Discovery of Novel Receptors for Lipid Mediators - a study leading to the identification of receptors involved in metabolism and the immune system

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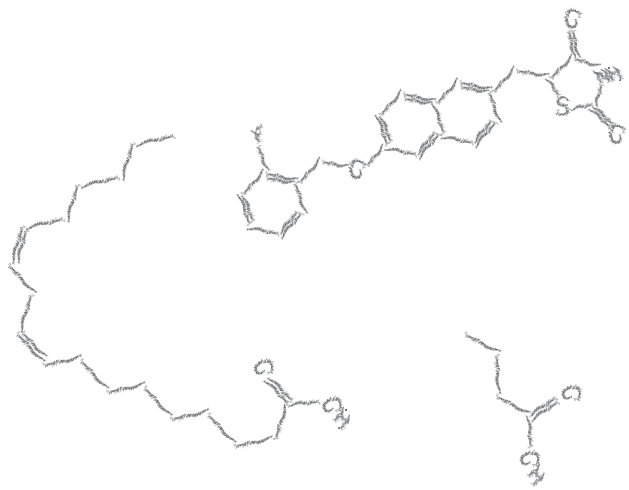

An academic dissertation regarding the

Discovery of Novel Receptors for Lipid Mediators –

a study leading to the identification of receptors involved in metabolism and the immune system

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Title and subtitle: **Discovery of Novel Receptors for Lipid Mediators** –
a study leading to the identification of receptors involved in metabolism and the immune system

Intercellular communication is of crucial importance in regulating physiology and G-protein coupled receptors (GPCRs) have evolved as an important mechanism in this process. Of the approximately 800 human GPCRs, about 160 are still considered to be "orphan" receptors for which an endogenous ligand remains to be identified. Since an estimated 50% of all clinical drugs act on 30 known GPCRs, the remaining orphan receptors provide excellent, potential new drug targets.

Orphan receptors were selected using known receptor sequences as templates and subsequently cloned into expressing plasmids that were then stably transfected into luciferase-based reporter cells. An orphan receptor was found to be the second GPCR, BLT_2, activated by the pro-inflammatory molecule leukotriene B_4.

Through use of a library of orphan receptors, potential ligands were screened for activity by applying reversed pharmacology. This approach led to the discovery of the novel receptor (FFA_1R) for medium- to long-chain free fatty acids, previously known as the orphan receptor GPR40. Significantly, this receptor was found to be expressed on e.g. pancreatic beta-cells and to mediate the fatty acid augmentation of glucose stimulated insulin secretion. The clinically used anti-diabetic drugs, thiazolidinediones, also activate FFA_1R expressed on reporter cells.

It was discovered that FFA_2R and FFA_3R (GPR43 and GPR41) are activated by short-chain fatty acids (SCFAs). Being abundantly expressed on blood leukocytes, FFA_2R may act as the mediator in SCFA-induced immune suppression in the intestinal tract. A recent proposal links FFA_3R to leptin secretion by adipose tissue.

Key words: Orphan, Receptor, GPCR, BLT2, GPR40, GPR41, GPR43, FFA1R, FFA2R, FFA, SCFA, Thiazolidinedione		
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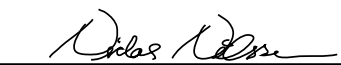
Signature:  Date: March 8, 2004

The beginning of knowledge is the discovery of something we do not understand.

Frank Herbert (1920-1986)

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The cover illustration shows the ligands, butyrate, linoleic acid and a synthetic compound, MCC-555, that activate the three fatty acid receptors.

The back cover illustrates the combination of scientific work and daily needs.

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Table of Contents

Abbreviations

7TM	Seven-transmembrane
AC	Adenylyl cyclase
ATP	Adenosine tri-phosphate
BLAST	Basic local alignment search tool
BLT_1	Leukotriene B_4 receptor 1
BLT_2	Leukotriene B_4 receptor 2
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CHO	Chinese hamster ovary cells
DNA	Deoxyribonucleic acid
EC_{50}	Half-effective concentration
EST	Expressed sequence tag
FFA	Free fatty acid
FFA_1R	Free fatty acid receptor 1
FFA_2R	Free fatty acid receptor 2
FFA_3R	Free fatty acid receptor 3
G-protein	GTP-binding protein
GDP	Guanosine diphosphate
GH	Growth hormone
GPCR	G-protein coupled receptor
GSIS	Glucose stimulated insulin secretion
GTP	Guanosine triphosphate
HeLa	Human cervix carcinoma cells derived from Henrietta Lacks
HUGO	Human genome organization
LA	Linoleic acid
LTB_4	Leukotriene B_4
NF-κB	Nuclear factor κB
NIDDM	Non-insulin dependent diabetes mellitus
ORF	Open reading frame
PPAR	Peroxisome proliferator-activated receptor
PTX	Pertussis toxin
SCFA	Short chain fatty acid
TM	Transmembrane
TZD	Thiazolidinedione

List of included publications

This thesis is based on the following articles. In addition, the entire thesis is available online, provided by Lund University, at http://www.lub.lu.se/dissdb/

Paper I **Cloning and characterization of cDNA encoding a novel human leukotriene B_4 Receptor.** Ylva Tryselius, Niclas E. Nilsson, Knut Kotarsky, Björn Olde and Christer Owman. *Biochem Biophys Res Commun 2000 Aug 2;274(2):377-382.*

Paper II **A human cell surface receptor activated by free fatty acids and thiazolidinedione drugs.** Knut Kotarsky*, Niclas E. Nilsson*, Erik Flodgren, Christer Owman and Björn Olde. *Biochem Biophys Res Commun 2003 Feb 7;301(2):406-410.*

Paper III **Identification of a free fatty acid receptor, FFA_2R, expressed on leukocytes and activated by short-chain fatty acids.** Niclas E. Nilsson*, Knut Kotarsky*, Christer Owman and Björn Olde. *Biochem Biophys Res Commun 2003 April 18;303(4):1047-1052.*

Paper IV **Characterization of GPR41 as a second receptor now designated FFA_3R belonging to the family of free fatty acid receptors and responding to short-chain fatty acids.** Niclas E. Nilsson, Knut Kotarsky, Christer Owman, and Björn Olde. *Manuscript.*

* These authors contributed equally to the work.

Introduction

(The abbreviations that appear in the text below are listed on page 6.)

This dissertation is a result of an exploratory voyage into the unknown that led to exciting and important discoveries, including the identification of previously unknown molecular mechanisms involved in metabolism and the immune system. The aim of this project was to discover new signal receivers (receptors) and to unravel their nature and function.

Receptors receive and transmit signals, which make them key regulators of physiological functions. They are involved in a multitude of different tasks - anything from detecting a photon to activating a muscle. In fact, a receptor of some kind is even required when cells need to communicate. When receptors display similar characteristics, they are placed into families. One of the larger of these families, in particular, consists of GPCRs. The specific aim of this investigation was to identify new GPCRs, set up a system to measure signal activation and to elucidate the role of these new GPCRs in human physiology.

The process of finding and identifying a new receptor varies and usually follows a long, winding and uncertain path. Our journey down this path was successful and resulted in the discovery of four new receptors related to lipid mediators (fatty acids and derivates). The results are summarized in four reports upon which this dissertation is based. The first describes the identification of a second receptor that is activated by LTB_4, a signal involved in inflammation. In the second report, we describe the discovery of an exceptionally interesting receptor which is activated by medium- to long-chain FFAs and anti-diabetic drugs, and plays a role in insulin secretion from the pancreas. The receptor identified in the third report is found on immune cells, activated by SCFAs and thought to play a crucial role in the immune system's tolerance of intestinal bacteria. Lastly, as related in the fourth report, a second receptor for SCFAs was discovered that has been recently suggested to regulate the endocrine function in adipose tissue (fat cells).

Explorations are full of surprises and one of the most exhilarating moments is when that unknown orphan receptor (a GPCR lacking a known endogenous activating substance, the ligand) suddenly responds to a stimulating compound. New receptor-ligand interactions open up doors to whole new fields of research. There is no doubt that the discovery of the FFA receptor family will have major implications for the understanding of fat signalling and metabolism. Particularly intriguing is the fact that the fatty acid receptor described in the second report is affected by the anti-diabetic drugs known as *Glitazones* (TZDs). Thus, this receptor is potentially involved in metabolic diseases such as obesity and diabetes.

- Professor Balthazar, a childhood scientific idol

G-Protein Coupled Receptors – GPCRs

A large and successful family of receptors

The importance of communication in a complex environment cannot be overemphasized, whether it is telephone communication between friends, the media in a society or molecules relaying signals about the physiological state of an organism. One of the most evolutionary successful groups of proteins is the super-family of GPCRs. The GPCRs have become a major part of the fulfilment of all complex living organisms' communicational needs [1]. GPCRs have evolved to accept a multitude of different intercellular signalling molecules, such as photons, odorants, hormones, neurotransmitters, chemo-attractants and nutrients. In addition, GPCRs are capable of orchestrating complex intracellular responses.

The human genome shares a similarity with that of other organisms. In both the fruit fly (*Drosophila melanogaster*) and the common nematode (*Caenorhabditis elegans*) a significant percent of all genes (5%) is estimated to be coding for GPCRs [1-3]. Although the genome of *Homo sapiens* is larger, still about 2% of all genes are devoted to GPCRSs [4]. Since GPCRs have a purpose even in basic organisms such as plants and yeast, it is apparent that this signalling mechanism is old and has been favoured by evolution [5, 6] (Fig. 1).

Figure 1. Examples of species utilizing GPCRs as signal receivers; *Homo sapiens* (Elvis), *Drosophila melanogaster* (fruit fly), *Caenorhabditis elegans* (nematode) and *Arabidopsis thaliana* (a plant).

GPCRs play a crucial role in monitoring and regulating the physiological state of an organism. By studying receptors, one can increase one's knowledge with regards to how both healthy and diseased bodies function. When the purpose of a receptor is understood, the physiology related to that receptor can be potentially modified by applying drugs to either stimulate or suppress an endogenous signal [7, 8].

A major reason to study GPCRs is their diverse and important role in human physiology, which can assist in the development of new drugs. Moreover, in-depth knowledge about drug targets can assist in the design of medicines that focus on the beneficial molecular mechanisms and minimize possible side-effects.

What does a GPCR look like?

In order to explain the exact function of GPCRs, a great amount of work has been put into creating a model of the actual protein structure. Even though we have been aware of this protein family for a long time, the first crystallized GPCR (rhodopsin) was presented only recently [9]. While trying to explain the mechanisms of other GPCRs, efforts have been made to superimpose the structure of the rhodopsin receptor onto them. However, the

amount of actual structure-function related data is not yet sufficient and the diversity of GPCR functions is too large to solve this problem thus far.

Typically, GPCRs have been recognized by their seven transmembrane-spanning α-helices [10]. These helices show a distinctive hydrophobicity pattern in the amino acid sequence, thus making the sequence identification of a putative GPCR fairly easy. Sitting in the cell membrane, a GPCR acts as a receiver of external molecules and relays the appropriate intracellular signal-responses.

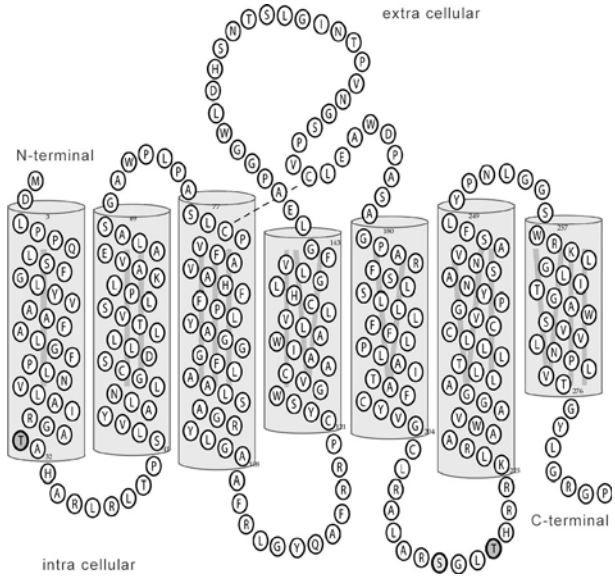


Figure 2. A GPCR, also known as a 7TM receptor, has seven α-helices that traverse the cell membrane. Here is an example of the human FFA_1R, the amino acid sequence showing unusually short N- and C-terminals. The FFA_1R sequence was aligned with that of the rhodopsin receptor, the only known crystallized GPCR, and mapped to its structure in order to produce this figure.

GPCRs usually consist of 300-1000 amino acid residues, while the size of the N-terminal, C-terminal and extra-cellular loops contribute to their varying sizes. An extreme example is the human GPCR for GABA (gamma aminobutyric acid), which consists of approximately 960 amino acid residues; the first 600 make up the extra-cellular N-terminal. The much smaller novel FFA_1R (described below), is comprised of 300 amino acids (Fig. 2)

The GPCR family is the largest of the cell surface protein families, with classification based on phylogeny and receptor function. The human GPCRs have been grouped into five sub-families: Rhodopsin, Glutamate, Adhesion, Frizzled/Taste and Secretin [11]. The Rhodopsin sub-family is the largest and it also contains most of the receptors related to lipid mediators. It is, therefore, our family of choice in the investigation of orphans receptors and putative new GPCRs for lipid mediators.

Most, but not all, of the rhodopsin-type GPCRs share some evolutionary conserved regions, or amino acids, thought to be important for the general function and conformation of the protein structure. The conserved regions include seven TM domains, a disulfide bond connecting the extra-cellular loop 2 and 3, an amino acid sequence DRY (aspartic acid, arginine and tyrosine) in the intracellular region of TM domain 3 and the 7^{th} TM domain which usually has an NPXXY (asparagines, proline, x, x and tyrosine) motif.

The existence of seven TM domains has been used as the primary feature to recognize GPCR genes, due to the fact that genomic sequences can relatively simply be screened for

ORFs exhibiting this feature. Due to their variable genomic structure, ORFs or non-translated regions either contain introns or are intronless. In the case of rhodopsin-like receptors, the ORF is often intronless [12-18] and we can make use of this feature in the cloning procedure. The four GPCRs described in this dissertation are examples of such intronless receptor genes. Figure 14 portrays the genomic organisation of GPR40, 41, 42 and 43, as well as their respective intronless ORFs. By designing primers at the start and end of the ORF, a complete intronless receptor gene can be PCR-amplified from e.g. human genomic DNA and subsequently cloned into a plasmid. This was the primary procedure used when cloning the orphan receptor genes in this project.

