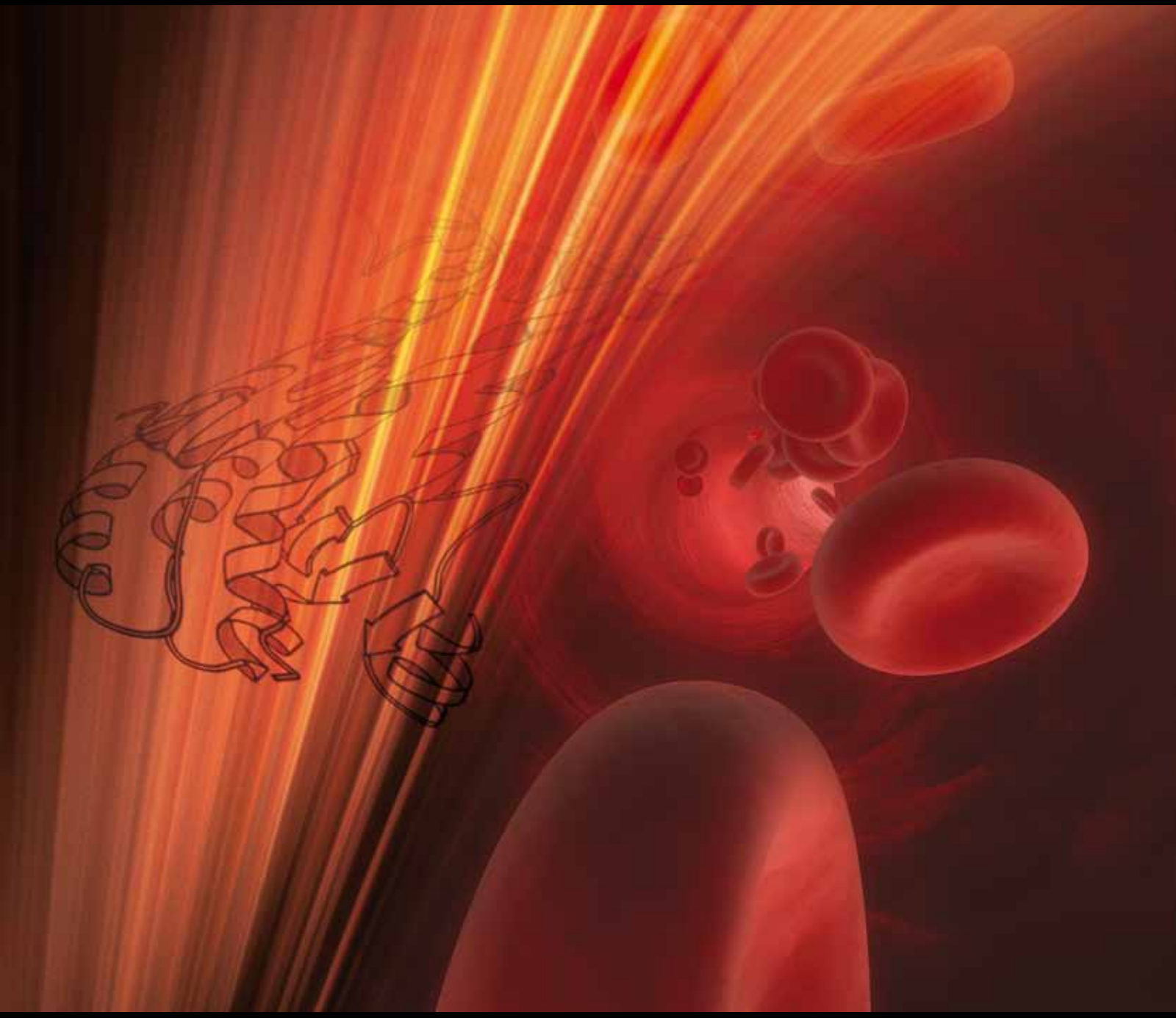
PPAR Research

Physiological and Nutritional Roles of PPAR across Species

Guest Editors: Massimo Bionaz, Gary J. Hausman, Juan J. Loor, and Stéphane Mandard



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Editorial Board

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Editorial

Physiological and Nutritional Roles of PPAR across Species

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There has been a tremendous amount of information produced on peroxisome proliferator-activated receptors (PPARs). The interest in PPARs was originally driven largely by their role in hypolipidemia and hepatocarcinogenesis, but it soon became evident that they played important roles in the metabolic syndrome and overall health of organisms including regeneration of tissues, differentiation, insulin signaling, overall lipid metabolism, and immune response (reviewed in [1–7]). From a nutritional standpoint, the PPARs are of extreme importance because of their ability to bind and be activated by long-chain fatty acids and their metabolites. Therefore, the PPARs are recognized as ideal candidates for therapeutic use in order to improve metabolism and overall health through diet. At present, there is substantial interest in therapeutic applications tailored to regulate PPARs via synthetic drugs (e.g., [8]), but the exploitation of dietary approaches is not a reality yet.

Most of our knowledge on PPARs has been produced by studies carried out in rodents and humans and little from other species, bovine and pig being the most studied among livestock species. The multitude of roles of PPARs and the possibility of regulating them through dietary approaches are also of interest in animal food production. Therefore, a comparative approach to bring together physiological and nutritional roles of PPARs across species appears critical.

For this reason, this special issue was dedicated to PPARs interspecies comparisons with a larger emphasis on livestock species compared to animal models or humans. Among the 6 papers published, 3 focused specifically on ruminants and one on chicken. The review from Bionaz et al. assembled all the information pertaining to ruminant PPARs, with emphasis on functions, activation, and potential targets for nutrigenomics approaches to improve animal production and wellbeing. The review underscored that the information about PPARs in ruminants accumulated quickly in the last decade owing to the recognition of their potential importance in those mammalian species. The functional comparison among ruminant, mouse, and human highlighted a similar role of PPAR isotypes on lipid metabolism between species. However, the data highlighted differences in the response to long-chain fatty acids. Monogastrics are more sensitive to unsaturated while ruminants, particularly bovine, are more sensitive to saturated long-chain fatty acids. Based on PPARs data generated in nonruminants and ruminants, they proposed an integrative and dynamic model encompassing the activation (by long-chain fatty acids) of the three PPAR isotypes in order to optimize the adaptation to lactation. Among others, they also reviewed the data supporting a role of PPARγ in controlling milk fat synthesis in ruminants and demonstrated that this feature is not shared by mouse or, likely, other monogastrics. A pivotal role of PPARγ in controlling milk fat synthesis was confirmed by the paper of Shi et al. published in the present special issue. Those authors demonstrated, using a combination of PPARγ specific activator, gene expression, luciferase-PPRE assay, and siRNA techniques, that this nuclear receptor controls the expression of milk fat-related genes also in primary goat mammary epithelial cells.

The activation of PPARγ using oral administration of 2,4-thiazolidinedione (TZD) in growing beef bulls was assessed by Arévalo-Turrubiarte et al. The authors aimed to test the effect of PPARγ activation on intramuscular fat (i.e., marbling). They observed a greater amount of TZD in liver of the treated animals, demonstrating an uptake of the drug via oral administration. The TZD treatment had no effect on carcass quality but had a strong effect on the expression of all three PPAR isotypes in liver (all decreased) and in muscle (increase only of PPARα). They observed also an overall increase in cell size and decrease of cellular synthesis in muscle and perirenal adipose tissue, but the opposite was observed in subcutaneous adipose. This effect was explained by higher insulin sensitivity due to the treatment. Activation of PPARγ with oral administration of a TZD in growing pigs also had no significant effect on marbling, but it did increase muscle fiber oxidative capacity regardless of fiber type [9]. As for bovine, the activation of PPARγ in swine may be useful to influence metabolism overall, but more studies are needed to examine this possibility. Takada and Kobayashi provided the first review of the three PPAR isotypes in poultry, particularly in chickens. Interestingly, they also provided a comparison with human PPARs both structurally and functionally. They uncovered several peculiar and unique functions in chicken PPARs and differences between chickens, and human PPARs. These data prompt for more chicken-specific studies in order to exploit the ability of PPARs to control lipid and glucose metabolism in this species.

Mandard and Patsouris reviewed recent evidence establishing that PPARs are critical regulators of inflammation in mammals. In the last decade, PPARs have emerged as modulators of inflammatory responses. Therefore, the potential therapeutic usefulness of PPARα and PPARγ activation in the control of obesity and diabetes-induced chronic (low-grade) inflammation has extensively been studied over the last couple of years using rodents. The authors discussed different aspects of the interaction of PPARγ with adipose inflammation. In the light of the recent findings, it has become clear that, besides activating PPARγ in the adipocyte, pharmacological activation of this receptor extends to a much broader range of cell types, such as T regulatory cells, which is likely beneficial in the suppression of obesity-associated inflammation in white adipose tissue, as far as rodents are concerned. The impact of the pharmacological activation of mouse PPARα in the context of obesity-induced hepatic inflammation is also reviewed as well as the potential relevance of PPARβ/δ as a molecular drug target to fight liver inflammation in the case of nonalcoholic fatty liver disease.

A growing body of evidence also indicates that PPARs are potent negative regulators of the acute-phase response in different species, ranging from mouse, rat, pig, cattle and humans. The review article by Mandard and Patsouris also expands on the potential beneficial use of PPAR (ant)agonists in the routine of livestock to prevent bacterial-induced excessive inflammatory reaction and associated diseases such as mastitis in dairy cows.

PPARα is also known to be critical for energy homeostasis. In line with this, the review paper by Ringseis et al. thoroughly summarizes the implication of PPARα in carnitine homeostasis in no less than six different species including rat, mouse, pig, cattle, chicken, and human. The comparative analysis performed by the authors led them to conclude that PPARα displays a key regulator role in carnitine homeostasis in general. It is the process of cellular carnitine uptake, with the key role of the PPARα target carnitine transporter novel organic cation transporter 2, that is particularly well conserved across the above-mentioned species.

All the papers in this special issue emphasized on one hand a similar function of PPARs among species, particularly related to lipid metabolism, but also, and more importantly, accentuated the differences and the species-specific functions and response to agonists.

In summary, the analysis of PPARs across species highlighted the following:

(i) interspecies conserved functional roles of those nuclear receptors (e.g., regulation of lipid metabolism and inflammation);

(ii) the potential for therapeutic intervention through nutritional modulation of PPARs in all species in order to prevent diseases and improve animal production;

(iii) differences between species that prompt for more species-specific studies in order to fully exploit the abovementioned therapeutic roles through nutrition.

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Review Article

Functional Role of PPARs in Ruminants: Potential Targets for Fine-Tuning Metabolism during Growth and Lactation

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Characterization and biological roles of the peroxisome proliferator-activated receptor (PPAR) isotypes are well known in monogastrics, but not in ruminants. However, a wealth of information has accumulated in little more than a decade on ruminant PPARs including isotype tissue distribution, response to synthetic and natural agonists, gene targets, and factors affecting their expression. Functional characterization demonstrated that, as in monogastrics, the PPAR isotypes control expression of genes involved in lipid metabolism, anti-inflammatory response, development, and growth. Contrary to mouse, however, the PPARγ gene network appears to controls milk fat synthesis in lactating ruminants. As in monogastrics, PPAR isotypes in ruminants are activated by long-chain fatty acids, therefore, making them ideal candidates for fine-tuning metabolism in this species via nutrients. In this regard, using information accumulated in ruminants and monogastrics, we propose a model of PPAR isotype-driven biological functions encompassing key tissues during the peripartal period in dairy cattle.

1. Introduction

In humans, mouse, and rat, nuclear receptors (NR), including PPARs, form a transcription factor family of 47–49 members [1]. Activity of NR allows for long-term (hours to days) control of metabolism because they can affect mRNA expression of target genes, including metabolic enzymes [2]. Thus, NR represent an important regulatory system in cells, tissues, and organs playing a central role in metabolic coordination of the entire organism.

Peroxisome proliferator-activated receptors (PPARs) were originally identified in *Xenopus* frogs [3] as novel members of the NR that induced the proliferation of peroxisomes in cells, a process that was accompanied by activation of the promoter of the acyl-CoA oxidase gene (*ACOX1*) encoding the key enzyme of peroxisomal long-chain fatty acid (LCFA) β-oxidation. The PPARα was the first member or isotype of the PPARs to be discovered in mammals during the search of a molecular target for liver peroxisome proliferators [4]. Those compounds include hypolipidemic drugs, that is, fibrates (e.g., clofibrate, fenofibrate, or Wy-14643), whose main effect is to lower blood triacylglycerol (TAG) and regulate cholesterol concentrations [5].

Initial characterization of PPARα (gene symbol *PPARA* in human and ruminants) in the adult mouse revealed that it was highly expressed in liver, kidney, and heart [4]. Shortly after PPARα was discovered, the isotypes PPARγ (gene symbol *PPARG*) and PPARβ/δ (gene symbol *PPARD*) were cloned [3, 6]. In monogastrics, *PPARA* is highly abundant in liver, intestine, heart, and kidney; *PPARG* is abundant in adipose and immune cells, while *PPARD* is ubiquitously expressed [7, 8]. In the mouse, both PPARγ isoforms γ1 and γ2 act in white and brown adipose tissue to promote adipocyte differentiation and lipid storage. While PPARγ2 is mainly expressed in adipocytes, PPARγ1 is expressed at modest levels also in other cells/tissues [9]. Expression of PPARβ/δ in murine resembled closely that of PPARα and was the sole isotype expressed in brain [6]. More recent studies in rats have established that PPARβ/δ is expressed ubiquitously throughout the body but is substantially more abundant in skeletal muscle than PPARα or PPARγ [7].

The PPARs form and function as heterodimers with retinoid-X-receptor (RXR). Once the ligand binds (e.g., LCFA, fibrates, thiazolidinedione (TZD)) to the ligand-binding domain (LBD), it produces a covalent modification of the PPAR structure [10] activating the NR. The activated PPAR/RXR binds to a specific DNA sequence (PPAR response element, PPRE) in the promoter region of specific target genes inducing or repressing their expression. The PPRE is a direct repeat of a hexanucleotide (AGGTCA) separated by a single nucleotide (i.e., DR-1). The DR-1 varies for each of the PPAR isotypes, thus conferring greater or lower strength to the PPAR/RXR complex for binding to PPRE and the strength of activation [11]. All PPAR isotypes are activated by ligand concentrations in the μM range or below, at least in nonruminants [12–14].

2. Role of PPAR in Monogastrics

The PPAR isotypes play multiple roles in mammals. There are a vast number of excellent reviews discussing those aspects in detail (e.g., [2, 5, 15–19]). Among others, the PPAR isotypes play important roles in regulating lipid and glucose metabolism, controlling inflammatory response, regulating tissue repair and differentiation, and cancer progression. Although with contrasting roles, PPAR isotypes affect blood vessel formation [20]. The PPARγ is pivotal in controlling the switch between adipogenesis and osteogenesis [17, 21] and insulin sensitivity [22], and it has an important neuroprotective role [23]. Similarly, it is well established that PPARα plays a crucial role in hepatic fatty acid catabolism in mitochondria, peroxisome, and microsomes [18]. The PPARβ/δ controls fatty acid catabolism in skeletal muscle and heart [2]. The PPAR isotypes are known to play important roles in all the reproductive tissues studied to date (reviewed in [24]). Due to the important functions played by the PPAR isotypes, PPARα and PPARγ have long been considered promising drug targets for human metabolic disorders as they regulate lipid and/or glucose homeostasis by controlling uptake, synthesis, storage, and clearance [25].

3. PPAR Isotype Expression in Ruminant Tissues

Judging from the published literature, the interest on PPAR isotypes in ruminants, particularly their role in lipid metabolism, has been modest compared to the vast literature in nonruminants, including human. Therefore, information about protein and gene expression abundance in ruminants is relatively scant. In order to help close this gap of knowledge we have performed Real-Time RT-PCR (qPCR) analysis to provide an evaluation of the relative distribution of PPAR isotypes in bovine tissues of adult Holstein dairy cows (i.e., three adipose depots, jejunum, liver, kidney, hoof corium, lung, placenta, and mammary), Holstein calves (semitendinosus muscle and rumen epithelium), longissimus muscle from Angus beef steers, and two cell lines obtained from adult bovines (Figure 1(a)). The data revealed that overall the relative distribution of PPAR isotypes in bovine tissues/cells is similar to other species.

3.1. PPARγ. This PPAR isotype has been the most-studied in ruminants. Our results from qPCR analysis (Figure 1(a)) indicated that *PPARG* expression is very high in all adipose tissues, followed by rumen, Madin-Darby Bovine Kidney cell line (MDBK), and placenta with moderate-to-low mRNA expression in small intestine, beef cattle longissimus muscle, hoof corium, lung, and mammary gland. In contrast, the lowest expression of *PPARG* was detected in liver, kidney, dairy calf semitendinosus muscle, bovine mammary alveolar cell line (MAC-T), and blood polymorphonuclear leukocytes (PMN) (Figure 1(a)). In an early study bovine PPARγ mRNA expression (via northern blot) was characterized in several tissues [29]. Similar to our data (Figure 1(a)), a greater expression of *PPARG* was detected in adipose tissue followed by spleen, lung, and ovary. Although lower, expression was also detected in mammary gland and small intestine. Expression was absent in pancreas and almost undetectable in liver. In other tissues the expression was very low or nondetectable. The *PPARG* is highly expressed in adipose tissue of mice [6], human [9], and chicken [30], all of which agree with the relative high expression in bovine adipose tissues (Figure 1(a)). Similar to mouse [6], human [9], pig [31], chicken [30], and beef bulls [32], the expression of *PPARG* in bovine liver, or other tissues such as kidney and intestine, was very low (Figure 1(a)).

We and others have previously detected expression of *PPARG* in bovine mammary tissue and the MAC-T cell line using qPCR [26, 33, 34]. In a recent study in our laboratory comparing gene expression between mammary gland and MAC-T cells, the former had greater expression of *PPARG* both during pregnancy and lactation [35]. The relatively high expression of *PPARG* in MDBK cells detected (Figure 1) confirmed previous observations [36]. Expression of *PPARG* was detected also in goat mammary, although at a significant lower level compared to bovine [37].

The *PPARG* is expressed at all stages during bovine embryo development (both in the inner mass and in the trophectoderm [38]) and in the placenta (cotyledons and caruncles) of bovine [39] and sheep [40], with an evident expression in the trophoblast [41]. Lutein cells [42] and uterus [43] express *PPARG*, but not bovine endometrial cells [44], while endometrial cells of pregnant ewes express this NR [41]. The expression of *PPARG* in ovary was confirmed in sheep [45] and the same study reported expression in pituitary gland but not hypothalamus. In previous studies it has been shown that this PPAR isotype is expressed in bovine aortic endothelial cells [46], beef cattle skeletal muscle (including intramuscular fat) [47], ovine intramuscular fat [48], bovine perimuscular preadipocytes [49], and bovine retinal pericytes [50]. In several beef cattle breeds, *PPARG* had a similar degree of expression in perirenal and omental adipose depots, followed by intramuscular fat and, in a minor quantity, in the longissimus muscle [47, 51].

The expression of various PPARγ isotypes in buffalo was recently evaluated [52] and found to be expressed in all tissues tested: ovary (follicles and corpus luteum), mammary gland, adipose tissue, liver, spleen, and lung. The isoforms PPARγ1a and 1b were highly expressed in ovarian tissue followed by

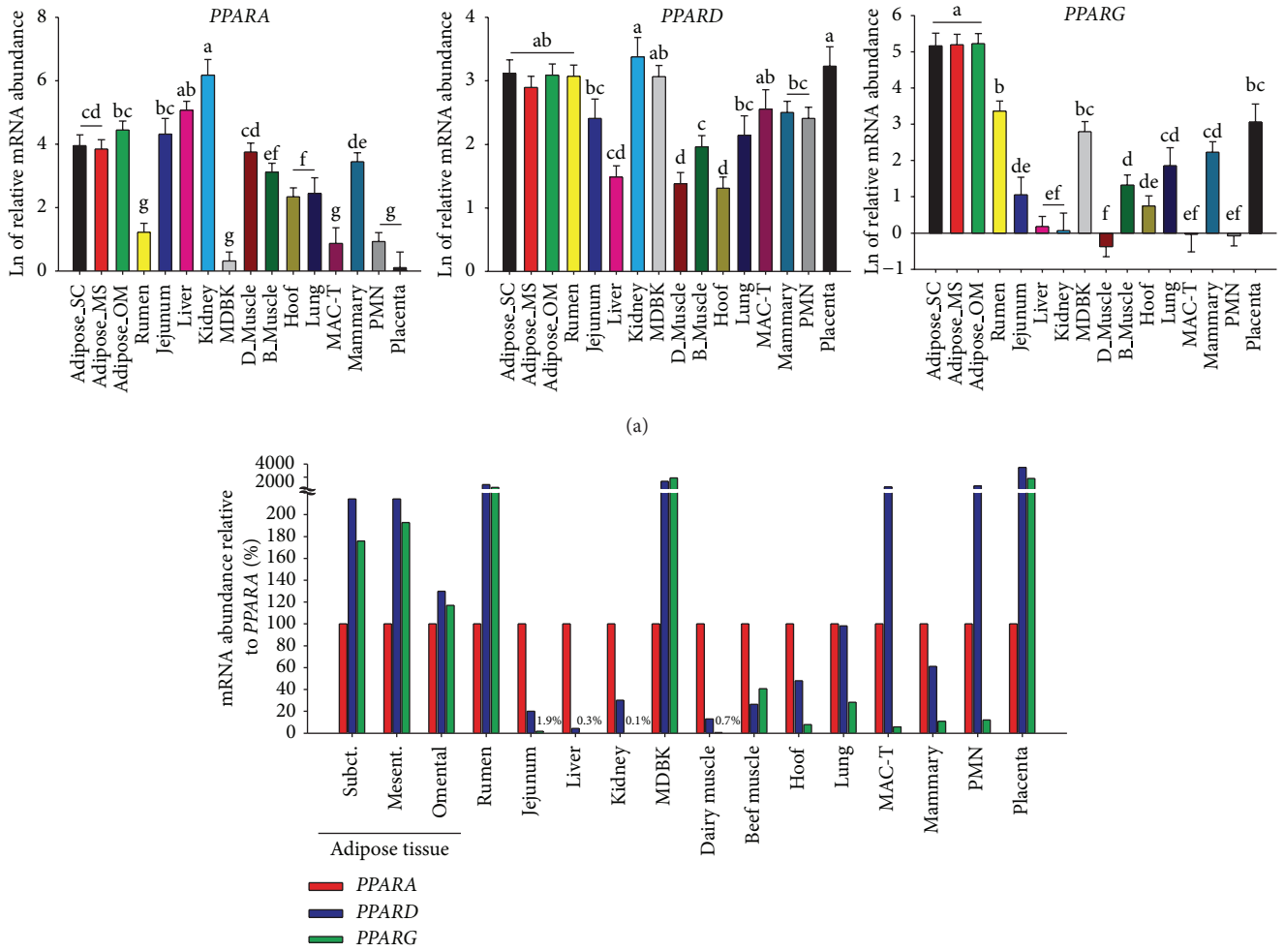


Figure 1: (a) Relative transcript abundance of each PPAR isotype in several bovine tissues and cells. We measured gene expression of PPAR isotypes in 14 different tissues including tissues from adult dairy cattle: adipose tissue (subcutaneous, mesenteric, and omental), small intestine (jejunum), liver, hoof corium, lung, kidney, mammary gland, blood polymorphonuclear leukocytes (PMN), and placenta; from dairy calves: rumen papillae and semitendinosus muscle (D-muscle); skeletal muscle of beef cattle (Longissimus *lombarum*); and two cell lines: Madin-Darby Bovine Kidney (MDBK) and bovine mammary alveolar cells (MAC-T). The total RNA was extracted and qPCR performed as previously described [26]. The qPCR data were normalized by the geometrical mean of 5 internal control genes (*PPP1R11*, *RPSI5A*, *ACTB1*, *MRPL39*, and *UXT*). For the difference of each PPAR isotype abundance between tissues, the qPCR data were transformed using a 6-point standard curve prior statistical analysis using PROC GLM of SAS (version 9.3) with tissue as main effect. Dissimilar letters denote significant differences ($P < 0.05$). (b) Tissue-specific relative mRNA abundance between PPAR isotypes. The % relative abundance of the three PPAR isotypes in each tissue was calculated using the delta Ct method as previously described [27]. The final data for *PPARG* and *PPARD* were obtained as % relative to *PPARA*. N.B.: the y-axis values in (a) are least square means of the Ct values transformed using the standard curve and then log2-transformed. The values in (b) are calculated without use of a standard curve. Therefore, the values in (a) are radically different compared to the values in (b) and the two cannot be compared.

spleen and mammary gland, respectively, while PPARγ2 was highly abundant in adipose tissue.

3.2. PPARα. This isotype has been less studied compared with PPARγ. The bovine PPARα gene is located in chromosome 5 in cattle [53]. The qPCR analysis of the relative mRNA abundance of *PPARA* highlighted, as in mice [6], human [54], and pig [31], that *PPARA* is very abundant in kidney (Figure 1(a)). Contrary to this general feature, even though the *PPARA* in liver of chicken is expressed at lower level than kidney, its expression in liver is similar to other tissues [30]. In contrast to what is observed in human [54], our data revealed that the relative abundance of *PPARA* was not statistically different between jejunum and adipose tissues of bovine (Figure 1(a)). In general the data in Figure 1(a) reveals a more widespread expression of this PPAR isotype among the tissues and cells evaluated compared to *PPARG*. The highest expression was observed in kidney and liver followed by adipose tissues, small intestine, and dairy cattle semitendinosus muscle. Beef cattle longissimus muscle and

mammary gland had relatively modest expression of *PPARA* followed by the least expression in hoof corium, lung, rumen, MDBK, MAC-T, PMN, and placenta (Figure 1(a)). We and others have consistently detected expression of *PPARA* in liver [55–60] and in MDBK cells in which also its activity was confirmed [28, 36, 61]. Partly corroborating our data (Figure 1), this PPAR isotype has been detected in bovine endothelial cells [62], skeletal muscle [63], rumen [64], uterus [43, 65], and neutrophils [66]. Similar to our data, it was observed very recently in young Limousin bulls that *PPARA* is expressed in liver, adipose, and muscle, with the greatest expression observed in liver, followed by semitendinosus muscle, and, then, intermuscular adipose tissue [32]. In ewes, its expression was detected in superficial endometrium and trophoblast during early pregnancy [41]. Lastly, expression of *PPARA* was demonstrated in sheep heart [67].

3.3. PPARβ/δ. As for nonruminants, the PPARβ/δ is the least-studied PPAR isotype also in ruminants, with few published information available. The results of our qPCR analysis indicate relatively similar *PPARD* mRNA expression in all the 14 tissues and cells assessed (Figure 1(a)); however, the greatest expression was observed in kidney and placenta followed by adipose tissues, rumen, and MDBK cells with the lowest expression observed in hoof corium, liver, and skeletal muscle (Figure 1(a)). The relative distribution of *PPARD* expression among cattle tissues/cells, even though similar to that in mouse [6], is rather curious particularly considering its low expression in skeletal muscle and the marked expression in blood neutrophils, placenta, and rumen tissue, that is, tissues that probably do not rely on LCFA oxidation as source of energy. Previous studies have observed expression of *PPARD* in bovine liver [56], aortic endothelial cells [68], mammary cells [69, 70], rumen [64], and uterus [43]. The *PPARD* was also shown to be expressed in longissimusmuscle of beef steers [47] and in both superficial endometrium and trophoblast of early pregnant ewes [41].

3.4. Relative Abundance between PPAR Isotypes in Cattle Tissues. To date, there is almost a complete lack of data available in the literature of a direct comparison of PPAR isotypes expression in ruminant tissues. Among the few available studies, it was observed that liver of dairy cows expresses a similar amount of *PPARA* and *PPARD* but does not express *PPARG* [44]. In a recent study where the expression of the three PPAR isotypes was evaluated in liver and muscle of beef bulls, the greatest expression was observed for *PPARA*, followed by *PPARG*, with the lowest expression for *PPARD* in liver, while, the largest expression in muscle was observed for *PPARG* [71]. This relative distribution among tissues is somewhat comparable to our data (Figure 1(b)). More numerous are the studies comparing mRNA abundance between PPAR isotypes in bovine cell culture. Those have revealed that bovine endometrial cells express *PPARA* and *PPARD* at a similar level, but not *PPARG* [44]. In addition, bovine aortic endothelial cells express both *PPARA* and *PPARG* [46] and mammary cells express both *PPARG* and *PPARD* [69].

When the relative mRNA abundance between the three PPAR isotypes was evaluated in several tissues from bovine (Figure 1(b)), we observed that the three adipose tissues along with rumen, MDBK cells, and placenta have a marked abundance of *PPARD* and *PPARG* compared with *PPARA*, whereas MAC-T cells and PMN were characterized by marked abundance of *PPARD* but very low abundance of the other two PPAR isotypes. Despite the relatively low abundance, at least *in vitro*, PPARγ appears to be functional in bovine neutrophils [72] and MAC-T cells [26]. Paradoxically, given its well-established function in monogastrics, with few exceptions (i.e., MDBK and beef cattle longissimus muscle), *PPARD* is more abundant than *PPARG*, even in the three adipose depots (Figure 1(b)). The *PPARA* instead was the more abundant PPAR isotype in small intestine, liver, kidney, skeletal muscle, hoof corium, lung, and mammary gland (Figure 1(b)).

Overall, the data in Figure 1 depict a distribution of PPAR isotypes that, similar to other species, seems to underscore the putative biological role of each PPAR isotype. For instance, the expression of *PPARA* is more abundant in tissues where LCFA oxidation is generally higher (e.g., liver and kidney) and *PPARG* is more abundant in lipogenic tissues (e.g., the three adipose tissues).

4. Sequence Homology, 3D Structure, and Activation of PPARα among Bovine, Mouse, and Human

We recently carried out an *in silico* analysis to compare the amino acid sequence homology of PPARα between bovine, mouse, and human [28]. The analysis revealed more than 90% conservation of this PPAR isotype between the three species, with bovine having greater overall homology to human (94.9%) than mouse (91.2%). When the four domains of the PPARα protein were compared, we observed lower conservation in the N-terminal A/B domain containing the ligand-independent activation function (AF-1), which was 86% conserved between bovine and human and 81% between bovine and mouse [17], and the largest conservation (i.e., 100%) in the DNA-binding domain. The latter suggests that the capacity of the domain for the recognition of the PPRE is highly conserved between species. This has been confirmed by the high responsiveness of rat PPRE when transfected in bovine endothelial cells [73].

The LBD is also highly conserved with greater homology of bovine with human (98%) than with mouse (92%). The lower conservation of the LBD and AF-1, which is common between species, could indicate a difference in interspecies sensitivity of PPARα activation [17] and a greater similarity between bovine and human than bovine and mouse. Surprisingly, when the transcription response of 30 putative PPARα target genes to the potent and specific PPARα agonist Wy-14643 were compared between mouse liver, human liver, and MDBK, we observed a greater number of genes with a common response between bovine and mouse (73%) than bovine and human (60%) [28]. Despite the limitation of comparing liver with kidney cells, those data indicate

a good degree of conservation of PPARα response between species. There are no published studies comparing PPARγ or PPARβ/δ response between ruminant and nonruminant species considering the same (or similar) tissue/cells. An attempt to compare the activation of PPARγ in mammary gland between dairy cattle and mouse is reported (see Section 9.2.1).

In order to further investigate the potential differences in PPARα between mouse and bovine we performed an *in silico* 3-dimensional (3D) structure analysis of the publicly available PPARα protein sequence [28]. The alignment analysis identified an overall high degree of conservation of PPARα amino acid sequence between the two species; however, when the overlap of the 3D structure of the PPARα of the two species was performed, we observed important differences in spatial structure of the LBD. In particular, the residues Leu462 and Tyr466 of the LBD in bovine result in a completely different spatial position compared with mouse (Figure 2). When the electrostatic potential of the surface was visualized, it was apparent that the bovine PPARα has an overall more neutral charge, particularly in the ligand pocket, compared with the highly negatively charged mouse PPARα. This allowed inferring that longer and more saturated LCFA (i.e., more neutrally charged and with a more straight configuration) might be more easily accommodated (Figure 2), hence, likely be better inducers in bovine.

It has been demonstrated, however, that the activation of PPAR isotypes is highly dependent on the A/B domain rather than the LBD [74]. This last observation could explain the interspecies differences observed, considering also that the A/B domain is the least-conserved between species and also between PPAR isotypes (see below). However, this does not fully explain the results from the comparison in PPARα response between bovine, mouse, and human [28] because the conservation of the A/B domain is lower between mouse and bovine than between human and bovine, despite the greater similarity in response between bovine and mouse compared to bovine and human [28].

