Discovery of Novel Receptors for Lipid Mediators

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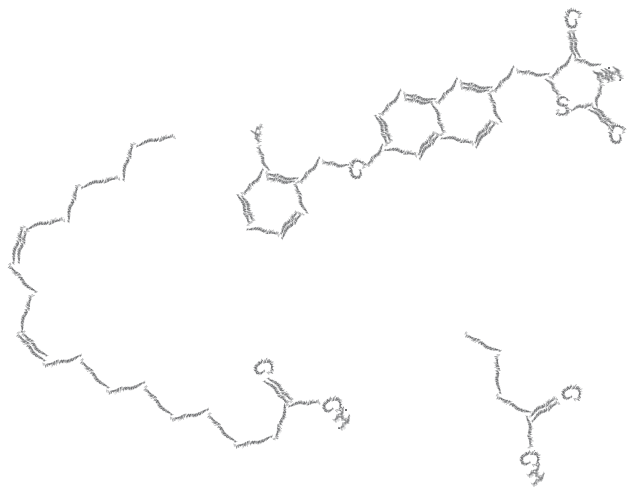

An academic dissertation regarding the

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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With the approval of the Faculty of Medicine, Lund University, to be presented for public examination at the BioMedical Center (BMC), GK-salen, April 23, 2004, at 13:15.

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Title and subtitle: **Discovery of Novel Receptors for Lipid Mediators** –
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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.

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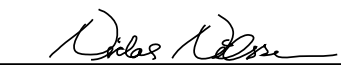
Signature:  Date: March 8, 2004

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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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.

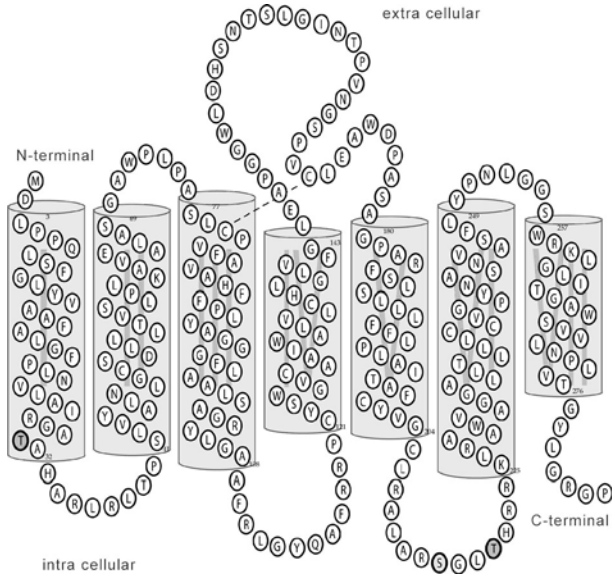


Figure 2. A GPCR, also known as a 7TM receptor, has seven α-helices that traverse the cell membrane. Here is an example of the human FFA_1R, the amino acid sequence showing unusually short N- and C-terminals. The FFA_1R sequence was aligned with that of the rhodopsin receptor, the only known crystallized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

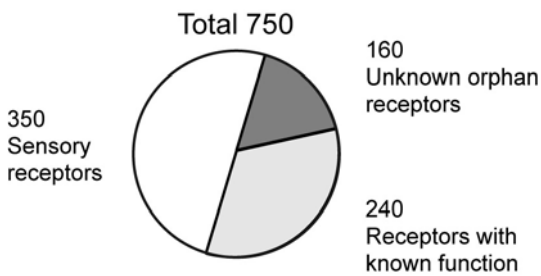


Figure 3. Pie chart representing all known GPCRs and the 160 orphan receptors in need of characterization (of which 110 are of Rhodopsin type). Of the 240 non-sensory receptors with known ligands/function it is remarkable that only about 30 are targets for today's successful drugs.

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.

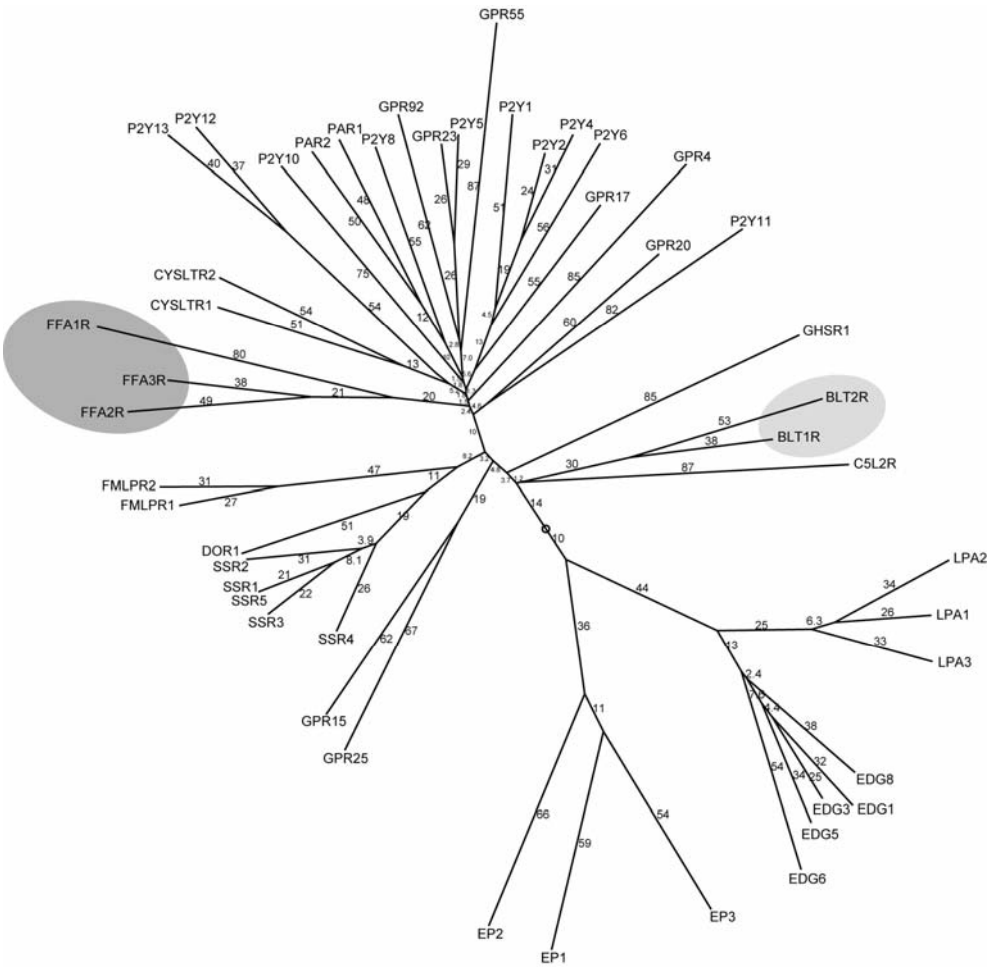


Figure 4. Phylogenetic illustration of a few selected receptors using the online resource, *AllAll's* PhyloTree function, at http://cbrg.inf.ethz.ch/Server/index.html. The dark grey circle highlights the sub-family of FFA receptors (paper II-IV), while the light grey points out the two LTB_4-activated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

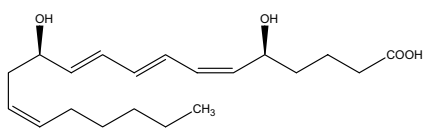


Figure 5. The structure of LTB_4.

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.

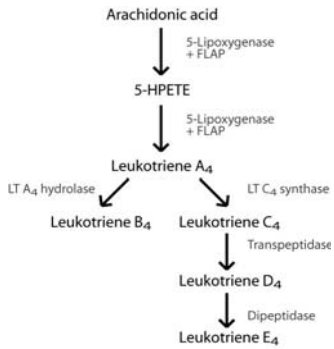


Figure 6. The leukotriene synthesis pathway, with arachidonic acid as the source. FLAP = 5-lipoxygenase activating protein. LTB_4 is mainly responsible for chemotaxis and leukocyte activation and activates two known GPCRs, BLT_1 and BLT_2 (as described in Paper I).

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.

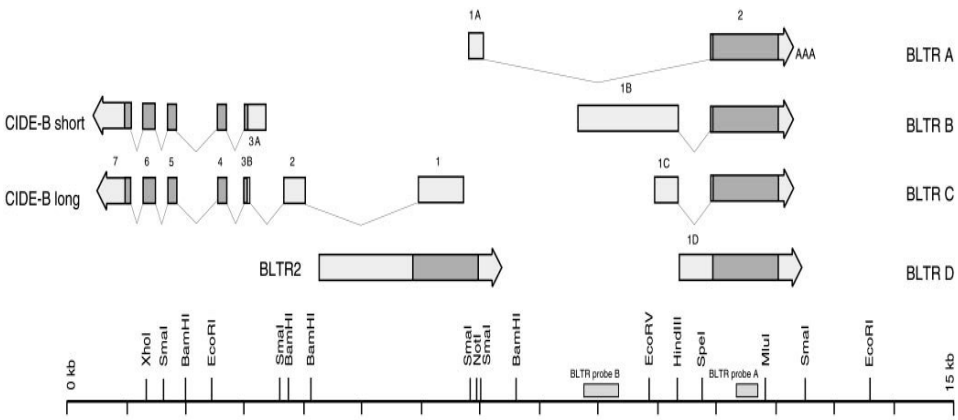


Figure 7. The organization of the *Blt1*/*Blt2* gene cluster indicating a highly complex and dense chromosomal region on 14q11.2-q12. Figure adapted from [18].

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].

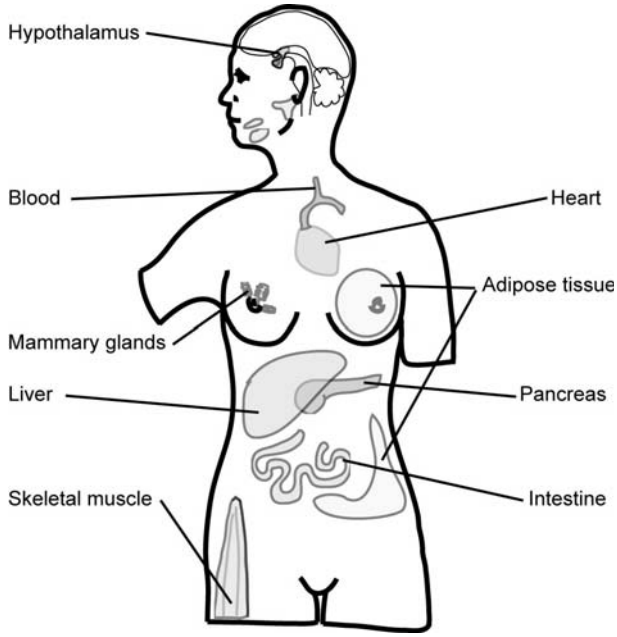


Figure 8. An illustration of the human body identifying some organs and tissues related to fatty acid signalling.

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.

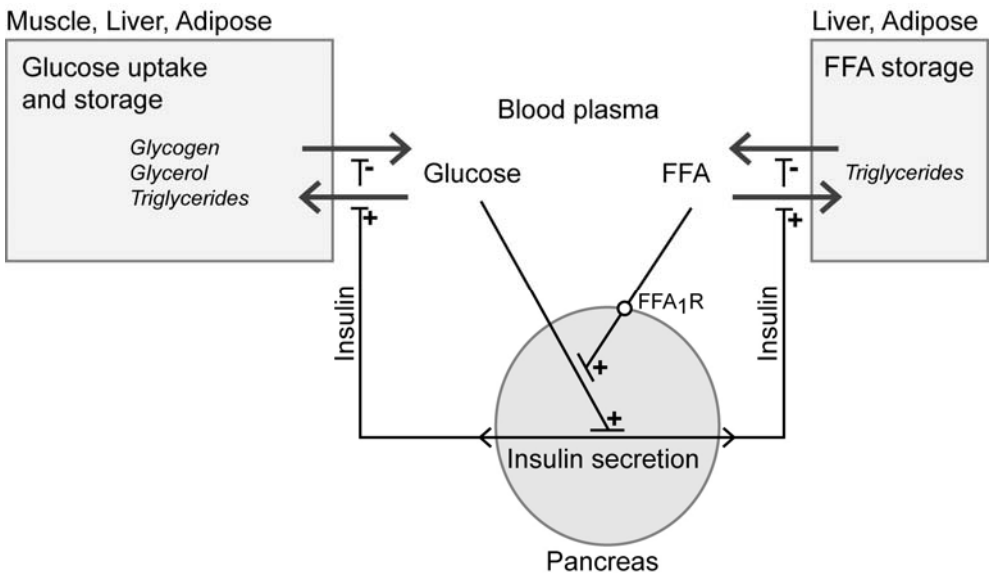


Figure 9. A simplified schematic of insulin's effect on glucose and FFA utilization. The pancreas regulates insulin secretion mainly by sensing the levels of glucose in blood plasma. This glucose-stimulated insulin secretion can be enhanced by FFAs. The newly discovered molecular mechanism, which acts 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

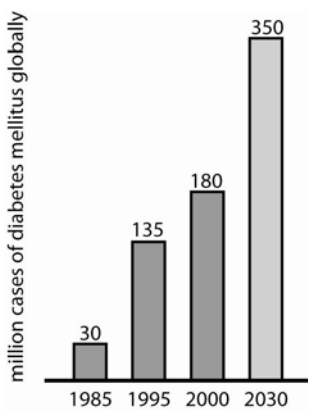


Figure 10. Number of people diagnosed with diabetes type 2 worldwide. The year 2030 estimate is based on current development trends. Data from the World Health Organization.

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].

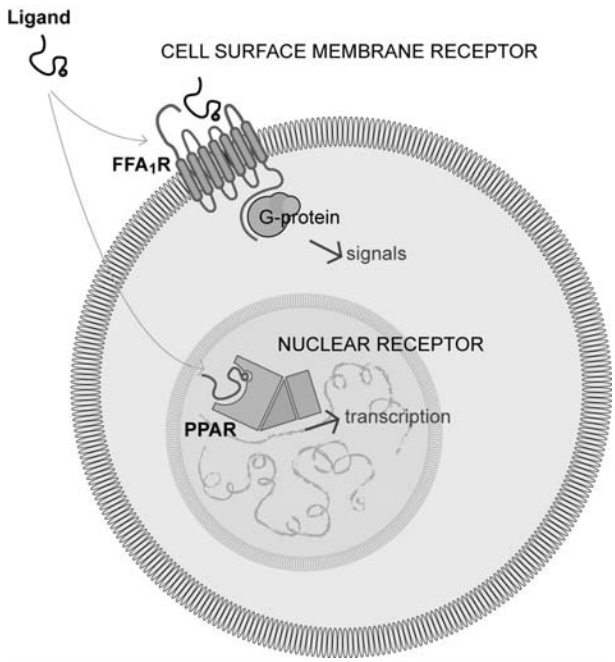


Figure 11. A cell displaying the two known forms of receptors for FFAs. PPAR is a transcription-regulating nuclear receptor activated by e.g. FFAs, prostaglandins, leukotrienes and TZDs. FFA_1R is the novel GPCR expressed on the cell surface and shares some ligands with PPARs, such as FFAs and TZDs.

