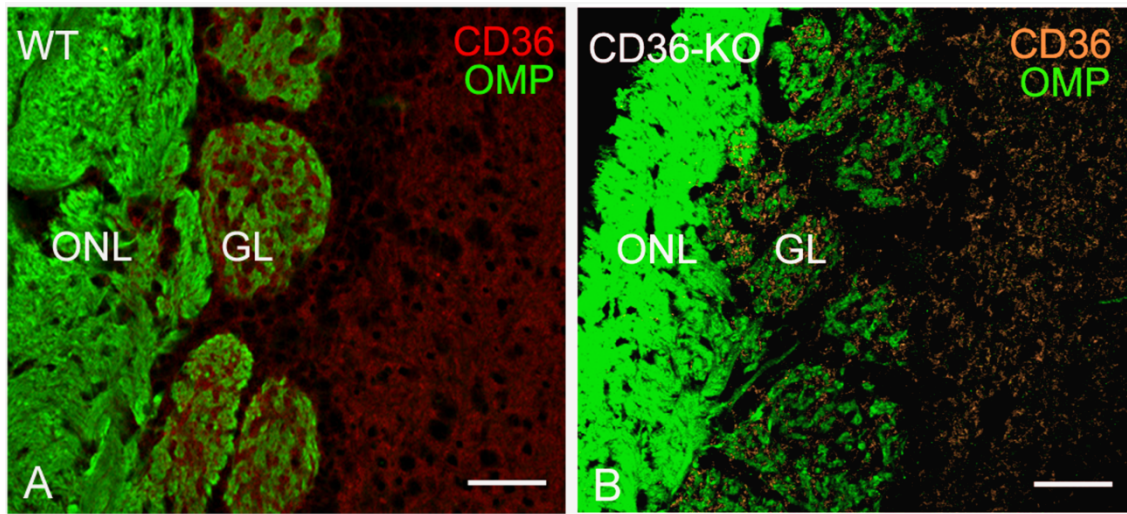


Fig. 5. Double immunostaining of the olfactory bulb from wild-type (WT) and CD36-knockout mice (CD36-KO) with AF2519 (red) and anti-OMP antibody (green). Axons forming the olfactory nerve layer (ONL) and glomeruli (GL) were negative for staining with AF2519. Bar: 20 μ m (A, B).

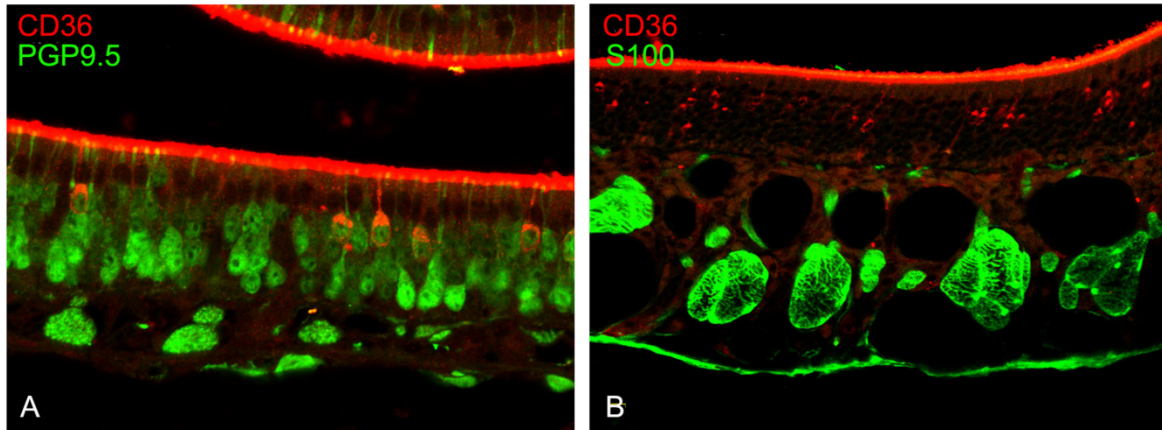


Fig. 6. Double immunostaining of the olfactory mucosa of wild-type mice with AF2519 (red) and anti-PGP9.5 antibody (green) or anti-S100 antibody (green). The red-coloured immunoreactivity for CD36 occurred in some ORCs but not evident in nerve bundles running in the lamina propria. Neuronal and glial cells are labelled green with antibodies for PGP 9.5 and S100, respectively. Bar: 20 μm (A, B).

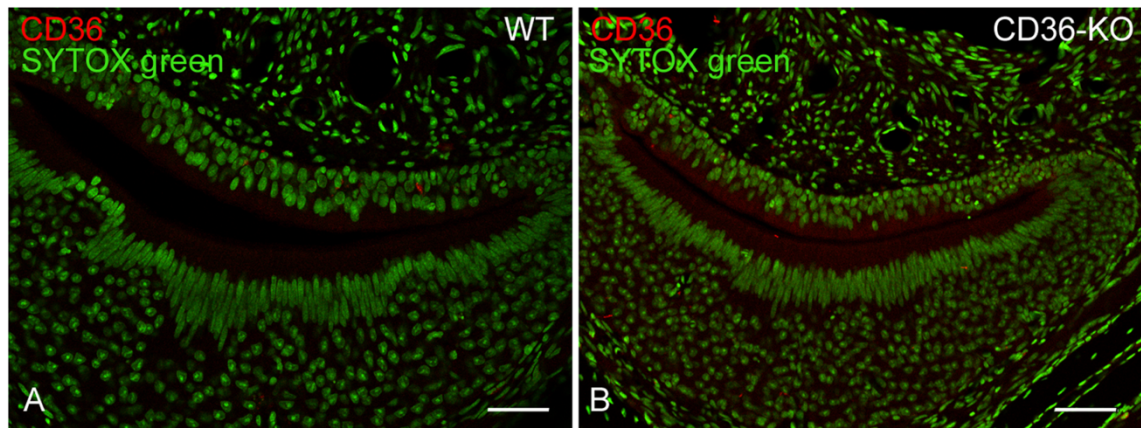


Fig. 7. Immunostaining of mouse VNO with AF2519. The red-coloured immunoreactivity for CD36 was not evident in either wild-type (WT) or CD36-knockout mice (CD36-KO). In both panels, nuclei are labelled with SYTOX (green). Sections were obtained using two each of wild-type and CD36-knockout littermates. Bar: 20 μ m.

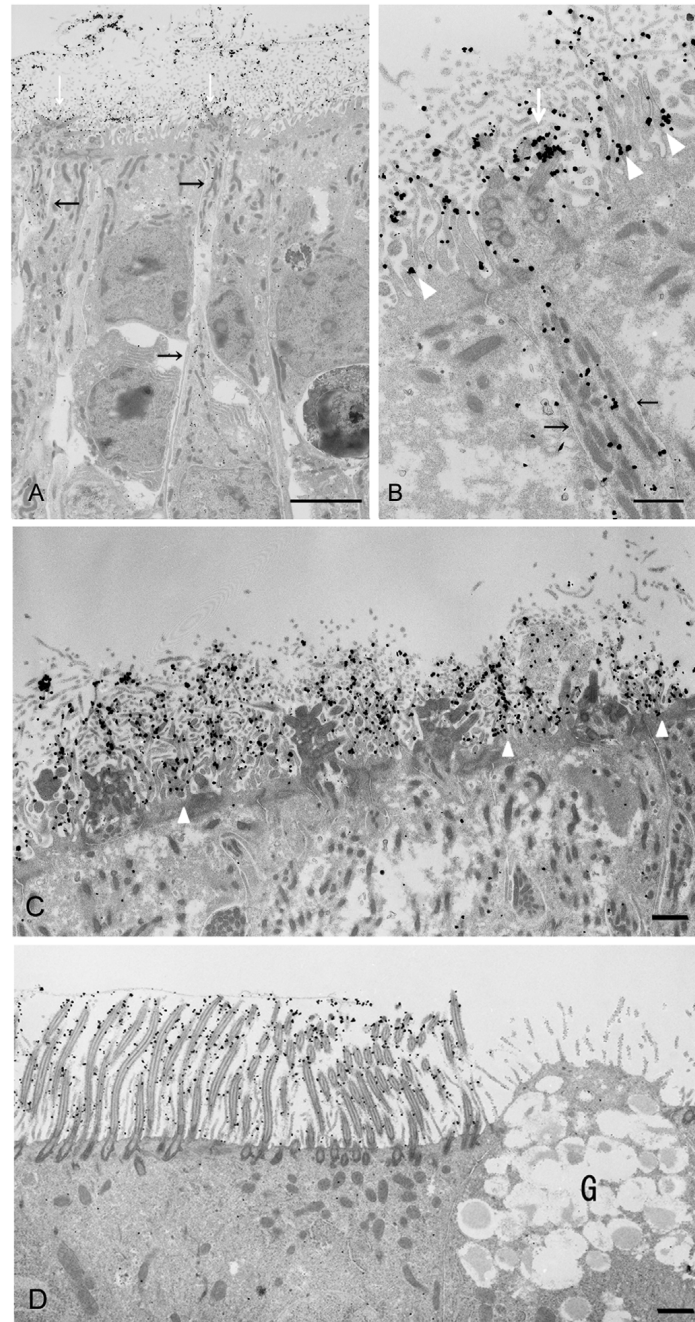


Fig. 8. Immunoelectron microscopic analysis of CD36 localisation in the nasal mucosa of wild-type mice. Immunoelectron microscopic analysis of the nasal olfactory (A, B and C) and respiratory (D) epithelium. Dotted particles showing the existence of CD36 distribute in the somata and dendrites of ORCs (black arrows in A and B). The particles on the olfactory cilia and on or around the microvilli of supporting cells are indicated by white arrows (A and B) and white arrowheads (B and C), respectively. G: goblet cells. Bar: 5 μ m (A), 1 μ m (B, C, D).