How signals are relayed by GPCRs

When an activating substance (i.e. a ligand) is bound, the receptor undergoes a conformational change or, rather, an active conformation of the receptor is favoured over an inactive. This structural change sends a signal through the cell membrane and into the cell cytoplasm that the receptor has been activated. This alternative conformation affects intracellular signalling molecules such as heterotrimeric G-proteins. The traditional G-protein complex consists of an α-and βγ-subunit which in its inactive state binds a GDP molecule. When activated, the GDP molecule is replaced by GTP and the messenger complex dissociates into free α- and βγ-subunits, which are capable of activating other intracellular targets and amplifying the ligand-induced signal [19]. The importance of GPCR signal transduction was acknowledged when Alfred G. Gilman and Martin Rodbell received the Nobel Prize in 1994 for their discovery of "G-proteins and the role of these proteins in signal transduction in cells". However, the traditional view of GPCR activation is not adequate enough to explain all instances of ligand-receptor interaction due to the fact that receptors can exist in more than just active or inactive conformations. Depending on the binding ligand, one of several active conformations is stabilized. Such a ligand-induced conformation could, consequently, induce a ligand-specific signal transduction [20].

The signalling ability of a GPCR depends upon a particular cell's repertoire of signal transduction proteins; therefore, activation of the same receptor on two different types of cells does not necessarily trigger the exact same response. The stable βγ-subunit consists of one β-subunit and one γ-subunit selected from 5 possible β-subunits and 12 possible γ-subunits. For a long time, the βγ-subunit was thought to be an inactive part, but it is now known that this subunit is also capable of eliciting a downstream signal [21]. The α-subunit of a heterotrimeric G-protein complex determines the variation in signal transduction for the most part. More than 20 G-protein α-subunits have been described and can be grouped into four classes: $G\alpha_{i/o}$, $G\alpha_{q/11}$, $G\alpha_{12}$ and $G\alpha_{s}$ [22].

The main members of the $G\alpha_{i/o}$-family are the three, abundantly expressed $G\alpha_{i}$-types which usually have an inhibitory effect on AC. The $G\alpha_{q/11}$-family includes $G\alpha_{q}$-types which are responsible for the PTX-insensitive activation of phospholipase C β-isoforms. This activation results in the formation of diacylglycerol and inositol triphosphate that usually triggers protein kinase and the release of Ca^{2+}, respectively. The $G\alpha_{12}$-family is small and thought to interact with c-Jun N-terminal kinase and phospholipase D. Activation of AC usually involves the G-protein of the $G\alpha_{s}$-family. AC is able to cleave ATP and produces cAMP. For example, this can lead to further activation of protein kinase A and the regulation of gene transcription by cAMP responsive elements [10, 22, 23]. In addition to the traditional view of GPCR signalling through G-proteins, new alternative

and G-protein independent pathways have been identified [24]. Even though most cells express several different GPCRs on the cell surface, signal specificity is maintained. This is a remarkable feature of G-proteins and illustrates the complexity and functional diversity of cell signalling which has evolved alongside GPCRs.

It is obvious that functionally screening for receptor activity involves a multitude of intricate, G-protein pathways and, in reality, one has to choose a specific method to assay a receptor system for potential activation. One of the most commonly used techniques is monitoring second messenger systems, for example, intracellular changes in Ca^{2+} concentration [25]. However, not all GPCR signalling is detectable by measuring Ca^{2+} and, depending on the cell type, the receptor itself and the assay method used, a potential signal can be missed. An alternative is to use a method capable of detecting a wide variety of signalling pathways, e.g. a reporter system with built-in signal amplification.

In order to support the investigation of orphan receptors, an in-house reporter system based on luciferase transcription was designed [26]. More precisely, a second generation reporter system [27] based on HeLa cells was stably transfected with plasmids containing orphan receptor genes and, subsequently, used for the main screenings.

Orphan receptors

Orphan receptors – a great potential for new discoveries

In the late-1990s, major efforts were made to sequence the human genome. This laid the technical foundation for the rapidly growing sequence databases. There were indications, even then, that the super-family of GPCRs was one of the larger ones, with the potential to grow. The joint-academic effort to sequence the entire human genome, called HUGO [28], enabled data mining in a completely new and revolutionary way.

Based on the completed HUGO project, the estimated total number of genes in the human genome is 35 000, of which approximately 750 are considered to be coding GPCRs [4]. Excluding the sensory receptors leaves about 400 GPCRs. An estimated 240 GPCRs have previously identified functions, while 160 are considered to be orphan GPCRs [29] (Fig. 3). However, the exact number of GPCRs in the human genome is still under debate. A recent bioinformatics study indicated more than 800 human GPCRs [11].

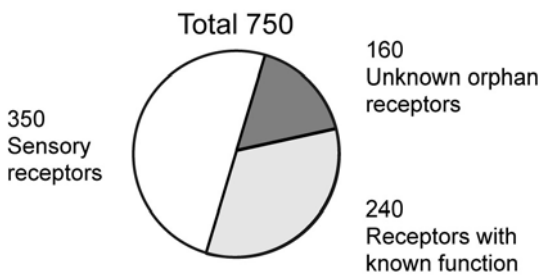


Figure 3. Pie chart representing all known GPCRs and the 160 orphan receptors in need of characterization (of which 110 are of Rhodopsin type). Of the 240 non-sensory receptors with known ligands/function it is remarkable that only about 30 are targets for today's successful drugs.

While it is estimated that 50% of all drugs with known mechanisms

exert their beneficial effects by targeting GPCRs, only 30 of the currently known GPCRs are, in fact, targeted by these drugs [30]. There is no doubt that a vast potential for new drug discoveries lies beyond the identification and characterization of orphan receptors.

Orphan receptor identification and selection

The available sequence databases, containing both EST [31] and genomic material, were subjected to data base mining. Initially, the amino acid sequence of the first receptor for LTB_4, BLT_1, was used as a BLAST [32] query when searching the GenBank [33] databases for genes and genomic sequences produced by the HUGO project. Potential orphan receptors were examined manually by comparing these sequences with those of known receptors. The ORFs of unrecognized sequences (previously unknown) were extracted using the software GeneJockey® and online resources such as transmembrane prediction, TMHMM [34]. Today, most of this work has been automated by sequencing facilities and the enormous task of post-genomic proteomics remains to determine the function of new, putative proteins.

By gathering sequences of orphan receptors that lacked known functions and ligands, a selection of potential receptors was phylogenetically analyzed (Fig. 4). Sequential homology and ESTs were the only available sources of information that could potentially aid the identification of a new orphan receptor. A noteworthy orphan receptor typically aligns itself in a group with known relatives, has a physiological relevant expression pattern and exhibits an amino acid sequence with typical characteristics (7TM). However, not all orphan receptors exhibit typical characteristics which can be used in the "de-orphanizing" procedure. In reality, very little information is available that links an orphan receptor to its function. Finding related sequences by means of BLAST alignment might not in itself expose any significant clues related to the activating substance or receptor function [35]. When trying to unravel the nature of an orphan receptor, additional information (if available), such as gene expression profiles, has to be taken into consideration.

Initial orphan receptor screening

During the course of the project, we selected orphan receptors that had a potential for identification. The orphan receptor genes were stably expressed in reporter cells [26] and subjected to a ligand library. We commenced our data mining using the sequence of the receptor BLT_1, which is activated by LTB_4. Thus, we were attempting to identify potential ligands related to similar substances, i.e. lipid mediators.

The prospective ligands used in our screening efforts included variations and metabolites of such compounds as leukotrienes, lipophosphatidic acids, long-chain fatty acids, prostaglandins and lipoxins. Crude chemical preparations and complex biological extracts were also used but found to be impractical due to the purifying process necessary in the identification of the functional ligand. The available literature was searched in order to find compounds related to lipid mediators with reported or suspected biological activity. Using a methodology called "reverse pharmacology" [30], an orphan receptor is subjected to a selection of compounds, the ligand library. This match-making attempt between an orphan receptor and several ligands is of the utmost importance in order to uncover new

physiological mechanisms which have the potential of being the future's new drug targets [29].

No matter how sophisticated the orphan receptor identification or how large the ligand library, a reliable measuring technique (assay) is essential in order to detect the unknown signal. For the majority of the orphan receptor screening, we utilized our own in-house developed reporter cell system, which has the ability to detect and amplify a wide range of intracellular signalling pathways, as well as produce extremely high signal-to-noise ratios [27]. Briefly, this reporter system is based on the activation of transcription factors, such as AP-1 and NF-κB. These transcription factors bind and activate the responsive elements TRE and NF-κB [36, 37], which drive the production of a luciferase enzyme. This enzyme's activity can be measured by means of its luminescence and is proportional to a GPCR's degree of activation.

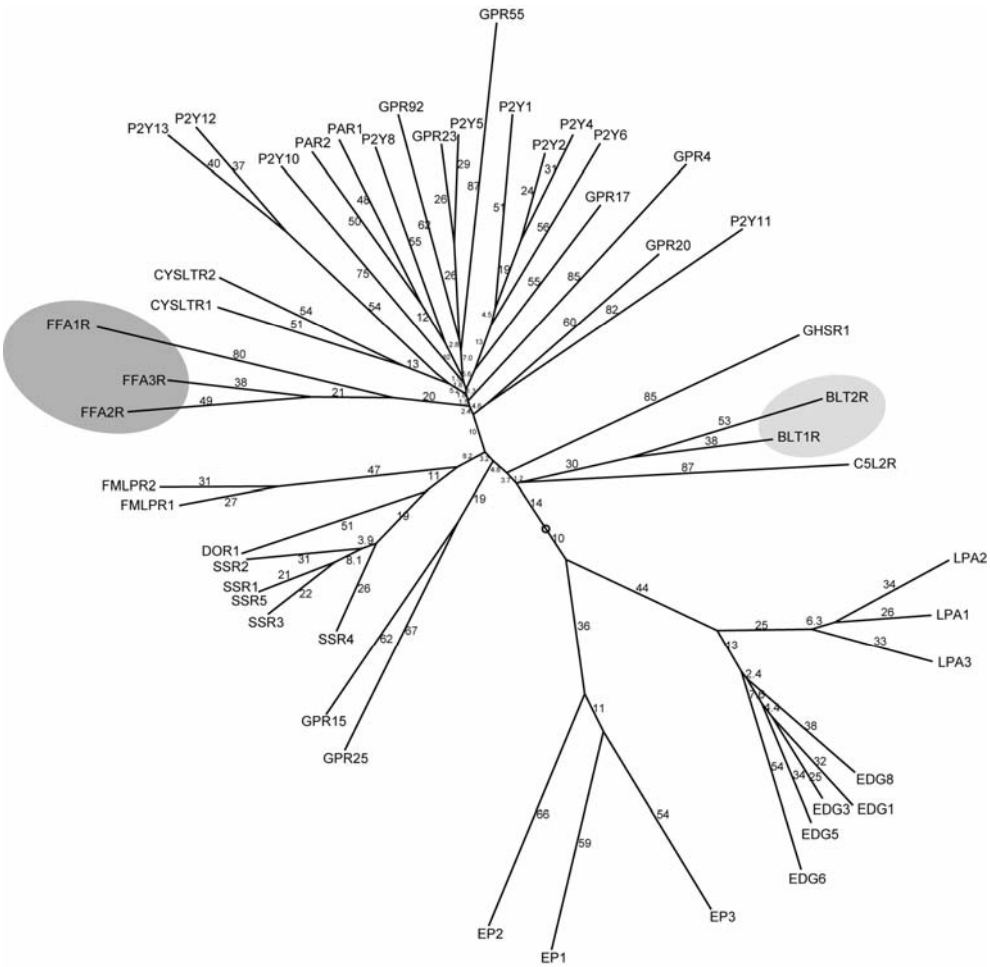


Figure 4. Phylogenetic illustration of a few selected receptors using the online resource, *AllAll's* PhyloTree function, at http://cbrg.inf.ethz.ch/Server/index.html. The dark grey circle highlights the sub-family of FFA receptors (paper II-IV), while the light grey points out the two LTB_4-activated receptors, BLT_1 and BLT_2 (paper I).

Current investigations - Deorphanized receptors

As previously described, several orphan receptors were cloned using genomic and EST information in order to produce specific primers for PCR amplification. During the course of this project, approximately 15 orphan receptors were screened for functionality. This resulted in the identification of four GPCRs which are activated by ligands related to lipid mediators: BLT_2 - the second receptor specifically activated by LTB_4, FFA_1R - the first receptor activated by medium- to long-chain FFAs, as well as FFA_2R and FFA_3R - two receptors with SCFAs acting as endogenous ligands.

The following chapters describe the exploratory work involved in identifying the activating ligands for these four receptors. The receptors' implicated physiological functions and the potential significance of these new findings are also compared to current understanding. For detailed information and methods refer to Appendix 2, which contains the four articles included in this dissertation.

The second receptor for leukotriene B_4 – BLT_2 (Paper I)

LTB_4 (Fig. 5) is a member of the eicosanoid family of ligands and, for more than 20 years, has been known to be involved in the immune system [38]. The eicosanoids have a carboxylic acid backbone containing 20 carbon atoms. In the case of leukotrienes, the last part of the word, "triene", indicates three sequential double bonds in the tail. LTB_4 is one type of leukotrienes, which includes LTA_4 (a precursor), LTC_4, LTD_4 and LTE_4. The latter three, also known as cysteinyl-leukotrienes, activate two GPCRs, $CysLT_1$ and $CysLT_2$ [39]. Leukotrienes are involved in the immune system, where they are produced for the most part. Leukocytes are the primary producers of LTB_4 and utilize an enzymatic pathway which employs arachidonic acid as a precursor molecule (Fig. 6). The process can be initiated by intracellular signals, such as Ca^{2+} and MAP kinase, and is preceded by activation of the immune system [39].

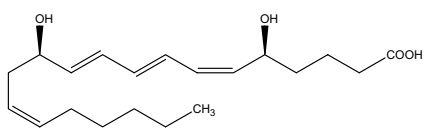


Figure 5. The structure of LTB_4.

Leukotriene B_4 is a GPCR-activating pro-inflammatory molecule

LTB_4 is a potent chemotactic mediator involved in mobilization of immune cells as part of the inflammatory process [40]. The ability to activate and recruit leukocytes to sites of inflammation is the primary function of LB_4. In fact, LTB_4 was discovered due to its highly potent chemotactic effect on neutrophils [41], but, later, it was also reported to recruit monocytes and macrophages [42].

Following the chemical gradient of LTB_4, the leukocyte eventually arrives at the site of inflammation. In addition, upon LTB_4-stimulaton, adhesion molecules [43], chemokines and reactive oxygen molecules are produced [44] in order to combat an infection. Moreover, phagocytosis [45] and degranulation [46] can take place as a result of LTB_4-induced leukocyte activation. LTB_4 also induces effects on lymphocytes, such as increased cytokine production [47], induced T-cell proliferation [48] and even early T-cell

recruitment [48]. Apart from its pro-inflammatory function, LTB_4 has also been reported to be involved in ovulation – suggested to resemble an inflammatory process [49].