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.

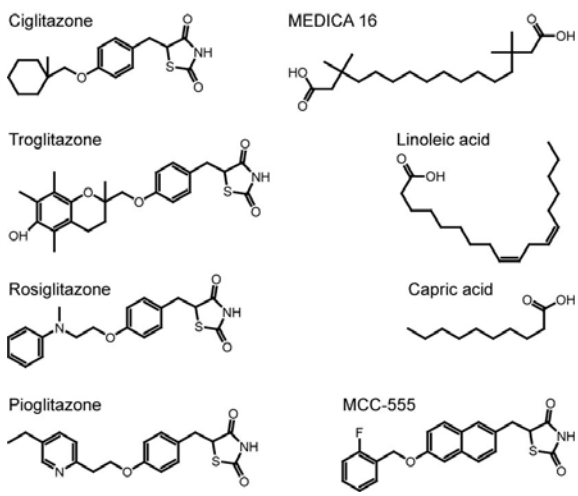


Figure 12. Two natural ligands for FFA_1R: linoleic acid (C18:2) and capric acid (C10:0). Representatives from the TZD group are Ciglitazone, Troglitazone, MCC-555, Rosiglitazone and Pioglitazone. The latter two are used clinically as anti-diabetic drugs. MEDICA 16 is an experimental anti-diabetic compound.

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.

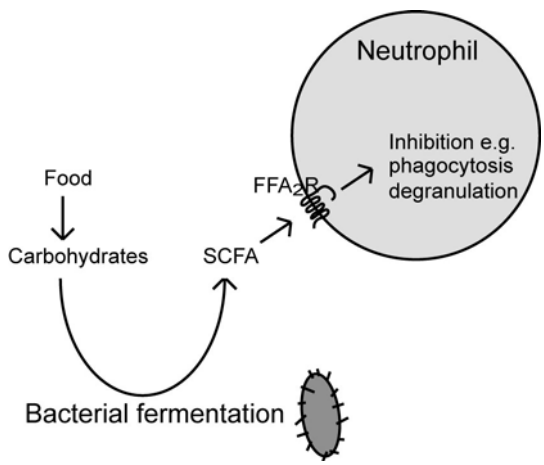


Figure 13. An illustration of the SCFA-induced regulation of the intestinal immune system. SCFAs are produced by bacterial fermentation and act as an indicator of the desired bacterial population.

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].

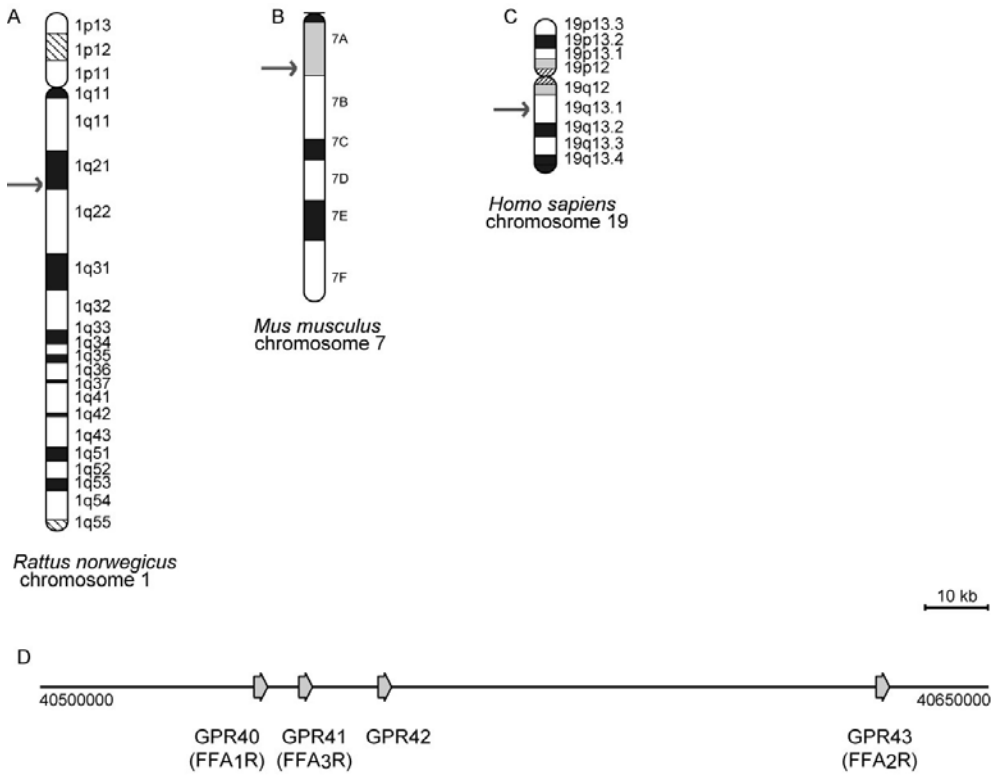


Figure 14. A) Rat, B) mouse and C) human chromosomes containing the DNA encoding of FFA-activated receptors. The arrows indicate the chromosomal position of the FFA-receptor gene cluster. D) 150 kb (thousand base pairs) part of the human chromosomal position at 19q13.1 (section 40,500 kb to 40,650 kb),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

Conclusions

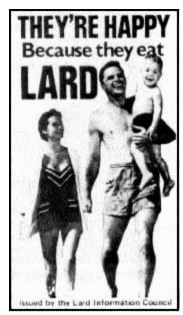
GPCRs are a large and important super-family of proteins involved in the regulation of physiological machinery. Approximately 800 GPCRs are known to exist; however, a large fraction of these (160) needs to be functionally identified as their activating ligands are unknown and they lack a physiological 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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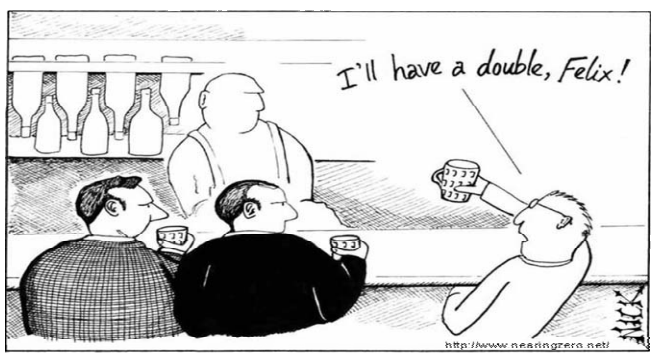
Discoveries require a good social environment (and beer).



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

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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	-	-	+	+		

Appendix 2 (Papers I – IV)

To boldly go where no one has gone before.

From *Star Trek*,
created by Gene Roddenberry

Paper I

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Cloning and Characterization of cDNA Encoding a Novel Human Leukotriene B_4 Receptor

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By homology screening using BLAST searches of expressed sequence tags (ESTs), we have found a previously unidentified cDNA encoding a putative seven-transmembrane receptor with highest similarity to the leukotriene B_4 receptor, BLTR. Analysis of calcium flow in transfected cells, along with sequence analysis, revealed that the EST encoded a functionally inactive protein, lacking the segment corresponding to the C-terminal part of the putative receptor protein. The missing segment was obtained by PCR amplification of a human leukocyte cDNA library and ligated to the truncated EST cDNA. The novel cDNA encodes a full-length receptor with 39% identity to the previously cloned BLTR. Studies of intracellular calcium flow of transfected HeLa cells exposed to various leukotrienes showed that also the novel BLTR-like receptor can be activated by leukotriene B_4, and it is therefore tentatively named BLTR2.

Key Words: **leukotriene B_4; BLTR; BLTR2; calcium assay; 7TM receptor.**

The leukotrienes (LTs) were discovered in the late seventies and shown to comprise a family of widely distributed and biologically highly active inflammatory mediators originating from arachidonic acid through the 5-lipoxygenase pathway (1). Their action is mediated through several pharmacologically defined membrane receptors that are classified into three separate groups based on the binding and actions of LTB_4, LTC_4, LTD_4 and LTE_4, alone or in combination with several antagonists (2). There is pharmacological evidence, however, for one or more additional classes of receptors (3). Not until 1996 was the first LT receptor cDNA cloned and characterised (4), and it was subsequently found to encode the LTB_4 receptor, BLTR (5, 6). It is a member of the leukocyte chemoattractant receptors (7) which belong to the superfamily of seven-transmembrane (7TM or, heptahelix) G-protein-coupled receptors (8). The chemoattractant function in the inflammatory response distinguishes LTB_4 from the other (cystein-containing) leukotrienes, whose main action is exerted in smooth musculature, vascular permeability and epithelial secretion (1). The cysteinyl leukotrienes have thus, for example, been implicated as important mediators in the pathophysiololgy of asthma (9). Interestingly, the human BLTR gene is located in chromosome 14 at q11.2–12 (4), close to one of the regions (defined as 14q11.2–13) reported to be linked to asthma-associated phenotypes (10).

The present paper describes the molecular cloning and functional characterization of a novel cDNA which encodes a putative 7TM receptor, having 39% peptide sequence identity with BLTR. The receptor can be activated by LTB_4, and the name proposed for the novel receptor is, hence, BLTR2. The two receptors show a limited overlap in the DNA sequence, meaning that also the BLTR2 gene is situated close to the "asthma locus" of chromosome 14.

MATERIALS AND METHODS

Identification and cloning of cDNA. An EST (Expressed Sequence Tag) clone, 444-J19 (IMAGE ID 223218), was identified by comparing the previously cloned BLTR receptor (4) to the NCBI EST database using searches with the BLAST algorithm (11). The image clone, an almost 2.7 kb long insert in a pT7T3D-Pac vector, was obtained from the UK HGMP Resource Centre at Hinxton, Cambridge, England. The insert was cleaved out with *Xho*I and *Not*I, purified on agarose gel and ligated to a *Not* I-digested mammalian expression vector, pcDNA3 (Invitrogen). The EST insert encodes a truncated, functionally inactive (data not shown), version of a putative heptahelix receptor. The missing C-terminal part of the gene was amplified by the polymerase chain reaction (PCR) using *Pwo* polymerase (Boehringer-Mannheim) and cDNA from a human leukocyte library (HL4050AH, Clontech) as template. The following two primers were used: 5′-TTCGTGCTTCCTTTCGGGCTGAT-3′ and 5′-AGTCTAGAGGGTCTGCTGTCAAAGG-3′.

The PCR product was ligated into the TA-cloning vector, pCR 2.1, from Invitrogen. A *Not*I/*Xba*I fragment from this product, containing the desired C-terminal part of the receptor, was cleaved out, purified on agarose gel, and subsequently ligated to the same restriction sites

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in the truncated pcDNA3 receptor clone to yield a complete coding sequence.

Exodeletions, subcloning, and sequencing. The complete pcDNA3 receptor construct was cleaved with *Bam*HI and *Kpn*I. Exodeletions were made from the linearised DNA using the Erase-a-Base System (Promega). DNA from individual exodeletion clones were prepared according to the alkaline lysis method (12). The DNA obtained was first treated with RNaseA (12) before being purified using the QIAquick PCR Purification kit from Qiagen. Sequencing was performed using the Big Dye Terminator Cycle Sequencing kit (PE Applied Biosystems). SP6 and T7 primers were used to sequence the exodeletions and full-length pcDNA3 clones. The 1.3 kb long *Apa*I fragment from the full pcDNA3 clone was subcloned into an *Apa*I site in pBluescript SK(+) from Stratagene, and sequenced using T3 and T7 primers.

Phylogenetic analysis. Amino acid sequences of related human G-protein coupled receptors and other known leukotriene receptors of different species were obtained from GenBank using BLAST and aligned using ClustalX version 1.8. The maximum parsimony (PROTPARS) method was applied to the alignment using the PHYLIP package to produce the phylogenetic tree (with bootstrap resampling of 100 subreplicates for statistical significance evaluation).

Cell transfection. HeLa cells were seeded in 6-well plates and incubated in DMEM medium (Gibco/BRL) with 0.5% penicillin, 0.5% streptomycin, 10% FBS and 1% Glutamax at 37°C. After 2–3 days the 60–80% confluent cells were transfected using Lipofectamine Plus (Gibco/BRL). DNA was prepared with the DNA Midiprep kit (Genomed). Full-length receptor plasmid, pcDNA3 (0.9 μg), and 0.4 μg of the photoprotein plasmid, pCDM.AEQ (a kind gift from Dr. Don Button, Stanford University School of Medicine, CA) (13), were mixed with 100 μl serum-free medium (OptiMEM, Gibco/BRL containing 0.5% penicillin, 0.5% streptomycin, 1% Glutamax) and 5 μl of Plus-reagent (Gibco/BRL) in an Eppendorf tube. In a second Eppendorf tube, 100 μl of the same serum-free medium and 9 μl of Lipofectamine (Gibco/BRL) were mixed. The two tubes were incubated at room temperature for 15 min and subsequently mixed, 800 μl serum-free medium was added, and the mixture was pipetted into the wells with HeLa cells that previously had been washed with serum-free medium. The cells were incubated with the DNA mixture at 37°C for 5.5 to 6 h. Half of the transfection solution was then removed and 3 ml of medium was added to each well (DMEM with 0.5% penicillin, 0.5% streptomycin, 10% FBS, 1% Glutamax). After 2 days of incubation at 37°C the cell medium was removed and the cells were washed with 2 ml PBS.

Cellular calcium measurements. Directly after transfection the cells were incubated for 4 h with 2 ml of a coelentrazine solution (DMEM with 0.5% penicillin, 0.5% streptomycin, 0.1% FBS, 1% Glutamax, 5 μM coelentrazine) at 37°C in the dark. After removal of the coelentrazine solution the cells were resuspended by treatment with PBS Dulbecco's medium without calcium and magnesium, with 2 mM EDTA, for 15 min. After a brief centrifugation and one wash they were resuspended in room-tempered extracellular buffer, ECB (140 mM NaCl, 20 mM KCl, 20 mM Hepes, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, 0.1 mg/ml BSA) at a concentration of approx. 400,000 cells/ml. Changes in cellular calcium levels were measured using a LUMIstar luminometer (LabVision) in a 96-well format. Hundred μl cells were pumped into a well containing 100 μl ligand mixed with ECB buffer, and the relative luminescence was measured and recorded. In each experiment the respective ligand was tested in triplicate. Identical volumes of ECB only or EtOH (diluted in ECB to the same concentration as in the leukotriene test solution), respectively, were administered in control experiments.

