CD36 involvement in orosensory detection of dietary lipids, spontaneous fat preference, and digestive secretions

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Rats and mice exhibit a spontaneous attraction for lipids. Such a behavior raises the possibility that an orosensory system is responsible for the detection of dietary lipids. The fatty acid transporter CD36 appears to be a plausible candidate for this function since it has a high affinity for long-chain fatty acids (LCFAs) and is found in lingual papillae in the rat. To explore this hypothesis further, experiments were conducted in rats and in wild-type and CD36-null mice. In mice, RT-PCR experiments with primers specific for candidate lipid-binding proteins revealed that only CD36 expression was restricted to lingual papillae although absent from the palatal papillae. Immunostaining studies showed a distribution of CD36 along the apical side of circumvallate taste bud cells. CD36 gene inactivation fully abolished the preference for LCFAs-enriched solutions and solid diet observed in wild-type mice. Furthermore, in rats and wild-type mice with an esophageal ligation, deposition of unsaturated LCFAs onto the tongue led to a rapid and sustained rise in flux and protein content of pancreatic secretions. These findings demonstrate that CD36 is involved in oral LCFAs detection and raise the possibility that an alteration in the lingual fat perception may be linked to feeding dysregulation.

Introduction
In Western diet, about 40% of daily caloric intakes is lipid, despite the fact that the recommended level is 10% lower. This high-fat supply greatly contributes to the prevalence of obesity and associated diseases (i.e., non–insulin-dependent diabetes, atherosclerosis, and hypertension). In humans, studies showed that obese subjects prefer lipid when compared with lean subjects (1, 2), suggesting that inappropriate lipid perception might influence obesity risk by impacting feeding behavior.

The regulation of lipid intake is a complex phenomenon controlled by instantaneous orosensory stimuli (i.e., texture, odor, and taste) and delayed postingestive signals (3). Until recently, the involvement of gustation in this phenomenon was neglected, dietary fat being thought to be detected only by trigeminal (texture perception) and retronasal olfactory cues (4). However, short-term behavioral studies, in which normal and anosmic rodents were allowed to choose between oil- or xanthan-enriched solutions (to mimic fat texture), strongly suggested that gustation plays a significant role in lipid perception (5, 6). Although dietary lipids consist mainly of triglycerides, compelling evidence from studies on the rat strongly suggests that long-chain fatty acids (LCFAs) may be responsible for the orosensory cue for fat. Indeed, adult animals exhibit a lower preference for triglycerides and short-chain fatty acids than for LCFAs (6, 7). Moreover, pharmacological inhibition of lingual lipase, the enzyme responsible for efficient LCFAs release from dietary triglycerides, profoundly decreases preference for lipids (8). Interestingly, lingual lipase level is especially high in the vicinity of taste buds, since it is locally secreted in the cleft of foliate and circumvallate papillae by the Ebner glands (8). Such an anatomical design may be sufficient to generate an LCFAs stimulus in taste receptor cells. In keeping with this assumption, unsaturated LCFAs were reported to inhibit, in rat taste bud cells, the delayed rectifying K⁺ channels known to be implicated in the transduction pathway of a variety of taste stimuli (9, 10). Moreover, rat lingual sensory epithelium expresses CD36 (also known as fatty acid transporter [FAT]) (11, 12), which binds LCFAs with an affinity in the nanomolar range (13, 14). The CD36 amino acid sequence predicts a ditopic glycoprotein with a large extracellular hydrophobic pocket (15, 16) between 2 short cytoplasmic tails. The C-terminal cytoplasmic tail has been shown to be associated with Src kinases (17), suggesting an involvement of CD36 in cell signaling. Together, these data support the existence of a chemical perception of LCFAs in the oral cavity.