The existence of a pharmacologically defined high affinity receptor for LTB_4 eventually led to the identification of BLT_1 [50, 51]. This happened shortly after its initial identification as a chemoattractant-like orphan receptor [52].

It has also been suggested that a low affinity target for LTB_4 exists [53, 54]. After the identification of BLT_1, the development and usage of antagonists acting on this GPCR supported the existence of a low affinity receptor for LTB_4 [55]. Subsequently, a second receptor for LTB_4 displaying low-affinity characteristics was cloned and identified independently by three research groups (Paper I) [56, 57]. It has been named BLT_2 [58], according to international nomenclature.

The initial identification of BLT_2 was performed by means of a homology screening, using the amino acid of BLT_1 as a template. The screening showed that a part of one particular EST clone shares a significant homology with BLT_1. Further sequencing of the EST clone yielded a partial ORF, lacking the GPCR C-terminal, rendering the receptor dysfunctional. Further database mining uncovered the missing sequence, which was then cloned and merged with the existing partial ORF – producing a previously unknown receptor sharing a 39% amino acid sequence identity with BLT_1.

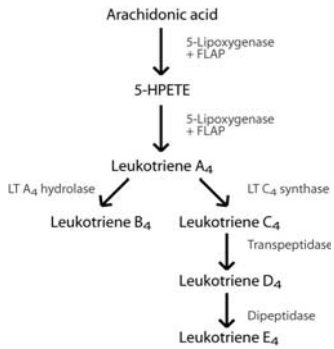


Figure 6. The leukotriene synthesis pathway, with arachidonic acid as the source. FLAP = 5-lipoxygenase activating protein. LTB_4 is mainly responsible for chemotaxis and leukocyte activation and activates two known GPCRs, BLT_1 and BLT_2 (as described in Paper I).

The complete ORF was transiently expressed in HeLa cells, thus enabling them to mobilize Ca^{2+} upon stimulation by LTB_4. On the other hand, cells transfected with a control plasmid encoding EGFP (enhanced green fluorescence protein) failed to do so. LTC_4, LTD_4 and LTE_4 were all unable to stimulate a significant BLT_2-induced Ca^{2+}-mobilization. Northern blotting revealed BLT_2 gene expression mainly in the liver, spleen, kidney, heart and skeletal muscle.

The discovery of an atypical ORF indicated the existence of two possible translational start positions – the first yielding an N-terminal of 51 amino acids and the second 20 amino acids. As it was not known which translational start position was being used *in vivo*, a complete sequence consisting of both start positions was initially employed. Later results (from unpublished experiments) obtained from using reporter cells pointed towards a functional receptor with a shorter N-terminal. While investigating the genomic region of BLT_1, it became apparent that the genes of the two LTB_4-activated GPCRs overlapped and were a part of a gene cluster of unusually high complexity [18] (Fig. 7).

The identification of BLT_2 led to an awareness of the existence of multiple target GPCRs and enabled more focussed pharmacological studies on the effects of LTB_4. Mouse knock-out studies indicated a primary role for BLT_1 and LTB_4-mediated chemotaxis to the site of infection, whereas BLT_2 was proposed to be involved in LTB_4-induced signalling at the site of inflammation, with higher concentrations of LTB_4 [59].

As LTB_4 is an important factor in the activation of the immune system, its potential involvement in inflammatory disease has been investigated. One example is the recent finding of LTB_4-induced recruitment of monocytes to atherosclerotic plaques [60]. LTB_4-antagonists reduced the clinical symptoms of arthritis in a mouse model, demonstrating the involvement of LTB_4 signalling in this disease [61]. A recent study correlates an increased expression of the 5-lipoxygenase activating protein with an increased risk of myocardial infarction and stroke [62]. This is particularly interesting as 5-lipoxygeanse is the enzyme responsible for the production of LTB_4 (Fig. 6) and the above-mentioned pathological risk is related to leukotriene-induced inflammation in the arterial wall.

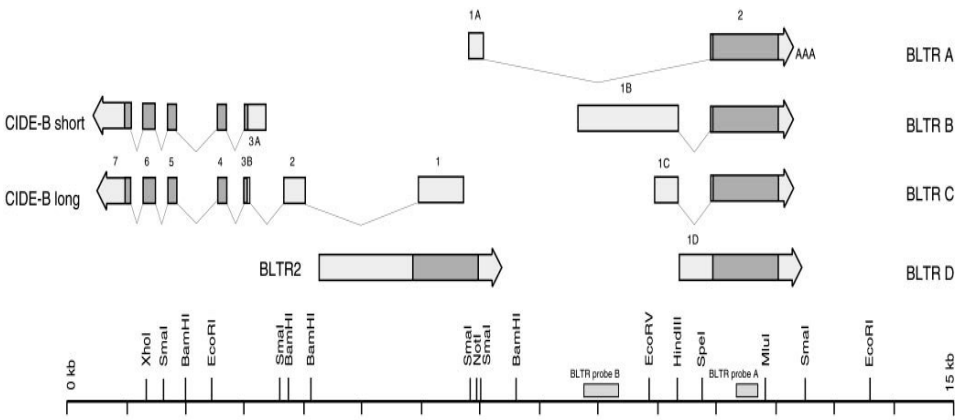


Figure 7. The organization of the *Blt1*/*Blt2* gene cluster indicating a highly complex and dense chromosomal region on 14q11.2-q12. Figure adapted from [18].

Free Fatty Acid Receptor 1 - FFA_1R (Paper II)

The nutritional role of lipids is well described and, in addition to their important energy function as dietary fatty acids, they act as signalling molecules. As they participate in normal metabolic regulation, fatty acids are potentially involved in pathophysiological conditions. The modern life-style – with its altered intake of fat – demonstrates a direct correlation between the new dietary trend and a number of diseases such as diabetes, cancer, atherosclerosis and obesity [63].

After the food intake, fat is generally transported from the intestine to its main sites of absorption, e.g. adipose tissue and the liver, in the form of triacylglycerols (three fatty acid chains bound together by a glycerol molecule). Fat is released from adipose tissue as FFAs, not esterified as triacylglycerols, and, therefore, is also known as non-esterified fatty acids (NEFAs). As with many other lipophilic molecules, FFAs are transported bound to albumin and under resting conditions, their average concentration is 150 mg per litre blood plasma [64]. As long-chain fatty acids consisting of 16 or 18 carbon atoms are the most common in the human body [65], this value roughly translates into a concentration of 0.5 mM long chain FFAs in blood plasma.

In Paper II [66], we describe the identification of a GPCR, which is activated by medium- to long-chain FFAs. This was a result of our orphan project, which attempted to match up bioactive compounds (potential ligands) with orphan receptors. Out of ten orphan receptors screened for various lipid mediators, including arachidonic acid (AA) and LA, only the reporter cells expressing GPR40 indicated activation by LA. The subsequent characterization of GPR40 revealed a GPCR with promiscuous activation by carboxylic acid and with a chain length ranging from 10 to 18 carbon atoms. Such a wide range of ligands is unusual for a GPCR, but this might reflect its physiological context, e.g. a nutrient sensor. Concentration-response assays using reporter cells showed that GPR40 was more potently activated by the medium-chain FFAs than the longer ones. Further studies, as well as taking into account two additional publications regarding GPR40 [67, 68], indicate that EC_{50}-values and levels of activation might depend on the actual assaying technique used. Varying methods, such as time of ligand stimulation, presence of fatty acid binding proteins, cell line and cell media, can affect the results. In general, the concentration of FFAs needed to activate the receptor is in the micro-molar range. The FFAs' relatively high EC_{50}-values indicate a low affinity receptor even though no functional binding assay has been able to confirm this. Still, the concentration range of the natural ligands is well in accordance with the plasma levels of circulating FFAs in the human body – approximately 500 μM under normal conditions and able to increase to well above 1000 μM during fasting [69, 70].

After the discovery of a range of FFAs acting as agonists on GPR40, we suggested renaming this orphan receptor; therefore, in the rest of this thesis it is referred to as FFA_1R. In order to continue the characterization of FFA_1R, we investigated the gene expression and looked for target organs which could reveal the physiological relevance of the new-found GPCR. A strong gene expression was found in the insulinoma β-pancreatic cell line, MIN6, as well as in the heart, liver and skeletal muscle. The predominant expression in MIN6 cells instigated a hypothesis concerning FFA and insulin secretion in β-pancreatic cells, while the actual FFA-induced Ca^{2+}-mobilization in MIN6 cells was confirmed. Three publications, including Paper II [67, 68], identify FFA_1R as an FFA-receptor and all point out that β-pancreatic cells are a major site of gene expression. However, there is a discrepancy within these articles regarding the existence of FFA_1R in other organs and tissue. For instance, only Briscoe *et al.* describe a high expression of FFA_1R in the human brain [67], implying the fact that several more physiological functions might be related to FFA_1R. Further in-depth expression studies and pharmacological characterization is needed in order to fully understand the involvement of FFA_1R in the complex system of lipid signalling. A few examples of FFA's participation in physiological regulation follow.

General functions of free fatty acids as signalling molecules

Circulating plasma FFAs have the ability to supply energy when needed, but also to act as signalling molecules, relaying information about the body's metabolic state to the organs involved [71]. FFAs have a wide range of functions in the human body. New breakthroughs, such as the discovery of FFA_1R, FFA_2R and FFA_3R (the two latter GPCRs, described below, were previously known as GPR43 and GPR41), are crucial to the overall understanding of lipid signalling and will enable new molecular explanations of known physiological effects. A few organs and tissues which have a documented relation to FFAs are: the hypothalamus, liver, mammary glands, adipose tissue, heart, muscle and pancreas

(Fig. 8). However, the precise involvement of FFA-activated receptors or other target molecules has to be considered in each particular physiological context.

The hypothalamus GH and somatostatin secretion are affected by FFAs [72-74]. Elevated levels of plasma FFAs, as seen in obesity, lead to chronically lower levels of GH [75], which in turn result in decreased adipose lipolytic activity. Therefore, excess weight is maintained through a direct link between FFAs and GH regulation [76]. Indication of FFA_1R expression in the hypothalamus exists in the form of an EST (clone BI603605, dbEST database [77]) and one research group has reported expression in the brain [67].

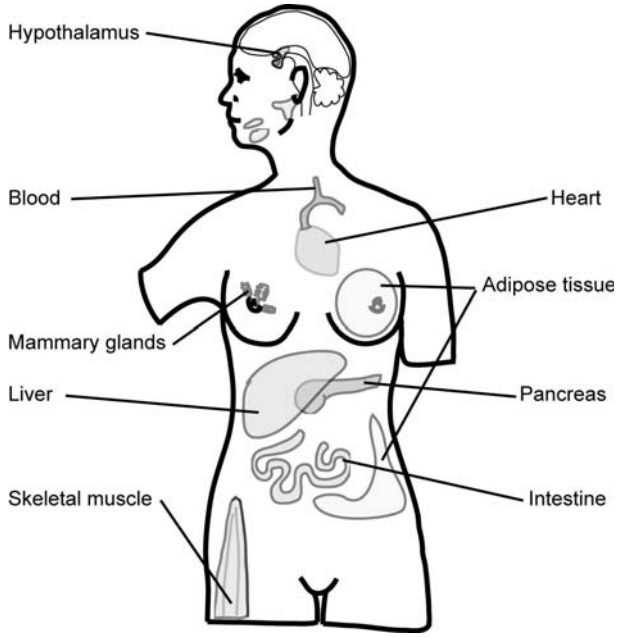


Figure 8. An illustration of the human body identifying some organs and tissues related to fatty acid signalling.

Salivary glands secrete fatty acids in saliva [78] and long-chain FFAs have been reported to regulate synthesis and secretion processes in the submandibular gland through Ca^{2+}-dependent mechanisms [79].

Fatty acids, mostly in the form of triacylglycerols, comprise the majority of the total milk volume produced by the mammary glands and have a role in the acute regulation of the process [80]. In addition, some FFAs, e.g. conjugated LA (CLA), demonstrate anti-tumour activity by inducing apoptosis in mammary tumour cells [81]. On a molecular level, FFA_1R was recently reported to be functionally expressed on the human breast cancer cell line, MCF-7 [82].

High levels of FFAs are related to hypertension and general heart failure [83, 84]. Coronary heart disease is also linked to fat intake, saturated FFAs in particular [85]; however, the beneficial effects of unsaturated FFAs might be linked to their anti-hypercholesterolemic ability [86]. Moreover, elevated plasma FFA concentrations have been associated with an increased risk of ischemic heart disease [87], although the exact mechanisms are still unclear.

In general, the liver has an important function in lipid metabolism as it degrades fatty acids into smaller molecules that are able to act as an immediate energy source and produces triglycerides, phospholipids and cholesterol [64]. FFAs can also affect the regulation of liver glucose metabolism and peripheral insulin concentration [88]. Hepatic

insulin resistance can be caused by increased levels of FFAs and is thought to play a role in the general development of NIDDM (diabetes of type 2) [88-90].

During both rest and exercise fatty acids are the major energy source for muscle activity. FFAs comprise a large fraction of fat fuel and have been shown to increase in plasma concentration during exercise [91]. FFA-induced skeletal muscle insulin resistance plays a significant role in the progressive development of NIDDM [90] by inhibiting insulin-stimulated glucose uptake [92]. Although we have identified FFA_1R-gene expression in muscle, its actual function needs to be addressed in detail.

The main function of adipose tissue is to store fatty acids as a future source of energy. The obvious advantage of being able to store excess energy in between periods of food intake is in contrast to the disadvantage of long-term, excessive storage which can lead to metabolic diseases. Excess energy is stored by adipose cells as triglycerides and released into the circulating plasma as FFAs when needed [64]. Not only is the passive role of adipose tissue important, but evidence for an important active endocrine function is growing [93]. A recent study has revealed that FFA_3R is involved in adipose endocrine function due to propionate's ability to increase circulating leptin levels [94] (FFA_3R is discussed in Paper IV). The involvement of PPARs in adipose tissue is discussed below.