Hybridisation to Northern blots and mRNA arrays. A 422 bp PCR fragment (Probe A) was amplified and cloned into pBluescript SK(+) (Stratagene) using the following two primers: 5′-TCCAGTTTTGCCCAGATGTGCTA-3′ and 5′-TTCCAGCTCAGCAGTGTCTCGTT-3′. This probe is located mainly in the BLTR2 untranslated leader sequence, but includes 142 bp of the open reading frame. Probe B is 673 bp long and is located within the leader sequence only. It was amplified and cloned into pBluescriptSK(+) (Stratagene) using the following primers: 5′-GAGAGGGCTGCTTCTTAGTATGT-3′ and 5′-TACTCCTGTCCTGTGCCTATCA-3′.

The cloned fragments were used as templates for PCR and the resulting bands were labelled with [α-^{32}P]dCTP using the Megaprime DNA labelling system (Amersham). Premade Northern blots were obtained from Invitrogen and Clontech. The mRNA multiple tissue expression array blot (MTE array) was purchased from Clontech. Hybridisations were performed according to the manufacturer's instructions, and autoradiographed using Kodak BioMax MS.

RESULTS AND DISCUSSION

In search of additional members of the leukotriene receptor family, the sequence encoding the first cloned leukotriene receptor (4) was run against various databases. One EST was found to have some similarity to the BLTR receptor. However, cell transfection followed by exposure to various leukotrienes showed the expressed protein to be silent in calcium flux assays. In fact, sequencing revealed that the EST clone lacked the segment encoding the C-terminal part of the putative 7TM receptor protein. Interestingly, 100 bp in the 3′-end of the EST clone were identical to the 5′-end of a cDNA that encoded BLTR (14). During the synthesis of the cDNA library from which the EST had been made (15), the cDNA had been cut with *Not*I before ligation into the cloning vector. A *Not*I site is also present 100 bp into the relevant BLTR leader sequence (14). We hypothesised that the two genes might be linked in the genome, and that the open reading frame of the novel gene continued into the BLTR leader sequence. Further analysis revealed that in the correct reading frame a stop codon was located 223 bp into the leader sequence. This would yield a protein of a size similar to that of BLTR. The missing segment was obtained by PCR amplification of a human leukocyte cDNA library, using one primer corresponding to the EST sequence and another primer corresponding to the BLTR leader sequence just downstream of the stop codon. Subsequent ligation of the cloned segment to the truncated cDNA yielded a novel, 2.8 kb long cDNA clone containing the complete coding sequence for a putative heptahelix receptor (Fig. 1). Based on the sequence characteristics and functional analysis described below we propose the name "leukotriene B_4 receptor 2," or BLTR2, for the novel receptor.

Characteristics of the Amino Acid Sequence

The full-length cDNA (Fig. 1) contains an open reading frame with two in-frame methionines preceding the first membrane-spanning helix region and an in-frame stop signal. We favour the first methionine as shown in Fig. 1 for initiation of translation, since Met-22 agrees less with the optimal Kozak sequence for translation

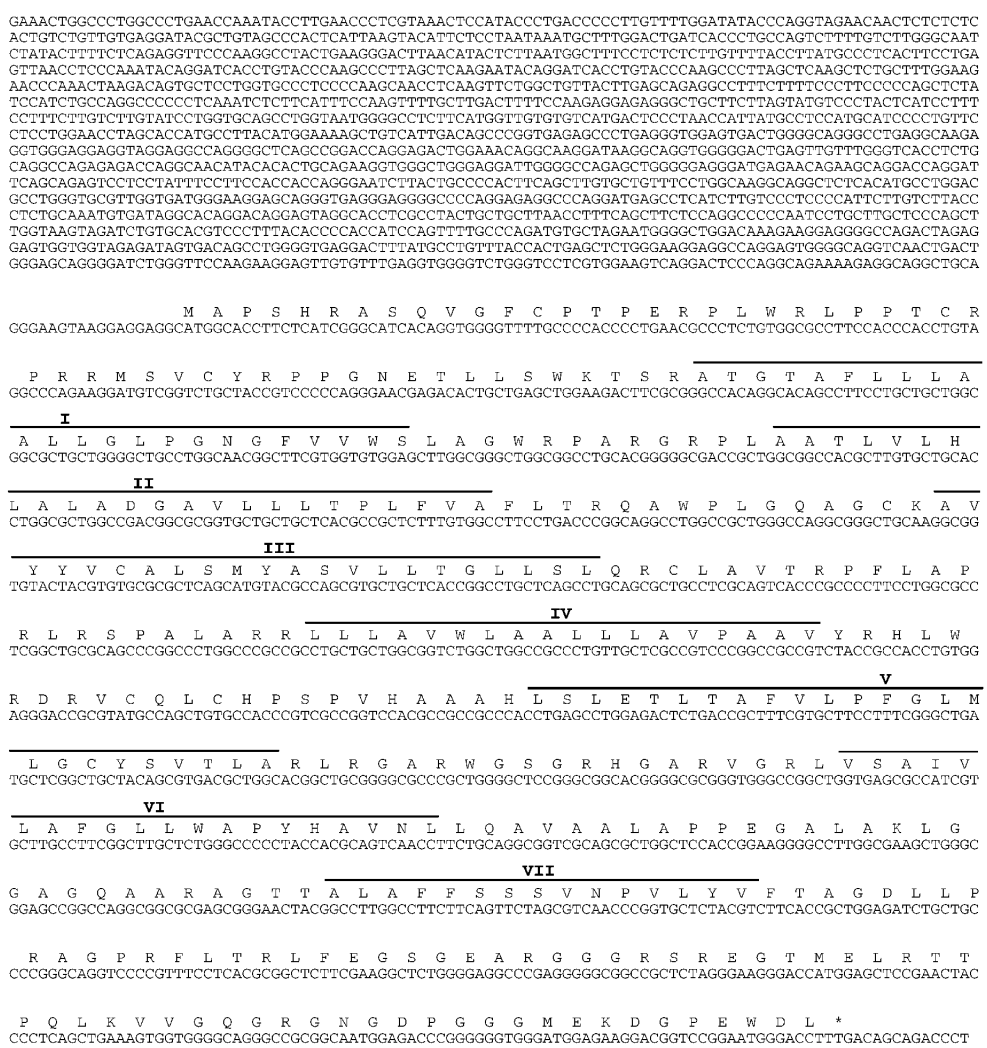

FIG. 1. The nucleotide and deduced amino acid sequences of the novel BLTR2 gene. The cloned cDNA is 2.8 kb long, and encodes a 389 amino acid residues long protein with seven putative transmembrane domains (I–VII). The sequence data has been deposited with the EMBL/GenBank databases, Accession No. AF277230.

(16). However we cannot exclude that the Met-22 also might function as a translation start, but probably at a lower frequency. The predicted polypeptide thus consists of 389 amino acid residues, giving a calculated relative molecular weight of approximately 42 kDa. The receptor protein shows several features common to the G-protein-linked heptahelix receptors, namely (a) consensus sequence for one N-linked glycosylation (Asn-X-Ser/Thr) at Asn-41 in the N-terminal extracellular tail; (b) conserved cysteine residues in each of the first (Cys-125) and second (Cys-199) extracellular loops providing the possibility to form a stabilising disulphide bond in the protein structure; (c) proline residues in all (but the third) membrane-spanning regions thought to induce flexibility within the helix structures; and (d) a C-terminus with serine and threonine residues (2/5) that might serve as substrate for serine/threonine protein kinases.

In the BLAST search (11) of the sequence databases, BLTR2 was clearly the receptor that presented the highest degree of similarity with BLTR, although there is only 39% overall amino acid sequence identity be-

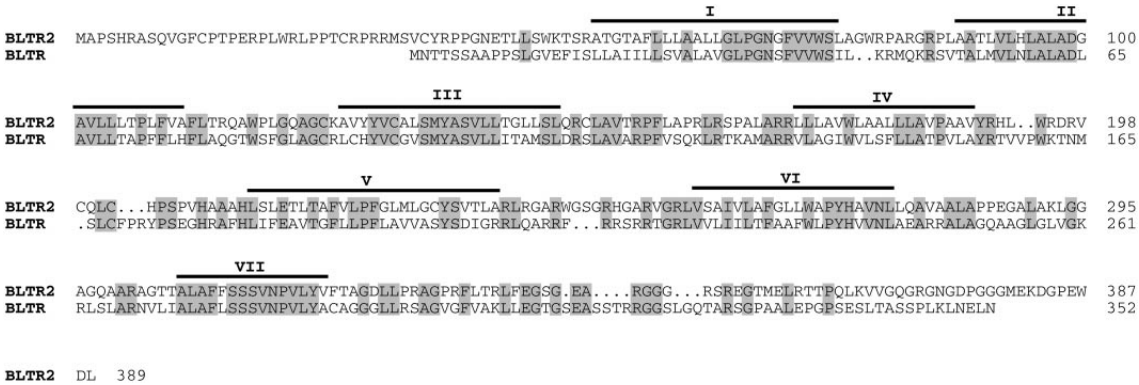

FIG. 2. Alignment of the BLTR2 and BLTR amino acid sequences. The shadowed regions indicate identical amino acid residues. The greatest similarity occurs within the putative transmembrane regions, but can also be found in some intra- and extracellular loops and in the C-terminus.

tween the two receptors (Fig. 2). However, the predicted transmembrane regions (I–VII in Fig. 2) are very similar, a few of them almost identical. Typically, subtypes within the same receptor family would exhibit 50–65% sequence identity, and higher within the transmembrane regions. But the NPY receptor family, for example, also includes subtypes that are considerably more divergent, with only 31–34% overall sequence identity (17). The most highly conserved motif in the heptahelix receptor superfamily is the DRY at the end of the third transmembrane region, the arginine residue being particularly well conserved. The corresponding QRC motif of BLTR2 is very unusual. Phylogenetic analyses indicate (Fig. 3) a close evolutionary relationship of BLTR2 to BLTR, whereas the other known leukotriene receptor, CysLT1R (18), shows a more significant relationship to the formyl peptide receptor-like (FPRL) group.

BLTR2 Can Be Activated by Leukotriene B_4

The new full-length receptor clone was tested in expression assays in a search for ligands that could activate the receptor. Thus, human HeLa cells were transfected with the receptor-pcDNA3 construct together with an aequorin-pcDNA3 reporter plasmid. When cellular calcium levels are elevated as a consequence of receptor activation, aequorin together with the substrate, coelentrazine, emits a transient light, that can be detected in a luminometer (13). Among several leukotrienes tested the new receptor was repeatedly and significantly activated by leukotriene B_4 (Fig. 4A). HeLa cells that had been mock-transfected with an EGFP-pcDNA3 construct together with aequorin-pcDNA3 were used as a negative control. The nature of the response resembled that of the previously cloned receptor for leukotriene B_4, BLTR. The calcium flux appears with a time-lag of some 5–7 s following addition of the ligand (Fig. 4B).

Expression Analysis

Tissue specific expression of BLTR2 was analysed by hybridisation of Northern blots and of a dot blot mRNA

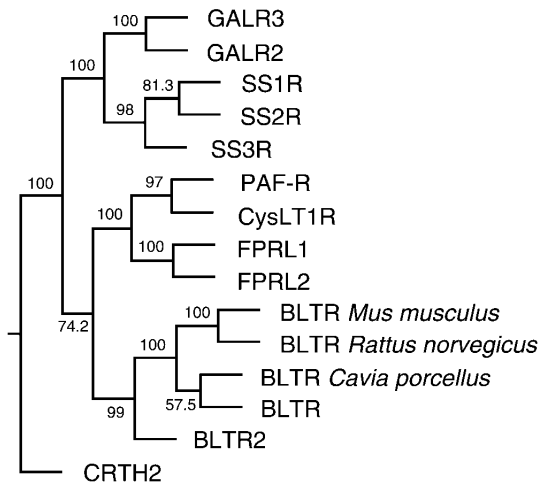

FIG. 3. Phylogenetic analysis of the BLTR2 protein. Numbers at the branch points are the results, in percent, of a bootstrap analysis and demonstrates the confidence of the inferred nodes. Abbreviations used and the GenBank Accession Nos. (in parentheses) of the receptors are as follows: GALR3, Galanin receptor type 3 (NP_003605); GALR2, Galanin receptor type 2 (O43603); SS1R, Somatostatin receptor 1 (NP_001040); SS2R, Somatostatin receptor 2 (NP_001041); SS3R, Somatostatin receptor 3 (NP_001042); PAF-R, Platelet activating factor receptor 1 (P25105); CysLT1R, Cysteinyl leukotriene 1 receptor (NP_006630); FPRL1, Formyl peptide receptor-like 1 (NP_001453); FPRL2, Formyl peptide receptor-like 2 (NP_002021); BLTR; Leukotriene B_4 receptor, *Mus musculus* (NP_032545); *Rattus norvegicus* (JC7096); *Cavia porcellus* (AAD42063); *Homo sapiens* (CAA67001); BLTR2, Leukotriene B_4 receptor 2, (AF277230); CRTH2, Chemoattractant receptor-homologous molecule expressed on TH2 cells (NP_004769).

