The Functional Role of CD36 Involved in Fatty Acid Transduction

Han Xu
Utah State University

Follow this and additional works at: https://digitalcommons.usu.edu/etd

Part of the Biology Commons

Recommended Citation
Xu, Han, "The Functional Role of CD36 Involved in Fatty Acid Transduction" (2014). All Graduate Theses and Dissertations. 2172.
https://digitalcommons.usu.edu/etd/2172

This Dissertation is brought to you for free and open access by the Graduate Studies at DigitalCommons@USU. It has been accepted for inclusion in All Graduate Theses and Dissertations by an authorized administrator of DigitalCommons@USU. For more information, please contact digitalcommons@usu.edu.
THE FUNCTIONAL ROLE OF CD36 INVOLVED IN FATTY ACID TRANSUDCTION

By

Han Xu

A dissertation submitted in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Biology

Approved:

Timothy A. Gilbertson, Ph.D.
Major Professor

David A. York, Ph.D.
Committee Member

MieJung Park-York, Ph.D.
Committee Member

Michael Lefevre, Ph.D.
Committee Member

Anhong Zhou, Ph.D.
Committee Member

Mark R. McLellan, Ph.D.
Vice President for Research and Dean of the School of Graduate Studies

UTAH STATE UNIVERSITY
Logan, Utah

2013
ABSTRACT

The Functional Role of CD36 Involved in Fatty Acid Transduction

by

Han Xu, Doctor of Philosophy

Utah State University, 2013

Major Professor: Dr. Timothy A. Gilbertson
Department: Biology

The multifunctional fatty acid (FA) binding protein, Cluster of Differentiation 36 (CD36), has been found to be expressed in a variety of tissues where it is involved in multiple fat-related biological processes, such as lipid metabolism in mammals and the detection of lipid-like pheromones in insects. As identified in the apical membranes of taste cells, along with functional evidence in behavioral and cellular level, its involvement in the gustatory FAs detection is suggested. Nonetheless, whether CD36 acts as a direct lipid sensor or as a chaperone protein that facilitates the function of FA-activated G protein coupled receptors (GPCRs), such as taste cell expressing GPR120, remains to be determined. To characterize the role of CD36 in FA signaling, either as a primary receptor or in concert with GPCRs, I utilized human embryonic kidney 293 (HEK293) cell lines that express the different combination of the LCFA receptor GPR120 and CD36. By using intracellular calcium imaging, the presence of CD36
increased the cell sensitivity to LA slightly in GPR120+ cells. Treating the
cells with CD36 inhibitor, sulfo-N-succinimidyl oleate (SSO), resulted
in a large reduction, but not abolishment of the LA activated response, which was absent
in CD36+GPR120- cells. To investigate the role of CD36 in FA transduction specifically
in taste, a mouse taste bud-derived (TBD) cell line, TBD-a1, was used. Knockdown of
CD36 by RNA interference in these cells reduced but did not eliminate their intracellular
calcium responses to LA. *In vivo*, isolated taste cells from CD36-KO mice and WT mice
were compared for their FA sensitivity. CD36-KO cells were capable of responding to
LA with the concentration-response curve not shifted significantly compared to WT cells.
However, SSO significantly reduced the LA response in WT mice. At the behavioral
level, responsiveness to LA in CD36-KO mice was not eliminated comparing to WT
mice after formation of a conditioned taste aversion to LA. These data suggest that CD36
is a protein that facilitates the activation of GPR120 by FAs instead of a primary receptor
for FAs itself. In the taste system, CD36 is not required but may facilitate activity in FAs
responsive pathways.

(116 pages)
The Functional Role of CD36 Involved in Fatty Acid Transduction

Han Xu

As one of the most epidemic health concerns in the world, obesity has become a widespread health issue especially for the developed world. It is related to various health conditions including heart disease, type 2 diabetes and certain types of cancer. It is also quite costly. In United States alone, the medical cost of obesity in 2008 was estimated to be $147 billion. Many underlying mechanisms of obesity have been studied in the past few decades. However, the most prevalent cause appears to be simply an imbalance of energy intake and expenditure, resulting in the accumulation of body fat. On one side of this imbalance, the increase in intake of dietary fat, the most energy dense nutrient, has been suggested to be a major player in the development of obesity. This relationship has illustrated the importance of understanding the mechanisms underlying the sensory perception of fat in food. The perception of fat has been considered to be conducted through somatosensory and olfactory system for many years. Since 1997, when Gilbertson et al. provided the first evidence that fatty acids activate taste cells by inhibiting delayed rectifying potassium channels, there has been accumulating evidence supporting the existence of a “taste of fat”. As part of the research on fat taste in Gilbertson lab in Utah State University, the study of this dissertation focused on the involvement of a taste cell expressing protein, cluster of differentiation 36 in fatty acid transduction, especially in taste system.
ACKNOWLEDGMENTS

I have been dreaming of this moment since I was a kid. What I have never dreamed of was the difficulty to actually come to this point. Looking back into the years I spent in Logan, I see so many twists. I went through the dramatic change from bathing in the naive self-confidence to being racked with self-doubt as a young scientist. Fortunately, the completing of this dissertation salvaged some confidence. Now all those painful nights of self-re-evaluating and criticizing turned out to be worthy. However, I will never forget the continuous help and encouragement I received from my dearest colleagues and friends, without them I would never come this far, and probably already drown in the depression by now.

I can never express enough my gratitude to my major professor, Dr. Timothy A. Gilbertson. It was his expectation that stopped me from quitting. It was his support that provided me the opportunity to explore around. I relied on his enormous patience, and guidance to overcome all the difficulties I faced during my Ph.D. work. He is also a great model for me as a real scientist, who is un-biased, open-minded and creative. Without his mentoring and modeling, I could never become an independent scientist, and this dissertation work would never become possible.

I would also like to thanks my committee members, Dr. David A. York, Dr. Mie-Jung Park-York, Dr. Michael Lefevre and Dr. Anhong Zhou, for their insightful suggestions and advices on my research. I want to express special thanks to Dr. Joseph K-K Li who was previously on my committee. I have received valuable suggestions from
him about my career, even after his retirement. I also want to thank Dr. MieJung Park-York, whose criticize made me re-think of the standards of a real scientist.

I would like to thank Dr. Dane Hansen sincerely, in addition to her enormous help on daily basis, the knockdown experiments in this dissertation work would not be possible without her help. From the design of experiment to the actual carrying out of the work, I cannot imaging finishing this work without her support.

The colleagues in Gilbertson lab, Bhavik, Pin (Cherry), Arian, Tian and Yan are the ones who continually nourish me with optimistic atmosphere in the lab. My dearest friend Yan and Tian gave me the most generous help both as a personal friend and a reliable colleague. Tian and Cherry basically taught me almost all the techniques I needed in this research. They are sweet friends and great teachers. Their friendship and help was the great supports that companies my years in Logan.

Also, I would like to thank my brilliant friends, special thanks to Gong, Zilong, and Jerpin. I had so much delightful talks with them. They are the great source of happiness.

Last, I bothered my parents with my capriciousness over years, and they replied me with continuous support, endless tolerance, and encouragement. I would like to express my deepest gratitude to them.

Han Xu
CONTENTS

ABSTRACT ....................................................................................................................... iii
PUBLIC ABSTRACT ........................................................................................................ v
ACKNOWLEDGMENTS ................................................................................................. vi
LIST OF TABLES ............................................................................................................. xi
LIST OF FIGURES .......................................................................................................... xii
LIST OF ABBREVIATIONS........................................................................................ xiii

1. INTRODUCTION .......................................................................................................... 1
   Rationale for the Proposed Research ................................................................. 1
   Taste Buds, Taste Receptor Cells and Taste Transduction.............................. 3
   Fat Taste ............................................................................................................. 5
   Senses that might contribute to the orosensory recognition of fats ............ 6
   Gustatory cue of fats ................................................................................... 8
   Gustatory nerves convey fat signals ....................................................... 101

   Taste Transduction Mechanisms of Free Fatty Acids........................................ 12
   Delayed rectifying potassium channels .................................................... 12
   Fatty acid activated G protein coupled receptors ...................................... 13
   CD36 ......................................................................................................... 15

   Working Model: Hypothesis for Fatty Acid Transduction involving CD36 .... 20
   Dissertation Outline .......................................................................................... 21
   References ................................................................................................. 23

2. CD36 IS INVOLVED IN GPR120/G\textsubscript{α16}-DEPENDENT FATTY ACID
   TRANSDUCTION PATHWAY .................................................................................. 37
   Abstract ............................................................................................................. 37
   Introduction ...................................................................................................... 38
   Materials and Methods .................................................................................. 40

   Construction and maintenance of the transfected HEK 293 cell lines...... 40
Calcium imaging .......................................................... 41
Solutions .......................................................... 42
Statistical analysis .......................................................... 43

Results .......................................................... 43

ind-GPR120/G_{\alpha16} cells respond to linoleic acid .................................................. 43
LA-induced [Ca^{2+}]_{in} increases are promoted by introducing CD36 in ind-GPR120/G_{\alpha16} cells .......................................................... 44
SSO inhibits LA-induced [Ca^{2+}]_{in} increases in ind-CD36/GPR120/G_{\alpha16} cells. .......................................................... 46
CD36 is not the main signaling component in LA-induced [Ca^{2+}]_{in} increases in transfected HEK293 cells .......................................................... 47

Discussion .......................................................... 48
References .......................................................... 50

3. CD36 IS NOT REQUIRED BUT MAY FACILITATE ACTIVITY IN FATTY ACID RESPONSIVE PATHWAYS IN THE TASTE SYSTEM .................................................. 59

Abstract .......................................................... 59
Introduction .......................................................... 60
Materials and Methods .......................................................... 64

Maintenance of TBD-a1 cells .......................................................... 64
siRNA construction and transfection .......................................................... 64
Quantitative real-time PCR .......................................................... 65
Animals .......................................................... 66
Taste cell isolation .......................................................... 66
Calcium imaging .......................................................... 67
Solutions .......................................................... 68
Conditioned taste aversion .......................................................... 69

Results .......................................................... 72

CD36 knockdown does not induce significant decrease of responsiveness to linoleic acid in TBD-a1 cells .......................................................... 72
CD36 deficiency does not eliminate the taste cell linoleic acid detection .......................................................... 73
SSO inhibits linoleic acid detection in mice taste cells .......................................................... 74
Mice lacking CD36 retain the sensitivity to linoleic acid .......................................................... 75

Discussion .......................................................... 77
References .......................................................... 82

4. SUMMARY AND FUTURE DIRECTIONS .......................................................... 90
Summary of Research ........................................................................................................... 90
What is the mechanism of reported CD36-dependence of fatty acid preference? 93
GPR120 dependence needs further examination ................................................................. 94
What causes the inconsistency between CD36 knock-out and SSO results? ......... 95
Is there any discrimination of CD36 involvement in fatty acid taste across
different types of taste cells? .......................................................................................... 95
What is the function of CD36 in fat perception? ............................................................... 96
Other limitations of the current study .............................................................................. 97
References ......................................................................................................................... 99
<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>32</td>
</tr>
</tbody>
</table>

Expression of FAs binding and G protein coupling of FA-activated GPCRs
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>33</td>
</tr>
<tr>
<td>1.2</td>
<td>34</td>
</tr>
<tr>
<td>1.3</td>
<td>35</td>
</tr>
<tr>
<td>1.4</td>
<td>36</td>
</tr>
<tr>
<td>2.1</td>
<td>54</td>
</tr>
<tr>
<td>2.2</td>
<td>55</td>
</tr>
<tr>
<td>2.3</td>
<td>56</td>
</tr>
<tr>
<td>2.4</td>
<td>57</td>
</tr>
<tr>
<td>2.5</td>
<td>58</td>
</tr>
<tr>
<td>3.1</td>
<td>85</td>
</tr>
<tr>
<td>3.2</td>
<td>86</td>
</tr>
<tr>
<td>3.3</td>
<td>87</td>
</tr>
<tr>
<td>3.4</td>
<td>88</td>
</tr>
<tr>
<td>3.5</td>
<td>89</td>
</tr>
</tbody>
</table>

1.1 Signal transmission in taste buds.
1.2 Structure of CD36.
1.3 $[Ca^{2+}]_{in}$ responses to fatty acids in CD36-positive and -negative cells.
1.4 Model for the transduction of fatty acids by taste receptor cells.
2.1 Illustration of the constructed plasmids for ind-CD36/GPR120/$\alpha_{16}$ cells and ind-GPR120/$\alpha_{16}$ cells.
2.2 HEK293 cell constructs expressing GPR120 respond to linoleic acid.
2.3 Comparison of responses to linoleic acid between ind-GPR120/$\alpha_{16}$ cells and ind-CD36/GPR120/$\alpha_{16}$ cells.
2.4 SSO inhibited LA-induced $[Ca^{2+}]_{in}$ increases in ind-CD36/GPR120/$\alpha_{16}$ cells.
2.5 Response on con-CD36 cells to linoleic acid and on ind-CD36/GPR120/$\alpha_{16}$ and ind-GPR120/$\alpha_{16}$ cells to myristic acid.
3.1 Fatty acids responses in TBD-a1 cells with CD36 knock down.
3.2 CD36 deficiency did not eliminate taste cell responsiveness to linoleic acid.
3.3 SSO pretreatment inhibited LA-induced intracellular calcium response in wild-type mice taste cells.
3.4 Sensitivity to linoleic acid is not affected by CD36 deficiency in mice.
3.5 Stimulus generalization between linoleic acid and oleic acid was found only in wild-type mice.
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>Adenosine-5′-triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>[Ca$^{2+}$]_in</td>
<td>Intracellular calcium concentration</td>
</tr>
<tr>
<td>CA</td>
<td>Caproic acid</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>CD36</td>
<td>Cluster of differentiation 36</td>
</tr>
<tr>
<td>CS</td>
<td>Conditioned stimulus</td>
</tr>
<tr>
<td>CTA</td>
<td>Conditioned taste aversion</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DB</td>
<td>Denatonium benzoate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DRK</td>
<td>Delayed-rectifying potassium</td>
</tr>
<tr>
<td>EC$_{50}$</td>
<td>Half maximal effective concentration</td>
</tr>
<tr>
<td>ENaC</td>
<td>Epithelial sodium channel</td>
</tr>
<tr>
<td>FAT</td>
<td>Fatty acid translocase</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acid</td>
</tr>
<tr>
<td>fMRI</td>
<td>Functional magnetic resonance imaging</td>
</tr>
<tr>
<td>fura-2 K5</td>
<td>Fura-2 pentapotassium salt</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon-like peptide-1</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrogen chloride</td>
</tr>
<tr>
<td>HCN4</td>
<td>Hyperpolarization-activated and cyclic nucleotide-gated ion channel</td>
</tr>
<tr>
<td>HEK293</td>
<td>Human embryonic kidney 293</td>
</tr>
<tr>
<td>HEPES</td>
<td>2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid</td>
</tr>
<tr>
<td>IP$_3$</td>
<td>Insitol 1,4,5-triphosphate</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>KCNA</td>
<td>Shaker family of delayed rectifying potassium channels</td>
</tr>
<tr>
<td>KCNB</td>
<td>Shab family of delayed rectifying potassium channels</td>
</tr>
<tr>
<td>KCNC</td>
<td>Shaw family of delayed rectifying potassium channels</td>
</tr>
<tr>
<td>KO</td>
<td>knock out</td>
</tr>
<tr>
<td>LA</td>
<td>Linoleic acid</td>
</tr>
<tr>
<td>LCFA</td>
<td>Long chain fatty acid</td>
</tr>
<tr>
<td>LiCl</td>
<td>Lithium chloride</td>
</tr>
<tr>
<td>MA</td>
<td>Myristic acid</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NCAM</td>
<td>Neural cell adhesion molecule</td>
</tr>
<tr>
<td>NE</td>
<td>Norepinephrine</td>
</tr>
<tr>
<td>NST</td>
<td>Nucleus of the solitary tract</td>
</tr>
<tr>
<td>OSN</td>
<td>Olfactory sensory neurons</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PA</td>
<td>Palmitic acid</td>
</tr>
<tr>
<td>PIP$_2$</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PKD1L2</td>
<td>Polycystic kidney disease 1-like protein 2</td>
</tr>
<tr>
<td>PLA$_2$</td>
<td>Phospholipase A$_2$</td>
</tr>
<tr>
<td>PLCβ2</td>
<td>Phospholipase C β2 isoform</td>
</tr>
<tr>
<td>PTK</td>
<td>Protein tyrosine kinase</td>
</tr>
<tr>
<td>PUFAs</td>
<td>Polyunsaturated fatty acids</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>siCD36</td>
<td>Small interfering RNA specific to CD36</td>
</tr>
<tr>
<td>siNEG</td>
<td>Nonspecific small interfering RNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SNMP</td>
<td>Sensory neuron membrane protein</td>
</tr>
<tr>
<td>SOC channels</td>
<td>Store operated calcium channels</td>
</tr>
<tr>
<td>Src-PTKs</td>
<td>sarcoma-protein-tyrosine kinases</td>
</tr>
<tr>
<td>SSO</td>
<td>Sulfo-$N$-succinimidyle oleate</td>
</tr>
<tr>
<td>STIM1</td>
<td>Stromal interaction molecule 1</td>
</tr>
<tr>
<td>TBD</td>
<td>Taste bud derived</td>
</tr>
<tr>
<td>TRC</td>
<td>Taste receptor cell</td>
</tr>
<tr>
<td>TRPM5</td>
<td>Transient receptor potential melastatin member 5</td>
</tr>
<tr>
<td>US</td>
<td>Unconditioned stimulus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>VGCC</td>
<td>Voltage-gated calcium channels</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>TLR1</td>
<td>Toll-like receptor 1</td>
</tr>
<tr>
<td>TLR2</td>
<td>Toll-like receptor 2</td>
</tr>
</tbody>
</table>
Rationale for the Proposed Research

Along with carbohydrates and protein, dietary fat, mainly in the form of triglycerides, represents one of the three primary energy sources. During digestion, triglycerides, consisting of three fatty acids and a glycerol backbone, are hydrolyzed by lipases into free fatty acids, mono- and di-glycerides and glycerol in the intestine. These metabolites resynthesized to triacylglycerol (TAG) and TAG then are carried in the lymphatic system to the liver, where it provide energy or may be stored. The energy per gram of fat provided by triglycerides is over twice that available from carbohydrates and protein, making it a very efficient energy source.