Discussion

Using *in situ* hybridisation and immunohistochemical analyses, the author provided compelling evidence for the production of CD36 by ORCs in the mouse olfactory epithelium. Strikingly, the author observed an intense immunoreactivity for CD36 in the surface layer throughout the olfactory epithelium (Fig. 4A). It seemed likely that these surface signals were, at least in part, due to CD36 protein produced in ORCs and transported to the cilia, as immunogold particles were present in the dendrites and somata of ORCs and cilia (Fig. 8A and 8B). Altogether, the author proposes that a major site of function for CD36 in the olfactory mucosa is on the cilia.

CD36 protein was rarely detectable in the nerve bundles running in the lamina propria of olfactory mucosa, the axons forming the olfactory nerve layer in the outermost layer of the bulb and axon terminals in the glomeruli (Figs. 4 and 5). The CD36 localisation pattern is distinct from that of G-protein-coupled odorant receptors, which are often observed in the axon termini as well as in somata, dendrites and cilia [21, 22]. On the other hand, the CD36 localisation pattern resembles that of certain SNMPs in the invertebrate olfactory organs. For example, Snmp-1 protein in the antenna of the silk moth localises to the cilia, dendrites and somata, but rarely to the axons or nerve bundles [14, 16]. Interestingly, CD36-related protein has been found to co-localise with G-protein-coupled odorant receptors and is postulated to act as a co-receptor [15]. These findings, together with the specific immunolabelling pattern in distinct ORCs (Fig. 4A), led the author to speculate that CD36 is produced only by ORCs expressing certain types of odorant receptors, and that it plays a supportive role for these receptors.

SNMPs have been found to serve as co-receptors for distinct types of pheromone receptors [15]. Furthermore, in the mouse brain, CD36 mRNA has been shown to express in the regions involved in reproductive behaviour (*e.g.*, bed nucleus of stria terminalis), which is thought to primarily rely upon the detection of pheromone by VNO [23]. These previous findings lead to the hypothesis that CD36 could play a role in the detection and/or delivery of pheromones in specific neural circuits of mice. However, CD36 is unlikely to serve to detect pheromones at the VNO of mice. Indeed, no significant signals for CD36 protein were detected in the sensory epithelium of VNO (Fig. 7). Reproductive behaviours in mice are also known to be affected by odorants

detected through main olfactory epithelium [24]. Involvement and role of CD36 in the murine reproductive behaviours await further investigation.

The author detected CD36 mRNA expression consistently in the superficial cell layer of the olfactory epithelium, where the supporting cells are localised (Fig. 1B). However, signal for CD36 protein was detected only on or around microvilli of the supporting cells (Fig. 8B and 8C). These findings, together with the intense signals observed throughout the surface layer of the olfactory epithelium (Fig. 4A), lead the author to assume that a number of CD36 molecules produced in olfactory supporting cells are released into the extracellular milieu (*e.g.*, into the mucous layer of the olfactory epithelium). In fact, CD36 is known to exist in the plasma fraction of blood, as a constituent of microparticles derived from platelets and other cells [25]. The destiny of CD36 produced in the olfactory supporting cells, including the formation of CD36-containing microparticles from the cells, awaits further investigation. The author also detected CD36 mRNA in the superficial cell layer of the nasal respiratory epithelium (Fig. 1B), and revealed many immunogold particles attached onto cilia of the ciliary cells (Fig. 8D). Certain SNMPs have been shown to exist in non-neuronal epithelial cells and in supporting cells around the olfactory sensory neurons [15, 16]. *Heliothis virescens* SNMP-2 is postulated to be involved in quick pheromone clearance of the cuticular sensilla lymph to allow for highly sensitive olfactory detection [26]. By analogy, the author suggests that CD36 expressed by the olfactory supporting cells and respiratory ciliary cells contribute to the clearance of noxious substances such as oxidised phospholipids present in the nasal cavity.

In summary, the author found that CD36 is produced by ORCs and that it occurs abundantly in the superficial layers of the olfactory epithelium. The site of production and the localisation, together with its ability to recognise a variety of amphiphilic molecules, lead to the proposition that CD36 is intimately involved in olfactory reception. In addition, CD36 expression in olfactory supporting cells and nasal respiratory ciliary cells suggests additional role(s) for this protein in the nasal cavity.

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CHAPTER 2

A role of CD36 in the conscious perception of an oxidised phospholipid species in mice

Introduction

Animals have highly developed chemosensory systems that enable efficient detection of diverse soluble or volatile compounds in the environment. Detection of exogenous molecules by taste bud cells and/or olfactory receptor cells transmits signals to the brain and consequently induces various behavioural responses in animals (*e.g.*, appetitive or aversive response to foods) [1, 2]. To better understand the mechanisms underlying chemosensory perception, efforts have been made to identify sensory proteins in the oral and nasal cavities [3, 4].

CD36 is a broadly expressed transmembrane protein associated with the recognition of multiple molecules (*e.g.*, polar lipids) [5, 6]. For example, in macrophages, CD36 contributes to the clearance of oxidised forms of low-density lipoprotein from the blood by recognising distinct phospholipid species on the surface of the particles [7, 8]. Furthermore, CD36 expressed by enterocytes appears to participate in not only recognition and absorption of long-chain fatty acids but also in formation of chylomicrons [9]. In addition to its well-characterised functions, CD36 has been postulated to play a role in the chemosensory detection of long chain fatty acids in the oral cavity, leading to an appetitive response to the lipid species in rodents. Several lines of evidence support this finding: CD36 is localised to the apical surface of taste bud cells in the circumvallate papillae of rats [10], long-chain fatty acids increase the concentration of intracellular free calcium in taste bud cells where CD36 is considered to be expressed [11], and mice with CD36 gene disruption exhibit a reduced preference to long-chain fatty acids such as linoleate [12].

A species of oxidised phospholipid, 1-(palmitoyl)-2-(5-keto-6-octene-diyl)-phosphatidylcholine (KODiA-PC), is one of the most potent ligands for CD36 in lipids [20]. Oxidised forms of phospholipids, including KODiA-PC, are believed to occur on the surface of low-density lipoproteins during periods of oxidative stress and on the

surface of cells undergoing apoptosis [8, 13]. Animals, including humans, might often encounter or even ingest such phospholipid species in the environment. It is, however, unclear whether animals consciously perceive these phospholipid species occurring outside the body and, if so, whether CD36 is involved in the process of perception. In this chapter, the author provided evidence that (i) mice could perceive KODiA-PC and (ii) CD36 could contribute to the sensing of a lower level of the oxidised phospholipid species in the animal. The author also demonstrates that the perception of KODiA-PC relies considerably on the olfactory system in the animal.

Materials and Methods

Animals.

CD36-knockout mice (C57BL6/J background) [14] were provided as described previously [15]. Wild-type and CD36-knockout littermates were obtained from the same breeding pair and thus have a genetically identical background. To genotype the mice lines, the author used the primer set described in a previous study [14]. Mice were bred under a constant temperature (22°C) and a 12 : 12 h light–dark cycle (dark phase of 18 : 00–6 : 00). One week prior to experiments, each of the mice was individually housed with *ad libitum* access to water and standard mouse chow (MF; Oriental Yeast, Japan). All mice used were between 8 and 12 weeks of age. All the tests including the training sessions were performed at 18 : 00 under food and water deprivation that began 1 h prior to the tests. This animal experiment was performed in accordance with the ethics guidelines of the Kyoto University Animal Experimentation Committee and was in complete compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Olfactory nerve transection (ONX).

The author conducted the transection of olfactory nerves as described previously [16]. Briefly, mice were anaesthetised with 1% sodium pentobarbital (10 µl/g body weight) and placed in a stereotaxic instrument using ear bars and an incisor holder to immobilise the head. The right and left olfactory bulbs were exposed, a 0.2-mm thick Teflon blade was inserted between the olfactory bulbs and cribriform plate, and then a bilateral transection of olfactory nerve fibres was conducted. The skin incision was closed using

a nylon suture. The surgical-control (sham) mice underwent the same surgical procedure with no transection of olfactory nerve fibres. The reduction or loss of olfaction by the nerve transection was verified as previously described [16].

Preparation of test fluids.

KOdiA-PC was purchased from Cayman Chemical (Ann Arbor, MI, USA). The original container contained 5 mg KOdiA-PC in 1 ml of ethanol. Another phospholipid species, 1-(palmitoyl)-2-(arachidonyl)-sn-phosphatidylcholine (PAPC) (a parent lipid of KOdiA-PC), was purchased from Hycult Biotech Inc. (Plymouth Meeting, PA, USA). A 5-mg aliquot of PAPC was dissolved in 1 mL of ethanol ($\geq 99.5\%$, Nacalai Tesque, Kyoto, Japan). The two phospholipid species in ethanol were stored at -70°C until use. Immediately prior to experiments, both the test fluids and vehicle control were prepared by adding of phospholipid species in ethanol and ethanol alone to sucrose (Nacalai Tesque) dissolved in distilled water at a concentration of 0.15 M (0.15 M sucrose solution), respectively.

Two-bottle choice test.