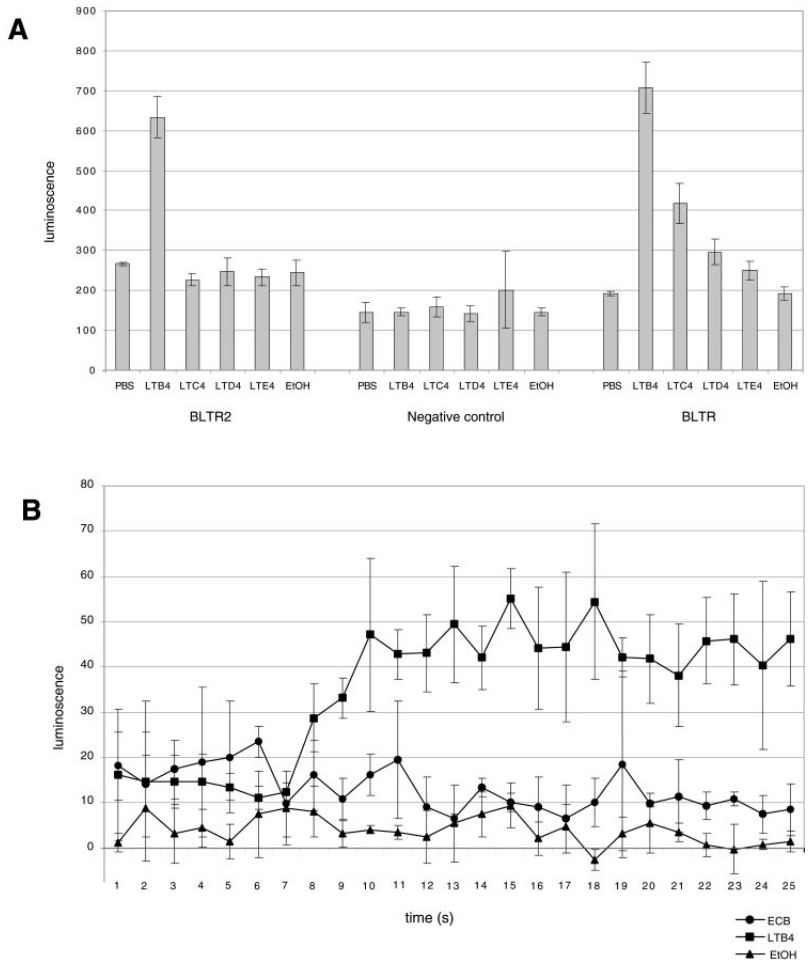

FIG. 4. BLTR2 can be activated by leukotriene B_4 in HeLa cells transfected for transient receptor expression. Transfection with cDNA encoding BLTR, BLTR2, or EGFP (enhanced green fluorescent protein, negative control), together with an aequorin plasmid. After loading cells with coelentrazine an elevation of intracellular calcium levels can be recorded as luminescence. A representative experiment is shown in A, where the columns depict integrated luminescence for 25 s after ligand addition. Means ± SD shown from triplet experiments. As can be seen in B it takes 5–7 s after addition of leukotriene B_4 until the calcium response appears. No calcium flux is seen after addition of ECB or the leukotriene solvent (EtOH). Values are means ± SD from triplicate tests.

array. The array (data not illustrated) showed a general low level of expression in most of the tissues represented. Highest expression levels were detected in liver, followed by heart, kidney, placenta, brain and spleen; but also the expression in adrenal gland, pancreas, testis, pituitary gland, stomach, lymph nodes, lung and peripheral blood leukocytes was above the background level. The Northern blots essentially confirmed the dot blot array data and showed expression in liver, lung and pancreas, with traces of expression in heart and placenta (Fig. 5A). Expression was also seen in skeletal muscle, brain, spleen and kidney (Fig. 5B). At least two transcript sizes are present (5.3 kb and 1.3 kb), and genome studies suggest that the gene might be regulated in a complex manner (19). Hybridisation of the same mRNA array with BLTR probes (data not shown) indicated a similar low level of expression in most tissues, with somewhat higher expression in adrenal gland, kidney, liver, heart and skeletal muscle. Northern blots hybridised to elucidate the distribution of BLTR message (4) have previously shown high expression in leukocytes, bone marrow, lymph nodes, spleen and thymus, and also in pancreas, skeletal muscle and heart. The partly overlapping expression pro-

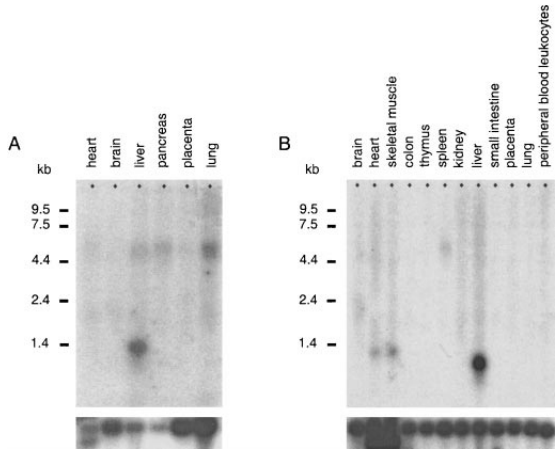

FIG. 5. Expression analysis of BLTR2 by Northern blot hybridisation. In blot A (approximately 2 μg mRNA/lane) transcripts can be detected in liver, lung, and pancreas. Traces of expression are also detected in heart and placenta. In blot B (1 μg mRNA/lane) transcripts can be detected in liver, heart, and skeletal muscle. Traces of expression can also be found in spleen, brain, and kidney. The mRNA signals should be related to the intensity of the actin signals included at the bottom for reference.

files of BLTR and BLTR2 is notable, and further studies will reveal if they function together as subtypes of the same receptor or whether they perform more separate functions as individual entities. Their close genomic localisation on chromosome 14 is interesting and suggests an evolutionary relationship (19). However, they are only 39% identical and although they both can be activated by leukotriene B_4, one cannot exclude that they might function differently in different types of—native or transfected—cells, or complement each other in different ways, e.g., through dimerisation.

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Paper II

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A human cell surface receptor activated by free fatty acids and thiazolidinedione drugs

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Abstract

Fatty acids, which are essential nutritional components, are also involved in cardiovascular and metabolic diseases. Here we report a human cell surface receptor that we name free fatty acid receptor (FFAR), because it is specifically activated by medium to long-chain free fatty acids. The receptor belongs to the class of seven-transmembrane, G-protein coupled receptors (GPCRs) and also mediates responses to antidiabetic drugs of the thiazolidinedione type. It is expressed in skeletal muscle, heart, liver, and pancreatic β-cells. Stimulation of FFAR increases the intracellular calcium concentration in cells expressing the receptor in a native (pancreatic β-cell line) or in a recombinant form. In view of the nature of the activating substances, their physiological role in the body, and the tissue distribution of FFAR we suggest the term ''nutrient sensing receptor'' for receptors acting at the interface between dietary components and signalling molecules.

Keywords: Receptor; Cell surface; GPR40; Fatty acid; Thiazolidinediones; Diabetes; Obesity; Arteriosclerosis; Reverse pharmacology; Drug evaluation; Preclinical

Lipids provide energy to the cell and contribute to cellular components, including organelles and plasma membrane, and also serve as chemical messengers in the body [1]. This highlights the role of lipids as both dietary components and potential disease factors [2,3]. Particularly free fatty acids (FFAs), e.g., poly-unsaturated FFAs, appear in a plethora of studies, which have focused on their metabolic function in all types of cells and tissues. Over the last few years, it has been noted that some fatty acids also have a messenger role [4], mediated via the nuclear peroxisomal proliferator-activated receptors (PPARs) [5]. However, not all biological effects reported can be explained by these mechanisms. Some of the mediator effects appear to be PPAR independent [6,7] and are rather characteristic of cell surface receptor involvement. Among FFAs, only arachidonic acid (AA) has been found to be a precursor of signalling molecules that acts through surface receptors, notably GPCRs [8]. Linoleic acid (LA) is one of the several medium- to long-chain essential fatty acids forming the starting point in liponeogenesis and fatty acid metabolism (including the formation of, i.a., AA and its metabolites [9]). In addition, these fatty acids are key components in dietary fat and are particularly abundant in plant oils and fish [10]. Taken together, this prompted us to target FFAs with a screening approach aimed at identifying potential surface receptors.

Materials and methods

Materials. Fatty acids and fatty acid derivatives were from the following commercial suppliers: Larodan AB, Malmö, Sweden; Sigma and Cayman Chemical, Ann Arbor, MI. Rosiglitazone was a gift from GlaxoSmithKline. Coelentrazine was purchased from BioSynth AG. Cell culture reagents, equipment, and media were obtained from Invitrogen and oligonucleotides from MWG Biotech.

Cloning. Cloning of the R10/GPR40 ORF from human genomic DNA was performed by PCR using the following primers:
forward: 5′-GGCGGATCCACCATGGACCTGCCCCCGCAGCTC TCCTTCG-3′;
reverse: 5′-GGCGGATCCTTACTTCTGGGACTTGCCCCCTTGC GTT-3′.
PCR conditions were: 96 °C for 3 min, 96 °C 45 s; 57 °C 1 min; and

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72 °C 1 min (30 cycles). The PCR product was restricted with *Bam*HI, subcloned into the pIRESpuro vector (Clontech), and sequenced.

Cell culture. HeLa, CHO, and MIN6 cells were grown in Dulbecco's modified Eagle's medium with Glutamax-I supplemented with 10% FBS, 0.5% streptomycin and penicillin. INS-1E cells were grown in RPMI 1640 with Glutamax-I supplemented with 10% FBS, 50 μM β-mercaptoethanol, 0.5% streptomycin and penicillin. All cells were maintained at 37 °C with 7% CO_2.

HFF11 reporter cells and assay. Construction of the HFF11 reporter cell lines (successor of the previously described HeLa based cell line, HF1) expressing the orphan GPCRs studied was performed as earlier reported [11]. They contain the reporter plasmid, pcFUS3, where the original promoter region has been extended by 6 NFκB and 6 STAT elements. On day 1, HFF11 reporter cells, with or without the test receptor, were seeded into 96-well plates at 20,000 cells per well in 100 μl growth medium. On day 3, the medium was removed and replaced by 90 μl serum-free medium. Substances and controls were added in the appropriate concentrations 24 h later. Stimulations were performed at 37 °C for 7 h. Cell lysates were assayed in a BMG Lumistar Galaxy luminometer as previously described [11]. "Relative response" was calculated as the ratio between the experimental value (agonist) subtracted by the background value (PBS) and the maximum response value (1 μM PMA) subtracted by the background value.

Aequorin reporter assay. Cells were seeded the day before transfection in a 6-well plate (400,000 cells/well). Then, 0.8 μg pCDM.AEQ reporter plasmid [12] and 1 μg pIRESpuro.R10 receptor plasmid, or pcEGFP for controls, were mixed with 100 μl OptiMEM containing 5 μl per transfection of PLUS reagent. After 15 min, 100 μl OptiMEM and 9 μl (HeLa cells) or 6 μl (CHO cells) of lipofectamine were added and incubated for another 15 min. Then, 800 μl OptiMEM was added, and the cells were washed and incubated with the transfection solution for 5 h. MIN6 and INS-1E cells were processed essentially as described for CHO cells, except that the pIRESpuro.R10 vector was omitted in the transfection. Two days after transfection, cells were loaded with 10 μM coelenterazine in serum-free PBS. Loaded cells were resuspended in serum-free ECB (extra cellular buffer) and injected into a 96-well plate containing test or control (vehicle) substances. Light emission (luminescence) was recorded for 1 min in a BMG Lumistar Galaxy luminometer and normalised to total emission using 0.1% Triton and 100 mM $CaCl_2$ for each well.

Northern blot. Two micrograms mRNA prepared from MIN6 cells and INS-1E cells was electrophoresed, together with an RNA size marker, and transferred to a nylon membrane according to standard procedures [13]. The membrane and a pre-made human multiple tissue northern blot were hybridised with the cloned R10 (FFAR) ORF according to standard (50% DMF at 42 °C) protocols [13] and subjected to autoradiography.

Data analysis. All data points represent measurements carried out in triplicates ($n = 3$) and each experiment was repeated three to six times. Statistical errors are shown as standard deviations. The data were statistically analysed using the GraphPad PRISM software package.

Results and discussion

Linoleic acid specifically activates receptor 10 (R10) expressing reporter cell lines

We used a highly sensitive reporter system [11] equipped with ten different putative human GPCRs for which the endogenous activating ligands are still unknown. The receptor sequences were selected from GenBank, based on a reasonably high sequence homology to the receptors for leukotriene B4 (an AA derivative), previously cloned in our laboratory [14,15]. During the initial screening, reporter cell lines with or without individual recombinant test receptors were challenged with FFAs, such as AA and LA, at 50 μM. Only the cell line expressing the candidate receptor, R10 (designated GPR40 [16] in GenBank), gave a strong and reproducible response to LA, with a Z-factor [17] of 0.8 (Fig. 1A). The LA response obtained in the R10 expressing cell line was partially sensitive to pertussis toxin (data not shown), indicating that the signal was transduced by a GPCR coupling to G-proteins of both G_q and G_i classes. In contrast, AA had no effect on R10 (Fig. 1B) or on any other test receptor, R1–R9 (data not shown).

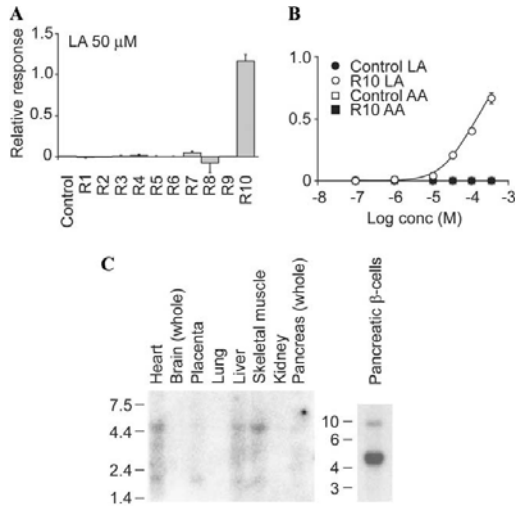

Fig. 1. Receptor activation and tissue expression. (A) Screening of 10 orphan receptors (R1–R10) expressed in HFF11 reporter cells and stimulated with LA, which activates only R10 (FFAR). Control is sham-transfected HFF11 cells. (B) Concentration–response curves for LA and AA using HFF11.pR10, control is sham-transfected HFF11 cells. Only LA activates the receptor, R10 (FFAR), in a concentration-dependent manner. (C) Autoradiograph of human multiple tissue northern blot and murine pancreatic β-cell insulinoma cell line (MIN6) showing expression of R10 (FFAR) in heart, liver, skeletal muscle, and pancreatic β-cells. Weak expression is also seen in placenta.

Tissue distribution

Northern blot analysis of select human tissues revealed that the corresponding gene is expressed mainly in the liver, heart, and the skeletal muscle. The receptor is also expressed in pancreatic β-cell insulinoma (Fig. 1C).

R10 transfected cells mobilise calcium upon ligand stimulation

In order to confirm the results, the ability of the receptor to mobilise calcium was investigated in two

different cell lines, CHO and HeLa, transiently co-transfected with expression vectors containing the R10 open reading frame and the calcium-activated photoprotein, aequorin. As shown in Fig. 2, only cells transfected with the R10-encoding vector, in contrast to sham-transfected cells, were capable of eliciting an instant calcium-mediated luminescence flash upon LA stimulation.