Literature on the physiological advantage(s) provided by such a putative orosensory detection system is scarce. A weak rise in the protein content of pancreatic juice has been reported within 10 minutes after oral delivery of LCFAs in esophagectomized rats, suggesting that the presence of lipids in the oral cavity contributes to the cephalic phase of pancreatic secretions (18). Findings demonstrate that taster paired with fat intake can also influence lipid metabolic fate. Indeed, prolonged elevation in blood triglyceride was observed in rats in which a small amount of oil was directly administered onto the tongue before an intragastric feeding (19). Longer-term metabolic changes have also been reported in healthy humans, in which a rise in plasma triglyceride level was observed 2 and 4 hours after a preload-

Nonstandard abbreviations used: ACBP, acyl-CoA–binding protein; FAT, fatty acid transporter; FATP, fatty acid transport protein; FABP, intestinal fatty acid–binding protein; LCFA, long-chain fatty acid; L-FABP, liver fatty acid–binding protein.

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The effect was lipid dependent, since no change was noticed with a lipid-free sham feeding (20).

Although all these observations argue in favor of a taste for fat, the nature and physiological function(s) of an oral lipid sensor remain elusive. In the rat, CD36 appears to be a plausible candidate for this function. To explore this hypothesis, experiments were conducted both in rats and in wild-type and CD36-null mice. As the sensitivity to basic tastes is species specific (21), expression of CD36 in papillae and surrounding nonsensory epithelium was first investigated and compared in mouse and rat. Then, impact of CD36 gene inactivation on short- and long-term fat preference and digestive secretions was explored. Data reported herein provide what we believe to be the first identification of a lipid sensor component in the oral cavity by demonstrating that lingual stimulation of CD36 by unsaturated fatty acids impacts both behavioral and digestive physiology.

Results

CD36 is specifically found at the apical side of lingual taste bud cells. In mammals, taste buds are found within papillae throughout lingual and palatal mucosa. They are especially concentrated in foliate and circumvallate papillae, respectively localized at the lateral and back sides of the tongue, and weakly present in fungiform papillae on the anterior tip of the lingual mucosa. In rat, CD36 has been found in circumvallate papillae (11, 12), raising the possibility of its involvement in the detection of dietary lipids. To explore whether CD36 expression was restricted to the oral sensory epithelium, papillae and the surrounding nonsensory epithelium were isolated from mouse lingual and palatal mucosa and analyzed by conventional and real-time RT-PCR. Oral cavity mapping of the expression pattern of lipid-binding proteins classically found in the digestive tract was also undertaken to determine whether CD36 exhibits specific oral localization as compared with other lipid-binding proteins. Presence of taste buds in the preparations was assessed using α-gust-
ducin gene expression, since this G protein is involved in the transduction of bitter and sweet tastes (22).

In the mouse, CD36 was strictly restricted to the lingual gustatory papillae, where its expression pattern correlates quite well with that of α-gustducin. Indeed, FAT/CD36 was highly expressed in circumvallate papillae, to a lesser extend in foliates, and rarely in fungiform papillae (Figure 1A). In contrast, CD36 was not found in palate, despite the presence of α-gustducin (Figure 1A). This was also true in the rat (data not shown).

The CD36 expression pattern was not shared by any of the other lipid-binding proteins tested (Figure 1B). Indeed, fatty acid transport protein–4 (FATP-4), a plasma membrane molecule with an acyl-CoA synthetase–like activity (23), and acyl-CoA–binding protein (ACBP), a housekeeping gene involved in cellular acyl-CoA trafficking (24), were found throughout the lingual and palatal mucosa. As expected, liver fatty acid–binding protein (L-FABP) and intestinal fatty acid–binding protein (I-FABP), which have previously been found to be expressed in tissues characterized by a high LCFA requirement (25), were undetectable in the oral mucosa (Figure 1B).