Prior to the experiments, each of the mice was first given a 0.3 M sucrose solution and distilled water for 10 min. This treatment was repeated for 3 days. Then, the mice were given *ad libitum* access to a 0.15 M sucrose solution and distilled water for 10 min. After mice learned to choose the 0.15 M sucrose solution for 2 consecutive days, they were tested. Mice were deprived of water and food 1 h prior to the test, during which test fluids were exposed to the mice for 10 min. The consumption of fluids in each cage was measured as a weight and was expressed as grams consumed *per* cage. In the tests using a fluid containing the lower concentration of KOdiA-PC, they were repeated for 3 days. To avoid developing a preference for a particular side (left or right), the positions of bottles were randomly altered every day throughout all training and test periods.

Licking test.

The principle and apparatus involved with the licking test have been previously described [17]. Briefly, prior to the experiments, mice were trained to ingest sufficient amounts of 0.15 M sucrose solution within 10 min. Mice were deprived of water and food for 1 h, and then 0.15 M sucrose solutions were presented to the animals in home

cages for 10 min. The training was repeated for 5 days. After training, mice were placed in the test chamber for 1 h without any fluids present for habituation to the test environment. The following day, each of the mice was placed without food and water for 1 h in the test chamber, during which time 0.15 M sucrose solution was presented to the mice for 10 min. During the presentation, the author recorded the number of licks. This was repeated for additional 4 consecutive days. After this 5-day period, mice were subjected to a licking test using a 0.15 M sucrose solution containing KODiA-PC. This was repeated for 3 days. The author calculated the initial licking rate by counting the number of licks for 1 min following after the first lick.

Statistical analysis.

Repeated measures analysis of variance (ANOVA) and Bonferroni *post hoc* test were conducted for statistical analyses by means of the aid of Prism 5 software package (GraphPad, San Diego, CA, USA). A *P*-value of less than 0.05 was regarded to be statistically significant.

Results

Investigation of the perception of KODiA-PC in mice

The author first assessed whether animals could consciously perceive KODiA-PC. For this, this author conducted a test where animals were offered the choice of a pair of fluids (two-bottle choice test). The conscious perception of a substance of interest would be measured by comparing the intake of fluids with or without it. The author initially performed a test in which C57BL6/J mice were given the choice of distilled water with and without KODiA-PC. It has previously shown that the concentration of KODiA-PC necessary to achieve half-maximal inhibition of oxidised LDL binding to a synthetic peptide-based mimic of mouse CD36 was 0.075 to 0.1 mM [18]. The author therefore, set the concentration of KODiA-PC at 0.15 mM, expecting the animals to perceive it. However, the author failed to evaluate the perception of KODiA-PC in the animals, because the intake of distilled water was low (data not shown). By referring to studies on the evaluation of accessibility of bitter compounds in animals [19, 20], the author then performed another two-bottle choice test using a sucrose solution as vehicle control. The author set the concentration of sucrose at 0.15 M, because the intake of the solution was significantly higher than that of distilled water (Fig. 1A, left panel). In mice (wild-type mice), the intake of the sucrose solution with 0.15 mM KODiA-PC was significantly less than that of the solution alone (Fig. 1B, left panel). Based on this data, the author suggests that the mice could perceive KODiA-PC and thus avoided or hesitated to ingest the fluid containing the lipid.

It is important to investigate the involvement and role of CD36 in the perception of KODiA-PC in the mice. For this, the author investigated the choice of the two fluids in C57BL6/J mice with CD36 gene disruption (CD36-knockout mice). If the mice ingested the fluids in a similar fashion, CD36 involvement in the perception of KODiA-PC in the wild-type mice would be indicated. Note that the intake pattern for distilled water and 0.15 M sucrose solution in the CD36-knockout mice was similar to that of the wild-type mice (Fig. 1A, right panel), indicating that CD36 does not affect the perception of sucrose. Like in the wild-type mice, the intake of the sucrose solution with KODiA-PC was apparently less than the intake of the solution alone in CD36-knockout mice (Fig. 1B, right panel). Two-way repeated measures ANOVA with genotype as the variable and test fluid as the repeated measures gave a *P*-value of more

than 0.5 for the KOdiA-PC $\times$ CD36 gene disruption interaction. The author suggests that the wild-type mice do not require CD36 for perceiving KOdiA-PC under this experimental condition.

Investigation of perception of a lower level of KOdiA-PC and the involvement of CD36 in mice

The author next assessed if mice could perceive a lower level of KOdiA-PC using the two-bottle choice test: with and without KOdiA-PC (7.5 μ M). The phospholipid at concentrations of less than 50 μ M has been shown to exhibit no apparent inhibition in the binding of oxidised low-density lipoproteins to the model CD36 in physiological buffered saline [18, 21]. For an accurate assessment, the author used 0.15 M sucrose solution as control vehicle.

On the first trial, the wild-type mice ingested both fluids in a comparable fashion (Fig. 2A, left panel). The author repeated the test for an additional 2 days. On the second and third trials of test, the intake of 0.15 M sucrose solution containing KOdiA-PC was less than that of the solution alone (Fig. 2A, left panel). Note that the intake volume of the sucrose solution increased as the days went on ($P = 0.024$, one-way repeated measures ANOVA), whereas that of the solution with KOdiA-PC did not ($P = 0.54$, one-way repeated measures ANOVA). These results led the author to suggest that the mice could perceive KOdiA-PC in the fluid and hesitated to ingest it. PAPC, the non-oxidised parent of KOdiA-PC, is known to be incapable of binding to CD36 [8, 18, 21]. The author found that the wild-type mice ingested 0.15 M sucrose solution with and without PAPC (6.4 μ M) in a comparable manner (Fig. 2B left panel). Therefore, the author concluded that the ingestive behaviour of mice in the two-bottle choice test using 7.5 μ M of KOdiA-PC is not an artifact. The wild-type mice were not likely to perceive KOdiA-PC at a concentration of 0.75 μ M in the sucrose solutions (data not shown).

The CD36-knockout mice were also offered the two-bottle choice test using 0.15 M sucrose solution with and without KOdiA-PC (7.5 μ M). The mice ingested the two fluids in a similar manner during at each trial (Fig. 2A, right panel). The author conducted two-way repeated measures ANOVA (with genotype as the variable and test fluid as the repeated measures) *per* trial to assess the effects of CD36 gene disruption on the choice of the two fluids. The P -value obtained in the first, second and third trials

were 0.52, 0.18 and 0.011 for the effect of KODiA-PC, 0.49, 0.53 and 0.70 for the effect of CD36 gene disruption and 0.62, 0.30 and 0.14 for the KODiA-PC $\times$ CD36 gene disruption interaction, respectively. Bonferroni *post hoc* test revealed a statistically significant difference between the intake volumes of the two fluids in the wild-type mice on the third trial (Fig. 2A). Collectively, the author suggests that CD36 may play a role in the perception of a lower level of KODiA-PC in mice. Like the wild-type mice, the CD36-knockout mice were found to consume 0.15 M sucrose solution with and without PAPC (6.4 μ M) in a comparable manner (Fig. 2B, right panel), suggesting that CD36 deletion does not affect the behavioural responses to PAPC.

Further investigation of CD36 involvement in the perception of a lower level of KODiA-PC by a licking test

To further support CD36 involvement in the perception of a lower level of KODiA-PC, the author performed a brief-access licking test where the acceptability of test fluids could be measured without considering post-ingestive influences. The wild-type and CD36-knockout mice were given a 0.15 M sucrose solution on the first 5 days. During the days, the initial licking rates were similar between the mouse lines (Fig. 3, Day 1 to 5). From the day 6 to 8, both lines of mice were given 0.15 M sucrose solution containing 7.5 μ M KODiA-PC. The initial licking rate in the wild-type mice decreased as the days went on, whereas that of the CD36-knockout mice remained unchanged (Fig. 3, Day 6 to 8). On the final trial, the rate of initial liking was significantly lower in the wild-type mice ($100 \pm 9/\text{min}$) than in the CD36-knockout mice ($177 \pm 21/\text{min}$). These results further support the importance of CD36 in the perception of a low level of KODiA-PC.

The effect of ONX on the perception of KODiA-PC in mice

The findings that the wild-type mice still noticed the lower level of KODiA-PC led the author to postulate that the olfactory system, responsible for highly sensitive detection of odorants [2, 3, 22], is critical for perception of the phospholipid. To test the hypothesis, the author examined the effects of ONX on the ingestion of fluid with KODiA-PC. The wild-type mice with sham surgery and those with ONX were given the choice of 0.15 M sucrose solution with and without KODiA-PC (7.5 μ M). It is noted that the surgical operation did not seriously affect the choice of distilled water and 0.15 M

sucrose solution in the mice (Fig. 4A).

On the first trial, the mice with sham surgery ingested the two fluids in a similar manner (Fig. 4B, left panel). On the second and third trials, the intake of the solution with KODiA-PC was less than that of the control vehicle (Fig. 4B, left panel). It is noted that unlike in no-surgery mice (refer to Fig. 2A, left panel), the intake volume of 0.15 M sucrose solution in the sham-operated mice did not increase during these trials ($P = 0.12$, one-way repeated measures ANOVA), indicating that the sham surgery resulted in a decline in the appetite for the solution. Regardless, the mean values for intake of fluid containing KODiA-PC at levels lower than those of the control vehicle on the second and third trials led to the suggestion that the sham-operated mice were still capable of perceiving the lipid.