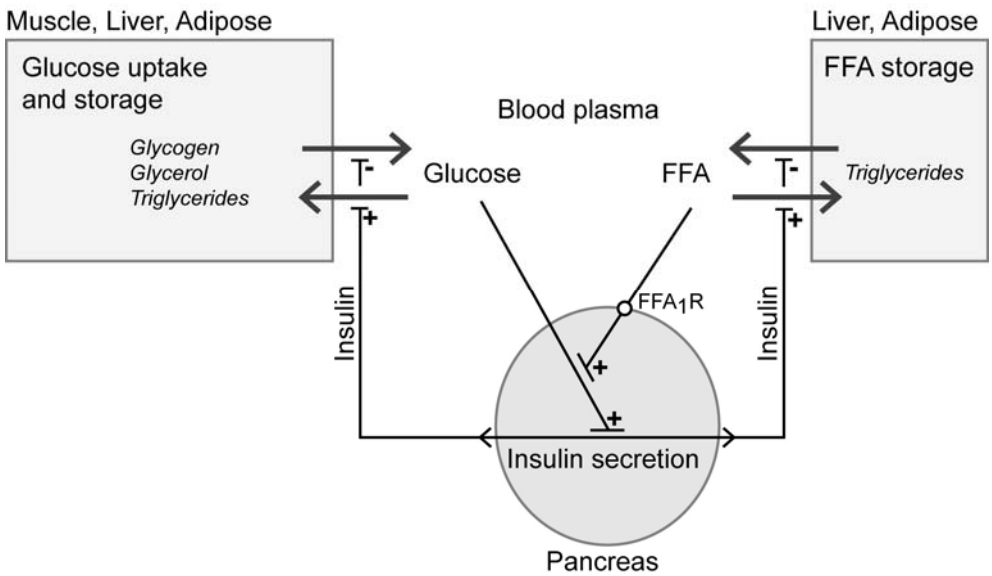


Figure 9. A simplified schematic of insulin's effect on glucose and FFA utilization. The pancreas regulates insulin secretion mainly by sensing the levels of glucose in blood plasma. This glucose-stimulated insulin secretion can be enhanced by FFAs. The newly discovered molecular mechanism, which acts at the interface between nutritional fatty acids and the pancreas, is the GPCR described in Paper II, FFA_1R. Increased levels of glucose promote glucose usage, while at the same time both FFAs and excess glucose are stored. Increased levels of FFAs further enhance this mechanism, as they prioritize the usage of glucose rather than FFAs.

FFA-regulation of vascular conditions is well known and can directly raise blood pressure, which in the long run, may contribute to the development of hypertension [95, 96]. An endothelial inability to dilate has been associated with FFAs and is thought to be the link between obesity, NIDDM and increased blood pressure [97].

The pancreas is the single organ that shows the highest expression of FFA_1R and is also the target with the most well-documented FFA-induced physiological effects. Circulating levels of plasma FFAs maintain and contribute to 30-50 % of basal insulin secretion [92], while acute stimulation of FFAs on pancreatic islets potentiates GSIS [98, 99]. Furthermore, there is a significant difference between short and long term FFA exposure on the endocrine function of the pancreas. Acute stimulation potentiates the GSIS, but long term exposure decreases the same function [100]. An exhaustion theory could possibly explain this phenomenon since an increase in sustained FFA-induced basal secretion is not matched by an equal increase in *de novo* insulin synthesis [101]. Pancreatic lipotoxicity is complex and other potential pathophysiological mechanisms exist [102]. The link between obesity, the endocrine pancreas, FFAs and NIDDM is discussed below.

Free fatty acids, obesity and non-insulin dependent diabetes mellitus

Several reports show a direct and acute effect on pancreatic insulin secretion when it is stimulated by FFAs [98, 103]. Not only does a sudden increase in the level of FFAs augment insulin secretion, but it also appears to play a significant role in the regulation of basal secretion [70, 104]. This is all part of a healthy physiological response to a momentary increase in FFA plasma concentration. The increase in plasma insulin promotes glucose usage rather than FFA usage, allowing fat to be stored mainly in adipose tissue as triglycerides (Fig. 9). On the other hand, a sustained elevated FFA plasma level has been shown to impair insulin secretion, induce β-pancreatic lipotoxicity and cause progressive cell failure [105]. Periods between food intake result in lower levels of insulin. A normal and functioning metabolic system responds to these levels by inhibiting the use of glucose in the liver and muscle. This, in turn, enhances the release of FFAs into the plasma as an alternative energy source.

There is a strong correlation between an increased level of circulating FFAs and diagnosis with diabetes, specifically type 2 - NIDDM [90]. Impaired insulin secretion renders the metabolic organs incapable of utilizing glucose and greatly up-regulate the break down of fat. Therefore, an FFA-induced reduction of the pancreatic ability to secrete sufficient amounts of insulin further increases the release of FFAs from adipose tissue even though there is a high presence of glucose in the blood plasma. If the pancreas and insulin dependent organs have become desensitized, this extra increase in FFAs could be sufficient enough to overcome the elevated threshold for insulin function. In fact, this is often the reason why obese people still may have a functioning glucose metabolism, even though they have elevated FFA levels. But, in the

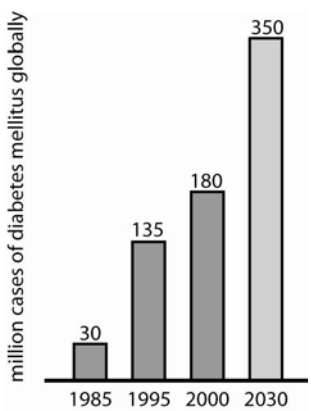


Figure 10. Number of people diagnosed with diabetes type 2 worldwide. The year 2030 estimate is based on current development trends. Data from the World Health Organization.

long run, chronically high FFA levels and a growing demand for even greater insulin levels causes pancreatic malfunction as the glucose and FFA-stimulated insulin secretion is unable to further compensate. In this case, insulin levels drop causing glucose and FFAs to accumulate to an even greater extent in the blood plasma. Peripheral insulin-resistance – a state in which organ metabolism, e.g. muscle metabolism, does not respond to an insulin signal – now creates a pathophysiological condition which precedes NIDDM.

The normal and well-defined function for FFAs – to stimulate an acute physiological response – can be hampered by a life-style high in fat-intake and low in exercise. This type of life-style might be able to induce chronically elevated fatty acid levels that over-stimulate or exhaust the GSIS mechanism [92, 106]. The outcome of this might be high levels of glucose, fatty acids and triacylglycerols. These high levels, in turn, speed up progressive beta-cell failure and greatly increase the risk for cardiovascular disease and several other systemic problem [107, 108]. According to the World Health Organization [109], 180 million people in the world have been diagnosed with NIDDM. This figure is expected to rise to 350 million by the year 2030 if the current rate of development continues (Fig. 10).

Obesity is another fat-related global problem that is reaching epidemic proportions [110]. In the last 10-15 years, public opinion on obesity has changed from it being a mostly cosmetic inconvenience to a serious health-threatening problem. Currently, it is estimated that two-thirds of the adult American population and about half of the people living in the UK are overweight [111, 112]. Not only adults living in the so-called western world exhibit an increase in body weight. The number of obesity and NIDDM cases related to life-style has been growing among children and Asian people in general [113, 114]. High food intake combined with inadequate exercise results in a growing amount of adipose tissue, which causes the systemic levels of FFAs to increase [115]. The chronically elevated levels of plasma FFAs related to obesity and excess fat storage seem to be linked to the increased prevalence of NIDDM in society [116, 117]. The functional expression of FFA_1R on β-pancreatic cells is described in Paper II, which indicates its role in insulin secretion and systemic metabolism. This role has also been independently confirmed by others [67, 68].

Peroxisome proliferator activated receptors and thiazolidinediones

The identification of FFA_1R as an FFA-activated cell surface receptor was completely novel and, even though there were previous indications of this, the actual pharmacological mechanism has remained concealed until now. This could potentially be due to the extensive research about and focus on a known group of FFA receptors, namely nuclear PPARs [118]. PPARs are expressed in the cell nucleus and are members of the transcription factor type of receptors, which use a fundamentally different signalling mechanism (Fig. 11).

Currently, three sub-types of PPARs have been identified: α, δ (also known as β) and γ isoforms [119]. Simply described, the PPAR protein forms a complex with the retinoid X receptor (RXR) and transcription is initiated when the ligand binds to its receptor. PPAR-α is expressed in brown adipose tissue, the liver and skeletal muscle and is thought to regulate uptake, activation and oxidation of fatty acids [120]. PPAR-δ is widely expressed; however, the research has focussed, for the most part, on the receptor's proposed role in

fertility and the central nervous system [120]. Due to its ability to bind the anti-diabetic drugs TZDs, PPAR-γ has received the most attention from a metabolic perspective [121]. PPAR-γ is mainly expressed in adipose tissue, but lower amounts have also been reported in various other cell types, such as macrophages and colon cells [122]. The main, endogenous function of PPAR-γ is to regulate adipogenesis and the "thrifty gene response" [122].

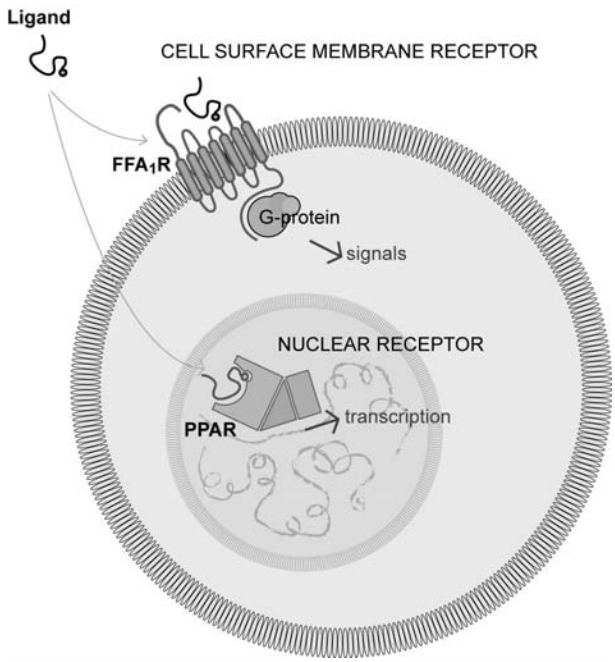


Figure 11. A cell displaying the two known forms of receptors for FFAs. PPAR is a transcription-regulating nuclear receptor activated by e.g. FFAs, prostaglandins, leukotrienes and TZDs. FFA_1R is the novel GPCR expressed on the cell surface and shares some ligands with PPARs, such as FFAs and TZDs.

PPARs are activated by a range of ligands related to fatty acids and are clearly involved in sensing the body's metabolic state [120]. In general, PPARs are poorly activated by saturated FFAs and a carbon chain-length of 14 or more is required to induce ligand binding [123]. Unsaturated FFAs, as well as prostaglandins, eicosanoids and leukotrienes, act as potent PPAR activators [123-125]. See Appendix 1 for a table illustrating natural and synthetic ligands and their effects on the three PPAR isoforms, as well as on FFA_1R, FFA_2R and FFA_3R.

Anti-diabetic drugs

The story of TZDs or "Glitazones" (Fig. 12) began with the observation of reduced plasma glucose and insulin resistance in obese-diabetic mice and rats when they were administered a newly synthesized compound, Ciglitazone [126, 127]. Other structurally similar compounds were synthesized and screened for anti-diabetic effects, for example, lowered glucose plasma and FFA concentrations. Troglitazone became the first TZD to be approved as an anti-diabetic drug for humans [128], followed by Pioglitazone [129] and Rosiglitazone [130]. However, various side effects, such as weight gain, have been observed and, more seriously, Troglitazone was removed from the market in 2000 after being associated with irreversible liver damage [131]. Typically, an 8 mg oral administration of Rosiglitazone results in 99% plasma bioavailability, with concentration peaking at roughly 600 ng/ml (corresponding to a theoretical concentration of 1.5 μM) after 0.5 h and an almost complete drug clearance after 24 h [132].

The discovery that PPAR-γ binds and is functionally activated by TZDs caused a flood of reports and research regarding these nuclear receptors and NIDDM. The main targets for the development of new anti-diabetic drugs are currently PPARs, in particular, the agonistic effect of TZD on PPAR-γ. The findings in Paper II, which describe FFA- and TZD-induced activity on FFA_1R, are controversial even though numerous PPAR-independent TZD effects have been reported [133-138]. Whether these PPAR-independent pathways are related to FFA_1R-signalling or other unknown mechanisms remains to be clarified.

Other compounds, unrelated to TZDs, have also been reported to induce PPAR-γ agonistic activity. The experimental drug, MEDICA 16 [139], is an example of such an activator. It also functions as a FFA_1R ligand and exhibits anti-diabetic activity in obesity-induced insulin-resistant rats [140]. However, other endogenous PPAR-γ ligands, such as Prostaglandin J2 (in particular, 15-deoxy-delta12,14-PGJ2), are potent agonists and effective adipogenic agents [141] and yet there have been no reports of insulin sensitizing effects, as with TZDs. In addition, other synthetic compounds, e.g. Indomethacin, are able to activate PPAR-γ (however less potently than by TZDs) and induce adipocyte differentiation [142], but no reports describing anti-diabetic effects exist.

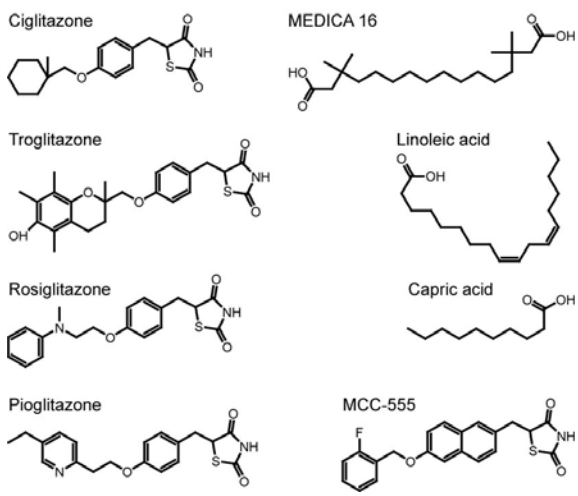


Figure 12. Two natural ligands for FFA_1R: linoleic acid (C18:2) and capric acid (C10:0). Representatives from the TZD group are Ciglitazone, Troglitazone, MCC-555, Rosiglitazone and Pioglitazone. The latter two are used clinically as anti-diabetic drugs. MEDICA 16 is an experimental anti-diabetic compound.

When TZDs were found to bind to PPAR-γ, their anti-diabetic effects were also suggested to be mediated through this receptor although a direct link to the regulation of glucose metabolism has not been proven. The lack of a direct connection is due mainly to the difficulty of finding relevant expression of PPAR-γ in skeletal muscle, where glucose uptake and utilization primarily takes place. Based on the primary expression profile of PPAR-γ, a model explaining the TZD-induced anti-diabetic effect on adipose tissue was put forward. In this model, the glycaemic effect is thought to be secondary to the effect of TZDs on PPAR-γ expressed in adipose tissue [143]. PPAR-γ-mediated adipogensis stimulates increased triglyceride storage, which could result in the removal of FFAs from the plasma [117]. However, it seems counterintuitive to treat NIDDM with a drug that promotes adipogenisis and increases body weight when obesity has been associated with insulin resistance [144]. Adding to the uncertainty, reports exist that argue for both adipose tissue dependent [145] and independent [146] TZD anti-diabetic activity. Furthermore,

newer synthetic compounds designed from a PPAR perspective and screened for anti-diabetic abilities do not necessarily display high PPAR-γ affinity [147-150]. Taking everything into consideration, it has also been suggested that insulin sensitization is in fact mediated through mechanisms that are independent from PPAR-γ signalling [138].

