The elusive endogenous adipogenic PPAR γ agonists: lining up the suspects

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Abstract

The nuclear receptor peroxisome proliferator-activated receptor γ (PPAR γ) is the key decisive factor controlling the development of adipocytes. Ligand-mediated activation of PPAR γ occurs early during adipogenesis and is thought to prime adipose conversion. Although several fatty acids and their derivatives are known to bind to and activate PPAR γ , the identity of the ligand(s) responsible for initiating adipocyte differentiation is still a matter of debate. Here we review recent data on pathways involved in ligand-production as well as possible endogenous, adipogenic PPAR γ agonists.

Keywords

PPAR γ

Adipogenesis

Polyunsaturated fatty acids

Cyclooxygenases

Lipoxygenases

Reactive oxygen species

Abbreviations

$\cdot\text{NO}$	Nitric oxide
$\cdot\text{NO}_2$	Dioxide radical
$\cdot\text{O}_2$	Superoxide
$\cdot\text{ONO}_2^-$	Peroxynitrite
15-dPGJ ₂	15-deoxy- ^{12,14} -prostaglandin J ₂
AA	Arachidonic acid

ADH	Alcohol dehydrogenase
AF-1	Ligand-independent activation function
AF-2	Ligand-dependent activation function
azPC	Azelayol phosphatidylcholine
BAT	Brown adipose tissue
CBR	Carbonyl reductase.
C/EBP	CCAAT/enhancer binding protein
ChREBP	Carbohydrate element binding protein
COX	Cyclooxygenase
CPA	Cyclic phosphatidic acid
cPLA ₂	Cytosolic PLA ₂
CYP	Cytochrome P450 monooxygenase
DHET	Dihydroxyeicosatrienoic acid
DPEP	Dipeptidase
EET	Epoxyeicosatrienoic
EH	Epoxide hydrolase
ELOVL3	Elongation of very long chain fatty acids 3
EPA	Eicosapentaenoic acid
eLOX3	Epidermis lipoxygenase type 3
Epac	Exchange protein directly activated by cAMP
FAD	Flavin adenine dinucleotide
FAS	Fatty acid synthase
FFA	Free fatty acid
GGT	Gamma-glutamyltransferase
GPC	Glycerophosphocholine
GPx	Glutathione peroxidase
HETE	Hydroxyeicosatrienoic acid

HODE	Hydroxyoctecenonic acid
HPETE	Hydroperoxyeicosatetraenoic acid
HPGD	15-hydroxyprostaglandin dehydrogenase
HPODE	Hydroperoxyoctadecadienoic acid
IBMX	Isobutyl-methylxanthine
KETE	Ketoeicosatetraenoic acid
LA	Linoleic acid
LBD	Ligand binding domain
LNO ₂	Nitrolinoleic acid
LOX	Lipoxygenase
LPA	Lysophosphatidic acid
LTC ₄ S	Leukotriene C ₄ synthase
MAPK	Mitogen-activated protein kinase
MEF	Mouse embryonic fibroblast
Mo-Co	Molybdenum cofactor
MUFA	Monounsaturated fatty acid
NOS	Nitric oxide synthase
NOX4	NADPH oxidase 4
NR	Nuclear receptor
PG	Prostaglandin
PGDH	15-hydroxyprostaglandin dehydrogenase
PGFS	Prostaglandin F synthase
PL	Phospholipase
PPAR γ	Peroxisome proliferator-activated receptor γ
PPRE	Peroxisome proliferator response element
pRB	Retinoblastoma protein
PTGDS	Prostaglandin D ₂ synthase

PTGES	Prostaglandin E synthase
PTGIS	Prostacyclin synthase
PTGR	Prostaglandin reductase
PTM	Post-translational modification
PUFA	Polyunsaturated fatty acid
RetSat	Retinol saturase
ROS	Reactive oxygen species
RXR	Retinoic X receptor
sEH	Soluble epoxide hydrolase
SREBP-1c	Sterol-regulatory element binding protein 1c
TBXAS	Thromboxane A synthase
TX	Thromboxane
TZD	Thiazolidinedione
UCP1	Uncoupling protein 1
WAT	White adipose tissue
XOR	Xanthine oxidoreductase

1. Introduction

Adipose tissue was long regarded as an organ solely used for energy storage and release. However, the onset of the obesity epidemic spurred great interest into understanding the biological function of adipose tissue and today it is clear that adipocytes are important regulators of whole body homeostasis [1].

Adipocytes function as energy storages. Millenniums of evolution have optimized their ability to efficiently store energy in times of plenty. This has according to the thrifty gene hypothesis given a survival advantage when supplies were scarce [2]. Today, however, where caloric overflow rather than shortage is a challenge in most societies, this feature has turned into a disadvantage. Indeed, enlarged and dysfunctional adipose stores as seen during obesity are clearly associated with the development of type II diabetes mellitus. This is not only due to perturbations in the energy flux into adipocytes which leads to systemic lipid depositions. Unbalanced secretion of cytokines from adipocytes, referred to as adipokines, affects processes such as appetite, energy consumption and insulin signaling.

Adipose tissues have traditionally been divided into unilocular white (WAT) and multilocular brown adipose tissue (BAT). This distinction was at least partly rooted in the expression of uncoupling protein 1 (UCP1) which was thought to be restricted to the classic brown adipose stores. UCP1 allows leakage of protons through the inner mitochondria membrane thereby dissipating the electrochemical gradient and producing heat instead of ATP.

It was, however, known that diet or cold stress could induce the appearance of UCP1 in the white compartments [3, 4]. The discovery that classic brown adipocytes in contrast to their UCP1-expressing white relatives share origin with myocytes [5, 6], led to the terms “beige” [7] or “BRITE” [8] to name these brown-in-white adipocytes. Interestingly, the UCP1-expressing adipocytes found in adult humans bare a closer resemblance to BRITE adipocytes rather than classic brown adipocytes [9, 10].

Research on adipose tissue development has revealed an essential role for the peroxisome proliferator-activated receptor γ (PPAR γ) in the control of adipocyte formation. Hence, it is often referred to as a master regulator of adipogenesis [11].

PPAR γ was originally cloned independently by the groups of Ronald Evans and Bruce Spiegelman [12, 13]. It belongs to the family of transcription factors denoted nuclear receptors (NRs). Two other members of this family share high homology with PPAR γ , namely PPAR α and PPAR δ /PPAR β . Interestingly, whereas PPAR γ is involved in lipid storage and anabolism, evidence points towards a role for both PPAR α and PPAR δ in lipid catabolism [14-16].

At the protein level PPAR γ exists as two different isoforms of the same gene, PPAR γ 1 and PPAR γ 2 (Figure 1) [17]. The two splice forms are almost identical with the exception that PPAR γ 2 has an extended N-terminal (28 amino acids in humans and 30 in mice). Whereas PPAR γ 1 is more widely expressed, PPAR γ 2 is almost exclusively present in the adipose tissues.

The N-terminal part of PPAR γ contains the A/B-domain harboring the ligand-independent activation function (AF-1) which is followed by the zinc-finger DNA-binding C-domain. These domains are tethered to the C-terminal region by a hinge region called the D-domain. The C-terminal contains the E-domain which contains the dimerization domain, the ligand-binding domain (LBD), and the ligand-dependent activation function (AF-2).