In the mice with ONX, the intake volumes of the two fluids were similar for each trial (Fig. 4B, right panel). The author analysed the effect of ONX on the choice of the two fluids by performing two-way repeated measures ANOVA (surgery as the variable and test fluid as the repeated measures) *per* trial. The P -value obtained in the first, second and third trials were 0.12, 0.018 and 0.94 for the effect of KODiA-PC, 0.58, 0.88 and 0.83 for the effect of ONX and 0.53, 0.090 and 0.10 for the KODiA-PC $\times$ ONX interaction, respectively. Bonferroni *post hoc* test revealed a statistically significant difference between the intake volumes of the two fluids on the second trial in mice with sham surgery but not in those with ONX (Fig. 4B, left panel). Collectively, the author suggests the importance of an intact olfactory system for perceiving a low level of KODiA-PC.

The author has shown the perception of KODiA-PC in mice by a two-bottle choice test using fluids with or without the lipid at a concentration more than an order of magnitude larger than 7.5 μ M (0.15 mM) (Fig. 1B). The author also sought to evaluate the involvement of olfactory system in the perception of the level of KODiA-PC. For this, the author conducted an experiment in which the wild-type mice with sham surgery and ONX were given the choice of 0.15 M sucrose solution with and without KODiA-PC (0.15 mM). The intake of the sucrose solution with KODiA-PC was less than that of the vehicle control in the mice receiving the sham operation (Fig. 4C, left panel). It is noted that the intake of sucrose solution alone was lower in the sham-operated mice (0.43 ± 0.027 g) than in the no-surgery mice (0.65 ± 0.013 g, refer to Fig. 1B, left panel), reinforcing the idea that the sham surgery affects the intake of the

solution. Regardless, the apparent difference between the intake volumes of the sucrose solutions with or without KODiA-PC conveys the perception of KODiA-PC in the sham-operated mice. The ONX mice ingested the two fluids in a similar manner (Fig. 4C, right panel). Two-way repeated measures ANOVA with surgery as the variable and test fluid as the repeated measures gave a *P*-value of less than 0.01 for the KODiA-PC $\times$ ONX interaction. These results suggest that the perception of higher levels of KODiA-PC also relies considerably on olfactory system in mice.

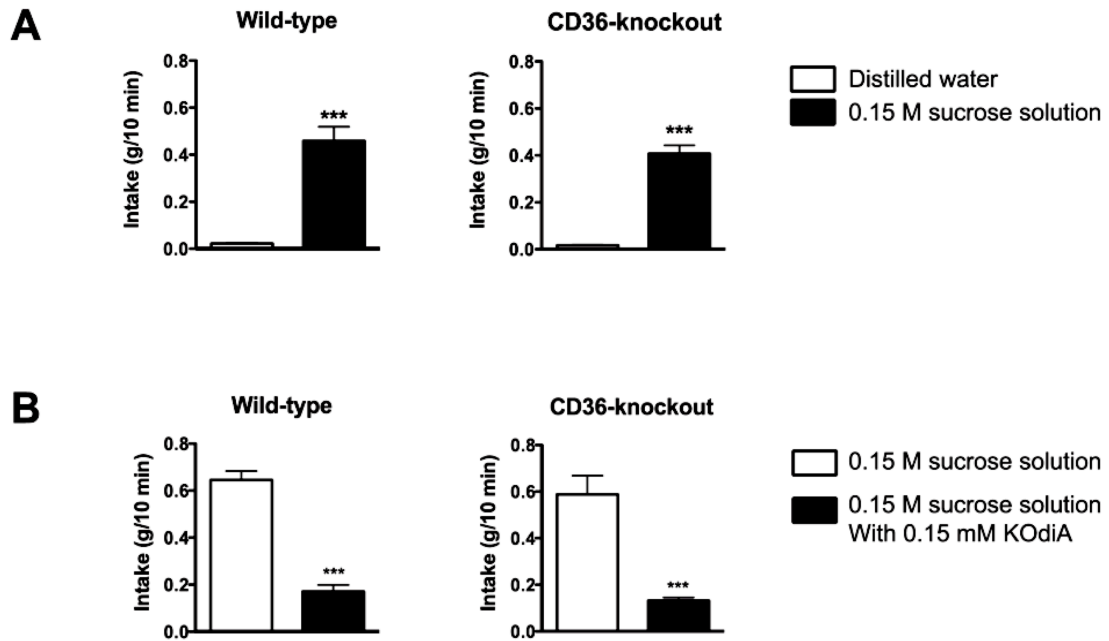


Fig. 1. Assessment of the perception of KODiA-PC (0.15 mM) and CD36 involvement in mice. Wild-type and CD36-knockout mice were given 0.15 M sucrose solution and distilled water (A) or 0.15 M sucrose solution with and without KODiA-PC (B). In each trial, mice were given fluids at the same time for 10 min. Intake (g) from each cage was measured and converted to the value *per* mouse. Each column and bar represents the intake $\pm$ SE of at least 8 cages. Statistical analysis was performed using two-way repeated measures ANOVA with genotype as the valuable and test liquids as the repeated measures and Bonferroni *post hoc* test. In (A), the *P*-values obtained were less than 0.0001 for the effect of sucrose, 0.41 for the effect of CD36 gene disruption and 0.47 for the sucrose $\times$ CD36 gene disruption interaction. In (B), the *P*-values obtained were less than 0.0001 for the effect of KODiA-PC, 0.23 for the effect of CD36 gene disruption and 0.86 for the KODiA-PC $\times$ CD36 gene disruption interaction. ***, *P* < 0.001 *versus* the intake of distilled water (A) or that of sucrose solution alone (B).

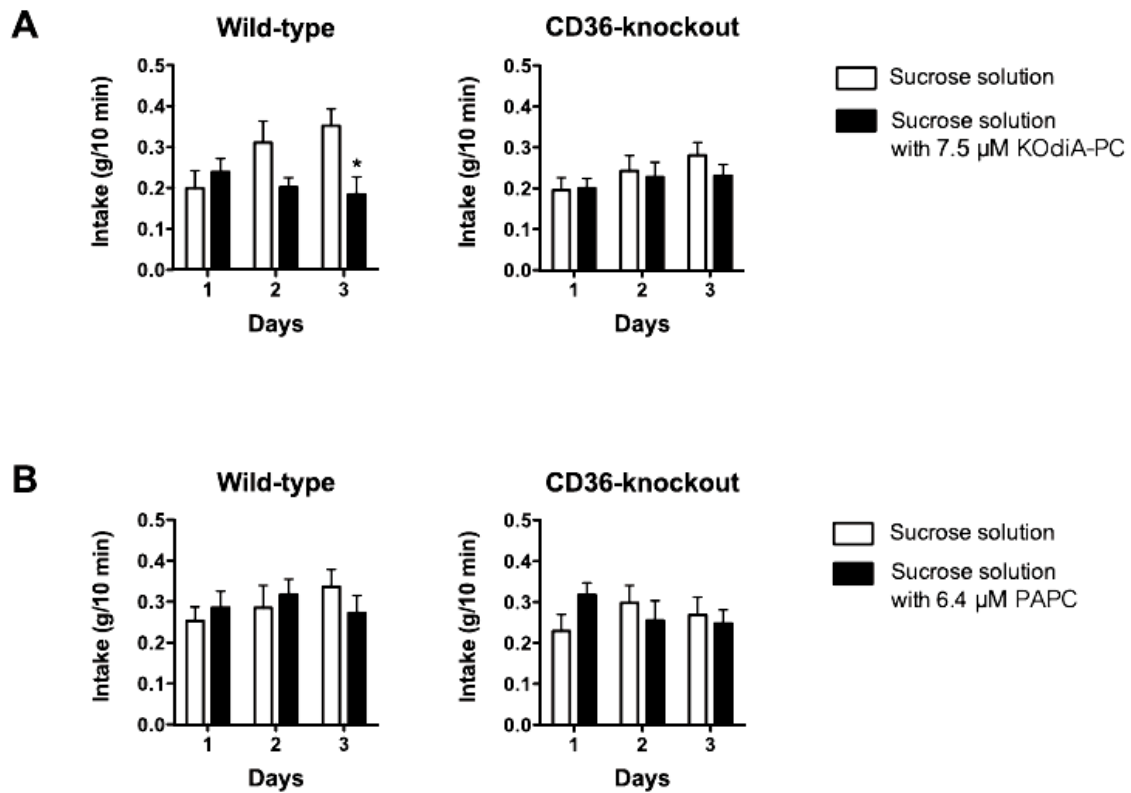


Fig. 2. Assessment of the perception of KODiA-PC (7.5 μM) and CD36 involvement in mice.

Wild-type and CD36-knockout mice were given 0.15 M sucrose solution with and without KODiA-PC (A) or that with and without PAPC (6.4 M) (B) at the same time for 10 min. The test was repeated for 3 days. Each column and bar represents the intake $\pm$ S.E. of at least 8 cages. In (A) and (B), the effects of CD36 gene disruption were analysed *per day* by using two-way repeated measures ANOVA with genotype as the valuable and test liquids as the repeated measures and Bonferroni *post hoc* test. In (A), the *P*-values obtained for the KODiA-PC effect, the CD36 gene disruption effect and the KODiA-PC $\times$ CD36 gene disruption interaction were described in the RESULTS section. $*P < 0.05$ versus the intake of sucrose solution alone. In (B), the *P*-value obtained in the context of the first, second and third trials were 0.20, 0.99 and 0.089 for the effect of PAPC, 0.20, 0.29 and 0.42 for the effect of CD36 gene disruption and 0.72, 0.50 and 0.40 for the PAPC $\times$ CD36 gene disruption interaction, respectively. Bonferroni *post hoc* test did not detect statistically significant differences between the intake volumes of the two fluids with or without PAPC on each trial in either wild-type or CD36-knockout mice.