It is imperative that the discovery of TZD-induced activation of reporter cells expressing FFA_1R leads to an in-depth study in order to differentiate PPAR- and FFA_1R-mediated signalling. Consequently, such a study will be able to promote the desired anti-diabetic effects and reduce the unwanted and malicious side effects. The growing amount of PPAR-independent reports further emphasizes the importance of clarifying the molecular mechanism of TZDs. In any case, there is no doubt that the new evidence for FFA_1R involvement in insulin secretion needs to be taken into consideration with regards to the general regulation of fat and glucose metabolism.

Free Fatty Acid Receptor 2 - FFA_2R (Paper III)

FFA_2R, the closest relative (although only sharing ~30% identical amino acids) to FFA_1R, is identified and characterized as a GPCR activated SCFAs in Paper III. GPR43, previously an orphan receptor, was cloned, expressed on reporter cells and found to be activated by various SCFAs (mainly acetate, propionate and butyrate). The results from the reporter cell assay were confirmed by measuring Ca^{2+}-mobilization and a significant gene expression was identified in human peripheral blood leukocytes. The physiological function of FFA_2R was pursued taking into consideration the nature of the activating ligands and the expression profile.

Short chain fatty acids as ligands

SCFAs, also known as short chain carboxylic acids, are comprised of 1 to (approximately) 6 carbon atoms with a terminal carboxylic group. There are reports suggesting that SCFAs are molecules involved in numerous regulatory functions [151-153]. In particular, investigations into the SCFA-effect on immune cells revealed a multitude of SCFA-induced physiological responses [154-156]. Even direct evidence for probable GPCR involvement was noted when propionic acid induced PTX-sensitive Ca^{2+}-mobilization in leukocytes [157-159].

Anaerobic bacterial fermentation usually produces high amounts of SCFAs which seem to interact with the immune system [160, 161]. Several studies have explored this system, and are as yet unable to clarify the molecular mode of action. Two examples of this physiological feature are: first, an unwanted bacterial infection which is able to evade the immune system and, second, a desired and healthy bacterial colonization in the intestine. It is possible that these features act through a common molecular mediator, as the existence of both implies the utilization of an SCFA-mediated immune-suppressing mechanism [162, 163] – a known SCFA capability to inhibit certain leukocyte functions, e.g. phagocytosis and degranulation [152, 163]. In Paper III, we identify FFA_2R as a GPCR expressed on human neutrophils which is functionally activated by SCFA.

One hypothesis attempts to explain the SCFA-induced inhibition of the immune system in gingivitis, in which the physical settlement of bacterial plaque in the mouth produces milli-molar amounts of SCFA [164]. In the initial stages of the disease, the bacteria initiate an inflammatory response, but, at the same time, SCFAs inhibit certain neutrophil activity

that results in a prolonged and sustained gingival inflammation [162]. It is possible that the bacteria are, to some extent, using a mechanism meant to allow a certain degree of desired bacterial colonization in the intestinal region.

The immune system requires balance in the intestine

Large amounts of bacteria (colon *bacilli*) are present in a healthy colon, participate in the complete digestion of cellulose and supply the body with absorbable nutrients. In herbivores, this mechanism serves an important energy supplying function, but in humans, the added energy produced by bacterial fermentation is not as significant. Instead, vitamin K, vitamin B_{12}, thiamin and riboflavin are the essential substances produced by bacteria in humans [64]. In addition, an important, passive result of this beneficial bacterial presence is the existence of a "controlled" environment protected from unwanted infections. Apparently, the intestine benefits from the presence of bacteria and, in-turn, a friendly environment is offered for bacterial colonization. The intestine is highly immunogenic and constantly exposed to a large amount of foreign biological material, which constitutes a challenge to the immune system. To avoid constant activation of the immune system in the intestine, it is essential to regulate a balanced inflammatory response [165]. The SCFAs produced by bacterial fermentation in the intestine have been suggested to play a crucial role in the balancing act of this host-bacterial symbiotic relationship [163]. During anaerobe bacterial fermentation, SCFAs are produced in high concentrations, mainly as acetate, propionate and butyrate [166] – the most potent agonists which activate FFA_2R (Paper III) [167, 168].

With regards to these observations, it is attractive to appoint the physiological role of SCFA-mediated acceptance of intestinal bacterial colonization to FFA_2R (Fig. 13). (1) There is a high abundance of SCFAs in the intestine which are produced by bacteria [166]. (2) A balance is required in the immune system's response to the bacterial presence in the intestine [163]. (3) SCFAs have a documented ability to inhibit certain neutrophil functions [161]. (4) FFA_2R has been identified as the missing GPCR activated by SCFAs and is expressed on neutrophils (Paper III) [167, 168].

Further investigations into the potential role of FFA_2R as an immune-regulatory GPCR might help to elucidate the cellular mechanism of bacteria-host relationships and, potentially, the process of over-active inflammatory diseases, for example, ulcerative colitis and Crohn's disease. Such knowledge could lead to the development of new medicines, with FFA_2R as the therapeutically immunosuppressive target.

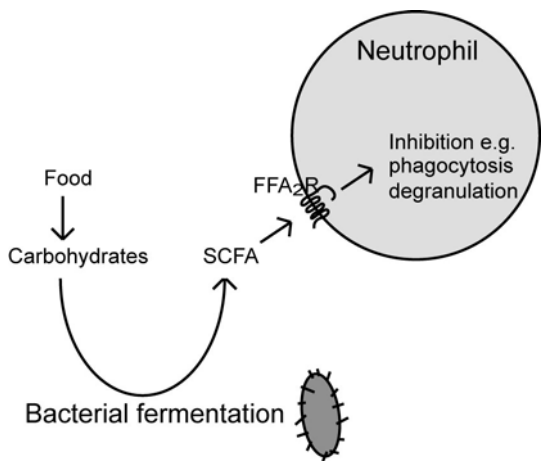


Figure 13. An illustration of the SCFA-induced regulation of the intestinal immune system. SCFAs are produced by bacterial fermentation and act as an indicator of the desired bacterial population.

Free Fatty Acid Receptor 3 - FFA_3R (Paper IV)

The first *in silico* discovery of orphan receptors GPR40-43 identified four genes coding for putative GPCRs [15]. Two of these orphan receptors, GPR41 and GPR42, have 98% of their amino acid sequence identities in common. Due to the technical nature of the cloning procedure and the overall resemblance of the nucleotide sequences, GPR41 was favoured and subsequently cloned. Initially, GPR41 was believed to be functionally identical to GPR42, but recent reports identify the latter as a pseudo-gene [167].

A second receptor activated by short chain fatty acids

Even though the amino acid sequence of the orphan receptor GPR41 indicated a relevant homology with FFA_2R (35% identical amino acid residues), it was not obvious that it would, in fact, constitute a second GPCR activated by SCFAs. However, cloning and expressing the gene encoding the human orphan receptor GPR41 revealed that the third member (FFA_3R) of the FFA-activated receptor family is also functionally activated by SCFAs.

There is a discrepancy in the FFA_2R- and FFA_3R-activating ligand spectra that can be utilized in order to differentiate the activity of the two receptors. The order of potency for SCFAs acting on FFA_2R is acetate ≥ propionate > butyrate and for FFA_3R: butyrate ≥ propionate > acetate. Using CHO cells, we compared the Ca^{2+}-mobilization ability of FFA_2R and FFA_3R. The results indicated similar activity to that seen when using reporter cells to measure FFA_2R and FFA_3R activity.

Reporter cells expressing either FFA_1R, FFA_2R or FFA_3R were challenged with Rosiglitazone. Only FFA_1R-expressing cells were activated by the TZD, Rosiglitazone. In addition, the mouse homologue to FFA_1R was able to mediate a Rosiglitazone-induced reporter cell signal (unpublished data). These results indicate that TZD-induced activity is limited and specific to FFA_1R-expressing reporter cells.

The cloning and identification of FFA_3R completed the characterization of the FFA-activated receptor family. The human genes of these three functional GPCRs are located in a cluster at chromosomal position 19q13.1 and the alignment of their amino acid sequences indicates a phylogenetical relationship (Fig. 4). Several reports describe clusters of adjacent genes that code for GPCRs, e.g. the IL-8 receptors [169], fMPL receptors [170], BLT_1/BLT_2 [18] and the FFA receptors [15]. In the human, mouse and rat genomes, the FFA receptors are located on chromosome 19, 7, and 1 respectively (Fig. 14). The proposed human pseudo-gene for GPR42 is not present in either the available mouse or rat genomic sequences, suggesting a recent human gene duplication that resulted in the two genes coding for GPR41 and GPR42.

Although these FFA-receptors exhibit phylogenetical relationships and are activated by ligands with similar chemical structures (fatty acids), they seem to have evolved in different physiological contexts. FFA_1R is involved in β-pancreatic insulin secretion, while FFA_2R-activation is likely involved in regulating immune system response. FFA_3R expression has been detected in adipose tissue and it was recently suggested that this receptor participates in the regulation of leptin secretion [94]. SCFAs were found to stimulate adipose tissue secretion of leptin – a hormone involved in energy homeostasis that regulates appetite and body weight [94, 171]. A deficiency in leptin signals – due to a

reduction in leptin-induced melanocyte stimulating hormone (MSH) secretion in the hypothalamus – could result in obesity. MSH inhibits the appetite; therefore, the chain of events that leads to reduced levels of this hormone could result in the absence of a "stop-eating signal" [172].

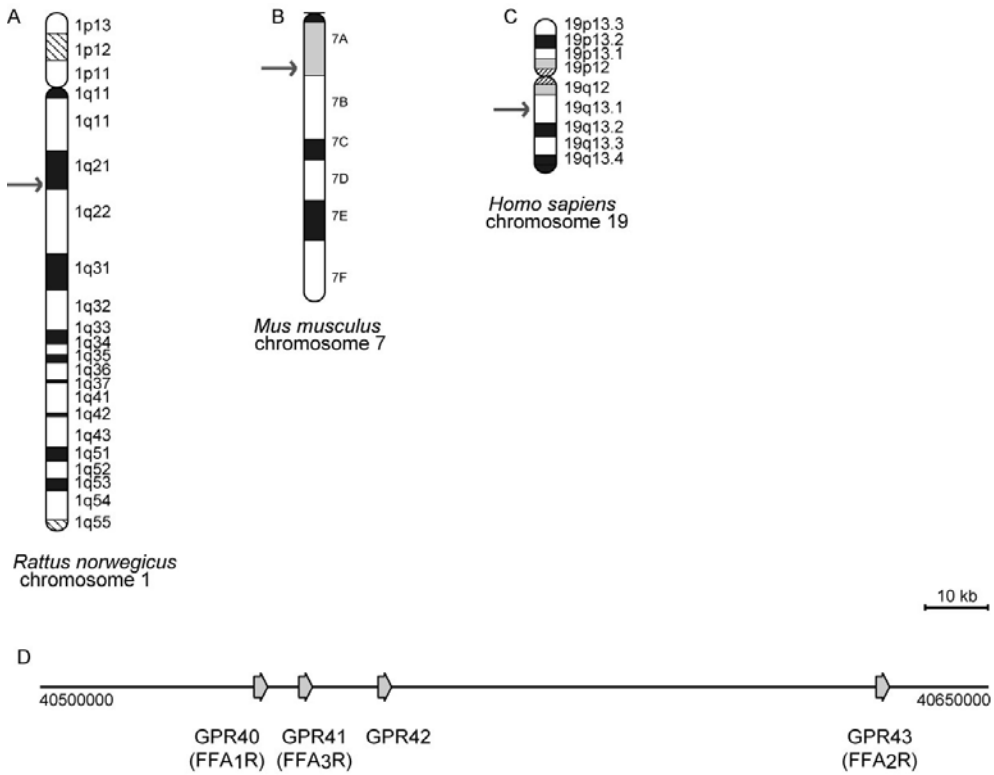


Figure 14. A) Rat, B) mouse and C) human chromosomes containing the DNA encoding of FFA-activated receptors. The arrows indicate the chromosomal position of the FFA-receptor gene cluster. D) 150 kb (thousand base pairs) part of the human chromosomal position at 19q13.1 (section 40,500 kb to 40,650 kb), containing genes of the FFA-receptors. Data from the *Ensembl genome browser* (http://www.ensembl.org)

Conclusions

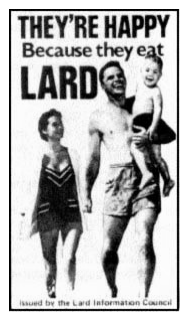
GPCRs are a large and important super-family of proteins involved in the regulation of physiological machinery. Approximately 800 GPCRs are known to exist; however, a large fraction of these (160) needs to be functionally identified as their activating ligands are unknown and they lack a physiological context. The so-called orphan receptors are potential new drug targets and have, therefore, received a great deal of attention. The importance of studying GPCRs in the regulation of physiological functions is emphasized by the fact that 50% of today's drugs exert their beneficial functions by acting on these receptors.

The aim of this project was to discover new receptors, identify their activating ligands and characterize their physiological role. The theoretical targets were orphan receptors which could be activated by lipid mediators. This approach led to the successful identification and characterization of four new GPCRs.

The discovery of an EST-clone that contained an amino acid sequence similar to that of the receptor, BLT_1, led to the identification of a second GPCR, BLT_2, which is activated by LTB_4. Leukotrienes are capable of mobilizing the immune system by inducing chemotaxis and other inflammatory processes. The existence of a low-affinity receptor for LTB_4 has been predicted and it has been suggested that BLT_2 fills that role.

LA was able to induce activation of reporter cells expressing the orphan receptor, GPR40. This GPCR was characterized and found to be promiscuously activated by medium- to long-chain FFAs (carboxylic acids with a carbon chain ranging from 10 to 18 atoms). Due to its proposed ability to act as a "nutrient sensor", the name FFA_1R was suggested. FFA_1R is expressed on β-pancreatic cells and FFA-induced activation causes an augmentation of insulin secretion. The activation of FFA_1R by the anti-diabetic drug, TZD (glitazones), implies a clinical connection to diabetes type 2.

Two GPCRs (FFA_2R and FFA_3R) were found to be functionally activated by SCFAs. FFA_2R expression was abundant on peripheral blood leukocytes and these results helped to explain the missing SCFA-activated GPCR. One hypothesis identifies FFA_2R as a mediator in the SCFA-induced immune suppression in the intestinal tract. This function is necessary in order to avoid an overactive immune system response related to the exogenous presence of bacteria. The third and final member of the FFA-activated receptor family is FFA_3R, which is also activated by SCFAs but pharmacologically distinguishable from its sibling, FFA_2R. Recent reports suggest that FFA_3R controls energy homeostasis through the regulation of leptin secretion by adipose tissue.