A wide spectrum of free fatty acids activate reporter cells expressing R10

A large number of FFAs were tested for their ability to activate the receptor in view of differences in chain length, substituents, and degree of saturation, using the R10-expressing reporter cell line, HFF11.pR10. Table 1 shows that fatty acids capable of receptor activation include saturated as well as mono- and poly-unsaturated FFAs, at concentrations within their physiological ranges [18]. Saturated FFAs comprising 10–18 carbon atoms were all able to activate the receptor, while medium-length saturated FFAs (10–12 carbon atoms) had the highest potency (order of potency: capric acid > lauric acid > myristic acid > palmitic acid). Short-chain FFAs were unable to activate the receptor at concentrations up to 1 mM (data not shown). The 18-carbon stearic acid was able to activate the receptor, but its low solubility in aqueous solutions makes it impossible to determine a reliable EC_{50} value. Unsaturated FFAs with a chain-length shorter than 20 carbon atoms also activated the receptor, but with a lower potency (order of potency: stearidonic acid > linolenic acid > linoleic acid > oleic acid). In view of the broad spectrum of FFAs capable of activating R10, we henceforward refer to this receptor as free fatty acid receptor, or FFAR.

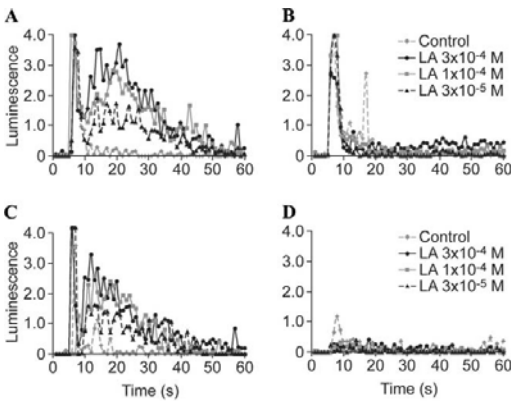

Fig. 2. Time-course of calcium mobilisation in aequorin-expressing CHO and HeLa cells transfected with receptor or sham-transfected cells, stimulated at different linoleic acid (LA) concentrations or given vehicle only (control). Cells were exposed to compounds after 5 s of measurement. (A) CHO cells transfected with R10 (FFAR). (B) Sham-transfected CHO cells. (C) HeLa cells transfected with R10 (FFAR). (D) Sham-transfected HeLa cells.

In addition to the essential FFAs, we also tested substances of clinical interest. 9-HODE (9-hydroxy, *trans*-10,*cis*-12-octadecanoic acid) is a hydroxy derivative of LA. It is a major component of oxo-LDL, associated with several aspects of arteriosclerosis [19]. CLA (conjugated LA) is a group of LA derivatives, comprising two conjugated double bonds in different locations and chirality. They are dietary components with a spectrum of biological functions, including anticarcinogenic effects [20]. Especially well studied is the 10-*trans*,12-*cis* CLA isomer which has been reported to increase fatty acid oxidation in preadipocytes and in hepatocytes [20]. As shown in Table 1, both 9-HODE and, to a lesser extent 10-*trans*,12-*cis* CLA, activated FFAR.

Binding studies

In binding experiments not shown, cell membranes from the FFAR-expressing HFF11 reporter cell line were used. Unlabelled capric acid or lauric acid was used in attempts to displace tritiated myristic acid. These binding experiments failed, most likely due to the low affinity of the receptor for myristic acid, as indicated by its high EC_{50} value. A radiolabelled ligand with higher

Table 1
FFAR response to a variety of active FFAs as measured with HFF11.pR10 reporter cells

Trivial name	Chain length:double bonds	EC_{50} in μM (means ± SD)	Systematic name
Capric acid	10:0	12.6 ± 0.4	Decanoic acid
Lauric acid	12:0	22.5 ± 0.3	Dodecanoic acid
Myristic acid	14:0	30.3 ± 0.6	Tetradecanoic acid
Palmitic acid	16:0	143.2 ± 11	Hexadecanoic acid
Oleic acid	18:1	123.1 ± 12	*cis*-9-Octadecenoic acid
Linoleic acid	18:2	38.4 ± 1.5	*cis*-9,*cis*-12-Octadecadienoic acid
Linolenic acid	18:3	27.1 ± 1.5	*cis*-9,*cis*-12,*cis*-15-Octadecatrienoic acid
γ-Linolenic acid	18:3	28.5 ± 2.6	*cis*-6,*cis*-9,*cis*-12-Octadecatrienoic acid
Stearidonic acid	18:4	8.9 ± 0.7	*cis*-6,*cis*-9,*cis*-12,*cis*-15-Octadecatetraenoic acid
CLA	18:2	77.3 ± 8	*trans*-10,*cis*-12-Octadecadienoic acid
9-HODE	18:2	3.4 ± 0.1	(±)-9-Hydroxy,*trans*-10,*cis*-12-octadecadienoic acid

affinity for FFAR is presently not commercially available.

Antidiabetic thiazolidinedione drugs and MEDICA16 activate cells expressing recombinant FFAR (R10)

The characteristics of tissue distribution of FFAR and the nature of the ligand spectrum prompted us to investigate certain pharmacologically active drugs, selected on the basis of their target tissues. Indeed, both, the experimental anti-obesity compound, MEDICA16 [21], and the antidiabetic thiazolidinediones, rosiglitazone [22] and MCC-555 [23], strongly activated HFF11 reporter cells expressing FFAR (Figs. 3A–C). However, the non-thiazolidinedione type antidiabetic drugs, glibenclamide and clofibrate, were unable to activate the receptor (data not shown). MEDICA16 (3,3,14,14-tetramethyl-hexadecanedioic acid) is an experimental drug that has been shown to exhibit antidiabetogenic and hypolipidemic actions in a rat model [24]. It has been reported to lower plasma levels of chylomicrons, triacylglycerols, and cholesterols. Its main site of action is thought to be the liver, although drug actions have also been observed in the heart and in pancreatic β-cells [25].

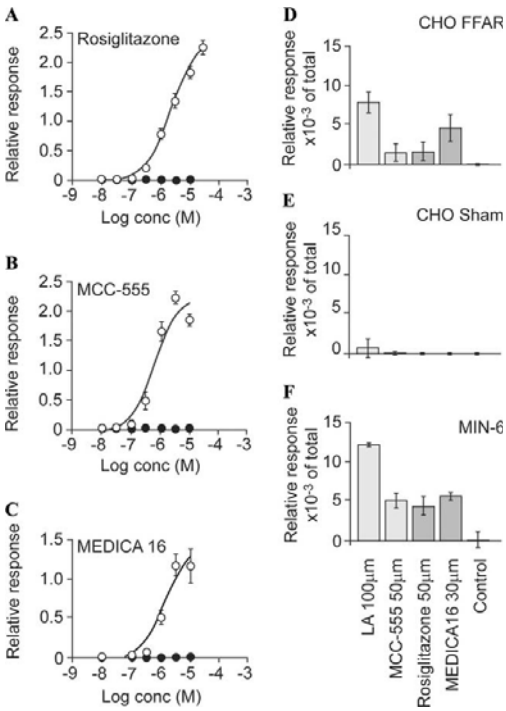

Fig. 3. Concentration–response curves from HFF11 reporter cells, FFAR transfected white circles and SHAM transfected as black circles (A–C), and bar-graphs from CHO and MIN6 cells (D–F) showing the receptor-mediated effects of various ligands. (A) The clinical anti-diabetic drug, rosiglitazone ($EC_{50} = 2.8 \pm 0.25\,\mu M$). (B) The experimental anti-diabetic drug, MCC-555 ($EC_{50} = 0.6 \pm 0.15\,\mu M$). (C) The experimental anti-obesity drug, MEDICA16 ($EC_{50} = 1.22 \pm 0.1\,\mu M$). (D) CHO cells transiently expressing FFAR. (E) Sham-transfected CHO cells. (F) MIN6 cells endogenously expressing the murine homologue of FFAR. The cells in (D–F) were stimulated with linoleic acid (100 μM), rosiglitazone (50 μM), MCC-555 (50 μM), MEDICA16 (30 μM), or vehicle. Bars show means ± SD of representative experiments performed in triplicate.

The thiazolidinediones (e.g., rosiglitazone and MCC-555) constitute a novel class of antidiabetic drugs that have been shown to reduce plasma glucose levels and improve insulin sensitivity in adipose tissue, liver, and muscle [26]. Although their exact mechanism of action is unknown, reports indicate that certain thiazolidinediones bind and activate the nuclear receptor, PPARγ [27]. Our observations are in accordance with recent reports of unexplained PPARγ-independent thiazolidinedione effects [28,29]. For example, MCC-555 which has a more potent antidiabetic effect than rosiglitazone poorly activates PPARγ [23]. The presence of a surface receptor, like FFAR, conforms with the direct and immediate effect of thiazolidinediones [30].

The ability of rosiglitazone, MCC-555, and MEDICA 16 to mobilise calcium was compared to that of linoleic acid in CHO cells transiently transfected with FFAR. Linoleic acid was equally potent in this and in the HFF11 transcriptional assay, whereas the synthetic ligands had a substantially weaker effect in the calcium mobilisation assay (Figs. 3D and E). This discrepancy may be explained by the concept of "agonist-directed trafficking of receptor signalling" [31], which has been reported for several other GPCRs [32]. Another explanation is based on the difficulty of determining the true maximal efficacy of low-affinity ligands thus making comparisons between systems more complicated. Signalling other than by calcium mobilisation, as displayed by the synthetic ligands, is reflected only in the HFF11 assay since it covers several effector pathways.

FFAR agonists induce calcium mobilisation in pancreatic β-cell lines

The physiological relevance of our findings on recombinant FFAR is supported by experiments using pancreatic β-cell lines, rat INS-1E (data not shown), and mouse MIN6, which endogenously express the homologues of FFAR (Fig. 1C). Linoleic acid, rosiglitazone, MCC-555, and MEDICA16 all elicited calcium release in these cell lines (MIN6: Fig. 3F, INS-1E data not shown) with the same pattern as in CHO cells transfected with FFAR (Fig. 3D).

To conclude, we have cloned and identified FFAR, a human cell surface receptor of the GPCR family that is activated by medium to long-chain FFAs at concentrations corresponding to their physiological plasma levels.

FFAR is also activated by certain anti-diabetic and anti-obesity drugs. The receptor is expressed in organs and tissues of major importance in fat and energy metabolism. Taken together, this suggests that FFAR serve a role as a "nutrient sensing receptor." Thus, the identification of this hitherto functionally unknown receptor further emphasises the pivotal role of FFAs in an important physiological–pathological context.

Acknowledgments

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Paper III

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Identification of a free fatty acid receptor, FFA_2R, expressed on leukocytes and activated by short-chain fatty acids

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Abstract

Short-chain fatty acids (SCFAs) have long been known to exert cellular effects on blood leukocytes. Acetate, propionate, and butyrate represent the most capable SCFA, inducing calcium mobilization which subsequently regulates leukocyte function in the immune system. We have cloned the previously described putative orphan G-protein coupled receptor, GPR43, and have functionally identified SCFA as the activating ligands. Acetate and propionate were found to be the two most potent ligands, although butyrate, formate, and valerate (in this order of potency) also were able to induce receptor activation. Both the human and mouse receptor homologues were found to share the same pattern of ligand activation. This finding, together with a high degree of amino acid sequence similarity between the mouse and human homologues, indicates an evolutionary conserved function. Upon ligand stimulation, the receptor mobilized intracellular calcium in both a recombinant system as well as in human granulocytes. We found the human gene to be predominantly expressed in peripheral blood leukocytes and, to a lesser extent, in spleen. We suggest the designation FFA_2R to this second receptor activated by free fatty acids. The first-described FFAR, now named FFA_1R, is activated by medium- to long-chain free fatty acids.

Keywords: Cell surface receptor; GPCR; GPR43; Fatty acid; SCFA; Acetate; Propionate; Pertussis toxin; PMNC; Immune system

The initial response to an invasion of pathogenic bacteria is provided by the innate immune system through infiltration of granulated leukocytes. Some pathogens, particularly gram-negative anaerobic bacteria are, however, able to evade this first line of defense [1]. The mechanism involved is not entirely understood, though numerous reports indicate an action of chemical mediators and, specifically, short-chain fatty acids (SCFA), having a documented ability to modify the immune response [2–5]. SCFA are known to exert a multitude of cellular effects on polymorphonuclear cells (PMNC), such as altering cytoplasmic pH, calcium concentration, oxygen metabolism, phagocytosis, cell proliferation, cytoskeletal actin distribution, granulocyte motility, and chemotaxis [2,6–8]. This plethora of SCFA-induced biological effects are, unquestionably, generated by several mechanisms where at least one indicates the involvement of G-protein mediated signaling [6,7].

Another physiological aspect of SCFA is their role in the digestive process of the intestine, where they comprise the end product of a tolerable anaerobic fermentation. A major function of the intestinal bacterial flora is to recover and ferment undigested carbohydrates and to provide a major energy source in the form of SCFA in millimolar concentrations [9–11]. As a result of the presence of the enteric microflora, the gastrointestinal tract is a highly immunogenic environment constituting a physiological challenge to the immune system. Thus, there is an apparent need for an intestinal regulation of the immunological response [12–14]. Recently, the importance of SCFA's anti-inflammatory activities in the intestine has been pointed out as being part of such a mechanism [15].

Following our identification of a G-protein coupled receptor (GPCR) designated FFAR (free fatty acid

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receptor), which is activated by medium- to long-chain free fatty acids [16], we expanded our investigation to also include SCFA. The fact that the putative "orphan" GPCR and FFAR homologue, GPR43, recently were reported to be highly expressed on human neutrophils and monocytes [17] and earlier reports indicating the involvement of a GPCR in SCFA-associated immune regulation led us to investigate the ability of GPR43 to be activated by SCFA.

Materials and methods

Cloning. Cloning of the GPR43 ORF (open reading frame) from genomic (human and mouse) DNA was performed by PCR using the following primers:

Human ORF:
forward: 5′-ATTGCGGCCGCAGGATGCTGCCGGACTGGAAGAGC-3′;
reverse: 5′-ATTGCGGCCGCACTGCTACTCTGTAGTGAAGTCCGA-3′.

Murine ORF:
forward: 5′-CGGAATTCCGGGGGACTCTCTACTCGGTGACAA-3′;
reverse: 5′-ATTGCGGCCGCCAGAATGACCCCAGACTGGCACAGT-3′.