Shortly after its cloning, it was shown that ectopic expression of PPAR γ could induce adipogenesis in fibroblasts; an effect augmented by the presence of relatively nonspecific PPAR γ ligands [18]. The later identification of PPAR γ as the endogenous target of the antidiabetic thiazolidinediones (TZDs) [19] provided an explanation for

earlier observations showing that the TZD pioglitazone increased adipocyte differentiation in preadipocyte cell lines [20, 21]. The efficacy by which PPAR γ induces adipogenesis is underscored by the fact that forced expression of PPAR γ causes adipocyte differentiation in both cultured myogenic and osteogenic cells [22, 23].

The requirement for PPAR γ in adipocyte differentiation is emphasized by the inability of PPAR γ -deficient embryonic stem cells [24] and mouse embryonic fibroblasts (MEFs) [25] to undergo adipogenesis. Complete knockout of PPAR γ is embryonic lethal at gestational day 10-10.5 due to placental dysfunction [25, 26].

Three groups have circumvented the early lethality by the use of different techniques. Spiegelman and colleagues made chimeric mice derived from wildtype and PPAR γ -deficient ES cells. Whereas PPAR γ null cells contributed to several tissues, adipose tissue was devoid of any PPAR γ -deficient cells [24]. Evans and colleagues tethered diploid embryos derived from PPAR γ ^{+/-} crosses to tetraploid embryos from wildtype mice. The tetraploid morulas did not contribute to the embryo but rescued the placental defect. The embryos were derived solely from the diploid cells. One PPAR γ -null pup survived until after term. It died shortly hereafter, but was completely devoid of adipose tissue [26]. Later, Mortensen and coworkers knocked out PPAR γ in the embryo except in trophoblasts that form the placenta. PPAR γ -deficient mice were born at a Mendelian ratio, but only 10 % of these survived to adulthood. These mice suffered from severe lipodystrophy and insulin resistance [27].

Mice heterozygous for PPAR γ have less adipose tissue when fed a high-fat diet although this could be correlated to increased energy expenditure [25, 28]. In contrast to what was expected based on the positive effect of TZDs on insulin sensitivity, PPAR γ ^{+/-} mice displayed decreased insulin resistance [25, 28, 29].

The conclusions drawn from genetic ablation studies have been strengthened by both *in vitro* and *in vivo* experiments with dominant negative versions of PPAR γ . A PPAR γ with a deletion of the 16 outermost C-terminal residues showed dominant negative effect towards PPAR γ and inhibited adipogenesis when ectopically expressed [30]. Based on homology with the thyroid hormone receptor β and the dominant negative effect observed when mutating analogous amino acids, Chatterjee and colleagues mutated two C-terminal residues (Leu496Ala and Glu499Ala) in human PPAR γ . As expected, this mutant was dominant negative towards PPAR γ and forced expression inhibited adipocyte differentiation of human preadipocytes [31]. Mutating only the homologous leucine in mouse PPAR γ resulted in a mutant with similar ability [32]. Knockin of this mutant in mice was embryonic lethal if homozygous and heterozygous mice were lipodystrophic and insulin resistant [33].

Not only is PPAR γ necessary for the differentiation of adipocytes. It is also required for their survival. Mice with adipose specific ablation of PPAR γ by Fatty acid binding protein 4 (*Fabp4*) promoter driven Cre-expression and excision of a floxed PPAR γ suffer from hypocellularity and lipodystrophy [34]. By making the Cre activity controllable it was later shown that adipose tissues are replenished with new adipocytes [35].

In an attempt to knockin the best described human polymorphism of PPAR γ 2 (Pro12Ala), Auwerx and colleagues unintended knocked down PPAR γ expression in WAT likely due to interference of the neomycin cassette with PPAR γ expression. Several of these mice, named PPAR $\gamma^{\text{hyp/hyp}}$, died early and the survivors suffered from severe lipodystrophy and absence of WAT. The more pronounced lipodystrophy seen in the

PPAR $\gamma^{hyp/hyp}$ mice properly originates from the absence of PPAR γ during adipocyte differentiation, whereas the *Fabp4*-driven knockouts loose PPAR γ after it has been active for a short period in the adipocyte [36].

In order to better understand the effect of the Pro495Leu human polymorphism, which is linked to lipodystrophy and insulin resistance [37, 38], the mutation was knocked-in in mice. Homozygous mice died during embryonic development whereas heterozygous mice survived. These heterozygous mice showed abnormal fat distribution and decreased brown adipose recruitment [39, 40]. Curiously, exactly opposite to human subjects with the polymorphism, heterozygous Pro495Leu mice had an increment in extra-abdominal adipose tissue at the expense of visceral adipose tissue. This could explain the normal insulin sensitivity in these mice as subcutaneous fat is related to higher sensitivity for insulin. Indeed, mice with the Pro495Leu polymorphism on a leptin-deficient background (*ob/ob*) developed insulin resistance. Here, the positive effect on insulin sensitivity of subcutaneous WAT likely drowned in the extreme adiposity [41].

As PPAR γ 2 expression is restricted to adipose tissue, it was speculated if only this isoform is required for adipogenesis. The question was since answered by the use of two different *in vitro* approaches – and with two different outcomes. By the use of engineered zinc-finger-mediated knockdown of PPAR γ , Camp and colleagues showed that only forced expression of PPAR γ 2 was able to restore adipogenesis [42]. On the contrary, Mueller et al. observed that PPAR γ 1 was able to restore adipogenesis in PPAR γ null MEFs albeit with a lower efficiency compared with PPAR γ 2 [43].

Two studies have addressed the same question *in vivo* by making specific PPAR γ 2 knockouts. Whereas both groups reported a decrease in insulin sensitivity in male mice, only Zhang et al. observed a decrease in WAT stores [44]. Medina-Gomez et

al. found a normal adipose development even though expression of lipogenic and β -oxidative genes was decreased [45]. Both groups reported a decrease in adipogenic potential when trying to differentiate PPAR γ 2-deficient MEFs *in vitro*.

Curiously, both groups reported a normal development of BAT in their respective knockouts. The PPAR $\gamma^{\text{hyp/hyp}}$ mice described earlier also developed BAT although it was more fat-laden compared with the BAT seen in wildtype mice due to the lack of WAT. The PPAR $\gamma^{\text{hyp/hyp}}$ mice express PPAR γ 1, and not PPAR γ 2 in their BAT. Taken together, these results suggest that PPAR γ 2 is not as important for development of BAT as it is for WAT. One interpretation of these results would be that merely increasing the level of PPAR γ 1 relative to PPAR γ 2 would favor BAT development at the expense of WAT. This is, however, not the case as mice lacking PPAR γ 2 did not express UCP1 in their WAT [44].

Finally, mice with a mutation at serine 112 (Ser112Ala) in PPAR γ , a site known to be phosphorylated by the mitogen-activated protein kinase (MAPK) [46], showed no increase in adipose mass, these mice still had increased insulin sensitivity and MEFs prepared from these mice had increased adipogenic potential indicating augmented PPAR γ activity [47].