Sammanfattning på svenska (Summary in Swedish)

Oberoende av om det är i ett samhälle, datornätverk eller organen i en mänsklig kropp, så är behovet av kommunikation stort i en komplex miljö. Eftersom den komplicerade människokroppen är beroende av kommunikation för att reglera funktioner, skickas otaliga mängder signaler oavbrutet. För att signalerna skall vara meningsfulla så måste de kunna tas emot och behandlas. Behovet av specifika mottagare för att kunna urskilja enskilda signaler är uppenbar, som t.ex. signalen att en muskel skall användas, näsan känner en doft eller att en infektion skall angripas. Kroppen har löst detta med hjälp av en stor mängd mottagarmolekyler (receptorer) som sitter på ytan av celler och fångar upp dessa signaler.

Receptorer fungerar som ett lås där en korrekt signalmolekyl agerar nyckel och låser up - aktiverar mottagarreceptorn. För att kunna reglera de komplexa mekanismerna i kroppen finns många olika typer av receptorer. Den största gruppen besläktade receptorer är de så kallade G-protein kopplade receptorerna. När den korrekta signalmolekylen aktiverar dessa receptorer så skickar de i sin tur vidare uppgifter om lämpliga åtgärder in i cellen via G-proteiner. Totalt i den mänskliga kroppen finns närmare tusen olika receptorer av den här typen, men många av dessa är helt okända och vi saknar kunskap om deras aktiverande signalmolekyl, var de finns och vilken roll de spelar den mänskliga fysiologin.

Ungefär hälften av dagens läkemedel fungerar genom att påverka dessa receptorer på ett eller annat sätt. T.ex. kan förhöjt blodtryck kompenseras genom att en receptors signalförmåga blockeras (t.ex. beta-blockare). Eftersom flera hundra receptorer idag inte är karakteriserade finns det en enorm läkemedelspotential inom denna grupp av receptorer.

Målet med detta arbete har varit att identifiera sådan okända receptorer, sätta in dem i ett molekylärt system för att hitta den korrekta signalmolekylen och klargöra hur och vilka av kroppens organ som påverkas. Detta är ett är projekt där det inte går att förutsäga processens gång, eller dess slutgiltiga mål, men som stimulerar till ett nyfiket utforskande.

Denna upptäcktsresa bland okända receptorer ledde fram till karakteriseringen av fyra nya receptorer, vilka beskrivs mera ingående i detta arbete. Den första identifierades som den andra receptorn för LTB_4, som är involverad i immunförsvarets inflammatoriska process. På ytan av pankreasceller påträffades vidare en receptor som aktiveras av mellanlånga till långa fettsyror (10 till 18 kolatomer i kedjelängd) och medverkar i regleringen av insulinfrisättningen. Även vissa moderna diabetesläkemedel visade sig kunna påverka denna receptor. Den tredje receptorn aktiveras av signaler i form av korta fettsyror (2 till 6 kolatomer i kedjelängd), typiskt sådana som produceras av tarmbakterier. Det har länge varit känt att sådana signaler har förmågan att hämma immunförsvaret och medverkar troligen till att kroppen tillåter en bakterienärvaro i tarmen. Slutgiltigen, identifierades en tredje aktör i den fettsyre-aktiverade receptorfamiljen. Även denna aktiveras av korta fettsyror och föreslogs nyligen att reglera vissa funktioner i fettceller. De tre receptorerna som aktiveras av olika typer fettsyror bildar tillsammans en besläktad underfamilj av G-protein kopplade receptorer.

Dessa upptäckter kan bidraga till förståelsen av fettmetabolism och utvecklingen av nya specifika läkemedel inom områden som diabetes, fetma och immunförsvar.

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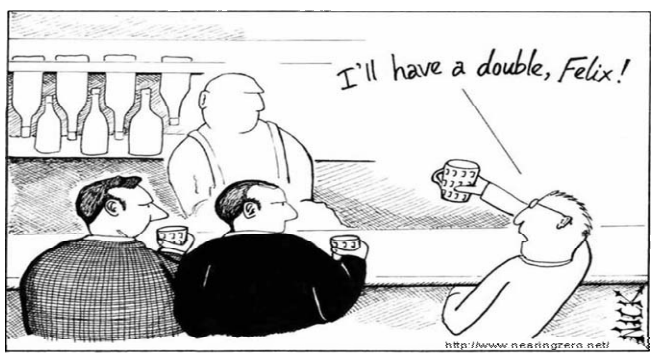
Discoveries require a good social environment (and beer).



Cambridge, 1953. Shortly before discovering the structure of DNA, Watson and Crick, depressed by their lack of progress, visit the local pub.

References

1. J. Bockaert and J. P. Pin. Molecular tinkering of G protein-coupled receptors: an evolutionary success. *Embo J* **18**, 1723-9 (1999).

2. C. I. Bargmann. Neurobiology of the Caenorhabditis elegans genome. *Science* **282**, 2028-33 (1998).

3. M. D. AdamsS. E. CelnikerR. A. HoltC. A. EvansJ. D. Gocayne*, et al.* The genome sequence of Drosophila melanogaster. *Science* **287**, 2185-95 (2000).

4. E. S. LanderL. M. LintonB. BirrenC. NusbaumM. C. Zody*, et al.* Initial sequencing and analysis of the human genome. *Nature* **409**, 860-921 (2001).

5. S. Plakidou-Dymock, D. Dymock and R. Hooley. A higher plant seven-transmembrane receptor that influences sensitivity to cytokinins. *Curr Biol* **8**, 315-24 (1998).

6. H. G. Dohlman, J. Thorner, M. G. Caron and R. J. Lefkowitz. Model systems for the study of seven-transmembrane-segment receptors. *Annu Rev Biochem* **60**, 653-88 (1991).

7. Y. Fang, J. Lahiri and L. Picard. G protein-coupled receptor microarrays for drug discovery. *Drug Discov Today* **8**, 755-61 (2003).

8. P. Ma and R. Zemmel. Value of novelty? *Nat Rev Drug Discov* **1**, 571-2 (2002).

9. K. Palczewski, T. Kumasaka, T. Hori, C. A. Behnke, H. Motoshima*, et al.* Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* **289**, 739-45 (2000).

10. R. J. Lefkowitz. The superfamily of heptahelical receptors. *Nat Cell Biol* **2**, E133-6 (2000).

11. R. Fredriksson, M. C. Lagerstrom, L. G. Lundin and H. B. Schioth. The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Mol Pharmacol* **63**, 1256-72 (2003).

12. M. Sawzdargo, T. Nguyen, D. K. Lee, K. R. Lynch, R. Cheng*, et al.* Identification and cloning of three novel human G protein-coupled receptor genes GPR52, PsiGPR53 and GPR55: GPR55 is extensively expressed in human brain. *Brain Res Mol Brain Res* **64**, 193-8 (1999).

13. D. K. Lee, S. R. George, R. Cheng, T. Nguyen, Y. Liu*, et al.* Identification of four novel human G protein-coupled receptors expressed in the brain. *Brain Res Mol Brain Res* **86**, 13-22 (2001).

14. B. F. O'Dowd, T. Nguyen, A. Marchese, R. Cheng, K. R. Lynch*, et al.* Discovery of three novel G-protein-coupled receptor genes. *Genomics* **47**, 310-3 (1998).

15. M. Sawzdargo, S. R. George, T. Nguyen, S. Xu, L. F. Kolakowski*, et al.* A cluster of four novel human G protein-coupled receptor genes occurring in close proximity to CD22 gene on chromosome 19q13.1. *Biochem Biophys Res Commun* **239**, 543-7 (1997).

16. B. P. Jung, T. Nguyen, L. F. Kolakowski, Jr., K. R. Lynch, H. H. Heng*, et al.* Discovery of a novel human G protein-coupled receptor gene (GPR25) located on chromosome 1. *Biochem Biophys Res Commun* **230**, 69-72 (1997).

17. L. F. Kolakowski, Jr., B. P. Jung, T. Nguyen, M. P. Johnson, K. R. Lynch*, et al.* Characterization of a human gene related to genes encoding somatostatin receptors. *FEBS Lett* **398**, 253-8 (1996).

18. N. E. Nilsson, Y. Tryselius and C. Owman. Genomic organization of the leukotriene B(4) receptor locus of human chromosome 14. *Biochem Biophys Res Commun* **274**, 383-8 (2000).

19. M. Rodbell. The role of hormone receptors and GTP-regulatory proteins in membrane transduction. *Nature* **284**, 17-22 (1980).

20. T. Kenakin. Ligand-selective receptor conformations revisited: the promise and the problem. *Trends Pharmacol Sci* **24**, 346-54 (2003).

21. D. E. Clapham and E. J. Neer. G protein beta gamma subunits. *Annu Rev Pharmacol Toxicol* **37**, 167-203 (1997).

22. S. Offermanns. G-proteins as transducers in transmembrane signalling. *Prog Biophys Mol Biol* **83**, 101-30 (2003).

23. U. Gether. Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. *Endocr Rev* **21**, 90-113 (2000).

24. K. L. Pierce, R. T. Premont and R. J. Lefkowitz. Seven-transmembrane receptors. *Nat Rev Mol Cell Biol* **3**, 639-50 (2002).

25. K. Kiselyov, D. M. Shin and S. Muallem. Signalling specificity in GPCR-dependent Ca2+ signalling. *Cell Signal* **15**, 243-53 (2003).

26. K. Kotarsky, C. Owman and B. Olde. A chimeric reporter gene allowing for clone selection and high-throughput screening of reporter cell lines expressing G-protein-coupled receptors. *Anal Biochem* **288**, 209-15 (2001).

27. K. Kotarsky, L. Antonsson, C. Owman and B. Olde. Optimized reporter gene assays based on a synthetic multifunctional promoter and a secreted luciferase. *Anal Biochem* **316**, 208-15 (2003).

28. V. A. McKusick. HUGO news. The Human Genome Organisation: history, purposes, and membership. *Genomics* **5**, 385-7 (1989).

29. A. Wise, K. Gearing and S. Rees. Target validation of G-protein coupled receptors. *Drug Discov Today* **7**, 235-46 (2002).

30. S. Wilson, D. J. Bergsma, J. K. Chambers, A. I. Muir, K. G. Fantom*, et al.* Orphan G-protein-coupled receptors: the next generation of drug targets? *Br J Pharmacol* **125**, 1387-92 (1998).

31. M. S. Boguski. The turning point in genome research. *Trends Biochem Sci* **20**, 295-6 (1995).

32. S. F. Altschul, W. Gish, W. Miller, E. W. Myers and D. J. Lipman. Basic local alignment search tool. *J Mol Biol* **215**, 403-10 (1990).

33. D. A. Benson, I. Karsch-Mizrachi, D. J. Lipman, J. Ostell and D. L. Wheeler. GenBank. *Nucleic Acids Res* **31**, 23-7 (2003).

34. A. Krogh, B. Larsson, G. von Heijne and E. L. Sonnhammer. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J Mol Biol* **305**, 567-80 (2001).

35. A. Gaulton and T. K. Attwood. Bioinformatics approaches for the classification of G-protein-coupled receptors. *Curr Opin Pharmacol* **3**, 114-20 (2003).

36. K. Kotarsky, N. E. Nilsson, B. Olde and C. Owman. Progress in methodology. Improved reporter gene assays used to identify ligands acting on orphan seven-transmembrane receptors. *Pharmacol Toxicol* **93**, 249-58 (2003).

37. S. J. Hill, J. G. Baker and S. Rees. Reporter-gene systems for the study of G-protein-coupled receptors. *Curr Opin Pharmacol* **1**, 526-32 (2001).

38. P. Borgeat and B. Samuelsson. Transformation of arachidonic acid by rabbit polymorphonuclear leukocytes. Formation of a novel dihydroxyeicosatetraenoic acid. *J Biol Chem* **254**, 2643-6 (1979).

39. S. W. Crooks and R. A. Stockley. Leukotriene B4. *Int J Biochem Cell Biol* **30**, 173-8 (1998).

40. B. Samuelsson, S. E. Dahlen, J. A. Lindgren, C. A. Rouzer and C. N. Serhan. Leukotrienes and lipoxins: structures, biosynthesis, and biological effects. *Science* **237**, 1171-6 (1987).

41. A. W. Ford-Hutchinson, M. A. Bray and M. J. Smith. Lipoxygenase products and the polymorphonuclear leucocyte. *Agents Actions* **10**, 548-50 (1980).

42. M. J. Smith, A. W. Ford-Hutchinson and M. A. Bray. Leukotriene B: a potential mediator of inflammation. *J Pharm Pharmacol* **32**, 517-8 (1980).

43. J. P. van Pelt, E. M. de Jong, P. E. van Erp, M. I. Mitchell, P. Marder*, et al.* The regulation of CD11b integrin levels on human blood leukocytes and leukotriene

B4-stimulated skin by a specific leukotriene B4 receptor antagonist (LY293111). *Biochem Pharmacol* **53**, 1005-12 (1997).

44. H. Sumimoto, K. Takeshige and S. Minakami. Superoxide production of human polymorphonuclear leukocytes stimulated by leukotriene B4. *Biochim Biophys Acta* **803**, 271-7 (1984).

45. P. Mancuso, P. Nana-Sinkam and M. Peters-Golden. Leukotriene B4 augments neutrophil phagocytosis of Klebsiella pneumoniae. *Infect Immun* **69**, 2011-6 (2001).

46. S. A. Rae and M. J. Smith. The stimulation of lysosomal enzyme secretion from human polymorphonuclear leucocytes by leukotriene B4. *J Pharm Pharmacol* **33**, 616-7 (1981).

47. H. Morita, K. Takeda, H. Yagita and K. Okumura. Immunosuppressive effect of leukotriene B(4) receptor antagonist in vitro. *Biochem Biophys Res Commun* **264**, 321-6 (1999).

48. K. A. Yamaoka, H. E. Claesson and A. Rosen. Leukotriene B4 enhances activation, proliferation, and differentiation of human B lymphocytes. *J Immunol* **143**, 1996-2000 (1989).

49. L. L. Espey, N. Tanaka and H. Okamura. Increase in ovarian leukotrienes during hormonally induced ovulation in the rat. *Am J Physiol* **256**, E753-9 (1989).

50. T. Yokomizo, T. Izumi, K. Chang, Y. Takuwa and T. Shimizu. A G-protein-coupled receptor for leukotriene B4 that mediates chemotaxis. *Nature* **387**, 620-4 (1997).

51. C. Owman, A. Sabirsh, A. Boketoft and B. Olde. Leukotriene B4 is the functional ligand binding to and activating the cloned chemoattractant receptor, CMKRL1. *Biochem Biophys Res Commun* **240**, 162-6 (1997).