Immunolocalization of CD36 in the mouse circumvallate papillae revealed a specific staining of the apical side of some taste bud cells lining the taste pores (Figure 2, A and B). Immunoreactivity for α-gustducin was found in a large number of taste bud cells (Figure 2, C and D). Taste receptors cells were unstained when the primary antibodies were absent (data not shown). In agreement with the real-time RT-PCR data (Figure 1A), no CD36 staining was detected in palatal papillae, despite the presence of several spindle-shaped cells immunoreactive for α-gustducin (Figure 2, E and F). Immunostaining analysis of mouse circumvallate papillae indicated that about 78% of α-gustducin positive taste buds were also immunopositive for CD36 (n = 72). In a taste bud slice, the number of cells positive for α-gustducin and CD36 was 8.0 ± 2.9 and 3.0 ± 0.9 cells (n = 150), respectively. Since an average of 20 cells/slice were counted using Hoechst nuclear staining, it can be estimated that about 40% of cells were positive for α-gustducin and 16% for CD36. It is important to note that the counting of CD36-positive cells was somewhat challenging due to the apical labeling of the cells. Therefore, it is difficult to accurately estimate the proportion of α-gustducin cells that

Figure 2
Immunolocalization of CD36 and α-gustducin in the mouse circumvallate papillae and palate. CD36 immunoreactivity was confined to the apical side of taste bud cells in the lingual epithelium (A and B), while α-gustducin was found throughout the taste buds in spindle-shaped cells (C and D). (E) Coexpression of CD36 and α-gustducin was found in some lingual taste receptor cells. Palatal taste buds were positive for α-gustducin (F) but negative for CD36 (G).
might also have been positive for CD36. Nevertheless, careful analysis of double-immunostained sections suggested that CD36 might be coexpressed with α-gustducin in some receptor cells (Figure 2E).

Invalidation of CD36 gene abolishes the spontaneous preference for LCFAs. To determine whether CD36 plays a role in the oral detection of lipids, wild-type and CD36-null mice were subjected to 2-bottle preference tests. Wild-type mice exhibited a strong preference for a 2% linoleic acid–enriched solution as compared with a control solution containing 0.3% xanthan gum to emulsify the fatty acid and mimic the lipid texture (Figure 3). Similar results were reproduced with 10% linoleic acid (data not shown). Such a linoleic acid–mediated effect is not species specific, since it has been already found in the rat (7). In contrast, CD36-null mice did not discriminate between the control and linoleic acid–enriched solutions (Figure 3). This loss of preference is strictly limited to the lipid detection, since wild-type and CD36-null animals exhibited a similar response for sucrose or quinine solutions (Figure 3). To explore whether this stereotypic behavior observed with long-term (48-hour) preference tests occurred independently of

Figure 4
The short-term preference for lipid-enriched beverages and meals found in the wild-type mice is CD36 dependent. (A) Fluid intake in 1-hour-water-restricted wild-type and FAT/CD36-null mice subjected for 0.5 hours to a 2-bottle preference test. Xanthan gum (0.3%) was used to emulsify 2% linoleic acid in water and to mimic the lipid texture. The control solution was water with 0.3% xanthan gum added. (B) Food intake in 12-hour-fasted wild-type and FAT/CD36-null mice subjected to a choice between a 5% linoleic acid– or paraffin oil–enriched diet for 1 hour. *P < 0.05.

Figure 5
Effect of lingual fatty acid load on bile flux in rats. Anesthetized rats with bile diversion and esophageal ligation to prevent any lipid ingestion were subjected to an oral load of different purified fatty acids (0.2 ml). Controls received 0.2 ml water by the same route. Filled circles and open squares correspond to control solution and fatty acids tested, respectively. Data represent mean ± SEM; n = 9. *P < 0.05; **P < 0.01.
postigestive cues, water-restricted mice were subjected for 0.5 hours to a 2-bottle test. As shown in Figure 4A, wild-type mice drank about 2-fold more linoleic acid solution than the control beverage containing xanthan gum. However, this fatty acid preference was not reproduced in CD36-null mice. Preference tests were next extended to a standard meal, with mice subjected to an optional ingestion of 2 diets with different LCFA contents. Results showed that CD36-null mice displayed a lower preference for solid diets enriched with LCFAs compared with wild-type controls (Figure 4B).