PCR conditions were: 96 °C for 3 min, 96 °C for 45 s; 58 °C for 1 min; and 72 °C for 1 min (30 cycles). The PCR products were restricted with *Not*I (human) or *Not*I/*Eco*RI (mouse), subcloned into the pIRESpuro vector (Clontech), and sequenced.

Cell culture. CHO cells were grown in Dulbecco's modified Eagle's medium (DMEM) with Glutamax-I supplemented with 10% FBS, 0.5% streptomycin and penicillin. HFF11 (HeLa) cells were grown in DMEM with Glutamax-I supplemented with 3% FBS, 0.5% streptomycin and penicillin. All cells were maintained at 37 °C with 7% CO_2.

HFF11 reporter cells and assay. Construction of the HFF11 reporter cell lines expressing the GPCRs was performed as earlier reported [18]. The HFF11 is based on the reporter plasmid, pcFUS3, where the original promoter region has been extended by 6 NF-κB and 6 STAT elements [19]. On day 1, HFF11 reporter cells, either expressing the recombinant receptor or sham-transfected, were seeded into 96-well plates at 20,000 cells per well in 100 μl growth medium. On day 3, the medium was removed and replaced by 90 μl serum-free medium. Substances and controls were added in the appropriate concentrations 24 h later. Stimulations were performed at 37 °C for 6 h. Cell lysates were assayed in a BMG Lumistar Galaxy luminometer as previously described [18]. Pertussis toxin (PTX) treatment was carried out at 100 ng/ml for 16 h. "Relative response" was calculated as the ratio of the experimental value (agonist) subtracted by the background value (PBS) over the maximum response value (1 μM PMA) subtracted by the background value.

Aequorin reporter assay. Cells were seeded the day before transfection in a 6-well plate (400,000 cells/well). Then, 0.8 μg pCDM.AEQ reporter plasmid [20] and 1 μg pIRESpuro.GPR43 plasmid, or pcEGFP for controls, were mixed with 100 μl OptiMEM containing 5 μl per transfection of PLUS reagent. After 15 min, 100 μl OptiMEM and 9 (HeLa cells) or 5 μl (CHO cells) lipofectamine (Invitrogen) were added and incubated for another 15 min. Then, 800 μl OptiMEM was added, and the cells were washed and incubated with the transfection solution for 5 h. Two days after transfection, cells were loaded with 10 μM coelenterazine and injected into a 96-well plate containing ligand or control (vehicle) substances. Light emission (luminescence) was recorded for 1 min in a BMG Lumistar Galaxy luminometer and normalized to total emission using 0.1% Triton and 100 mM $CaCl_2$ for each well.

Fura-2 assay on human PMNC. Fresh human PMNCs from three different individuals were prepared using polymorphoprep. The PMNC were harvested, washed, and resuspended in RPMI-medium. The cells where loaded with 2 μM Fura-2 in the presence of 2.5 mM probenecid for 1 h, washed, and subsequently analyzed in a 96-well plate fluorometer (Fluostar Galaxy, BMG, Germany). Cell suspensions were pumped into wells, already containing the test substances and controls, at the appropriate concentrations. Changes of the intracellular calcium concentrations were recorded for 1 min and normalized to the maximal response (10 mM acetate).

Northern blot. A pre-made human multiple tissue Northern blot (Clontech) was hybridized with a labeled human GPR43 ORF according to standard protocols (50% formaldehyde buffer) [21] and then subjected to autoradiography. The same blot was stripped and reprobed using a β-actin control provided by the manufacturer.

Data analysis. All data points represent measurements carried out in triplicates ($n = 3$) and each experiment was repeated three to four times. Statistical errors are shown as standard deviations (SD). The data were statistically analyzed using the GraphPad PRISM software package. ClustalX was used for sequence alignment and TMHMM 2.0 [22] for transmembrane predictions.

Results and discussion

SCFA-induced activation of human GPR43

The previously identified putative GPCR ORF, GPR43 (GenBank Accession No. AF024690) [23] was cloned and stably expressed in the recombinant reporter cell line, HFF11, which is the second-generation reporter cell line [19]. A spectrum of SCFAs activated the receptor, but failed to induce any response in sham-transfected reporter cells. High concentrations, in the millimolar range, of SCFA, such as acetate (carbon chain length = C2), propionate (C3), and butyrate (C4), activated the reporter cells in a concentration-dependent manner (Fig. 1A). As high concentrations are required to reach the maximal activation of the receptor, an accurate determination of the half-effective concentration (EC_{50}) was difficult. Using the reporter cells, approximate EC_{50} values for the human receptor were determined to be 2.6 ± 0.6 mM for acetate, 3.1 ± 0.7 mM for propionate, and 1.0 ± 0.5 mM for butyrate. Formate (C1) and valerate (C5) were also able to activate the receptor but to a lesser extent; while caproate (C6), lactate, and β-hydroxy butyrate were unable to induce any measurable activation in concentrations up to 30 mM. This spectrum of SCFA potency is similar to previous reports regarding SCFA's ability to induce physiological changes in human PMNC, such as their effect on cytoskeletal F-actin [6].

The SCFA-mediated signal in neutrophils is known to involve a PTX receptive pathway [7,24]. In order to investigate the possible involvement of such a PTX-susceptible signaling pathway, reporter cells were pretreated with PTX for 10 h and then assayed. The

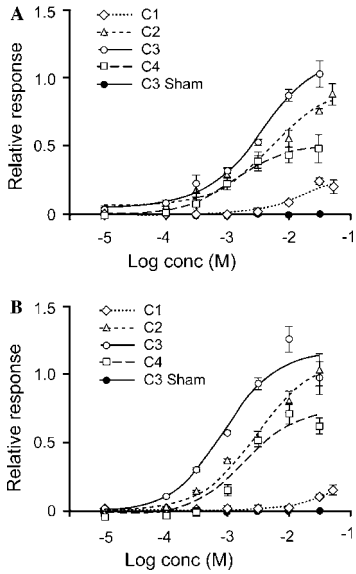

Fig. 1. Concentration–response curves obtained by SCFA stimulation of (A) the human receptor and (B) the mouse homologue. GPR43 (FFA_2R)-transfected HFF11 reporter cells are depicted by open symbols and sham-transfected controls (propionate stimulation only) by filled circles. C1; formate, C2; acetate, C3; propionate, and C4; butyrate. Values are means ± SD ($n = 3$).

propionate-induced signal was reduced by approximately 70% when pretreated with PTX (Fig. 4C), indicating the involvement of a G-protein belonging to the G_i/G_0-family. The previously reported PTX-sensitive SCFA-induced calcium mobilization was reduced by 90% when subjecting human neutrophils to 5 mM propionic acid [24].

Species homology and functional assay of the mouse homologue

Protein sequence alignment of a mouse sequence (GenBank Accession No. NM146187) indicated a strong relationship with human GPR43, as the two sequences display 85% amino acid sequence identity (Fig. 2). Also a putative rat homologue was identified on chromosome 1 (NW043364) and alignment indicated a 96% identity to the mouse sequence.

The mouse ORF was cloned as described above, expressed in reporter cells, and tested with SCFA. The results revealed similar activation patterns for both the human (Fig. 1A) and mouse (Fig. 1B) receptors. The mouse EC_{50} values were calculated to be: 2.6 ± 1.1 mM for acetate, 0.9 ± 0.4 mM for propionate, and 1.9 ± 1.0 mM for butyrate (Fig. 1B).

The human and murine sequences thus shared a high sequence homology and showed similar ligand activation spectra of the cloned mouse and human receptors. This indicated an evolutionary preserved ability for GPR43 to be activated by SCFA. We therefore assign this new GPCR the designation free fatty acid 2 receptor, FFA_2R, and in consequence with this the previously named FFA receptor (FFAR) [16] should now be designated FFA_1R.

Tissue expression

In order to validate its identification, the human GPR43 (FFA_2R) ORF was hybridized on a Northern blot expressing various mRNAs (Fig. 3). Two transcripts with the sizes of 2.8 and 3.8 kb were found to be expressed predominantly in peripheral blood leukocytes but also in spleen. A shorter transcript, approximately 2.4 kb, was weakly expressed in skeletal muscle and heart. These results are in accordance with previous reports, indicating the existence of a cell surface receptor activated by SCFA and expressed on human neutrophils [6]. Also, the GPR43 gene was recently reported to be present in hematopoietic and related immune cells [17].

Calcium mobilization assays

The SCFAs ability to induce immediate calcium mobilization was investigated in CHO cells which were transiently transfected with expression plasmids containing either the human FFA_2R or EGFP (as control) genes together with vector encoding the aequoringene [20]. Only cells transfected with FFA_2R showed an increase in the intracellular calcium concentration following exposure to SCFA (Fig. 4A). In sham-transfected cells the intracellular calcium levels remained unchanged for all SCFA tested in concentrations up to 10 mM. Acetate and propionate were the two most potent activators, showing almost equal responses. Using data from three independent assays, all including triplicates for each measured value, the EC_{50} values were determined to be 0.8 ± 0.3 mM for acetate and 0.9 ± 0.4 mM for propionate. As in the assay on reporter cells, butyrate showed less efficacy, with an approximate EC_{50} of 1.2 ± 0.5 mM. Valerate did not induce a detectable signal.

Human PMNC were isolated from fresh blood and subjected to calcium assay using the Fura-2 method. In accordance with the findings of FFA_2R-expressing CHO cells, both acetate and propionate generated a strong concentration-dependent calcium signal, while butyrate only induced a partial response (Fig. 4B). The EC_{50} values were slightly higher (acetate 2.0 ± 0.8 mM and propionate 1.8 ± 0.6 mM) than for the recombinant CHO system, but in the same order of magnitude. Previous reports have shown acetate and propionate to be the two most potent SCFA in evoking calcium

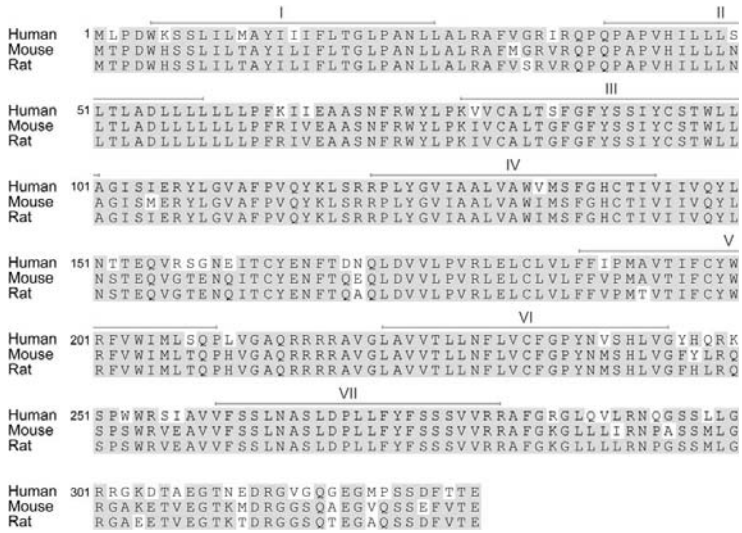

Fig. 2. Amino acid sequence alignment (ClustalX) of the human, mouse, and rat FFA_2R homologues. The human sequence originates from GenBank Accession No. AF024690, mouse NM146187, whereas the rat sequence is a novel ORF identified on chromosome 1 (rat genome contig NW043364). Identical amino acid residues are in shaded boxes. Horizontal lines indicate the seven (I–VII) putative transmembrane regions, as calculated using TMHMM 2.0.

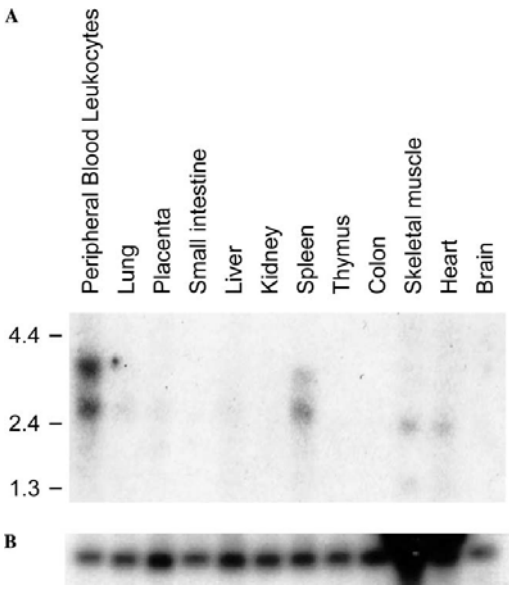

Fig. 3. (A) Autoradiograph of human multiple tissue Northern blot revealing a strong expression of GPR43 (FFA_2R) in peripheral blood leukocytes, but also in spleen, and weak expression in skeletal muscle and heart. (B) Control autoradiograph using a β-actin probe.

mobilization that reaches an activation plateau at above 10 mM and with an approximated EC_{50} value of 1–2 mM [7,24].

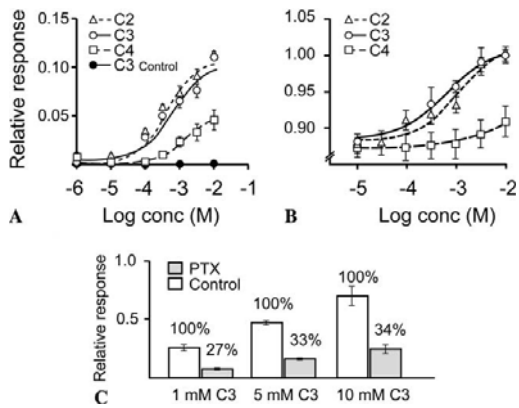

Fig. 4. (A) Calcium mobilization in GPR43 (FFA_2R)-expressing CHO cells (using aequorin) indicated as open symbols and sham-transfected control cells shown as filled circles. (B) Calcium mobilization in human PMNC (using Fura-2). C2; acetate, C3; propionate, and C4; butyrate. (C) FFA_2R-expressing reporter cells activated by C3 (propionate) in the presence of PBS (control) or pertussis toxin (PTX). The percentual reduction in the relative response in the presence of PTX is indicated. Values are means ± SD ($n = 3$).

These observations together with the results of the aequorin assay indicated that expression of recombinant FFA_2R mediates calcium mobilization in a similar manner as reported for human PMNC [7] expressing the receptor endogenously.