52. C. Owman, C. Nilsson and S. J. Lolait. Cloning of cDNA encoding a putative chemoattractant receptor. *Genomics* **37**, 187-94 (1996).

53. D. W. Goldman and E. J. Goetzl. Heterogeneity of human polymorphonuclear leukocyte receptors for leukotriene B4. Identification of a subset of high affinity receptors that transduce the chemotactic response. *J Exp Med* **159**, 1027-41 (1984).

54. A. H. Lin, P. L. Ruppel and R. R. Gorman. Leukotriene B4 binding to human neutrophils. *Prostaglandins* **28**, 837-49 (1984).

55. H. J. Showell, E. R. Pettipher, J. B. Cheng, R. Breslow, M. J. Conklyn*, et al.* The in vitro and in vivo pharmacologic activity of the potent and selective leukotriene B4 receptor antagonist CP-105696. *J Pharmacol Exp Ther* **273**, 176-84 (1995).

56. T. Yokomizo, K. Kato, K. Terawaki, T. Izumi and T. Shimizu. A second leukotriene B(4) receptor, BLT2. A new therapeutic target in inflammation and immunological disorders. *J Exp Med* **192**, 421-32 (2000).

57. S. Wang, E. Gustafson, L. Pang, X. Qiao, J. Behan*, et al.* A novel hepatointestinal leukotriene B4 receptor. Cloning and functional characterization. *J Biol Chem* **275**, 40686-94 (2000).

58. C. Brink, S. E. Dahlen, J. Drazen, J. F. Evans, D. W. Hay*, et al.* International Union of Pharmacology XXXVII. Nomenclature for leukotriene and lipoxin receptors. *Pharmacol Rev* **55**, 195-227 (2003).

59. A. M. Tager and A. D. Luster. BLT1 and BLT2: the leukotriene B(4) receptors. *Prostaglandins Leukot Essent Fatty Acids* **69**, 123-34 (2003).

60. R. J. Aiello, P. A. Bourassa, S. Lindsey, W. Weng, A. Freeman*, et al.* Leukotriene B4 receptor antagonism reduces monocytic foam cells in mice. *Arterioscler Thromb Vasc Biol* **22**, 443-9 (2002).

61. F. Tsuji, K. Oki, K. Fujisawa, A. Okahara, M. Horiuchi*, et al.* Involvement of leukotriene B4 in arthritis models. *Life Sci* **64**, PL51-6 (1999).

62. A. Helgadottir, A. Manolescu, G. Thorleifsson, S. Gretarsdottir, H. Jonsdottir*, et al.* The gene encoding 5-lipoxygenase activating protein confers risk of myocardial infarction and stroke. *Nat Genet* (2004).

63. R. H. Unger. Lipotoxic diseases. *Annu Rev Med* **53**, 319-36 (2002).

64. A. C. Guyton and J. E. Hall. *Textbook of medical physiology* (Saunders, Philadelphia, 1996).

65. M. C. Linder. *Nutritional biochemistry and metabolism : with clinical applications* (Elsevier, New York, 1991).

66. K. Kotarsky, N. E. Nilsson, E. Flodgren, C. Owman and B. Olde. A human cell surface receptor activated by free fatty acids and thiazolidinedione drugs. *Biochem Biophys Res Commun* **301**, 406-10 (2003).

67. C. P. Briscoe, M. Tadayyon, J. L. Andrews, W. G. Benson, J. K. Chambers*, et al.* The orphan G protein-coupled receptor GPR40 is activated by medium and long chain fatty acids. *J Biol Chem* **278**, 11303-11 (2003).

68. Y. Itoh, Y. Kawamata, M. Harada, M. Kobayashi, R. Fujii*, et al.* Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40. *Nature* **422**, 173-6 (2003).

69. S. Nielsen, J. O. Jorgensen, T. Hartmund, H. Norrelund, K. S. Nair*, et al.* Effects of lowering circulating free fatty acid levels on protein metabolism in adult growth hormone deficient patients. *Growth Horm IGF Res* **12**, 425-33 (2002).

70. R. L. Dobbins, M. W. Chester, M. B. Daniels, J. D. McGarry and D. T. Stein. Circulating fatty acids are essential for efficient glucose-stimulated insulin secretion after prolonged fasting in humans. *Diabetes* **47**, 1613-8 (1998).

71. E. A. Nunez. Biological complexity is under the 'strange attraction' of non-esterified fatty acids. *Prostaglandins Leukot Essent Fatty Acids* **57**, 107-10 (1997).

72. P. S. Van Dam, H. E. Smid, W. R. de Vries, M. Niesink, E. Bolscher*, et al.* Reduction of free fatty acids by acipimox enhances the growth hormone (GH) responses to GH-releasing peptide 2 in elderly men. *J Clin Endocrinol Metab* **85**, 4706-11 (2000).

73. F. F. Casanueva, L. Villanueva, C. Dieguez, Y. Diaz, J. A. Cabranes*, et al.* Free fatty acids block growth hormone (GH) releasing hormone-stimulated GH secretion in man directly at the pituitary. *J Clin Endocrinol Metab* **65**, 634-42 (1987).

74. R. M. Senaris, M. D. Lewis, F. Lago, F. Dominguez, M. F. Scanlon*, et al.* Effects of free fatty acids on somatostatin secretion, content and mRNA levels in cortical and hypothalamic fetal rat neurones in monolayer culture. *J Mol Endocrinol* **10**, 207-14 (1993).

75. C. Dieguez, E. Carro, L. M. Seoane, M. Garcia, J. P. Camina*, et al.* Regulation of somatotroph cell function by the adipose tissue. *Int J Obes Relat Metab Disord* **24 Suppl 2**, S100-3 (2000).

76. N. Briard, M. Rico-Gomez, V. Guillaume, N. Sauze, V. Vuaroqueaux*, et al.* Hypothalamic mediated action of free fatty acid on growth hormone secretion in sheep. *Endocrinology* **139**, 4811-9 (1998).

77. M. S. Boguski, T. M. Lowe and C. M. Tolstoshev. dbEST--database for "expressed sequence tags". *Nat Genet* **4**, 332-3 (1993).

78. K. Yashiro, S. O. Shin, Y. Sakashita, A. Himejima, A. Mori*, et al.* [Changes in the free fatty acid composition of rat salivary glands induced by chronic administration of isoproterenol]. *Gifu Shika Gakkai Zasshi* **17**, 547-56 (1990).

79. N. Fleming and L. Mellow. Arachidonic acid stimulates intracellular calcium mobilization and regulates protein synthesis, ATP levels, and mucin secretion in submandibular gland cells. *J Dent Res* **74**, 1295-302 (1995).

80. M. C. Neville and M. F. Picciano. Regulation of milk lipid secretion and composition. *Annu Rev Nutr* **17**, 159-83 (1997).

81. C. Ip, M. M. Ip, T. Loftus, S. Shoemaker and W. Shea-Eaton. Induction of apoptosis by conjugated linoleic acid in cultured mammary tumor cells and premalignant lesions of the rat mammary gland. *Cancer Epidemiol Biomarkers Prev* **9**, 689-96 (2000).

82. T. Yonezawa, K. Katoh and Y. Obara. Existence of GPR40 functioning in a human breast cancer cell line, MCF-7. *Biochem Biophys Res Commun* **314**, 805-9 (2004).

83. B. M. Egan. Insulin resistance and the sympathetic nervous system. *Curr Hypertens Rep* **5**, 247-54 (2003).

84. S. C. Hendrickson, J. D. St Louis, J. E. Lowe and S. Abdel-aleem. Free fatty acid metabolism during myocardial ischemia and reperfusion. *Mol Cell Biochem* **166**, 85-94 (1997).

85. S. Renaud and D. Lanzmann-Petithory. Dietary fats and coronary heart disease pathogenesis. *Curr Atheroscler Rep* **4**, 419-24 (2002).

86. G. Wolfram. Dietary fatty acids and coronary heart disease. *Eur J Med Res* **8**, 321-4 (2003).

87. M. Pirro, P. Mauriege, A. Tchernof, B. Cantin, G. R. Dagenais*, et al.* Plasma free fatty acid levels and the risk of ischemic heart disease in men: prospective results from the Quebec Cardiovascular Study. *Atherosclerosis* **160**, 377-84 (2002).

88. J. Svedberg, G. Stromblad, A. Wirth, U. Smith and P. Bjorntorp. Fatty acids in the portal vein of the rat regulate hepatic insulin clearance. *J Clin Invest* **88**, 2054-8. (1991).

89. P. Shah, A. Basu and R. Rizza. Fat-induced liver insulin resistance. *Curr Diab Rep* **3**, 214-8 (2003).

90. G. Boden and G. I. Shulman. Free fatty acids in obesity and type 2 diabetes: defining their role in the development of insulin resistance and beta-cell dysfunction. *Eur J Clin Invest* **32 Suppl 3**, 14-23 (2002).

91. M. D. Jensen. Fate of fatty acids at rest and during exercise: regulatory mechanisms. *Acta Physiol Scand* **178**, 385-90 (2003).

92. G. Boden. Effects of free fatty acids (FFA) on glucose metabolism: significance for insulin resistance and type 2 diabetes. *Exp Clin Endocrinol Diabetes* **111**, 121-4 (2003).

93. J. B. Prins. Adipose tissue as an endocrine organ. *Best Pract Res Clin Endocrinol Metab* **16**, 639-51 (2002).

94. Y. Xiong, N. Miyamoto, K. Shibata, M. A. Valasek, T. Motoike*, et al.* Short-chain fatty acids stimulate leptin production in adipocytes through the G protein-coupled receptor GPR41. *Proc Natl Acad Sci U S A* (2004).

95. B. M. Egan, E. L. Greene and T. L. Goodfriend. Nonesterified fatty acids in blood pressure control and cardiovascular complications. *Curr Hypertens Rep* **3**, 107-16 (2001).

96. A. Fagot-Campagna, B. Balkau, D. Simon, J. M. Warnet, J. R. Claude*, et al.* High free fatty acid concentration: an independent risk factor for hypertension in the Paris Prospective Study. *Int J Epidemiol* **27**, 808-13 (1998).

97. H. O. Steinberg, M. Tarshoby, R. Monestel, G. Hook, J. Cronin*, et al.* Elevated circulating free fatty acid levels impair endothelium-dependent vasodilation. *J Clin Invest* **100**, 1230-9 (1997).

98. E. P. Haber, H. M. Ximenes, J. Procopio, C. R. Carvalho, R. Curi*, et al.* Pleiotropic effects of fatty acids on pancreatic beta-cells. *J Cell Physiol* **194**, 1-12 (2003).

99. S. M. Parker, P. C. Moore, L. M. Johnson and V. Poitout. Palmitate potentiation of glucose-induced insulin release: a study using 2-bromopalmitate. *Metabolism* **52**, 1367-71 (2003).

100. G. Paolisso, A. Gambardella, L. Amato, R. Tortoriello, A. D'Amore*, et al.* Opposite effects of short- and long-term fatty acid infusion on insulin secretion in healthy subjects. *Diabetologia* **38**, 1295-9 (1995).

101. L. C. Bollheimer, R. H. Skelly, M. W. Chester, J. D. McGarry and C. J. Rhodes. Chronic exposure to free fatty acid reduces pancreatic beta cell insulin content by increasing basal insulin secretion that is not compensated for by a corresponding increase in proinsulin biosynthesis translation. *J Clin Invest* **101**, 1094-101 (1998).

102. T. A. Buchanan. Pancreatic beta-cell loss and preservation in type 2 diabetes. *Clin Ther* **25 Suppl B**, B32-46 (2003).

103. E. C. Opara, V. S. Hubbard, W. M. Burch and O. E. Akwari. Characterization of the insulinotropic potency of polyunsaturated fatty acids. *Endocrinology* **130**, 657-62 (1992).

104. G. Boden, X. Chen and N. Iqbal. Acute lowering of plasma fatty acids lowers basal insulin secretion in diabetic and nondiabetic subjects. *Diabetes* **47**, 1609-12 (1998).

105. S. Kashyap, R. Belfort, A. Gastaldelli, T. Pratipanawatr, R. Berria*, et al.* A sustained increase in plasma free fatty acids impairs insulin secretion in nondiabetic subjects genetically predisposed to develop type 2 diabetes. *Diabetes* **52**, 2461-74 (2003).

106. I. Rustenbeck. Desensitization of insulin secretion. *Biochem Pharmacol* **63**, 1921-35 (2002).

107. G. A. Bray. Risks of obesity. *Endocrinol Metab Clin North Am* **32**, 787-804, viii (2003).

108. A. Steinmetz. Treatment of diabetic dyslipoproteinemia. *Exp Clin Endocrinol Diabetes* **111**, 239-45 (2003).

109. The World Health Organization. *http://www.who.int*.

110. M. Thearle and L. J. Aronne. Obesity and pharmacologic therapy. *Endocrinol Metab Clin North Am* **32**, 1005-24 (2003).

111. National Institute for Diabetes and Digestive and Kidnery Diseases. *http://www.niddk.nih.gov*.

112. International Association for the Study of Obesity. *http://www.iotf.org*.

113. R. J. Harris. Nutrition in the 21st century: what is going wrong. *Arch Dis Child* **89**, 154-8 (2004).

114. C. S. Yajnik. Early life origins of insulin resistance and type 2 diabetes in India and other Asian countries. *J Nutr* **134**, 205-10 (2004).

115. J. P. Felber and A. Golay. Pathways from obesity to diabetes. *Int J Obes Relat Metab Disord* **26 Suppl 2**, S39-45 (2002).

116. R. N. Bergman and M. Ader. Free fatty acids and pathogenesis of type 2 diabetes mellitus. *Trends Endocrinol Metab* **11**, 351-6 (2000).

117. P. Arner. The adipocyte in insulin resistance: key molecules and the impact of the thiazolidinediones. *Trends Endocrinol Metab* **14**, 137-45 (2003).

118. M. Gottlicher, E. Widmark, Q. Li and J. A. Gustafsson. Fatty acids activate a chimera of the clofibric acid-activated receptor and the glucocorticoid receptor. *Proc Natl Acad Sci U S A* **89**, 4653-7 (1992).

119. S. Kersten, B. Desvergne and W. Wahli. Roles of PPARs in health and disease. *Nature* **405**, 421-4 (2000).

120. J. Berger and D. E. Moller. The mechanisms of action of PPARs. *Annu Rev Med* **53**, 409-35 (2002).

121. J. M. Lehmann, L. B. Moore, T. A. Smith-Oliver, W. O. Wilkison, T. M. Willson*, et al.* An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma). *J Biol Chem* **270**, 12953-6 (1995).

122. J. Auwerx. PPARgamma, the ultimate thrifty gene. *Diabetologia* **42**, 1033-49 (1999).

123. H. E. Xu, M. H. Lambert, V. G. Montana, D. J. Parks, S. G. Blanchard*, et al.* Molecular recognition of fatty acids by peroxisome proliferator-activated receptors. *Mol Cell* **3**, 397-403 (1999).