While an excellent energy source, the excessive intake of fat contributes greatly to the prevalence of obesity, non–insulin-dependent diabetes, atherosclerosis, and hypertension [1,2]. The over consumption of fat is due, at least in part, to its addictive properties in both animals [3,4,5] and humans [6,7,8]. Many strains of rats and mice spontaneously prefer lipids [4,5]. Until recently, this preference has been considered to be attributable mainly to postingestive signals involved in long-term preference and reinforcing effects [9], olfactory cues [10,11] and somatosensory (textural) cues [12,13,14]. Taste was not believed to be a major factor in the sensory recognition of dietary fat.

However, accumulating data suggest that the contribution of gustatory cues to fat perception is substantial. Mice show significant preference for corn oil over mineral oil
which mimics the texture of corn oil but not the taste or flavor of it. Further, Fukuwatari and colleagues found that when olfactory and textural effects were minimized, the mice could still recognize oleate [16]. Interestingly, they also found that the free fatty acid (FFA) rather than triglyceride is important for the gustatory recognition of fat, which is consistent with the observation by Kawai that inhibition of lingual lipase results in a markedly diminished preference for pure triglycerides in mice [17]. Based upon these and other data, it appears likely that the gustatory recognition of fat depends on the detection of very small amounts of free fatty acids which are either contained in fat-containing food or are generated by lingual lipase from triglycerides. Free fatty acids can be generated in the oral cavity within a time frame consistent with taste perception [17]. Lingual lipase is locally secreted in the cleft of foliate and circumvallate papillae by the von Ebner’s glands [17] so that its concentration is likely significantly higher in the vicinity of taste buds and may be sufficient for FFA sensing.

Other compelling results also support the involvement of gustation in the sensing of dietary fat. Cell-based assays in rat taste receptor cells show that applying FFAs extracellularly inhibits delayed-rectifying potassium (DRK) channels [18], which are known to be implicated in the transduction pathway of a variety of taste stimuli. This report was the first one to implicate the presence of a sensory transduction mechanism for fat on the tongue (discussed below). Validation of the ability of fatty acids to be sensed in the oral cavity has been achieved in human studies. Westerterp-Plantenga [19] and Mattes [20] did a series of well controlled psychophysical investigations, both suggesting the oral detection of FFA with minimal input from the olfactory and viscosity-sensing
systems. Furthermore, the known fatty-acid translocase Cluster of Differentiation 36 (CD36) which plays a role in fatty acid transport in a variety of tissues is located on the apical (chemoreceptive) side of the taste bud in the circumvallate papillae [21]. Taken together, there is mounting evidence that fatty acid activation of taste bud cells may contribute to the taste of fat and further contribute to the regulation of food intake. My dissertation research was therefore designed to study the involvement of CD36 in mammalian fat taste transduction in mouse taste cells and two model systems, including transfected Human Embryonic Kidney 293 cells (HEK293) cells and taste bud derived (TBD) cell lines.

**Taste Buds, Taste Receptor Cells and Taste Transduction**

Four types of papillae are present on the surface of mammalian tongue: filiform (non-sensory structures), fungiform, foliate and circumvallate. The later three are responsible for the taste detection to different chemicals, eliciting the five basic tastes: sweet, sour, salty, bitter and umami. Onion shaped taste buds containing ~50-150 cells are located in these sensory papillae. It is commonly accepted that there are at least three types of cells present in each taste bud. Type I cells have been proposed as “supporting cells” which modulate the local environment within taste buds for the normal functioning of Type II and Type III cells. Type II cells, which are generally referred to as receptor cells, express chemical receptors on the membrane of apical side of the cell and all the necessary signaling components of G protein-coupled receptor (GPCR)-mediated pathways within the cells GPCRs are believed to function as the primary receptors for sweet, bitter and umami perception [22,23]. Type III cells (presynaptic cells) are thought
to be the output cells of the taste bud which transmit the taste signals to the afferent nerve through synapses [24,25]. However, the role of the different cell types and the mechanisms underlying intercellular signaling within the taste buds are still controversial subjects. The apical membrane of taste receptor cells (TRCs) is exposed to the environment of the oral cavity. Taste compounds interact with receptors or ion channels situated on the apical membranes of TRCs. A popular model of taste transduction is illustrated in Figure 1.1 [26]. In this model, Type II cells are narrowly tuned and Type III cells have broad responses to tastants. With GPCRs, T1Rs and T2Rs [23], Type II cells (receptor cells) are capable of detecting sweet, bitter and umami tastes, but not salt and sour tastes, and data support the idea that each cell is responsive primarily to one taste quality [26]. Type III cells isolated from the taste buds which are not in connection with Type II cells only respond to sour and salty stimuli [25]. In contrast, Type III cells not separated from the taste buds are broadly responsive to taste stimuli (two or more taste qualities), including sweet, bitter and umami [26], although they express none of the taste GPCRs nor their downstream effectors. Those GPCR-dependent responses rely on cell-to-cell signaling. Type II cells secrete ATP through Ca-activated pannexin 1 hemichannels during taste stimulation [25] acting on the purinergic P2Y4 receptor [27] on Type III cells. Thus, in some cases, the signals generated in Type II cells are passed into Type III cells. With both the direct salt and sour stimuli and ATP signal, Type III cells release serotonin (5-HT) and norepinephrine (NE) [27], possibly transmitting the taste signal to connected gustatory afferent neurons. The taste signals generated by the TRCs are transmitted from taste buds to the connected gustatory afferent neurons and the
then directly connected neurons in the nucleus of the solitary tract. Passing through the nucleus of the solitary tract, the information reach several nuclei and cortical areas in the brain.

**Fat Taste**

As mentioned previously, currently it is commonly believed that there are five basic tastes, which are sweet, sour, salty, bitter, and umami. However, the concept of basic tastes always faces challenges. Robert P. Erickson pointed out that the current concept of five basic categories of taste is strongly influenced by our semantics, which differs from culture to culture, and was generated from daily experiences and merely function as a general descriptor rather than a concept rooted in scientific terms. Furthermore, Erickson claimed that this concept of basic tastes was first hypothesized from some poorly designed investigations and lacked subsequent empirical validation [28].

Despite the debate concerning the nature of the basic taste primers, studies at behavioral, molecular and cellular levels provide strong support for the ability of the gustatory system to be able to sense fats consistent with the idea that fat has a taste. The following paragraphs will focus on the increasing evidence that supports a role for fatty acids as the proximate stimuli for fat taste along with sweet, sour, bitter, salty, and umami stimuli.

*Senses that might contribute to the orosensory recognition of fats*

When we talk about the sensing of fat in food, we usually use the non-scientific descriptions such as “greasy,” “sticky,” “oily,” or “slippery.” Indeed, fats in food do alter
palatability and arouse various sensations, which allow us to distinguish fat rich foods from the ones with less fat. Spontaneous preference to dietary fat during ingestion have been well studied with 2-bottle preference tests in both rats [4,29,30,31] and mice [32]. With free choice, most animals tend to prefer high fat diet to a more nutritionally balanced chow diet [29], long chain fatty acids solutions to their control solutions [4], flavors mixed into corn oil emulsion over flavors presented in water [30], and fluid containing triglyceride oil over the same fluid without oil [31]. In humans, high-fat foods tend to elevate natural opiate levels in the brain, and are the most preferred [33]. Oral exposure to fat alters postprandial lipid metabolism as well [34]. Although post-oral pathways are possibly contributing to this spontaneous preference [35,36,37], it is clear that there must be effective and efficient sensors that allow us and animals to distinguish low-fat foods from high-fat foods immediately during ingestion.

From the various sensory modalities purported to be important, visual, olfactory, somatosensory, which one(s) is (are) significantly contributing to this complex sensory process surrounding the recognition of dietary fat? Further, how much is taste involved in this process? To address this question, eliminating or minimizing the effects of visionary, olfactory, and somatosensory cues is quite necessary, especially in behavioral studies.

The visual cue in behavioral studies in animals, for example, preference tests with solutions, is naturally eliminated since the solutions used are usually colorless. And in human sensory tests, the visual cues are minimized often by blindfolding the participants or using red light [38]. Textural cues may be minimized by the use of agents that provide
texture in the absence of other sensory cues. For example, xanthan gum was used to mask the textural effect of oil in solutions in rat preference tests [5,16].

**Olfactory cue of fats**

In case of the olfactory system, before we address the question of eliminating olfactory cues in behavioral tests of fat taste, it is necessary to clarify the positive evidence of the olfactory attributes of fat. The existing evidence supports odor cues of rancid fats quite well, but are surprisingly limited for odor cues of nonoxidized fats. Olfaction is shown to contribute to the spontaneous preference to high-fat food in mice [11]. However, electrophysiological recording failed to show any increase of neural activity with nasal exposure to caprylic acid [39], lauric or linoleic acid [40] in rhesus macaques. Bilateral bulbar lesions on most of the presumed fatty-acids-responsive areas did not impair the ability of rats to discriminate between acetic and caproic or propionic acids [41]. With treatments blocking olfaction such as zinc sulfate irrigation or olfactory bulbectomy, rats and mice exhibited attenuated preference to oil and long-chain fatty acids with higher concentrations comparing to the non-treated animals [4,5,10,16]. Human studies did not find any effect of eliminating the olfactory input on detecting of fat [12]. Thus, with limited contribution of odor cue in fat detection, there are only two sensory cues left that could be responsible for oral sensory of fat, that is tactile cues and taste cues.

**Tactile cue of fats**

Texture/tactile perceptions of dietary fat have been studied in human sensory tests. It is often described with terms pertaining to viscosity by sensory testers, such as buttery,
fatty, greasy, oily, creamy, and clinging [42], and often considered as a predominant contribution in subjective ratings by sensory panels [43]. In electrophysiological studies in primates, viscosity is found to arouse responses in certain amygdala neurons [40]. The non-viscosity texture representations elicit responses in a population of single neurons in the orbitofrontal cortex, which cannot be stimulated with free fatty acids, indicating its textural rather than gustatory function [44]. This finding is consistent with other evidence from a human fMRI study [45]. Furthermore, fat-produced viscosity and non-fat-produced viscosity are dissociated with different texture channels [44]. Lubricity may also be a texture attribute contributing to oral detection of fat [44,46]. Thus, texture contributing to fat oral detection is well supported, but the transduction mechanism underlying is still under debate. In addition to the predominant view that the textural properties of fats activate pressure-sensitive free endings and corpuscular receptors, which we have just discussed, free fatty acids might arouse somatosensory sensations by activating lingual nerve-innervated trigeminal neurons, inducing intracellular calcium rise from releasing calcium stores from the endoplasmic reticulum [47]. Fatty acids were also reported to modulate DRKs [18] on trigeminal neurons as in chemosensory mechanisms.

**Gustatory cue of fats**

Accumulating data strongly suggest that the contribution of gustatory cues to fat perception is substantial. Fushiki and colleagues did a series of preference tests on mice. Most mice strains show a significant preference for corn oil over mineral oil which mimics the texture of corn oil but not the taste or flavor of it [15]. Further, when olfactory and textural effects are minimized, mice can still recognize oleate [16]. Interestingly, it is
the free fatty acids rather than triglyceride that are important for the gustatory recognition of fat, which is consistent with the observation that inhibition of lingual lipase results in a markedly diminished preference for pure triglycerides in mice [17]. Based upon these data and data from other researches [4,16], it appears likely that the gustatory recognition of fat depends on a very small concentration of free fatty acids, which are either already contained in food or hydrolyzed from triglycerides by lingual lipase. Hydrolysis of triglycerides into free fatty acids occurs over a time frame consistent with taste perception [17]. The same authors reported that lingual lipase is locally secreted in the cleft of foliate and circumvallate papillae by the von Ebner’s glands [17], so that its concentration is likely significantly higher within the vicinity of taste buds and may be sufficient for FFA sensing. Consistent results have been developed in human studies. Westerterp-Plantenga [19] and Mattes [20] did a series of well controlled psychophysical investigations, both suggesting the oral detection of FFA with minimal input of olfactory and viscosity-sensing systems.

Despite the mounting evidence, the validity of fat as a basic taste has not been conclusively demonstrated. First, low concentrations of fatty acid oxidation products may reflect possible effective stimuli contributing to the detection and preference by animals [31]; something that has not been completely controlled in any of the previously mentioned studies. Second, evidence of the contribution of human lingual lipase activity is questionable. Although a recent study shows that inhibiting lingual lipase activity can influence oral sensitivity in humans in predictable ways [48], its activity is only detectable at very low levels by enzymatic assay, while Western blots failed to confirm
its expression [49]. Thus, gustatory detection of fat in humans might actually require free fatty acid presence in foods, which can be introduced with cooking and released by mastication.

The contribution of oxidation products can be well controlled with short term behavioral tests. With only very brief presentation of stimuli, a postingestive effect is virtually eliminated, and oxidation of fatty acids is also minimized. In combination with conditioned taste aversion (CTA), it is possible to tell more definitively whether fatty acids are effectively detected. Animals are averely conditioned to a stimulus (the conditioned stimulus, CS), such as fatty acids, by injecting LiCl immediately after CS oral exposure. Subsequent brief exposure to the CS will lead to rejection, which suggests effective oral detection of this stimulus. Failure to show any aversive response to CS indicates that tested CS is not effectively sensed by the animals. Following this strategy, McCormack et al. found that rats are able to detect non-esterified oleic acid and linoleic acid in concentration as low as 66 µM [50]. As little as 1.5% of viscosity change induced by these fatty acids and the lack of aversion to ethanol (vehicle used to dissolve fatty acids) odor were used to exclude viscosity and olfaction from contributing to the detection. This result is coincident with the CTA study reported by Liu et al. on mice [51].