Concluding remarks

What the actual physiological significance is and how SCFA activation of FFA_2R affects the immune system, primarily the granulocytes, remain to be further investigated. SCFA have a multitude of cellular and immunoregulatory effects and, in fact, all related functions of PMNC, except cell adhesion, appear to be modulated by SCFA [25]. We have identified the previous orphan GPR43 as a receptor that is functionally activated by SCFA—most efficiently by acetate and propionate, but not by lactate—and with an intracellular signaling pathway that is sensitive to PTX. GPR43 was found to be highly expressed on human PMNC and had the ability to elicit calcium signaling in a recombinant model in accordance with results on human PMNC [7,24]. Our findings help clarify the "missing PTX-susceptible SCFA-activated" GPCR that has been pharmacologically acknowledged but not identified at the protein level [25]. SCFA has been classified into three different modes of action [6,7]: (i) transiently changing the pH without engaging G-proteins, (ii) modulating cell functions, such as pH level, calcium concentration, and actin through pathways not involving G-proteins, and (iii) altering cell function through calcium, IP3, and actin distribution through pathways involving G-proteins. Our findings identify GPR43, henceforth named FFA_2R, as the first GPCR mediating the SCFA effect through this third mode of action.

As previously mentioned, a physiologically relevant site of FFA_2R activation is the gut, where the bacterial presence produces SCFA in the millimolar range, which modulates the normal leukocyte activity [5]. Recent studies indicate a key regulatory function of SCFA in maintaining a healthy and controlled intestinal environment [15]. The present identification of FFA_2R will help elucidate the intricate symbiotic relationship between enteric bacteria and the immune system in gastrointestinal function. In a pathophysiological situation, SCFA-induced immunoregulation might contribute to the bacterial evasion of the immune system. High concentrations of SCFA have been reported in connection with severe anaerobic infections [26–28]. Other clinical disorders were SCFA/FFA_2R interactions might be involved include inflammatory bowel diseases, gingivitis, and propionic acidemia. It is feasible that FFA_2R provides a molecular link between SCFA and immune cells in both normal and pathophysiological conditions. This mechanism may even contribute to the beneficial effect of certain modern so-called functional foods containing specific strains of bacteria [29].

Acknowledgments

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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

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Abstract

Here, we report the cloning and identification of the third member of the free fatty acid (FFA) -activated receptor family, belonging to the rhodopsin-like subgroup of G-protein coupled receptors (GPCR). The amino acid sequence of the orphan receptor, GPR41, displayed significant homology to those of the previously identified receptors FFA_2R/GPR43 and FFA_1R/GPR40. Reporter cells expressing the GPR41 receptor up-regulated an inbuilt luciferase gene strongly in response to propionate (C3) and butyrate (C4). Also acetate (C2) and caproate (C6) activated the receptor, but less efficaciously. Similar results were achieved when measuring calcium mobilization in CHO cells using the aequorin technique. In order to investigate the ability of different FFAs to activate the three different members of the receptor family, we cross-stimulated reporter cells expressing GPR41, FFA_1R or FFA_2R with FFAs of varying chain lengths. All receptor-mediated signals were susceptible to pertussis toxin (PTX), but to a varying extent. We also tested Rosiglitazone, a thiazolidinedione-type drug, on reporter cells expressing either of the three receptors. In contrast to the FFA_1R, neither FFA_2R, nor GPR41 expressing reporter cells were activated by this compound. Accordingly we suggest the name FFA_3R for the third member of the FFA-activated receptor family

Introduction

Free fatty acids (FFAs), ranging from short- to long chain carboxylic acids, have a well documented and important physiological role, particularly related to nutrition and metabolism in both health and disease. The identification of new intracellular signaling mechanisms of these substances has emerged with the novel discovery of interaction with certain so-called orphan receptors, previously known as GPR40 [1-3] and GPR43 [4-6]. We have suggested that these receptors are re-named Free Fatty Acid Receptor 1 (FFA_1R) and Free Fatty Acid Receptor 2 (FFA_2R) because of their ability to be specifically activated by a range of FFAs.

Short-chain fatty acids (SCFAs) are produced during anaerobic metabolism in the gut and are absorbed to yield high quantities of in the blood system [7, 8]. Well known effects are seen in the colonic epithelium and in the immune system, both as sources of energy and in immune system regulation [9]. Based on the recent identification of the G-protein coupled receptor (GPCR), FFA_2R, as being activated by SCFA, it has been suggested that this receptor explains the immune regulatory ability of SCFAs [4-6]. SCFAs are known to exert a plethora of physiological effects, but not all of them can be understood by the above-mentioned mechanisms involving FFA_2R. For instance, it has been reported that SCFAs have an ability to affect adipose tissue [10] as well as regulating pancreatic insulin secretion in some species, such as ruminants [11], but not in humans [12].

In this report we describe and confirm the existence of a second molecular target for SCFAs. It is a GPCR that is highly sequentially related to FFA_2R but with a partly different range of activating ligands. The identification was achieved by cloning the orphan receptor, GPR41 [1], which was expressed in reporter cells containing a sensitive promotor functionally linked to the expression of a reporter gene [13, 14]. As the receptor was found to be functionally activated by SCFAs, we consequently suggest that this newly characterized

GPCR is renamed to Free Fatty Acids Receptor 3, FFA_3R.

Material and methods

Cloning. Cloning of the GPR41 ORF from human genomic DNA was performed by PCR using the following primers:

forward: 5′- ATTGCGGCCGCACCATGGATACAGGCCCCGACCAGT -3′;

reverse: 5′- ATTGCGGCCGCACCTAGCTTTCAGCACAGGCCACCT -3′.

PCR conditions were: 96°C for 3 min, 96°C 45 sec; 59°C 1 min; 72°C 1 min (30 cycles). The PCR products were restricted with *Not*I, subcloned into the pIRESpuro vector (Clontech), and sequenced. Cloning of the human GRP40 and GPR43 has been described previously [4, 15].

HFF11 reporter cells and assay. Construction of the HFF11 reporter cell lines expressing the GPCRs was performed as earlier reported [13, 14]. The HFF11 is based on the reporter plasmid, pcFUS3, where the 9 x TRE promoter region has been extended by 6 NFκB and 6 STAT elements [14]. On day 1, HFF11 reporter cells, either expressing the recombinant receptor or sham-transfected, were seeded into 96-well plates at 20,000 cells per well in 100 µl growth medium. On day 3, the medium was removed and replaced by 90 µl serum-free medium. Ten µl active substances or solvent controls were added in the appropriate concentrations 24 h later. Stimulations were performed at 37°C for 6 h. Cell lysates were assayed in a BMG Lumistar Galaxy luminometer [13]. Pertussis toxin (PTX) treatment was carried out at 100 ng/ml for 16 h. “Relative response” was calculated as the ratio of the experimental value (agonist) subtracted by the background value (PBS) over the maximum response value (1 µM PMA) subtracted by the background value [16].

Cell culture. CHO cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) with Glutamax-I supplemented with 10% FBS, 0.5% streptomycin and penicillin. HFF11

(HeLa) cells were grown in DMEM with Glutamax-I supplemented with 3% FBS, 0.5% streptomycin and penicillin. All cells were maintained at 37°C with 7% CO_2.

Aequorin reporter assay. Cells were seeded the day before transfection in a 6-well plate (400,000 cells/well). Then, 0.8 µg of pCDM.AEQ reporter plasmid[17] and 1 µg pIRESpuroGPR41 plasmid, or pcEGFP for controls, were mixed with 100 µl OptiMEM containing 5 µl per transfection of PLUS reagent. After 15 min, 100 µl OptiMEM and 9 µl (HeLa cells) or 5 µl (CHO cells) of lipofectamine (Invitrogen) were added and incubated for another 15 min. Then, 800 µl OptiMEM were added, and the cells were washed and incubated with the transfection solution for 5 h. Two days after transfection, cells were loaded with 10 µM coelenterazine and injected into a 96-well plate containing ligand or control (vehicle) substances. Light emission (luminescence) was recorded for 1 min in a BMG Lumistar Galaxy luminometer and normalized to total emission using 0.1% Triton and 100 mM $CaCl_2$ for each well.

Data analysis. All data points represent measurements carried out in triplicates (n=3), and each experiment was repeated three times. Statistical errors are shown as standard deviations (SD). The data were statistically analyzed using the GraphPad PRISM (GraphPad Software, San Diego, CA, USA) software package.

Phylogenic study. For receptor amino acid sequence alignment the human sequences for the FFAR family (GPR40, GPR41 and GPR43) Genbank [18] entries AF024687, AF024688 and AF024690 respectively, were used. The same order of mouse homologues sequences were AF539809, XM145470 and NM146187. For rat, the Genbank sequence AF539810 was used for GPR40 (FFA_1R), while GPR43 (FFA_2R) and GPR41 (FFA_3R) were identified in the chromosome 1 supercontig NW047557 using WU-blast [19]. Sequences for the human receptors GAL_1R, GAL_2R, GAL_3R, BLT_1R, BLT_2R, $CYSLT_1R$ and $CYSLT_2R$ were obtained

from GenBank. ClustalX [20] was used for sequence alignments and phylogenic studies. TMHMM 2.0 was used to analyze putative transmembrane (TM) domains [21].

Results and Comments

GPR41/FFA$_3$R belongs to the family of free fatty acid receptors

All free fatty acid receptor sequences from the three species; human, mouse and rat, were aligned and presented in a phylogenic tree (Fig. 1B). It is apparent that in all species the receptors form a family where FFA$_2$R and FFA$_3$R are most related, second to FFA$_1$R. This homology relationship is reflected in the activating ligand spectrum. FFA$_2$R and FFA$_3$R are both activated by short-chain fatty acids, while FFA$_1$R responds to medium- to long chain free fatty acids (Fig. 2B). As illustrated in Figure 2B, acetate (C2) and propionate (C3), used as SCFA representatives, does not cross-activate FFA$_1$R. The medium-chain length fatty acid, capric acid (C10) as well as the long-chain fatty acid, lionleic acid (C18:2) only activate FFA$_1$R transfected reporter cells and no activation of FFA$_2$R or FFA$_3$R occurs. The three human receptors share a great degree of homology (Fig.1A). A striking feature is the very short N-terminal and FFA$_1$R's unusually short C-terminal. FFA$_2$R and FFA$_3$R both have longer C-terminals, which could imply a different usage of intracellular messenger proteins. These features are conserved between species (data not shown). Preserved between all receptors and species is a longer sequence between transmembrane (TM) regions 4 and 5, resulting in a longer extra-cellular loop. It should be noted that the so-called rat GPR41 mentioned in a report [22] regarding apoptosis during ischemic hypoxia, is not the rat homologue to the human GPR41, but a different and unrelated receptor. The rat homologue sequence used in the alignment was identified using BLAST and extracted from the rat

genome. The receptor protein sequence known as GPR42 is almost identical to GPR41, but reports indicate that it might be a dysfunctional gene, a possibly inactive product of gene duplication. A recent study introduced mutations in GPR41, yielding a more GPR42-like sequence, which turned the receptor inactive, like the GPR42 receptor itself [5]. Hence, GPR42 is not to be considered a functional member of the FFAR-family and could even be the result of an evolutionary late gene duplication, as it does not appear to have a counterpart in either the mouse or rat genome. BLAST [19] analysis of available mouse and rat genomic sequences indicates the organization of a gene cluster similar to the human, but lacking a fourth (GPR42) gene. The human FFAR gene cluster is located on chromosome 19, the known mouse and rat homologues are found on chromosome 6 and 1, respectively.

GPR41/FFA$_3$R is activated by SCFAs

Based of the sequential homology with GPR43/FFA$_2$R, the putative orphan GPR41 was challenged with short-chain fatty acids (SCFAs). The reporter cells, stably expressing the human receptor, gave a significant response to SCFAs, having chain lengths ranging from 2 to 6 carbon atoms, but not sham-transfected control cells (Fig. 2A). Propionate (EC_{50}=0.25±0.1 mM) and butyrate (EC_{50}=0.4±0.15 mM) were the most potent and efficacious activators in the reporter system, followed by caproate (EC_{50}=0.3±0.05 mM) and acetate (EC_{50}=4±0.1 mM), though both with significantly lower efficacy. None of the compounds induced any signal in sham-transfected reporter cells, and propionate was chosen as a control example in Figure 2A. FFA chains comprising more than 6 carbon atoms were not able to activate FFA$_3$R. Compared to its closet relative, FFA$_2$R, the receptor activates the reporter cells less efficaciously when correlated to the stimulus of 1 μM PMA. The FFA$_3$R's inability to activate the reporter construct to a similar extent could be due to an alternative usage of signaling molecules and might also be cell-type specific. The same relationship has

been reported by others, also when testing different cell lines, such as HeLa, CHO and HEK293, and using different measuring methods [5].

Another distinctive feature of FFA_1R is its functional activation in HFF11 reporter cell system when exposed to the thiazolidinedione-type drug, Rosiglitazone. The response is specific in that FFA_2R and FFA_3R fail to induce any signal with Rosiglitazone in concentrations as high as 100 μM (Fig. 2B).

As with FFA_2R [4] it is important to point out that neither lactate nor β-hydroxybutyric acid were found to active FFA_3R, nor was the di-acid, succinate (data not shown). This points towards a specific discrepancy in the ligand-induced response possible derived from the physiological importance of discriminating between short-chain fatty acids as metabolites, on the one hand, and ketone bodies, on the other.

Calcium mobilization assays with the two SCFA activated receptors

In order to validate the ability of FFA_3R to induce cellular response upon SCFA stimulation and compare to FFA_2R, CHO cells were transiently transfected with expression plasmids containing the human FFA_2R, FFA_3R or EGFP (Enhanced Green Fluorescence Protein) genes together with vector encoding the aequorin gene [17]. Whereas the EGFP-transfected cells failed to induce calcium mobilization, both FFA_2R and FFA_3R responded to SCFA stimulation in a concentration-response dependent manner (Fig. 3). As with the reporter cells, FFA_3R signaled less efficiently in calcium mobilization assays when tested in CHO cells compared to both FFA_1R and FFA_2R. This might indicate a difference in the coupling to G-proteins and may well be of physiological relevance. The order of potency observed in this assay agrees with the above-reported order for the reporter system. We also found that FFA_1R and FFA_2R were able to mediate calcium response in HEK293 cells, whereas FFA_3R failed to do so (data not shown). This also indicates a possible difference in

signaling pathways between the receptors, which was also indicated in the following PTX studies.