5. Structural Similarity between PPAR Isotypes in Bovine

Approximately 80% of the 34 amino acid residues in the binding cavity of the three PPAR isotypes (α, β/δ, and γ) are conserved in humans and rodents [75]. The main features dictating the ligand specificity across the PPAR isotypes appear to be the topology of the ligand binding cavity; for example, the PPARβ/δ cavity is much narrower than PPARα and PPARγ and, thus, cannot accommodate bulky polar heads found in thiazolidinedione (TZD) [75, 76]. In contrast, TZD is a potent ligand of PPARγ. Once inside the cavity, the side chains of the ligand (e.g., hydrogen, carboxyl groups) interact with the amino acid residues to achieve a stable configuration.

In bovine, the three PPAR isotype proteins have low conservation overall, with PPARα being more similar to PPARβ/δ (59%) than PPARγ (52%) [28]. The three proteins have a large degree of conservation in the DNA binding domain (>80%), but a low degree of conservation in the A/B domain (<21%) [28]. The PPARα has a greater degree of conservation in the LBD with PPARβ/δ (71%) than PPARγ (64%) [28]. This last observation suggests that among the three isotypes, the expected response to agonists should be more similar between PPARα and PPARβ/δ as it is the case in nonruminants [2]. This would imply that activation of PPARα and PPARβ/δ could result in similar outcomes, for example, fatty acid catabolism.

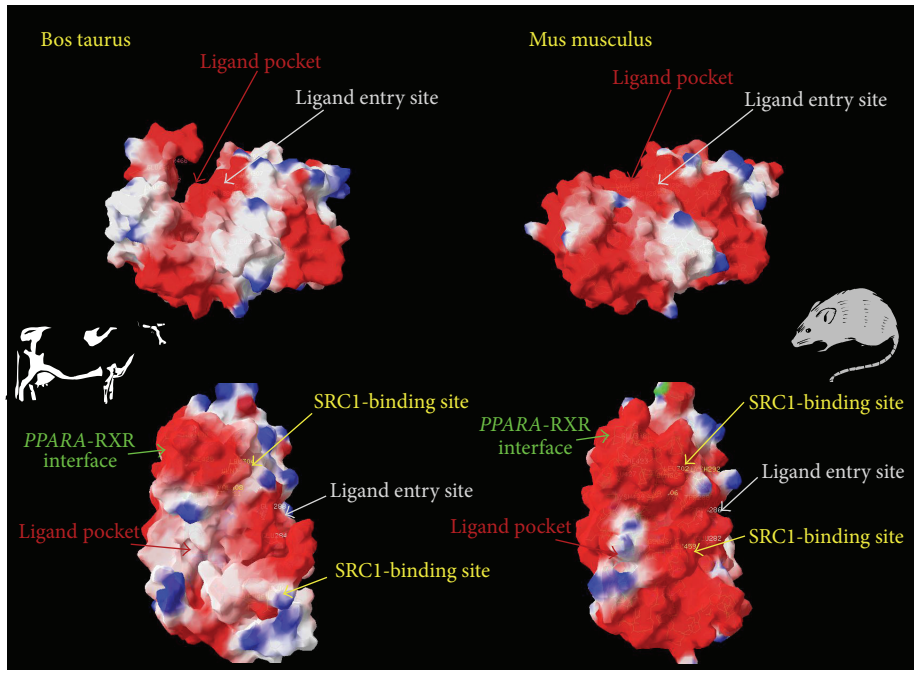
The 3D depiction of the bovine PPAR isotypes surface reveals a difference in the ligand pocket (Figure 2) [28]. The PPARα appears to have a larger pocket compared with the other two PPAR isotypes. In addition, analysis of the electrostatic potential of the surface indicates a greater negative charge in PPARγ than PPARα and PPARβ/δ, with the latter being mostly positively charged. Those observations suggest a greater capacity of PPARα for binding neutrally charged and/or more structurally rigid compounds. Clearly, this inference is only speculative.

6. Ruminant PPAR Response to Synthetic and Natural Agonists

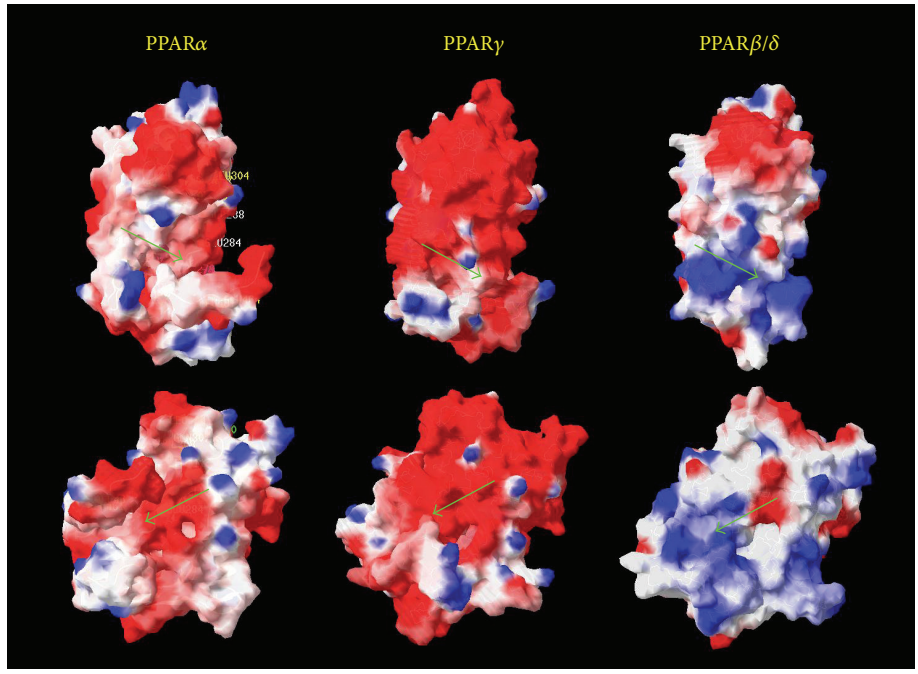
The effect of PPAR agonists in nonruminants has been tested in different models using *in vitro* systems with specific assays such as the Coactivator-Dependent Receptor Ligand Assay (CARLA) [18] or the transfection of PPRE with firefly luciferase (e.g., [96]). An additional assay available today is the direct measurement of activation of PPAR isotypes after nuclear isolation by the presence of PPRE immobilized onto the bottom of cell culture wells; however, such assays have not been developed for ruminants [61]. The use of these techniques with greater sensitivity, precision, and reliance in ruminants has been scant [61]. Most of the studies performed in ruminants are based on measurements of changes in expression of genes or proteins after treatment with PPAR isotype-specific agonists.

6.1. Ruminant PPAR Response to Synthetic Agonists. Several synthetic PPAR agonists are available today for nonruminants [18]. Among the most commonly used are Wy-14643 and fenofibrate as PPARα agonists and TZD and rosiglitazone as PPARγ agonists. Very few synthetic agonists of PPARβ/δ are known (e.g., GW501516). Besides agonists, a few antagonists have been developed, for example, the PPARγ specific antagonists GW9662 [97] and BADGE [98], the PPARα antagonists T0070907 [99] and GW6471 [100], and the PPARβ/δ antagonists GSK0660 [101] and GSK3787 [102]. The use of the specific agonists in combination with antagonists could be a valid, though indirect, approach to uncover both the existence of an active PPAR isotype in cells or tissues and PPAR target genes.

Supplementary Table 1 (see Supplementary Material available online at http://dx.doi.org/10.1155/2013/684159) contains a summary of studies performed to date using specific PPAR agonists in ruminants. From the data, it is evident that most of the studies dealt with bovine with few ones in sheep and goat. A large amount of the bovine studies were performed with bovine endothelial cells. Those cells have been widely used as a model to study endothelial physiology and



(a)



(b)

Figure 2: Inter-species and inter-isotypes three-dimensional PPAR protein structure comparisons. (a) Three-dimensional surface structure of bovine (residue 202–470; UniProtKB/TrEMBL Q5EA13) and mouse (residue 202–468; UniProtKB/TrEMBL P23204) PPARα ligand binding domain (LBD). The upper and lower panels include two views of the 3D structure of the PPARα protein in bovine and mouse species. The 3D structure is in full alignment between species. From the comparison, the difference in the ligand pocket of the PPARα between the two species is evident, with a larger and more pronounced pocket in bovine compared with mouse. In addition, the bovine PPARα appears to be more neutrally charged compared with the same protein in mouse. (b) Three-dimensional surface structure comparisons between PPARα (residue 202–470; Q5EA13), PPARγ (residue 234–505; O18971), and PPARβ/δ (residue 171–441; A4IFL4) LBD of bovine. Shown is the ligand pocket domain (green arrow) in two diverse views for each of the PPAR isotypes. The comparison highlights the larger and more neutrally charged ligand pocket in PPARα compared with the more negatively charged PPARγ ligand pocket and positively charged and small PPARβ/δ ligand pocket. The images were modified from [28]. Legend: red = negative charge; white = neutral charge; blue = positive charge. The 3D analyses were performed using Swiss-Pdb Viewer software (freely available at http://spdbv.vital-it.ch/).

pathology, particularly for the inflammatory status related to arteriosclerosis, that is, with a clear biomedical purpose and not to understand ruminant biology. Overall those studies established important roles of PPAR in endothelial cells [46, 92, 103, 104]. In particular the activation of PPARγ and PPARα appears to have a protective role for endothelium (Supplementary Table 1).

The first study performed using a PPAR agonist with a clear aim to understand the biology of ruminants was performed in 1998 by a German group [42] where it was observed that PPARγ controls progesterone synthesis in lutein cells isolated from dairy cows. Subsequent studies in granulosa cells of sheep confirmed the role of PPARγ in controlling progesterone synthesis [45].

In 1998, a Japanese group demonstrated that activation of PPARγ is central for adipogenic differentiation of vascular stromal cells from bovine adipose tissue [105] and intramuscular fibroblast-like cells [106]. In 2001, another Japanese group demonstrated that *in vivo* injection of the PPARγ agonist 2,4-TZD partially reversed the insulin resistance induced by TNFα in dairy steers. The phenomenon was explained by the activation of PPARγ in adipose tissue [107]. A year later a group of researchers from a pharmaceutical company fed the PPARα agonist Wy-14643 to lactating goats [108]. The authors reported an overall increase in hepatic β-oxidation and aromatase activity by Wy-14643 and decreased cholesterol in blood (with numerical decrease of TAG as well). No effects were observed on liver size, milk composition, or content of hepatic cytochrome P450. The low magnitude of changes and the unexpected lack of effect of the treatment on P450 led the authors to conclude that the goat is a weak responder to PPARα agonists.

The two studies *in vivo* mentioned above were critical for animal bioscientists interested in PPAR because they demonstrated that PPARα in liver and PPARγ in adipose tissue of ruminants are active and likely play similar roles as in monogastrics: regulation of β-oxidation for PPARα and regulation of adipogenesis and insulin sensitivity for PPARγ. Since then, few additional *in vivo* studies using PPAR agonists with agricultural aims have been performed (Supplementary Table 1). Recently, we tested the effects of oral administration for 14 days of the PPARα agonist clofibrate on liver of weaned dairy calves [78] (see also Supplementary Table 1). The treatment had several expected effects such as the increase in expression of several PPARα target genes (see Section 7 for details about PPAR targets in ruminants), but the magnitude of response was lower than usually observed in rodents; thus, we concluded, as for the work performed on goats, that the bovine hepatic PPARα is a weaker responder compared to rodents.

The above observations from *in vivo* studies of a weak response in ruminants might be explained by the inherent differences in digestive physiology. Contrary to monogastrics, in ruminants, the digestion of any feed is markedly affected by the process of fermentation in the rumen via microorganisms. None of the above studies have assessed the effect of the rumen on PPAR agonists. In this regard, it could have been interesting to measure the blood concentration of the agonists. Interestingly, the human PPAR isotypes also appear to have a lower response compared with rodents [19]. It can also be the case that Wy-14643, a recognized potent PPARα agonist in rodents, is not as potent in ruminants. In accord with this, we have observed in bovine cells a greater increase in expression of PPARα target genes by saturated LCFA compared to Wy-14643 [28]. Those responses indicate a species-specific response to PPAR induction and a different effect of agonists between species.

The results obtained during the *in vivo* study of the Japanese group mentioned above led to a series of *in vivo* experiments in pregnant and lactating dairy cows [82, 84, 109, 110]. The purpose of those studies was to evaluate the effects of PPARγ activation on preventing metabolic problems typical of the peripartal period. The specific PPARγ agonist 2,4-TZD was used (via injection) for that purpose (Supplementary Table 1). The treatment with 4 mg/kg BW daily of 2,4-TZD during the last two or three weeks prepartum until parturition decreased substantially the NEFA post-partum. Such effect was ascribed to enhanced insulin sensitivity and *PPARG* expression in adipose. In addition, the treatment improved the overall metabolic health postpartum, as reflected in greater feed intake, lower hepatic lipid accumulation, and greater glycogen content in the liver. Overall, the data also suggested an improved fertility (i.e., lower open days) in cows treated with 2,4-TZD.

This series of *in vivo* experiments reported above (see also Supplementary Table 1) was the first demonstration that PPAR isoforms can play a pivotal role in the physiology and metabolism of dairy cattle. It also underscores the concrete possibility of fine-tuning the PPAR isotype activity through appropriate treatments in order to improve overall performance and health of dairy cattle.

An elegant *in vivo* study performed recently in pregnant sheep involved the injection of rosiglitazone into the fetuses for >10 days beginning at ca. 25 days before term [81]. The experiment demonstrated that activation of PPARγ had a similar effect on fetuses as overnutrition of the pregnant mother, which is known to induce obesity in later life in offsprings. For instance, rosiglitazone treatment increased expression of lipoprotein lipase and adiponectin in adipose tissue and *PPARA* and PPARγ coactivator 1 alpha (*PPARGC1A*) in liver of fetuses (Supplementary Table 1).

Several *in vitro* studies using synthetic agonists have demonstrated that activation of PPAR isotypes (except gamma) affects fertility by increasing the expression and/or production of prostaglandins, for example, prostaglandin (PG) F2α, and PGE2 in bovine endometrial cells [44, 77]. Other *in vitro* studies were carried out in order to test the response to PPAR isotypes in two bovine cell lines (MDBK and MAC-T) with the purpose of determining PPARα and PPARγ target genes [26, 28, 36, 61]. Besides target genes, those studies also uncovered several biological functions of PPAR isotypes in ruminants. For instance, the activation of PPARγ in MAC-T cells with rosiglitazone provided a demonstration that PPARγ controls expression of several genes known to be involved in milk fat synthesis [26] while activation of PPARα controls lipid metabolism at the cellular and organismal level

(i.e., by controlling expression of several signaling molecules) [28].

All the above studies clearly demonstrated an active role of PPAR isotypes in ruminants. The studies also established that PPAR isotypes can be manipulated by using synthetic agonists; however, from a practical stand-point the suggestion of using synthetic agonists is not feasible, namely, because of the high costs that would be incurred. Clearly that could be circumvented if natural ligands are identified.

6.2. Ruminant PPAR Response to Natural Agonists

6.2.1. LCFA. The great interest in PPARs in the area of nutrition stems from the ability to bind and be activated (or inhibited) by LCFA or chemically related derivatives [18, 111, 112].

Monogastrics. In monogastrics all PPAR isotypes are sensitive to fatty acids, particularly LCFA. Although the potency varies with each PPAR isotype, the most-potent PPAR endogenous ligands in nonruminants are linoleic acid, linolenic acid, arachidonic acid, and also derivatives of arachidonic acid such as leukotriene B4 (LTB4) or PG [12]. In general it is safe to conclude that PPAR isotypes in most monogastrics species studied to date have a greater sensitivity towards unsaturated than saturated [17, 18]. However, in nonruminants both saturated and unsaturated LCFA enhance PPAR transactivation *in vitro* (e.g., [12, 113, 114]).

In vivo data have been more variable and in some instances high dietary fat activated PPAR target genes regardless of whether the dietary lipid was mostly polyunsaturated (PUFA), monounsaturated, or saturated (e.g., [115]). At the cellular level studies with endogenous ligands such as free LCFA or LCFA-CoA (i.e., activated 16:0, 18:2n-6, 18:3n-3, and 20:4n-6) have demonstrated (at least for PPARα) that both forms of the FA exhibit high affinity (i.e., low nanomolar dissociation values) for the ligand-binding domain of PPAR [114]. This point is important because intranuclear concentrations of free LCFA and LCFA-CoA range between 120–500 nM and 8 nM, respectively [116].

From a mechanistic standpoint it is important to point out that FA binding proteins (FABP, particularly FABP1 and FABP4) are important in channeling intracellular nonactivated (i.e., without addition of the CoA group) LCFA not only to the various organelles but also to the nucleus where the LCFA can activate PPAR. The essential role of FABP in transporting LCFA into the nucleus for the activation of PPAR isotypes was first reported in rodent liver where the amount of FABP1 protein significantly correlated with transactivation of PPAR in response to LCFA (linoleic acid, linolenic acid, and arachidonic acid) as well as other chemical ligands [117].

Ruminants. To our knowledge there are only two published studies where PPRE luciferase was used to test activation of PPAR isotypes in bovine cells [62, 68]. In one study, however, only activation of PPARβ/δ was assessed and no LCFA were tested. In another study the activation of PPARα by free LCFA or oleic acid was demonstrated in bovine aortic endothelial cells [62]. So far the effect of LCFA on ruminant PPAR activity has been evaluated primarily in an indirect way through measuring changes in expression of target genes after addition of specific LCFA. This model has limitations, one being the capacity of LCFA to bind and activate additional transcription factors (TF). Besides PPARs, also Hepatic Nuclear Factor 4 (HNF4α), Liver X Receptor (LXR), and RXR can bind LCFA, as shown in human, mouse, and rat [118]; however, in those species the LXRβ and the RXRα appear to be weakly activated by natural LCFA while PPARα, PPARβ/δ, and PPARγ are strongly activated [119]. The greater sensitivity of PPAR compared with other TF provides some support for the use of target gene expression as a proxy for evaluating activation of PPARs by LCFA. Another limitation of the indirect approach is the inability to distinguish the activation between PPAR isotypes. Using the above indirect approach it was demonstrated that ruminant PPAR are activated by several physiologically relevant LCFA (Table 1).

The LCFA experiments in ruminants were mainly performed with MAC-T and MDBK cells and focused on PPARα and PPARγ [26, 28, 36, 61]. In both cell types the LCFA clearly induced expression of genes previously shown using specific agonists (Wy-14643 and rosiglitazone) to be PPARα and PPARγ target genes (see Table 2 and Section 7 for details). The potency of saturated was greater than unsaturated LCFA. In particular, in MDBK cells we observed weaker induction of target genes as the degree of unsaturation increased [28]. Above all it was observed that palmitate and stearate induced a very strong activation of transcription of PPARα and PPARγ target genes [26, 28]. Those data were suggestive of an evolutionary adaptation of the PPAR in ruminants to respond to saturated LCFA, which are the most abundant LCFA in the circulation of ruminants [120, 121] compared to monogastrics [122, 123] due to extensive ruminal hydrogenation of unsaturated LCFA. However, our studies suggested that the LCFA activated gene expression not only through PPAR isotypes but also other TF, probably the ones mentioned above, or even other unknown TF [28]. This point, as well as the role of coactivators and their relative abundance [76], deserves further investigation in order to select with greater confidence the most suitable mixture of LCFA for modulating metabolism in ruminants.

Because intracellular LCFA pools are a mixture of saturated and unsaturated LCFA, it is interesting that PPARγ (and maybe other PPAR isotypes) is capable of binding two LCFA simultaneously, at the least in monogastrics [124]. This suggests that there could exist a mechanism whereby the composition of LCFA in the cytosol dictates the "strength" of the response, that is, the ability to bind two LCFA simultaneously could allow PPARγ to give a graded response to the varying composition of the intracellular LCFA pool [124].

6.2.2. Glucose. Besides LCFA, it has been also reported that glucose binds and activates PPARα in mouse connecting glucose with lipid metabolism [125]. This has not been confirmed in ruminants; however, it has been shown that ruminant PPARβ/δ binds and is activated by glucose [68].

Table 1: Activation of PPAR isotypes in ruminants by main long-chain fatty acids or glucose.

LCFA/glucose	Effect on PPAR isotype[@]			Method[#]	References
	PPARα	PPARγ	PPARβ/δ		
16:0	+++	+++	n/a	Indirect	[26, 28, 61]
18:0	+++	+++	n/a	Indirect	[26, 28]
c9-18:1	++	+	n/a	Indirect/Luciferase	[26, 28, 62]
t10-18:1	n/a[&]	+	n/a	Indirect	[26]
18:2	+	n/a	n/a	Indirect	[26, 28]
c9,t11-18:2	+	n/a	n/a	Indirect	[26, 28]
t10,c12-18:2	+	±	n/a	Indirect	[26, 28]
CLAmix[$]	+	n/a	n/a	Indirect	[36, 77]
20:0	++	n/a	n/a	Indirect	[26, 28]
20:4n-6	++	n/a	++[*]	Indirect/Luciferase	[68, 77]
20:5n-3	++	++	n/a	Indirect	[26, 28]
22:6n-3	+	n/a	n/a	Indirect	[26, 28]
Glucose	No	n/a	++	Luciferase	[68]

[@]+++: strong agonist; ++: agonist; +: weak agonist; ±: mixture between agonist and antagonist.
[*]The 12-HETE, a metabolite of the 20:4n-6 is the actual agonist.
[#]Indirect: the effect on PPAR isotype target genes was uncovered by the use of specific PPAR synthetic agonists; luciferase: the use of the PPRE-luciferase construct to test activation of PPAR by agonists.
[$]A mixture (ca. 50% each) of the t10,c12- and c9,t11-conjugated 18:2
[&]Not available.

Specifically, it was demonstrated in bovine endothelial cells that when PPARβ/δ is activated by glucose, it downregulates glucose transport in order to prevent hyperglycemia.

6.2.3. Other Natural Agonists/Antagonists. As with nonruminants, PPARγ in bovine vascular endothelial and mammary cells is activated by PGJ2 [46, 69]. The PPARγ is inhibited and its expression decreased by the oxidative stress intermediate H_2O_2 in bovine endothelial cells [94, 126]. Nitric oxide appears to be an inhibitor because it decreased the expression of the *PPARGC1A*, a known PPARγ target gene [94]. This compound decreased the expression of *PPARGC1A* during the first 12 h after treatment but increased the expression of the same gene in the longer term (>24 h) [127]. The increase in expression of *PPARGC1A* was demonstrated to be crucial for the mechanism of protection from oxidative stress [127]. In bovine articular chondrocytes, the presence of oxidized LDL increased expression of vascular endothelial growth factor (VEGF) through PPARγ [128].

7. PPAR Isotype Target Genes in Ruminants

In several of our studies, the overall response of PPARα and PPARγ in bovine cells was strong and consistent [26, 28, 61, 129]. Those studies allowed uncovering several bovine-specific PPARα target genes (Table 2), and several were already established as PPARα targets in other species. Among bovine-specific PPARα target genes, the osteopontin (*SPP1*) gene had a large increase in expression after Wy-14643 treatment in bovine kidney cells [28] contrary to what has been observed in human and mouse [130, 131]. Between bovine, human, and mouse, only 67% of the putative PPARα target genes tested responded in a similar fashion, suggesting a species-specific response of PPAR [28].

The activation of PPARα by Wy-14643 resulted in a general increase in lipid metabolism-related genes including several involved in lipid synthesis, such as lipin 1 (*LPIN1*) and sterol regulatory element binding transcription factor 1 (*SREBF1*) [28]. Interestingly, expression of both genes was not induced in a previous study using the same model [61]. The only difference between the two studies was the addition of insulin in the latter [28]. In support of a potentially important role of insulin for PPAR activation, in a recent study with MDBK, we observed a faster response in expression of PPARα target genes after addition of insulin [61]. Therefore, insulin in bovine seems essential for PPAR activation but may be more crucial for some genes (e.g., *LPIN1* and *SREBF1* versus carnitine palmitoyltransferase 1A (*CPT1A*)) [28, 61].

The increased expression of *SREBF1* with Wy-14643 in the MDBK study might also be due to the activation of PPARγ because we observed that activation of PPARγ with rosiglitazone increased expression of *SREBF1* in MAC-T cells [26]. The activation of PPARγ in MAC-T cells appeared to be robust [26]; however, the use of 10 μM TZD for 12 h in MDBK cells did not affect expression of any gene tested using microarray technology, suggesting that activity of PPARγ in MDBK is extremely low or inexistent (Bionaz et al. unpublished data). This observation is intriguing considering that overall expression of *PPARG* in MDBK is relatively high compared with other tissues/cells (Figure 1(a)), and higher than *PPARA* (Figure 1(b)). Furthermore, the response to PPARα agonists is consistently high in those cells [28]. Therefore, it cannot be excluded that the increase in expression of *SREBF1* after addition of Wy-14643 was due exclusively to PPARα activation.

Compiled data from our and other groups in Table 2 suggest that there are some inconsistencies in the response of target genes between tissues or cells, or even between

Table 2: PPAR isotype target genes in ruminants grouped by main biological function.

Gene	HUGO gene name	Tissue/cells[1]	PPAR[2]	Reference
	Fatty acid import and activation			
ACSL1	Acyl-CoA synthetase long-chain family member 1	MDBK	⇑PPARα	[28, 61]
		Liver	⇑PPARα	[78]
ACSL3	Acyl-CoA synthetase long-chain family member 3	MDBK	⇑PPARα	[28]
CD36	Thrombospondin receptor	MDBK	⇑PPARα	[28]
		BAEC	⇑PPARγ	[79]
		MAC-T	⇔PPARγ	[26]
		Muscle	⇑PPARγ	[80]#
FABP4	Fatty acid binding protein 4	MDBK	⇑PPARα	[28]
		MAC-T	⇔PPARγ	[26]
		sP. adipose	⇑PPARγ	[81]
LPL	Lipoprotein lipase	MAC-T	⇔PPARγ	[26]
		bS. adipose	⇔PPARγ	[82]
	Fatty acid synthesis			
ACACA	Acetyl-CoA carboxylase alpha	MAC-T	⇑PPARγ	[26]
FASN	Fatty acid synthase	MAC-T	⇑PPARγ	[26, 83]
		bS. adipose	⇔PPARγ	[82]
		bS. adipose	⇓PPARγ	[84]
INSIG1	Insulin induced gene 1	MAC-T	⇑PPARγ	[26]
SCD	Stearoyl-CoA desaturase (delta-9-desaturase)	MDBK	⇑PPARα	[28]
SREBF1	Sterol regulatory element binding factor 1	MAC-T	⇑PPARγ	[26]
		MDBK	⇑PPARα	[28]
	Fatty acid oxidation			
ACADVL	Acyl-CoA dehydrogenase, very long chain	MDBK	⇑PPARα	[61]
		Liver	⇑PPARα	[78]
ACOX1	Acyl-coenzyme A oxidase 1	MDBK	⇔PPARα	[61]
		Liver**	⇑PPARα	[78]
CPT1A	Carnitine palmitoyltransferase 1A (liver)	MDBK	⇑PPARα	[28, 36, 61]
		Liver**	⇑PPARα	[78]
CPT2	Carnitine palmitoyltransferase 2	PAEC	⇑PPARγ	[85]
CRAT	Carnitine O-acetyltransferase	PAEC	⇑PPARγ	[85]
CYP4A11	Cytochrome P450, family 4, subfam. A, polypeptide 11	Liver	⇑PPARα	[78]
	Triacylglycerol synthesis			
AGPAT6	1-Acylglycerol-3-phosphate O-acyltransferase 6	MAC-T	⇑PPARγ	[26]
DGAT1	Diacylglycerol O-acyltransferase 1	MAC-T	⇑PPARγ	[26]
LPIN1	Lipin 1	MAC-T	⇑PPARγ	[26]
		MDBK	⇑PPARα	[28, 61]
LPIN3	Lipin 3	MDBK	⇑PPARα	[28, 78]
	Cholesterol synthesis			
HMGCR	3-Hydroxy-3-methylglutaryl-CoA reductase	MDBK	⇑PPARα	[28]

Table 2: Continued.

Gene	HUGO gene name	Tissue/cells[1]	PPAR[2]	Reference
SREBF2	Sterol regulatory element binding transcription factor 2	MAC-T	⇑PPARγ	[26]
		Signaling molecules		
ANGPTL4	Angiopoietin-like 4	Liver	⇑PPARα	[56, 86]$^{++}$
		MDBK	⇑PPARα	[28]
FGF21	Fibroblast growth factor 21	Liver	⇑PPARα	[86]$^{++}$ [87]
EDN1	Endothelin 1	BAEC	⇓PPARα	
			⇓PPARγ	
LEP	Leptin	bS. adipose	⇑PPARγ	[84]
NOS3	Nitric oxide synthase 3 (endothelial cell)	BAEC	⇑PPARα	[88]
PTGS2	Prostaglandin-endoperoxide synthase 2	BEND	⇑PPARα	[44]
			⇑PPARγ	[44]
		pBESC	⇑PPARα	[77]
		MAC-T	⇔PPARγ	[26]
SPP1	Osteopontin	MDBK	⇑PPARα	[28]
VEGF	Vascular endothelial growth factor	BAEC	⇑PPARγ	[89]
		Other functions		
CDKN2A	Cyclin-dependent kinase inhibitor 2A	BAEC	⇓PPARγ	[90] [91]
GAPDH$^{\$}$	Glyceraldehyde-3-phosphate-dehydrogenase	s. ASC	⇑PPARγ	
			⇑PPARβ/δ	
OLR1	Oxidized low density lipoprotein receptor 1	BAEC	⇑PPARα	[92]
			⇓PPARγ	[46]
PC	Pyruvate carboxylase	Hepatoma*	⇑PPARα	[93]
		MDBK	⇔PPARα	[28]
SLC2A1	Solute carrier family 2, member 1	BAEC	⇓PPARβ/δ	[68]
TERF2	Telomeric repeat binding factor 2	BAEC	⇑PPARγ	[90]
		PPAR activation-related functions		
PPARA	Peroxisome-proliferator-activated receptor alpha	BAEC	⇑PPARα	[62]
		MDBK	⇔PPARα	[28, 61]
		Liver	⇑PPARγ	[81]
		Muscle**	⇑PPARγ	[81]
PPARG	Peroxisome-proliferator-activated receptor gamma	bEPC	⇑PPARγ	[94]
		MAC-T	⇔PPARγ	[26]
PPARGC1A	PPARγ, coactivator 1 alpha	s. Muscle	⇑PPARγ	[81]

[1] Acronyms: BAEC: Bovine Aortic Endothelial Cells; BEND: Bovine Endometrial Cells; bEPC: bovine renal Epithelial cells; BRCP: Bovine Retinal Capillary Pericytes; bS. Adipose: bovine subcutaneous adipose; pBESC: primary (16-day cycle) bovine endometrial stromal cells; MDBK: Madin-Darby Kidney Cell Line; PAEC: ovine pulmonary arterial endothelial cells; sP.adipose: sheep perirenal adipose; [1]s. ASC: sheep adipose stem cells; s. Muscle: sheep muscle.

[2] The PPAR activated by the treatment with a different effect on expression of the target gene (⇑ induction; ⇓ inhibition; ⇔ no change).

* Rat hepatoma was transfected with bovine PC promoter region.

** The increase in expression was with $P < 0.10$ but $P > 0.05$.

$ The activity and not the mRNA expression of GAPDH was measured.

Inferred based on the high correlation of expression between *PPARG* and *FABP4*.

++ Inferred based on hepatic mRNA expression in studies with peripartal cows and undernutrition ketosis [56, 86, 95] (see main body of the paper for details).

the same tissue/cell. This is not surprising considering that several conditions can change the activity of PPAR isotypes, for example, the addition of insulin mentioned above. However, another important factor that might explain the different response between cell types or experiments is the abundance and activity of coregulators [132].

Some unexpected findings can be seen from data reported in Table 2. For instance, the well-established PPARγ target in nonruminants *FABP4* [133] does not appear to be affected by activation of PPARγ in ruminants, at least in MAC-T cells [26] but was induced by activation of PPARα in MDBK cells [28]. In a study performed in intramuscular fat of growing beef steers, it was observed a very high correlation between the expression of *FABP4* and *PPARG* suggesting a dependence of *FABP4* expression from PPARγ [80]. Contrary to such observation, in a recent study in pregnant overfed versus normal fed energy dairy cows, no change in expression of *FABP4* was observed but a greater expression of *PPARG* in subcutaneous adipose [134]. As for others, this unexpected finding in ruminant cells needs to be further confirmed; however, it underscores the limitation of using nonruminant data in the context of bovine.