**Gustatory nerves convey fat signals**

As mentioned before, taste cells on tongue are innervated by the chorda tympani nerve and the glossopharyngeal nerve. The chorda tympani nerve conveys signals from the anterior tongue, including fungiform and anterior foliate papillae. And the glossopharyngeal nerve conveys signals from circumvallate papillae and posterior foliate
papillae located at the back of the tongue. They transmit taste signals to the nucleus of the solitary tract (NST) in the brain stem. If fats are perceptible in gustation, nerve activity is expected in these peripheral gustatory nerves when animals are orally exposed to fats. Positive evidence has been published with whole nerve recording in mice exposed to FFAs. The glossopharyngeal nerve shows stronger activity than the chorda tympani, however, both are dependent on the expression of fatty acid activated G protein coupled receptors GPR120 and GPR40 [52]. Comparable evidence has been published in CD36 knockout and wild-type mice [53].

In addition to directly recording the nerve activity, their transection has also been used to determine the involvement of a gustatory nerve in oral fat detection. In rodents, nerve transection has been reported many times to reduce the preference and conditioned taste aversion to fats [53,54,55,56]. Glossopharyngeal nerve cuts diminish licking responses to corn oil but not to glucose [55]. Oral fat exposure induced pancreatic exocrine secretion is also found to be reduced after nerve cuts [53]. However, caution needs to be taken when interpreting these experiments, since nerve transection may also alter salivation and lingual lipase secretion, and also because that chorda tympani and glossopharyngeal are mixed nerves carrying both taste and somatosensory signals.

Taken together, with data from animal behavioral tests, electrophysiological studies and from human studies, it is safe to state that free fatty acids are capable of activating the gustatory system consistent with the perception of dietary fat. The following paragraphs will discuss the current studies of this detection system within the oral cavity, including the fatty acid receptor candidates that convey fatty acids signals in
Taste cells; these include delayed rectifying potassium channels, fatty acid activated GPCRs, and fatty acid binding protein CD36, as well as their downstream signaling pathways.

**Taste Transduction Mechanisms of Free Fatty Acids**

**Delayed rectifying potassium channels**

The first receptors implicated in the detection of free fatty acids in taste cells were DRK channels identified by Gilbertson et al. [18]. Free fatty acid-sensitive DRK channels are found to be expressed in various systems, such as smooth and skeletal muscle and cardiac cells, where their inhibition by free fatty acids activates these systems [57,58,59]. Similar results are found in taste cells. DRK channels help repolarize the cell membrane following its depolarization. Electrophysiological experiments indeed proved that their inhibition promotes and prolongs the depolarization of rat taste cells [18]. This enhancing effect is also supported by preference test in rats. Subthreshold concentrations of saccharin are not preferred by animals when given alone, but are significantly preferred when presented together with a sub-threshold concentration of fatty acids [60].

Taste receptor cells express a variety of DRK channels encompassing members of the KCNA, KCNB and KCNC families. Of these, the most highly expressed channels, assessed by quantitative real time PCR, are the KCNA5, KCNB1, KCNB2, and KCNC1 channels. Heterologous expression of these channels was used to assess their fatty acid sensitivity to try and identify the primary fatty acid-sensitive channel. In general, members of the KCNA family appear to be highly sensitive, KCNB channels are
moderately sensitive and the KCNC family appears insensitive to polyunsaturated fatty acids (PUFAs), as determined by patch clamp recording.

Interestingly, the relative expression of these DRK channel subtypes appears to be correlated with fatty acid responsiveness and overall dietary fat preference. Molecular experiments revealed that taste cells from high fat diet obesity-resistant rats express a greater ratio of fatty acid-sensitive DRK channels than an obesity-prone strain. Further, in electrophysiological experiments, taste cells from obesity-resistant rats are more responsive to fatty acids suggesting an inverse correlation between peripheral responsiveness to fatty acids and dietary fat preference [60]. The oral fat-sensing pathways also appear to be sensitive to dietary experience. High fat diets (or the development of obesity) altered the expression of DRK channels (i.e. decreased the ratio of fatty acid-sensitive DRK channels) and reduced correspondingly the responsiveness of the taste cells to fatty acids. Taken together, DRK channels appear to be a viable candidate receptor for fatty acids in the gustatory transduction of dietary fat.

However, given the fact that free fatty acids act as open channel blockers and only a very small portion of DRK channels are open at rest, it is hard to explain the large response to free fatty acids, unless there are other fatty acid receptors on the membrane that could provide the initial activation (depolarization) of the cells, which in turn could open these channels.

**Fatty acid activated G protein coupled receptors**

With the possible exception of salty and sour taste transduction, the initial receptive events involved in taste reception all involve the activation of G protein-
coupled receptors (GPCRs). Recent research had identified some previously orphan GPCRs as being responsive to different kinds of fatty acids. GPR120 expressed on circumvallate papillae and fungiform papillae on the tongue as well as in enteroendocrine cells in mice and humans \( [61,62,63,64] \) are reported to be receptors for unsaturated long-chain fatty acids \( [62] \). Functionally, activation of GPR120 by dietary fat results in the secretion of glucagon-like peptide-1 (GLP-1) in endocrine cells \( [62] \) and taste cells \( [65] \). GPR40 is indicated to be a receptor of medium- to long-chain fatty acids \( [64] \) and is expressed in pancreatic islets \( [66] \). The short chain fatty acids are ligands for GPR41 and GPR43 \( [67] \), which are highly expressed in adipose tissue \( [68] \), enteroendocrine cells, mucosal mast cells \( [69] \) and lymphatic tissues \( [67,70,71] \). More recently, GPR84 has been identified as a medium-chain fatty acid receptor where it is expressed in monocytes and macrophages \( [72] \). Interestingly, its mRNA level can be markedly elevated by lipopolysaccharide treatment, suggesting its possible role in monocytes/macrophage activation and hosting immune response.

The coupling with G proteins is different across the fatty acid-activated GPCRs and as a result, leads to various downstream cell signaling pathways. GPR40 couples with \( G_{q/11} \), which leads to the protein kinase A and C (PKA and PKC) activities and the elevation of intracellular calcium levels (\([Ca^{2+}]_{in}\)). Interestingly, cAMP generated by PKA is reported to close DRK channels. GPR41 coupling to \( G_{i/o} \) and GPR43 coupling to \( G_q \) or \( G_{i/o} \) have the downstream pathway as the formation of inositol 1,4,5-trisphosphate (IP\(_3\)). GPR84 couples primarily to \( G_{i/o} \) and elicits a cAMP increase in its downstream pathway. Long chain fatty acids activating GPR120 resulting in the inhibition of caspase-
3 activity is revealed to be via the G$_q$ pathway [73]. The properties of fatty activated GPCRs are summarized in Table 1.1.

In the current research, I have focused on the function of CD36 in fatty acids transduction. It is necessary also to look further into the function of GPR120, as it shares the same ligand as CD36, long chain fatty acids. GPR120 co-localizes with phospholipase-C$\beta$2 (PLC$\beta$2) and $\alpha$-gustducin in mice taste bud cells [64]. It mediates taste preference and nerve responses for fatty acids in mice, as demonstrated in its knock out animals [52] and its dysfunction leads to obesity in both mice and humans [74]. This accumulating evidence led me to include this protein in our hypothesized model of fatty acid taste transduction.

**CD36**

*The Identification of CD36*

CD36 was first isolated from platelets in 1989 [75] as a thrombospondin-binding protein [76, 77]. A rat homolog of CD36, also known as fatty acid translocase (FAT), was identified in 1993 [78]. Immediately, the 2432 bp cDNA of this 88-kDa membrane protein was isolated [79]. The sequence of CD36 protein is highly conserved between the cloned human and rodent proteins: the rat protein FAT is 85% homologous with human CD36 and reacts with a polyclonal antibody against human CD36 [80]. Thus, the term “FAT/CD36” is usually used when referring to either homolog in publications, which does not mean that heterogeneous protein complex. Both homologs (rat FAT and human CD36) are integral membrane proteins with two transmembrane domains, two very short intracellular segments and a heavily glycosylated extracellular domain which forms a
hydrophobic pocket (Figure 1.2 A) [79]. CD36 is involved in angiogenesis, atherosclerosis, inflammation, and lipid metabolism [81]. Transfected fibroblasts with FAT/CD36 showed increased rates of fatty acid uptake, indicating that the protein plays a key role in fatty acid transport [82]. This transport of fatty acid on rat adipocytes can be irreversibly inhibited by the covalent labeling on FAT/CD36 with N-sulfosuccinimidyl esters of long chain fatty acids by 75% [78]. The purified FAT/CD36 from adipose tissue reversibly binds native long chain fatty acids (LCFAs) at nanomolar concentration range, with a saturating fatty acid-protein ratio near 3, giving direct evidence of its involvement in the fatty acid metabolism [83].

The Involvement of CD36 in Fatty Acid Perception

CD36 is expressed on a variety of cell types including endothelium, erythrocytes, platelets, adipocytes, dendritic cells, neutrophils, monocytes/macrophages, microglia, muscle cells and, interestingly from our perspective, the apical side of taste buds [21,84,85,86,87,88,89,90,91,92]. As a multifunctional receptor, CD36 plays an active role in a variety of physiological and pathological processes. It was reported to play a main role in adipocytes and macrophages to recognize and degrade oxidized low density lipoprotein [86]. In platelets, it functions as a receptor for thrombospondin-1 and collagen type I/IV [76,93]. It was also shown that CD36 participates in phagocytic clearance as a cofactor of Toll-like receptors by facilitating the recognition of anionic phospholipids of bacteria in human monocytes [94,95] and dendritic cells [96], rat retinal pigment endothelium [97], and Drosophila hemocytes [98].
The first hint of CD36’s participation in the sensation or absorption of dietary fat was observed by Poirier et al. [99] that FAT in the jejunal mucosa is expressed in the brush border of epithelial cells, and FAT mRNA in the small intestine is increased by a LCFA-rich diet. This observation in jejunum was soon followed by one on the taste organ by Fukuwatari [21], in which CD36 was revealed to be specifically localized in the apical side of taste bud cells in the circumvallate papillae, strongly suggesting the participation of CD36 in oral fat perception. CD36 knockout mice are reported to exhibit reduced fat preference and decreased fat consumption [100], providing behavioral support of CD36 mediation of a gustatory component to fat preference.

Beginning in 2005, Laugerette and colleagues published a series of papers focusing on the role of CD36 in gustatory fatty acid transduction. Initially, they observed that the inactivation of CD36 gene fully abolished the preference for LCFA-enriched solutions and solid diet which is observed in wild-type mice. In addition to the preference tests, the flux and elevation of protein level in pancreatobiliary juice can be another indicator of fat perception. An oral lipid load was sufficient to enhance the protein content of pancreatobiliary juice in rats [101] and flux, with their esophagus ligated to prevent nutrient ingestion. Here Laugerette and colleagues found that the linoleic acid-mediated induction of both flux and protein content of pancreatobiliary secretions observed in wild-type mice was fully abolished in CD36-null mice [102].

In their research published in 2008, CD36-positive cells were selected from isolated mice circumvallate taste bud cells [103] and changes in the $[Ca^{2+}]_{in}$ levels inducted by LCFAs were investigated in both selected (CD36-positive) and non-selected
(CD36-negative) cells. Rapid and robust increase in the $\text{[Ca}^{2+}\text{]}_{\text{in}}$ was observed in CD36-positive cells, which could be inhibited by 400 µM sulfo-N-succinimidyl oleate (SSO) (Figure 1.3 B), while in CD36-negative cells the $\text{[Ca}^{2+}\text{]}_{\text{in}}$ remained at a low level (Figure 1.3 A). They also found that the neuronal activation of the NST triggered by oral stimulation was absent in CD36-null mice, which can be observed in wild-type animals.

In conclusion, they believed that fatty acid perception involves the activation of CD36. In a very recent human study, the genotypes of the CD36 gene showed correlation with gustatory sensitivity to oleic acid and triolein. The subjects homozygous for the allele that associates with low expression of CD36 had higher detection thresholds to these fats [48].

*Is CD36 a Chaperone Protein rather than a Primary Receptor?*

The mechanistic basis of CD36 interacting with its ligands and the downstream signaling is still poorly understood in any biological system. Besnard and colleagues provided evidence that supports CD36 as a receptor of long chain fatty acids that directly transduces fatty acid signals to its downstream signaling pathways. Their work shows that CD36 is required for inositol 1,4,5-triphosphate (IP$_3$) production, capacitative calcium influx and protein tyrosine kinase (PTK) phosphorylation in response to linoleic acid, and that its inhibitor SSO curtails these responses [104]. And this CD36 dependent calcium signaling might involve multiple phospholipase A$_2$ (PLA$_2$) isoforms and stromal interaction molecule 1 (STIM1), which regulates the store operated Ca$^{2+}$ channels (SOC) [105]. However, our data contradicts this theory. We found that the linoleic acid response in the form of intracellular calcium rise is not exclusively observed in type II taste cells
that are CD36 positive, which is the population studied in their research. This drives us to search for other possible roles of CD36 in fatty acid taste.

In 2007, an insightful study on *Drosophila* olfactory sensory neurons (OSN) cilia was suggested a different role for CD36 [106]. The authors found that sensory neuron membrane protein (SNMP), a *Drosophila melanogaster* CD36 homologue, is required in the detection of fatty-acid derived odorant pheromone. They also showed strong evidence of SNMP acting in concert with other transmembrane odorant receptors. OSNs shows different electrophysiological patterns when SNMP (or the pheromone receptor) was mutated, both of which are required for the normal function of the neurons in the response to the pheromone.

Interestingly, a previous study showed that the ectopically expressed pheromone receptor could still be activated by directly applying the pheromone on OSN not expressing SNMP. However, in this 2007 paper, when pheromones are presented in air to the receptor in its native environment, SNMP turned out to be essential. Similar results have been revealed by our lab using a heterologous expression system. CD36 does not seem to be a *requirement* for the G protein coupled receptors response to fatty acids, but may help facilitate the response. Parallel to the case in *Drosophila*, the role of CD36 as a co-factor of GPCR seems to represent a viable alternative to the role of CD36 in fatty acid transduction.

Further support for the theory suggesting CD36 may function as a co-receptor comes from studies in the immune system, in which the CD36-dependent recognition of
specific lipid-derived pathogens is coupled with the Toll-like receptors to initiate the innate immune response.

**Working Model: Hypothesis for Fatty Acid Transduction involving CD36**

Our general model for the transduction of free fatty acids by taste receptor cells is shown in Figure 1.4. Briefly, fatty acids specifically bind to and activate G protein coupled receptors, coupled to the activation of PLCβ2, which cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and IP3. The latter then activates IP3 receptors on the endoplasmic reticulum and releases intracellular Ca2+, which activates the cell membrane located cation channel transient receptor potential melastatin member 5 (TRPM5) and induces the influx of cations, eliciting a depolarizing receptor potential. The membrane depolarization activates DRK channels, a subset of which is blocked by fatty acids, reinforcing the depolarization. This enhanced depolarization is necessary to open voltage-gated calcium channels (VGCC) leading to a rise of intracellular Ca2+ level, which triggers the release of neurotransmitters. The role of CD36 in this transduction cascade, we hypothesize, is to initially bind free fatty acids and present them in a proper orientation to the fatty acid-activated GPCRs and/or fatty acid-sensitive DRK channels. The experiments included in this dissertation will test the hypothesis that **CD36 is not the primary receptor for fatty acids, but rather helps facilitate fatty acid binding to the fatty acid-activated G protein coupled receptors.**
Dissertation Outline

This dissertation research explores the role that CD36 plays in fatty acid gustatory sensation transduction. Given the abundant evidence of two competing receptors of long chain fatty acids found in taste cells, CD36 and GPR120, my research is focused on the following questions in general: Is CD36 crucial for fatty acid taste? Are these two receptors involved in the same transduction pathway? If they are, what is their function in the pathway? To answer the first question, I have made the use of genetically deficient mouse strains lacking CD36. I have investigated both the difference between taste cell responsiveness to fatty acid isolated from CD36-KO mice and wild type mice. I have also compared taste sensitivity to fatty acids of these two types of animals at the behavioral level. To study the mechanism of fatty acid transduction involving CD36 and GPR120 in vitro, I have used the constructed HEK293 cell lines transfected expressing GPR120 and Gα16 with or without CD36. I compared their intracellular calcium responses to fatty acids in CD36-positive cells and CD36-negative cells. Further, both transfected HEK293 cells and isolated taste cells were treated with CD36 specific inhibitor SSO to isolate the involvement of CD36 in these cells. In addition, a newly developed mouse taste bud derived cell line (TBD-a1 cells) was used as an in vitro system to perform the RNA interference of CD36, which cannot readily be performed in the primary taste cells.