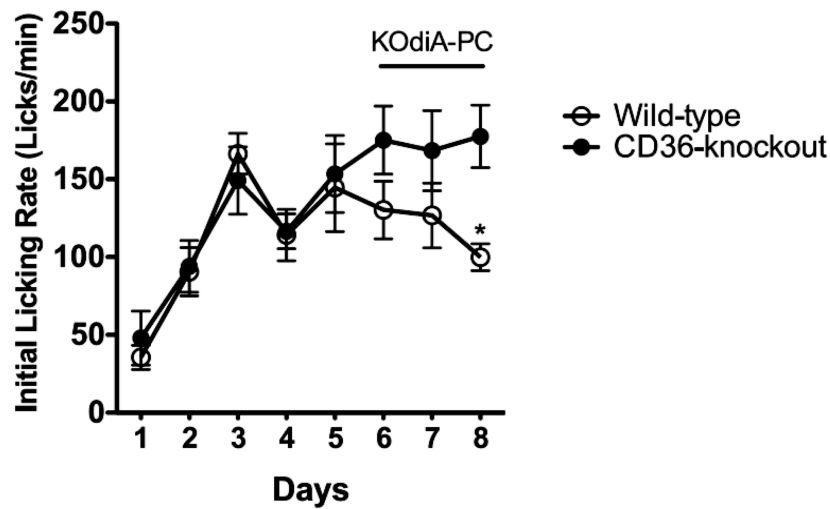


Fig. 3. Assessment of CD36 involvement in the perception of KODiA-PC using a licking test. In this test, 8 and 9 of wild-type and CD36-knockout mice, respectively, were given 0.15 M sucrose solution for 5 days and the solution containing 7.5 μ M KODiA-PC for 3 days. The initial licking rates were calculated as described in Materials and methods. Data are expressed as mean $\pm$ SE. Statistical analysis was performed using two-way repeated measures ANOVA with genotype as the valuable and time as the repeated measures and Bonferroni *post hoc* test. The *P*-values obtained were 0.25 for the effect of CD36 gene disruption, less than 0.0001 for the effect of day and 0.070 for the CD36 gene disruption $\times$ day interaction. *, *P* < 0.05 *versus* the initial licking rate in CD36-knockout mice at day 8.

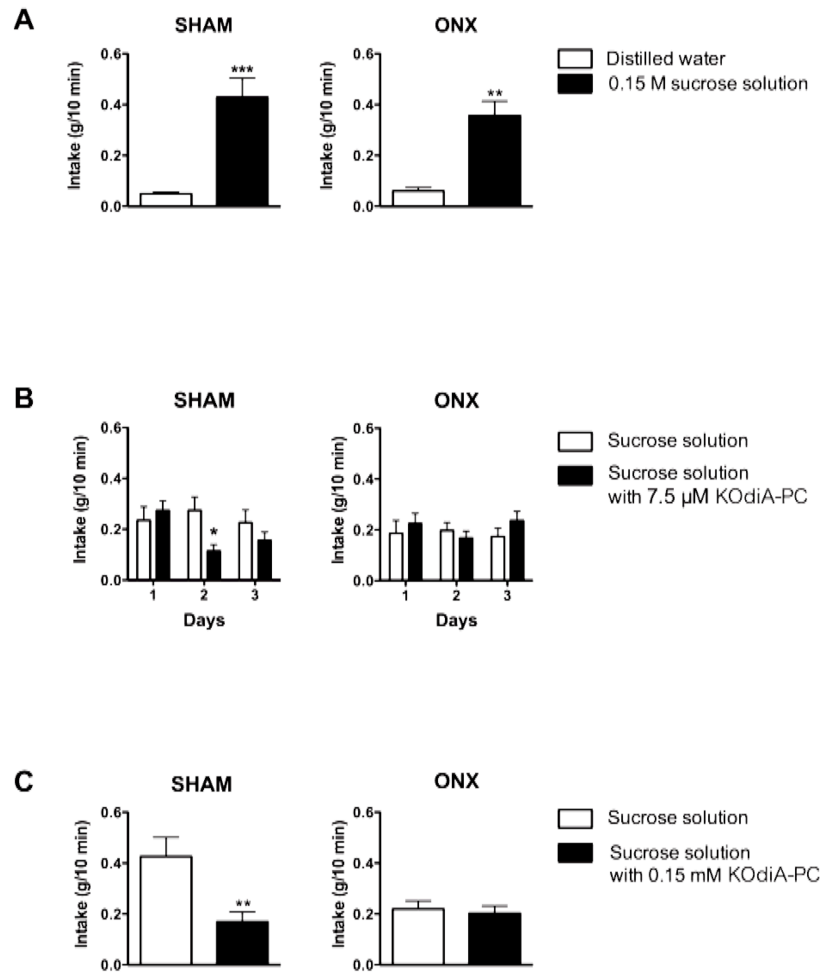


Fig. 4. Effect of ONX on the perception of KODiA-PC in mice. Wild-type mice with sham operation (SHAM) and those with ONX were offered the choice of 0.15 M sucrose solution and distilled water (A), 0.15 M sucrose solution with and without KODiA-PC (7.5 μ M) (B) or 0.15 M sucrose solution with and without KODiA-PC (0.15 mM) (C). Each column and bar represents the intake $\pm$ SE of at least 8 cages. Statistical analysis was performed using two-way repeated measures ANOVA with surgery as the valuable and test fluid as the repeated measures and Bonferroni *post hoc* test. In (A), the *P*-values obtained were less than 0.0001 for the effect of sucrose, 0.22 for the effect of ONX and 0.13 for the sucrose $\times$ ONX interaction. ****P* < 0.001, ***P* < 0.01 *versus* the intake of distilled water. In (B), the effects of ONX were assessed *per trial*. The *P*-values obtained for the KODiA-PC effect, the ONX effect and the KODiA-PC $\times$ ONX interaction were described in the RESULTS section. **P* < 0.05 *versus* the intake of sucrose solution alone. In (C), the *P*-values obtained were 0.0019 for the effect of KODiA-PC, 0.049 for the effect of ONX and 0.0095 for the KODiA-PC $\times$ ONX interaction. ***P* < 0.01 *versus* the intake of sucrose solution alone.

Discussion

In this chapter, the author showed that mice avoided or hesitated to ingest fluids containing KOdiA-PC. The findings provide evidence for conscious perception of the oxidised phospholipid species in the animal. Considering the radical nature of products of lipid oxidation, the author infers that may produce an irritant and/or unpleasant sensation in the animal. Furthermore, the author provided evidence that CD36 could contribute to the perception of KOdiA-PC in mice. However, the receptor appeared to be required only for an efficient recognition of the lipid by the animals. In fact, the effects of CD36 deletion on the ingestive response to the KOdiA-PC-containing fluid became detectable only when the lipid was applied at a lower level (Figs. 1 to 3). There is a family of membrane-bound proteins in insects called sensory neuron membrane proteins (SNMPs) that have similar structural features to CD36 [23, 24]. Of these, SNMP-1 is considered to facilitate the detection of pheromones *via* a G-protein coupled receptor expressed by the sensory neurons [25]. By analogy, the author suggests that CD36 may play a supportive role in the detection of KOdiA-PC by an unknown signal transduction receptor(s). Indeed, CD36 is postulated to serve as a cofactor of the toll-like receptor 2 and to enhance recognition of lipoteichoic acid by this receptor [26].

It is notable that mice did not hesitate to ingest the fluid containing 7.5 μ M KOdiA-PC at the first trial but did hesitate at the second and third trials (Fig. 2A). One explanation for this is that the mice did not perceive the KOdiA-PC in the fluid as unpleasant at the initial trial and that it is not until the animals perceives some discomfort after ingestion of the fluid that they considered it hazardous during the following trials. In other words, an aversive feedback mechanism is necessary to elicit a behavioural response. However, direct addition of KOdiA-PC to the gastrointestinal tracts or inside the body did not appear to modulate the feedback. This idea is supported by the observation that the mice demonstrated no or little latency to ingest a saccharin solution even when conditioned with intragastric KOdiA-PC in a conditioned taste aversion test (unpublished observation). Rather, the data obtained support the significance of chemosensory inputs through the oral and/or nasal cavity to assess the biological value of KOdiA-PC. In fact, mice with ONX did not hesitate to ingest the fluid containing 7.5 μ M KOdiA-PC (Fig. 4B). The author has not yet tried to evaluate the importance of gustatory sensation for KOdiA-PC, because a sucrose solution was

used as control vehicle, the content of which is perceived primarily by gustatory neurons [1, 2]. The author assumes that gustatory nerve transection would cause a significant reduction in intake of sucrose solutions in mice, which would make it difficult to assess the aversive effects of KOdiA-PC *per se*. Indeed, transections of distinct gustatory nerves have been demonstrated to result in marked declines in the appetite or sucrose in rats [27]. The involvement of the gustatory system in the perception of KOdiA-PC awaits further investigation.