Another cause of discrepancy might be due to methodological differences between studies, such as the methods used to perform qPCR. Most of the target genes reported in Table 2 were uncovered using qPCR. This technique relies on the identification and use of proper internal control genes [135], which is seldom conducted. As a result, some of the data generated by qPCR may lack accuracy prompting for a more routine application of all quality controls. In order to overcome several of the critical limitations often found in work reporting qPCR data, the minimum information for Publication of quantitative Real-Time PCR experiments (MIQE) [136] was created. Adherence to those guidelines will help standardize protocols, thus, enhancing data reliability. The use of such guidelines should be required by a greater number of scientific journals.

8. Effect of NEFA, Energy in the Diet and Fetal Reprogramming, on PPAR Isotypes

8.1. NEFA. The provision of LCFA to mammalian cells is from NEFA originating from adipose tissue lipolysis or from lipolysis of chylomicron or very low density lipoproteins (VLDL). The activation of bovine PPARα by NEFA was demonstrated recently in bovine aortic endothelial cells, where it was observed that PPARα activity was increased by release of free FA from VLDL via the action of lipoprotein lipase (LPL) [62]. In the same experiment it was demonstrated that ~10 μM of released NEFA in the media activated PPARα by ca. 80% compared to 10 μM of the specific PPARα agonist Wy-14643. A similar concentration of oleic acid alone activated bovine PPARα up to ca. 60% compared to Wy-14643. The activation of PPARα was due to free FA uptaken by the cells as demonstrated by the strong linear relationship between activation of PPARα and uptake of LCFA [62]. In addition, the activation of PPARα was proportionally inhibited by amount of albumin in the medium [62]. The results from the same study also indicated that the free FA released by the LPL, and not the circulating plasma FA (i.e., albumin-bounded NEFA), are the ones able to activate PPARα. The authors explained this by proposing that the high concentration of LCFA needed for PPARα activation can be achieved only by local release by lipase of LCFA from lipoproteins. Those results need to be further confirmed because of their important implications in the fine-tune activation of PPARs by dietary approaches.

The activation of PPAR by FA entering the cells via the unsaturable process is supported by the fact that endogenous activation of PPARα *in vivo* seems to occur mainly with high levels of LCFA that occur under fasting conditions in nonruminants [137]. In addition, we have shown in bovine cells that the expression of PPARα target genes is faster and more pronounced if cells are treated with free palmitate instead of palmitate bound to albumin [61].

The above-mentioned findings are relevant to dairy cattle soon after parturition when the hypoinsulinemia due to negative energy balance (NEB) reduces insulin sensitivity, and uncoupling of the growth hormone-insulin-like growth factor-1 axis results in substantial increase in NEFA, a mixture of LCFA whose composition can be partly altered through dietary approaches.

Evidence of increased activation and/or expression of PPARs due to the surge in NEFA has been reported in cattle. In particular, it has been observed that during the transition from pregnancy to lactation, characterized by a large surge of plasma NEFA, there is upregulation in expression of several PPAR target genes (e.g., *CPT1A*, *ACOX1*, see Table 2) in liver of dairy cattle, with a concomitant increase in expression of *PPARA* [57, 138, 139]; however, not all the studies found this to be a consistent response [140].

8.2. Nutrient Restriction. Nutrient restriction in dairy cows, causing a concomitant increase in blood NEFA, enhanced expression of *PPARA* and *PPARD* in liver [56] and protein expression of PPARγ in the hypothalamus [141]. Similarly, a 60-day period of body weight loss in beef cows was associated with greater expression of all three PPAR isotypes in biceps femoris muscle and several PPAR target genes, compared with cows that maintained body weight [142]. Overall, the data indicated that the NEB, with a consequent increase in NEFA, appears to induce expression and activation of all PPAR isotypes, but particularly of *PPARA* and *PPARD*.

8.3. High Dietary Energy. High dietary energy during pregnancy in dairy cows was associated with lower expression of liver *PPARA* early post-partum [143]. High dietary energy in weaned Angus steers, but not Angus × Simmental steers, was associated with lower expression of *PPARD* in Longissimus *lumborum* muscle [47].

8.4. Dietary Energy and Fetal Reprogramming. In ovine, nutrient restriction in ewes during early pregnancy (between 28 to 80 days gestation) increased expression of *PPARA* in the adipose tissue of the near-term fetus [144]. However, this was true only if the ewes were fed to requirements after this period of pregnancy; the adipose tissue of fetuses from ewes fed ad libitum from 80 days of pregnancy to term had lower *PPARA*

expression [144]. The above data clearly indicate that level of energy in the diet of the mother has a strong effect on the fetal transcriptome, that is, fetal reprogramming.

The fetal reprogramming of PPAR due to dietary energy level also has been observed when animals were overfed energy during pregnancy, such that fetuses of those dams had greater expression of *PPARG* and other lipogenic genes [145]. In contrast, either control or a high-energy diet in the periconception period or during pregnancy did not affect expression of *PPARG* in perirenal, omental, or subcutaneous adipose tissue of 4-month-old lambs [146]. Interestingly, intrafetal administration of a PPARγ agonist, rosiglitazone, increased expression of *LPL*, a putative PPARγ target gene, in perirenal adipose tissue of sheep fetuses [81]. No effect was observed for *PPARG* itself. In contrast, in the same study rosiglitazone increased expression of *PPARA* in liver.

9. Biological Effects of PPAR Activation in Ruminants

Most of the biological roles of PPAR uncovered in monogastrics can likely be extrapolated to ruminants; however, before those roles can be considered established also in ruminants, experiments need to be performed. Due to the modest amount of research performed to date, the biological significance of PPAR isotypes in ruminants is not well established, but the studies so far conducted have confirmed the existence of conserved roles between monogastrics and ruminants. In this section we provide an overview of the biological roles suggested by most of the experiments on PPAR carried out in ruminants besides those mentioned above on bovine endothelial cells.

9.1. Control of Adipogenesis and Lipid Metabolism

9.1.1. PPARγ. As for nonruminants [21], PPARγ plays a pivotal role in adipogenesis in ovine and bovine [91, 147], and in dairy cows its expression is high in adipose tissue (Figure 1) and appears to control lipogenesis by acutely responding to energy level in the diet [82, 134, 148, 149]. The importance of PPARγ in adipogenesis has been highlighted also by the identification of this as one of the candidate genes related to bovine marbling [150]. Besides lipogenesis, PPARγ might also play a role in LCFA oxidation as recently observed in lamb pulmonary arterial endothelial cells [85]. In that study it was demonstrated that PPARγ controls the expression of carnitine palmitoyltransferase 2 (*CPT2*) and carnitine O-acetyltransferase (*CRAT*), both genes involved in the entry of LCFA into the mitochondria, while it controls the translation of *CPT1A* but not its expression [85].

9.1.2. PPARα. The activation of goat PPARα *in vivo* increased fatty acid oxidation in liver [108]. The oral administration of Wy-14643 increased palmitate oxidation in liver of dairy calves with a concomitant increase in expression of several genes known to be PPARα targets (see Table 2) involved in FA oxidation in nonruminants [78]. Therefore, it is apparent that the activation of PPARα in ruminants controls catabolism of fatty acids. Other pieces of evidence supporting that conclusion include the fact that FA catabolism in mitochondria and peroxisome increases during the transition from pregnancy into lactation [151]. This appears to be consequence of the large surge of NEFA and the concomitant increase in expression of few key genes rather than an increase of overall pathway flux [152]. However, the expression of *PPARA* in liver of dairy cattle increases from pregnancy to early post-partum [57, 138]. In the same time, several PPARα target genes involved in lipid metabolism have a similar increase in expression as *PPARA* in liver during the transition from pregnancy to lactation; those include *ACOX1* and acyl-coenzyme A dehydrogenase, medium chain (*ACADM*) [57, 138]. Finally, the use of Wy-14643 in MDBK cells increased expression of several genes involved in lipid catabolism [28, 61] (Supplementary Table 1). One of those key genes is the well-known PPARα target *CPT1A* [57, 138].

9.1.3. PPARβ/δ. Compared with PPARα and PPARγ, the role on lipid metabolism of PPARβ/δ activation in ruminants is less clear. The PPARβ/δ was shown to have a role in adipogenesis in sheep because its activation increased activity of GAPDH [91]. An involvement of PPARβ/δ in adipogenesis also was reported by several experiments performed in monogastrics [2]. However, a contrasting role of PPARγ and PPARβ/δ was observed in primary bovine mammary cells, where several PPARγ ligands reduced the expression of PPARβ/δ [69]. PPARα unarguably has a primary role in controlling fatty acid oxidation in rodents; however, PPARβ/δ also controls fatty acid oxidation in skeletal muscle, heart, and brown and white adipose tissue [2]. Several data indirectly suggest a similar role in ruminants. It was observed that during nutrient restriction [56] and during body weight loss in muscle of beef cows [142], both situations that enhance LCFA oxidation, there was a concomitant increase in expression of *PPARA* and *PPARD*.

In summary, the pivotal role of PPARγ in controlling adipogenesis and lipogenesis in adipose tissue, which was clearly established in nonruminants, can also be considered established in ruminants. The control of fatty acid oxidation by PPARα in ruminants appears supported by the data published to date. The few data available also suggest a role for PPARβ/δ in lipid catabolism in ruminants.

9.2. Control of Milk Fat Synthesis by PPARγ in Dairy Cattle. Milk fat synthesis in dairy cows appears to be controlled at least in part by PPARγ. This was originally suggested by the increase in expression of *PPARG* in mammary gland of dairy cows between pregnancy and lactation [33]. In the same study, a large increase in expression of a network of genes potentially involved in milk fat synthesis and for the most part putative PPARγ target genes was observed. Based, on those data we then tested, and demonstrated, the hypothesis that PPARγ controls expression of key genes involved in milk fat synthesis, including *SREBF1* [26].

A pivotal role of milk fat synthesis regulation by SREBP1 has been originally proposed based on the consistent reduction of *SREBF1* expression by *t10,c12*-CLA, a minor unsaturated FA produced during ruminal biohydrogenation of

long-chain polyunsaturated FA [153]. The activity of SREBP1 is largely due to its abundance, which is controlled by the transcription and posttranscriptional regulation, and abundance and activation of the cofactors SREBP cleavage-activating protein (*SCAP*) and insulin induced gene 1 and 2 (*INSIG1* and *INSIG2*) [2, 33]. The INSIGs protein blocks SREBP1 activity when the level of oxysterol is high (see references in [33]). The reduced activity of SREBP1 by *t10,c12*-CLA is also controlled at the posttranslational level [2], but in this regard it is interesting that *t10,c12*-CLA consistently decreases the expression of *SREBF1*. Considering the unidirectional response of SREBP1 to *t10,c12*-CLA (i.e., inhibition of milk fat synthesis), and the inability of this TF to bind and be activated by other LCFA, it appears obvious that other TF must be involved in the positive response of milk fat synthesis to LCFA. Hence, it is remarkable that the activation of PPARγ by rosiglitazone in MAC-T cells was accompanied by a significant increase in expression of *SREBF1*, demonstrating that *SREBF1* is a PPARγ target gene in ruminants [26]. Our overall data [26, 33] suggest a concerted action of SREBP1 and PPARγ in controlling milk fat synthesis but underscore a more fundamental role of PPARγ, the only one among the two that is able to be activated by LCFA.

The evidence supporting a role of PPARγ in controlling milk fat synthesis has recently been dismissed [153] using three different arguments; here we briefly outline those arguments and present the counterarguments.

(1) The ca. 2-fold increase in expression of *PPARG* in bovine mammary gland from pregnancy to lactation [33] was interpreted as "related to differentiation and the initiation of milk synthesis rather than the regulation of milk fat synthesis during established lactation" [153]. The PPARγ is known to be involved in differentiation, but almost exclusively of the adipose tissue where it plays an essential role [21, 154]. For the rest, it is known that PPARγ has a negligible role in the differentiation of epidermis, one among several epithelial tissues [155]; however, a role for this PPAR isotype in differentiation of sebaceous gland after skin injury has been reported [15]. Although a role for PPARγ in the differentiation of mammary gland cells cannot be fully discarded, it has not yet been reported.

(2) The authors based their conclusions on the fact that CLA are activators of PPARγ in monogastrics, *de facto* disregarding the findings showing that ruminant PPARγ does not seem to be activated by CLA, especially in mammary epithelial cells [26] (see Table 1).

(3) The most critical misinterpretation dealt with the observed increase in expression of genes related to milk fat synthesis in MAC-T cells after treatment with the PPARγ agonist rosiglitazone [26]. The data clearly pointed to an active role of PPARγ in controlling milk fat synthesis. The authors, using the above argument about activation of PPARγ by CLA, interpreted those data exclusively from a milk fat depression angle; that is, activation of PPARγ by CLA should be responsible for depressing milk fat synthesis. That was neither what the data suggested nor our conclusions [26].

In an *in vivo* experiment the activation of PPARγ pre-partum by TZD affected adipose tissue post-partum but, apparently in contrast to the above data, decreased milk fat production [109]. This result is not completely surprising considering that the TZD treatment was provided pre-partum when there is a large abundance of PPARγ in adipose tissue and a low abundance in mammary gland [33], whereas, when PPARγ is expected to increase in mammary gland due to the onset of lactation [33], the TZD was no longer supplemented and the amount of NEFA, which could have played a role in activating PPARγ, was decreased in cows treated with TZD [109]. In addition, the adipose tissue competes with mammary gland for lipogenic substrates, especially if the insulin sensitivity is high, as demonstrated by the reduced milk fat by injection of insulin in cows [156]. From this point of view it would be interesting to test the effect of TZD injection post-partum on milk fat synthesis in dairy cows.

Besides PPARγ and SREBP1, data from another laboratory suggested that LXR also plays a role in controlling *de novo* FA synthesis [157]. It is recognized that in order to demonstrate the central role of PPARγ, SREBP1, LXR, or their combination in controlling milk fat synthesis in dairy cows, there is need for more fundamental studies, for instance, via gene-specific knock-outs. Recently, two studies from the same laboratories [158, 159] used siRNA specific for *SREBF1* in order to define the role on controlling milk fat synthesis of this transcription factor. From the studies it was shown that basal transcription of genes involved in *de novo* FA synthesis in bovine mammary epithelium is partly under control of SREBP1. Some of the same genes were induced when LXR was activated using a specific agonist. Studies using siRNA specific for *PPARG* in bovine mammary cells are lacking. In the context of milk fat synthesis regulation, we deem more relevant the unbiased discovery of the role of LCFA in affecting the transcriptome by binding specific TF than demonstrating a more crucial role of one or another TF.

9.2.1. Is PPARγ Crucial for Milk Fat Synthesis Also in Mouse? Contrary to dairy cows [33], in mouse the mammary *PPARG* expression decreased between pregnancy to lactation [160], also after accounting for the large disappearance of adipose tissue [161]. In porcine mammary gland, the *PPARG* was not affected by lactation [162]. The expression of *PPARG* in mouse and pig mammary gland suggests that PPARγ likely does not control milk fat synthesis in monogastrics. In order to further study the role of PPARγ on milk fat synthesis in monogastrics, we have performed an *in vitro* experiment in mouse mammary epithelial cells (HC11; Figure 3). The experiment also was performed with the purpose of comparing the data previously generated with bovine mammary cells [26]. For this reason, the experiment was performed in HC11 with the same experimental design as the one previously performed in MAC-T cells [26]. Most of the treatments in HC11 were the same as in MAC-T cells with the exception of the PPARγ inhibitor GW9662.

As observed in MAC-T cells, the saturated LCFA palmitate increased expression of several lipogenic genes in HC11 but, differently than in MAC-T cells [26], the effect appeared to be PPARγ-independent due to the extremely low

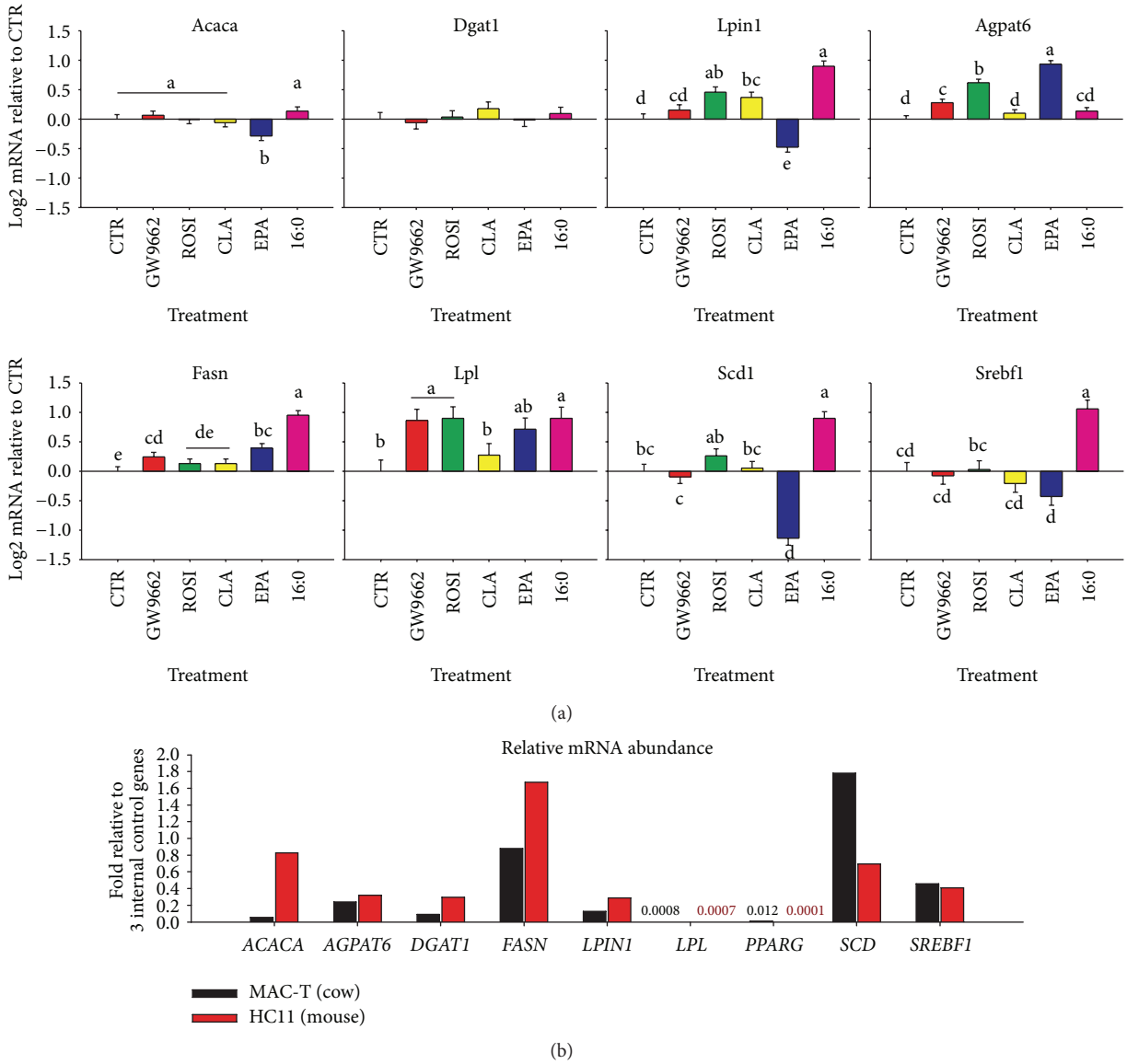


Figure 3: Effect of PPARγ activation on genes coding for proteins involved in milk fat synthesis in mouse mammary epithelial cells HC11. The experiment was performed with the purpose to test the effects of 50 μM of the PPARγ activator rosiglitazone, the PPARγ inhibitor GW9662, or 100 μM of several long-chain fatty acids (*trans*-10,*cis* 12-conjugated linoleic acid (CLA), eicosapentaenoic acid (EPA), or palmitate (16:0)) for 12 hours in HC11 cells and compare the data with results using the same experimental design (except the GW9662 treatment) in MAC-T cells [26]. All the procedures with few modifications were as previously described [26]. The RNA was extracted and qPCR performed for several genes known to be involved in milk fat synthesis and significantly upregulated by rosiglitazone in MAC-T cells and the same 3 internal control genes used [26]. In (a), the effect of treatments on HC11 cell is reported. For that experiment, the qPCR data were calculated as fold change relative to control and log2 transformed prior statistical analysis using Proc GLM of SAS with treatment as main effect and replicate as random. Dissimilar letters denote significant differences between treatments ($P < 0.05$). In (b), a comparison in mRNA abundance between measured genes in the control group of HC11 and MAC-T cells is presented. The relative mRNA abundance was calculated as previously described [26] but as fold difference relative to the geometric mean of the median Ct values of the 3 internal control genes instead as % relative abundance. The same analysis was performed for the MAC-T cells using data previously published [26]. The *PPARG* was detectable only for few samples in HC11 cells and *LPL* was barely detectable in both HC11 and MAC-T cells.

expression and activity of PPARγ (Figure 3(a)). Those findings are intriguing because, together with the greater abundance of *PPARA* compared with *PPARG* in MAC-T cells (Figure 1(b)), suggests that the observed increase in mammary lipogenic genes due to palmitate are via PPARα or other TF rather than PPARγ in immortalized mammary cells from cattle and mouse.

Contrary to what was observed in MAC-T cells [26] and *in vivo* in mouse mammary gland [163], the *t10,c12*-CLA failed to inhibit the expression of lipogenic genes in HC11 (Figure 3(a)). This observation is surprising considering that the *Srebp1* expression is relatively high and with similar level in HC11 compared with MAC-T cells (Figure 3(b)). Only EPA decreased expression of few lipogenic genes in HC11; among

those the *SCD* was downregulated by EPA also in MAC-T cells [26]. The relative abundance of genes measured in HC11 compared to MAC-T cells (Figure 3(b)) revealed that lipogenic gene expression is overall greater in HC11 than MAC-T, with exception of *SCD* that is more abundant in MAC-T cells. The *PPARG* had low expression in both cell lines but was virtually absent in HC11, while clearly detectable in MAC-T cells. This observation likely accounted for the fact that the PPARγ agonist rosiglitazone and the inhibitor GW9662 had little effect on the expression of most genes in HC11 (Figure 3(a)). On the contrary, rosiglitazone increased the expression of all those genes in MAC-T cells [26].

The virtual absence of *Pparg* expression in HC11 (Figure 3(b)) together with the lack of decrease in expression of milk fat-related genes by CLA despite the large expression of *SREBF1* seems to indicate a role of PPARγ, and more likely PPARγ-SREBP1 crosstalk, in translating the lipogenic inhibition, and particularly milk fat depression effect, of CLA (and likely EPA) usually observed *in vivo*. However, the data also point to a more complex nutrigenomics response to LCFA, likely involving additional TF besides SREBP1 and PPARγ.

Overall, the comparison between the mouse and the bovine mammary epithelial cell lines, with all the limitations of *in vitro* experiments, highlights a crucial difference between rodents and bovine in the genomic control of milk fat synthesis. The data clearly uncovered no roles for PPARγ in controlling milk fat synthesis in mouse. Those observations suggest caution when inferring physiological responses using data from a different species.

9.3. Control of Inflammatory Response. The activation of PPARγ, PPARα, and PPARβ/δ has anti-inflammatory effects in nonruminants [19, 164] and some data are available in ruminants suggesting a similar effect. The first demonstration that PPARγ might play an anti-inflammatory role in ruminants was carried out by a Japanese group by injecting for 9 days human recombinant TNFα plus TZD in dairy steers. They observed that the TZD treatment partially reversed the insulin resistance caused by TNFα [107]. The TZD effect was probably due to enhanced insulin signaling through PPARγ activation by also counteracting the effect of TNFα [165]. The anti-inflammatory effect of PPARγ in ruminants is elicited not only by counteracting the effect of TNFα, but also by reducing the production of this cytokine. This was demonstrated recently when treatment of bovine peripheral blood mononuclear cells with 100 μM of *t10,c12*-CLA or 10 μM of rosiglitazone attenuated the production of TNFα *in vitro*, with a stronger effect observed in cells treated with rosiglitazone [166].

In bovine primary mammary epithelial cells (bMEC), the activation of PPARγ by several agonists caused downregulation of several proinflammatory cytokines and increased expression of the chemokine *CCL2* and TNFα [69]. In contrast, PGJ2 enhanced markedly the expression of both interleukin 8 (*IL8*) and chemokine (C-X-C motif) ligand 6 (*CXCL6*) and had no effect on other cytokines [69]. The same study also demonstrated that the generation of proinflammatory mediators in bMEC treated with lipopolysaccharide (LPS) can be modulated by synthetic PPARγ agonists. These findings support a role of PPARγ in mastitis resistance in dairy cows.

Some additional evidences support an anti-inflammatory role of PPAR in ruminants. The activation of PPARα has shown to limit leukocyte adhesion to the bovine endothelium [167]. The expression of *PPARG* is reduced by intramammary infection with *Escherichia coli* [168] and PPAR signaling was evidently inhibited by intramammary infection with *Streptococcus uberis* [169]. The *PPARG* and *PPARA* were also markedly downregulated in PMN soon after an inflammatory challenge; however, the expression of *PPARD* increased markedly and was substantially more abundant than the other isotypes (Moyes et al. unpublished data). In contrast, the expression of *PPARA* and *PPARG* in liver was not affected after intramammary treatment with *Escherichia coli* that induced a strong hepatic acute-phase reaction [170]; however, the most-impacted biological effect of the treatment was the reduction of lipid metabolism in the liver, particularly steroid synthesis and PPAR signaling [171]. The involvement of PPARβ/δ in the process of inflammation was recently underscored when an intramammary infusion of LPS led to marked upregulation of *PPARD* and several proinflammatory genes in liver of dairy cows (e.g., *TNF*, *NFKB1*) [172].

The potential role of PPAR isotypes on inflammation can also be inferred by the fact that the expression of the PPARα agonist *ANGPTL4* (Table 2) increases markedly in response to inflammation not only in mouse liver [173] but also in bovine liver [172], and it has been proposed to serve as a positive acute phase protein (+APP) [173]. In that context, it is interesting that the expression of *ANGPTL4* in adipose tissue increases markedly after parturition [134, 174], when the animals experience inflammatory-like conditions [175, 176]. Whether the upregulation of *ANGPTL4* in adipose tissue after parturition denotes a response of the tissue to an inflammatory state remains to be determined; however, there is evidence of activation of immune-related pathways in adipose tissue soon after parturition [177].

9.4. Control of Intertissue Metabolic Adaptations during Changes in Nutritional Status and Physiological State. In monogastrics, the PPARα targets angiopoietin-like 4 (*ANGPTL4*) [178] and fibroblast growth factor 21 (*FGF21*) [179, 180] have been identified as extra-hepatic signals (hepatokines) that play an important role in the coordination of tissue adaptations to fasting, undernutrition, and the transition into lactation in bovine [56, 95, 174]. Although direct proof of bovine PPARα activation as the trigger for the marked upregulation of liver *FGF21* after parturition [86, 95] is not available, the fact that the upregulation of *FGF21* was observed in animals with greater NEFA [86] is suggestive of *FGF21* as a PPARα target in bovine. The link between PPARα activation and *ANGPTL4* was previously discussed with data from cows suffering from undernutrition-driven ketosis [56] and was partly confirmed *in vitro* [28]. However, it was recently observed that hepatic *ANGPTL4* and *PPARD* (not *PPARA*) expression was upregulated during acute inflammation suggesting that in bovine this PPAR isotype also may regulate expression of the hepatokine [172]. Specific

molecular work would need to be carried out to clarify the validity of the observed relationship in terms of a functional link.

9.5. Other Roles. The use of PPARγ agonists decreases protein synthesis, but as demonstrated in bovine aortic endothelial cells, the mechanism appears to be independent of PPARγ [181]. As with nonruminants, the activation of PPARγ improves insulin sensitivity in dairy cows [82]. The activation of PPARα in the liver might also increase gluconeogenesis. This was inferred by the impaired gluconeogenesis in PPARα-null mice [182]; however, none of the main enzymes involved in gluconeogenesis are known to be PPARα targets in nonruminants [182]. One of the three known promoter regions of bovine pyruvate carboxylase (*PC*), a key enzyme in gluconeogenesis, was activated by Wy-14643 when transfected as a construct with firefly luciferase into rat hepatoma cells, indicating a potential control of expression of this enzyme by PPARα in ruminants [93]. However, the *PC* expression was not induced in MDBK cells treated with Wy-14643 or single LCFA [28]. The expression of *PC* was instead induced by cocktails of LCFA and particularly the concentration mimicking NEFA composition in dairy cows around parturition [183]. Therefore, an increase in gluconeogenesis via the activation of PPARα in ruminants still needs to be fully proven.

It has been demonstrated that the high-glucose-induced downregulation of the glucose transport system in bovine endothelial cells is mediated by PPARβ/δ [68]. It was shown that activation of PPARβ/δ inhibits the expression of the solute carrier family 2 member 1 (or facilitated glucose transporter GLUT1) coupled with an increase in expression of calreticulin, a protein that increases degradation of GLUT1 mRNA. The condition tested in the study (i.e., high glucose) has probably little implication for ruminants, considering the low level of circulating glucose compared with nonruminants (<4 mM in dairy cows [176] versus ca. 5 mM in human and >6 mM in mouse [184]). However, the control of glucose transport by PPARβ/δ could have implications in milk synthesis, considering that GLUT1 is one of the most important glucose transporter and its expression increases drastically during lactation in mammary tissue of dairy cows [185]. Thus, this PPAR isotype could play a pivotal role in provision of glucose for lactose synthesis. Interestingly, in mammary gland during lactation, *PPARD* is significantly downregulated [186] concomitant with an increase in expression of several glucose transporters, including GLUT1 [70, 185]. If the suggested link is real, this offers the opportunity of using PPARβ/δ antagonists in order to improve milk production.

More recently, it was demonstrated that *PPARD* transcript in rumen epithelium of neonatal dairy calves is substantially more abundant than *PPARA* (see also Figure 1(b)), and its expression increased markedly from the milk-fed stage to the roughage-fed (i.e., high-structural fiber) stage at ~10 weeks of age [64]. The increase correlated with greater mass of the rumen, which suggested a potential link between PPARβ/δ and mechanisms driving ruminal epithelial cell development and proliferation [64].

10. What Controls Abundance of PPAR in Tissues?

The sensitivity of various tissues to PPAR isotype-specific agonists is closely related with the abundance of the specific isotype and other essential factors such as the abundance of coactivators or corepressors, LCFA, and hormones [76, 187–189]. As for nonruminants, the abundance of various isotypes in tissues appears to be directly related with the specific function they perform; for example, PPARγ abundance is relatively high in lipogenic tissues while PPARα is relatively high in tissues with elevated FA catabolic capacity (see Figure 1(a)). Besides tissue-specific distribution, other factors can control the abundance of PPAR isotypes in tissues.

Among factors controlling PPAR isotypes expression in ruminants (Supplementary Table 2), it is evident that several lipid molecules, some nutritionally relevant such as LCFA and retinoids, and propionate (likely indirectly via glucose and insulin) can affect expression of PPAR isotypes, with a different sensitivity based on tissue type. The expression of ruminant PPAR isotypes is also affected by physiological status, level of energy in the diet, mechanical cues (e.g., laminar flow, mechanical load), oxygen and peroxide levels, hormones, and other growth factors (Supplementary Table 2). In addition, data from several groups also suggest that the activation of PPARγ increases expression of its own gene and, in the case of sheep, also the expression of *PPARA* (Supplementary Table 2). Interestingly, in bovine mammary epithelial cells several PPARγ agonists decreased the expression of *PPARD*, with one case (ciglitazone) in which *PPARG* also was downregulated [69].

Overall the data presented in Supplementary Table 2 suggest that it is possible to increase or decrease the abundance, hence the sensitivity, of PPAR isotypes in ruminant tissues. Among the factors affecting the PPAR isotype expression, the more interesting from a nutrigenomics point of view are the LCFA and the level of dietary energy because they can be easily manipulated.

11. PPAR Isotype Activation during the Peripartal Period in Dairy Cattle: A Hypothesis

11.1. The Peripartal Condition. The transition from pregnancy into lactation (also called simply "transition period") is one of the most stressful stages of the life of dairy cattle [190]. Physiologically, the transition period is a complex phenomenon intertwining various metabolic activities (e.g., lipid, glucose, protein) and functions (e.g., inflammatory response) of several organs and tissues (e.g., adipose tissue, mammary, liver, uterus, and immune system) [152, 190]. A key feature of the transition period from a metabolic and health standpoint is the increase in plasma of NEFA and ketone bodies (KB), both of which can be toxic above certain thresholds, and by a general decrease in both insulin sensitivity (except for the mammary gland) and blood insulin concentration [191]. The transition period is also characterized by inflammatory-like conditions as consequence of

the release of proinflammatory cytokines, which along with NEFA affects directly liver functionality leading to poor performance [192].