In Chapter 2, in order to test my hypothesis that CD36 facilitates the fatty acid-activated GPCRs-dependent pathways, the GPR120 and Gα16 were heterologously expressed in HEK293 cells with or without CD36. First I found that in this heterologous system, GPR120/Gα16 was sufficient to activate intracellular calcium response to long
chain fatty acid linoleic acid. In addition to this working pathway, introducing CD36 into this system made the cells slightly, though not significantly, more sensitive to linoleic acid. In addition, treating the CD36-positive cells with SSO significantly reduced the responses. Thus, my conclusion is that in this heterologous expression system, CD36 is involved in GPR120/Gα16-dependent fatty acid transduction. But its role in this transduction is not a critical one.

In Chapter 3, I first made use of the TBD cells as an *in vitro* taste cell model. Specifically knocking down the expression of CD36 using RNA interference led to a reduction in linoleic acid-induced responses. However, this reduction in responsiveness was not statistically significant. Similar results were also obtained in isolated taste cells from CD36-KO mice, which had slightly smaller responses to linoleic acid than taste cells isolated from wild type mice. However, treatment with SSO significantly reduced the linoleic acid responsiveness in wild-type cells. I also performed conditioned taste aversion tests on CD36-KO and wild type mice. By examining their ability to establish taste aversions through oral exposure to a linoleic acid solution followed by intraperitoneal injection of LiCl solution to induce gastric distress, the capability of the animals to orally detect linoleic acid was assessed. I found that CD36 deficiency did not impair their linoleic acid sensitivity.

Chapter 4 is the summary of this dissertation research. I also discuss the remaining questions and the future directions for this research.
References


Table 1.1  Expression of FAs binding and G protein coupling of FA-activated GPCRs

<table>
<thead>
<tr>
<th></th>
<th>Expression</th>
<th>FAs</th>
<th>G-protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP</td>
<td>Pancreatic islets</td>
<td>Medium</td>
<td>Gq/11</td>
</tr>
<tr>
<td>R40</td>
<td>- long</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GP</td>
<td>Adipose tissue, enteroendocrine cells</td>
<td>short</td>
<td>Gq/o</td>
</tr>
<tr>
<td>R41</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GP</td>
<td>Lymphatic tissues</td>
<td>short</td>
<td>Gq or Gq/o</td>
</tr>
<tr>
<td>R43</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GP</td>
<td>Monocytes &amp; macrophages</td>
<td>Medium</td>
<td>Gq/o</td>
</tr>
<tr>
<td>R84</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GP</td>
<td>Enteroendocrine cells</td>
<td>Long</td>
<td>Gq</td>
</tr>
<tr>
<td>R120</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 1.1 Signal transmission in taste buds. Type II cells are receptor cells for bitter, sweet, and umami. Type III cells are receptor cells for salty and sour taste. Type II cells secret ATP upon taste stimulus. ATP then act on Type III cells so the signals generated in Type II cells are passed into Type III cells. With salt and sour stimuli and ATP signal, Type III cells release 5-HT and NE to connected gustatory afferent neurons [26].
Figure 1.2 Structure of CD36. CD36 is a membrane protein with two integral transmembrane domains. Both the amino-terminus and carboxy-terminus are short and intracellular. And extracellular domain is heavily glycosylated and forms a hydrophobic pocket [107].
Figure 1.3  $[\text{Ca}^{2+}]_\text{in}$ responses to fatty acids in CD36-positive and -negative cells [53].
See text for details.
Figure 1.4 Model for the transduction of fatty acids by taste receptor cells. CD36 initially binds free fatty acids and present them in a proper orientation to the fatty acid-activated GPCRs and/or fatty acid-sensitive DRK channels. Fatty acids activate G protein coupled receptors, coupled to the activation of PLCβ2, which cleaves PIP2 into DAG and IP3. IP3 opens IP3R on ER and releases intracellular Ca2+, which activates TRPM5 on cell membrane and induces the influx of cations, eliciting a depolarizing receptor potential. The membrane depolarization activates DRK channels, a subset of which is blocked by fatty acids, reinforcing the depolarization, upon which VGCC is opened, leading to a rise of intracellular Ca2+ level, which triggers the release of neurotransmitters.
CHAPTER 2

CD36 IS INVOLVED IN GPR120/Gα16-DEPENDENT FATTY ACID TRANSDUCTION PATHWAY

Abstract

The multifunctional fatty acid binding protein Cluster of Differentiation 36 (CD36) has been shown to play a role in a variety of fat-related biological processes in vertebrate animals including angiogenesis, atherosclerosis, inflammation and lipid metabolism. It was first identified in rat adipocytes as a long chain fatty acid transporter and shows a very high affinity for long chain fatty acids. CD36 was initially identified in taste cell apical membranes by Fukuwatari et al. in 1997. Evidence from behavioral approaches supported a role of CD36 in the gustatory recognition of fatty acids. Mice lacking CD36 did not show preference for fatty acids (linoleic acid) in 48-h preference tests [1]. Two competing, though not mutually exclusive, theories have emerged concerning the role of CD36 in fatty acid transduction in taste cells. In one, it is believed to directly activate a downstream signaling pathway. In the other theory, CD36 is hypothesized to play a role as a chaperone protein that binds and orients free fatty acids to the fatty acid-activated G protein coupled receptors (GPCRs) and/or fatty acid-sensitive delayed rectifying potassium channels. To characterize the role of CD36 in fatty acid signaling, either as a primary receptor or in concert with GPCRs, I have utilized human embryonic kidney 293 (HEK293) cell lines that inducibly express the long chain fatty acid receptor GPR120.
with or without CD36 or CD36 alone. Using ratiometric intracellular calcium imaging, I was able to compare the response of these cells to different concentrations of linoleic acid which activates GPR120. The presence of CD36 shifted the concentration-response curve to the left slightly. Treating the CD36 expressing cells with CD36 inhibitor, sulfo-N-succinimidyl oleate (SSO), resulted in a large reduction, but not abolishment of linoleic acid activated intracellular response. And this responsiveness to linoleic acid was absent in cells that only express CD36 and lack GPR120. Thus, I conclude that CD36 is a protein which facilitates the activation of GPR120 by fatty acids instead of a primary receptor for fatty acids itself.

**Introduction**

Fats were widely accepted to be tasteless and their salient chemosensory cues were their texture and odor. The first evidence of fat taste was provided by Gilbertson et al. in 1997 that fatty acids elicit responses in rat taste cells by blocking a subfamily of delayed rectifying potassium (DRK) channels while they are open [2,3,4]. However, given the fact that only a small portion of these DRK channels are open at resting membrane potentials, the existence of upstream signaling pathways which can provide the prerequisite of DRK channel opening was hypothesized. During the search for these primary fatty acids receptors, two compelling receptors for long chain fatty acids, CD36 and GPR120 have emerged.

CD36 is a multifunctional receptor, which plays an active role in various physiological and pathological processes. It is an 88-kDa integral membrane protein with two transmembrane domains, two short intracellular segments, and an extracellular
domain, which is highly glycosylated and forms a hydrophobic pocket [5]. It was found to be expressed on a variety of cell types, such as endothelium, erythrocytes, platelets, dendritic cells, neutrophils, monocytes/macrophages, microglia, muscle cells and adipocytes [6,7,8,9,10,11,12,13,14]. In adipocytes and macrophages, it was reported to recognize oxidized low density lipoprotein [8]. It binds to thrombospondin-1 and collagen type I/IV in platelets [15,16]. It functions as a cofactor of Toll-like receptors in monocytes and dendritic cells, as well as retinal pigment epithelium cells by facilitating the recognition of anionic phospholipids [17,18,19]. Evidence supports its key role in fatty acid transport [20,21], and the purified protein reversibly binds long chain fatty acids [21]. The expression of CD36 in taste organs was first observed by Fukuwatari et al. in 1997. Using immunocytochemical approaches, they found that CD36 was specifically localized in the apical side of taste bud cells in the circumvallate papillae, which suggested the protein’s participation in oral perception of fats [22]. The participation of CD36 in fatty acid taste perception was further revealed by Besnard et al. in 2005 [1]. They found that CD36 deficiency abolished the spontaneous preference for long chain fatty acids, as well as the fatty acid-induced flux of pancreatobiliary secretion [1], which is an indicator of fat perception [23]. At the cellular level, the purified CD36-positive circumvallate taste cells had a rapid and robust increase in the $[\text{Ca}^{2+}]_{\text{in}}$, which was much smaller in the CD36-negative cells [24]. They found that linoleic acid-induced IP$_3$ production, capacitative calcium influx and Src-protein-tyrosine kinases (Src-PTKs) phosphorylation was CD36 dependent [25], and might involve stromal interaction molecule 1 (STIM1) [26] and store-operated calcium (SOC) channels [25]. All this
evidence strongly supports the idea that CD36 might serve as a primary receptor in fatty acid taste.

Recently, several previously orphan GPCRs have been identified as responsive to different kinds of fatty acids. As a receptor of unsaturated long-chain fatty acids, GPR120 was reported to be expressed on circumvallate papillae and fungiform papillae on the tongue [27,28,29,30]. The dietary fat-induced activation of GPR120 led to the secretion of glucagon-like peptide-1 (GLP-1) in taste cells [31]. Moreover, deletion or dysfunction of GPR120 was reported to impair the spontaneous fatty acid preference as well as gustatory nerve responses [32], and furthermore, led to obesity in mouse [33].

In this present study, I used HEK293 cells transfected with constructed GPR120/Gα16 or CD36/GPR120/Gα16 plasmids to test a hypothetical integration of these two pathways, in which CD36 facilitates the signaling transduction of GPR120. With ratiometric functional calcium imaging, I found that GPR120/Gα16 cells were able to respond to linoleic acid. This response was slightly facilitated in the CD36/GPR120/Gα16 cells. Applying CD36-specific inhibitor SSO on the CD36/GPR120/Gα16 cells decreased the [Ca^{2+}]_in response, indicating the involvement of CD36 in this linoleic acid-induced response, which was GPR120/Gα16-dependent.

**Materials and Methods**

*Construction and maintenance of the transfected HEK 293 cell lines*

The fatty acid receptor cell lines were constructed by and a generous gift of International Flavor and Fragrances Inc. (IFF Inc.). For inducible (ind)-CD36/GPR120/Gα16 cells, Cd36, Gpr120 of *Mus musculus* and guanine nucleotide-
binding protein subunit alpha-16 ($G_{\alpha16}$) of *Homo sapiens* were constructed into the plasmids. For inducible-GPR120/$G_{\alpha16}$ cells, only $G_{\alpha16}$ and Gpr120 were included. Both plasmids had an inducible promoter assembled upstream to the target genes, Cd36, $G_{\alpha16}$, and Gpr120, as illustrated in Figure 2.1. For constitutive (con)-CD36 cells, a constitutive promoter was assembled prior to the Cd36 gene. These plasmids are transfected into HEK293 cells with the use of Invitrogen’s Flp-In and Trex Flp-In Systems.

All cells were cultured in DMEM + Glutamax (Invitrogen) with 10% Tet-Free fetal bovine serum (Fisher), 10 $\mu$g/ml Blasticidin S HCl (Invitrogen) and 100 $\mu$g/ml Hygromycin B (Invitrogen). For inducible cell lines -- ind-CD36/GPR120/$G_{\alpha16}$ and ind-GPR120/$G_{\alpha16}$ cells -- to induce the expression of transfected receptor genes, cells were washed with DMEM + Glutamax and incubated in induction medium for 48 hours before experiments. The induction medium was DMEM + Glutamax mixed with 10% Tet-free fetal bovine serum and 0.5 $\mu$g/ml doxycycline (Sigma).

**Calcium imaging**

Intracellular calcium was measured by ratiometric calcium imaging using fura-2/AM on a high speed imaging system (High Speed InCyt, Intracellular Imaging Inc., Cincinnati, OH). Cells were plated onto 12 mm or 15 mm coverslips at least 8 h before the experiment, which then were washed in Tyrode’s buffer. The plated cells were then loaded with 4 $\mu$M fura-2 AM (Invitrogen) in Tyrode’s buffer with 0.25% pluronic acid for 60 min at 37°C in the dark and then rinsed in FBS free medium for 30 min so that the acetoxy methyl ester group of fura-2 AM was cleaved by nonspecific esterases. The cells were then mounted into imaging chamber (RC-25F or RC-26Z, Warner Instruments) and
placed on an inverted Nikon TE-100 microscope, where they were continuously perfused with Tyrode’s buffer with or without fatty acids. Images were recorded with a monochrome integrating CCD camera through a 20x objective lens of an inverted Nikon TE-100 microscope. Benthan FGS 150 changing monochromator emitted the excitation wavelengths of 340 nm and 380 nM with an emission wavelength ~510 nM. Images were captured every 3 seconds and analyzed in InCyt Im2 software (Intracellular Imaging Inc.). The 340 nm/380 nm fluorescence ratio of each cell was converted to Ca\(^{2+}\) concentration directly within the software, based on the calcium standard curve generated with fura-2 K5 (Invitrogen) and calcium calibration buffer kit (Invitrogen). Data analyses were based on the peak amplitude or area under the curve in the presence or absence of fatty acid stimuli. Area under the curve of each response is obtained with Gaussian multi-peak function provided by the analytical software Origin 7 (OriginLab, Northampton MA).

**Solutions**

Tyrode’s saline contained 140 mM NaCl, 5 mM KCl, 1 mM MgCl\(_2\), 1 mM CaCl\(_2\), 10 mM HEPES, 10 mM glucose, and 10 mM Na pyruvate, adjusted to pH 7.4 with NaOH and 310 mOsm with NaCl. Fatty acids stocks were made in 100% ethanol and stored under nitrogen and mixed into fresh Tyrode’s immediately before experiments. Stock solutions of SSO (a generous gift from IFF Inc.) were dissolved in dimethyl sulfoxide (DMSO) and diluted with Tyrode’s for pre-treatment to the cells. The final concentration of DMSO in test solutions was kept under 0.1%. Fatty acid perfusion was followed by 1 mg/ml fatty acid-free bovine serum albumin (BSA, Sigma) solution in Tyrode’s.
Statistical analysis

The significant effects of all the treatments compared to their controls were determined by a two-tailed Student’s t-test ($\alpha = 0.05$). Data are presented as mean ± S.E.M., unless otherwise indicated.

Results

In the present study I have used ratiometric calcium imaging to test the hypothesis that the presence of CD36 leads to a potentiation of the GPR120-mediated fatty acid-induced responses in HEK293 cells. Since the promiscuous $G_{a16}$ is not the G protein found in native tissue that couples with GPR120 and functions as part of its signaling cascade, it was necessary to first test the functionality of this combination of transfected proteins, GPR120 and $G_{a16}$ in HEK293 cells.

*ind-GPR120/G$_{a16}$ cells respond to linoleic acid*

The effectiveness of doxycycline inducible expression of transfected genes was validated with functional tests using fura-2-based calcium imaging. As shown in Figure 2.2, native HEK293 cells had minimal responses to the GPR120 agonist linoleic acid at 30 µM ($\Delta[Ca^{2+}]_{in} = 31.69 \pm 8.803 \text{ nM, } n = 49; 9.773 \pm 2.818 \text{ nM, } n = 22; 14.79 \pm 4.606 \text{ nM, } n = 38$), cultured with or without 0.5 µg/ml or 5 µg/ml doxycycline, respectively. This was similar to the values recorded from non-induced ind-GPR120/G$_{a16}$ cells ($\Delta[Ca^{2+}]_{in} = 11.11 \pm 3.735 \text{ nM, } n = 28$). In contrast, ind-GPR120/G$_{a16}$ cells treated with either 0.5 µg/ml or 5 µg/ml doxycycline responded to 30 µM linoleic acid with average intracellular calcium rise exceeding 100 nM, ($\Delta[Ca^{2+}]_{in} = 124.1 \pm 14.24 \text{ nM, } n = 66$ for 0.5 µg/ml doxycycline and 113.1 ± 11.28 nM, $n = 66$ for 5 µg/ml doxycycline). Thus,
cotransfection of GPR120 and G_{α16} in HEK293 cells can functionally trigger a downstream calcium rise in response to stimulation with physiological concentrations of linoleic acid. Other fatty acids with different chain lengths and degrees of unsaturation were also tested on ind-GPR120/G_{α16} cells. As shown in Figure 2.2 C and D, as expected, the short-chain fatty acid butyric acid (C4:0) and the medium-chain fatty acid capric acid (C10:0), which are not ligands for GPR120, both failed to trigger intracellular calcium changes in these cells (Δ[Ca^{2+}]_{in} = 2.25 ± 0.8725 nM for butyric acid 30 µM and 1.714 ± 0.5505 nM for capric acid 30 µM, n = 56). The same cells, however, showed significant responses to 30 µM linoleic acid (Δ[Ca^{2+}]_{in} = 115.7 ± 6.565 nM, n = 56), which is consistent with reported GPR120 specificity [34]. This further confirmed that GPR120 is the primary component in this intracellular calcium response to long chain fatty acid in ind-GPR120/G_{α16} cells and validated the use of these cell line constructs for this research project.