It remains unclear whether the reduction or loss of aversion to the fluid containing 7.5 μ M KOdiA-PC upon ONX could be directly attributed to the lack of signals detected in a CD36-dependent fashion. Regardless, the ingestion of the sucrose solution with and without KOdiA-PC in the wild-type mice with ONX (Fig. 4B, right panel) was not considerably different from that found in CD36-knockout mice (Fig. 2A, right panel). Based on these results, the author postulates that CD36 plays a relevant role in the olfactory detection of the oxidised phospholipid, KOdiA-PC. The precise site(s) of function of CD36 in the perception of KOdiA-PC are also unclear. Strotmann and Breer previously have shown that CD36 mRNAs are detected in the mouse olfactory epithelium [28]. Furthermore, the author has found the occurrence of CD36 protein in distinct olfactory receptor cells and its abundance in the ciliary layer (*i.e.*, mucus layer) of the epithelium in mice [29]. There exist soluble proteins capable of fixing odorants with low detection thresholds in olfactory mucus (known as odorant binding proteins) [24]. The olfactory mucus may provide an environment for efficient binding between these proteins and odorants. It is tempting to speculate that CD36 effectively captures KOdiA-PC on the surface of olfactory epithelium, thereby contributing to the sensing of the phospholipid.

The aversive response of mice to the fluid containing 0.15 mM KOdiA-PC appeared to rely upon the olfactory system but not necessarily by CD36. One explanation for this is that KOdiA-PC at higher concentrations directly stimulates olfactory sensory neurons in the nasal epithelium in a cell-surface binding proteins-independent manner and generates signals leading to aversive responses. The author cannot also exclude the possibility that distinct olfactory receptor(s) in the sensory neurons are responsible for KOdiA-PC recognition. The putative unknown receptor(s) may not necessarily require the assistance of CD36 for the detection of high levels of KOdiA-PC.

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CHAPTER 3

CD36 involvement in the olfactory perception of an odour-active volatile compound, oleic aldehyde, in mice

Introduction

Chemosensory systems are responsible for detection of soluble or volatile chemicals. The two main systems in mammals, gustatory and olfactory systems, distinguish nutritive compounds from harmful ones within the oral and nasal cavities [1]. The sense of smell is particularly important for avoiding harmful chemicals in foods prior to ingestion [2]. The olfactory perception starts from the binding of odorants to specific G-protein-coupled receptors on olfactory sensory neurons (OSNs) located within the olfactory epithelium of the nasal cavity [3]. However, for many of odorants, the precise mechanisms of olfactory detection in mammals remain to be studied.

CD36 is a membrane-bound receptor with two transmembrane spans that is produced and expressed by a variety of sites in the body of vertebrates [4]. The receptor is involved in lipid binding and uptake within tissues, such as the small intestinal, adipose and muscle tissues [5]. CD36 has also been postulated to play roles in the gustatory system. Indeed, CD36 has been shown to mediate the detection of long-chain fatty acids in the oral cavity, driving the consumption of the lipid species in rodents [6, 7]. More recent studies demonstrated a novel role of CD36 — the role in the olfactory system. This is exemplified by (i) the localisation of the receptor on the cilia of OSNs in the main olfactory epithelium [8], (ii) a weaker response to oleic acid in the olfactory sensory neurons of CD36-knockout mice compared to wild-type littermates [9] and (iii) a reduction of preference for a lipid mixture odour through dysfunction of CD36 in mice [10]. The author also demonstrated that mice with olfactory nerve transection failed to perceive 1-(palmitoyl)-2-(5-keto-6-octanedioyl)phosphatidylcholine (KOdiA-P C), one of the most potent ligands for CD36 among oxidised phospholipids [11]. However, these studies focused on lipids with no or very weak odour. The involvement and role of CD36 in animal behaviours toward odour-active volatile compounds has not yet been investigated so far.

In this chapter, *via* ethological tests using CD36-deficient mice and their wild-type littermates, the author aimed to examine whether CD36 is involved in the olfactory perception of odour-active volatile compounds present in foods. The author attempted to use oleic aldehyde as a model odorant in foods (known to be present in smoked fish), because the compound has been found to display the highest CD36 ligand activity among tested aldehydes in foods [12]. The author showed that (i) wild-type mice but not CD36-deficient ones discriminated between a sucrose solution with oleic aldehyde and the sucrose solution alone in a feeding test and (ii) wild-type mice appeared to perceive the aldehyde as an odorant, whereas CD36-knockout mice did not, in an exploration test. Results obtained prompted the author to propose that CD36 is involved in the olfactory perception of distinct odour-active volatile compounds in the environment, including foods.

Materials and Methods

Animals.

CD36-knockout mice (C57BL6/J background) [13] were provided as previously described [14]. Wild-type and CD36-knockout littermates were obtained from the same breeding pair and thus have a genetically identical background. To genotype the mice lines, the author used the primer set described in a previous study [14]. Mice were bred under a constant temperature (22°C) and a 12:12 h light–dark cycle. All mice used were between 8 and 12 weeks of age. Two weeks prior to experiments, each mouse was individually housed with *ad libitum* access to water and standard mouse chow (MF; Oriental Yeast, Japan). This animal experiment was performed in accordance with the ethics guidelines of the Kyoto University Animal Experimentation Committee and was in complete compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Preparation of test fluids.

Oleic aldehyde (or *cis*-9-octadecenal) was prepared by the chemical conversion of oleic acid as described in a previous study [12]. Briefly, oleic acid was once converted into oleic alcohol and then the purified alcohol by silica gel column chromatography was converted into oleic aldehyde by treatment with 2-iodoxybenzoic acid. More than 98% of purity of the oleic aldehyde was dissolved to 1% in ethanol ($\geq 99.5\%$; Nacalai Tesque, Kyoto, Japan). It was stored at -70°C until use.

Two-bottle choice test.

Mice were bred under a 12:12 h light–dark cycle (dark phase of 18:00 – 6:00). All the tests including the training sessions were performed at 18:00 under food and water deprivation for 1 hour prior to the sessions. Prior to the experiments, each of the mice was first given a 0.3 M sucrose (Nacalai Tesque) solution and distilled water for 10 min. This was repeated for 3 days. The mice were then given *ad libitum* access to a 0.15 M sucrose solution and distilled water for 10 min. After mice learned to select the 0.15 M sucrose solution for 2 consecutive days, they were regarded to be eligible for tests. Both the test and control fluids were prepared by the 0.15 M sucrose solution with the oleic aldehyde dissolved in the ethanol and ethanol alone, respectively. In tests using fluids

with and without the oleic aldehyde, they were repeated for 3 days. The fluid consumption in each cage was measured as a weight and was expressed as grams consumed *per* cage. To avoid developing a preference for a particular side (left or right), the positions of fluids were randomly altered every day throughout all training and test periods.

Exploration test.

Mice were bred under a 12:12 h light–dark cycle (dark phase of 6 : 00 – 18 : 00). All the tests including the training sessions were performed at 10 : 00. Food was deprived for 18 hours and provided only for 6 hours after a session. Prior to the experiments, each mouse was habituated to the exploration cage, A $22.5 \times 22.5 \times 29.5$ cm plexiglas box with solid transparent walls and a hole of 1 cm of diameter in one of the sides, located 8 cm above the cage ground. The cage ground was covered with a layer of Alpha-Dry bedding. A stainless cover with a square transparent window was set during the tests to avoid odour dissipation. The video recording system is fixed above the exploration cage at a distance of 30 cm from the cage ground. After each test, the mouse was returned to the home cage and the exploration cage was thoroughly wiped clean in order not to influence the following tests. The mice were first exposed to the general environment and duration of exploration test for three days (habituation) prior to training and test sessions. On session, the mice were allowed a 3-min free exploration for stabilising behaviour [15]. Next, a filter paper containing 40 μ l of 1% (v/v) ethanol (vehicle control) was presented in front of the hole for 1 min. Note that the filter paper was placed at the position where the mice were unable to directly touch. The first exposure to the vehicle control is followed by 2 more successive exposures to ethanol on new paper. After the 3 trials with the vehicle control, the mice were exposed to a filter paper containing 40 μ l of odorant that dissolved in 1% ethanol. The exposure to the odorant stimulus lasts for 1 min. An inter-trial interval was given between trials for 2 min without stimulus. It is important to set an adequate length of interval to measure stable exploratory behaviours of mice in each trial [16, 17]. In experiment, the total time of exploratory behaviour was assessed for every trial through video recordings. Exploratory behaviours regarded as ones related to olfactory perception includes smelling, sniffing or heading the nose towards the hole at a close distance.

Statistical analysis.

Repeated measures analysis of variance (ANOVA), and Bonferroni *post hoc* test were used for statistical analyses by means of the aid of Prism 5 software package (GraphPad, San Diego, CA, USA). A *P*-value of less than 0.05 was considered to be statistically significant.

Results and discussion

Assessment of the perception of oleic aldehyde and CD36 involvement in mice via two-bottle choice test.

The author initially assessed whether animals could recognise oleic aldehyde in feeding, if so, whether CD36 is involved in the sensing. For this, the author examined the choice of the two fluids with or without oleic aldehyde in wild-type and CD36-knockout mice. It has been shown that (i) mice can consciously perceive KODiA-PC included in a sucrose solution at a concentration of 7.5 μM [11] and (ii) the CD36 ligand activity is about 3.5 times lower in oleic aldehyde than in KODiA-PC [12]. Based on these data, the author set the concentration of oleic aldehyde at 35 μM , expecting that animals certainly perceive it. It is also noted that the author used a 0.15 M sucrose solution as a vehicle fluid to ensure minimum intake in animals [11].