The metabolic load placed on the liver of periparturient cows is exacerbated by this inflammatory-like conditions and also by the decrease in feed intake and the ensuing NEB, which often occurs as early as 10 days prior to parturition (reviewed in [193]). All of the above increase the risk of dairy cattle for developing metabolic disorders such as fatty liver [194] and ketosis [195, 196], but more importantly these disorders are tightly connected with other typical peripartal diseases [197]. Therefore, a smooth transition period is an important target in order to optimize performance and overall welfare of dairy cows. Interestingly, most of the above-described conditions (e.g., high NEFA, insulin insensitivity, fatty liver, inflammatory-like conditions) with the exception of the NEB are common to the metabolic syndrome that afflicts human [198].

11.2. PPAR Isotype Activation to Help Transition Dairy Cattle. It has been proposed previously that the PPAR isotypes are ideal targets for the prevention and cure of the metabolic syndrome in humans [199]. The use of PPARγ agonists is a clinical approach currently in practice to treat insulin resistance, one of the main problems related with the metabolic syndrome [22, 200]. Similarly, it was proposed earlier that PPAR isotypes play a pivotal role in the physiological adaptation of dairy cattle to the transition period [36, 201]. It was proposed that fine-tuning the activity of PPARα and PPARγ, in particular, by nutritional approaches at specific time/s during the transition period might be a way to prevent and/or help the cows overcome metabolic disorders. Among nutritional approaches in order to affect PPARs, the saturated LCFA appear to be the most promising based on *in vitro* data (see above and [28]). The effects of saturated LCFA on PPARs activation and the consequent improvement of lipid metabolism appear to be supported by recent *in vivo* data [202]. In that study it was observed that the adaptations in lipid metabolism in dairy cows fed high-saturated fat compared with a low-fat control diet or a high-linseed diet (high in unsaturated LCFA) for up to 5 weeks pre-partum was better.

In Figure 4 a qualitative hypothetical model describing the potential role of PPAR isotypes in transition dairy cows is depicted. That model rests on the well-established fact that the liver, adipose, rumen, skeletal muscle, immune system, and mammary gland play a crucial role in the adaptations leading to the onset of lactation. Other organs such as uterus, kidney, and pancreas also are crucial in this context but less is known about their molecular adaptations to lactation. In particular, data partly reviewed above strongly support a pivotal role of PPAR isotypes in the regulation of fertility and pregnancy; however, the overall effect of PPAR isotypes activation on fertility is not fully clear. In addition, the PPAR isotypes likely play a more important role before pregnancy compared with early lactation, when the cows are not yet cycling. Once the role of PPAR isotypes is better defined for the reproductive organs, it can become an important component of the overall model proposed.

Dairy cattle during the transition from pregnancy into lactation experience a multitiered set of adaptations aimed at allowing the mammary gland to begin and maintain lactogenesis. From a physiological perspective, the inherently low capacity of animals to consume enough dietary energy and the detrimental inflammatory-like conditions due to release of proinflammatory cytokines lead to the marked release of LCFA into the bloodstream from the adipose tissue. Those LCFA are mostly metabolized by the liver. A greater level of dietary energy in the form of nonstructural carbohydrate provided to the animal early postpartum can partly alleviate the negative shortfall in energy status; such approach would enhance production of short-chain fatty acids (SCFA), of which propionate metabolism via gluconeogenesis could serve as a trigger for greater insulin secretion [193]. The latter has been shown to promote rumen epithelial cell proliferation and might work in concert with PPARs to coordinate metabolism and development of these cells [64]. During the peripartal period, proinflammatory cytokines are released and induce the liver to produce +APP [176], taking away hepatic resources for normal liver functions (e.g., glucose synthesis, lipid metabolism, and ureagenesis) [175, 176, 197]. This condition effectively exacerbates the tissue's capacity to coordinate appropriately metabolism of lipid and to provide the required glucose to mammary gland for milk synthesis. The marked NEFA concentration is only partly oxidized by liver with the rest accumulating as TAG. The TAG are then packed into VLDL for release into the bloodstream, but at a lower rate relative to monogastrics [203]. An excessive accumulation of TAG can have detrimental effects on liver function [194].

We propose that the increased abundance pre-partum of PPAR isotypes and the timely and isotype-specific activation pre- or post-partum might be beneficial in preparing and allowing the animal to face the above-described conditions favoring a smooth transition into lactation. In particular the following:

(i) the greater abundance and activation of PPARγ prepartum in adipose tissue can prevent the large NEFA surge due partly to an increase in insulin sensitivity, leading to reduced lipid overload on the liver with a consequent reduction of fatty liver, ketone body production, and any potential satiety effects (as consequence of high FA oxidation) [204]. The activation of PPARγ postpartum in mammary gland can allow to increase or to maintain the amount of milk fat in the early stages of lactation when NEFA provide exogenous LCFA for the mammary gland. Several pieces of evidence support such expected effects in adipose tissue [82, 109] and mammary tissue [26, 33, 186];

(ii) the greater abundance and activation of PPARα in liver and skeletal muscle in postpartum relative to prepartum can increase oxidation of NEFA leading to a lower accumulation of lipid in the liver. The greater oxidation capacity of liver (i.e., increase ketone body synthesis per unit of NEFA oxidized) would help prevent any substantial alteration in the production of

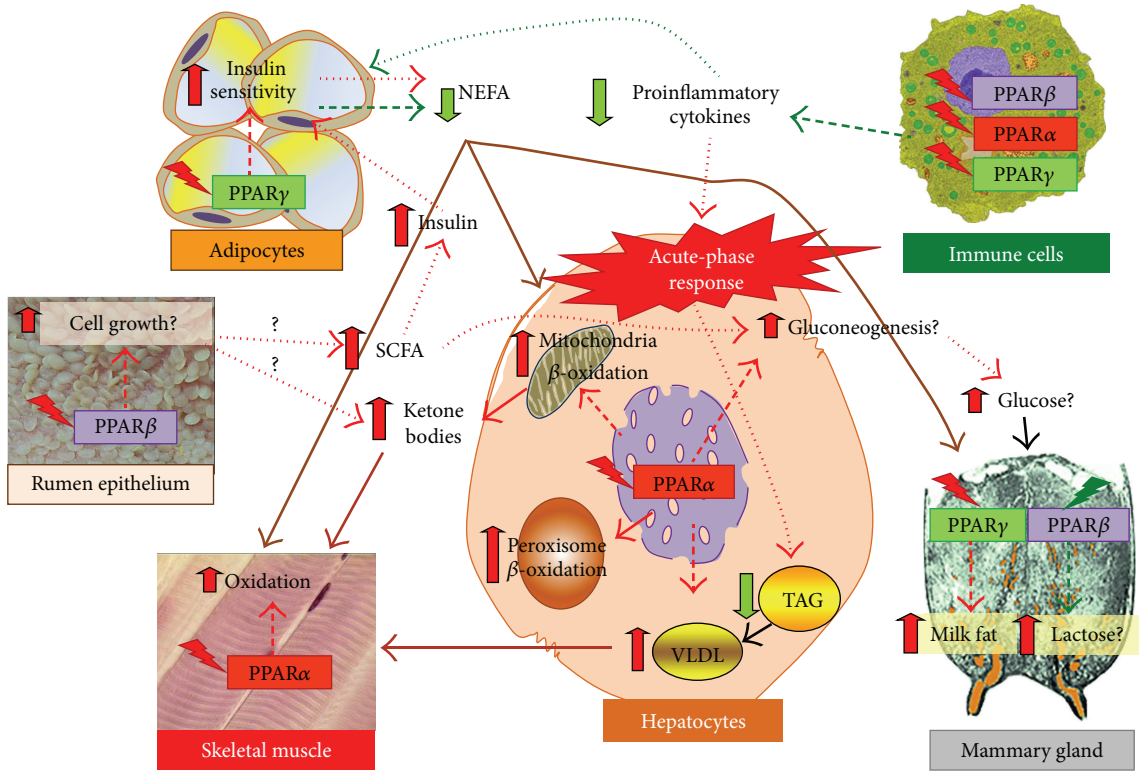


Figure 4: Improving transition from pregnancy into lactation in high producing dairy cows by nutrigenomics approach through PPAR isotypes: a hypothesis. The liver buffer cells from the excessive concentration of circulating nonesterified fatty acids (NEFA) by both catabolizing long-chain fatty acids (LCFA) with production of ketone bodies (KB) and esterifying them as triacylglycerol (TAG). The TAG are then accumulated in lipid droplets and packed into VLDL for release into the bloodstream. The liver is also induced by proinflammatory cytokines to produce positive acute phase proteins (+APP) taking away hepatic resources for normal liver functions. Despite the decrease in peripheral insulin concentration postpartum, the activation of PPARγ prior to parturition can decrease NEFA postpartum through greater insulin sensitivity primarily on the adipose tissue. The activation of PPARβ/δ (PPARβ in the figure) via LCFA can increase rumen epithelium growth with consequent larger production of short-chain fatty acids (SCFA) including propionate, which stimulate insulin production, and butyrate, augmenting the KB in blood. The increased activation of PPARα just before parturition and during the first 14 days postpartum in the liver and muscle can increase NEFA oxidation with greater proportion of KB produced per amount of NEFA uptake. The activation of PPARα in the liver has the potential to increase gluconeogenesis and VLDL synthesis. The KB can serve as fuels by skeletal muscle instead of NEFA and glucose; both molecules are substrates for mammary gland. In this tissue, the activation of PPARγ postpartum should increase or maintain milk fat. In addition, the inhibition of PPARβ/δ postpartum can potentially increase glucose import with a consequent increase in lactose synthesis, and hence, milk yield. The activation of PPAR isotypes just prior to parturition and during the first two weeks post-partum should diminish the inflammatory-like conditions preventing, on one hand, the stimulation of NEFA release and, on the other hand, hepatic acute-phase reaction, both determined by proinflammatory cytokines. This coordinated set of reactions should provide an ideal metabolic situation leading to a smoother transition from pregnancy into lactation, that is, allow the liver to allocate its resources for "normal" functions. As a consequence of this, the incidence of diseases typical of the peripartal period would be reduced, and hence, cows with higher performance and more healthy. Regular dashed arrows represent "effect on" due to PPAR isotype activation/inhibition, and round dot arrows denote secondary (or indirect) effects of PPAR isotype activation. In both cases red = activation or increase and green = inhibition or decrease.

ketone bodies due to the systemic decrease of NEFA as a consequence of PPARγ activation. The activation of PPARα in liver might also increase gluconeogenesis rate, an essential process in ruminants particularly for milk synthesis. Another expected response would be increased VLDL synthesis and secretion by preventing the negative effect of the acute-phase reaction as a consequence of inflammatory-like conditions on apolipoproteins and other molecules involved in VLDL synthesis and TAG export. This suggestion is based on several pieces of evidence such as the observed negative association between apolipoprotein B100 or other VLDL components with inflammatory-like conditions in ruminants [170, 175, 176, 194, 205, 206];

(iii) the activation of PPARα, PPARγ, and particularly PPARβ/δ in immune (e.g., neutrophils, macrophages) and endothelial cells might contribute to a reduction of the NEFA surge induced by proinflammatory cytokines [207] and also increase insulin sensitivity and prevent the negative effect of acute phase reaction on liver functionality [166, 176].

The hypothetical model for fine-tuning PPAR isotypes for prevention of metabolic disorders in transition dairy cows we propose (Figure 4) is indirectly supported by several *in vivo* and *in vitro* studies, but a number of major details remain to be understood. One of the most important pertains to the effects of LCFA on PPAR isotype activation and, particularly, on how they could be used to target activation of a particular PPAR isotype at a particular stage of the transition period. More detailed and mechanistic studies with LCFA, for example, effective dose/s of individual LCFA or mixtures, are essential in order for these nutrients to have practical application as proposed in our model (Figure 4).

12. Conclusions and Perspectives

The understanding of physiological roles of PPAR isotypes in ruminants has advanced incrementally over the last decade. There is enough direct and indirect evidence compiled to conclude that these NR are biologically relevant in this species. The data suggest that the harmonized activity of PPAR isotypes across tissues is one facet of the multitiered set of control points that evolved to coordinate metabolism and physiological responses to endogenous and exogenous ligands. The transition from pregnancy to lactation provides the clearest example of the need for control points to ensure the nourishment of the neonate offspring, while ensuring the fitness of the dam. At a fundamental level, the functional activity of PPARs during this physiological state provides an elegant example of the multitiered concept because it links biological molecules with cellular responses that encompass several tissues. The model proposed based on the most-current knowledge is quite complex and its full evaluation obviously requires an integrative systems approach, that is, several tissues at various levels (e.g., cells and the underlying molecular networks) need to be studied simultaneously considering their dynamic adaptation.

If the model/hypotheses proposed hold, it would become the first "true" nutrigenomics application in dairy cattle biosciences. Benefits would go beyond simply establishing the physiological role of PPAR isotypes in ruminants. Improving the transition from pregnancy into lactation means to provide benefits for farmers, dairy cattle, and the society as a whole. We envisage farmers modulating the LCFA in the diets of dairy cattle to fine-tune metabolism through PPAR isotypes. Today, the continuous development of high-throughput technologies and bioinformatics tools permits the study of complex phenomena as is the case of the transition from pregnancy to lactation in dairy cattle [152]. This is an exciting era for expanding scientific knowledge and, apparently, the proper one for nutrigenomics.

Abbreviations

+APP: Positive acute phase proteins
ACOX1: Acyl-coenzyme A oxidase 1, palmitoyl
AF-1: Activation Function 1 or ligand-independent activation function
ANGPTL4: Angiopoietin-like 4
bMEC: Bovine primary mammary epithelial cells
CARLA: Coactivator-dependent receptor ligand assay
CCL2: Chemokine (C-C motif) ligand 2
CLA: Conjugated linoleic acid
CPT1A: Carnitine palmitoyltransferase 1A (liver)
CPT2: Carnitine palmitoyltransferase 2
CRAT: Carnitine O-acetyltransferase
CXCL6: Chemokine (C-X-C motif) ligand 6
EPA: Eicosapentaenoic acid
FA: Fatty acid(s)
FABP: Fatty acid binding protein
FGF21: Fibroblast growth factor 21
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
GLUT1: Glucose transporter 1
IL-8: Interleukin 8
INSIG1: Insulin induced genes 1
INSIG2: Insulin induced genes 2
KB: Ketone bodies
LBD: Ligand binding domain
LCFA: Long-chain fatty acid(s)
LCFA-CoA: LCFA-coenzyme A (i.e., activated LCFA)
LDL: Low density lipoprotein(s)
LPIN1: LiPIN 1 (coding for a phosphatidate phosphatase)
LPL: Lipoprotein lipase
LPS: Lipopolysaccharide
LXR: Liver X receptor
MAC-T: Bovine mammary alveolar cells transfected with simian virus-40 (SV-40) large T-antigen
MDBK: Madin-Darby bovine kidney cells
MIQE: Minimum Information for Publication of Quantitative Real-Time PCR Experiments
NEB: Negative energy balance
NEFA: Nonesterified fatty acid(s)
NFKB1: Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
NR: Nuclear receptor(s)
PC: Pyruvate carboxylase
PG: Prostaglandin(s)
PGJ2: 15-Deoxy-delta-12,14-prostaglandin J2
PMN: Polymorphonuclear cells
PPAR: Peroxisome proliferator-activated receptor
PPRE: PPAR response element
PUFA: Polyunsaturated fatty acid(s)
qPCR: Quantitative Real-Time reverse transcription polymerase chain reaction
RXR: Retinol X receptor
SCAP: SREBP cleavage-activating protein
SCD: Stearoyl-CoA desaturase
SCFA: Short-chain fatty acid(s)
siRNA: Short interference RNA
SREBF1: Sterol regulatory element-binding transcription factor 1
TAG: Triacylglycerol(s)
TF: Transcription factor(s)
TNF: Tumor necrosis factor(s)
TZD: Thiazolidinedione

VEGF: Vascular endothelial growth factor
VLDL: Very low density lipoprotein(s)
Wy-14643: Pirinixic acid.

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Research Article

PPARγ Regulates Genes Involved in Triacylglycerol Synthesis and Secretion in Mammary Gland Epithelial Cells of Dairy Goats

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To explore the function of PPARγ in the goat mammary gland, we cloned the whole cDNA of the PPARγ gene. Homology alignments revealed that the goat PPARγ gene is conserved among goat, bovine, mouse, and human. Luciferase assays revealed that rosiglitazone enhanced the activity of the PPARγ response element (PPRE) in goat mammary epithelial cells (GMECs). After rosiglitazone (ROSI) treatment of GMECs, there was a significant ($P < 0.05$) increase in the expression of genes related to triacylglycerol synthesis and secretion: *LPL, FASN, ACACA, PLIN3, FABP3, PLIN2, PNPLA2, NR1H3, SREBF1*, and *SCD*. The decreases in expression observed after knockdown of PPARγ relative to the control group (Ad-NC) averaged 65%, 52%, 67%, 55%, 65%, 58%, 85%, 43%, 50%, and 24% for *SCD, DGAT1, AGPAT6, SREBF1, ACACA, FASN, FABP3, SCAP, ATGL,* and *PLIN3*, respectively. These results provide direct evidence that PPARγ plays a crucial role in regulating the triacylglycerol synthesis and secretion in goat mammary cells and underscore the functional importance of PPARγ in mammary gland tissue during lactation.

1. Introduction

Lactation is a process highly demanding of lipid synthesis and transport. Although peroxisome proliferator-activated receptor γ (PPARγ) is known to promote lipogenesis and adipogenesis in adipose tissue [1], its role in the lactating mammary gland is less clear. Many candidate genes that regulate lipid synthesis have been identified during the lactation cycle [2]. Researchers have evaluated the expression profiles of 54 genes associated with bovine milk fat synthesis through various periods during lactation and built a regulatory network [3]. Their data showed that PPARγ might be the main factor that regulates the nuclear transcription factor, sterol regulatory element-binding transcription factor 1 (SREBF1), which also affects the expression of some fatty acid metabolism genes during lactation [3, 4].

Much data have been published regarding PPARγ's role in milk fat synthesis in bovine [5–7], while there is a lack of data on its role in the dairy goat. Whether PPARγ also plays the same critical role in regulation of milk fatty acid synthesis during the lactation process in dairy goat remains to be determined. In the present study, we first identified the sequence of PPARγ in dairy goat mammary tissue and evaluated the activity of the PPRE via luciferase assays. Its function in dairy goat mammary epithelial cells (GMECs) was also investigated through the use of the pharmaceutical ligand rosiglitazone (ROSI) and adenovirus-mediated RNA interference.

2. Materials and Methods

2.1. cDNA Cloning. The primers used in the amplification of the goat PPARγ transcript sequence (*PPARG*) used for cDNA cloning are reported in Table 1. Primers were designed based on the consensus conserved sequences between humans (AB472042) and bovines (BC116098). The PCR reaction was performed with goat mammary epithelial cell cDNA as a template. The cDNA cloning of the 5′ and 3′UTR was implemented according to the manufacturer's protocols of

Table 1: Primer pairs used in PCR for amplification of goat *PPARG* from mammary cDNA.

Name of fragment	Sequence	Product length
PPARγ CDS	Forward: 5′-ATGGTTGACACAGAGATGCCG-3′	1413 bp
	Reversal: 5′-GTAGATTTCCTGTAGAAGTGGGTGG-3′	
PPARγ 3′RACE	Outer: 5′-AAGTAACTCTCCTAAAATACGGCG-3′	516 bp
	Inner: 5′-CCAGAAAATGACGGACCTCAGGCAGA-3′	160 bp
PPARγ 5′RACE	GSP1: 5′-CGGTGATTTGTCTGTCGTCTTTC-3′	750 bp
	GSP2: 5′-GATACAGGCTCCACTTTGATTGC-3′	260 bp

Table 2: Characteristics of shRNA used in the experiment.

Name of shRNA	Sequence
sh500-sense	5′-GATCC GGAGGACGATCAGATTGAA **gagtactg TTCAATCTGATCGTCCTCC**TTTTTTC-3′
sh500-antisense	5′-TCGAGAAAAAAGGAGGACGATCAGATTGAA **cagtactc**TTCAATCTG**A**TCGTCCTCCG-3′
Sh614-sense	5′-GATCC**GGATGTCTCATAACGCCAT gagtactg ATGGCGTTATGAGACATCC**TTTTTTC-3′
sh614-antisense	5′-TCGAGAAAAAA**GGATGTCTCATAACGCCAT cagtactc ATGGCGTTATGAGACATCC** G-3′
Sh1006-sense	5′-GATCC**GGCTTTGTGAACCTTGACT gagtactg AGTCAAGGTTCACAAAGCC** TTTTTTC-3′
sh1006-antisense	5′-TCGAGAAAAAA**GGCTTTGTGAACCTTGACT cagtactc AGTCAAGGTTCACAAAGCC** G-3′

Three shRNAs (numbers stand for their position in cDNA) were designed, and each shRNA was added with restriction sites *BamH* I and *Xho* I. The loop domain (lower-case nucleotides) contained a *Scal* I site.

the 5′RACE system Ver.2.0 (Invitrogen, USA) and 3′-full RACE core set Ver.2.0 (Takara, Japan). The nested gene-specific primers for *PPARG*, designed based on its open read fragment (ORF), were used for 3′RACE. Similarly, the nested gene-specific primers (Table 1) were also designed for 5′RACE. All the PCR fragments were cloned into pMD-19T plasmid vectors (Takara, Japan) and then sequenced at a commercial facility (Invitrogen, Shanghai, China). The PPARγ protein structure was predicted using PHYRE2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index).

2.2. Vector Construction and shRNA. The luciferase vector (pGL3-basic) containing three copies of PPRE was designed as described before [8]. The shRNA sequences were designed using the WI siRNA Selection Program (http://sirna.wi.mit.edu/home.php) and BLOCK-iT RNAi Designer (http://rnaidesigner.invitrogen.com/rnaiexpress/) using the goat PPARγ gene sequence (HQ589347.1). We selected the highest-ranked shRNA sequences. Additionally, a BLAST search against all EST sequences in GenBank was performed to ensure that the selected sequences were specific for goat PPARγ. Meanwhile, those sequences were selected and synthetized at a commercial facility (Invitrogen, Shanghai, China) with *Bam*H I and *Xho* I restriction sites suitable for the cloning process (see Table 2). Lastly, three shRNA were generated by heat treatment annealing and constructed into *pENTR/CMV-GFP/U6*-shRNA. The CDS of PPARγ was subcloned into the *pDsRed1-C1* plasmid vector between the *Xho* I and *Eco*R I restriction sites to generate *pDsRed1-C1-PPARγ*.

2.3. Cell Culture and Treatments. Goat mammary epithelial cells isolated from a Xinong Saanen goat at peak lactation [9] were allowed to grow in 60 mm culture dishes (NUNC, Denmark) in DMEM/F12 medium (HyClone, China). Routine cultures were incubated at 37°C in 5% CO_2 and air. Culture medium was changed every 24 h. Medium was composed of DMEM/F12 with insulin (5 mg/L, Sigma, USA), hydrocortisone (5 mg/L, Sigma, USA), penicillin/streptomycin (10 kU/L, Harbin Pharmaceutical Group, China), epidermal growth factor (1 mg/L, Sigma, USA), and fetal bovine serum (10%, Gibco, USA). ROSI (BioVision, USA) was resuspended in DMSO (Sigma, USA) at a concentration of 50 mmol/L. Cells cultured in 60 mm culture dishes and subcultured to 90% confluence were treated with 50 μmol/L ROSI and harvested at 0, 12, and 24 h after treatment to extract total RNA. The 293A cells for preliminary testing of shRNA and generating recombinant adenovirus were cultured in the basal medium containing 10% fetal bovine serum and 90% DMEM (Gibco, USA).

2.4. Preliminary Screening of shRNA Sequences. In order to get the most effective shRNAs for targeting PPARγ gene, an experiment was done as follows. 293A cells at 80% confluence in 12 plates were transiently transfected with 1.0 μg of three *pENTR/CMV-GFP/U6*-shRNAs with *pDsRed1-C1*-PPARγ, at a ratio of 3 : 2 using FuGENE HD Transfection Reagent (Roche, Switzerland). The *pDsRed1-C1-PPARγ* vector also was transfected alone as a control in the same amount as above. All the steps were performed in accordance with the manufacturer's protocol. The GFP fluorescence was monitored by using a Leica fluorescent microscope (DMI4000B, Germany).

2.5. Adenovirus Generation. shRNA expression cassettes with an EGFP reporter gene in the pENTR vector were switched into an adenoviral vector (pAd/PL-DEST) using the Gateway technique (Invitrogen, USA) to generate pAd-shRNA vectors. *Pac* I linearized adenoviral plasmids were transfected into 293A cells to generate the adenovirus. About

Table 3: Characteristics of primer pairs used, amplicon length, and efficiency of reaction in the RT-qPCR.

Accession[#]	Gene	Primer sequence (5′ to 3′)	Product length (bp)	Efficiency
JN236219.1	*ACACA*	Forward: CTCCAACCTCAACCACTACGG Reversal: GGGGAATCACAGAAGCAGCC	171	2.09
JI861797.1	*AGPAT6*	Forward: AAGCAAGTTGCCCATCCTCA Reversal: AAACTGTGGCTCCAATTTCGA	101	2.17
X91503[#]	*CD36*	Forward: GTACAGATGCAGCCTCATTTCC Reversal: TGGACCTGCAAATATCAGAGGA	81	2.18
DQ380249.1	*DGAT1*	Forward: CCACTGGGACCTGAGGTGTC Reversal: GCATCACCACACACCAATTCA	101	2.11
NM_001009350	*FABP3*	Forward: GATGAGACCACGGCAGATG Reversal: GTCAACTATTTCCCGCACAAG	120	2.14
DQ915966.3	*FASN*	Forward: GGGCTCCACCACCGTGTTCCA Reversal: GCTCTGCTGGGCCTGCAGCTG	226	2.13
AJ431207	*GAPDH*	Forward: GCAAGTTCCACGGCACAG Reversal: GGTTCACGCCCATCACAA	249	2.16
DQ997818	*LPL*	Forward: AGGACACTTGCCACCTCATTC Reversal: TTGGAGTCTGGTTCCCTCTTGTA	169	2.18
GU332719	*NR1H3*	Forward: CATCAACCCCATCTTCGAGTT Reversal: CAGGGCCTCCACATATGTGT	163	2.13
HQ846826	*PLIN2*	Forward: TACGATGATACAGATGAATCCCAC Reversal: CAGCATTGCGAAGCACAGAGT	203	2.16
HQ846827	*PLIN3*	Forward: GGTGGAGGGTCAGGAGAAA Reversal: TCACGGAACATGGCGAGT	170	1.13
GQ918145	*PNPLA2*	Forward: GGAGCTTATCCAGGCCAATG Reversal: TGCGGGCAGATGTCACTCT	226	2.24
HQ589347.1	*PPARG*	Forward: CCTTCACCACCGTTGACTTCT Reversal: GATACAGGCTCCACTTTGATTGC	145	2.21
DV935188[#]	*SCAP*	Forward: CCATGTGCACTTCAAGGAGGA Reversal: TGTCGATCTTGCGTGTGGAG	108	2.10
GU947654	*SCD*	Forward: CCATCGCCTGTGGAGTCAC Reversal: GTCGGATAAATCTAGCGTAGCA	257	2.10
HM443643.1	*SREBF1*	Forward: CTGCTGACCGACATAGAAGACAT Reversal: GTAGGGCGGGTCAAACAGG	81	2.20

Annealing temperature for all primers in this table is 60°C.
ACACA, acetyl-coenzyme A carboxylase alpha; *AGPAT6*, 1-acylglycerol-3-phosphate O-acyltransferase 6; *CD36*, thrombospondin receptor; *DGAT1*, diacylglycerol acyl transferase 1; *FABP3*, fatty acid binding protein 3; *FASN*, fatty acid synthase; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *LPL*, Lipoprotein lipase; *NR1H3*, liver X receptor α; *PLIN2*, perilipin2; *PLIN3*, perilipin3; *PNPLA2*, patatin-like phospholipase domain containing 2; *PPARG*, peroxisome proliferator-activated receptor γ; *SCAP*, cleavage activating protein; *SCD*, stearoyl-CoA desaturase; *SREBF1*, Sterol regulatory element-binding transcription factor 1.
[#]The primer sequences are from bovine.

8 to 10 days after transfection, the recombinant virus was collected and subjected to two rounds of amplification in 293A cells. The viral titers were determined in transduced 293A cells through GFP expression as previously described [10–12].

2.6. Luciferase Assays. To assess the degree of PPARγ activation, goat mammary epithelial cells at 80% confluence in 96-well plates were transiently transfected with 0.08 μg of PPRE×3-Luc reporter plasmid along with a *Renilla* vector (pRL-TK) as a control using the FuGENE HD transfection reagent at a ratio of 25 : 1. After a 24 h recovery period in medium, cells were treated with 0, 10, 25, 50, and 100 μmol/L ROSI. Forty-eight hours later, cells were harvested and lysates were made using reporter lysis buffer (Promega, USA) according to the manufacturer's instructions. Luciferase activity in the cell extract was determined using luciferase assay buffer and luciferase assay substrate according to the manufacturer's protocol (Promega, USA) in a luminometer (BHP9504, China).

2.7. Adenovirus Transduction. Goat mammary epithelial cells at 70–80% confluence were transduced with adenovirus supernatant at a multiplicity of infection (MOI) of 200. The medium was replaced with fresh medium 6 h later. The shRNA negative control adenovirus (Ad-NC) was used as a control. Cells were harvested 48 h after transduction.

2.8. RNA Extraction and Real-Time RT-PCR (qPCR). Total RNA was extracted from cells using RNAprep pure cell kit (Tiangen, China). The first-strand cDNA of different treatments was synthesized from 0.5 μg of purified total RNA using the PrimeScript RT kit (Takara, Japan) according to the manufacturer's instructions. Sufficient cDNA was prepared to

run all the selected genes (Table 3). Primers were designed to span exon-exon boundaries according to BLAST against bovine genome in order to avoid amplification of genomic DNA using Primer 5.0 software. The specificity of the primers was tested using the same protocol as for qPCR in a simple thermocycler (S1000, Bio-rad, USA), and the PCR product was run in a 15 g/L agarose gel. In addition, a dissociation protocol was performed in the RT-qPCR. Only primers with a single band on the agarose gel, a unique peak in the dissociation curve after the RT-qPCR, and devoid of primer-dimers were selected. The efficiency of each primer pair was tested using a standard curve as previously described [3]. All the amplicons were sequenced in order to assess the right amplified genes. Characteristics of all primers used in the RT-qPCR reaction are described in Table 3. RT-qPCR reactions were performed according to the manufacturer's instructions (SYBR Premix Ex Taq II, Perfect Real Time, Takara, Japan). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was selected as an internal control gene [13]. Although we did not verify additional genes as internal controls, *GAPDH* was used partly because it has been used previously in a goat mammary tissue study [13], and also because it has been widely used as the sole control gene in bovine cell studies [14]. However, we understand the limitation of using a single internal control gene because more reliable data requires the verification and use of at least 3 internal controls [15].

2.9. Western Blot. Whole cell proteins were extracted with RIPA buffer (Solarbio, China) supplemented with PMSF (Pierce, USA). Western blotting was performed using the following primary and secondary antibodies: anti-PPARγ (Abcam, ab19481, Hong Kong, 1 : 400) and goat anti-rabbit IgG (Tiangen, China, 1 : 1000). All antibodies were used according to the manufacturer's recommendations. Signals were detected using the chemiluminescent ECL Western blot detection system (Pierce, USA).

2.10. Statistical Analysis. Each treatment was replicated 3 times, and results are expressed as mean ± SD. Data of RT-qPCR was analyzed relative to the control using the $2^{-\Delta\Delta Ct}$ method. The statistical significance for ROSI treatment was determined by the ANOVA test using SPSS 19.0 software. Treatment means for shRNA interference were separated using Fisher's least significant difference pair-wise comparisons. Significance was declared at $P < 0.05$.

3. Results and Discussion

3.1. Molecular Cloning and Sequence Analysis of Dairy Goat PPARγ. PPARγ is a member of the nuclear hormone receptor superfamily of transcription factors. It has been fully confirmed in humans and mice that PPARγ directly regulates adipose cell proliferation, maturation, and differentiation [16, 17]. A potential role of PPARγ in controlling milk fat synthesis also has been reported in bovine due to the increase of its expression between pregnancy and lactation [2] and the increase in expression of genes involved in milk fat synthesis after activation with ROSI [5]. However, its role, if any, on milk fat synthesis in the mammary gland of the goat remains relatively unknown. In this study, we cloned the dairy goat PPARγ CDS and then used 5′RACE and 3′RACE procedures to obtain the full-length cDNA. The whole goat PPARγ gene contains a 5′UTR of 114 bp, an ORF of 1428 bp, and a 3′UTR 215 bp. Homology alignment (BLASTN) revealed that the dairy goat PPARγ gene (HQ589347.1) shares 90%, 89%, 98% and 98% identity with human (AB472042), mouse (NM_001127330.1), sheep (NM_001100921), and bovine (BC116098), respectively. Figure 1(a) shows their genetic relationship. The structure prediction using online software revealed that there are two zinc finger structures and a ligand binding domain in the dairy goat PPARγ protein (Figure 1(b)). It was also predicted (PredictNLS online software) that the nuclear localization signal sequence (-KKSRNKC-) of the dairy goat PPARγ gene does not exist in either ends of the peptide chain, but it is present in the protein internal compartment.