The effects of PPAR γ germline or adipose specific knockouts are summarized in table 1. Collectively, all data emphasize the importance for PPAR γ in adipocyte development and function, rightfully earning PPAR γ the title of the “master regulator of adipogenesis”. There appears to be an almost strict correlation between PPAR γ activity and adiposity, whereas the relation between PPAR γ and insulin sensitivity is more complex [48, 49].

1.1 Regulation of transcription

PPAR γ functions as a transcription factor through sequence-specific binding to DNA followed by either hindering the access or increasing the recruitment of the transcriptional machinery. Binding to DNA is coordinated with other transcription factors. PPAR γ forms heterodimers with its obligate partner, the transcription factor retinoid X receptor (RXR) [50]. Together they recognize and bind the consensus AGGTCA sequence repeated once and separated by a nucleotide, the direct repeat (DR-1), commonly referred to as the peroxisome proliferator response element (PPRE).

Besides the RXRs, PPAR γ also has intimate relations with the family of CCAAT/enhancer binding proteins (C/EBPs). During the early stages of adipose conversion, C/EBP β and C/EBP δ are thought to prime the binding of PPAR γ to DNA [51]. At the later stages, a tight collaboration with C/EBP α is essential for formation of fully functional adipocytes [52-54].

It is, however, likely that interplay of PPAR γ with other transcription factors extends even beyond that of the RXRs and the C/EBPs. In the initiation of adipogenesis, the formation of hotspots with the concomitant binding of numerous transcription factors is a prerequisite for effective adipocyte differentiation [55].

Besides its interplay with other transcription factors, PPAR γ binds numerous other proteins involved in the regulation of transcription. The general term cofactors have been linked to these auxiliary proteins. The hunt for cofactors led to the identification of PPAR γ cofactor 1 α (PGC-1 α) [56]. Although dispensable for adipocyte differentiation, this coactivator is together with its close relative, PGC-1 β , essential for mitochondrial biogenesis in adipocytes [57]. The constantly growing list of cofactors has revealed that

these proteins not only are involved in regulating adipogenesis, but also have a decisive role in determining the “color” of the formed adipocyte [58].

The simplified model for how NRs control gene expression states that in the absence of ligand or in the presence of antagonist, the NR recruits a corepressor complex to a given promoter thereby repressing transcription actively. In the presence of ligand, the corepressor complex is exchanged with a coactivator complex which ultimately initiates transcription. This description is, however, too simplified as a potent agonist can also lead to repression of genes. Therefore, it is evident that the coregulator state of a NR depends on both the promoter and the given cell type [59].

The promoter-dependent recruitment was elegantly demonstrated in adipocytes by Lazar and colleagues. Guan et al. showed that in mature adipocytes the PPRE in *Fabp4* promoter was bound by coactivators whereas the PPRE in the glycerol kinase (GyK) had corepressors recruited. Upon administration of PPAR γ ligands, coactivators were recruited to the GyK PPRE and expression of the gene was heavily increased, whereas expression of *Fabp4* was minimally induced [60]. The barely detectable increase of *Fabp4* in adipocytes when treated with rosiglitazone contrasts the situation in immature dendritic cells, where *Fabp4* expression increased dramatically in response to the same drug [61]. The strong induction of *Fabp4* suggests binding of corepressors to the *Fabp4* PPRE in the absence of ligand in this cell type. The recent delineations of genes up- or downregulated, combined with mapping of transcription factor binding elicited by a strong PPAR γ agonist in adipocytes is a crucial first step in understanding the mechanism by which PPAR γ orchestrates its ligand-mediated and promoter-specific induction or repression of transcription [62, 63].

The activity of PPAR γ has also been shown to be regulated by several post-translational modifications (PTMs). Phosphorylation of serine 112 by MAPK repressed the activity of PPAR γ [46, 64]. A mutation preventing phosphorylation of serine 112

resulted *in vivo* in smaller adipocytes and increased insulin sensitivity [47]. Curiously, cyclin dependent kinases (CDKs)-mediated phosphorylation of the same serine residue was reported to increase the activity of PPAR γ [65, 66]. It is therefore likely, that other phosphorylation events modulate the outcome of serine 112 phosphorylation. Of note, CDK5 is reported to be responsible for the obesity-induced phosphorylation of PPAR γ at serine 273 which modulates the activity of PPAR γ [67]. Other PTMs affecting the activity of PPAR γ include acetylation [68], SUMOylation [69] and glycosylation [70].

1.2 Ligands and PPAR γ

A unifying feature of most NRs is their activation by small, hydrophobic ligands. Several fatty acids and their oxidized or nitrated derivatives are known to act as ligands for PPAR γ [71]. Besides the known naturally occurring ligands, the earlier mentioned TZDs are potent agonists for PPAR γ [19]. This promiscuity towards agonists can at least partially be explained by the large ligand-binding pocket of PPAR γ [72, 73] that even allows concomitant binding of two ligands [74].

Interestingly, combining ligands with affinities for separate regions within the pocket, one with and one without an active cysteine residue, synergizes in PPAR γ activation [75, 76]. Adding to the intricacy of PPAR γ ligand binding was the recent description of an alternative binding site for several synthetic PPAR γ agonists including but not limited to MRL20, MRL24 and SR9034. The TZDs showed little potency for this alternate site [77]. It is therefore evident that the binding of ligands to PPAR γ is very complex.

The complexity is however not limited to the binding of ligands. Even activation of the PPAR γ AF-2 differs between ligands. This has classified some agonists as partial

as they even at saturating concentrations do not elicit the same activation level as full agonists, such as TZDs. This seems to be caused by differences in the conformational changes induced by ligand-binding. Whereas full agonists lock the 12th of the 13 α -helices in the LBD leading to efficient cofactor binding, this is not the case for partial agonists which stabilize other regions of the LBD [72, 78].

Hitherto, research on the functional impact of PPAR γ ligand binding has mainly focused on the ability to recruit or dislodge core transcriptional machinery constituents. Therefore, the recent identification of ligands with little to no classic transcriptional agonism activating PPAR γ in adipose tissue of obese mice by preventing an inhibitory PPAR γ phosphorylation was surprising [67, 79]. It is however still unknown if endogenous ligands have similar capabilities.

Ablation of adipocyte differentiation by PPAR γ antagonists strongly suggests a requirement for ligand activation of PPAR γ during adipogenesis [80-82]. In addition, administration of potent full PPAR γ agonists such as rosiglitazone induces browning of mouse [8] as well as human white adipocytes [83, 84] adding another aspect to ligand-dependent activation of PPAR γ in the context of adipocyte function.

Generally, ligands activate PPAR γ by inducing conformational changes in AF-2, thereby easing the replacement of co-repressors with co-activators. However, while the AF-2 domain activity is increased by ligands it is not entirely quiescent in their absence. The fact that a PPAR γ mutant without ligand binding capacity could restore adipogenesis as efficient as the wild type PPAR γ in PPAR γ -deficient MEFs suggested that ligand binding might not be required for the initiation of adipogenesis [85]. As the production of PPAR γ ligands happens early during adipogenesis when PPAR γ expression is at its lowest [82, 86], the early boost in AF-2 activity normally provided by a ligand was likely

circumvented by the high ectopic expression of either wild type or ligand-binding deficient PPAR γ . Notably, a mutant entirely lacking the AF-2 function was unable to rescue differentiation [85]. This inability of the AF-2 mutant to restore adipogenesis is thus consistent with PPAR γ antagonist experiments, both underscoring a requirement for the normal ligand-activated AF-2 function of PPAR γ .