124. B. M. Forman, J. Chen and R. M. Evans. Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta. *Proc Natl Acad Sci U S A* **94**, 4312-7 (1997).

125. B. Desvergne and W. Wahli. Peroxisome proliferator-activated receptors: nuclear control of metabolism. *Endocr Rev* **20**, 649-88 (1999).

126. T. Fujita, Y. Sugiyama, S. Taketomi, T. Sohda, Y. Kawamatsu*, et al.* Reduction of insulin resistance in obese and/or diabetic animals by 5-[4-(1-methylcyclohexylmethoxy)benzyl]-thiazolidine-2,4-dione (ADD-3878, U-63,287, ciglitazone), a new antidiabetic agent. *Diabetes* **32**, 804-10 (1983).

127. A. R. Diani, T. Peterson, G. A. Sawada, B. M. Wyse, B. J. Gilchrist*, et al.* Ciglitazone, a new hypoglycaemic agent. 4. Effect on pancreatic islets of C57BL/6J-ob/ob and C57BL/KsJ-db/db mice. *Diabetologia* **27**, 225-34 (1984).

128. T. Yoshioka, T. Fujita, T. Kanai, Y. Aizawa, T. Kurumada*, et al.* Studies on hindered phenols and analogues. 1. Hypolipidemic and hypoglycemic agents with ability to inhibit lipid peroxidation. *J Med Chem* **32**, 421-8 (1989).

129. T. Sohda, Y. Momose, K. Meguro, Y. Kawamatsu, Y. Sugiyama*, et al.* Studies on antidiabetic agents. Synthesis and hypoglycemic activity of 5-[4-(pyridylalkoxy)benzyl]-2,4-thiazolidinediones. *Arzneimittelforschung* **40**, 37-42 (1990).

130. B. C. Cantello, M. A. Cawthorne, G. P. Cottam, P. T. Duff, D. Haigh*, et al.* [[omega-(Heterocyclylamino)alkoxy]benzyl]-2,4-thiazolidinediones as potent antihyperglycemic agents. *J Med Chem* **37**, 3977-85 (1994).

131. E. J. Murphy, T. J. Davern, A. O. Shakil, L. Shick, U. Masharani*, et al.* Troglitazone-induced fulminant hepatic failure. Acute Liver Failure Study Group. *Dig Dis Sci* **45**, 549-53 (2000).

132. P. J. Cox, D. A. Ryan, F. J. Hollis, A. M. Harris, A. K. Miller*, et al.* Absorption, disposition, and metabolism of rosiglitazone, a potent thiazolidinedione insulin sensitizer, in humans. *Drug Metab Dispos* **28**, 772-80 (2000).

133. Y. Hattori, S. Hattori and K. Kasai. Troglitazone upregulates nitric oxide synthesis in vascular smooth muscle cells. *Hypertension* **33**, 943-8 (1999).

134. M. Wang, S. C. Wise, T. Leff and T. Z. Su. Troglitazone, an antidiabetic agent, inhibits cholesterol biosynthesis through a mechanism independent of peroxisome proliferator-activated receptor-gamma. *Diabetes* **48**, 254-60 (1999).

135. S. N. Wu, L. L. Ho, H. F. Li and H. T. Chiang. Regulation of Ca(2+)-activated K+ currents by ciglitazone in rat pituitary GH3 cells. *J Investig Med* **48**, 259-69 (2000).

136. B. Brunmair, F. Gras, S. Neschen, M. Roden, L. Wagner*, et al.* Direct thiazolidinedione action on isolated rat skeletal muscle fuel handling is independent of peroxisome proliferator-activated receptor-gamma-mediated changes in gene expression. *Diabetes* **50**, 2309-15 (2001).

137. I. Gouni-Berthold, H. K. Berthold, A. A. Weber, Y. Ko, C. Seul*, et al.* Troglitazone and rosiglitazone induce apoptosis of vascular smooth muscle cells through an extracellular signal-regulated kinase-independent pathway. *Naunyn Schmiedebergs Arch Pharmacol* **363**, 215-21 (2001).

138. C. Furnsinn and W. Waldhausl. Thiazolidinediones: metabolic actions in vitro. *Diabetologia* **45**, 1211-23 (2002).

139. J. Bar-Tana, G. Rose-Kahn and M. Srebnik. Inhibition of lipid synthesis by beta beta'-tetramethyl-substituted, C14-C22, alpha, omega-dicarboxylic acids in the rat in vivo. *J Biol Chem* **260**, 8404-10 (1985).

140. N. Mayorek, B. Kalderon, E. Itach and J. Bar-Tana. Sensitization to insulin induced by beta,beta'-methyl-substituted hexadecanedioic acid (MEDICA 16) in obese Zucker rats in vivo. *Diabetes* **46**, 1958-64 (1997).

141. S. A. Kliewer, J. M. Lenhard, T. M. Willson, I. Patel, D. C. Morris*, et al.* A prostaglandin J2 metabolite binds peroxisome proliferator-activated receptor gamma and promotes adipocyte differentiation. *Cell* **83**, 813-9 (1995).

142. J. M. Lehmann, J. M. Lenhard, B. B. Oliver, G. M. Ringold and S. A. Kliewer. Peroxisome proliferator-activated receptors alpha and gamma are activated by indomethacin and other non-steroidal anti-inflammatory drugs. *J Biol Chem* **272**, 3406-10 (1997).

143. D. E. Moller. New drug targets for type 2 diabetes and the metabolic syndrome. *Nature* **414**, 821-7 (2001).

144. T. M. Larsen, S. Toubro and A. Astrup. PPARgamma agonists in the treatment of type II diabetes: is increased fatness commensurate with long-term efficacy? *Int J Obes Relat Metab Disord* **27**, 147-61 (2003).

145. L. Chao, B. Marcus-Samuels, M. M. Mason, J. Moitra, C. Vinson*, et al.* Adipose tissue is required for the antidiabetic, but not for the hypolipidemic, effect of thiazolidinediones. *J Clin Invest* **106**, 1221-8 (2000).

146. C. F. Burant, S. Sreenan, K. Hirano, T. A. Tai, J. Lohmiller*, et al.* Troglitazone action is independent of adipose tissue. *J Clin Invest* **100**, 2900-8 (1997).

147. Y. Fukui, S. Masui, S. Osada, K. Umesono and K. Motojima. A new thiazolidinedione, NC-2100, which is a weak PPAR-gamma activator, exhibits potent antidiabetic effects and induces uncoupling protein 1 in white adipose tissue of KKAy obese mice. *Diabetes* **49**, 759-67 (2000).

148. S. Rocchi, F. Picard, J. Vamecq, L. Gelman, N. Potier*, et al.* A unique PPARgamma ligand with potent insulin-sensitizing yet weak adipogenic activity. *Mol Cell* **8**, 737-47 (2001).

149. P. Misra, R. Chakrabarti, R. K. Vikramadithyan, G. Bolusu, S. Juluri*, et al.* PAT5A: a partial agonist of peroxisome proliferator-activated receptor gamma is a potent antidiabetic thiazolidinedione yet weakly adipogenic. *J Pharmacol Exp Ther* **306**, 763-71 (2003).

150. D. Dey, S. Medicherla, P. Neogi, M. Gowri, J. Cheng*, et al.* A novel peroxisome proliferator-activated gamma (PPAR gamma) agonist, CLX-0921, has potent antihyperglycemic activity with low adipogenic potential. *Metabolism* **52**, 1012-8 (2003).

151. C. Laurent, C. Simoneau, L. Marks, S. Braschi, M. Champ*, et al.* Effect of acetate and propionate on fasting hepatic glucose production in humans. *Eur J Clin Nutr* **49**, 484-91 (1995).

152. C. Eftimiadi, E. Buzzi, M. Tonetti, P. Buffa, D. Buffa*, et al.* Short-chain fatty acids produced by anaerobic bacteria alter the physiological responses of human neutrophils to chemotactic peptide. *J Infect* **14**, 43-53 (1987).

153. M. Wajner, K. D. Santos, J. L. Schlottfeldt, M. P. Rocha and C. M. Wannmacher. Inhibition of mitogen-activated proliferation of human peripheral lymphocytes in vitro by propionic acid. *Clin Sci (Lond)* **96**, 99-103 (1999).

154. B. A. Brunkhorst, E. Kraus, M. Coppi, M. Budnick and R. Niederman. Propionate induces polymorphonuclear leukocyte activation and inhibits formylmethionyl-leucyl-phenylalanine-stimulated activation. *Infect Immun* **60**, 2957-68 (1992).

155. H. W. Stehle, B. Leblebicioglu and J. D. Walters. Short-chain carboxylic acids produced by gram-negative anaerobic bacteria can accelerate or delay polymorphonuclear leukocyte apoptosis in vitro. *J Periodontol* **72**, 1059-63 (2001).

156. C. R. Cavaglieri, A. Nishiyama, L. C. Fernandes, R. Curi, E. A. Miles*, et al.* Differential effects of short-chain fatty acids on proliferation and production of pro- and anti-inflammatory cytokines by cultured lymphocytes. *Life Sci* **73**, 1683-90 (2003).

157. P. H. Naccache, N. Faucher, A. C. Caon and S. R. McColl. Propionic acid-induced calcium mobilization in human neutrophils. *J Cell Physiol* **136**, 118-24 (1988).

158. R. I. Fonteriz, A. Sanchez, F. Mollinedo, D. Collado-Escobar and J. Garcia-Sancho. The role of intracellular acidification in calcium mobilization in human neutrophils. *Biochim Biophys Acta* **1093**, 1-6 (1991).

159. N. Faucher and P. H. Naccache. Relationship between pH, sodium, and shape changes in chemotactic-factor-stimulated human neutrophils. *J Cell Physiol* **132**, 483-91 (1987).

160. C. Eftimiadi, M. Tonetti, A. Cavallero, O. Sacco and G. A. Rossi. Short-chain fatty acids produced by anaerobic bacteria inhibit phagocytosis by human lung phagocytes. *J Infect Dis* **161**, 138-42 (1990).

161. O. D. Rotstein. Interactions between leukocytes and anaerobic bacteria in polymicrobial surgical infections. *Clin Infect Dis* **16 Suppl 4**, S190-4 (1993).

162. R. Niederman, J. Zhang and S. Kashket. Short-chain carboxylic-acid-stimulated, PMN-mediated gingival inflammation. *Crit Rev Oral Biol Med* **8**, 269-90 (1997).

163. M. D. Saemann, G. A. Bohmig and G. J. Zlabinger. Short-chain fatty acids: bacterial mediators of a balanced host-microbial relationship in the human gut. *Wien Klin Wochenschr* **114**, 289-300 (2002).

164. R. Niederman, Y. Buyle-Bodin, B. Y. Lu, C. Naleway, P. Robinson*, et al.* The relationship of gingival crevicular fluid short chain carboxylic acid concentration to gingival inflammation. *J Clin Periodontol* **23**, 743-9 (1996).

165. L. Biancone, I. Monteleone, G. Del Vecchio Blanco, P. Vavassori and F. Pallone. Resident bacterial flora and immune system. *Dig Liver Dis* **34 Suppl 2**, S37-43 (2002).

166. P. B. Mortensen and M. R. Clausen. Short-chain fatty acids in the human colon: relation to gastrointestinal health and disease. *Scand J Gastroenterol Suppl* **216**, 132-48 (1996).

167. A. J. Brown, S. M. Goldsworthy, A. A. Barnes, M. M. Eilert, L. Tcheang*, et al.* The Orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. *J Biol Chem* **278**, 11312-9 (2003).

168. E. Le Poul, C. Loison, S. Struyf, J. Y. Springael, V. Lannoy*, et al.* Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. *J Biol Chem* **278**, 25481-9 (2003).

169. S. K. Ahuja, T. Ozcelik, A. Milatovitch, U. Francke and P. M. Murphy. Molecular evolution of the human interleukin-8 receptor gene cluster. *Nat Genet* **2**, 31-6 (1992).

170. Y. Le, P. M. Murphy and J. M. Wang. Formyl-peptide receptors revisited. *Trends Immunol* **23**, 541-8 (2002).

171. R. S. Ahima, D. Prabakaran, C. Mantzoros, D. Qu, B. Lowell*, et al.* Role of leptin in the neuroendocrine response to fasting. *Nature* **382**, 250-2 (1996).

172. S. Forbes, S. Bui, B. R. Robinson, U. Hochgeschwender and M. B. Brennan. Integrated control of appetite and fat metabolism by the leptin-proopiomelanocortin pathway. *Proc Natl Acad Sci U S A* **98**, 4233-7 (2001).

Appendix 1

Various ligands acting on PPARs and FFA_1R [67, 68, 123-125], [66] (Paper II). Worthy of note is the discrepancy between PPARs and FFA_1R when subjected to the medium-chain FFAs (capric acid and lauric acid) and the ability of the synthetic anti-diabetic compounds, MEDICA16, Ciglitazone, Rosiglitazone and MCC-555, to activate both PPAR-γ and FFA_1R. To completely illustrate the family of FFA-receptors and the spectra of activating ligands, the data regarding FFA_2R and FFA_3R from Papers III and IV, as well as other reports [167, 168], has been included in this table. (+) represents an active ligand, (-) a tested but inactive ligand and an empty field indicates a lack of information.

Ligand	PPAR-α	PPAR-δ	PPAR-γ	FFA_1R	FFA_2R	FFA_3R
SCFAs						
Formiate (C1)					+	-
Acetate (C2:0)				-	+	+
Propionate (C3:0)				-	+	+
Butyrate (C4:0)				-	+	+
Capric acid (C6:0)				-	-	+
Caprylic acid (C8:0)	-	-	-	-	-	-
Medium/Long saturated FFAs						
Capric acid (C10:0)	-	-	-	+	-	-
Lauric acid (C12:0)	-	-	-	+		
Myrisitc acid (C14:0)	+	+	-	+		
Palmitic acid (C16:0)	+	+	-	+		
Unsaturated FFAs						
Oleic acid (C18:1)	+	+	+	+		
Linoleic acid (C18:2)	+	+	+	+	-	-
Arachidonic acid (C20:4)	+	+	+	+		
Other endogenous ligands						
Prostaglandin A_1	+	+	+			
Prostaglandin J_2	+		+			
15-deoxy-$\Delta^{12,14}$-Prostaglandin J_2	-	-	+	-		
9-hydroxyoctadenoic acid	-	-	+	+		
Leukotriene B_4	+			-		
Synthetic ligands						
Indomethacin			+	-		
MEDICA 16	-	-	+	+	-	-
Ciglitazone	-	-	+	+	-	-
Rosiglitazone	-	-	+	+	-	-
MCC-555	-	-	+	+		