3.2. A PPARγ Ligand Enhanced Activity of PPARγ Response Element in GMECs. PPARγ is a ligand-dependent nuclear transcription factor, and several unsaturated fatty acids in mammalian tissue are its natural ligands [18]. Binding of ligands to the PPARγ ligand binding domain causes conformational changes in the receptor [16, 19]. Once activated, PPARγ forms a heterodimeric complex with retinoid X receptor (RXR) and binds to the PPRE upstream of target genes [8]. In the present study, dairy goat mammary epithelial cells were incubated with rosiglitazone, a chemosynthetic ligand, which has a high affinity for PPARγ and enhanced its activity. As shown in Figure 2, treatment with ROSI caused an activation of PPARγ in GMECs. The luciferase levels between the treatment group and the control group (treatment with 0 μmol/L ROSI) were statistically significant ($P < 0.05$). Data also indicated that the activation of the PPARγ by ROSI reached a peak at 50 μmol/L dose.

3.3. Activation of PPARγ by ROSI Affects Expression of Genes Related to Triacylglycerol Synthesis and Lipid Droplets in GMECs. Genes related to *de novo* fatty acid synthesis (acetyl-coenzyme A carboxylase alpha (*ACACA*), fatty acid synthase (*FASN*)), desaturation (Stearoyl-CoA desaturase (*SCD*)), TAG synthesis (Diacylglycerol acyl transferase 1, (*DGAT1*)) and other genes including fatty acid binding protein 3 (*FABP3*) and Perilipin2 (*PLIN2*) were upregulated in adipose tissue of rats [20], humans [21], and bovine mammary epithelial cells [5] treated with ROSI. As summarized in Figure 3, treatment with ROSI increased the expression of *ACACA*, *FASN*, *SCD, FABP, LPL,* and also those associated with lipid droplet formation and hydrolysis (*PLIN2* and patatin-like phospholipase domain containing 2, *PNPLA2*), and transcription regulators (*SREBF1*; liver X receptor α, *NR1H3*) (Figure 3). The significant ($P < 0.05$) increase in gene expression suggests that these genes are putative PPARγ target genes in goat mammary gland. In a previous study, the expression of genes associated with long-chain fatty acid uptake or intracellular activation and transport, including *LPL*, was not affected by ROSI treatment of bovine mammary

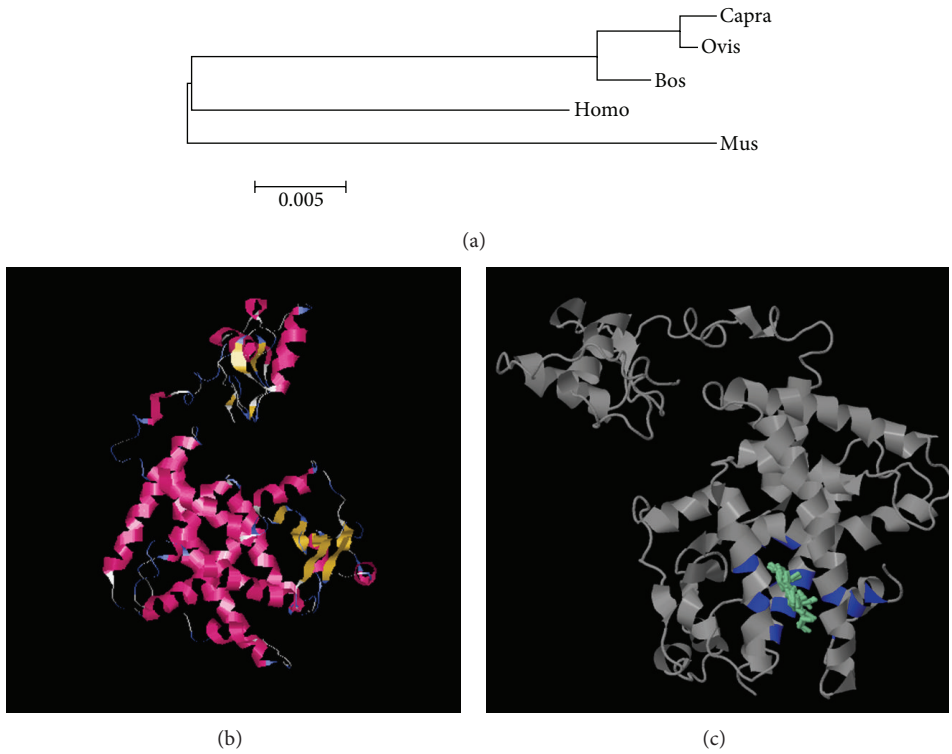

Figure 1: Structure prediction and phylogenetic alignment analysis of the dairy goat PPARγ gene. (a) Phylogenetic tree showing the relatedness of PPARγ CDS sequences of mouse (Mus), human (Homo), bovine (Bos), sheep (Ovis), and goat (Capra). The alignment was performed with ClustalW. The digital "0.005" is the genetic ruler. (b) The tertiary structure prediction of goat PPARγ. Alpha helices are colored in crimson, beta sheets in yellow, turnings in blue, and irregular curl in white. (c) The ligand binding domain prediction of goat PPARγ. The amino acids involved in the binding sites are colored in blue. The ligands colored in laurel green. In grey is the predicted tertiary structure of the goat PPARγ protein.

cells for 12 h [5]; however, our results revealed that *LPL* was upregulated significantly with ROSI treatment but only after 24 h. These contrasting responses may be related at least in part with inherent species differences in the regulatory mechanism via PPARγ [5, 22].

3.4. Preliminary shRNA Screening and Adenovirus Generation. The GFP protein on the *pENTR/CMV-GFP/U6-shRNA* vector was used to assess the efficacy of transduction via intensity of green fluorescence inside the cells. The 293A cells were either transfected with only the *pDsRed1-C1-PPAR*γ construct (red fluorescent cells) or cotransfected with both constructs. Once the shRNA enters the cell, if specific for PPARγ, it would enhance *pDsRed1-C1-PPAR*γ construct with a concomitant reduction of red fluorescence. In this way, the shRNA efficacy in knocking down PPARγ was assessed by the disappearance of red fluorescence in the cells. As shown in Figure 4, sh1006 and sh614 were more efficient than sh500 to silence PPARγ (Figures 4(b2) and 4(c2)). There was more dsRED fusion protein being coded and detected in the sh500 group (Figure 4(a2)), indicating that sh500 had weaker silencing effect on goat *PPARG*. This was probably also due to the lower transfection observed for the sh500 construct (Figure 4(a3)).

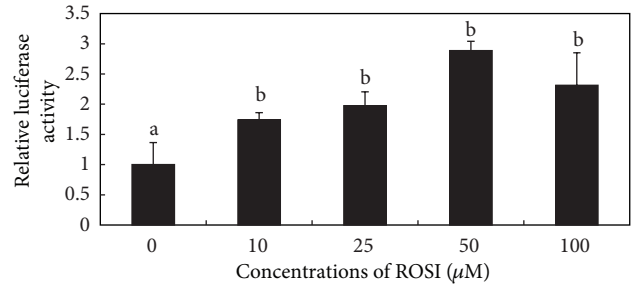

Figure 2: ROSI activated the PPARγ response element (PPRE) effectively in GMECs. DMECs were transfected with pGL3-basic-PPRE×3 and pRL-TK vectors. After transfection, cells were treated with different concentration of ROSI. Luciferase and *Renilla* luciferase assays were performed in triplicate, and the results were expressed relative to the control (0 μmol/L). Luciferase activity data were normalized with *Renilla* luciferase activity. The data represent mean ± SD of three independent experiments. $^{b}P < 0.05$ versus the control group.

Although the approach depicted in Figure 4 is not quantitative, it represents a relatively easy way to screen efficient shRNAs.

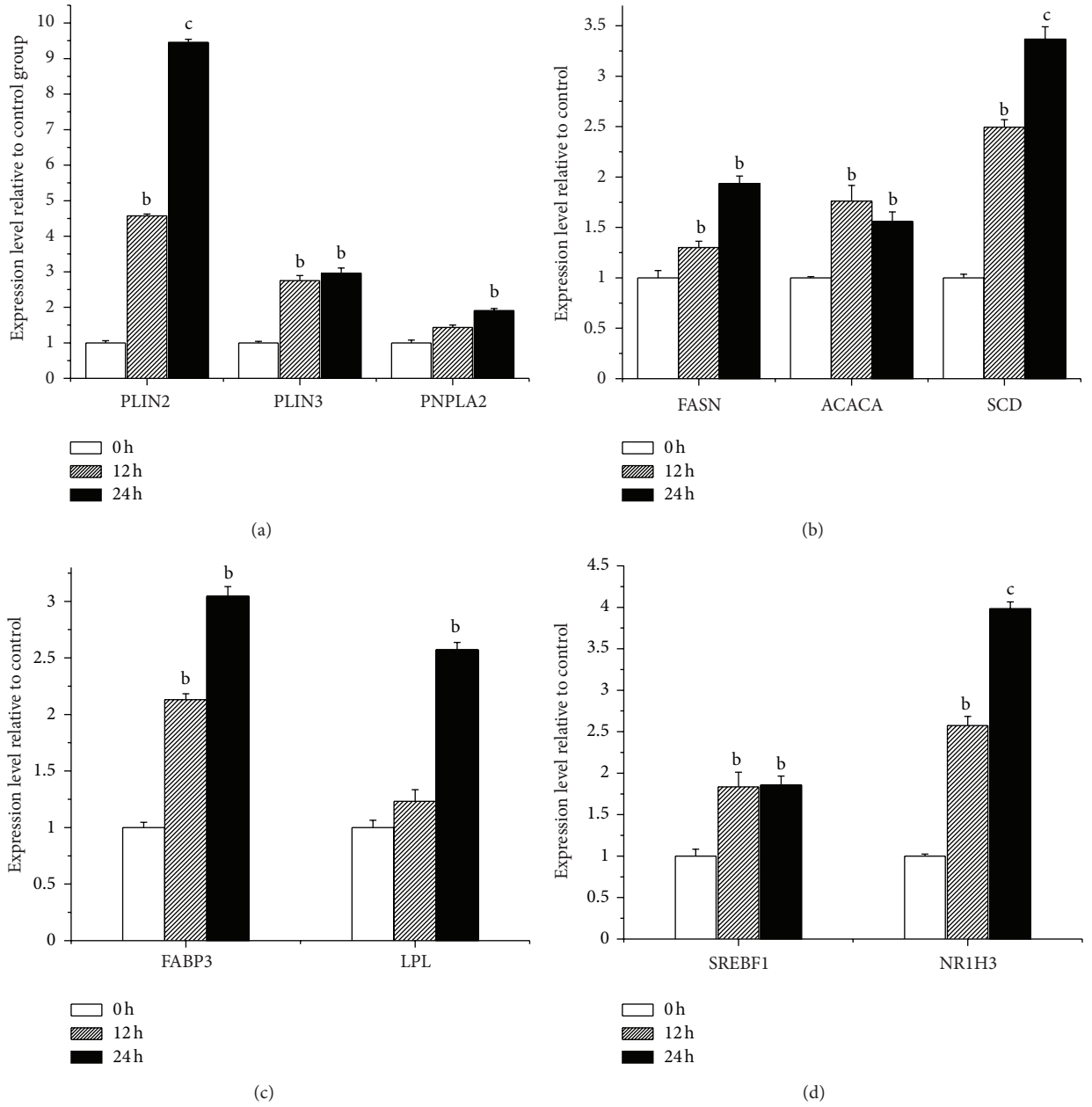

Figure 3: ROSI affects the expression of genes coding for proteins involved in lipid synthesis in GMECs through PPARγ signaling. Dairy goat mammary epithelial cells were treated with ROSI and harvested at 0, 12, and 24 h. (a) Genes related to lipid droplet formation (*PLIN2* and *PLIN3*) and hydrolysis of triacylglycerols (*PNPLA2*). (b) Genes related to fatty acid synthesis (*FASN* and *ACACA*) and desaturation (*SCD*). (c) Genes related to cellular fatty acid uptake (*FABP3*, *LPL*). (d) Genes related to regulation of transcription (*SREBF1* and *NR1H3*). The data are mean ± SD of three independent experiments. $^{b}P < 0.05$ versus the control group (0 h). $^{c}P < 0.01$ versus the control group (0 h).

According to the results of the preliminary screening, sh1006 and sh614 were selected to generate adenovirus Ad-sh614 and Ad-sh1006. Judging by the RT-qPCR and western blot analysis (Figure 5), compared with Ad-sh614 (about 20%), the Ad-sh1006 (about 60%) was more efficient in knocking down goat *PPARG*.

3.5. Knockdown of Goat PPARγ in GMECs Affects Expression of Genes Involved in Triacylglycerol Synthesis and Lipid Droplet Formation in GMECs. Based on the above results, the Ad-sh1006 was selected to block expression of *PPARG* in GMECs, and expression analysis of genes known to be involved in milk fat synthesis and lipid droplet formation was evaluated (Figure 6). Results demonstrated that *FASN* (−58%), *ACACA* (−65%), and *SCD* (−65%) decreased significantly after *PPARG* knockdown (Figure 6(a)). With the exception

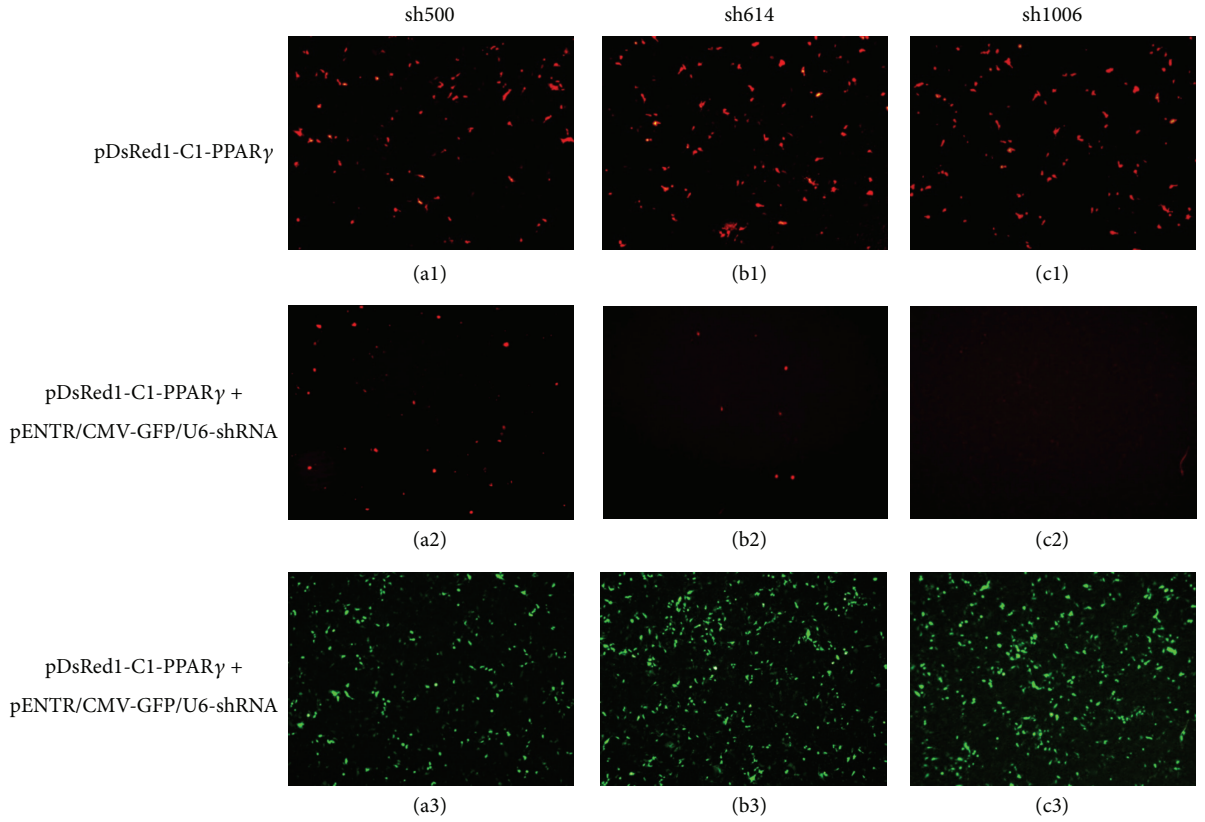

Figure 4: Efficacy screening of the three designed shRNA via images analysis. *pDsRed1-C1-PPARγ* vector was transfected as a control ((a1), (b1), and (c1)). The three tested shRNA (sh500, sh614, and sh1006) as *pENTR/CMV-GFP/U6*-shRNA construct were cotransfected with *pDsRed1-C1-PPARγ* vector. The transduction efficiency was estimated by the level of green fluorescent protein (GFP) expression ((a3), (b3) and (c3)). Shown are representative images of the PPARγ expression (in red) after a 48 h cotransfection. (a1), (b1), and (c1) show high transfection and expression of PPARγ construct vector. (a2), (b2), and (c2) show reduction of PPARγ expression after addition of shRNA construct, while (a3), (b3), and (c3) show efficacy of shRNA transfection as shown by the green color (i.e., GFP). Images were obtained by a fluorescence microscope (Leica, DMI4000B, Germany) at 100x magnification. The images clearly show that the sh1006 had the highest effect on PPARγ vector expression (c2).

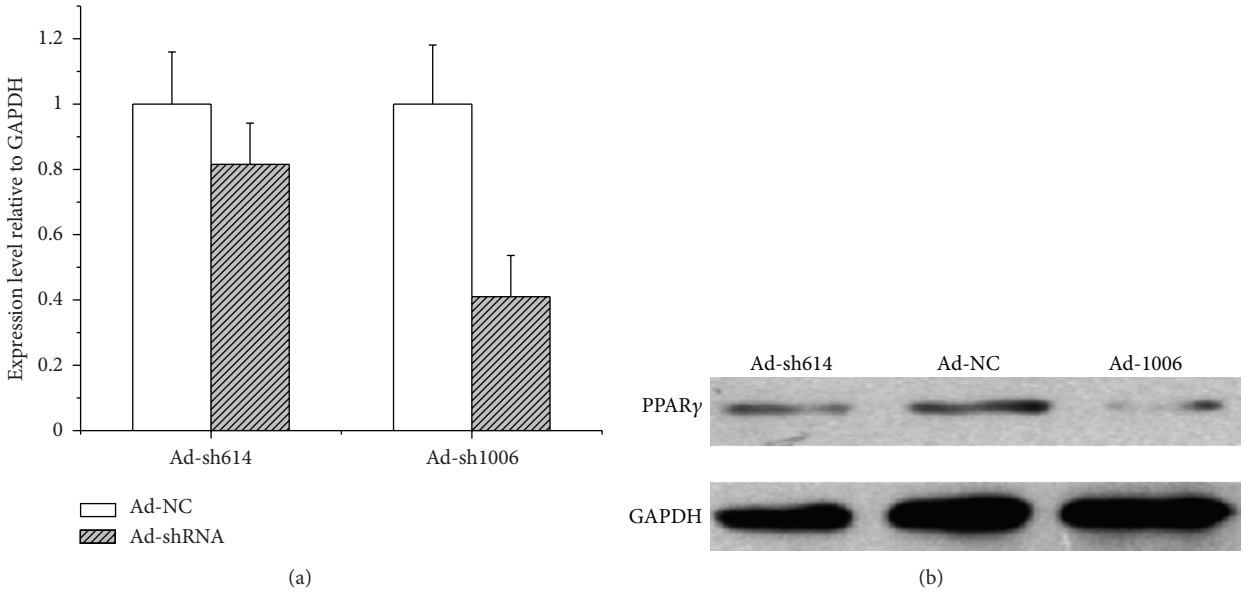

Figure 5: Efficacy screening of the two designed shRNA via RT-qPCR and western blot. The efficiency of Ad-sh614 and Ad-sh1006 (transduced with two adenoviruses at 200 multiplicity of infection for 48 h) in decreasing *PPARG* expression in dairy goat mammary epithelia cells was assessed by RT-qPCR (a) and western blot (b). The data revealed that Ad-sh1006 had the highest knockdown of PPARγ transcript and protein; thus, it was used in the subsequent experiments.

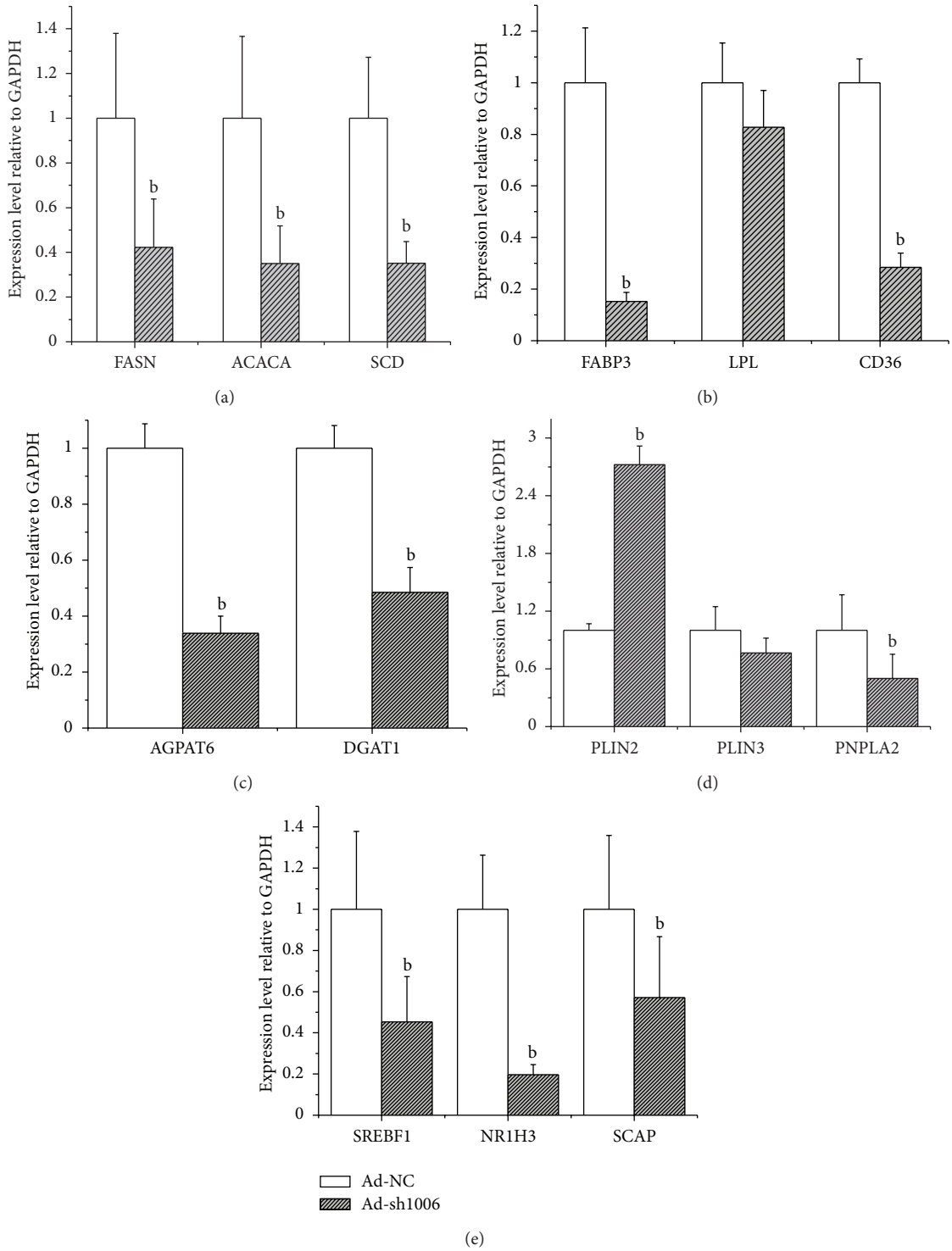

Figure 6: Effect of PPARγ knockdown on genes coding for proteins involved in milk fat synthesis in GMECs. The expression of genes related to fatty acid synthesis (a), cellular fatty acid uptake (b), triacylglycerol synthesis (c), lipid droplet formation and triacylglycerol hydrolysis (d), and transcriptional regulation (e) was assessed in goat epithelial cells (GMECs) after transduction with Ad-sh1006 at 200 MOI for 48 h. The data represent the mean ± SD of cells transfected with control (Ad-NC) or Ad-sh1006 vector in triplicate per experiment. $^{b}P < 0.05$ versus the control group.

of *SCD*, those data are in agreement with observations in bovine [5] and suggest that PPARγ regulates *de novo* fatty acid synthesis and desaturation in goat mammary cells.

In bovine mammary cells, SREBF1 has attracted much attention because of its regulation of FASN and SCD expression and the major role played in milk fat synthesis [6, 23]. PPARγ indirectly regulates SREBP1 protein activity through regulation of the expression of insulin-induced gene 1 (INSIG1) and directly regulates SREBF1 expression in adipose cells of mice [4]. We observed that the expression of *SREBF1* and *SCAP* decreased by 50% and 43% after knockdown of *PPARG* (Figure 6(e)). The mRNA of NR1H3 gene also was reduced by 75% when *PPARG* was knocked down (Figure 6(e)). Our data agree to a large extent with a previous bovine study, where an increase of *SREBF1* expression after ROSI treatment was observed [5]. We speculate that there might be two different signaling networks regulating *de novo* fatty acid synthesis in ruminant mammary cells. One pathway is under direct regulation of PPARγ and encompasses genes such as *LPL, NR1H3*, and *FABP3* (Figures 6(b) and 6(e)); another is under indirect regulation of PPARγ through SREBF1 and NR1H3 (Figure 6(e)) which would, in turn, participate in upregulation of the transcription of *FASN* and *ACACA* [23–25]. Regardless of the specific mechanism, our data support the previous hypothetical milk fat synthesis transcriptional networks proposed for bovine mammary [3]. In agreement with that previous proposal, our data support a complex regulatory network that controls mammary triacylglycerol synthesis in goat mammary cells such that several protein factors serve as putative checkpoints to regulate milk fat synthesis. PPARγ appears to be one of those factors in dairy goats.

PPARγ plays multifaceted roles in the regulation of triacylglycerol synthesis and secretion besides the *de novo* synthesis of fatty acids. As an adiposity factor, PPARγ is able to regulate triacylglycerol synthesis and deposition and then dominate the process of differentiation of fat cells [26]. In the present study, the mRNA expression of genes related to triacylglycerol synthesis *DGAT1* (−52%) and *AGPAT6* (−67%) decreased greatly after infection with Ad-sh1006 (Figure 6(b)), which suggests that PPARγ regulates triacylglycerol synthesis in mammary cells as in fat cells.

Triacylglycerols are deposited in fat cells, while in the mammary cells they are secreted in the form of lipid droplets in milk. To investigate the role of PPARγ in transcription of milk fat globule protein genes, we measured the mRNA expression of *PLIN2, PLIN3,* and *PNPLA2* after PPARγ knockdown (Figure 6(d)). The expression of *PLIN2* was largely induced while the expression of *PLIN3* and *PNPLA2* decreased approximately 24% and 50%, respectively, in cells transfected with Ad-sh1006, while it is not extremely for *PLIN3*. Previous data from humans [27] indicated that there is a PPRE on the promoter of the PLIN2 gene; thus, it is considered as a downstream target and would be decreased after PPARγ knockdown. However, our data showed that the expression of *PLIN2* had an unexpected increase. Such response might have been caused by compensatory effects of other unidentified transcription factors.

Other data also support the evidence [28] that PPARγ could affect not only the genes related to fatty acid transport, but also genes that control triacylglycerol hydrolysis in goat mammary cells (Figure 6(c)). For instance, expression of *PNPLA2* is significantly increased during lactation in bovine mammary tissue [29]. However, judging by differences in milk fatty acid profiles between goat milk and bovine milk [30], goat mammary lipid synthesis differs in some respects from bovine. From a mechanistic standpoints the upregulation of *PNPLA2* after PPARγ activation may be functionally related with the unique characteristics of goat milk.

Our data showed that even if there is great similarity between two ruminant dairy species such as goat and cow [5], there are still some inherent differences between them. Such differences may at least in part be caused by different target genes of PPARγ in each species. Attempts to compare *in vitro* data among studies performed in different laboratories are obviously challenging because of differences in cell culture conditions (e.g., culture medium, absence of prolactin in our study and not in bovine [31]) and also different protocols. The comparisons of data from the present study with data generated in bovine mammary [31] are likely also slanted because of the use in the present study of *GAPDH* as the only internal control for RT-qPCR normalization versus multiple genes used in the bovine study.

4. Conclusions

In the present studies, we cloned the PPARγ gene in dairy goat mammary gland and explored its function *in vitro*. As proposed in bovine mammary gland, PPARγ plays a multifaceted role in regulating the overall process of fatty acid and triacylglycerol synthesis and secretion. Our overall data indicate that PPARγ in goat mammary plays a role in controlling milk fat synthesis directly or via the activation of the transcription regulators *SREBF1* and *NR1H3*. Together, our data provide strong evidence that PPARγ is the key regulator of milk fat synthesis in ruminants. Hence, controlling PPARγ activation may prove useful in regulating milk fat production in the lactating dairy goat.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Review Article

Nuclear Control of the Inflammatory Response in Mammals by Peroxisome Proliferator-Activated Receptors

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Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that play pivotal roles in the regulation of a very large number of biological processes including inflammation. Using specific examples, this paper focuses on the interplay between PPARs and innate immunity/inflammation and, when possible, compares it among species. We focus on recent discoveries establishing how inflammation and PPARs interact in the context of obesity-induced inflammation and type 2 diabetes, mostly in mouse and humans. We illustrate that PPARγ ability to alleviate obesity-associated inflammation raises an interesting pharmacologic potential. In the light of recent findings, the protective role of PPARα and PPARβ/δ against the hepatic inflammatory response is also addressed. While PPARs agonists are well-established agents that can treat numerous inflammatory issues in rodents and humans, surprisingly very little has been described in other species. We therefore also review the implication of PPARs in inflammatory bowel disease; acute-phase response; and central, cardiac, and endothelial inflammation and compare it along different species (mainly mouse, rat, human, and pig). In the light of the data available in the literature, there is no doubt that more studies concerning the impact of PPAR ligands in livestock should be undertaken because it may finally raise unconsidered health and sanitary benefits.

1. Introduction

The peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that play critical roles in very different biological pathways such as lipid, protein, glycerol, urea, glucose, glycogen and lipoprotein metabolism, adipogenesis, trophoblast differentiation, and cell migration [1–6]. Notably, PPARs are also required to balance cell proliferation and cell death and therefore impact skin wound healing and proliferative diseases such as cancer [7–9]. PPARs are also prominent players in inflammation control [10, 11]. PPARα, the first PPAR isotype identified in mouse, was originally cloned in the early 1990s as a novel member of the steroid hormone receptor superfamily [12]. Shortly after, a rat version of PPARα as well as three novel members related to each other (xPPARα, xPPARβ, and xPPARγ) and to mouse PPARα have been subsequently cloned from *Xenopus* (frog) [13]. Since then, substantial efforts have been made to identify other related receptors; several additional PPAR isoforms and variants have been therefore isolated in a wide range of species including mammals (human, rabbit, mouse, rat, pig, rhesus and cynomolgus monkey, dog, guinea pig, hibernating ground squirrel, and hamster), fishes (grass carp, cobia not only but also marine fish such as the teleost red sea bream (*Pagrus major*) and the mullet *Chelon labrosus*), marine gastropod mollusks (*Cyclostoma*), reptiles (leopard gecko, crocodile, and turtle), and birds (domestic chicken, goose) [14–51].

Since PPARs are ligand-activated transcription factors, a large part of our knowledge about their biological importance is coupled to the function of their target genes. At the molecular level, it was shown that PPARs readily heterodimerize with

the Retinoid X Receptor (RXR) prior to ligand binding [52]. In all species tested so far, *Pparα*, *Pparβ/δ*, and *Pparγ* show specific time- and tissue-dependent patterns of expression (Table 1).