Even though PPAR γ is best known for its role in adipogenesis, the true nature of its ligand during adipose conversion still remains elusive. Importantly, incubation of a lipid extract of media from differentiating adipocytes with GST-PPAR γ , but not GST alone, was able to remove the compound(s) that increased the activity of the PPAR γ LBD [82]. Therefore, the endogenous ligand is not only able to diffuse out of the cell but it also has the ability to bind and activate PPAR γ indicating the presence of one or more PPAR γ ligands during initiation of adipocyte differentiation.

With the identification of PPAR γ as the target of the insulin-sensitizing TZDs, there has been a huge interest in identifying agonists with lower side-effect burdens as compared to TZDs. This led to a surge in papers describing novel synthetic agonists, some of which have progressed to clinical tests. Furthermore, it is now evident that several natural, dietary compounds have PPAR γ activating capabilities. The scope of this review is solely to delineate the cellular pathways currently suggested to be involved in the generation of PPAR γ ligands during adipocyte differentiation. For a detailed description of the synthetic and natural product PPAR γ agonists we refer to excellent reviews [87-89].

2. Fatty acids

Most fatty acids can activate PPAR γ at micromolar concentrations [90-92]. An exception is erucic acid that does not activate PPAR γ [91, 93]. In general, polyunsaturated fatty acids (PUFAs) are better activators of PPAR γ than monounsaturated fatty acids (MUFAs) and saturated fatty acids [90-92]. Although the transactivation capacity of fatty acids is relatively poor it mirrors their ability to stimulate 3T3-L1 cell differentiation [94]. Activation of PPAR γ by very long-chain fatty acids has been suggested to be necessary for adipogenesis as knockdown of elongation of very long chain fatty acids 3 (ELOVL3) decreases adipose conversion of 3T3-L1 preadipocytes [95]. However, the expression of ELOVL3 does not follow the kinetics for ligand production [82, 86] with ELOVL3 expression being lowest at the onset of differentiation [95].

Of several fatty acids, arachidonic acid (AA) was reported to be the most potent binder and activator of PPAR γ [91]. As fatty acid derivatives in general are better activators of PPAR γ than the fatty acids themselves [94], we will discuss AA-derived compounds in relation to PPAR γ activation in detail below. Furthermore, AA is the best described substrate of three families of lipid-modifying enzymes suggested to be involved in PPAR γ ligand production. It is, however, important to note that other fatty acids such as linoleic acids (LA) and eicosapentaenoic acid (EPA) can also function as a substrate for these enzymes.

3. Oxidized Polyunsaturated Lipids

The best described naturally occurring ligands are oxidized lipids derived from AA i.e. the eicosanoids, and there is strong evidence that the presence of an α,β -unsaturated electrophile is critical for covalent binding to the cysteine residue 285 (Cys-285) within the PPAR γ ligand binding pocket and potent activation of the receptor

[96]. Three enzyme families are capable of generating such eicosanoids by oxygenation of AA: cyclooxygenases (COXs), lipoxygenases (LOXs), and cytochrome P450 monooxygenases (CYPs) (Figure 2).

The COX and LOX enzymes both dioxygenate AA and thereby resemble lipid auto-oxidation by reactive oxygen species (ROS). However, whereas the latter results in random outcomes, the enzymatic control provides stereo and positional specificity to the oxygen addition [97, 98]. The two COX enzymes, i.e. the prostaglandin endoperoxide isomerases, produce prostaglandin (PG) H₂ which can be transformed to a wide array of prostanoids and thromboxanes (TX) by unique synthases.

Most LOXs yield hydroperoxyeicosatetraenoic acids (HPETEs), which can be further transformed by glutathione peroxidase (GPx) to hydroxyeicosatrienoic acids (HETEs) or by the epidermis lipoxygenase type 3 (eLOX3), a hydroperoxide isomerase or epoxyalcohol synthase, to yield α,β -unsaturated γ -hydroxy epoxides [99]. These enzyme families can also metabolize LA to produce hydroperoxyoctadecadienoic acids (HPODEs) yielding their respective hydroxyoctadecenoic acids (HODEs). While 15-LOX is enantiospecific, yielding the 13-S-HODE [100], COX metabolism produces R/S mixtures of both the 9- and 13-HODEs [101]. The further metabolism of HETEs and HODEs by the so-called 13-HODE dehydrogenase results in the formation of fatty acid ketones [74, 102], which are themselves putative PPAR γ ligands.

The fatty acid CYP monooxygenases come in two forms, CYP2 epoxygenases and CYP4 omega-hydroxylases, which add one oxygen to AA generating either epoxyeicosatrienoic (EETs) or ω and ω -1 hydroxyhydroeicosatetraenoic acids (e.g. 20-HETEs), respectively [103]. The role for these monooxygenases in adipogenesis remains virtually unexplored. While the 20-HETE, EETs and dihydroxyeicosatrienoic acids (DHETs) derived from the EETs by the soluble epoxide hydrolase (sEH) are reported activators of PPAR α [104] and PPAR γ [105], chemical inhibition of this family of

enzymes does not affect adipogenesis [86]. Moreover, the finding that EETs inhibited adipogenesis argues against an involvement of this class of fatty acid derivatives in the ligand-mediated activation of PPAR γ during adipogenesis [106-108]. In contrast, expression of the ω -hydroxylases CYP4A11 and CYP4F2 are reported to decrease mesenchymal stem cell-derived adipocytes, and 20-HETE-dependent adipogenic stimulation has been reported to be dependent on cyclooxygenase-2 metabolism [109].

3.1. Prostaglandins

The discovery that several prostaglandins are able to activate the PPAR γ -LBD indicated for the first time a possible interplay between this family of lipids and the nuclear receptor [92]. Shortly after, the laboratories of Evans and Lehmann showed that the PGD₂ metabolite, 15-deoxy-^{12,14}-prostaglandin J₂ (15-dPGJ₂) acted as a PPAR γ agonist with adipogenic-stimulating effect, maturing the suggested interplay into a dogma [110, 111]. Interestingly, while the binding-affinity of 15-dPGJ₂ for PPAR γ is low compared to TZDs, equimolar concentrations of either is required in a number of cellular models to elicit the same response [112].

Recent data, however, argue against PGD₂-derived products as the endogenous ligand associated with adipocyte differentiation. In particular, the concentration of 15-dPGJ₂ in media and cells of differentiating 3T3-L1s is several orders of magnitude lower than the exogenous concentration required to induce adipogenesis [113, 114]. Also, it is not generated in a timely fashion with its production peaking late during differentiation [115]. Furthermore, while PGD₂-derived ¹²-PGJ₂ can act as a PPAR γ ligand and induce adipogenesis, its levels increase gradually during adipocyte differentiation (Figure 3) [114, 116]. Since PGs and other fatty acids are bound to albumin within culture media, thus, lowering the effective concentration of an exogenous fatty acid such data must be

interpreted cautiously. Still, lipid extracts of media from differentiating adipocytes containing PPAR γ -ligand(s) did not co-migrate with 15-dPGJ₂ in a thin-layer chromatographic analysis [82]. Therefore, data reported to date render it unlikely that 15-dPGJ₂ is the endogenous PPAR γ ligand active during initiation of adipocyte differentiation.