The changes in pancreatobiliary secretions mediated by oral delivery of fatty acids are CD36 dependent. A potential physiological advantage of an oral lipid detection is the functional preparation of the digestive tract to incoming lipid. This assumption is supported by recent reports showing that an oral lipid load was sufficient to enhance the protein content of pancreatobiliary juice in esophagostomized rats (18). To further explore this hypothesis, the effects of an oral load with different types of fatty acids on pancreatobiliary flux and composition were assessed in rats with an esophageal ligation to prevent nutrient ingestion. As shown in Figure 5, a rapid rise in bile flux occurred following oleic acid, linoleic acid, or linolenic acid deposition in the oral cavity. This effect was highly dependent on both the length of carbon chain and the presence of 1 or several double bonds, since it was not reproduced with medium-chain and saturated LCFAAs. If no change in pancreatobiliary flux was observed (e.g., 10.0 ± 2.1 vs. 7.4 ± 1.0 µl/30 min/rat in controls after linoleic acid load onto lingual epithelium; n = 9; P = NS), a strong increase in the protein content of pancreatic juice was observed in rats stimulated by polyunsaturated fatty acids (Figure 6). The contribution of CD36 to these effects was assessed using intact and CD36-null mice with esophageal ligation. As shown in Figure 7, the linoleic acid–mediated induction of both flux and protein content of pancreatobiliary secretions found in wild-type mice was fully abolished in CD36-null mice. Similar data were obtained in rats: the rise in bile flux triggered by the direct deposition of linoleic acid onto the tongue (56.1 ± 39.7 vs. 44.2 ± 73.0 µl bile/30 min/rat in control; P < 0.05; n = 9) was not observed when this fatty acid was applied onto the soft palate, i.e., onto a CD36-negative sensory mucosa (51.2 ± 66.0 vs. 52.7 ± 73.0 µl bile/30 min/rat in control; n = 5; P = NS).

Discussion

Recent studies strongly suggest the involvement of gustation in the spontaneous preference for lipids both in rats and mice (7, 26). Herein, we report the first demonstration to our knowledge of the crucial role played by the integral membrane glycoprotein CD36 in this phenomenon. Indeed, CD36 deficiency fully abolished the high palatability of the LCFA-enriched solutions observed in wild-type mice. This effect was specifically restricted to lipid detection, since the preference for sucrose and the aversion for quinine remained unchanged in wild-type and CD36-null mice. This behavioral specificity might be accounted for by involvement of CD36 in a specialized lipid detection system localized within the oral cavity and/or by postigestive stimuli leading to an increase in lipid intake. Short-term preference tests and anatomical expression within the mouse oral mucosa supported the role of CD36 as a specific oral lipid sensor. Indeed, wild-type mice subjected to a 2-bottle test for only 0.5 hours displayed a higher preference for LCFA-enriched solution than did CD36-null mice. This experimental design excluding postigestive cues highlights the role of CD36 in the preference for fat. Moreover, in circumvallate papillae, CD36 expression appears to be restricted to cells lining the taste pores. Such an apical localization in papillae known to have the highest taste bud density is particularly adaptive to generate a fat stimulus. Indeed, CD36 is directly exposed to an extracellular medium enriched in LCFAAs as a result of locally increased lingual lipase levels (8). These data are also in good agreement with our previous observation that CD36 is confined to the apical microvilli of enterocytes (27). The role of CD36 as lipid sensor is further supported by its predicted protein structure. CD36 has a large extracellular lipid-binding pocket and an intracellular tail that has previously been shown to be associated with kinases (15–17). Additional studies are required to determine whether the interaction between LCFAAs and CD36 can transduce a signal at the origin of fat taste. An alternative possibility might be that LCFAAs are direct-
A ary secretions triggered by lingual linoleic acid deposition in intact suppressed qualitative and quantitative changes in pancreato-biliary flux, in turn, to firing of afferent nerve fibers. Only when they are applied extracellularly (28), CD36 might of taste receptor cells, facilitating the closure of K₃+ channels found in taste receptor cells and responsible for transduction of some taste 