After ligand treatment, the PPAR/RXR heterodimer stably binds on genomic DNA at specific sites called Peroxisome Proliferator Response Element (PPRE) and upregulates gene transcription. Consensus PPREs are formed by two hexameric core binding motifs (AGGTCA) in a direct repeat orientation with an optimal spacing of one nucleotide (DR1). Molecular investigations have demonstrated that PPAR occupies the 5′ motif of the DR1 [53]. Recent analyses have further revealed that even if DR1 PPREs can be located within the promoter sequences of target genes, about 50% of all target sites are located within genes (introns, exons) as well as in 3′ downstream sequences of the target genes [4, 7, 54–58]. The PPARα (NR1C1), PPARβ/δ (NR1C2), and PPARγ (NR1C3) genes encode proteins that share a highly conserved structure and molecular mode of action, yet the array of genes regulated by each PPAR isotype is divergent and may also differ from one species to another [59]. An extended analysis of the cross-species (mouse to human) conservation of PPREs brought support to this hypothesis because it revealed only limited conservation of PPRE patterns [60]. Strengthening this observation, only a minor overlap between the Wy14,643 (Wy: a specific PPARα agonist) regulated genes from mouse and human primary hepatocytes was found by Rakhshandehroo et al. demonstrating that some, but not all, genes are equally regulated by PPARα in mouse and human hepatocytes [61]. In this review, we explore and focus on the role of PPARs in the control of chronic (mediated by obesity) or acute (as a result of bacterial infection) inflammation in different species, mainly from human, mouse, rat, pig, and cow.

2. PPARs and Obesity-Induced Inflammation: Interplay with Adipose Tissue Macrophages

2.1. PPARα. In spite of the relative weak expression level of *Pparα* in white adipose tissue (WAT, mainly in adipocytes and not in stromal-vascular cells), several lines of evidence support the notion that PPARα and PPARα agonists could play a functional role in the control of obesity-induced chronic inflammatory response *in vivo*. For instance, treatment of obese diabetic KKAy mice with Wy decreased the mRNA levels of *Tnf-α* (tumor necrosis factor-α), *Mcp-1* (monocyte chemotactic protein-1, also referred to as chemokine (C-C motif) ligand 2, *CCL2*), and *Mac-1* (macrophage antigen-1, also known as cluster of differentiation molecule-11b, *Cd11b*) in epididymal fat, suggesting a reduction in macrophage infiltration [62]. In addition, expression of inflammatory genes in adipose tissue such as *Tnf-α*, *Mcp-1*, and *IL-1β* (Interleukin-1 beta) as well as that of specific macrophage markers such as *Cd68* (macrophage antigen *Cd68*, also known as scavenger receptor class D member 1, *Scard1*), *F4/80* (also referred to as lymphocyte antigen-71, *Ly71*), and *Adam8* (ADAM metallopeptidase domain 8, also known as cluster of differentiation molecule-156, *Cd156*) in the stromal vascular fraction was more pronounced in *Pparα*-deficient mice compared to WT (wild-type) mice rendered obese with a high-fat feeding,

Table 1: Tissue distribution of the various PPARs in different species.

Specie	Tissue	Expression
	PPARα (NR1C1)	
Cow/cattle	Liver	++ [237]
	WAT	N.D.
	GI tract	N.D.
	Brain	N.D.
	Spleen/thymus	N.D.
Chicken	Liver	++ [23]
	WAT	+ [82]
	Brain	++ [82]
	Spleen	+ [82]
Human	Liver	+++ [20, 61, 118, 162, 175]
	Primary hepatocytes	± to +++ [61, 134]
	HepG2 hepatoma cells	+ [54]
	HepaRG hepatoma cells	++ [134]
	WAT	+ [20, 118, 238]
	Isolated adipocytes	± [20]
	GI tract	++ [20, 118, 175, 239]
	Brain	+ [118, 175, 240]
	Monocytes	+ [241, 242]
	Dendritic cells	++ [241, 242]
	Kidney	++ [20, 118]
	Heart	+++ [118]
Pig	Liver	± [243]
	WAT	+ [243]
Mouse/rat	Liver	+++ [61, 83, 244–247]
	Hepatocytes	++ [61]
	GI tract	++ [Nursa] [175]
	Brain	+ [Nursa]
	Spleen/thymus	− [83]
	Macrophages (BMDM)	− [244]
	FAO hepatoma cells	++ [54]
	WAT	+ [62, 248]
	PPARβ/δ (NR1C2)	
Cow/cattle	Liver	N.D.
	WAT	N.D.
	GI tract	N.D.
	Brain	N.D.
	Spleen/thymus	N.D.
Chicken	Liver	N.D.
	WAT	N.D.
	GI tract	N.D.
	Brain	N.D.
	Spleen/thymus	N.D.

Table 1: Continued.

Specie	Tissue	Expression
Human	Liver	± [20]
	HepG2 hepatoma cells	++ [54]
	WAT	± [20]
	Isolated adipocytes	± [20]
	Large intestine	+++ [20]
	Small intestine	+ [20]
	Colon mucosae (adult)	++ [239]
	Brain	N.D.
	Monocytes	++ [241]
	Macrophages	+++ [249]
	Dendritic cells	+ [241]
	Kidney	+ [20]
	Skeletal muscle	± [20]
Pig	Liver	+ [250]
	WAT	++ [250]
	Stomach	++ [250]
	Brain	++ [250]
Rabbit	Liver	± [251]
	GI tract	+ [251]
	Brain	++ [251]
	Spleen/thymus	± [251]
Mouse/rat	Liver	+ to ++ [Nursa] [50, 83, 246, 247]
	FAO hepatoma cells	++ [54]
	WAT	+ [Nursa] [50]
	GI tract	+++ [Nursa] [50, 175]
	Brain	+++ [Nursa] [50, 83, 193, 252]
	Macrophages (BMDM)	++ [244]
	Colon	++ [83]
PPARγ (NR1C3)		
Cow/cattle	Liver	− [253]
	WAT	+++ [253, 254]
	Spleen/thymus	++ [253]
	Small intestine	± [253]
	Mammary gland	[235]
Chicken	Liver	− [82]
	Spleen/thymus	+ [82]
	Brain	+ [255]
	WAT	+++ [255]
Human	Liver	+ [20, 256, 257]
	HepG2 hepatoma cells	+ [54]
	HepaRG cells	± [134]
	Primary hepatocytes	± [134]

Table 1: Continued.

Specie	Tissue	Expression
	WAT	+++ [20, 54, 256, 257]
	Isolated adipocytes	+++ [20]
	Simpson-Golabi-Behmel Syndrome (SGBS) adipocytes	+++ [84]
	Large intestine	+++ [20]
	Small intestine	± [20]
	Brain	N.D.
	Monocytes	+++ [241]
	Dendritic cells	+++ [241]
	Kidney	+ [20]
	Skeletal muscle	± [20]
Pig	Liver	− [243]
	WAT	++ [243]
Rabbit	Liver	− to + [251, 258]
	WAT	+++ [258]
	GI tract	+++ [251]
	Brain	− [251]
	Spleen/thymus	++ [251]
Mouse/rat	Liver	+ to − [Nursa] [83, 246, 247]
	Hepatocytes	+ [259]
	FAO hepatoma cells	− [54]
	WAT	+++ [Nursa] [83, 256, 260]
	3T3-L1 adipocytes	+++ [84]
	GI tract	+ [Nursa] [83]
	Brain	+ [Nursa] [83, 261, 262]
	Spleen/thymus	++ [83]
	Macrophages (BMDM)	+++ [244]

Abbreviations: GI: gastrointestinal; WAT: white adipose tissue; N.D.: not determined. BMDM: bone marrow-derived macrophages.
Symbols: −: absent; ±: barely detectable; +: weak; ++: moderate; +++: high. the citation link for Nursa is http://www.nursa.org/10.1621/datasets.02001.

reinforcing the notion that PPARα is required for the control of the adipose inflammation process [63]. Another study has also examined the effects of fibrates on the inflammatory changes induced by the interaction between adipocytes and macrophages in obese adipose tissue. Systemic administration of Wy or fenofibrate to genetically obese *ob/ob* mice significantly reduced *Tnf-α* and *Mcp-1* mRNA expression in WAT [64]. Similar observation was also reported using adipose tissue explants from *ob/ob* mice suggesting a direct effect of PPARα agonists. To check for the definitive involvement of PPARα in the effects of Wy-mediated reduction in the production of proinflammatory cytokines by white fat pads, adipose tissue explants obtained from PPARα-deficient

mice were also used [64]. Compared to WT mice, induction of *Mcp-1* mRNA expression by TNF-α (a major paracrine mediator of inflammation in adipocyte) was much robust in adipose tissue explants from *Ppar*α-deficient mice, suggesting that PPARα is constitutively required to control the steady-state level of adipose *Mcp-1* mRNA levels. Intriguingly, induction of adipose *Mcp-1* mRNA expression by TNF-α was also suppressed by Wy in explants from *Ppar*α-deficient mice, suggesting that Wy can act independently of the presence of the receptor in fat, at least for the control of the inflammation process [64]. Because *Ppar*γ is expressed in both mature adipocytes and macrophages, we cannot rule out that part of the effects of fibrates on adipose inflammation are mediated through this other PPAR isotype. Moreover, treating 3T3-L1 mouse adipocytes with Wy or fenofibrate suppressed bacterial lipopolysaccharides-(LPS-) mediated increased in *Mcp-1* mRNA levels, indicating a cell autonomous effect [62]. Interestingly, pharmacological activation of PPARα also reduced LPS-mediated induction of *Mcp-1* mRNA level in peritoneal macrophages. Therefore, it is possible that PPARα agonists mediate reduction of the inflammatory response in both adipocytes and infiltrated macrophages in WAT. Whether adipose PPARα is a critical factor for the control of adipose inflammation remains a matter for further study. To close this gap, it could be interesting in the future to check for the consequence of the selective deletion of *Ppar*α in WAT, using the Cre/loxP strategy and the adipocyte/macrophage-specific aP2 (a-FABP) promoter [65].

2.2. PPARβ/δ. While ubiquitously expressed, probably in all cells found in WAT, PPARβ/δ is also the isotype whose exact roles in the control of WAT function and type-2 diabetes in general are the least clear. Firstly, PPARβ/δ undoubtedly displays anti-inflammatory properties in numerous cell types present in WAT, such as macrophages, adipocytes, and endothelial cells [66]. In agreement, it was found that activation of PPARβ/δ prevents LPS-induced NF-κB (a key regulatory proinflammatory transcription factor) activation by regulating ERK1/2 (Extracellular signal-Regulated Kinases) phosphorylation in adipocytes and WAT in mice [67]. PPARβ/δ may therefore represent an interesting target for the treatment of inflammatory diseases such as atherosclerosis [68]. Secondly, several investigations aiming to determine the role of PPARβ/δ in WAT mass have demonstrated that it probably only plays a moderate role in adipogenesis and an indirect role in the control of WAT mass [69–72]. For instance, feeding murine models of obesity and diabetes with a PPARβ/δ agonist decreases their adiposity [73]. Yet, these effects are most likely mediated by *Ppar*β/δ expression in other nonadipose tissues such as liver and skeletal muscle because WAT *Ppar*β/δ conditional knockout mice do not exhibit any apparent adipose tissue phenotype [70]. Furthermore, this indirect role of PPARβ/δ is also provided in mice overexpressing *Ppar*β/δ in skeletal muscle because these mice display decreased adiposity and adipocyte size [74]. Regarding WAT inflammation, several publications have led to discrepant findings as well. For instance, reconstitution with *Ppar*β/δ null bone marrow of irradiated WT mice to generate *Ppar*β/δ null animals lacking *Ppar*β/δ in hematopoietic cells had no clear effects on WAT inflammation and insulin sensitivity. If any benefits on insulin sensitivity were seen, these were different according to the genetic background of the mice and likely mediated by the liver where PPARβ/δ switches the phenotype of Kupffer cells (liver macrophages-like cells) into an anti-inflammatory phenotype (also called M2 phenotype; this phenotype is acquired after cell activation by cytokines such as Interleukin-4 and Interleukin-13) [66, 75]. Classically, activated macrophages (also known as M1 type) express high levels of proinflammatory mediators that elevate inflammation to a low, but chronic, grade and contribute to insulin resistance [76, 77]. In contrast, M2 "alternatively" activated macrophages are characterized by low production of proinflammatory cytokines (including IL-1β, TNF-α, and IL-6) and high production of anti-inflammatory cytokines (including IL-10), by a gene expression profile distinct from other macrophage populations and by their capacity to scavenge debris, to promote angiogenesis, tissue repair, and remodeling [78]. However, the observations evoked above contrast with that of Kang et al. who describe that PPARβ/δ is required for the polarization of adipose tissue macrophages (ATMs) into an M2 phenotype [79]. In summary, the exact role of PPARβ/δ in the control of WAT inflammation requires further investigations.

2.3. PPARγ. In response to an inappropriate diet, insulin resistance settles in WAT further limiting its capacity to store fat. Consequently, excess fatty acids overflow into other organs such as skeletal muscle and liver (ectopic fat), which in turn alters proper functioning of these tissues [80]. PPARγ is strongly associated with obesity because it is highly expressed in white fat depots and it serves as a target for certain anti-diabetic drugs. A substantial amount of *Ppar*γ*1* mRNA level is detected in many tissues including white and brown adipose tissue, skeletal muscle, liver, colon, bone, and placenta and cell types such as pancreatic β-cells and macrophages in different species ranging from humans to rodents, sheep and cattle [81]. The other *Ppar*γ isoform, *Ppar*γ*2*, is highly expressed in WAT in rodents (mainly rats and mice) as well as in humans, chicken, and sheep [20, 82–86].

A wealth of studies has established the critical role of PPARγ in adipose tissue biology and it is now widely accepted that PPARγ is a predominant nuclear receptor regulating the process of adipose differentiation both *in vivo* and *in vitro* [87–89]. However, it now appears that it is more specifically the low-grade systemic inflammation associated with obesity that is central to the etiology of the disease. During development of obesity, the expansion of WAT is accompanied with increased infiltration of macrophages that accumulate around stressed mature adipocytes [90]. Several genetic and pharmacological manipulations have further revealed situations in which obesity and inflammation were disconnected, demonstrating that obesity as such does not necessarily leads to type-2 diabetes as long as inflammation does not occur [77, 91–93]. In the context of obesity, adipocytes are exposed to excessive concentrations of free fatty acids. We and others have recently demonstrated that various fatty acids, especially arachidonic acid, induce the murine adipose transcription and secretion of chemokines such as MCP-1,

Regulated upon Activation, Normal T-cell Expressed and Secreted/chemokine (C-C motif) ligand 5 (RANTES/CCL5), and the chemokine Keratinocyte Chemoattractant (KC, also known as CXCL1) [94–96]. As chemokines govern the recruitment of leukocytes such as macrophages, high-fat diets providing elevated levels of fatty acids are likely to cause the adipose secretion of chemokines. In turn, these chemokines will induce the recruitment of macrophages in WAT and elevate local inflammation (Figure 1).

Detailed analysis of the molecular mechanisms involved revealed that the activation of the Toll-like receptor 4 pathway (TLR4) by the fatty acids was required. Surprisingly, activation of this pathway causes the decreased expression level of *Pparγ*, which was prevented by the cotreatment with ER stress inhibitors [94]. This observation adds up to other publications demonstrating the key, yet unstable, role played by this specialized organelle in maintaining an adequate cellular response to metabolic stresses [97, 98]. Together, this led us to establish a model in which fatty acids, through a TLR4/ER stress-dependent pathway, induce the recruitment of leukocytes by increasing the secretion of chemokines [99].

In spite of decreased *Pparγ* mRNA levels, pharmacological activation of PPARγ with rosiglitazone (RSG), a thiazolidinedione (TZD)/PPARγ agonist, prevents fatty acid-mediated adipose induction of chemokines expression and secretion [94, 100]. These observations were strengthened by *in vivo* experiments where treatments of mice fed a high-fat diet by RSG increased adiposity but decreased the expression of chemokines by adipocytes, the classically activated adipose tissue macrophages (M1 type) content and WAT inflammation [77, 94, 101]. Therefore, PPARγ maintains the expression of chemokines to a minimal level in adipocytes. As a member of the nuclear hormone receptor superfamily, PPARγ displays both transactivational and transrepressional activities [59, 102]. Interestingly, it is likely through transrepressional activity that PPARγ affects chemokines secretion by adipocytes [94]. In line with this, it is worth mentioning the recent discovery of MBX-102/JNJ39659100, a member of a novel non-TZD class of selective partial PPARγ agonist with weak transactivational activity, yet high transrepressional activity for PPARγ, that conserves insulin-sensitizing properties without inducing well-known major side effect [103]. As PPARγ transrepressional activity is involved in the repression of proinflammatory cytokines and chemokines, it is tempting to think that part of TZDs therapeutic properties on type-2 diabetes could be explained by their anti-inflammatory properties. Therefore, developing agents able to disconnect the transactivational activity of PPARγ from its transrepressional activity may represent an effective strategy to treat different inflammatory diseases such as type-2 diabetes. This hypothesis raises the fundamental question about how does PPARγ transrepressional activity work? Elucidation of the basic mechanism on how PPARγ controls inflammation has derived primarily from work performed in macrophages [104–106]. As PPARγ transrepressional activity is also involved in the repression of proinflammatory cytokines in the stromal vascular cells of WAT (i.e., the macrophage containing cellular fraction), similar molecular mechanisms of regulation may also occur in adipocytes and macrophages, but it is a nonproven hypothesis at the moment. The scenario is probably as follows: in resting situation, constant binding of corepressors complexes such as nuclear receptor corepressor (NCOR) and silencing mediator for retinoid and thyroid hormone receptors (SMRT) on the gene promoter sequence of these cytokines and chemokines prevent their expression [106]. When an inflammatory stimulus is applied, NCOR becomes ubiquitinated further excluding these complexes from the nucleus. In addition, coactivators are recruited to the promoter of cytokines and transcription of the gene occurs. However, when activated by an agonist of the TZD family, PPARγ becomes SUMOylated and docked to the corepressor complexes [107, 108]. Association between PPARγ and NCOR prevents its ubiquitination further maintaining the expression of chemokines and cytokines in a repressed state. The contribution of PPARs in disconnecting obesity and inflammation is illustrated in genetic models where PPAR isotypes were selectively invalidated in macrophages and bone marrow-derived cells. First, when *Pparγ* is invalidated in macrophages, mice become more susceptible to develop insulin resistance, a state that is accompanied with elevated local inflammation in liver, adipose, and skeletal muscle tissues [109, 110]. All the above observations were explained by the shift of macrophages into a proinflammatory (M1 type) phenotype [110]. In consequence, one major role of PPARγ in macrophages is to maintain this population in an alternative anti-inflammatory state (M2 type) expressing genes such as the anti-inflammatory cytokine Interleukin-10, the IL-1 receptor antagonist (IL1-Ra), and arginase I [111, 112].

Another mechanism by which PPARγ controls adipose tissue macrophage polarization in coordinating the metabolism of macrophages. Indeed, classical (M1) activation of macrophages is a highly energy demanding state, which is sustained by glycolytic activity. Alternative (M2) activation of macrophages is less energy demanding and represents a state in which energy supplies are provided by oxidation of fatty acids and glucose. Interestingly, Odegaard and Chawla demonstrated that PPARγ is required to coordinate the oxidative genetic program in macrophages [113]. In support of this notion, it was also demonstrated that the expression of *Pparγ* in macrophages is under the control of the pro-M2 cytokine Interleukin-4, which further involves the activation of STAT6 (signal transducer and activator of transcription 6). Finally, PPARγ requires the transcriptional coactivator PGC-1β (peroxisome proliferator-activated receptor-gamma coactivator-1) in order to induce the oxidative program supporting macrophages alternative activation. Altogether, this series of observations illustrates that macrophage polarization involves different metabolic pathways that are necessary to sustain their energetic demand, and that PPARγ is coordinating this metabolic activity [113, 114].

Besides macrophages, invasion of WAT by neutrophils, eosinophils, B cells, T cells, and mast cells has been also reported. Recently, a small subset of T lymphocytes, the CD4 (+) Foxp3 (+) T regulatory (Treg), were abundantly found in the WAT of normal (lean) but not in different mouse models of obesity [115]. Interestingly, elegant studies have demonstrated that Treg cell depletion in the abdominal adipose tissue led to the induction of proinflammatory

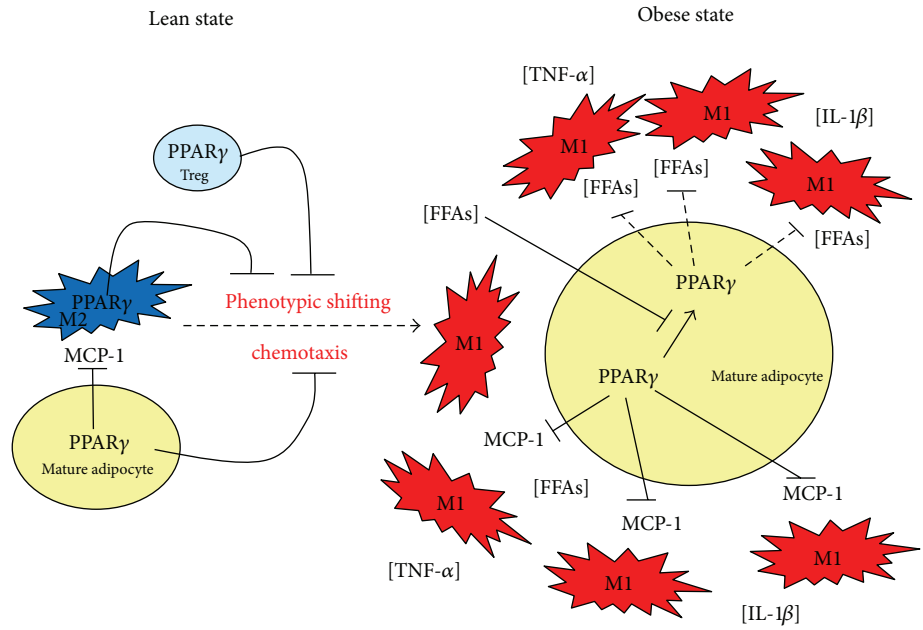

Figure 1: Contribution of the anti-inflammatory roles of PPARγ in the onset of WAT inflammation in the context of obesity and insulin resistance. In the lean state, PPARγ activity maintains homeostasis in mature adipocytes in preventing the secretion of chemokines such as MCP-1. In addition, alternatively activated macrophages (M2) and Treg cells are resident leukocytes in WAT coordinating numerous biological activities such as stimulating angiogenesis and cleaning of dead cells. The role of PPARγ in these cells is to prevent classical activation of macrophages and local inflammation to develop. When obesity is reached, mature adipocytes are exposed to excessive concentrations of free fatty acids (FFAs), which decrease *Pparγ* expression. In consequence, insulin sensitivity is also decreased in adipocytes, which elevates even more local FFAs concentrations as adipocytes are no longer able to properly store fatty acids and lipolysis also becomes activated. Furthermore, these FFAs activate macrophages shifting into an M1 phenotype, promoting the release of proinflammatory cytokines such as TNF-α and IL-1β. Secondly, as PPARγ transrepressional activity is decreased, adipocytes secrete high concentrations of chemokines (MCP-1), further promoting the recruitment of macrophages. Occurrence of this feed forward amplification loop between adipocytes and macrophages eventually leads to the elevation of local inflammation, further exacerbating local insulin resistance, which will turn systemic in the long term. MCP-1: monocyte chemoattractant protein-1; treg cells: regulatory T cells; FFA: free fatty acids; PPARγ: peroxisome proliferator-activated receptor γ; TNF-α: tumor necrosis factor-alpha; IL-1β: Interleukin-1 beta.

transcripts and enhanced inflammatory state of murine WAT [115]. Very recently, Cipolletta et al. found that deleting mouse *Pparγ* in Treg cells markedly influences the number of Treg cells residing specifically in WAT and pioglitazone, a synthetic/TZD agonist of PPARγ, and increases substantially the WAT Treg cell population in WT obese animals fed a high-fat diet [116, 117]. Furthermore, the ability of TZDs to downregulate the inflammatory state of WAT and to improve insulin sensitivity was impaired in specific *Pparγ*-deficient Treg cells. In conclusion, this information indicates that regulatory T cells expressing *Pparγ* are engaged in suppressing adipose tissue inflammation in obesity. Furthermore, PPARγ not only plays an important role in adipose macrophages but also in Treg cells. Further studies are required in order to test whether PPARγ may play a role in other immune cells controlling adipose tissue inflammation and whether this finding can be translated in other species such as humans.

3. PPARs and Inflammation in Liver

3.1. PPARα. In rodents, *Pparα* is abundantly expressed in liver where it regulates a whole array of genes involved in the uptake, binding and degradation of fatty acids by mitochondrial and peroxisomal β-oxidation, as well as in lipoprotein assembly, transport and inflammation [118, 119]. More than a decade ago, as PPARα is the nuclear receptor for the eicosanoid leukotriene B4 but also for the palmitoylethanolamide (the naturally occurring amide of palmitic acid and ethanolamine), a role for this nuclear receptor in modulating inflammation was evoked [11, 120, 121]. Since then, a solid body of evidence has implicated PPARα in the duration of inflammation control because prolonged inflammatory response was observed in mice lacking *Pparα*, suggesting anti-inflammatory actions for this nuclear receptor [11, 122].

3.1.1. Role of PPARα in the Control of Obesity-Induced Inflammation in Liver. The role of PPARα in inflammation has also been studied in the context of obesity-induced chronic low-grade inflammation, which is characterized by increased circulating inflammatory cytokines and acute-phase proteins [123, 124]. Elegant experiments with Sv129 mice lacking the nuclear receptor *Pparα* and rendered obese by chronic high-fat feeding displayed an increased abundance of macrophages in liver [63]. In agreement with this observation, mRNA

levels of proinflammatory genes were markedly increased in *Pparα*-deficient mice fed high fat diet. Because PPARα is a master regulator of fatty acid β-oxidation, PPARα may indirectly inhibit inflammation by preventing fat accumulation in liver. However, treatment of mice under nonsteatotic conditions with Wy supports the notion that PPARα is able to downregulate expression of inflammatory genes in liver independently of its effect on hepatic lipid storage [63]. Hence, by reducing hepatic lipid storage (and therefore lipotoxicity) and by suppressing proinflammatory gene expression in liver, PPARα may protect mice from steatohepatitis. These findings were further strengthened by the work of Lalloyer and collaborators who studied the impact of Pparα deletion in apoE2-KI mice (a human like hyperlipidemic mouse model) that were subjected to a Western diet supplemented or not with fenofibrate [125]. These ApoE2-KI *Pparα*-knockout (−/−) mice displayed exaggerated liver steatosis and inflammation. Notably, reduced expression of inflammatory markers and macrophage content was observed in WT mice fed fenofibrate but not in *Pparα*-knockout mice, highlighting the functional role of PPARα in hepatic inflammation control. Because fenofibrate treatment immediately reduced the expression of inflammatory genes, it was proposed that the beneficial effect of fenofibrate on hepatic lipid disorders (nonalcoholic steatohepatitis) could partly be due to its inhibitory effect on proinflammatory genes [126].

Inasmuch as PPARα is a critical regulator of the hepatic inflammation process, the understanding of how *Pparα* expression in the hepatocyte is regulated could provide substantial clues to fight inflammation. In mice, liver *Pparα* expression and PPARα activity are strongly reduced by IL-1β, a cytokine produced by Kupffer cells, the resident macrophages of the liver [127]. From a molecular point of view, the inhibitory effect of IL-1β on *Pparα* promoter activity is mediated by the binding of NF-κB to two NF-κB binding sites located in the promoter of the *Pparα* gene. Noteworthy, similar molecular mechanism is also observable with the human version of the *PPARα* promoter, suggesting possible translation to the human situation. Therefore, strategies aiming at reducing Kupffer cell-derived IL-1β could theoretically limit the expansion of inflammation, at least in liver.

3.1.2. PPARα and the Control of Inflammatory Gene Expression by Transrepression. In addition to upregulation of gene expression, a growing body of evidence in the scientific literature indicates that PPARα also displays significant transrepressional activities on inflammatory genes. In agreement, PPARα has been shown to interfere with several proinflammatory transcription factors including STAT, activator protein-1 (AP-1), nuclear factor-kappa B (NF-κB), and nuclear factor of activated T cells (NFAT). NF-κB activity is tightly controlled by the degradation of the inhibitory protein IκB-alpha (IκBα) that retains NF-κB dimers in a nonactive form in the cytoplasm. It is worth recalling that PPARα upregulates the expression of IκBα in human aortic smooth muscle cells as well as in primary human hepatocytes [128]. Upon activation of IκBα, the nuclear translocation and DNA-binding activity of the proinflammatory transcription factor NF-κB is suppressed. Induction of IκBα expression can be seen as one of the mechanisms that contribute to the anti-inflammatory activities of PPARα activators. It was also reported that pharmacologically activated PPARα was capable to sequestrate the coactivator glucocorticoid receptor-interacting protein-1/transcriptional intermediary factor-2 (GRIP1/TIF2), leading to a reduced activity of the proinflammatory transcription factor CAATT/enhancer binding proteins (C/EBP) that ultimately cannot anymore transactivate the fibrinogen-β gene in liver [129]. By virtue of their anti-inflammatory abilities, glucocorticoids are among the most commonly prescribed medications for the treatment of acute and chronic inflammatory diseases. Simultaneous activation of PPARα and glucocorticoid receptor alpha (GRα) enhances transrepression of NF-κB-driven gene expression and additively represses proinflammatory cytokine production [130]. This finding paves the road for new approaches for the treatment of inflammatory diseases where the additive effect of PPARα and GRα activation could repress to a larger extent the inflammatory gene expression program.

3.1.3. Direct Upregulation of Anti-Inflammatory Genes by PPARα. PPARα has been first described as a ligand-activated transcription factor across species and as such it directly upregulates a certain array of genes. In addition to downregulating expression of proinflammatory genes, PPARα could therefore theoretically suppress the inflammatory response by direct upregulation of gene(s) with anti-inflammatory properties. Surprisingly, only a very limited number of inflammatory genes have been identified so far as direct PPARα positive targets. Searching for novel direct PPARα reguled genes in liver, we previously identified the Interleukin-1 receptor antagonist (IL1-Ra) gene as an additional mechanism for PPARα to negatively regulate the APR in mouse liver [131]. It is noteworthy that upregulation of *IL-1ra* by PPARα was conserved in human (HepG2 hepatoma cells and human monocyte/macrophage THP-1 cell line) supporting the notion that similar regulation likely occurs in humans [131, 132]. Furthermore, using mice deficient in *Pparα* combined with pharmacological activation of PPARα by the synthetic PPARα agonists Wy, fenofibrate, or clofibrate, two different groups found that the liver expression of *Vanin-1* (a glycosylphosphatidylinositol-linked membrane-associated pantetheinase that promotes the production of inflammatory mediators by intestinal epithelial cells) was directly regulated by PPARα in mice [118, 133]. Treatment of primary human hepatocytes or HepaRG cells (a cell line derived from a liver tumor of a female patient) with two different PPARγ agonists (RSG and troglitazone) also modulate the mRNA levels of *Vanin-1* indicating that similar to PPARα, *Vanin-1* could be regulated by PPARγ [134]. *In vivo* upregulation of *Vanin-1* in the liver of mice by the di(2-ethylhexyl) phthalate (DEHP), a synthetic PPARγ ligand, has been also reported [135]. The question arises, why an anti-inflammatory transcription factor such as PPARα would increase the expression of *Vanin-1* that rather promotes the inflammation process. At present, it is hard to reconcile the Wy-mediated upregulation of *Vanin-1* mRNA level in liver with the anti-inflammatory role of PPARα. Follow-up investigations are eagerly awaited to partly

close this gap. Additionally, the group of S. Kersten also reported on the direct and critical role of human PPARα in the hepatic regulation of the mannose-binding lectin (*MBL*) gene, a soluble mediator of innate immunity [136]. Given that *MBL* is an important player in complement cascade activation as part of the first-line host defense, the positive regulation *MBL* fits within the role of PPARα as important regulator of inflammation and innate immunity.