More recently, another α,β -unsaturated keto-prostanoid, 15-keto-PGE₂, was implicated as a PPAR γ ligand linked to adipogenesis [117-119]. In this respect, it is interesting that its precursor PGE₂, not PGD₂, is the most prominent PG in 3T3-L1 cells [120]. Exogenous 15-keto-PGE₂ was able to circumvent the need for the cAMP-elevating agent, isobutyl-methylxanthine (IBMX), during adipogenesis of 3T3-L1 preadipocytes [117], where cAMP-dependent signaling promotes the production of PPAR γ ligands [82]. Importantly, the level of 15-keto-PGE₂ peaks early during adipose conversion (Figure 3) [119] in accordance with the ligand-mediated activation of PPAR γ [82, 86]. Furthermore, ectopic expression of prostaglandin reductases that remove the α,β -unsaturation converting 15-keto-PGE₂ into the PPAR γ -binding-deficient derivative, 13,14-dihydro-15-keto-PGE₂, abolishes adipogenesis [117, 119]. Moreover, prostaglandin reductase 2 (PTGR2) has been identified by differential display during the late phase of 3T3-L1 adipocyte differentiation [121].

In stark contrast to these positive effects of PGs, most prostanoids are reported to block adipogenesis, likely through the activation of their classic target, G-protein-coupled receptors [122-128]. Additionally, knockdown or sustained expression of COX-2 augments or represses adipogenesis, respectively, suggesting a negative effect of PGs on adipose conversion [86, 129]. In this respect, it is intriguing that COX-2 is transiently induced shortly after initiation of adipocyte differentiation of 3T3-L1 preadipocytes [86, 113]. This short burst may however be involved in regulating the fate of the adipocytes,

as COX-2-deficiency prevents the development of beige fat cells that express uncoupling protein 1 [130, 131].

We therefore still have much to learn before we fully understand the complexity of PG signaling during adipocyte differentiation. Nevertheless, all studies but one have reported either no or a positive effect of chemical ablation of the COX enzymes on adipogenesis [86, 113, 122, 132-136]. Therefore, it is difficult to maintain the notion that PGs are either solely responsible or in fact essential for ligand-mediated activation of PPAR γ during adipogenesis.

3.2. Epidermis lipoxygenase type 3 and xanthine oxidoreductase

A link between LOXs and PPAR γ through the production of a ligand for PPAR γ is well documented [137-140]. However, few reports address the function of LOXs in adipogenesis. Chemical inhibition of LOX decreased adipogenic conversion of preadipocytes [86, 141]. Interestingly, to block adipogenesis, the LOX inhibitor had to be present within the period where 3T3-L1 cells produced a PPAR γ -ligand, and the production was sensitive to LOX inhibition [86]. This blockade of PPAR γ ligand production was later contradicted [82], although the authors did not report if their batch of the unstable LOX-inhibitor blocked adipocyte differentiation.

3T3-L1 preadipocytes only express one LOX, the hydroperoxide isomerase eLOX3. We have shown that forced expression of eLOX3 is able to restore adipogenesis in retinoblastoma protein (pRB)-deficient MEFs. These MEFs require an exogenous PPAR γ ligand to differentiate [142]. Furthermore, eLOX3 appears essential for adipogenesis, as short hairpin RNA-mediated knockdown of eLOX3 blocks adipocyte differentiation of 3T3-L1 preadipocytes. Additionally, the products of eLOX3, hepoxilins, are able to bind to and activate the PPAR γ -LBD in fibroblasts. Importantly,

hepoxilins are present early during adipogenesis fitting the profile for the PPAR γ -ligand (Figure 3) [143]. Like the α,β -unsaturated ketones, these α,β -unsaturated epoxides are prone to covalent adduction with reactive cysteines [144], and thus, possess the appropriate chemical reactivity to act as a potent PPAR γ ligand.

Since eLOX3 is unable to use unsaturated fatty acids as substrates, it cannot act alone to generate a PPAR γ -ligand during adipogenesis, and the enzymatic partner providing the fatty acid hydroperoxide has yet to be identified. As eLOX3 is promiscuous [99, 145], substrate specificity is of little help in eliminating candidates and we must consider the entire spectrum of biological hydroperoxide generation. While LOXs are attractive candidates, 5(S)-LOX, 8(S)-LOX, 12(S)-LOX (i.e. 12/15-LOX) and 12(R)-LOX expression were not detected in either 3T3-L1 cells [86] or human adipocytes [146], although others have reported 5-LOX expression at 5-days post initiation of differentiation of 3T3-L1 preadipocytes [147]. Consistent with this, both adipocytes and adipose stromal vascular cells were found to produce 5-LOX-dependent leukotrienes [148]. Similarly, while COX activity which could yield HPODEs is present in these cells and expression co-occurs with ligand activation as reported above, COX ablation does not prevent adipogenesis. Therefore, ROS-dependent sources of fatty acid hydroperoxides must be taken into account [149]. Among these candidates are the NADPH oxidase 4 (NOX4) [150] and the xanthine oxidoreductase (XOR).

NADPH oxidases are expressed in preadipocytes and small interfering RNA against NOX4, but not NOX1, inhibits adipocyte differentiation and lipid droplet accumulation in 3T3-L1 adipocytes [149, 151]. In addition, differentiation itself reduces NOX4 expression [152] as does the PPAR γ ligand rosiglitazone [153]. Interestingly, whole body NOX4 ablation leads to basal adipose hypertrophy in mice, despite a marked decrease in PPAR γ expression [150]. Collectively, NOX4 remains an interesting candidate for ROS-initiated formation of oxidized lipids acting as PPAR γ ligands.

Along the same vein, Friedman and colleagues have demonstrated that XOR is involved in the production of a PPAR γ -ligand during adipogenesis [154]. Although XOR was originally named for its role in purine catabolism, it can produce ROS [155, 156]. This enzyme can be converted to one of two forms, an oxidase or dehydrogenase, through the reduction of cysteine residues [157] and its expression is regulated by transcription factors classically associated with oxidative stress [158]. Interestingly, reduced expression of XOR leads to increased adiposity and sensitivity to high fat diets. Expression of NOX4 was increased in the adipose tissue of mice heterozygous for XOR [159] indicating a possible redundancy between the two ROS-generating enzymes.

On the other hand, complete XOR ablation reduces adiposity by 50% [154]. However, XOR-deficient mice fail to thrive and die before 6 weeks of age [160]. The decrease in adipose mass could therefore be a secondary effect of the failure to thrive. This is indicated by the lower level of serum free fatty acid (FFA) in mice lacking XOR compared to wild type. Lipodystrophy is normally accompanied by an increase in serum FFA.