**LA-induced [Ca^{2+}]_{in} increases are promoted by introducing CD36 in ind-GPR120/G_{α16} cells**

In order to determine the potential enhancing effect of CD36 on GPR120-dependent fatty acid responses, ind-CD36/GPR120/G_{α16} cells were tested in comparison to ind-GPR120/G_{α16} cells for the ability of linoleic acid, the prototypical fatty acid stimulus, to induce intracellular calcium responses (Figure 2.3). In both types of cells an intracellular calcium rise was observed in response to 30 µM linoleic acid (Figure 2.3A and B). The average peak amplitude of responses across 33 ind-CD36/GPR120/G_{α16} cells (Δ[Ca^{2+}]_{in} = 131.3 ± 8.911 nM) was slightly higher than the one of 18 ind-GPR120/G_{α16}
cells ($\Delta[Ca^{2+}]_\text{in} = 106.1 \pm 19.64 \text{nM}$). However, Student’s t-test of this two groups did not show statistical significance of this difference ($p = 0.19$), probably due to the small sample size.

It is possible that the 30 µM linoleic acid represented a saturating dose causing a ‘ceiling effect’ in the fatty acid response. To test for this possibility, five different concentrations of linoleic acid were tested on these two types of cells in order to obtain a concentration–response curve, from which $EC_{50}$ of these two cells lines in response to linoleic acid could be calculated. In every experiment, a specific concentration of linoleic acid was perfused on cells for 2.75 min, washed with 1 mg/ml fatty acid-free BSA until the calcium level returned to near baseline levels, and then perfused again with 40 µM linoleic acid. The peak response amplitude of the first stimulus was normalized to the second one (to 40 µM linoleic acid) within each cell. 5 µM, 10 µM, 20 µM, 40 µM and 60 µM linoleic acid were tested to generate the curve. By normalizing to the response of the same stimulus (linoleic acid), the variation across cells and preparations could be minimized. As shown in Figure 2.3 C, $EC_{50}$ of ind-CD36/GPR120/Gα16 cells (7.6 µM) was lower than the one of ind-GPR120/Gα16 cells (12.8 µM), indicating higher sensitivity of these cells to linoleic acid. The average responses to 5 µM, 10 µM and 40 µM were significantly different in the two types of cells ($p = 0.037$ for 5 µM, $p = 0.023$ for 10 µM and $p = 0.041$ for 40 µM). However, the comparison of individual points was not meaningful, since they were merely response ratio and did not reflect the true response amplitudes.
**SSO inhibits LA-induced \([Ca^{2+}]_{in}\) increases in ind-CD36/GPR120/\(G_{\alpha16}\) cells.**

As shown in Figure 2.3, the EC\(_{50}\) of LA-activated intracellular calcium response in ind-CD36/GPR120/\(G_{\alpha16}\) cells was slightly lower than in ind-GPR120/\(G_{\alpha16}\) cells, suggesting an increase in affinity introduced by coexpression of CD36 with the functional GPR120/\(G_{\alpha16}\) system. To further investigate the involvement of CD36 in this LA-induced response in these two constructed cell lines, I used SSO to inhibit the function of CD36. The sulfo-N-succinimidyl moiety of sulfo-N-succinimidyld esters is highly reactive and modifies fatty acid binding proteins covalently [35]. SSO binds to FAT/CD36 protein specifically, which results in an arrest of the transport function of this protein [35,36,37], making it a powerful tool in the functional assessment of CD36.

In the present study, 500 μM SSO was added in the cell incubating culture medium 20 minutes immediately prior to regular ratiometric calcium imaging. The control group was exposed to the same concentration of DMSO (0.1%), which was used to dissolve SSO prior to use. In addition to ind-CD36/GPR120/\(G_{\alpha16}\) cells, ind-GPR120/\(G_{\alpha16}\) cells were also tested to control the possible nonspecific effects of SSO. The peak amplitude of intracellular calcium response to 40 μM linoleic acid was recorded and summarized in Figure 2.4. Within all four groups of cells, the only group that showed a significant effect of SSO on linoleic acid-induced responses was the SSO pretreated ind-CD36/GPR120/\(G_{\alpha16}\) cells, in which the LA-induced \(\Delta[Ca^{2+}]_{in}\) was significantly smaller than the others (\(\Delta[Ca^{2+}]_{in} = 100.5 \pm 8.585\) nM, \(n = 22\); \(p = 0.025\) compared with untreated ind-CD36/GPR120/\(G_{\alpha16}\) cells, \(p < 0.001\) with SSO pretreated ind-GPR120/\(G_{\alpha16}\) cells, and \(p < 0.001\) with untreated ind-GPR120/\(G_{\alpha16}\) cells). The untreated ind-
CD36/GPR120/G<sub>α16</sub> cells, SSO treated and untreated ind-GPR120/G<sub>α16</sub> cells had no significant difference in the response to linoleic acid stimulus (Δ[Ca<sup>2+</sup>]<sub>in</sub> = 175.5 ± 30.9422 nM, n = 13; Δ[Ca<sup>2+</sup>]<sub>in</sub> = 249.5 ± 26.07177 nM, n = 20; and Δ[Ca<sup>2+</sup>]<sub>in</sub> = 205.76923 ± 22.72249, n = 26, respectively). The lack of effect of SSO on the cells lacking CD36 is consistent with the specificity of SSO on CD36 and no other fatty acid signaling elements. Thus, by applying CD36 specific inhibitor SSO, ind-CD36/GPR120/G<sub>α16</sub> cells had reduced responses to linoleic acid, indicating the involvement of CD36 in LA-activated Δ[Ca<sup>2+</sup>]<sub>in</sub> in these transfected cells.

**CD36 is not the main signaling component in LA-induced [Ca<sup>2+</sup>]<sub>in</sub> increases in transfected HEK293 cells**

I have demonstrated that CD36 contributed to the linoleic acid induced signaling in these transfected HEK293 cells with GPR120/G<sub>α16</sub> expressed, leading to an intracellular calcium rise. However, it was still not clear whether it is the primary signaling component. It is possible that CD36 contributes to this calcium rise by adding a separate pathway to the GPR120/G<sub>α16</sub> dependent signaling. Alternatively, CD36 may promote the responsiveness by facilitating the GPR120/G<sub>α16</sub> dependent pathway. To test these two possibilities, I performed the same ratiometric calcium imaging as previously described on HEK293 cells transfected constitutively with CD36 but lacking GPR120/G<sub>α16</sub>, which was previously proven to be functional in LA-induced [Ca<sup>2+</sup>]<sub>in</sub> increases. Thus, if these cells respond to the linoleic acid stimulus, CD36 may contribute as a fatty acid signaling pathway in parallel to GPR120/G<sub>α16</sub>. However, as shown in Figure 2.5 A, these cells failed to generate any intracellular calcium rise in response to 30
μM linoleic acid (0 out of 20 cells), indicating that LA-evoked intracellular calcium rise was GPR120/Gα16 dependent. This conclusion was further supported by comparing ind-CD36/GPR120/Gα16 and ind-GPR120/Gα16 cells on their response to a medium chain fatty acid, myristic acid, which is a ligand of GPR120 [28], but not of CD36. The calcium rise peak amplitude in response to 30 μM myristic acid was normalized to the response peak amplitude of 30 μM linoleic acid within each cell. And as shown in Figure 2.5B, the ratio of response to myristic acid and linoleic acid in two cell lines had no significant difference (MA/LA = 0.269 ± 0.068, n = 51 for ind-CD36/GPR120/Gα16 cells and 0.2542 ± 0.05146, n = 86 for ind-GPR120/Gα16 cells, p = 0.8524).

**Discussion**

In the present study, I first demonstrated the capability of ind-GPR120/Gα16 cells to respond to linoleic acid in the absence of CD36. Then by introducing CD36 in these transfected cells, the responsiveness to linoleic acid was slightly enhanced. The specific CD36 inhibitor can partially block this response in CD36 expressing cells. However, the cells that were only transfected with CD36 were not responsive to linoleic acid. These results suggest that CD36 promote the responsiveness to linoleic acid in heterologous systems by enhancing the co-expressed GPR120 pathway.

The difference in responsiveness to linoleic acid between ind-GPR120/Gα16 cells and ind-CD36/GPR120/Gα16 cells was not significant. The response to 30 μM linoleic acid in ind-CD36/GPR120/Gα16 cells was only ~23% higher than in ind-GPR120/Gα16 cells, a difference that was not statistically significant. In addition to this, the EC50 of linoleic acid-induced intracellular calcium rise was slightly lower in ind-
CD36/GPR120/G_{a16} cells than in ind-GPR120/G_{a16} cells. However in ind-CD36/GPR120/G_{a16} cells, pre-treatment with SSO resulted in a reduction in linoleic acid responsiveness by \(~43\%\). The remaining response, which can be considered as CD36-independent, was significantly smaller than the one in ind-GPR120/G_{a16} cells. This contradiction requires us to consider the possibility that the expression of CD36 might alter the existing GPR120/G_{a16} signaling pathway in other ways than directly working as a component in it. For example, although both cell lines were induced with the same dose of doxycycline and with the same period of time, the protein level of GPR120 might be altered by CD36 expression. Furthermore, CD36 was reported to regulate actin polymerization in microglial [38] and in macrophages [39]. Thus, by introducing CD36 in these HEK293 cells, the cytoskeletal structure, the cytokinesis, as well as various cell signaling events might be altered. Nonetheless, the significant decrease of linoleic acid response in SSO treated ind-CD36/GPR120/G_{a16} cells comparing to the untreated ind-CD36/GPR120/G_{a16} cells did prove CD36’s involvement in linoleic acid transduction.

Is this involvement of CD36 in response to linoleic acid in parallel with GPR120/G_{a16} pathway or GPR120/G_{a16}-dependent? I answered this question with results from functional calcium imaging in cells that were only transfected with CD36. Similar to the non-transfected HEK293 cells, these con-CD36 cells failed to generate any calcium response to linoleic acid. This result does not suggest that CD36 cannot function as a primary fatty acid receptor that has its downstream pathway independent from GPR120 in fatty acid taste transduction \textit{in vivo}, since these transfected HEK293 cells might lack the crucial components of the putative pathway that are present in taste cells. However, in
these transfected HEK293 cells, this result does indicate the GPR120/Gα16-dependence of CD36’s participation.

In conclusion, in this CD36/GPR120/Gα16 system, GPR120/Gα16 is crucial for linoleic acid-induced cell response, in which CD36 is involved if present, but not essential.

References


Figure 2.1 Illustration of the constructed plasmids for (A) ind-CD36/GPR120/\(G_{\alpha16}\) cells and (B) ind-GPR120/\(G_{\alpha16}\) cells. (IND: inducible promoter).
Figure 2.2    HEK293 cell constructs expressing GPR120 respond to linoleic acid. (A) and (B) Intracellular calcium rise responding to 30 µM linoleic acid in HEK293 or ind-GPR120/Gα16 cells cultured with 0, 0.5, or 5 µg/mL doxycycline (Published in [40]). (A) The typical responses to 30 µM linoleic acid of single cells treated with different concentration of doxycycline. (B) Mean response (peak amplitude of intracellular calcium rise) ± S.E.M. to linoleic acid in HEK293 cells treated with 0, 0.5 µg/, and 5 µg/mL doxycycline, and in ind-GPR120/Gα16 cells, after 0, 0.5 µg/ml and 5 µg/ml treatment of doxycycline, respectively. Error bars indicate standard error. (C) A typical ind-GPR120/Gα16 cell response to capric acid, linoleic acid and butyric acid 30 µM, summarized in (D).
Figure 2.3  Comparison of responses to linoleic acid between ind-GPR120/G_{a16} cells and ind-CD36/GPR120/G_{a16} cells. (A) Typical intracellular calcium response to linoleic acid 30 μM, summarized in (B), mean response peak amplitude of intracellular calcium rise ± S.E.M. to 30 μM linoleic acid in cell lines. (C) Concentration-response function and EC_{50} for LA-activated Δ[Ca^{2+}]_{in} in both cell lines.
Figure 2.4 SSO inhibited LA-induced $[\text{Ca}^{2+}]_{\text{in}}$ increases in ind-CD36/GPR120/$G_{\alpha 16}$ cells. LA-activated $\Delta[\text{Ca}^{2+}]_{\text{in}} \pm \text{S.E.M.}$ in ind-CD36/GPR120/$G_{\alpha 16}$ cells and in ind-GPR120/$G_{\alpha 16}$ cells pre-treated with either 500 μM SSO or 0.1% DMSO as control. Asterisks indicate significant differences.
Figure 2.5  Response on con-CD36 cells to linoleic acid and on ind-CD36/GPR120/Go16 and ind-GPR120/Go16 cells to myristic acid. (A) con-CD36 cells typically do not respond to linoleic acid (30 µM). (B) Mean relative response peak amplitude to 30 µM myristic acid normalized to the peak amplitude of 30 µM linoleic acid-induced response within each cell ± S.E.M.
CHAPTER 3

CD36 IS NOT REQUIRED BUT MAY FACILITATE ACTIVITY IN FATTY ACID RESPONSIVE PATHWAYS IN THE TASTE SYSTEM

Abstract

The fatty acid binding protein, Cluster of Differentiation 36 (CD36), has been found to be expressed in a variety of tissues where it is involved in multiple fat-related biological processes including angiogenesis, atherosclerosis, inflammation and lipid metabolism in mammals as well as in the detection of lipid-like pheromones in insects. Its identification in the apical membranes of taste cells using immunocytochemical approaches in 1997 by Fukuwatari et al., lead to the suggestion of its involvement in the detection of fatty acids in the gustatory system. Consistent with this, CD36-null mice have a reduced spontaneous preference for linoleic acid, which is commonly observed in wild-type mice [1,2]. At the cellular level, CD36-negative cells failed to generate intracellular calcium ([Ca\(^{2+}\)\(_{in}\)) increase in response to long chain fatty acids [3]. Nonetheless, whether CD36 acts as a direct lipid sensor or as a chaperone protein that facilitates the function of fatty acid-activated G protein coupled receptors (GPCRs), such as GPR120, which are also expressed in taste cells, remains to be determined. To investigate the role of CD36 in fatty acid taste transduction, a mouse taste bud-derived (TBD) cell line, TBD-a1, was used. Knockdown of CD36 by RNA interference in these cells reduced but did not eliminate their intracellular calcium responses to linoleic acid.
In vivo, taste cells from CD36 knockout mice and WT mice were isolated and compared for the ability of fatty acids to elicit a rise in $[Ca^{2+}]_{in}$. Cells from knock out (KO) mice were capable of responding in the absence of CD36. The concentration-response curve was not shifted significantly with the present of CD36. However, the inhibitor of CD36, sulfo-$N$-succinimidyle oleate (SSO) induced significant reduction in response to linoleic acid in wild type mice. At the behavioral level, responsiveness to linoleic acid in CD36-null mice was not eliminated comparing to wild type mice after formation of a conditioned taste aversion to linoleic acid. These data suggest CD36 is not required but may facilitate activity in fatty acid responsive pathways in the taste system.