CD36 wild-type and CD36-null mice with pancreato-biliary diversion and esophageal ligation to prevent any lipid ingestion were subjected to an oral load with linoleic acid (0.2 ml). Controls received 0.2 ml water by the same route. Black and white bars correspond to pancreato-biliary flux before and after lingual linoleic acid deposition, respectively. The functional validity of preparations was assessed by a duodenal HCl infusion (gray bars). Data represent mean ± SEM; n = 9. *P < 0.05; **P < 0.01; ***P < 0.001.

Figure 7
Impact of CD36 gene invalidation on the changes in pancreato-biliary flux (A) and protein levels (B) triggered by a lingual linoleic acid deposition. Anesthetized wild-type and CD36-null mice with pancreato-biliary diversion and esophageal ligation to prevent any lipid ingestion were subjected to an oral load with linoleic acid (0.2 ml). Controls received 0.2 ml water by the same route. Black and white bars correspond to pancreato-biliary flux before and after lingual linoleic acid deposition, respectively. The functional validity of preparations was assessed by a duodenal HCl infusion (gray bars). Data represent mean ± SEM; n = 9. *P < 0.05; **P < 0.01; ***P < 0.001.

The possibility for an additional modality directed to fat has often been suggested but remains a matter of debate. The results presented herein constitute the first molecular support for the existence of an orosensory receptor for lipids in rodents. Overconsumption of dietary fat greatly contributes to the current epidemic of obesity. Mice to which corn oil was given as an optional supplement to standard laboratory chow over a long period exhibit a chronic excessive caloric intake and develop obesity (34). Our data raise the possibility that CD36-mediated fat perception in food may contribute to the obesity risk. Further experiments are now required to understand the events underlying transduction of LCFA signaling in taste bud cells and to explore the neural pathway affecting behavioral and digestive physiology.

Methods
Experimental procedures. French guidelines for the use and the care of laboratory animals were followed, and experimental protocols were approved by the animal ethics committee of Burgundy University. Wistar rats and FAT/CD36 wild-type or -null mice (backcrossed 6 times to C57BL/6J mice) (35) were housed individually in a controlled environment (constant temperature and humidity, darkness from 8 pm to 8 am) and fed ad libitum a standard laboratory chow (UAR A04; Usine d’Alimentation Rationnelle). The strategy used to invalidate CD36 eliminated a portion of introns 2 and 3 and the entire third exon. No regulatory element is known in these intronic sequences (36). In the region of chromosome 5
where CD36 is located, the nearest genes up- or downstream are about 0.05 Mb away. Additionally, there are no genes or expressed sequence tags overlapping with the CD36 gene.

In a first set of experiments, rats and C57BL/6J wild-type mice (Charles River Laboratories) were used to establish the expression pattern throughout the lingual and palatal epithelia of major lipid-binding proteins found in the digestive tract: CD36 (15), FATP-4 (35), I-FABP and L-FABP (37), and ACBP (38). Fungiform, foliate, and circumvallate papillae were isolated according to previously published procedures (39, 40). In brief, lingual epithelium was separated from connective tissue by enzymatic dissociation (elastase and dispase mixture, 2 mg/ml each in Tyrode buffer: 140 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM CaCl2, 10 mM glucose, 1 mM MgCl2, 10 mM Na pyruvate, pH 7.4) and papillae dissected under a microscope. Epithelium surrounding the papillae was also collected to serve as nonsensory control tissue. Palatal mucosa was isolated by peeling. Intestinal mucosa was used as positive control for lipid-binding proteins. Samples were snap-frozen in liquid nitrogen and stored at −80°C until RNA extraction.