Pertussis toxin affects the signaling of all three fatty acid receptors

To investigate the possible involvement of G-proteins belonging to the Gi/Go-family reporter cells expressing either FFA_1R, FFA_2R or FFA_3R were pre-treated with PTX and stimulated with an activating ligand. The linoleic acid-induced FFA_1R signal was reduced by 30% indicating a major signaling pathway other than Gi/Go in the HeLa reporter cells (Fig. 4). FFA_2R or FFA_3R expressing reporter cells were stimulated using propionate; after pre-treatment with PTX the induced signal was reduced by 65% and 75%, respectively. This suggests a more prominent involvement of the Gi/Go signaling pathway for both FFA_2R and FFA_3R. Other reports indicate that cellular signaling might differ between cell lines, as FFA_2R calcium-signaling was not inhibited by PTX in HEK293 cells [5]. In CHO cells FFA_2R was suggested to have dual Gi/o and Gq signaling pathways, while FFA_3R only seemed to function through Gi/o [6].

Discussion

FFA_1R has been shown to play a role in the insulin secretion in pancreatic islets [2, 3, 15] whereas FFA_2R is involved in the function of immune cells [4-6], but the function of FFA_3R is yet unknown. Undoubtedly it is being activated by SCFA in a similar, but yet pharmacologically distinguishable, manner as its closest relative, FFA_2R. However, the receptors do not appear to share the same physiological role.

Some speculations can be instigated based on the nature of the activating ligands as well as the proposed expression in adipose tissue [5]. Propionate has been found to be able to acutely regulate adipose tissue gene expression. A nutrient challenge, e.g. propionate

infusions in ruminants affects gene expression, lipogenesis and adipose tissue metabolism [23]. These studies where based on previous reports of propionate having acute and dose-dependent effects on the plasma levels of insulin and glucagons. Propionate infused at physiological levels stimulates the secretion of insulin from the pancreas [24]. Earlier studies also indicate similar findings in goats, where butyrate and propionate, but not acetate or β-hydroxybutyrate, augment insulin secretion [11, 25]. SCFAs with branched carbon chains show a greater insulin-releasing activity than their isomers with straight chain lengths [26]. Even though the pharmacological profile of the of the insulintropic SCFAs seen in ruminants does indeed appear to match the one of FFA_3R it should be noted that all the above-mentioned studies have been reported only in ruminants It has been suggested that this is a special feature of the ruminant pancreas, since only small changes in the propionate-stimulated insulin secretion was noted in rat [11]. Efforts trying to confirm the same physiological function in humans have failed [12]. Another aspect of a metabolically related effect of propionate and butyrate is the inhibitory effect on growth gormone (GH) secretion [27].

The FFA_3R is the third functional member in the GPCR family activated by free fatty acids, and it shows the highest homology with FFA_2R, which are both activated by SCFAs. FFA_1R is activated by medium- to long-chain free fatty acids and, importantly, the only of the three receptors that is affected by thiazolidinedione-type drug, Rosiglitazone.

In the course of our work with the complete family of fatty acid-activated receptors, two publications reported similar findings [5, 6]. Our findings agree with these, but determined using alternative methods. This report summarizes the existence of a GPCR family consisting of three previously unknown receptors, sharing similar amino acid sequence homology as well as related ligand identity.

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Fig. 1. (A) Alignment of the three human fatty acid activated receptors. Identical amino acids are indicated by a black box, while similar ones are grey. Grey lines above the amino acid sequences illustrate predicted FFA_1R TM regions, whereas consensus TM regions for FFA_2R and FFA_3R are indicated with a line below the sequences. FFA_1R share approximately 30% amino acid sequence identity with both FFA_2R and FFA_3R, whilst the two latter have 35% identical amino acid residues. (B) Phylogenetic study of the three free fatty acid-activated receptors. Sequences from three species were included in the study; human, mouse and rat. The sequences of a few other human GPCRs (galanin receptor 1-3, leuktriene receptor 1-2 and cysteinyl receptor 1-2) were also included. Numbers on the branches indicate boot strap values in percent (n=1000). While

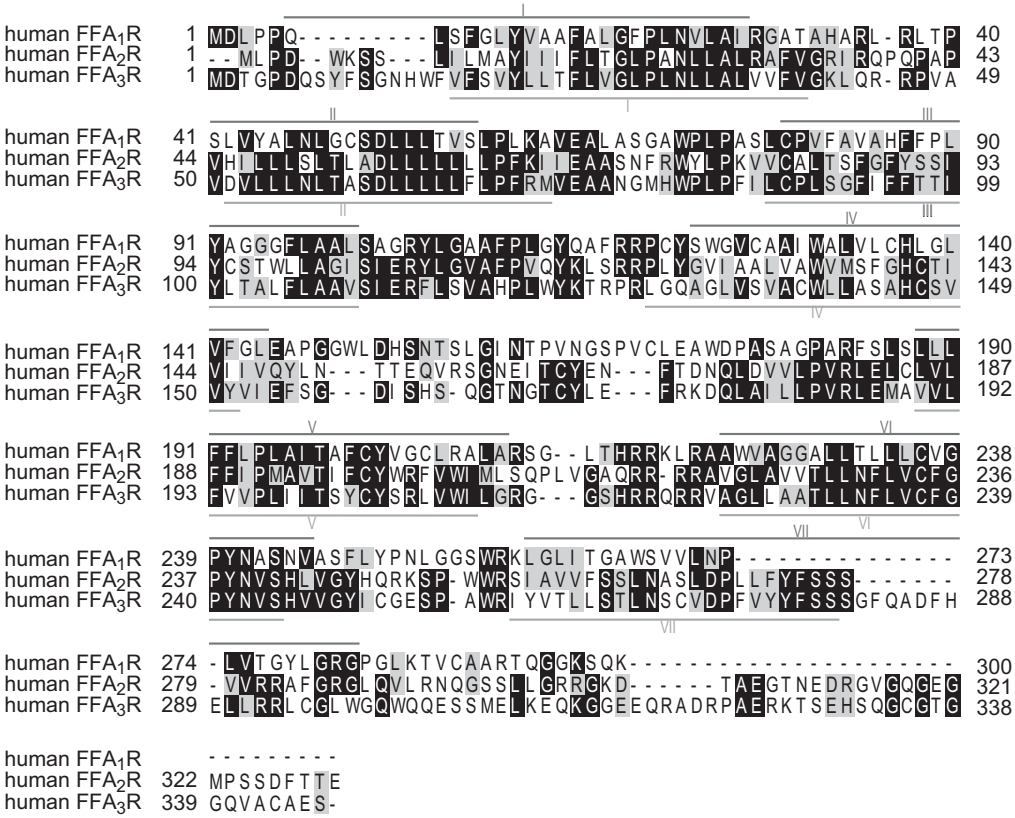
Fig. 2. (A) FFA_3R (GPR41) is activated by a range on SCFAs. Reporter cells stably transfected with the plasmid encoding human GPR41 generate a luciferase response when stimulated with propionate (C3), butyrate (C4), acetate (C2) and caproate (C6). Sham-transfected reporter cells fail to respond to any of the SCFAs (propionate used as an example). (B) Reporter cells transfected with FFA_1R, FFA_2R, FFA_3R or plasmid only (sham), were all challenged with 10 mM acetate (C2), 3 mM propionate (C3), 50 μM capric acid (C10), 50 μM linoleic acid (C18:2) and 10 μM Rosiglitazone. FFA_2R and FFA_3R only respond to SCFAs (acetate and propionate) while medium- to long-chain fatty acids (capric acid and linoleic acid) are required to activate FFA_1R. Rosiglitazone only affects FFA_1R and none of the ligands induce a signal in sham cells. Values are means ± standard deviation (SD) (n=3).

Fig. 3. Calcium mobilization in CHO cells (using aequorin). Light grey bars represent fluorescence from CHO cells expressing FFA_2R (GPR43), dark grey FFA_3R (GPR41) and white sham transfected. Each receptor was assayed with three concentrations of acetate, propionate, butyrate and vehicle only (PBS). Only CHO cells expressing FFA_2R and FFA_3R respond with calcium mobilization due to SCFA stimulation. The measured FFA_3R signal is overall lower compared to FFA_2R. A pharmacological difference is noticeable in the order of ligand activity; FFA_2R: Acetate ≥ Propionate > Butyrate and FFA_3R: Butyrate ≥ Propionate > Acetate.

Fig. 4. Pertussis toxin inhibits signaling in HeLa reporter cells. Pre-treatment with PTX resulted in a signal level of approximately 70% in the case of FFA_1R, 35% for FFA_2R and respectively 25% for FFA_3R. Varied susceptibility to PTX may indicate alternative down stream signaling mechanisms.

Fig. 1

A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human FFA2R 1 --ML PD--WKSS---LILMAYIIIFLTGLPANLLALRAFVGRIRQPQPAP 43
human FFA3R 1 MDTGPDQSYFSGNHWFVFSVYLLTFLVGLPLNLLALVVFVGKLQR-RPVA 49
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human FFA2R 44 VHILLLSLTLADLLLLLLPFKIIEAASNFRWYLPKVVCALTSFGFYSSI 93
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human FFA2R 94 YCSTWLLAGISIERYLGVAFPVQYKLSRRPLYGVIAALVAWVMSFGHCTI 143
human FFA3R 100 YLTALFLAAVSIERFLSVAHPLWYKTRPRLGQAGLVSVACWLLASAHCSV 149
human FFA1R 141 VFGLEAPGGWLDHSNTSLGINTPVNGSPVCLEAWDPASAGPARFSLSLLL 190
human FFA2R 144 VIIVQYLN---TTEQVRSGNEITCYEN---FTDNQLDVVLPVRLELCLVL 187
human FFA3R 150 VYVIEFSG---DISHS-QGTNGTCYLE---FRKDQLAILLPVRLEMAVVL 192
human FFA1R 191 FFLPLAITAFCYVGCLRALARSG--LTHRRKLRAAWVAGGALLTLLLCVG 238
human FFA2R 188 FFIPMAVTIFCYWRFVWIMLSQPLVGAQRR-RRAVGLAVVTLLNFLVCFG 236
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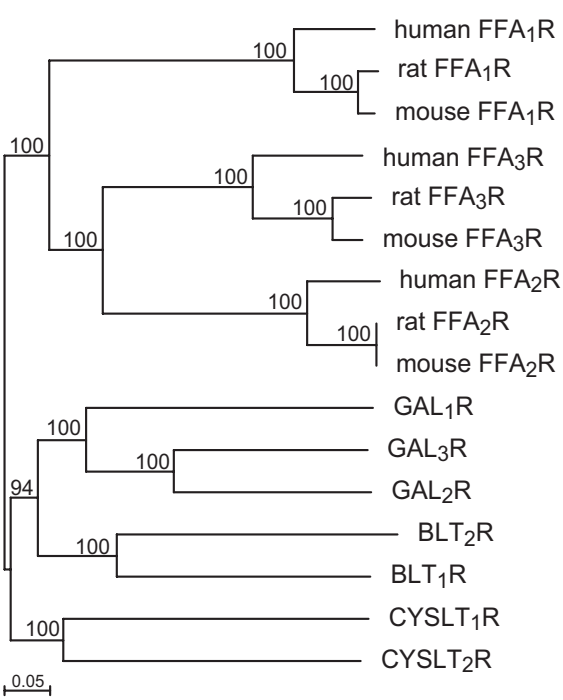
B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GAL2R
BLT2R
BLT1R
CYSLT1R
CYSLT2R
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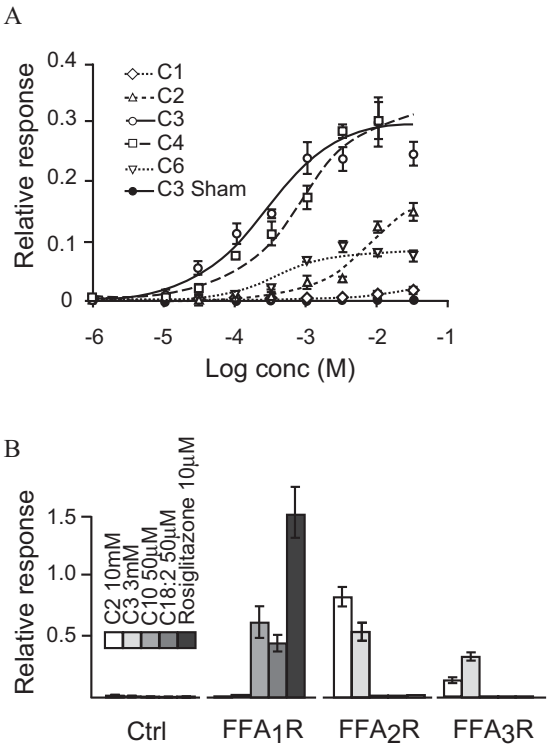
94
0.05

Fig 2


A
Relative response
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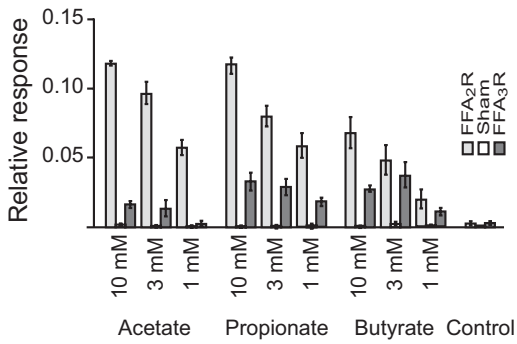
0.3
0.2
0.1
0
C1
C2
C3
C4
C6
C3 Sham
-6
-5
-4
-3
-2
-1
Log conc (M)
B
Relative response
1.5
1.0
0.5
C2 10mM
C3 3mM
C10 50µM
C18:2 50µM
Rosiglitazone 10µM
Ctrl
FFA1R
FFA2R
FFA3R

Fig 3


Relative response
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0.10
0.05
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Sham
FFA3R
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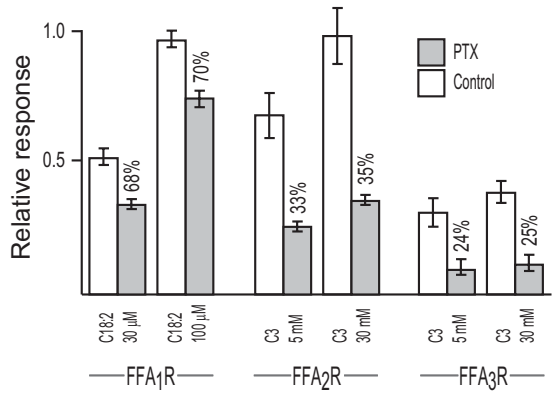
1 mM
10 mM
3 mM
1 mM
10 mM
3 mM
1 mM
Acetate
Propionate
Butyrate
Control

Fig 4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PTX
Control
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70%
33%
35%
24%
25%
C18:2 30 µM
C18:2 100 µM
C3 5 mM
C3 30 mM
C3 5 mM
C3 30 mM
FFA1R
FFA2R
FFA3R

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