Furthermore, unpredictable effects on adipogenesis by systemic imbalances in ROS-levels through perturbations in NOX4 or XOR may mask the true involvement of these enzymes in adipocyte differentiation *in vivo*.

It is therefore possible that NOX4-regulated XOR cooperates with eLOX3 in the production of the PPAR γ -ligand during differentiation. In this scenario, stimulation of XOR by NOX4 leads to generation of ROS. Auto-oxidation of AA by ROS results in the generation of isoprostanes, malondialdehydes, 4-hydroxy-nonenals, and HPETEs [161] of which the last can be converted into hepxilins by eLOX3 (Figure 4). As ROS-dependent oxygenation is undirected [97] and lacks substrate specificity, several hydroperoxides would presumably be generated by XOR. eLOX3 would then convert

these into hepoxilin and hepoxilin-like products of which at least hepoxilin A3 and B3 have the ability to activate PPAR γ .

The crystal structures of PPAR in complex with 9-HODE and 13-HODE showed that two molecules can bind simultaneously to the receptor [74]. As discussed by the authors, while the one 9-HODE and 13-HODE molecule is adjacent to helix 12 and forms interactions typical of synthetic full agonists, the second 9-HODE and 13-HODE molecule is distant to helix 12 and the area that accommodates it overlaps with that occupied by synthetic partial PPAR γ agonists. This suggests that partial PPAR γ agonists may exploit part of the signalling mechanism of the natural ligands. Docking results on hepoxilin A3 and B3, which bare structural similarities to 9-HODE, demonstrate similar binding mode to the latter [143].

Curiously, in keratinocytes hepoxilins activate PPAR α but neither PPAR δ nor PPAR γ [162]. Both keratinocytes and COS7 cells (kidney fibroblasts) contain an epoxide hydrolase (EH) activity converting hepoxilins into their trihydroxy derivatives (trioxilins) [163]. These trioxilins were just as efficient as their hepoxilin precursors in activating PPAR α in keratinocytes [162]. It is possible that differences in EH activity exist in various cell types and that conversion of the PPAR γ ligands, hepoxilins, to the PPAR α ligands, trioxilins, occurs at a higher rate in cells with high levels of EH activity. In fact, the sEH is induced by PPAR γ ligands and coordinately expressed with Perilipin A and B, with expression increasing at day 4 post-differentiation and beyond [164].

During the conversion of HPETEs into hepoxilins eLOX3 creates ketoeicosatetraenoic acid (KETE) as a byproduct [99]. Interestingly, lipids containing ketone groups are reported to covalently bind to residues in the PPAR γ ligand binding cavity [74] suggesting that the KETEs can at least theoretically also induce adipogenesis by direct binding to PPAR γ . Furthermore, through interaction with glutathione such keto

lipids can be converted into cystineyl-leukotriene like compounds [165]. Independently, the non-cysteine containing leukotriene B4 is a 5-LOX metabolite and potent PPAR α ligand [166] that can be produced by adipose tissue [148]. As PPAR α can induce adipogenesis in the presence of a strong PPAR α ligand [167], it is possible that eLOX3 can augment adipocyte differentiation through activation of both PPAR γ and PPAR α by hepoxilins and leukotriene B4, respectively. Although eLOX3 presumably can increase differentiation through PPAR α , the LOX likely exerts its normal adipose effect through PPAR γ as PPAR α , in contrast to PPAR γ , normally is regarded as dispensable for adipogenesis [168].

It is also possible that the retinol saturase (RetSat) is involved in orchestrating the ROS metabolism during adipocyte differentiation. RetSat has been shown to be implicated in survival to oxidant exposure [169]. Interestingly, this enzyme has been shown to be indispensable for adipogenesis in a manner independent of its normal product, 13,14-dihydroretinol [170]. In contrast to this, but in line with the conflicting phenotypes of NOX4 ablation *in vitro* and *in vivo*, RetSat-deficient mice display increased adiposity [171].

4. Other Fatty Acid Metabolites

Besides the eicosanoids, a variety of other naturally occurring lipids have been reported to act as PPAR γ ligands in relation to adipogenesis. These include the endocannabinoids, nitrated lipids, and anionic phospholipids.

4.1. Endocannabinoids

The endogenous ligands of the cannabinoid receptors are produced by adipocytes and reportedly influence adipogenesis. In particular, the AA metabolites 2-

arachidonoylglycerol and anandamide (i.e. N-arachidonylethanolamine) are reported to activate PPAR γ and promote adipogenesis [172]. Moreover, leptin, which suppresses lipogenesis [173], also inhibits adipocyte endocannabinoid production [174]. The levels of 2-arachidonoylglycerol and anandamide peak around day 4 during differentiation of 3T3-F442A preadipocytes in the presence of insulin (Figure 3) [175]. The timing of these changes in endocannabinoid levels coincides with the window needed for ligand-dependent promotion of adipocyte differentiation [82, 86]. However, it is still questionable whether 2-arachidonoylglycerol and anandamide stimulate adipogenesis via activation of PPAR γ or via activation of the cannabinoid CB1 receptor and the subsequent signaling cascade [176].

4.2. Nitrolinoleic acid

Nitrated linoleic and oleic acid are reported to act as high-affinity ligands with the ability to induce adipogenesis in 3T3-L1s [177-179]. Measured in red blood cells these modified fatty acids are present in the blood in concentrations well above what is required to activate PPAR γ [180]. Moreover, nitric oxide synthase activity increases upon adipocyte differentiation, and blockade of this activity interferes with this process [181]. Nitrolinoleic acid (LNO₂) is extremely labile in an aqueous environment. It is however stable when esterified in phospholipids and embedded into a lipid bilayer.

Generation of nitrated fatty acids relies on attack of fatty acids by metabolites of nitric oxide ($\cdot$ NO) for example $\cdot$ NO₂ and peroxynitrite [182]. It is interesting that an enzyme previously shown to be involved in the production of a ligand is capable of producing $\cdot$ NO, namely XOR. Not only can XOR produce $\cdot$ NO but it is also able to synthesize peroxynitrite through reaction of $\cdot$ NO with XOR-generated $\cdot$ O₂ [183]. Peroxynitrite and $\cdot$ NO₂ can also form a lipid radical when attacking an unsaturated fatty

acid, which is converted into either a dioxygenated or nitrated lipid depending on the oxygen tension (Figure 4) [184].

The formation of $\cdot\text{NO}$ by XOR occurs at two distinct catalytic sites. Generation of $\cdot\text{NO}$ from inorganic nitrate and nitrite takes place at the molybdopterin (Mo-Co) site, whereas only organic nitrite can be converted into $\cdot\text{NO}$ at the flavin adenine dinucleotide (FAD) site [183]. Only by blocking the activity of the FAD site was the production of a PPAR γ -ligand during adipogenesis abolished [154]. Notably, the FAD site also produces ROS.