**Introduction**

Until recently, fat was believed to be tasteless. It was widely accepted that the texture and the smell were the most salient cues that animals use to detect the components in fat during ingestive behavior, until Gilbertson et al. provided the first direct evidence that taste cue was elicited by fatty acids in rat taste cells in 1997 [4]. In this research, fatty acids were found to activate taste cells by inhibiting a subfamily of delayed rectifying potassium (DRK) channels. The follow up studies at both the cellular level and behavioral level supported the finding that fatty acids activate taste cells by blocking directly open DRK channels [5,6]. However, only a small portion of these DRK channels are open at resting membrane potentials. This drove the search for an upstream signaling pathway that can provide the prerequisite of DRK channel opening and subsequent cell membrane depolarization.
Multiple mechanisms have been proposed as the cognate receptors for free fatty acids. Compelling evidence implicates the multifunctional protein CD36 as a gustatory lipid sensor. It is expressed in mouse taste bud cells [2,7]. CD36 gene inactivation impaired spontaneous fat preference in mice [1,2] and neuronal activation in the gustatory area of the nucleus of the solitary tract elicited by a lingual deposition of LCFA was found to be CD36-dependent [8]. Within taste cells, linoleic acid induced intracellular calcium rise was reported to be exclusive in CD36-positive cells and was via an IP3-dependent mechanism, although the direct evidence of coupling of CD36 to phospholipase-C (PLC) activation was still not available. The downstream release of monoamine neurotransmitters 5-hydroxytryptamine and noradrenalin was also CD36-dependent. These cellular responses were found to require the function of store-operated calcium (SOC) channels and phosphorylation of Src-protein-tyrosine kinases (Src-PTKs), indicating a possible signaling pathway of CD36-dependent long-chain fatty acid (LCFA) transduction [3]. All these findings strongly support the idea that CD36 plays a crucial role in gustatory perception of fats.

On the other hand, fatty acid-activated GPCRs were also implicated as an essential component in fatty acid taste transduction. The long chain fatty acid receptors GPR40 and GPR120 are expressed in gustatory epithelium in mice, and their gene deletion impaired the spontaneous fatty acids preference as well as their gustatory nerve responses [9]. The dysfunction of GPR120 was also found to be associated with development of obesity in both mouse and human [10]. Moreover, fatty acid induced taste cell responses are G protein-PLC-dependent, which indicates the involvement of
GPCRs in fatty acids transduction [11]. Along with the results of my previous research in transfected human embryonic kidney 293 (HEK293) cells, (see Chapter 2), in which cells only expressed GPR120 were able to produce responses to fatty acids, it is reasonable to question their respective roles in fat taste.

Does CD36 serve as a crucial element in LCFA taste transduction? Alternatively, is it a chaperone protein which promotes the function of the other signaling pathways such as GPR120? Or do they both contribute to the LCFA taste transduction with independent downstream pathways?

The results from transfected HEK293 cells (Chapter 2) suggest that expressing CD36 alone in HEK293 cells does not endow the cells the responsiveness to linoleic acid in the form of intracellular calcium rise, though it does promote the responsiveness in ind-GPR120/Gα16 cells. However, there still remains the possibility that CD36 pathway includes components that are absent in HEK293 cells. After all, HEK293 cells have quite different protein expression from native taste cells and provide information only on those components that are expressed heterologously. Thus, in order to isolate the function of CD36 in fatty acid taste transduction, I have attempted to validate our findings in native taste cells or an in vitro model system that more faithfully recapitulates chemosensory cells.

Recently, a set of clonal taste bud-derived cell lines were established from p53-defecient mice. With RT-PCR, most of these cell lines were shown to express gustducin, the markers of type II taste cells, and/or a type III cell marker neural cell adhesion molecule (NCAM). In addition, taste receptors, such as T2R8, T1R3, PKD1L3, HCN4,
and ENaC, which comprise bitter, sweet, sour and salty pathways, were also found to be expressed in some of these cell lines [12]. These results made these cell lines a useful in vitro model of mammalian taste cells.

Therefore, in order to start to validate our previous findings, with preliminary linoleic acid responsiveness screening, I utilized one of these cell lines, TBD-a1 cells, to investigate the involvement of CD36 in fat taste transduction in the first part of the present study. CD36 knockdown was performed in these cells, which induced a decrease of responsiveness to linoleic acid. However, this reduction of responses was not statistically significant.

In addition to the studies in TBD-a1 cells, I also investigated the differences in responsiveness to fatty acids in CD36-KO and wild-type mice at both cellular level and animal behavioral level. Given the broad range of physiological functions of CD36, various alternations of physiological and pathological functions have been described in CD36-KO mice. Lipid metabolism is impaired in these mice associated to lipid uptake [13] and lipolysis dysfunction [14]. Accordingly, CD36-KO mice show increased plasma cholesterol, free fatty acid and triacylglycerol [15]. In addition to lipid metabolism, pathological progresses, such as atherosclerotic lesions [16], hyperlipidemia [17], corneal neovascularization [18] and choroidal involution [19], are altered in CD36-KO mice. In my specific field, taste, CD36-KO mice were reported to lose the response to oral fatty acid stimulation in the form of pancreatobiliary secretions [2] and neuronal activation of the nucleus of the solitary tract (NST) [8].
In the current study, for the first time the whole population of circumvallate taste cells isolated from CD36-KO mice and wild-type mice were compared for their response to the prototypical polyunsaturated LCFA, linoleic acid. I found that the CD36 did not cause significant changes in responses to linoleic acid in taste cells, which confirmed the exhibition of transduction pathways in addition to CD36 in fatty acid gustatory transduction. Moreover, I found that at the behavioral level, the animal’s oral sensitivity to linoleic acid was not significantly impaired in CD36-deficient mice, which further validated my hypothesis that CD36 was not a required component in the sensory transduction of dietary fat.

Materials and Methods

Maintenance of TBD-a1 cells

TBD cell lines were a generous gift from Dr. Y. Tomooka (Department of Biological Science and Technology and Research Center for RNA Science, Tokyo University of Science, Chiba, Japan). Cells were cultured in DMEM/F12 (HyClone) with 10% fetal bovine serum (Fisher), 10 µg/ml insulin (Sigma), 10 µg/ml transferrin (Sigma) and 1 µM forskolin (Sigma) at 37°C and 5% CO₂.

siRNA construction and transfection

For small interfering RNA experiments, Silencer® select pre - designed siRNA targeted against CD36 (Ambion) was used. The 50 nM, 100 nM and 150 nM of siCD36 were tested to achieve the maximum knockdown. siNEG, a nonsense construct, was used as a negative control as well. TBD-a1 cells were reverse transfected with siCD36 using
Lipofectamine® 2000 transfection reagent (Invitrogen) 24 h prior performing functional assays or performing quantitative PCR.

**Quantitative real-time PCR**

RNeasy Plus mini kit (Qiagen) and iScript™ cDNA synthesis kit (BIO-RAD) were used to synthesize first-strand cDNA from harvest TBD-a1 cells (12000 cells per well) according to manufacturer’s instructions. For real-time PCR, SmartCycler™ (Cepheid) was used to follow the PCR reaction in real time. Final reaction cocktail contains 1X reaction buffer, 2.5 mM Mg²⁺, 0.2 mM dNTPs, 60 nM forward and reverse primers of GAPDH, 200 nM GAPDH probe, CD36 TaqMan® gene expression assay (Life Technologies), 2 µM template cDNA and 10 U/µl HotMaster Taq. GAPDH was detected in Texas Red channel and CD36 in FAM. The level of CD36 mRNA was compared to GAPDH and represented as ∆Ct. The mean ∆Ct from different treatment groups, 50 nM, 100 nM and 150 nM siCD36, and siNEG were compared to the one of Opti-MEM and were used to derive ∆∆Ct, indicating the difference introduced by these siRNA treatments. R is the amount of target (CD36), normalized to the endogenous reference (GAPDH) and relative to a reference sample (Opti-MEM).

\[
\Delta C_t = C_{t_{CD36}} - C_{t_{GAPDH}}
\]

\[
\Delta \Delta C_{t_{treatment}} = \Delta C_{t_{treatment}} - \Delta C_{t_{Opti-MEM}}
\]

\[
R = 2^{\Delta \Delta C_t}
\]

The treatments that induce 80% lower expression of CD36 (\(2^{\Delta \Delta C_t}\)) were considered effective knockdown treatments. I chose the treatment with the lowest expression level of CD36 as the optimized knockdown treatment.
**Animals**

The CD36-KO mouse strain has been described in detail previously [15]. All experiments were performed on adult (2-6 months) male C57BL/6J or CD36 knockout mice that were maintained on a 12-h: 12-h day/night cycle with normal mouse chow and water provided *ad libitum*. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Utah State University and were performed in accordance with American Veterinary Medical Association guidelines.

**Taste cell isolation**

The basics of the isolation procedure have been adapted from those used elsewhere [20]. Briefly, the tongue was removed and placed in a Tyrode’s solution consisting of (in mM): NaCl, 140; KCl, 5; CaCl₂, 1; MgCl₂, 1; HEPES, 10; glucose, 10; Na pyruvate, 10; pH 7.4. The tongue was then injected between the epithelium and muscle layers with an enzyme cocktail containing: 2.4 mg/ml dispase II (Roche), 1.1 mg/ml collagenase A (Roche) and 1.0 mg/ml trypsin inhibitor (Type I-S; soybean; Sigma) in Tyrode’s saline. The tongue was incubated in Tyrode’s solution and bubbled with O₂ for 40 min at room temperature. Following the incubation, the tongue was washed with saline. The lingual epithelium was removed from the underlying muscle layer with forceps, pinned out in a Sylgard™-lined petri dish and re-incubated for 10 min with the same enzyme cocktail. The enzyme cocktail was then removed from the epithelium and replaced with Ca-Mg free Tyrode’s. After a 5-min room temperature incubation in Ca-Mg free Tyrode’s, individual taste cells which are still attached in taste buds were removed by gentle suction with a 100-150 µm firepolished pipette under low
magnification (×50). Taste cells isolated in this manner were then plated onto 15 mm glass coverslips coated with Cell-Tak Cell and Tissue Adhesive (BD Biosciences) for functional imaging.

**Calcium imaging**

Stimulus-induced changes in intracellular calcium of mice taste cells were measured by ratiometric calcium imaging using fura-2/AM on a high speed imaging system (High Speed InCyt, Intracellular Imaging Inc., Cincinnati, OH). Cells were plated onto 12 mm or 15 mm coverslips at least 8 h before the experiment, which then were washed in Tyrode’s buffer. The plated cells were then loaded with 4 μM fura-2 AM (Invitrogen) in Tyrode’s buffer with 0.25% pluronic acid for 60 min at 37°C in the dark and then rinsed in FBS free medium for 30 min so that the acetoxyethyl ester group of fura-2 AM could be cleaved by nonspecific esterases. The cells were then mounted into imaging chamber (RC-25F or RC-26Z, Warner Instruments) and placed on an inverted Nikon TE-100 microscope, where they were continuously perfused with Tyrode’s buffer or FA-containing solutions. Images were recorded with a monochrome integrating CCD camera through a 20x objective lens of an inverted Nikon TE-100 microscope. A Benthan FGS 150 fast-changing monochromator emitted the excitation wavelengths of 340 nm and 380 nm with an emission wavelength ~510 nM. Images were captured every 3 s and analyzed in InCyt Im2 software (Intracellular Imaging Inc.). The 340 nm/380 nm fluorescence ratio of each cell was converted to Ca²⁺ concentration directly within the software, based on the calcium standard curve generated with fura-2 K5 (Invitrogen) and calcium calibration buffer kit (Invitrogen). Data analyses were based on the peak
amplitude or area under the curve in the presence or absence of fatty acids stimulus. Area under the curve of each response is obtained with Gaussian multi-peak function provided by the analytical software Origin 7 (OriginLab, Northampton MA).

**Solutions**

Tyrode’s saline contained 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM HEPES, 10 mM glucose, and 10 mM Na pyruvate, adjusted to pH 7.4 with NaOH and 310 mOsm with NaCl. Fatty acids were prepared as stock solutions in 100% ethanol and stored under nitrogen and mixed into fresh Tyrode’s immediately before experiments. In some experiments, the sodium salt form of the fatty acid was used eliminating the need for ethanol dilution. No differences were noted depending upon the form of fatty acid used (free versus salt form). Stock solutions of SSO were generated by dissolving in dimethyl sulfoxide (DMSO) and diluted with Tyrode’s for use in the cell-based assays. The final concentration of DMSO was did not exceed 0.1%. Fatty acid perfusion was followed by 1 mg/ml fatty acid free bovine serum albumin (BSA, Sigma) solution in Tyrode’s saline. Calcium-magnesium free saline (Ca-Mg free Tyrode’s) contained (in mM): 140 NaCl, 5 KCl, 2 BAPTA, 10 HEPES, 10 glucose, and 10 Na pyruvate; pH 7.40 adjusted with NaOH; 310 mOsm. High potassium saline contained 45 mM NaCl, 100 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, 10 mM glucose and 10 mM Na pyruvate, adjusted to pH 7.4 with KOH and 310 mOsm with KCl. Taste mixture was Tyrode’s based solution of 20 mM saccharin, 100 μM SC45647, 3 mM denatonium benzoate, 100 μM cycloheximide and 5 mM monosodium glutamate. Fatty acid stimuli for calcium imaging were also made in Tyrode’s. The significant effects of all the
treatments compared to their controls were determined by a two-tailed Student’s t-test (α = 0.05). Data are presented as mean ± SEM, unless otherwise indicated.

Conditioned taste aversion

General strategy

Conditioned taste aversion (CTA) assays were performed in order to test if the deficiency of CD36 in mice affected their linoleic acid perception. Details of the CTA behavioral tests have been described previously [21]. The strategy of this test can be described in brief: each group of mice (CD36-KO and wild-type mice) was assigned to two groups: a CTA group and a control group. Mice received an intraoral application of the conditioned stimulus (CS), linoleic acid (sodium salt) solution, which was immediately followed by an intraperitoneal injection of LiCl (156 mg/kg), the unconditioned stimulus (US), to induce gastric distress (the CTA group) or a saline injection at the same concentration as a control condition. If the conditioned mice (i.e. LiCl groups) were capable to detect linoleic acid, these mice should be able to establish a conditioned taste aversion to linoleic acid, while the control group should not be affected. Thus, the establishment of this aversion could be presented as the difference in performance between the CTA group and the control group, which indicated the capability of the animals to orally detect linoleic acid. And the requisite role of CD36 in generating this aversion was estimated by comparing the CD36 null mice and the wild-type mice. In present study, I measured their licks to brief presentations of stimuli as the indicator of gustatory performance.
Behavioral training and CTA paradigm

All mice had *ad libitum* access to water until 24 h prior to conditioning and testing at which time the mice were placed on a 23.5-h water restriction schedule for the duration of the experiment. All mice were given 30-min access to water on each of the restriction days at 4 pm, more than 2 h after the training/conditioning/testing. Mice were first trained to lick during water stimulus trials in the Davis Rig for 3 consecutive days prior to the initial conditioning day. The mice that failed to adapt to the Davis Rig after the 3-day training were removed from the experiment.

Following training, taste aversions were conditioned through pairings of the CS (linoleic acid) and the US (LiCl or NaCl injections): for 3 consecutive days at 9:30 AM. Mice received US or saline injection right after an additional intraoral application of CS. The US injections (150 mM LiCl or 150 mM NaCl) were dose dependent on body weight (20 ml/kg). The CS of 200 μM LA (prepared with linoleic acid sodium salt) was selected based on preliminary behavioral data in our lab (not published). All mice receiving a LiCl injection showed behavioral signs of gastric malaise, the unconditioned response, within 20 min of the injection.

Testing procedures

Following the third conditioning day, three consecutive days of testing in the MS-160 Davis Rig gustatory behavioral apparatus assessed the formation of conditioned and generalized taste aversions. A fan was located near the chamber in order to direct constant airflow along the longitudinal axis of the stimulus delivery tray serving to reduce olfactory cues for any given stimulus. Each daily test session consisted of two
blocks of 15 trials including a water trail with stimulus durations of 5 s, wait times for the first lick of 120 s, and inter-stimulus intervals of 7 s. Each block included 1 trial of each test stimulus and 1 trial of water stimuli. The stimulus order within each block was randomly assigned. Before each trial, a 2 s access to water is provided to rinse the animal’s oral cavity. Total number of licks per stimulus was recorded and averaged across the two trials and then normalized to the average licks to water, in order to account for individual variances in the water-restricted motivation across the mice. All mice included in the data analysis sampled each stimulus at least once during each daily test session. Trials in which the mouse did not lick were excluded from analysis. Differences between LiCl and saline-injected mice within each genetic group were analyzed for statistical significance using an unpaired, two-tailed Student’s t-test and significance was set at $\alpha = 0.05$.