In a second set of experiments, the effect of CD36 gene invalidation on the spontaneous preference for lipid-enriched solutions (7, 26) was assessed using the 2-bottle preference test. After a training period of 3 days, individually caged wild-type and CD36-null mice were subjected for 2 days to a choice between 2% purified linoleic acid emulsified in 0.3% xanthan gum and palatal epithelia by real-time RT-PCR (ABI PRISM 7700; Applied Biosystems). α-Gustducin, a G protein considered as a molecular marker specific for taste bud cells, was also assayed to assess the purity of papillae samples. RNA levels were normalized against 18S ribosomal RNA. Primer/probe sets were designed with Primer Express software 1.00A (Applied Biosystems) using gene sequences from the GenBank database. Optimized PCR consisted of 40 cycles at 95°C for 15 seconds followed by amplification at 60°C for 30 seconds. PCR amplification was done using SYBR Green PCR Master Mix (Applied Biosystems) and the following primer sequences: FAT/CD36 forward, 5′-GATGACGTGCAAAGAACAG-3′; reverse, 5′-CTCGTCGGTGCTCATGTTAT-3′; α-gustducin (forward, 5′-GAGAGCAAGAGAATCCAGGACCAG-3′; reverse, 5′-GTGCTTTTCCAGATTCACC-3′), 18S ribosomal RNA (forward, 5′-TAAGTCCCTGCCCCTTGATACA-3′; reverse, 5′-GATCCGGAGGCTCACTAAAC-3′). The comparative 2−∆∆CT method (41) was used to determine CD36 and α-gustducin expression levels.

Comparison of expression pattern of CD36 with other lipid-binding proteins (FATP-4, L-FABP, I-FABP, and ACBP) was determined by RT-PCR amplification using a Whatman Biometra thermocycler and agarose gel electrophoresis. Stock PCR reaction mixtures (20 μl) were prepared on ice and contained 0.1 μg cDNA, 1.8 μl dNTP (2.5 mM dNTP), 2 μl x10 PCR buffer, 0.5 μl each primer (30 μM), and 0.3 μl Taq polymerase (1,000 U, QIAGEN). Amplification cycles consisted of 30 seconds at 94°C, 30 seconds at the specific melting temperature of each probe, and 1 minute at 72°C. The following primers were used: FATP-4 (forward, 5′-AAAAGGAGCTGCTCTTG-3′; reverse, 5′-AGAGGACCTATGACAACC-3′), L-FABP (forward, 5′-GAAGGAAACACTTATG-3′; reverse, 5′-CCCTTGGTCTTAAATCTTCTTGC-3′), I-FABP (forward, 5′-TCTTAGAGACACACACACAG-3′; reverse, 5′-CTTAGCTCTTGCGGTG-3′), ACBP (forward, 5′-CCCTGGCTCTTG-3′; reverse, 5′-GGCATATTGTCCTCAG-3′), CD36 (forward, 5′-TCAATTGCAGTACATGG-3′; reverse, 5′-TCAAGGCTTTGTGTCAG-3′), and α-gustducin (forward, 5′-AAGCTTACGGAGGATGC-3′; reverse, 5′-AAAGGAGATTGTTGCTTAC-3′).

Immunohistochemistry. Excised circumvallate and palatal papillae were embedded in OCT medium (Tissue-Tek, Oxford Instruments) and snap-frozen in isopentane chilled with liquid nitrogen. Cryostat sections (14 μm) were air dried for 2 hours at room temperature, fixed in 95% ethanol for 5 minutes, and rehydrated in 0.1 M PBS (pH 7.4) for 10 minutes. Rehydrated sections were blocked in 5% goat serum (Sigma-Aldrich) and 0.2% Triton X-100 in PBS for 15 minutes at room temperature and incubated overnight at 4°C with a 1:100 dilution of the anti-mouse CD36 antibody. We would also like to thank A. Holley, P. Macleod, and B. Schaal for critical reading of the manuscript and M.-C. Monnot for her excellent technical assistance. This work was supported by the National Institute of Agronomic Research.
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