Collectively, nitrated fatty acids exemplified by LNO_2 are potent PPAR γ ligands with the ability to induce adipocyte differentiation [179]. Still, whether LNO_2 or other PPAR γ -binding nitrated fatty acids are present early and transiently during adipose conversion in sufficient concentrations to activate PPAR γ remain unknown. The crystal structure of the PPAR γ LBD bound to nitrated linoleic acid has been solved [178]. The PPAR γ LBD forms a dimer and each LNO_2 molecule occupies approximately 40% of the ligand binding pocket. The acidic head group forms several hydrogen bonds with the surrounding PPAR γ residues, including Gln286 from helix 3 (Figure 5A and 5B). These interactions are in agreement with a crucial conserved mechanism for ligand-mediated activation of PPAR γ seen in synthetic full agonists, such as rosiglitazone. The pharmacophore shown in Figure 5C that is modeled on the 3D structure of LNO_2 , shows the spatial arrangement of the molecular features (hydrogen-bond acceptor, hydrophobic, negatively ionizable) that are essential for the binding of LNO_2 to PPAR γ . Molecules with similar features to LNO_2 located within the range of the above features (i.e. within the colored spheres) are expected to be able to bind to PPAR γ in a similar manner.

4.3. Anionic phospholipids

Preservation of the phosphate group in membrane-released lipids renders them anionic and some of these are known to act as PPAR γ ligands. Lysophosphatidic acid (LPA) is suggested to signal through plasma membrane-associated receptors but LPA is also reported to function as a PPAR γ ligand [185]. It is further interesting to note that the soluble epoxide hydrolase, is a bifunctional enzyme with both epoxide hydrolase and lipid phosphate phosphatase activity [186], functioning as an effective cytosolic LPA-phosphatase [187]. It is, however, questionable whether LPA is an endogenous ligand during adipose conversion as LPA inhibits adipogenesis [188]. Adding to this notion is that, the lysophospholipid cyclic phosphatidic acid (1-acyl-2,3-cyclic-glycerophosphate, CPA) binds to PPAR γ but works as an antagonist by stabilizing the PPAR γ -corepressor complex and thereby block adipogenesis [189].

The anionic azeloyl-phosphocholine (azPC), originally found in oxidized LPLs, is shown to be a potent PPAR γ ligand [190]. The effect of this fatty acid on adipose conversion is unknown. Yet, the plausible generation of ROS by XOR renders it possible that it could be generated during adipocyte differentiation.

Even intact phospholipids are reported to bind and activate PPAR γ . In their decipherment of the necessity for fatty acid synthase (FAS) in adipogenesis, Semenkovich and colleagues discovered that the voluminous lipid 1-O-octadecenyl-2-palmitoyl-3-glycerophosphocholine (18:1e/16:0-GPC) was tethered to PPAR γ at the onset of adipocyte differentiation [191]. Upon ablation of FAS, 18:1e/16:0-GPC was no longer found associated with PPAR γ . In this respect, it is interesting that ectopic expression of a transcriptional regulator of FAS expression, the transcription factor sterol-regulatory element binding protein 1c (SREBP-1c) was previously shown to augment the production of PPAR γ ligand(s) [192]. Similarly, it was recently reported that carbohydrate element binding protein (ChREBP) in a FAS-dependent manner enhanced

PPAR γ -mediated transactivation [193]. It remains, however, unresolved if 18:1e/16:0-GPC would be able to circumvent the requirement for FAS in adipocyte differentiation and if there would be a timely production/release of 18:1e/16:0-GPC during adipogenesis.

5. A possible involvement of clonal expansion

A common feature for all the described scenarios for the ligand-mediated activation of PPAR γ is that the lipids must be freely accessible to the transcription factor. This can occur through exogenous addition or mobilization of internal stores of a precursor or the ligand itself. Differentiation *in vitro* normally occurs in the presence of serum mimicking the situation *in vivo* rendering the surroundings an obvious source of lipids [194]. However, as even 3T3-L1 preadipocytes can differentiate in serum-free conditions the preadipocytes must also be able to supply the lipids needed for PPAR γ activation [195].

Concomitant with the early generation of PPAR γ ligands during adipogenesis most murine cells undergo two rounds of mitosis commonly referred to as clonal expansion. This process is shown to be essential for efficient adipocyte differentiation [196]. Of note, adipose conversion of murine C3H10T1/2 fibroblasts and human cells does not seem to rely on clonal expansion, but require an exogenous supply of a PPAR γ ligand to differentiate [197, 198]. These observations indicate an intimate link between proliferation and PPAR γ ligand production.

Interestingly, not only did chemical ablation of cell division prevent the generation of PPAR γ ligands. An exogenous PPAR γ agonist was able to circumvent the block in adipogenesis imposed by the chemical prevention of proliferation [199, 200].

The fatty acids that can function as precursors for known PPAR γ ligands are present in phospholipids embedded in cellular membranes. Their release is mainly regulated by the actions of phospholipases (PLs). Of the known families of phospholipases, PLA₂, PLC and PLD, only PLA₂ releases the free fatty acid directly. Interestingly, the activity of cytoplasmic PLA₂ (cPLA₂) is upregulated during proliferation [201]. This phospholipase is present early during adipocyte differentiation [202]. The activity and localization of cPLA₂ is regulated by an activating phosphorylation by MAPKs. This phosphorylation leads to the translocation of cPLA₂ to the nuclear membrane [203]. Activation of MAPK is necessary for both clonal expansion and adipocyte differentiation [199]. Interestingly, cAMP, the second messenger inducing PPAR γ agonist generation during the initial stages of adipocyte differentiation, is responsible for the activation of MAPK early during adipogenesis [199]. Based on our previous findings we hypothesize that cAMP elevates MAPK activity through activation of the exchange proteins directly activated by cAMP (Epacs) [204]. It is possible that the extensive remodeling of the nuclear membrane during proliferation is a prerequisite for efficient PUFA release and that activation of cPLA₂ by cAMP through MAPK is heavily involved in this release.

6. PPAR γ ligand production *in vivo*

The reports on a timely and transient activation of PPAR γ -LBD constructs showed that differentiating adipocytes themselves are able to produce PPAR γ ligands *in vitro* [82, 86]. *In vivo*, adipose tissue is constituted by a number of different cell types. The hydrophobic nature of PPAR γ ligands allows diffusion across cell membranes permitting them to act in a paracrine manner. Nearby cells and even the blood can thereby affect differentiation of adipose tissue-embedded preadipocytes. This also means

that the effect of a dysfunctional ligand-producing pathway may be masked as ligands produced nearby can exert a redundant function. Also, this transcellular biosynthesis could even be responsible for the opposite *in vitro* and *in vivo* findings for NOX4, XOR and RetSat through compensatory mechanisms.

eLOX3 and several other LOXs are expressed in the adipose tissue [86]. The presence of other LOXs indicates that eLOX3 may cooperate with other family members *in vivo* in control of adipocyte development in contrast to the suggested partnership with XOR *in vitro*. Further studies are however required to elucidate the *in vivo* function of eLOX3 in adipose development.

XOR expression is elevated in the adipose tissue of obese mice [154]. It is, however, not the only 'NO-producing enzyme which is elevated during obesity. Both endothelial and inducible nitric oxide synthase (eNOS and iNOS, respectively) are upregulated in obese individuals [205]. Interestingly, body fat correlates with serum 'NO levels [206]. These data collectively indicate a possibly increased production of nitrated lipid radicals in obese individuals.