On the first day, the wild-type mice ingested both fluids in a comparable manner (Fig. 1, left panel). The author repeated the test for an additional 2 days. On the second day, the intake of the sucrose solution with oleic aldehyde was less than that of the solution alone (Fig. 1, left panel). This result suggests that the mice could consciously perceive oleic aldehyde in the fluid and avoid ingesting it. This aldehyde is considered to be generated through the oxidation of fats. The odour of distinct volatile compounds generated from fat oxidation may often be attractive to mammals (especially to humans). However, these are carcinogenic and teratogenic when introduced to and accumulated in the bodies [19]. Considering the potentially harmful effect of aldehydes, the author infers that oleic aldehyde served as an unpleasant stimulus for the animals. The reasons that mice did not avoid oleic aldehyde on the first day are unclear (Fig. 1, left panel). Senses of smell and taste play more than just sensory perception, but also metabolic perception. The sensory perception interacts with post-metabolic feedback to determine the biological value of sensory stimulus. Therefore, in the tests, the mice evaluated a quality of oleic aldehyde referring to metabolic reflexes from the ingestion on the first day then expressed aversive response to the oleic aldehyde on the next day. On the third day of tests, the intake by mice was indifferent between the test fluids with and without oleic aldehyde (Fig. 1, left panel), suggesting that the less consumption of oleic aldehyde on the second day did not provide a significant metabolic feedback.

The author conducted the two-bottle choice test on CD36-knockout mice. The mice ingested the two fluids in a similar pattern during three consecutive days (Fig. 1, right panel). These results provided evidence that CD36 is involved in the perception of oleic aldehyde in feeding. Referring to the typical functions of CD36 scavenging oxidative compounds in the body [20, 21], the author speculates that CD36 perceives oleic aldehyde as a hazardous oxidised lipid and reflects the feeding behaviour. The author conducted two-way repeated measures ANOVA *per* trial to assess the effects of CD36 gene disruption on the choice of the two fluids. The *P*-value obtained in the context of the first, second and third trials were 0.13, 0.08 and 0.61 for the effect of oleic aldehyde, 0.52, 0.42 and 0.64 for the effect of CD36 gene disruption and 0.63, 0.16 and 0.94 for the oleic aldehyde $\times$ CD36 gene disruption interaction, respectively. Student's *t*-test detected a statistically significant difference between the intake volumes of the two fluids in the wild-type mice on the second trial ($P = 0.01$, Fig. 1, left panel), but not in CD36-knockout mice ($P = 0.80$, Fig. 1, right panel). Overall, the author suggests that CD36 takes a part in the recognition of oleic aldehyde in mice.

Assessment of the olfactory detection of oleic aldehyde and CD36 involvement in mice via exploration test.

The ability of mice to perceive oleic aldehyde, coupled with the volatile nature of the compound, led the author to hypothesise that the olfactory system plays a critical role in the perception of oleic aldehyde. However, the author cannot rule out the possibility that oleic aldehyde detected by taste bud cells contributed to the feeding behaviours. Therefore, the author attempted to test olfaction-driven instinctive behaviours in mice by an exploration test. The author assessed the time of exploratory behaviours in wild-type and CD36-knockout mice when exposed to the odour of oleic aldehyde. The exposure to the vehicle control in the first three trials is subject to habituate mice that reduce or sustain exploratory interest to the vehicle control; generate interest in the novel odorant stimulus on the fourth trial. The author defined the olfactory perception with the time showing exploratory behaviours toward oleic aldehyde significantly longer or shorter than that toward the non-odorant stimulus.

Prior to the tests with oleic aldehyde, the author executed the training session with acetic acid, which was reported to be recognised in rodents by sniffing [22]. It was to lead the mice understand where an odorant is introduced, since the animals were less

responsive to oleic aldehyde without training (data not shown). The author conducted the training twice on consecutive days. On the first day, wild-type and CD-knockout mice exhibited a longer exploration time for acetic acid than that for vehicle control. The *P*-value obtained from two-way repeated measures ANOVA *per* trial is 0.02 for the effect of trial, suggesting both genotypes of mice were properly trained for the exploration test. This observation argues against the previous studies where acetic acid led shorter exploratory behaviour than vehicle control in rodents [22, 23]. However, Ko and co-workers work revealed that the starvation modulates olfactory behaviour toward acetic acid and cause an increase in exploratory behaviour in fruit flies [24]. Likewise, the author suggests in this test, 18-hour food deprivation prior to training session allowed the strong exploratory behaviour in the mice.

On the second training with the same concentration of acetic acid, the exploration times for acetic acid and vehicle control were comparable in both mouse types, indicating relatively less responsiveness to acetic acid. Adaptation to smells is a phenomenon that constant or repetitive exposure to an odour reduces perception of it [25]. The results observed in the second training may be due to desensitisation toward the same odour, acetic acid. The author also found that the CD36-knockout mice became to spend longer time exploring both control vehicle and the acetic acid on the second training, compared to the wild-type mice. The author conducted two-way repeated measures ANOVA to assess the effects of CD36 gene disruption on the exploration times. The *P*-values obtained for the CD36 gene disruption effect was 0.02. It has been reported that CD36-knockout mice travel longer time and distance than wild-type mice in the anxiety tests [26]. Altogether, the author suggests that CD36 deficiency affected a higher locomotive activity in the present exploration test.

In the test session, the author found that the wild-type mice exhibited consistent exploratory behaviour during trial 1, 2 and 3. On the trial 4 where 35 μ M oleic aldehyde was presented, the exploration time significantly increased, compared to the exploration time for vehicle control on trials of 1, 2 and 3 (Fig. 2A, left panel). The one-way repeated measures ANOVA were conducted to assess the effects of trial on the exploration times and obtained *P*-values at 0.01 in wild-type mice. Although the alleviated intake of oleic aldehyde in the two-bottle choice test implied avoidance in the mice, this test demonstrated an increased exploratory behaviour toward oleic aldehyde. Previously, Endres and co-workers showed that trimethylthiazoline, which is

categorised as a repellent, was reported to induce a strong exploratory behaviour in rodents and suggested the long exploration time for the trimethylthiazoline is a behavioural response for information acquisition rather than theoretical represent of a positive hedonic value [27]. Accordingly, the author suggests that the longer exploratory behaviour reflects a need for caution to oleic aldehyde in mice.

Contrarily, CD36-knockout mice showed steady exploration times during the four trials, suggesting specific impairment of oleic aldehyde-elicited behavioural responses (Fig. 2A, right panel). These results imply that mice could perceive oleic aldehyde in the nasal cavity and CD36 would participate in the part of the odorant perception. It has recently become clear that a specific subpopulation of olfactory neurons express CD36 [10], suggesting CD36 detects a particular group of odorants. Similar to the training session, CD36-knockout mice showed longer exploration time toward vehicle control than wild-type mice in this test session, suggesting the behavioural characteristics of CD36-knockout described above.

To evaluate the involvement of CD36 in the perception of the level of oleic aldehyde, the author performed the exploration test using vehicle control and 0.35 mM oleic aldehyde. Like the earlier test with 35 μ M, the wild-type and CD36-knockout mice showed a stable exploration times for vehicle control on trial 1, 2 and 3 (Fig. 2B). On trial 4, wild-type mice exhibited a significant increase in the exploration time for 0.35 mM oleic aldehyde (Fig. 2B, left panel). The *P*-value was 0.002 for the effect of trial and statistically significant differences were detected between the exploration time for vehicle control and that for oleic aldehyde in the wild-type mice. Slight increment of exploration time but no significant change was found in the CD36-knockout mice on trial 4, compared to exploration time for vehicle control (Fig. 2B, right panel). Student's *t*-test displayed a little difference between the exploration time for vehicle control on trial 3 and that for oleic aldehyde in CD36-knockout mice (*P* = 0.18). The author speculates that sensors other than CD36 are involved in the olfactory perception and their involvement is dependent on the concentration of odour-active molecules. In insects, CD36-related protein has been found to be co-localised with olfactory receptors and suspected to serve as a co-receptor or transporter for the main odorant receptor proteins [28, 29]. By analogy, the author hypothesises that oleic aldehyde at low concentration requires a transfer to reach its specific olfactory receptor, while the aldehyde at high concentration can directly bind to the olfactory receptor.

In this chapter, the author provided evidence that CD36 is involved in the olfactory perception of oleic aldehyde. This reinforces the idea that CD36 plays a part in the mammalian olfactory system through its ability to recognise specific odorants, thereby affecting the food choice in mammals. Also, this finding would make a contribution to expanding the knowledge of the mechanism by which mammals recognise and discriminate a variety of volatile compounds in feeds and foods.

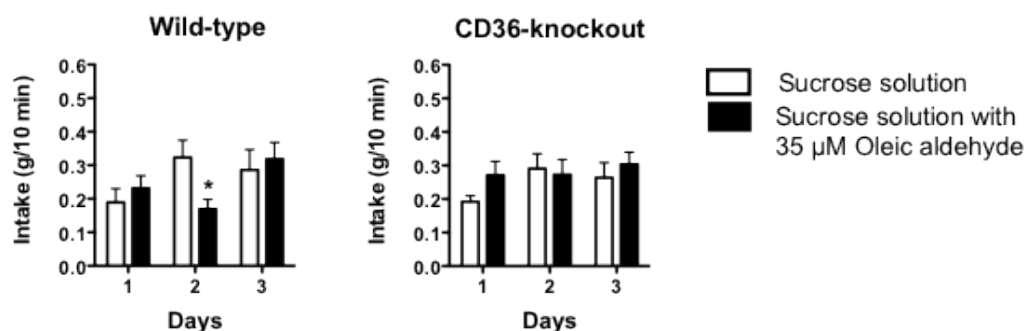


Fig. 1 Assessment of the perception of oleic aldehyde and CD36 involvement in mice via two-bottle choice test. Wild-type and CD36-knockout mice were given 0.15 M sucrose solution with and without 35 µM oleic aldehyde at the same time for 10 min. The test was repeated for 3 days. Each column and bar represents the intake $\pm$ S.E. of 8–10 cages. The effects of CD36 gene disruption were analysed *per* day by using two-way repeated measures ANOVA and Student's *t*-test. The *P*-values obtained for the oleic aldehyde effect, the CD36 gene disruption effect and the oleic aldehyde $\times$ CD36 gene disruption interaction were described in the RESULTS section. **P* < 0.05 *versus* the intake of sucrose solution alone.