3.2. Possible Role of PPARβ/δ in the Control of Inflammation Process in Liver. Similar to PPARα, the nuclear hormone receptor *Pparβ/δ* is expressed in the liver and displays anti-inflammatory activities. For instance, mice fed the PPARβ/δ agonist L-165041 are partially protected from chronic ethanol-mediated hepatic injury and inflammation [137]. Yet, others have reported that PPARβ/δ would promote hepatic stellate cell proliferation during acute and chronic liver inflammation, favouring the onset of hepatic tissue injury [138]. Therefore, the role of PPARβ/δ in liver is not fully understood and it deserves further investigations. In an attempt to define the functional role of PPARβ/δ in the liver in mice, the group of S. Kersten and collaborators has used Affymetrix microarrays to compare the RNA populations of normally fed wild-type mice *versus* mice deficient in the *Pparβ/δ* isoform [139]. *Pparβ/δ* deletion was associated with enrichment of gene sets involved in various innate immunity and inflammation-related processes including natural killer cell-mediated cytotoxicity, antigen processing and presentation, and Toll-like receptor pathway. Significant higher expression of genes reflecting enhanced nuclear factor-kappa B (NF-κB) activity was found in *Pparβ/δ* null mice [139]. Elevation of Kupffer cell (the resident macrophages in liver) marker gene expression was also observable. Enhanced expression of proinflammatory genes that are regulated by the NF-κB signaling was also noted in *Pparβ/δ* null mice following administration of the prototypical liver-specific toxicant carbon tetrachloride (CCl4) administration [140]. Of interest, normal-diet fed mice infected by adenovirus overexpressing *Pparβ/δ* in liver displayed reduced hepatic proinflammatory cytokines/chemokines (*IL-1β*, *Tnf-α*, *Ifn-γ* (*interferon-γ*), and *Mcp-1*) gene expression by the activated proinflammatory M1 macrophages [141]. In contrast, markers for the alternative anti-inflammatory M2 macrophage activation such as *Mrc1* (mannose receptor, C type 1, also known as Cluster of differentiation molecule-206, *Cd206*) and *Mgl1* (*galactose-type C-type lectin 1,* also referred to as *Cluster of differentiation molecule-301*, *Cd301*) were upregulated in the liver. Others have also reported that genetic deletion of *Pparβ/δ* in mice impaired the alternative anti-inflammatory M2 activation of hepatic macrophages (Küppfer cells) [75]. It was concluded that PPARβ/δ transcriptional signaling was required for the maintenance of alternative anti-inflammatory M2 activation of Kupffer cells in liver and for the decreased production of proinflammatory cytokines by the proinflammatory M1 macrophages. Curiously and in agreement with findings from Staels' group, these regulations were lost in mice fed a high-fat diet, casting doubt on the real impact of PPARβ/δ in decreasing obesity-induced hepatic inflammation in mice [141].

Inflammatory processes are generally considered to follow the transition of steatosis (simple fatty liver) to nonalcoholic steatohepatitis (NASH) and are therefore regarded as a characteristic finding of NASH. Intriguingly, it was recently found that the PPARβ/δ agonist GW0742 could attenuate hepatic steatosis by reducing liver triglyceride content and proinflammatory cytokines liver gene expression on a type-2 diabetic rat model [142]. However, this study did not aim at determining the impact of Kupffer cells on hepatic triglyceride storage and liver tissue inflammation. Consequently, unlike for PPARα, whether GW0742 involves some actions on Küppfer cells to prevent NASH is not documented.

Supporting further PPARβ/δ's anti-inflammatory activity, treatment of mice with GW0742 or KD3010, two PPARβ/δ agonists, significantly reduced copper-induced proinflammatory and APR cytokines in liver of mice [143, 144]. In contrast, blockade of the PPARβ/δ signaling pathway by the PPARβ/δ antagonist GSK0660 reverted copper-induced liver damages. Together, these findings support the notion that pharmacological activation of PPARβ/δ could become an important tool in the management of liver inflammation.

3.2.1. Humanized Mice for hPPARβ/δ: Role in Inflammation Control in Liver. In order to investigate whether the human version of PPARβ/δ also displays similar anti-inflammatory properties, a mouse model humanized for the PPARβ/δ isoform (PPARβ/δ KI) was established in a C57BL/6J-stabilized genetic background [145]. Subsequent experiments have shed light on the role of human PPARβ/δ on liver inflammation in the context of diet-induced obesity in mice. Similar to PPARα, pharmacological activation of PPARβ/δ (both of human and mouse origins) by the synthetic GW0742 compound led to the comparable induction of the liver *IL1-Ra* mRNA levels in WT and PPARβ/δ KI C57BL/6J mice. Moreover, it similarly decreased the gene expression of the proinflammatory cytokine *Tnf-α* and that of the APR proteins *fibrinogen-α* and *fibrinogen-β* [145]. These observations support the notion that the mouse *IL1-Ra* gene is likely transcriptionally regulated by the multiple PPAR isotypes and that PPARβ/δ plays anti-inflammatory functions in liver.

3.3. PPARγ: Role in the Control of Inflammation Process in Liver. A wealth of study has previously established a link between obesity and inflammation in the liver. Notably, excessive neutral lipids (triglycerides) accumulation in the liver can first lead to steatosis that may progress to steatohepatitis and ultimately to cirrhosis. In an effort to selectively study the functional role of liver PPARγ in obesity-induced hepatic inflammation, mice deleted of *Pparγ* in hepatocytes using the cell type-specific gene-knockout technology were recently established [146, 147]. While these mutant mice were protected against high-fat diet-induced hepatic steatosis, the number of liver inflammatory foci and the concentration of circulating inflammatory markers such as TNF-α and MCP-1 were similar as to control mice. These data argue against a predominant role of the liver form of PPARγ in controlling proinflammatory cytokine gene expression in the context of obesity-induced inflammation.

Many of the effects of TZDs are independent of PPARγ [148]. Supporting this notion, 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2), a natural PPARγ agonist, was found to reduce the recruitment of bone marrow-derived monocyte/macrophages (BMDM) in the liver of mice suffering from cholestasis-induced hepatic inflammation [149]. The suppression of BMDM migration did not result from the direct activation of PPARγ because the inhibitory effect of 15d-PGJ2 on BMDM migration was not affected by the pharmacological antagonization of PPARγ. Rather, 15d-PGJ2 reduced BMDM migration through ROS formation. Therefore, it should be acknowledged that some of the effects of TZDs on the inflammation process are independent of PPARγ.

4. PPARs and the APR across Species

The complex series of reactions initiated in response to infection and inflammation, trauma, burns, ischemic necrosis, and malignant tumors is called the APR. It is present in all animal species and constitutes a core component of the innate immune system. These alterations are mostly mediated by proinflammatory cytokines, and if prolonged, they contribute to a variety of ailments such as dyslipidemia, atherogenesis, diabetes, mitochondrial dysfunction, and muscle mass loss. Interconnections between APR and PPARs are illustrated by the reduction of PPAR expression in response to bacterial LPS exposure in numerous tissues such as liver, heart, kidney, and WAT [150–152]. This observation actually extends to most of type II Nuclear Hormone Receptors (NHRs) [153–155]. The prevalently accepted anti-inflammatory role for PPARs suggested that their agonists may be able to counterbalance APR-induced inflammation. In particular, the protective roles of PPARs were evaluated in response to endotoxemia induced by *Escherichia coli* LPS.

4.1. PPARα and the APR. Regarding PPARα, treating mice model of endotoxemia with fenofibrate or Wy surprisingly elevated TNF-α levels in plasma [156]. This elevation was not observed in *Pparα* knockout mice, further establishing a functional role of PPARα in mediating this effect of LPS [157]. Furthermore, some authors reported that C57BL/6 mice injected intraperitoneally with 100 μg of LPS (*Escherichia coli* LPS, serotype 055:B5) displayed a marked reduction in *Cyp4a10* (cytochrome P450, family 4, subfamily a, polypeptide 10) mRNA levels in the kidney [158]. Intriguingly, LPS-mediated reduction of *Cyp4a10* expression was still observable in the kidneys of *Pparα*-deficient mice. This finding suggests that mouse PPARα does not trigger the effects of LPS on *Cyp4a10* expression in the kidney [158]. Surprisingly, others found that injection of purified LPS (*Escherichia coli* LPS, serotype 0127:B8) in mice was inducing cytochrome *Cyp4a10 and Cyp4a14* (cytochrome P450, family 4, subfamily a, polypeptide 14) expression in kidney, in a PPARα-dependent manner [159]. Downregulation of *Cyp2a5*, *Cyp2c29,* and *Cyp3a11* by LPS was also comparatively reduced in *Pparα* null mice, suggesting that PPARα is somehow required for LPS-mediated gene regulation and could serve the purpose of LPS-mediated inflammation [159].

A profound role of PPARα in counteracting inflammation during APR is also illustrated by the fact that wild-type C57BL/6 mice injected intraperitoneally with proinflammatory cytokines such as TNF-α and IL-1β (two potent inducers of APR) display a significant reduction in hepatic mRNA levels of *Pparα* and its obligate partner *Rxrα* [155]. Similar results were also obtained using the human hepatoma Hep3B cell line; these data are in agreement with those reported by Stienstra and colleagues who recently disentangled the molecular mechanisms responsible for this reduction in *Pparα* mRNA levels [160]. Notably, further analysis revealed that the DNA binding of the heterodimer PPARα/RXRα to cognate peroxisome proliferator-responsive elements was significantly reduced [155]. This interesting piece of data explains, at least partially, why the expression of well-known *Pparα*-regulated transcripts is also concomitantly reduced [155]. Thus, by downregulating *Pparα* expression and PPARα activity in liver, LPS challenge may limit fatty acid β-oxidation. As a consequence, LPS would favor a metabolic shift in fatty acid metabolism by promoting their esterification and accumulation in the liver, ultimately leading to sepsis-induced hypertriglyceridemia.

In humans, it was recently shown that fenofibrate did not perform better than placebo in a cardiometabolic inflammation model where healthy adults were treated with LPS [161]. However, several observations also indicated that PPARα had beneficial effects against endotoxemia in humans. In spite of the relative low hepatic expression of *PPARα* in human, its pharmacological activation using fenofibrate or bezafibrate has been shown to decrease plasma levels of several APR proteins that are normally increased during inflammatory conditions [162–164]. Furthermore, PPARα activation by fenofibrate also prevents myocardial dysfunction during endotoxemia in rats [165].

Another line of evidence connecting PPARα to the control of inflammation gene expression came with the use of a liver-restricted *Pparα* expression mouse model that was treated with bacterial LPS [166]. Using mice deficient in *Pparα* in all tissues except the liver, a specific liver action of PPARα was highlighted because the hepatic expression and circulating levels of proinflammatory cytokines were comparatively lower in the mutant animals [166]. These findings support the notion that PPARα readily reduces the stimulation of the acute phase response (APR).

Hence, while PPARα is likely a factor playing a determinant role in the control of hepatic inflammation, its ability to control APR still deserves to be clearly unraveled.

4.2. PPARβ/δ and the APR. Information on the role of PPARβ/δ in the pathophysiology of sepsis-induced organ dysfunction and injury still remain fragmentary at the moment. In an effort to better investigate the role of PPARβ/δ in murine model of LPS-induced sepsis, WT and *Pparβ/δ*-deficient mice-previously subjected to LPS, were given the selective PPARβ/δ ligand (GW0742). Notably, GW0742 attenuated the degree of LPS-induced pulmonary inflammation, as well as cardiac and renal dysfunction [156, 167]. In further support of a role of PPARβ/δ in endotoxemia, LPS-treated WT and *Pparβ/δ*-deficient mice were also given

GSK0660 (a synthetic PPARβ/δ antagonist). Interestingly, most of the beneficial effects of GW0742 on the reduction of the septic shock was abolished [167]. PPARβ/δ may therefore represent an attractive method to counteract APR.

4.3. PPARγ and the APR. Similar to PPARα, results obtained on the role of PPARγ led to inconsistent observations, at least in rodents. The protective roles of PPARγ were particularly evaluated in response to endotoxemia induced by *Escherichia coli* LPS. It is worth recalling that RSG-induced activation of PPARγ in rats subjected to *Escherichia coli* LPS challenge alleviates LPS-mediated proinflammatory cytokine production in lungs inflammation models [168, 169]. Other studies performed with male Wistar rats also concluded that the beneficial protection of the 15d-PGJ2 against the multiple organ failure caused by endotoxin was mediated partially through PPARγ [170]. It was proposed that once activated, PPARγ would attenuate LPS-induced release of high mobility group box 1 in blood, a well-known late proinflammatory mediator of sepsis [171]. However, it should be stressed that others found that pharmacological activation of the PPARγ isotype was not useful for the treatment of acute inflammation in lean or *db/db* mice, raising doubts about the routine use of PPARγ agonists as anti-inflammatory agents in clinical applications [172]. The picture is even more complex because treating weaned pigs with RSG has been shown to be effective to protect them from LPS-induced intestinal damage, as the probable consequence of the inhibited production of intestinal proinflammatory mediators [173]. In conflict with these data, activation of PPARγ with RSG did not ameliorate and even worsened proinflammatory cytokine production in weaned pigs after *Escherichia coli* LPS challenge, casting doubts about the prevalently accepted anti-inflammatory role for PPARγ activation [174].

5. PPARs in Inflammatory Bowel Disease (IBD)

Characterized by an unrelenting destruction of the gut mucosa, the global prevalence rate of IBD is rising steadily. Ulcerative colitis and Crohn's disease are the two major forms of idiopathic IBD. These complex inflammatory diseases are usually developed in the second and third decades of life. Several players are involved in the onset of the disease among which not only different intestinal cells (intestinal epithelial cells, Paneth and goblet cells), second innate (macrophages, dendritic cells), and adaptive immune cells (lymphocytes), but also luminal bacteria. Collectively, scientific publications on IBD have established that the disease appears to involve maladaptive responses of the body to the intestinal flora, which also depends on individual genetic susceptibility.

Interestingly, all three PPAR isotypes are detected in the gastrointestinal tract. In rodents, *Pparα* is highly expressed in the proximal part of the small intestine (duodenum, jejunum) and colon but to a much lesser extent [175–177]. Expression of human *PPARα* expression also peaks in the small intestine and is less in the colon [118, 175]. Regarding mouse *Pparβ/δ*, its expression is highest in the epithelial cells of the colon and much less in small intestine [176].

5.1. PPARα in IBD. The role of PPARα during colonic inflammation has been well documented in several studies. In a model of IBD in mice, proinflammatory cytokines formation such as TNF-α and IL-1β was significantly higher in colon samples from *Pparα*-deficient mice compared with those of WT mice [178]. Furthermore and as it could be expected, administration of Wy or fenofibrate to mice suffering from colitis decreased mortality as well as mRNA levels of proinflammatory cytokines (*Ifnγ*, *Tnf-α*, *IL-6*, *IL-1β*, and *Interleukin-17*) in the distal colon leading to an overall delay in the onset of the disease [177]. Notably, the Wy lowering degree of colitis is PPARα dependent [179]. Together, these results indicate that PPARα and PPARα ligands may play an important role in controlling colonic inflammation through the activation of PPARα.

5.2. PPARβ/δ in IBD. Concerning *Pparβ/δ*, its deletion in mice resulted in exacerbated dextran sulfate sodium-induced colitis suggesting that this nuclear receptor could play a functional role against inflammatory colitis [180]. However, pharmacological activation of PPARβ/δ did not protect against dextran sulfate sodium-induced colitis pointing towards a ligand-independent anti-inflammatory effect of PPARβ/δ. More studies need to be done in order to clarify its role in the reduction of IBD.

5.3. PPARγ in IBD. With respect to *Pparγ*, its expression is restricted to the distal part of the intestine, especially caecum and colon [83, 176, 181–184]. Supporting a potential role of PPARs in IBD, colonic epithelial cells from ulcerative colitis patients express considerably lower levels of *PPARγ* [185]. In line with a role of PPARγ in the management of IBD, it is worth recalling that natural (such as conjugated linoleic acid) or synthetic PPARγ agonists provide effective treatments of colitis in rodent experimental models of the disease, but whether only PPARγ-dependent mechanisms are involved remains an open issue [186]. Illustrating the close ties between PPARγ and IBD, mice with targeted disruption of the *Pparγ* gene in intestinal epithelial cells displayed increased susceptibility to dextran sodium sulfate-induced colitis as well as higher mRNA levels of proinflammatory markers in the colon [187].

Notably, physical association of PPARγ with the transcription factor NF-κB (p50-Rel A heterodimer) has also recently emerged as a novel crucial mechanism by which PPARγ could also limit inflammation in epithelial cells of the gut exposed to *Bacteroides thetaiotaomicron*, a chief component of commensal gut microflora and a prevalent anaerobe of the human intestine [188]. The newly formed PPARγ/NF-κB p50-Rel A complex is rapidly exported from the nucleus resulting in the attenuation of NF-κB-mediated inflammation gene expression. Pharmacological modulation of this PPARγ-dependent anti-inflammatory mechanism might be promising for fighting IBD.

Given the critical role of PPARγ in controlling the activity of NF-κB, it is surprising that none of the 22 human PPARγ genetic variants identified and tested by Mwinyi et al. was associated with IBD susceptibility or disease course; in view of these results, the question still comes up, if PPARγ is

indeed a true modulating risk factor for IBD in humans [189].

Whereas *Pparγ* is abundantly expressed in intestinal epithelial cells, it is also highly expressed in macrophages and T cells. Genetic rodent models where *Pparγ* has been specifically invalidated in these cells have clearly indicated that PPARγ has protective effects on IBD [190–192]. Different mechanisms have been proposed so far however PPARγ anti-inflammatory property appears to be central to its benefits. In intestinal epithelial cells, different reports established that the ability of PPARγ to alter TLR2 and TLR4 signaling is an important factor. This is an interesting observation given the role of luminal flora in IBD because TLR2 and TLR4 are receptors sensing microbe components such as LPS of gram-negative bacteria. In addition, goblet and Paneth cells are also implicated in IBD. Whereas Paneth cells have a protective role against Crohn's disease, goblet cells protect against colitis. Whether PPARs have a role in the function of these cells in IBD remains unclear at the moment.

6. PPARs and Central Inflammation

Diseases of the central nervous system (CNS) present a challenge for the development of new therapeutic agents. *Pparγ*, *Pparα,* and *Parβ/δ* isoforms are expressed in the CNS at different levels, with *Parβ/δ* being the most abundant [86, 193–196].

6.1. PPARα in Central Inflammation. In the CNS, the expression of *Pparα* has been described in brain and spinal cord [193, 196, 197]. To evaluate the possible role for PPARα at the CNS level in mediating peripheral inflammation, the PPARα agonist GW7647 was intracerebroventricularly injected in mice subjected to carrageenan-induced paw edema [198]. Interestingly, specific activation of central PPARα controls inflammation in the spinal cord as well as in the periphery. It was concluded to the existence of a centrally mediated component for the anti-inflammatory effects of PPARα agonists.

6.2. PPARβ/δ in Central Inflammation. There are several lines of evidence supporting that PPARβ/δ serves a critical role in central inflammation. For instance, pharmacological activation of PPARβ/δ in rat aggregating brain cells cultures with the synthetic compound GW501516 decreased IFNγ-induced TNFα and INOS in a similar manner to what has been reported in isolated cultures [199]. Further supporting anti-inflammatory function for PPARβ/δ, oral administration of the selective PPARβ/δ agonist GW0742 in a mouse of experimental autoimmune encephalomyelitis, reduced astroglial and microglial inflammatory activation as well as IL-1β levels in brain [200]. Activation of PPARβ/δ by the gemfibrozil molecule (an FDA-approved lipid-lowering drug) was also recently shown to be beneficial for the correction of bacterial LPS-mediated inflammation in human microglia, suggesting that central PPARβ/δ could be a novel interesting molecular target [201]. Follow-up studies have thereafter investigated if central PPARβ/δ could indeed play a role in the control of CNS inflammation. Supporting this hypothesis, it was found that mice with specific deletion of *Parβ/δ* in hypothalamic neurons exhibited elevated markers of hypothalamic inflammation such as IL-6 and IL-1β [202]. Mutant mice fed a high-fat diet were also found to be resistant to further activation of hypothalamic inflammation. Central PPARβ/δ appeared therefore as a critical transcription factor in the management of CNS inflammation and lipid accumulation [202].

6.3. PPARγ in Central Inflammation. Over the past few years, PPARγ has been investigated for its action in ameliorating the development and progression of a number of CNS diseases. Because PPARγ agonists exhibit potent anti-inflammatory effects, the hypothesis was raised that they could display direct neuroprotective actions. Animal models of Alzheimer's disease or Parkinson's disease fed pioglitazone, a PPARγ agonist of the TZDs family, indeed displayed reduction in central inflammation and limited progression of the disease [203, 204]. The availability of FDA-approved agonists of this receptor should facilitate the rapid translation of these findings into clinical trials for a number of CNS diseases.

7. PPARs and Cardiac Inflammation

Heart failure patients show elevated plasma levels of proinflammatory cytokines suggesting that chronic inflammation could play an important role in cardiac diseases such as the development of cardiac hypertrophy. Cardiac hypertrophic and inflammatory pathways are intrically connected because they both activate NF-κB. PPARs isoforms are all present in cardiac muscle cells of mice and rats even though the *Pparγ* isoform is expressed at relatively low level [205].

7.1. PPARα in Cardiac Inflammation. Not only is *Pparα* highly expressed in liver, it also plays a very important role in cardiac inflammation. One illuminating set of experiments carried out with hypertensive rats, fed or not the PPARα activator fenofibrate, brings support to the notion that PPARα is also capable to decrease expression of inflammatory genes associated with NF-κB [206]. The anti-inflammatory effect of PPARα was further supported by other studies conducted in hearts of WT and *Pparα*-deficient mice. Notably, deletion of *Pparα* had a marked effect on the expression of genes related to inflammation and immunity [207]. In the context of cardiac hypertrophy (which is characterized by induction of inflammatory pathways), mRNA levels of genes, known to be under the dependence of the transcription factor NF-κB and therefore involved in inflammation and immunity, were decreased in neonatal rat cardiomyocytes treated with Wy or infected with adenoviruses overexpressing *Pparα* [208, 209]. Together, these data point to a pivotal role of PPARα in limiting the inflammatory response by transrepression of NF-κB in cardiomyocytes.

7.2. PPARβ/δ in Cardiac Inflammation. Interestingly, adenoviral-mediated overexpression of *Parβ/δ* in cultured neonatal rat cardiomyocytes substantially inhibited LPS-induced *Tnfα* expression [210]. In support of this result, pharmacological activation of the PPARβ/δ isotype with the GW501516 molecule prevented the proinflammatory profile induced by

lipids in heart and human cardiac AC16 cells [211]. Global and cardiomyocyte-restricted deletion of *Pparβ/δ* in mice has also definitively been instrumental in identifying PPARβ/δ as a critical nuclear receptor controlling proinflammatory cytokines production in response to LPS treatment in cardiomyocytes [210, 211]. It was concluded that absence of *Pparβ/δ* in cardiomyocytes further exaggerated LPS and lipid-induced proinflammatory cytokine production in heart.

7.3. PPARγ in Cardiac Inflammation. Besides metabolic effects, activation of PPARγ may also promote anti-inflammatory responses in heart. In agreement with this, mice infected by *Trypanosoma cruzi* (also known as *Schizotrypanum cruzi*) display intense inflammatory infection in cardiomyocytes. Supporting the assertion that PPARγ is a potent modulator of the inflammatory process, its selective activation by the 15d-PGJ2 inhibited the expression and activity of different inflammatory enzymes and proinflammatory cytokines in neonatal mouse *Trypanosoma-cruzi*-infected cardiomyocytes [212, 213].

8. PPARs, Inflammation, and Endothelium

8.1. PPARα and the Control of Endothelial Inflammation. Pharmacological activation of endogenous PPARα from porcine pulmonary-arterial endothelial cells or from human vascular endothelial cells with selective agonists reduced TNF-α –mediated induction of inflammatory transcription factors *NF-κB* and *AP-1* and expression of their target genes *Vcam-1* and *IL-6*. This piece of data suggests that irrespective of the species, PPARα is a molecular target that, once activated, reduces the proinflammatory phenotypes in endothelial cells [214, 215].

8.2. PPARβ/δ and the Control of Endothelial Inflammation. While the function of the PPARβ/δ isotype largely remained an enigma until the last century, probably because of the lack of connection with evident clinical manifestations, knowledge concerning its impact on inflammation in endothelial cell has tremendously increased over the last few years. Supporting this statement, treatment of primary vascular endothelial EAhy926 cells with the Merck ligand PPARβ/δ activator L-165041 suppressed TNFα-induced adhesion molecule (such as VCAM-1 and MCP-1) through significant reduction in the nuclear translocation of NF-κB [216, 217]. Furthermore, treating human umbilical vein endothelial cells (HUVEC) with the same molecule reduced the levels of C-reactive protein-mediated increase of Interleukin-6 (IL-6) and IL-8 [218]. Using the selective PPARβ/δ agonist GW501516, others also reported the critical role of PPARβ/δ in the suppression of IL-1β-induced *VCAM-1* and *E-selectin* expression in HUVECs [219]. At the molecular level, chromatin immunoprecipitation assays showed that ligand activation of PPARβ/δ in HUVECs switched the association of B cell lymphoma-6 (BCL-6), a transcription repressor and anti-inflammatory regulator, from PPARβ/δ to the vascular promoter of *VCAM-1* [219]. Such an unconventional ligand-dependent transcriptional pathway in which PPARβ/δ controls an inflammatory switch through its association and disassociation with the transcriptional repressor BCL-6 has been previously abundantly illustrated in macrophages foam cells [220].

Another way to limit the inflammatory response by the nuclear receptor PPARβ/δ in endothelial cells could partially involve its physical interaction with the Extracellular signal-Regulated Kinases (ERK). Notably, ERK was found to serve as an anti-inflammatory signal that suppresses expression of NF-κB-dependent inflammatory genes by inhibiting IKK activity in endothelial cells [221]. Furthermore, ERK1, 2, and 5 enhance PPARβ/δ transcriptional activity in C2C12 murine myoblasts leading to a reduction in cytokine-mediated NF-κB activation [67, 222]. Perhaps a similar molecular scenario could also take place in endothelial cells but it has not been documented yet. PPARβ/δ may therefore serve as a potent therapeutic target in inflammatory therapy.

8.3. PPARγ and the Control of Endothelial Inflammation. The nuclear receptor *Pparγ* is also expressed in vessel wall tissue including endothelial cells, which are, together with macrophages and smooth muscle cells, key players in atherosclerosis development [223, 224]. A wealth of studies has previously shown that PPARγ agonists can modulate the expression of many proinflammatory cytokines, chemokines, and adhesion molecules in endothelial cells [225, 226]. However, some PPARγ-independent effects have been reported for certain PPARγ agonists. Therefore, to circumvent the receptor-independent effect that individual PPARγ agonists may display, a constitutively ligand-independent active mutant form of PPARγ1 was delivered into human umbilical cord veins endothelial cells (HUVECs) [215]. Importantly, AP-1 and NF-κB pathways were inhibited by the constitutively active form of PPARγ1 in endothelial cells, leading to the prevention of endothelial activation, leucocyte recruitment, and synthesis of proinflammatory adhesion molecules. Definitive evidence that PPARγ plays a functional role in regulating the inflammatory process *in situ* in endothelial cell comes with the establishment of LDL receptor-deficient mice deleted from *Pparγ* especially in endothelial cells [227]. Lack of *Pparγ* in primary endothelial cells leads to increased inflammation (as shown by the robust increased expression of *Tnf-α*, *Mcp-1*, and *IL-1β*) in vessel wall of mutant mice treated with LPS or challenged with high-cholesterol diet. In agreement with these findings, others have also recently reported that the genetic deletion of *Pparγ* in endothelium in mice was upregulating LPS signaling as the consequence of induction of NF-κB activity [228].

Together, these data reinforce the notion that the pharmacological activation of PPARγ is likely beneficial by limiting inflammation at the level of the endothelial cell as well.

In summary, all three PPARs isotypes display an anti-inflammatory role by inhibiting the production of inflammatory cytokines in a large set of syndromes and diseases (Figure 2).

9. Dairy Cattle and Mastitis: PPAR Modulators as Future Promising Treatment?

In livestock species in general, data describing the use of synthetic PPAR agonists are very limited. Considering the

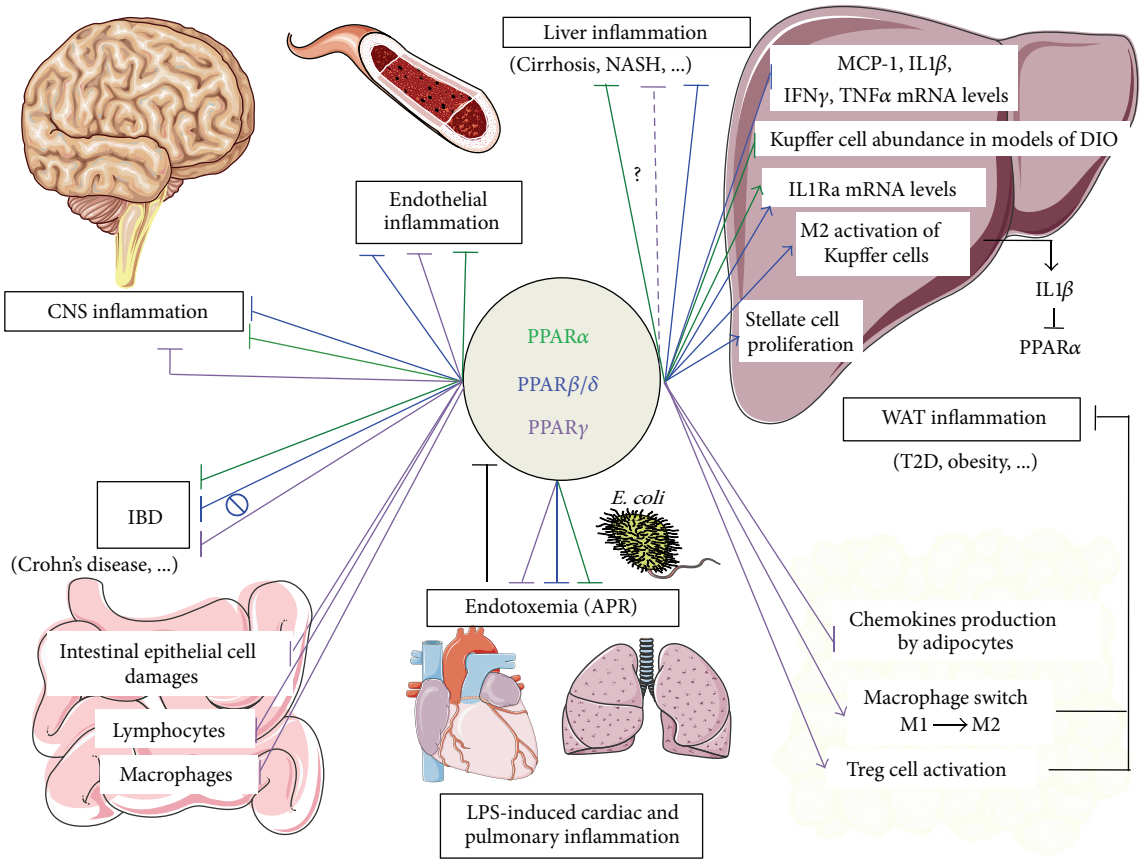

Figure 2: Representative illustration of PPAR main targets in inflammatory diseases. PPARα mostly displays anti-inflammatory properties in the context of liver inflammation. Its reported liver targets are hepatocytes and Küppfer cells [131]. IL-1β produced by Küppfer cells potently suppresses *Ppar*α expression and activity *via* NF-κB–dependent inhibition of PPARα promoter activity [160]. Besides downregulating gene expression of proinflammatory mediators such as *Mcp-1*, *Tnf-*α, *Ifn-*γ, *IL-1*β, and PPARα also directly controls expression of IL1-ra in liver [131, 163]. Küppfer cell activation is also dependent on PPARβ/δ, which also targets stellate cells and therefore prevents liver fibrosis [75, 138]. In addition, PPARβ/δ has well-established anti-inflammatory properties in diseases associated with CNS inflammation. In CNS, PPARβ/δ has also proven anti-inflammatory properties in neurons, glial cells, and astrocytes [200–202]. PPARγ anti-inflammatory properties are mainly illustrated in T2D and IBD. PPARγ serves as the molecular target of the insulin-sensitizing TZD drugs and plays a key role in T2D, adipogenesis and obesity. In WAT, mature adipocytes, Treg cells and macrophages have been identified as key cellular targets for PPARγ [66, 75, 116, 117]. Macrophage-specific deletion of PPARγ leads to specific reduction in alternatively activated macrophages (M2 state) in WAT leading to local inflammation [110]. Moreover, Treg-cell-specific deletion of *Ppar*γ was shown to reduce the abundance of Treg cells in WAT resulting in the increase of WAT infiltration by proinflammatory macrophages (M1) and monocytes [116, 117]. In IBD, PPARγ acts in intestinal epithelial cells, macrophages and lymphocytes [190–192]. Note that endotoxemia represses the mRNA expression level of *Ppars* (see black bar) [150–155]. Furthermore, multiple lines of evidence indicated that PPARγ is very important in endothelial cells, because it inhibits the *in situ* production of proinflammatory molecules such as vascular adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and MCP-1 [215, 223–228]. Similar conclusions were also drawn for the PPARα and PPARβ/δ isotypes [214, 216–220]. Finally, PPARs display protective effets against endotoxemia [166, 167, 169, 236]. NASH: nonalcoholic steatoHepatitis; T2D: type-2 diabetes; CNS: central nervous system; Treg cells: Foxp3$^+$ CD4$^+$ regulatory T cells; DIO: diet-induced-obesity; APR: acute phase response; green lines: action of PPARα; blue lines: action of PPARβ/δ; purple lines: action of PPARγ; ?: Some PPARγ-independent effects of PPARγ activators have been proposed [146, 147]; $\emptyset$: pharmacological activation of PPARβ/δ did not protect against dextran sulfate sodium-induced colitis pointing towards a ligand-independent anti-inflammatory effect of PPARβ/δ [180].

high-amino acid identities ranging from 95 to 98% for PPARs proteins in all species, one can think that bovine and porcine PPARs could also be targeted with existing synthetic PPAR agonists [229]. On the other hand, because only a minor overlap between the Wy-regulated genes from mouse and human primary hepatocytes was found and since PPRE are not fundamentally conserved along species, we have to admit that activation of PPARs does not necessarily activate the same array of genes in one species *versus* another [60, 61].