As mentioned, the destiny of the nitrated lipid radicals depends on the oxygen tension. Either they become dioxygenated lipids or nitrated fatty acids both with the potential to activate PPAR γ (Figure 4). This is of interest as adipose tissue in obese individuals is considered to be hypoxic suggesting that there is a shift in the fate of nitrated lipid radicals in adipose tissue when going from lean to obese. Both the increased generation of 'NO and the hypoxic state of the adipose tissue are likely to lead to increased levels of nitrated fatty acids. These potent PPAR γ ligands would presumably contribute to onset of the hyperplasia of the obese adipose tissue.

Other possible sources of PPAR γ ligands during obesity are macrophages [137] as the obese adipose tissue is heavily infiltrated by this cell type [207]. Furthermore, mast cells have been shown to induce adipogenesis through secretion of 15-dPGJ₂ [208].

Expression of autotaxin, a gatekeeper in LPA production, is also upregulated in the adipose tissue of obese mice [209]. Even though LPA is shown to be a PPAR γ ligand, increased LPA could contribute to perturbed PPAR γ signaling as treatment of adipocytes with LPA lowers sensitivity to other PPAR γ agonists [188].

7. Conclusion

With the linkage of XOR to PPAR γ ligand production during adipose conversion, Friedman and colleagues provided a theoretical platform for ROS-mediated generation of oxidized or nitrated lipids in differentiating adipocytes. Whether lipids like PGs, LNO₂ or hepoxilins are responsible for ligand-mediated activation of PPAR γ on their own or in collaboration, still remain to be resolved. The ligand-mediated activation of PPAR γ is even further complicated *in vivo*, where transcellular biosynthesis of ligands may have a major impact on PPAR γ -mastered adipogenesis and the regulation of both ligand formation and destruction is paramount for regulated adipogenesis.

Determination of the exact ligand(s) responsible for adipocyte formation may evoke little interest as so many ligands are already known. However, the PPAR γ agonists currently used in the clinic suffer from untoward side-effects. More knowledge on natural ligands, their mode of activating PPAR γ and which physiological processes they individually orchestrate will undoubtedly lead to generation of safer PPAR γ agonists with fewer side effects. With the work pioneered by the laboratories of Spiegelman and Griffin on how ligands can prevent phosphorylation of PPAR γ , it is obvious that we still have a way to go before we fully understand the impact of endogenous ligands on PPAR γ function.

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Figure legends

Figure 1. Schematics of PPAR γ 1 and PPAR γ 2. PPAR γ is conventionally divided into 4 domains: The N-terminal A/B-domain that harbors the ligand-independent activation function (AF-1), the C-domain with the DNA binding zinc-finger, the hinge region in the D-domain, and finally the E-domain containing regions responsible for dimerization, ligand binding and ligand-dependent activation function (AF-2).

Figure 2. The heart of AA metabolism. Derivatives of AA and the metabolizing enzymes. Protein names are within squares and fatty acids are depicted as circles. Orange dots are fatty acids which are reported to both activate PPAR γ and induce adipogenesis. Orange proteins induce adipocyte differentiation whereas blue proteins are reported to repress. Modified from the KEGG database [210, 211] using Cytoscape [212]. PGDH, 15-hydroxyprostaglandin dehydrogenase. GGT, gamma-glutamyltransferase. DPEP, dipeptidase. LTC4S, leukotriene C4 synthase. LTA4H, leukotriene A4 hydrolase. CBR, carbonyl reductase. TBXAS, thromboxane A synthase. PGFS, prostaglandin F synthase. PTGDS, prostaglandin D2 synthase. PTGIS, prostacyclin synthase. PTGES, prostaglandin E synthase.

Figure 3. Profiles of endogenous PPAR γ ligands during adipogenesis. Where available, we have plotted the relative ligand levels during adipose conversion. Dotted lines indicate activity of the PPAR γ LBD as described by Madsen et al. [86] and Tzameli et al. [82].

Figure 4. Proposed pathways for generation of PPAR γ and PPAR α ligands during adipocyte differentiation under normoxic and hypoxic conditions. Under normoxic conditions, NOX4 and/or XOR generate ROS which can convert AA into hydroperoxides (e.g. 12-HpETE). These are further transformed into the PPAR γ -activating hepoxilins by eLOX3 or KETEs by GPx and alcoholdehydrogenase (ADH). Trioxilins generated by sEH from hepoxilins can activate PPAR α which also can augment adipocyte differentiation. Conversely, under hypoxic conditions, XOR will produce both superoxide('O_2) and 'NO , which recombine to form peroxynitrite ('ONO_2), and ultimately the nitrating species nitrogen dioxide radical ('NO_2). Interaction of unsaturated lipids with 'NO_2 will produce nitrated fatty acids which themselves are potent PPAR γ activators.

Figure 5. A close view of LNO $_2$ bound to PPAR γ . A) Figure reproduced from PDB: 3cww with the use of CLC Drug Discovery Workbench software. LNO $_2$ molecule is shown in magenta, with the carboxyl and nitro groups depicted in red. B) Interactions between carboxyl and nitro groups of LNO $_2$ with amino acids of the PPAR γ binding pocket. C) Pharmacophore of LNO $_2$ based on its interactions with PPAR γ . Green: HB-acceptor, Cyan: hydrophobic, Blue: negatively ionizable. (Created by Biovia Discovery Studio software.)

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Table 1**Germline or adipose-specific genetically engineered mouse models for PPAR γ**

Description	Overall phenotype	Adipose phenotype	References
PPARγ^{-/-}	Embryonic lethal	-	[25, 26]
PPARγ^{-/-} tetraploid rescue	Early death due to lipodystrophy and hemorrhage	Lipodystrophy	[26]
Wildtype and PPARγ^{-/-} chimeric mice	-	No contribution of PPAR γ ^{-/-} cells to adipose tissue	[24]
PPARγ^{-/-} embryos except in the trophoblasts	Insulin resistance and hypotension	Lipodystrophy	[27]
PPARγ^{+/-}	Increased insulin sensitivity and energy expenditure	Smaller adipose tissues	[25, 28, 29]
PPARγ2^{-/-}	Decreased insulin sensitivity in male but not female mice	Less WAT, but normal BAT, development	[44]
PPARγ2^{-/-}	Decreased insulin sensitivity in male but not female mice	Normal WAT and BAT development although with decreased expression of lipogenic genes	[45]
Adipose-specific PPARγ^{-/-} (Fabp4-Cre)	Liver steatosis and insulin resistance	Lipodystrophy	[34, 35]

PPARγ^{hyp/hyp}	High lethality Increased β -oxidation in muscle	Lipodystrophy	[36]
PPARγ^{Leu496Ala/+} (PPARγ^{Leu496Ala/Leu496Ala} is embryonic lethal)	Liver steatosis and insulin resistance	Lipodystrophy	[33]
PPARγ^{Pro495Leu/+} (PPARγ^{Pro495Leu/Pro495Leu} is embryonic lethal)	Insulin resistance in Leptin-deficient background	Abnormal WAT distribution and decreased BAT development	[39-41]
PPARγ^{Ser112Ala/Ser112Ala}	Increased insulin sensitivity	Smaller adipocytes in WAT	[47]