**Stimuli**

All taste stimuli were mixed daily from reagent grade chemicals and presented at room temperature. Fatty acid stimulus concentrations were selected to be similar to concentrations previously shown to activate taste cells [5]. In addition to water, there were 10 test stimuli consisting of 0.3, 1, 10, 30, 100 and 200 μM LA (linoleic acid sodium salt), 100 μM oleic acid, 100 μM palmitic acid, 100 μM caproic acid, 100 μM lauric acid, 100 mM sucrose, 3 mM denatonium benzoate, 100 mM NaCl and pH 2.4 citric acid.
Results

*CD36 knockdown does not induce significant decrease of responsiveness to linoleic acid in TBD-a1 cells*

In the first part of the present study, I did a preliminary functional screening on four TBD cell lines with calcium imaging, TBD-a1, TBD-a5, TBD-a7 and TBD-c1 to check their responsiveness to linoleic acid. Among these cell lines, TBD-a1 cells showed the highest responsiveness to linoleic acid (data not shown). Along with the real-time PCR results showing its expression of GPR120 and CD36 as well as transient receptor potential melastatin member 5 (TRPM5, data not shown), TBD-a1 cells were validated as an appropriate *in vitro* cell model for investigating fat taste transduction.

To examine the involvement of CD36 in fatty acids induced activation of TBD-a1 cells, these cells were transfected with siRNA designed specific to CD36 (siCD36) or with nonsense sequence as negative control (siNEG). Concentration of siCD36 was optimized to 100 nM. As a result of this treatment, CD36 mRNA level was reduced to 0.019% of the level in siNEG treated cells with quantitative real-time PCR (Figure 3.1 A and B, p < 0.001). I was unable to measure changes in CD36 protein level since my attempts to find the appropriate CD36 antibody failed. Intracellular calcium rise was recorded accordingly in response to 90 µM palmitic acid, myristic acid or linoleic acid in both siCD36 and siNEG treated cells. The knockdown treatment did not affect the response to either palmitic acid or myristic acid, but reduced the response to linoleic acid from 121.3 ± 15.89 nM (n = 121) to 92.51 ± 7.477 (n = 106) nM, although this reduction was not statistically significant (p = 0.08236, Figure 3.1 C).
**CD36 deficiency does not eliminate the taste cell linoleic acid detection**

Previous research showed that LCFAs-activated intracellular calcium rise was at least partially CD36 dependent in taste bud cells [3]. However, in this research, the CD36-negative cells did exhibit LCFAs responsiveness, which was relatively smaller than the CD36-positive cells. In addition, these CD36-positive cells comprised approximately 16% of taste cell population [3], while our previous studies have shown that fatty acid responsive cells comprised a much larger proportion of taste cells (unpublished data). It is reasonable to hypothesize that an alternative pathway is involved in LCFA taste cell detection. To test this hypothesis, I compared the responsiveness of the whole population of circumvallate taste cells collected from CD36-null mice and wild-type mice. Taste cells from CD36-KO mice and wild-type mice were perfused with the following stimuli in sequence: high potassium saline, taste mixture containing denatonium benzoate, monosodium glutamate, cycloheximide, SC45647 and saccharin, 30 and 100 μM linoleic acid, 100 μM caproic acid (C6:0) and 100 μM palmitic acid (C16:0). Both type of cells had similar responsiveness to high potassium saline and taste mixture (Figure 3.2 A, B). 30 μM and 100 μM linoleic acid elicited calcium rise in both types of cells, 89.29% of wild-type cells and 92.98% of CD36-KO cells responded to 100 μM linoleic acid. Within the LA-responsive cells, the difference of average response amplitude was not significant (summarized in Figure 3.2 C; $\Delta[Ca^{2+}]_{in} = 40.79 \pm 6.698$ nM, and $57.02 \pm 10.23$ nM, for 30 μM linoleic acid in CD36-KO mice and wild-type mice cells; $\Delta[Ca^{2+}]_{in} = 172.8 \pm 19.29$ nM and $193.4 \pm 21.39$ nM for 100 μM linoleic acid in
CD36-KO and wild-type mice cells, respectively; n = 34 for CD36-KO cells and n = 38 for wild-type cells; p = 0.1997 for 30 μM and 0.4880 for 100 μM).

Concentration-response curves were also generated with six concentrations of linoleic acid, 5 μM, 10 μM, 20 μM, 40 μM, 80 μM and 100 μM, in both type of taste cells. Each stimulus was applied to cells once, washed out with BSA and followed by a second stimulus of 100 μM as a positive control. Only the cells that responded to the second 100 μM linoleic acid were included in final data analyses. As shown in Figure 3.2 D and E, EC\textsubscript{50} to linoleic acid in circumvallate taste cells was not significantly altered by CD36 deficiency (EC\textsubscript{50} = 31.72 μM for CD36-KO cells and 36.94 μM for wild-type cells). Thus, the taste cells responsiveness to linoleic acid, as determined by affinity of fatty acids for GPR120, was not significantly affected by CD36 deficiency.

SSO inhibits linoleic acid detection in mice taste cells

I also tested the dependence of the linoleic acid induced intracellular calcium rise on CD36 using the CD36 inhibitor SSO in taste cells. Isolated taste cells from either CD36-KO or wild-type mice were loaded with fura-2 AM for 40 min and treated with 100 μM SSO or 0.1% DMSO as control for 20 min prior to experiments. Thirty μM linoleic acid was perfused to cells and the intracellular calcium increase peak amplitude was recorded, which is summarized in Figure 3.3. In cells isolated from wild-type mice, SSO pretreatment inhibited the intracellular calcium response significantly (p = 1.245 × 10\textsuperscript{-5}; Δ[Ca\textsuperscript{2+}]\textsubscript{in} = 35.28 ± 3.744 nM, n = 113 for SSO and 86.14 ± 7.692 nM, n = 185 for DMSO). Pretreatment with SSO did not affect the CD36 independent response, as in CD36-KO cells, the SSO group and the control group showed no significant difference in
response magnitude to LA (p = 0.9382; Δ[Ca^{2+}]_{in} = 68.25 ± 20.28 nM, n = 44 for SSO and 70.5 ± 18.79 nM, n = 74 for DMSO). Interestingly, wild-type cells with control treatment did not show significant difference from CD36-KO cells, while the SSO treated wild-type cells had much smaller responses to linoleic acid than treated and control CD36-KO cells. These results suggest that CD36 pathway contributes partially to the taste cell response to linoleic acid. Moreover, the cells are able to respond to linoleic acid through a CD36-independent pathway, which largely compensates for the loss of response induced by CD36 deficiency.

**Mice lacking CD36 retain the sensitivity to linoleic acid**

CD36 deficient mice were reported to have lost [2] or partially lost [1] their spontaneous preference for LCFAs. However, it is not clear whether this reduction in preference was due to a decrease in sensitivity to LCFAs or some additional post-ingestive effect. In order to elucidate the effect of LCFA sensitivity at the level of the gustatory system, we used a short term, taste-specific assay of behavior. CTA assays can be utilized to achieve this goal. By pairing an unpleasant stimulus to the target oral stimulus, linoleic acid, the animals should be able to establish conditioned aversion to linoleic acid, if they can detect it. The effect of spontaneous preference should be eliminated with adequate pairing of US and CS.

In the present study, CD36-KO mice and wild-type mice were compared after conditioning. Following a direct oral application of 200 μM linoleic acid solution (water based), the animals received an intraperitoneal injection of LiCl to induce gastric distress or a NaCl saline injection as a control condition. After conditioning, animals were put in
Davis Rig gustatory behavioral apparatus and the licks per tastant trial were recorded as the parameter of animal response to the taste stimuli. With only a brief access to the stimuli, the post-ingestive cues for fatty acids were minimized. By comparing the LiCl group and the NaCl group, the formation of conditioned and generalized taste aversions were assessed. The results were consistent with the one from the cellular assays mentioned before: in the first testing day, CD36 deficient mice successfully developed significant aversion to linoleic acid at a concentration as low as 10 μM, suggesting that their sensitivity to linoleic acid was retained (Figure 3.4, p = 0.04604 for 10 μM, 0.00485 for 100 μM and 0.01187 for 200 μM linoleic acid, n = 8 for LiCl and n = 5 for NaCl) as the wild-type mice (p = 0.0402 for 30 μM and 0.0167 for 100 μM linoleic acid, n = 10 for both LiCl and NaCl groups). In day 2 the aversion reduced in both types of mice and disappeared by day 3, confirming that this aversion was specifically established during conditioning. These results suggest that linoleic acid oral detection in mice is not CD36 dependent.

Interestingly, a stimulus generalization between linoleic acid and oleic acid observed in wild-type mice reported previously [22] was replicated in wild-type mice but absent in CD36-deficient mice in current study (Figure 3.5). In the first testing day, wild-type LiCl group had significantly less licks to oleic acid (100 μM) than the NaCl group (p = 0.0287, n = 10), which was not shown by the CD36 null mice. The generalization was not observed to other fatty acids tested, which included a long chain saturated fatty acid, palmitic acid (100 μM), a short chain fatty acid caproic acid (100 μM) and a medium chain fatty acid lauric acid (100 μM), which were comparable to the cellular imaging
results. These results suggest that CD36 might be necessary for either detecting oleic acid in mice or contributing to the mechanism of generalization between linoleic acid and oleic acid.

**Discussion**

In this study, I focused on the differences in responsiveness to fatty acids induced by either CD36 knockdown in the taste cell line TBD-a1 or CD36 deficiency in mouse taste cells. I showed that CD36 knockdown did not eliminate the responsiveness to linoleic acid in TBD-a1 cells. Similarly, taste cells from CD36-deficient mice were still capable of responding to fatty acids. The concentration-response curve was not shifted significantly with the absence of CD36. However, the inhibitor of CD36, SSO induced a significant reduction in response to linoleic acid in WT mice. I will propose several hypothesis to explain this discrepancy. At the behavioral level, responsiveness to linoleic acid in CD36-null mice was not eliminated compared to WT mice after formation of a conditioned taste aversion to linoleic acid. These data suggest CD36 is not required but may facilitate activity in fatty acid responsive pathways in the taste system.

The first finding was that TBD-a1 cells with over 99.98% less CD36 mRNA as a result of knockdown treatment showed smaller responsiveness to linoleic acid. However, this difference in responses was only 23.73% of the responses in control cells. This result suggested that the existent of CD36-independent signaling pathways, which compose the main part of LCFA signaling in TBD-a1 cells. However, although the gene expression of GPR120, CD36 and TRPM5 were verified in this cell line, the expression of potential downstream signaling pathway components of CD36, such as PLC and SOCs was not
checked. Whether these components affect the responsiveness to FAs in TBD-a1 cells remains to be tested.

Another limitation of this knockdown experiment was that the protein level of CD36, especially the functional mature protein docked on membrane, was not measured. The efficiency of my knockdown procedure was only assessed at the mRNA level. The reason for this obvious limitation was the failure to find an effective antibody against mouse CD36, which prevented me from protein level assessments such as immunoprecipitation and Western blot. For the same reason, I was not able to test the difference of CD36 membrane level between CD36-null mice and wild-type mice with immunofluorescence. Evidence has shown that CD36 is an acutely regulated protein. The regulation could be at transcriptional, post-transcriptional, and post-translational. As for post-translational modifications, CD36 has three sites for palmitoylation turning the precursor protein into its mature form, which is crucial for protein targeting to membrane lipid rafts [23]. The turnover of the protein is also regulated by fatty acids and insulin [24]. Its post-translational stabilization involves caveolae and lipid rafts containing structural caveolin proteins [25]. Thus, the post-translational regulations to this protein might be the factors that affect the current result and requires further investigation. These regulations vary in different tissues or cell lines, with functional CD36 half-life inconsistent across cell types [23,24]. Thus, the estimation of protein level in current research in TBD-a1 cells cannot be made. For this reason, it is conceivable that post-transcriptional and post-translational regulations might affect the functional expression level of CD36 and further, the cell function of responding to linoleic acid, which might
explain the discrepancy of SSO treatment and CD36 knockdown. Thus, protein level assessments are necessary to be included in future study.

Taste cells from CD36-null mice and wild-type mice had similar responsiveness to linoleic acid, confirmed with concentration-response curves and EC$_{50}$, which was inconsistent with previous research done by Gaillard et al. In their study published in 2008, they found that LA induced a rapid increase in intracellular calcium in purified CD36-positive taste cells, while only a weak response in CD36-negative cells [3,8]. This discrepancy might result from the small population of CD36-positive cells in the whole circumvallate taste cell population. Only 11% circumvallate taste cells were CD36 positive [8], while the CD36-negative cells were still able to respond to linoleic acid with a ~200 nM intracellular calcium rise. This made the mean response I collected from the whole population of circumvallate taste cells less different between the CD36-KO mice and wild-type mice.

I also found that CD36 inhibitor SSO induced a significant reduction in LA-induced intracellular calcium response in wild-type mice taste cells, indicating the involvement of CD36 in linoleic acid taste perception. However, as mentioned above, the responsiveness of taste cells from CD36-KO and wild-type mice was not significantly different. This discrepancy might imply that the reduction induced by deficiency of CD36 could be compensated by other fatty acid signaling pathways. This possibility might also explain our CTA result that mouse oral sensitivity to linoleic acid was not CD36 dependent, in disagreement with Gaillard’s result that neuronal activation triggered by oral stimulation with linoleic acid is CD36-dependent [8].
The linoleic acid preference was first reported by Laugerette et al. to be eliminated in CD36-KO mice [2]. This result was later confirmed by Sclafani et al. on oil-naive mice [1]. These result seemed to suggest a complete deficiency in fatty acid oral detection induced by CD36 knock out, which my present CTA results contradict. However, both preference tests were performed with 2% linoleic acid emulsion (64.3 mM), which was 300 times higher than the concentrations that I chose for the CTA tests, 200 µM. With such a high concentration, the palatability might decrease as described in a recent study [26], which affected the preference test results. Actually, Sclafani et al. did find that CD36-deficiency did not affect the strong preference of mice to linoleic acid at much lower concentration, 0.25 - 0.5% (8 – 16 mM) [1]. Thus, the elimination of preference to high concentrations of linoleic acid reported previously might not be induced by impairment of fatty acid oral detection but other alternations introduced by CD36-deficiency, such as post-oral effects, which is highly unlikely in current study since the 200 µM linoleic acid solution has a very low nutritional content.

In the current CTA study, the conditioned aversions were only exhibited to 30 µM and 100 µM linoleate in the WT mice in the first testing day, which was not very convincing. It was surprising that the mice did not show statistically significant aversion to the strongest CS, linoleate solution at 200 µM. However, previous study in our lab showed that strong aversions could be established on WT mice [27]. In the CD36-KO mice, although strong aversions were observed at as low as 10 µM, the aversion was not statistically significant at 30 µM concentration. Nonetheless, the trend of dose-dependent
aversion was quite convincing in CD36-KO mice, indicating the ability of CD36-KO mice to orally detect linoleate.

In the present CTA experiment, sodium linoleate was used as a substitute of non-esterified linoleic acid, in order to eliminate the recognition to the vehicle, ethanol. The concentrations of linoleate used in this experiment would produce sodium ion concentrations less than 200 µM, which should not present a taste stimulus confound as 100 µM is far below the threshold of sodium detection (5 - 10 mM). However, oleic acid was still carried by ethanol. Nonetheless, the effect of ethanol might be negligible, because the animals were conditioned to non-ethanol-carried linoleate solution and that linoleate stimulus does not generalize with ethanol [22]. Thus, the generalization between linoleic acid and oleic acid was unlikely to be induced by ethanol.

The texture difference between linoleic acid solutions and water was not controlled with xanthan gum in the current study. However, the viscosity difference between 88 µM linoleic acid and water was reported to be negligible [22]. Since the lowest concentration at which the CD36-KO mice exhibited oral detection of linoleic acid was 10 µM in current study, it is unlikely that these mice detected linoleic acid solely on the basis of textual cues.