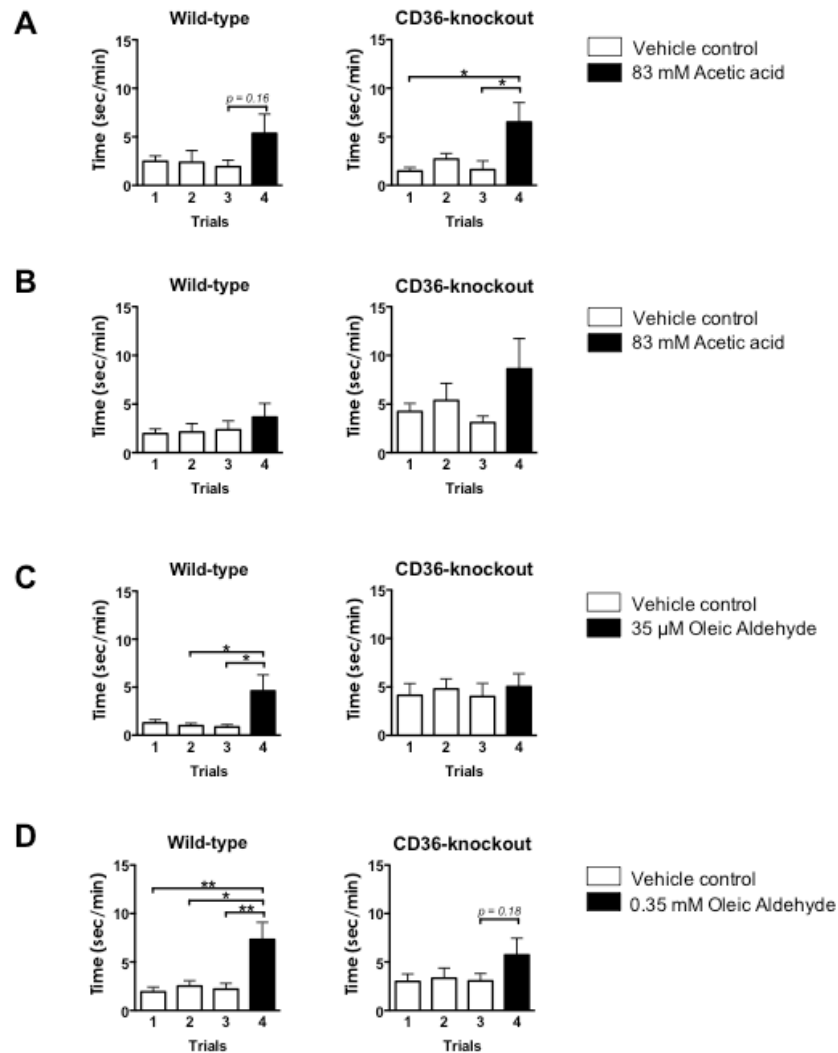


Fig. 2 Assessment of the olfactory detection of oleic aldehyde and CD36 involvement in mice *via* exploration test. Wild-type and CD36-knockout mice were subject to the training session with 83 mM acetic acid. After the training session which was conducted twice, wild-type and CD36-knockout mice were subject to the tests with 35 μ M and 0.35 mM oleic aldehydes on two consecutive days. During the each test session, the mice were exposed to a filter paper that contained 40 μ l of vehicle control for 1 min. It was followed by 2 more successive exposures. After the exposure to the vehicle control in 3 trials, the mice were exposed to a filter paper containing 40 μ l of oleic aldehyde dissolved in the vehicle control for 1 min. Each column and bar represents the olfactory exploration activity $\pm$ S.E. of 8–10 cages expressed by the exploration time (sec / min) on each trial. The effects of CD36 gene disruption and trials were analysed *per* trial by using repeated measures ANOVAs and Bonferroni's test and described in the text. * $P < 0.05$, ** $P < 0.01$ *versus* the exploration time for oleic aldehyde.

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SUMMARY

Chapter 1

In this chapter, to provide evidence for the involvement of CD36 in mammalian olfactory perception, the author investigated the localisation of this protein in the mouse olfactory mucosa. *In situ* hybridisation analysis using an antisense oligonucleotide to *CD36* mRNA detected scattered signals within the deeper epithelial layer of olfactory mucosa. The signals for the mRNA were also detected consistently in the superficial layer of the olfactory epithelium, which is occupied by supporting cells. Immunostaining with an anti-CD36 antibody showed that CD36 localises in the somata and dendrites of distinct olfactory receptor cells and that it occurs abundantly on the surface of olfactory epithelium. On the other hand, immunoreactive CD36 protein was rarely detectable in the nerve bundles running in the lamina propria of olfactory mucosa, the axons forming the olfactory nerve layer in the outermost layer of the bulb and axon terminals in the glomeruli. The author also obtained electron microscopic evidence for the association of CD36 protein with olfactory cilia. Altogether, the author suggests that CD36 plays a role in the mammalian olfaction. Signals for CD36 protein were also detected on or around the microvilli of olfactory supporting cells and the cilia of nasal respiratory epithelium, suggesting a role for this protein other than olfaction in the nasal cavity.

Chapter 2

In this chapter, the author assessed whether animals consciously perceive a ligand of CD36, 1-(palmitoyl)-2-(5-keto-6-octanedioyl)phosphatidylcholine (KODiA-PC), and if so, whether CD36 is involved in sensing the oxidised phospholipid species. The author found that mice avoided or hesitated to ingest fluids containing KODiA-PC, suggesting a conscious perception of the lipid in the animals. The author next assessed the involvement and role of CD36 in the KODiA-PC perception by comparing the behavioural responses of wild-type and CD36-deficient mice to the test fluids, and provided evidence that the protein could play a role in the sensing of a lower level of the lipid. The author also found that transection of the olfactory nerve of wild-type mice

resulted in an inability to perceive KOdiA-PC, showing the significance of olfactory system in the lipid sensing. These findings, coupled with the recent finding of CD36 expression in the mouse olfactory epithelium, led the author to predict that the site of CD36 action in the KOdiA-PC sensing plausibly lies within the nasal cavity of the animal.

Chapter 3

In this chapter, the author investigated if CD36 is involved in the perception (olfactory perception) of an odour-active volatile compound present in foods, oleic aldehyde, *via* ethological tests using CD36-deficient mice and their wild-type littermates. The author attempted to use oleic aldehyde as a model odorant in foods (known to be present in smoked fish), because the compound has been found to display the highest CD36 ligand activity among tested aldehydes in foods. The author initially assessed whether animals could recognise oleic aldehyde in feeding, if so, whether CD36 is involved in the sensing. For this, the author examined the choice of the two fluids with or without oleic aldehyde in wild-type and CD36-knockout mice. The author showed that wild-type mice but not CD36-deficient ones discriminated between a sucrose solution with oleic aldehyde and the sucrose solution alone in a feeding test. Also the author assessed the time of exploratory behaviours in wild-type and CD36-knockout mice when exposed to the odour of oleic aldehyde, and provided evidence that wild-type mice appeared to perceive the aldehyde as an odorant, whereas CD36-knockout mice did not, in an exploration test. Results obtained reinforce the idea that CD36 plays a part in the mammalian olfactory system through its ability to recognise specific odorants, thereby affecting the food choice in mammals.

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Shinhye Lee

LIST OF PUBLICATIONS

Main papers

1. Lee S, Eguchi A, Tsuzuki S, Matsumura S, Inoue K, Iwanaga T, Masuda D, Yamashita S, Fushiki T. (2015) Expression of CD36 by olfactory receptor cells and its abundance on the epithelial surface in mice. PLoS One. 10, e0133412.
2. Lee S, Eguchi A, Sakamoro K, Matsumura S, Tsuzuki S, Inoue K, Masuda D, Yamashita S, Fushiki T. (2015) A role of CD36 in the perception of an oxidised phospholipid species in mice. Biomed Res. 36, 303-11.
3. Lee S, Tsuzuki S, Inoue K. CD36 involvement in olfactory perception of an odour-active volatile compound, oleic aldehyde, in mice. Manuscript in preparation.

Related papers

1. Sakamoto K, Matsumura S, Okafuji Y, Eguchi A, Lee S, Adachi S, Fujitani M, Tsuzuki S, Inoue K, Fushiki T. (2015) Mechanisms Involved in Guiding the Preference for Fat Emulsion Differ Depending on the Concentration. J Nutr Sci Vitaminol (Tokyo). 61, 247-54.
2. Zheng M, Lee S, Tsuzuki S, Inoue K, Masuda D, Yamashita S, Iwanaga T. (2016) Immunohistochemical localization of fatty acid transporters and MCT1 in the sebaceous glands of mouse skin. Biomed Res. 37, 265-70.