In conclusion, in TBD-a1 cells, I found that linoleic acid responses were reduced but not eliminated by CD36 knockdown. In mice, based on the calcium imaging and conditioned taste aversion tests, I found that CD36-deficiency affected neither the overall performance of the whole population of mice circumvallate taste cells in response to linoleic acid nor animal oral detection of linoleic acid. Along with the reduction in
response induced by CD36 inhibitor on wild-type taste cells, the involvement of CD36 in fatty acid gustatory perception is confirmed, however it does not appear from my results that CD36 is essential for fatty acid taste.

References


Figure 3.1  Fatty acids responses in TBD-a1 cells with CD36 knock down. (A) $\Delta$Ct with 16 hours treatment with 50 nM, 100 nM, and 150 nM siCD36, siNeg, and optiMEM. (B) Expression level of CD36 as $2^{\Delta\Delta C_t}$ with different treatment. (C) Mean response peak amplitude of intracellular calcium rise ± S.E.M. to 90 μM palmitic acid, myristic acid or linoleic acid in TBD-a1 cells treated with 100nM siCD36 or siNeg.
Figure 3.2  CD36 deficiency did not eliminate taste cell responsiveness to linoleic acid. Responses to high potassium, taste mixture, 30 μM linoleic acid, 100 μM linoleic acid, 100 μM caproic acid and 100 μM palmitic acid in CD36-KO taste cells (A) and wild-type taste cells (B). (C) Mean responses (peak Δ[Ca^{2+}]_{in}) ± S.E.M. to linoleic acid 30 and 100 μM, 100 μM caproic acid and palmitic acid. Concentration-response curve of LA-induced intracellular calcium rise in CD36-KO taste cells (D) and wild-type taste cells (E).
Figure 3.3 SSO pretreatment inhibited LA-induced intracellular calcium response in wild-type mice taste cells. Mean response (peak Δ[Ca^{2+}]_{i}) ± S.E.M. to linoleic acid 30 μM in isolated circumvallate taste cells from CD36-KO mice and wild-type mice, pretreated with 100 μM SSO or 0.1% DMSO as control for 20 min before experiments. Asterisk indicates significant difference (P < 0.05).
Figure 3.4  Sensitivity to linoleic acid is not affected by CD36 deficiency in mice. Mean lick ratios ± S.E.M for linoleic acid in wild-type and CD36-KO male mice on day 1, 2 and 3 after CTA. Asterisks indicate significant differences between LiCl-injected (black) and NaCl-injected (red) groups (P < 0.05).
Figure 3.5   Stimulus generalization between linoleic acid and oleic acid was found only in wild-type mice. Mean lick ratio ± S.E.M. for 100 μM oleic acid, palmitic acid, caproic acid and lauric acid after CTA to 200 μM linoleic acid in day 1, 2 and 3. Asterisks indicate significant difference between LiCl-injected group (light grey) and NaCl-injected (dark grey) group (P < 0.05).
CHAPTER 4

SUMMARY AND FUTURE DIRECTIONS

Summary of Research

Fats were widely believed to be tasteless until recently. They were considered to be detected through their texture and olfactory cues. In 1997 Gilbertson et al. found that by blocking a subfamily of delayed rectifying potassium (DRK) channels, fatty acids were able to elicit responses in rat taste cells [1,2,3], which brought the taste of fats into view. During the search for the primary fatty acids receptors, two compelling receptors for long chain fatty acids, CD36 and GPR120, have emerged. Both of these competing candidate receptors of long chain fatty acids are found abundantly expressed in taste cells. Both of them have accumulating evidence that support their crucial roles in fatty acid perception at the cellular and behavioral level in a variety of species.

In this dissertation, my research aimed to answer the following questions: Is CD36 crucial for fatty acid taste? Are these two receptors involved in the same transduction pathway? If they are, what are their respective functions in the pathway?

To answer the first question, both in vitro and in vivo studies were performed. During the course of my dissertation research, several taste cell lines derived from p53-deficient mice were developed that we have made use of in this research. One of these mouse taste bud derived (TBD) cell lines, TBD-a1 cells expressed all the components of the fatty acid transduction pathway (cf. Fig. 1.4) and were used as an in vitro system, in which RNA interference of CD36 can be easily performed. Successful knock down CD36
did reduce the linoleic acid responses slightly (Figure 3.1). However, this effect of knock
down treatment was not statistically significant, suggesting CD36’s non-crucial
involvement in fatty acid taste perception. Similar results were also obtained from the in
vivo study. Taste cells were isolated from CD36-deficient mice and wild type mice. From
these isolated taste cells, linoleic acid concentration – response curves were generated,
from which EC50 of linoleic acid was calculated in both types of cells. The CD36-
deficient taste cells had a slightly right shifted concentration-response function and a
greater EC50, suggesting a reduction in linoleic acid sensitivity. However, at a
concentration around EC50, the CD36-KO cells showed this reduction of linoleic acid
responsiveness statistically insignificant (Figure 3.2). Furthermore, at the behavioral level,
I performed conditioned taste aversion (CTA) tests on CD36-KO and wild type mice. By
examining their capability to establish a taste aversion through oral exposure to linoleic
acid solution followed by intraperitoneal injection of LiCl solution to induce gastric
distress, the animals’ oral sensitivity to linoleic acid, presumably taste sensitivity, was
assessed. Consistent with the cell functional results, I found that CD36 deficiency did not
impair their linoleic acid sensitivity (Figure 3.4). These results suggest that CD36 is not a
crucial component in fatty acid taste transduction in mouse.

However, after treatment with the irreversible CD36 specific inhibitor sulfo-N-
succinimidyl oleate (SSO), the isolated wild type taste cells had a significant drop in
linoleic acid responsiveness (Figure 3.3), which clearly suggests the involvement of
CD36 in linoleic acid taste transduction pathway. And the discrepancy with result from
CD36-deficient cells indicates the existence of CD36-independent pathway, presumably GPR120, and its compensatory effect in fatty acid taste.

To answer the next questions: is CD36 involved in the GPR120 transduction pathway and what is their function, I used the constructed HEK293 cell lines transfected with GPR120 and $G_{\alpha 16}$ with or without CD36. First I found that in this heterologous system, GPR120/$G_{\alpha 16}$ was sufficient to activate intracellular calcium response to long chain fatty acid linoleic acid (Figure 2.2). In addition to this working pathway, introducing CD36 into this system made the cells slightly more sensitive to linoleic acid (Figure 2.3). In addition, treating the CD36-positive cells with SSO significantly reduced the responses (Figure 2.4). Thus, my conclusion is that in this heterologous expression system, CD36 enhances GPR120/$G_{\alpha 16}$-dependent fatty acid transduction. But its role in this transduction is not a central one. According to these results, my conclusion of this research can be summarized as follow: 1) CD36 is not crucial for fatty acid taste transduction in mouse; 2) when present, it is involved in fatty acid transduction signaling; 3) this signaling pathway might be GPR120 dependent. Despite these conclusions, several questions remain from my research. First, there is a discrepancy between my results presented here with the existing evidence that shows a more significant dependence of CD36 in fatty acid taste. Second, my data suggest that there is a significant GPR120 dependence on fatty acid taste, though this was not specifically addressed in this research that needs to be further examined. Third, what underlies the apparent inconsistency between the CD36 knock out results and SSO results? Fourth, linoleic acid responses were observed in ~90% of taste receptor cells, which brings the
question that what types of taste cells are activated by fatty acids? And finally, what is
the mechanism of CD36’s involvement in GPR120-dependent pathway in taste cells?
These open questions will be discussed below.

What is the mechanism of reported CD36-dependence of fatty acid preference?

This research was initially proposed based on reported evidence that supports
CD36’s critical role in fatty acid taste transduction. At behavioral level, CD36 deficiency
in mice was reported by Laugerette et al. in 2005 to abolish the spontaneous preference
for 2% linoleic acid emulsion to control emulsion [4]. However, Sclafani et al. reported
in 2007 that the spontaneous preference remains in CD36-KO mice, although smaller
than in wild-type mice [5]. Thus, the direct evidence for CD36-dependence of fatty acid
taste was not conclusive. In addition, as mentioned in Chapter 3, the concentration of
linoleic acid used in these two studies was much higher than the one that I used in my
CTA tests. Indeed, Sclafani et al. did find that the preference for the 0.25-0.5% linoleic
acid emulsions was not affected by CD36 deficiency [5], which is consistent with my
CTA result that CD36-deficiency does not diminish linoleic acid oral sensitivity in mice.
Furthermore, in Laugerette’s paper, when linoleic acid emulsions were present to the
animals for 48 hours, the difference between wild-type mice and CD36-KO mice was
much bigger than when present for only 0.5 h. This might suggest that the CD36
deficiency-dependent preference lost involves some long term mechanisms other than the
rather immediate taste perception which was assessed in the CTA test in Chapter 3.
Possible mechanisms might include the neuronal fatty acid sensing in hypothalamus
ventromedial nucleus (VMH). These neurons were reported to responsive to oleic acid,
which can be inhibited by CD36 inhibitor SSO [6]. This hypothesis can be tested by site specific knock down of VMH CD36 followed by long-term and short-term preference tests.

**GPR120 dependence needs further examination.**

In this dissertation research, the GPR120 dependence was only examined in the heterologous expression system in combination with $G_{\alpha16}$. Thus, the following questions rises: in this system, is CD36’s involvement in fatty acid induced fatty acid response GPR120-dependent or $G_{\alpha16}$-dependent? Is this dependence the same in taste cells?

Existing direct evidence of GPR120’s crucial involvement in fatty acid taste is still limited at animal behavioral and nerve activity level. At the behavioral level, GPR120-KO mice were found to be indifferent to linoleic acid or oleic acid comparing to vehicle control in 48-h and 0.5-h two bottle preference test, suggesting a profound effect of GPR120 on fatty acid detection in mice [7]. Linoleic acid-induced response is weakened in glossopharyngeal nerve (GL) and diminished in *chorda tympani* (CT) nerve by GPR120 deficiency [7]. In other research, these knockout mice were found to be obesity-prone [8]. At cellular level, G-proteins inhibitor guanosine-5’-O-(2-thiodiphosphate) (GDP-$\beta$-S) and phospholipase C (PLC) blocker U73122 both significantly reduces linoleic acid-induced inward current in isolated taste cells, suggesting the requirement of G-protein-PLC pathway in linoleic acid-induced responses [9]. However, this G-protein dependence is not limited to GPR120 coupled G-protein, as GDP-$\beta$-S is a general G-protein inhibitor. And indeed, GPR40, another long chain fatty acid-activated G protein-coupled receptor (GPCR), is also found in taste cells [7].
Therefore, further investigation specific to these fatty acid-activated GPCRs in taste cells is required, which might be accomplished with the usage of RNA interference in TBD cells as well as their knock out animals.

**What causes the inconsistency between CD36 knock-out and SSO results?**

In Chapter 3, a major discrepancy occurred in cell functional experiment when examine the involvement of CD36 in linoleic acid-induced response with CD36-deficiency and CD36 inhibitor treatment. The reduction in linoleic acid response induced by CD36 knockout was not significant in isolated taste cells (Figure 3.2), but was significant when inhibited by SSO treatment (Figure 3.3). This inconsistency might result from the following reasons: 1) as mentioned in Chapter 2, the CD36-positive cells comprise only a small population of taste cells [10]. When I investigated the whole population of taste cells, the effect of CD36-deficiency was masked by the fatty acid responsive CD36-negative cells. 2) CD36-independent pathway compensates for the effect of CD36-deficiency, for example, by regulating the protein level of signaling pathway components. The later one seems to be more reasonable, since SSO treated wild-type cells were found to be less responsive to linoleic acid than CD36-KO cells (Figure 3.3).

**Is there any discrimination of CD36 involvement in fatty acid taste across different types of taste cells?**

My data in Chapter 3 in isolated taste cells shows that both high potassium-responsive cells and taste mixture-responsive cells are able to respond to linoleic acid,
suggesting that type II and type III taste cells respectively are both contributing to this linoleic acid-induced intracellular calcium response (Figure 3.2). However, according to the unpublished data from our lab by Liu, CD36 was not evenly expressed in these two types of taste cells but rather exclusive in type II cells, in contrast with GPR120, which is found in both type II and type III cells in circumvallate taste cells. Therefore, different mechanisms of fat perception might exist in type II and type III cells, making the involvement of CD36 differ across cell types. This, in addition to the small population of CD36-positive cells, makes the single-cell based investigations rather urgent, which might include calcium imaging or patch-clamping followed by single-cell RT-PCR. Furthermore, nerve recording on CD36-KO mice will help finally determine the role of CD36 in fat perception.

**What is the function of CD36 in fat perception?**

My data suggest that CD36 is involved in fat perception in mouse. However, its absence does not abolish fat taste sensitivity. Then why is it expressed in taste cells? What function does it carry? How is it involved in fatty acid taste? Several mechanisms can be investigated in future research.

CD36 might help the binding of fatty acid to receptors. A quite recent research elucidated the crystal structure of a lipid sensing class A G protein-coupled receptor (GPCR), sphingosine 1-phosphate receptor 1 (S1P1). In this receptor protein, the access of the ligand binding pocket is buried within the transmembrane region, making the ligand infusion into the membrane necessary [11]. Although the crystal structure of GPR120 is still not clear, giving that it is also class A GPCR [12] and is fatty acid-
activated, I would hypothesize that its activation might need diffusion of fatty acid into or across the cell membrane, in which CD36 might facilitate. This facilitating effect may require the co-localization of CD36 and GPR120 or other fatty acid receptors, which can be unraveled with high-quality immunocytochemical experiments. Approaches such as Raman spectra and fluorescence resonance energy transfer (FRET) can also help in revealing the dynamic interaction between CD36 and other fatty acid receptors. In addition, lipid rafts might be the necessary structure that allows this interaction to happen. CD36 is reported to form heterotypic receptor complexes with Toll-like receptor 1 and 2 (TLR1 and TLR2) in lipid raft in human vascular endothelial cells [13]. Future research on this can start with disturbing lipid rafts with drugs, such as methyl-β-cyclodextrin.

CD36 might alter the fat perception in taste cells by regulating the cytoskeleton structures and downstream signaling coupled to cytoskeletons [14,15]. To test this, disruption of actin polymerization or tubulin assembling would be a starting point. Investigation into the potential coupling/regulation of CD36 to signaling pathway components downstream to GPR120 in taste cells, such as PLC, is also warranted.

**Other limitations of the current study**

As I mentioned in Chapter 3, the lack of prove of CD36 protein expression level in either animal tongue tissue or TBD-a1 cells is one of the limitations of this research. Due to the limitations on time and expense, I could not find an effective antibody against CD36, with which immunofluorescence could be performed on animal tissue in order to review the position and possible overlapping of CD36 and GPR120. The absence of this crucial antibody also stopped me from assessing the membrane expression level of CD36...
on TBD-a1 cells with knockdown against CD36. Given the fact that CD36 protein has post-translational modulations which regulates its transportation between endoplasmic reticulum and cell membrane and further regulates its half-life [16], its protein level on TBD-a1 cells in knockdown experiment is rather urgent to be measured.

The other limitation of this study is that fatty acid stimulus was limited to linoleic acid. In Chapter 3, on TBD-a1 cells and mice taste cells, myristic acid and caproic acid are tested. However, they are not reported ligands of either CD36 or GPR120. The shared ligands of these two proteins, such as oleic acid, docosahexaenoic acid (DHA) and stearic acid are not tested. Furthermore, although palmitic acid is a ligand of both proteins, neither TBD-a1 cells nor isolated taste receptor cells showed sensitivity to it (Figure 3.1 and 3.2). Thus, the conclusions of this research were drawn upon linoleic acid as a single stimulus. For this reason, further study involving various long chain fatty acids are warranted.

It is also worth to mention that the kinetics of calcium response to fatty acid stimulus was not monitored in this study. However, all three type of cells studied: transfected HEK293 cells, isolated mice taste cells and TBD-a1 cells did show rather identical response lag (1-1.5 min after starting of linoleic acid stimulus). No significant plateau shape of response to linoleic acid was noticed across all three cell types, either. Thus, the response kinetics might not be a significant issue to focus on in the future studies.
References