One of the most common diseases in dairy cattle in the world is mastitis, which can be defined as an inflammation of the mammary gland tissue, resulting from the introduction and multiplication of pathogenic microorganisms. Mastitis

is one of the most important health problems and is very costly for the dairy industry [230]. While treatment is possible with long-acting antibiotics, farmers have to wait until drug residues have left the cow's system before milk from such cows becomes again marketable. Several main causative bacteria that include *Escherichia coli* are responsible for the induction of inflammation of the udder tissue in dairy cattle. We have illustrated above that PPARγ activation, which typically results in the downregulation of inflammatory response, is suggested to be beneficial in inflammatory diseases not only in humans, but also in rats and pigs. We now question and discuss whether PPARγ activation could mitigate immunological stress of livestock, such as mastitis. As its function is to recognize pathogens that have not been encountered before, the innate immune system is the first line of defense against intramammary infection by bacteria [231]. It is generally accepted that emigration from the blood vessel of neutrophils (also known as polymorphonuclear neutrophil leucocytes) into the infected tissue, where they will deliver antimicrobial agents, is a hallmark of bacterial infection. Given that during the APR, reduction of the neutrophil flux into the mammary gland is believed to promote the incidence of severe *Escherichia coli*-induced mastitis, it could perhaps be envisioned to counterbalance this effect by treatment with existing PPARγ agonists [232]. Using two different mouse models of sepsis (cecal ligation and puncture as well as intraperitoneal injection of purified bacterial gram-negative LPS) it was rather shown that PPARγ inactivation with the GW9662 compound significantly (i) reversed the suppression of chemotaxis observed following LPS administration and (ii) increased recruitment of PMNs in the peritoneal cavity of mice subjected to cecal ligation and puncture [233]. Therefore, PPARγ displays two facets: once activated, it would dampen the massive production of proinflammatory cytokines in response to bacterial gram-negative LPS injection, by transrepressional mechanisms; at the same time, it would accentuate the suppression of chemotaxis further interfering with the recruitment of PMNs to the site of infection, two early key events for fighting against bacterial infection. Given that PPARγ is also a pivotal NHR involved in adipocyte differentiation and fat mass, modulating its activity could also affect fat depots important for meat quality. Therefore, pharmacological interventions in dairy cattle based on the use of PPARγ (anta) gonists may not offer an overall favorable therapeutic benefit, unless PPARγ ability to control inflammation and interfere with PMN recruitment is disconnected in these pharmacological reagents. The recent generation of pigs, which display physiological and anatomical similarities with humans, in which one allele of the *Ppar*γ gene has been disrupted could be partly informative concerning the real involvement of PPARγ in the etiology of mastitis in livestocks [234].

Applications of PPARα agonists could be of interest to decrease inflammation in the udder but since PPARα signaling is decreased in bovine mammary tissue challenged with bacteria, and because fatty acid oxidation is under the dependence of PPARα in the liver, the routine use of such molecules remains largely speculative [235].

10. Concluding Remarks

PPARs are lipid sensing transcription factors that were originally targeted in order to normalize metabolic issues. However, it also turned out that these NHRs were as well potently involved in switching off inflammation. Thanks to their respective and well conserved expression in numerous tissues amongst species, the prevalence of inflammatory diseases could be reduced by the use of a combination of different PPARs agonists. Quite surprisingly though, only a limited number of anti-inflammatory genes have been identified so far as direct and classical PPAR targets with a functional PPRE in genomic DNA, which could appear a bit puzzling at first glance. However, mechanisms involved in the anti-inflammatory properties of PPARs are broader than what might have been thought originally. Such properties are the reflect of a much elaborated transrepressional activity. The mechanisms behind this activity are currently being studied and remain more or less elusive at the moment. Therefore, it will be a major challenge for the future, in terms of therapeutic applications, to fully understand how these NHRs work and control inflammation. Compared to other anti-inflammatory strategies such as that involving glucocorticoids and its receptors, PPARs agonists may be responsible for limited drawbacks, yet their use also revealed controversial results in terms of efficacy and side effects.

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Review Article

Structural Features and Transcriptional Activity of Chicken PPARs (α, β, and γ)

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While an understanding of lipid metabolism in chickens is critical for a further improvement of food production, there are few studies concerning differences in lipid metabolism mechanisms between chickens and other species at a molecular level. Chickens have three PPAR gene subtypes (α, β, and γ) that function differently from those present in humans and mice. The chicken PPAR-gamma (cPPARγ) gene is shorter than that in humans and lacks a γ2 isoform. Moreover, in serum-free media, cPPARγ shows high transcriptional activity without exogenous ligands. Luciferase reporter assays were used to examine the effect of sera on cPPAR transcriptional activities and showed that adult bovine serum and chicken serum highly activate cPPARα and β functions. Moreover, we found that bezafibrate induces the transactivation function of cPPARβ, but not human PPARδ (human PPARβ ortholog). This ligand selectivity relies on one amino acid residue (chicken: Val419, human: Met444). These results show the possibilities for unique functions of cPPARs on chicken-specific lipid glucose metabolism. As such, a better understanding of the molecular mechanisms of lipid metabolism in chickens could result in higher productivity for the poultry industry.

1. Genomic Organization of Chicken PPARS (cPPARs)

The mechanisms of lipid and glucose metabolism for energy storage and homeostasis differ between species in ways that are not fully understood. To improve food production, comprehending the molecular basis for lipid and glucose metabolism in domestic animals is a significant issue. In avian species, lipids, especially triglycerides, are thought to be stored in adipocytes, hepatocytes, and growing oocytes. Excessive accumulation of lipids in the adipose tissue of broilers is a major concern for producers, because excessive fat deposits result in lower meat yields.

In 2000, we reported the cloning of three chicken PPAR subtype genes (α, β, and γ) from cDNA libraries prepared from 2.5-day-old chicken embryos and adult adipose tissue [1]. For cPPARγ, amino acid residues predicted for phosphorylation by MAP kinase [2] (Ser82 in cPPARγ) and Cdk5 [3] (Ser243 in cPPARγ) were conserved between mammals and chicken PPARγ. Moreover, predicted SUMOylation target residues (Lys77 and Lys365 in cPPARγ) were also conserved in cPPARγ [4]. This shows the possibility that the transcriptional activity of cPPARγ regulates growth factors and that cPPARγ activation has an anti-inflammatory effect. However, we were unable to isolate the PPARγ2 splicing variant identified in mammals [5, 6]. This variant is likely absent in chickens because Sato et al. also could not isolate chicken PPARγ2 by 5′-RACE [7] and because no sequence similar to a PPARγ2 isoform has been found in the chicken genome database (Figure 1). The PPARγ2 isoform has not been reported in *Xenopus* and zebrafish either. Furthermore, while the structural organization of PPARα and β/δ genes is largely conserved from humans to chickens, the PPARγ isoform is not. The cPPARγ gene (Ensembl ID: ENSGALG00000004974) has 6 exons and extends across ~50 kilobases (kb) of genomic DNA (Figure 1, upper panel). In contrast, the human PPARγ gene (Ensembl ID: ENSG00000132170) consists of 8 and 7 exons for the γ1

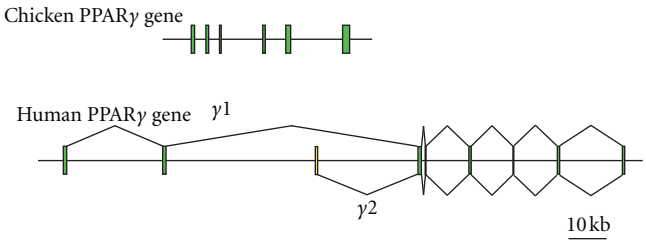

Figure 1: Structural organization of chicken (upper panel) and human (lower panel) PPARγ genes. Exons are shown as green boxes with the γ2-specific exon colored in yellow.

and γ2 subtypes, respectively, and extends across more than 160 kb of genomic DNA (Figure 1, lower panel). Other species such as squirrel (Ensembl ID: ENSSTOG00000012778), hedgehog (ENSEEUG0000006334), Chinese softshell turtle (ENSPSIG00000011100), and Anole lizard (ENSACAG00000013360) have the chicken-type organization of PPARγ genes.

In mammals, several polymorphisms of PPAR genes have been associated with metabolic disorders. In chicken, single nucleotide polymorphism (SNP) analysis has been conducted to find the association with lean and fat broilers [8]. In this study, PPARγ c-75G>A is reported to be associated with abdominal fat weight and also correlates with SNPs of other lipid metabolism-related genes (Fsbp4, C/ebpα, etc.).

Promoter analysis of the cPPARγ gene revealed that transcription factor binding sites (such as C/EBPα, Sp1 and AP1) exist as is the case with mammalian PPARγ1 and PPARγ2 promoters. Interestingly, structure of cPPARγ promoter is more similar to the mammalian PPARγ2 promoters compared to PPARγ1 promoters [9]. These results suggest that C/EBPα-PPARγ pathway is conserved across the species and that PPARγ2 gene first appeared in the course of evolution when additional PPARγ isoforms have been acquired.

2. Expression Patterns of cPPAR mRNAs

The mRNA expression patterns of cPPARs are similar to rodents and primates. In adult chicken, cPPARα is highly expressed in the heart, liver, and kidney [10]. As is the case for PPARβ or δ in other species, cPPARβ is expressed ubiquitously. Meanwhile, high expression of cPPARγ is observed in fat tissue as well as the kidney, heart, brain, liver, and intestine [7, 11]. Recent studies using renal-specific PPARγ knockout mice have shown that the gene regulates body weight by controlling water channel activities [12]. Thus, cPPARγ may also regulate body weight in chickens.

Hojo et al. examined cPPAR mRNA expression patterns in digestive organs during embryonic development in chickens [13]. In embryonic day 6 (E6), E9, and E12 embryos, PPARα is expressed in the epithelium, smooth muscle, and small intestine. PPARβ is expressed in the epithelium and moderately in mesenchymal tissue on E6, E9, and E12. PPARγ is weakly expressed in the epithelium on E9 and E12 and expressed in the medial and basal parts of the small intestine villi, the villi of PH3 and 14, and the crypt epithelium of the large intestine.

3. Regulators of cPPAR mRNAs

PPAR mRNA levels are known to be regulated in aging, metabolic disorder, and cancer [14]. In general, mRNA levels of PPARs or their target genes are upregulated in obesity because of increased fatty acids. Several regulators of PPAR mRNAs have recently been identified in chicken.

In adipocyte, accumulation of nonesterified fatty acids (NEFA) induces cPPARγ mRNA, and a cPPARγ downstream gene (adipocyte fatty acid binding protein (A-FABP)) controls the concentration of lipid and NEFA [15]. In fatty broiler chickens, cPPARγ mRNA expression is induced in liver but not in adipose [16]. Because PPARγ mRNA is induced in adipose tissue of obese mammals [17, 18], the result indicates a divergence of PPAR signal transduction mechanisms between avians and mammals.

Regulation of cPPARα mRNA is also reported. Comparison of gene expression profiles in the intramuscular fat of two broiler lines showed that PPARα and MAPK pathway are enhanced in both Beijing-you (BJY), which is a slow-growing Chinese breed possessing high meat quality, and Arbor Acres (AA), a commercial fast-growing broiler line [19]. Upon feed restriction, mRNA expression levels of cPPARα and one of its target gene, peptide transporter 1 (PepT1), are induced in the jejunum [20].

In myoblasts, isoproterenol (a β-adrenergic receptor agonist) and fatty acid enhance the phosphorylation of cPPARα. The mRNA of UCP3 is induced through cPPARα and AMPK-PKA pathway [21]. In spleen, cPPARα and γ mRNAs are induced by lutein, a plant carotenoid, and reduced by lipopolysaccharide (LPS) [22]. These results indicate that some regulators of cPPARs mRNA expression are conserved between chicken and mammals.

4. Comparison of the Amino Acid Sequences between Chicken and Mammalian PPARs

The amino acid sequence in the DNA binding domain (DBD) of cPPARs shows high similarity with mammals (cPPARα versus hPPARα: 95%; cPPARβ versus hPPARδ: 98%; cPPARγ versus hPPARγ: 100%). The similarity of the ligand binding domain (LBD) is also high for PPARα and PPARγ (cPPARα versus hPPARα: 96%; cPPARγ versus hPPARγ: 96%). However, the LBD of cPPARβ shows lower similarity between chicken and humans (90%), mice (86%), and *Xenopus* (78%). Because this diversity implies different ligand responses, we conducted luciferase reporter assays to examine the ligand-inducible transcriptional activity of cPPARs.

5. Transcriptional Activities of cPPARs

We first generated cDNA expression vectors of cPPAR LBDs fused to GAL4 DBD. The ligand-inducible transcriptional

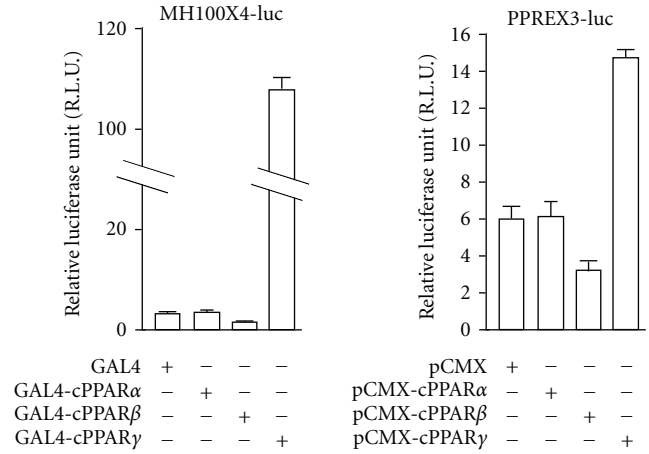

Figure 2: Transactivation function of cPPARs in serum-free media. After transfection with each plasmid mixture, cells were cultured for 36 hrs, lysed, and luciferase reporter assays were performed. MH100X4-luc is a GAL4 responsive luciferase reporter, and PPREX3-luc is a PPAR responsive luciferase reporter. Data are derived from triplicate points from two independent experiments and represented as the mean ± SE; $n = 6$. The results presented in this figure have been published [1].

activities of them were examined by luciferase reporter assays. We used serum-free conditions ((DMEM/ITB; DMEM with 5 μg/mL insulin, 5 μg/mL transferrin, and 0.01% fatty acid-free bovine serum albumin (BSA)) to reduce the effect of lipids contained in serum.

Rhesus monkey kidney CV-1 cells cultured in 24-well cluster tissue culture plates were transfected with expression vectors carrying GAL4-cPPAR LBDs (25 ng/well), pCMX-βGAL (100 ng/well, as a control for transfection efficiency), and the MH100X4-tk-luc reporter plasmid (150 ng/well) using calcium phosphate precipitation method [23].

Interestingly, GAL4-cPPARγ LBD showed high transcriptional activity without exogenous ligands (Figure 2, left panel). This activation was also observed for full-length cPPARγ (Figure 2, right panel), indicating that this activation is cPPARγ dependent. Moreover, cPPARβ suppressed basal transcriptional activity without ligands in DMEM/ITB (Figure 2, both panels). These findings show the possibility of a distinct ligand-independent transactivation function of cPPARs.

We next investigated the effect of serum on the transcriptional activities of cPPARs. It was found that adult bovine serum induces cPPARα transcriptional activities to a greater extent than fetal bovine serum and calf serum (Figure 3(a)). Interestingly, chicken serum (ChS) highly potentiated the transcriptional activities of cPPARα and β as compared to bovine serum. ChS also activated the transactivation function of hPPARs (Figure 3(b)). Furthermore, using lipid extracts from chicken serum by a previously established method [24], we showed that the lipid extracts retain the enhancement effects of chicken serum for the transcriptional activities of cPPARα and β (Figure 3(c)).

These results show that serum from adult animals (chicken and bovine) contains more endogenous PPARβ ligands than that from fetal or young animals (bovine). Although the identity of PPARβ ligand(s) in adult serum has not yet been revealed, fatty acids and their derivatives are strong candidates.

6. Bezafibrate Is a Ligand for Avian and Xenopus PPARβ

We next examined the effect of known PPAR activators (including fibrates and thiazolidinediones) on the transactivation function of cPPARs. Bezafibrate, a known ligand for *Xenopus* PPARβ (xPPARβ) [25], enhanced the ligand-inducible transactivation function of xPPARβ in our hands (data not shown) and cPPARβ, but not hPPARδ [1].

This result shows that bezafibrate selectively activates cPPARβ despite the relatively high structural similarity between cPPARβ and hPPARδ LBDs. We looked for the amino acids that are conserved between human and mouse PPARδ, but not chicken PPARβ and found that 15 amino acid residues were altered between chicken and mammals (Figure 4(a)). To identify the amino acid residue(s) critical for bezafibrate-dependent activation, we generated cDNA expression vectors carrying point mutations of relevant hPPARδ and cPPARβ residues. Luciferase reporter assays in CV-1 cells were performed, and it was found out that a single amino acid alteration in cPPARβ(Val419Met0029 abrogates bezafibrate-dependent activation. When the corresponding Met residue in hPPARδ was changed to Val, the mutant, hPPARδ(Met417Val) enhanced bezafibrate-dependent activation. Human PPARα has a Val residue at this position and when a point mutation was introduced, hPPARα(Val444Met) abrogated bezafibrate responsiveness (Figure 4(b)). However, another PPARβ/δ activator, carbaprostacyclin, activates both hPPARδ(Met417Val) and cPPARβ(Val419Met). These results suggest that the cPPARβ gene evolutionarily changed into the PPARδ type, which is restricted to fibrate responsiveness. Although distinct endogenous ligands for chicken PPARβ and human/mice PPARδ have not been identified, this amino

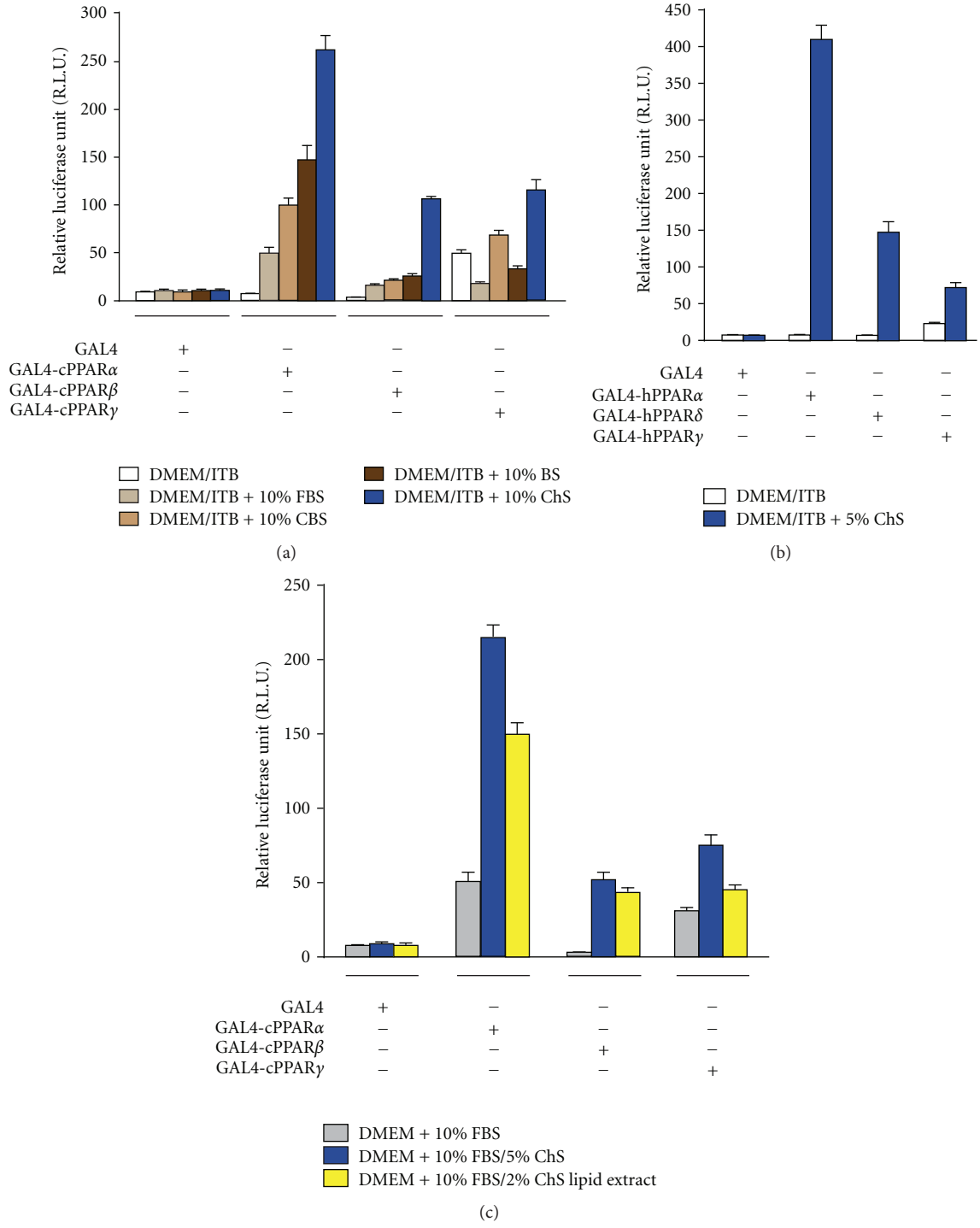

Figure 3: Effect of serum on the transactivation function of cPPARs. (a), (b). Effects of serum on the transactivation function of PPARs. Serum-induced transactivation function of cPPARs (a) or hPPARs. (b) After transfection with each plasmid mixture, cells were cultured with indicated serum conditions for 36 hrs, then lysed, and luciferase reporter assays were performed as described previously [1]. FBS: fetal bovine serum, CBS: calf bovine serum, BS: adult bovine serum, and ChS: chicken serum. (c) The effect of lipid extract from chicken serum (ChS) on the transactivation function of cPPARs. After being transfected with each plasmid mixture, cells were cultured for 36 hrs in DMEM/10%FBS with or without 5% ChS or ChS lipid extract equivalent of 2% ChS. Then cells were lysed and luciferase assays were performed. Data are derived from triplicate points from two independent experiments and represented as the mean ± SE; $n = 6$.

Figure 4: (a) Alignment of amino acid residues of chicken, mouse, and human PPARβ/δ LBDs. LBD is located at the C-terminus of chicken (amino acid residues 243 to 441) and mammalian (245 to 443) PPARβ/δ proteins. Amino acid residues conserved between mouse and human but not in chicken are highlighted in orange (mammals) and green (chicken). The amino acid residues critical for bezafibrate-dependent response of PPARβ/δ are indicated by a purple box. Accession numbers are as follows: cPPARβ, AAF80480; mPPARδ, NP_035275; hPPARδ, NP_001165289. (b) Bezafibrate (Beza) and carbaprostacyclin (cPGI) responsiveness of point mutated chicken PPARβ(Val419Met), human PPARα(Val444Met), and PPARδ(Met417Val) as previously reported [1].

acid change may reflect the difference between chicken and mammals in the regulation of energy storage [26].

Moreover, identification of amino acid residues involved in ligand interactions will help to generate PPARδ-specific ligands and facilitate the development of drugs to treat metabolic syndromes. Thus, investigation of species-selective PPAR ligand activity is significant for understanding species-specific lipid metabolism and development of new drugs.

7. Effects of Fibrate on Hens

There are few *in vivo* analyses of chickens fed with PPAR ligands. A previous report showed that hens treated with clofibrate (cPPARα ligand) had lower food intake and lost body weight during the 5-week feeding period, which in turn reduced egg production due to the absence of large follicle(s) and the presence of few small yellow and white follicles in the ovary [27]. Moreover, triglyceride levels in the livers of clofibrate-treated hens were reduced and mRNA levels of known PPARα target genes such as Acyl-CoA oxidase (ACO), hepatic lipase, and lipoprotein lipase (LPL) were induced. In contrast, mRNAs of insulin-induced genes (Insig-1, SREBP-2, FAS, HMG-CoA reductase, and LDL receptor) were downregulated by clofibrate treatment. These results show that, as with mice and humans, cPPARα regulates lipid metabolism in the liver and controls appetite [28].

8. Conclusions

In this paper we discussed the properties of chicken PPARs based on their genomic structural organization and ligand-inducible and -independent transactivation functions. Evolutionary changes in the PPARγ genomic structure may be one critical factor for species-dependent lipid homeostasis. Because there are few reports concerning the promoter activity of PPARγ gene in chickens and other nonmammals, more detailed analyses will be required. Interestingly, the ligand-inducible transcriptional activities of cPPARs do differ from mammalian PPARs. Because a single amino acid change leads to a selective ligand response, the spectrum of endogenous ligands of PPARs in avian species may be different from that of mammals. As such, further analysis will be required to understand the comparative endocrinology and this information will be useful for industrial applications in the poultry and other food industries.

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Research Article

Effect of 2,4-Thiazolidinedione on Limousin Cattle Growth and on Muscle and Adipose Tissue Metabolism

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The main adipogenic transcription factor PPARγ possesses high affinity to 2,4-TZD, a member of the Thiazolidinedione family of insulin-sensitizing compounds used as adipogenic agents. We evaluated 2,4-TZD's effect on bovine growth and PPAR tissue expression. Seventeen Limousin bulls (18 month-old; 350 kg body weight (BW)) were assigned into 2 treatments: control and 2,4-TZD (8 mg/70 kg BW) and were fed until bulls reached 500 kg BW. They were weighed and their blood was sampled. DNA, RNA, and protein were determined in liver; skeletal muscle; subcutaneous (SC), omental, perirenal adipose tissues (AT) to determine protein synthesis rate and cellular size. Expression of PPAR mRNA was measured in liver and muscle (PPARα, -δ, and -γ) and SC adipose tissue (γ) by real-time PCR. No significant differences were found ($P > 0.1$) in weight gain, days on feed, and carcass quality. Muscle synthesis was greater in controls ($P < 0.05$); cell size was larger with 2,4-TZD ($P < 0.05$). PPARα, -δ, and -γ expressions with 2,4-TZD in liver were lower ($P < 0.01$) than in muscle. No differences were found for PPARγ mRNA expression in SCAT. The results suggest the potential use of 2,4-TZD in beef cattle diets, because it improves AT differentiation, liver, and muscle fatty acid oxidation that, therefore, might improve energy efficiency.

1. Introduction

Peroxisome proliferator-activated receptors (PPAR) are ligand-activated transcription factors that belong to the nuclear hormone receptor superfamily. Three isotypes have been identified in lower vertebrates and mammals: PPARα or NR1C1; PPARβ/δ or NR1C2, also called NUC-1 or FAAR; PPARγ or NR1C3. These receptors exhibit different tissue distribution and functions and, to some extent, different ligand specificities. Mechanistically, they form heterodimers with the retinoid X receptor (RXR) and activate transcription by binding to a specific DNA element, termed the peroxisome proliferator response element (PPRE), in the regulatory region of a variety of genes encoding proteins that are involved in lipid metabolism [1] and energy balance [2–4]. The Thiazolidinediones (TZDs) are PPARγ agonists that possess clinical antidiabetic efficacy, mainly through their actions in adipose tissues [3]. The influence of TZD on adipose differentiation has been demonstrated through modifications on adipose tissue deposition and increases of intramuscular (IM) fat in meat animals [5, 6]; on the other hand, Michalik et al. [3] mentioned that some TZD may act on different PPAR, especially PPARα.

Marbling or IM fat has been positively correlated with meat quality [7] because of the improvement in beef tenderness and palatability [8]. The development of IM fat primarily depends on the animal's breed, gender, age, and nutrition [9, 10]. Therefore, adipose tissue and its metabolic

regulation have been studied in order to improve meat characteristics during fattening in feedlots.

The observations suggest that the quality and value of beef cattle carcasses could be increased through the utilization of these compounds as promoters of marbling and that, additionally, they might improve their energy efficiency.

2. Materials and Methods

2.1. Animals, Treatment, and Diet. Procedures involving animals were approved by the Institutional Committee for Experimental Animal Care of the Universidad Nacional Autónoma de México (UNAM) [11]. Eight bulls served as Controls (Cs) and nine as the treated group (T), which were fed 2,4-TZD (8 mg/70 kg BW); the dose was adjusted to the animal's total weight; this was according to previous pharmacological and clinical studies in humans treated by oral administration (rosiglitazone 8 mg/day), which demonstrated important changes in the metabolism of glucose during insulin resistance treatments, due to its impact on muscular and adipose tissues [12, 13]. In order to test 2,4-TZD by a practical method, we decided to use oral administration as given to humans and mice; to date, this is the first study to employ it within the food in ruminants; other studies have used intravenous administration [14]. Animals were assigned to treatments in a completely randomized design and housed pairwise on dirt-floor pens. Prior to the initiation of the experiment, they were immunized against the bovine respiratory disease complex (BRDC), the bovine viral diarrhea virus (BVDV), and leptospirosis (Cattlemaster 4; Pfizer Animal Health, Exton, PA, USA), injected with vitamins A, D, and E (Vigantol ADE; Merck KgaA, Darmstadt, Germany), implanted with 140 mg trenbolone acetate and 20 mg 17 beta estradiol (Revalor; Intervet/Schering-Plough Animal Health), and protected against ectoparasites (Tiguvon Spot-on, Bayer, Germany). Animals with >90 days of participation in the experiment were reimplanted and reinjected with the vitamins. The diet consisted in forage (alfalfa hay 80% Dry matter (DM)) and a supplement (89% DM, with 15% CP; 2.8 Mcal ED/kg DM), at a 2 : 1 relationship, respectively. The amount offered was based on 3% of BW (ENm: 1.5 Mcal/kg; ENg: 0.9 Mcal/kg, 14.9% protein). Feed was provided twice a day. Treated animals (T) received 2,4-Thiazolidinedione 90% (2,4-TZD) (Sigma Aldrich, St. Louis, MO, USA) in a dose of 8 mg/70 kg BW per animal [12] mixed within the supplement. Animals were individually weighed and their blood was sampled from the coccygeal vein (10 mL) at the 28-day intervals throughout the 196-day study; blood collection and weight were taken before receiving their first meal (on fasting) on the sampling day. Samples were collected in Vacutainer tubes (Hunan, China) without anticoagulants. They were centrifuged (at 13,000 rpm) and the serum was recovered and frozen at −70°C until analysis.

2.2. Slaughter, Sampling, and Analysis. As animals reached 500 kg of weight, C and T animals were sent to a nearby federal inspection-type abattoir (TIF) operated under Mexican federal inspection laws [15, 16]. Samples of liver, muscle, and adipose tissue from omental, perirenal, and SC depots were dissected, collected in CryoTubes (Nunc Cryo Tube vials; Roskilde, Denmark), frozen in liquid nitrogen, and stored at −70°C for subsequent analysis. Carcasses were chilled at −2°C for 32–36 h and quality and yield grades were evaluated, with assessment including the following: external fat thickness; *longissimus dorsi* muscle (LM) area at 7th and 8th rib; meat and fat color; marbling, according to North American Meat Processors Association (NAMPA) [17] standards.

2.3. Total RNA and DNA Isolation. Total RNAs from all tissue samples (100 mg each) were purified using 1 mL of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA was eluted in 40 μL of diethylpyrocarbonate- (DEPC-) treated water and quantified by spectrophotometer; samples were then stored at −70°C until further molecular biology experiments. DNA extraction (1 mL/50 mg of tissue) of DNAzol reagent (Invitrogen) was made according to the manufacturer's protocol and eluted in 50 μL of 8 mM sodium hydroxide (NaOH). Spectrophotometry reading was performed (Nano Drop 1000; Thermo Fisher Scientific, Inc., Wilmington, DE, USA) and samples were stored at −20°C. RNA integrity was verified by agarose gel electrophoresis (Seakem LE Agarose, Rockland, ME, USA).

2.4. Total Protein Isolation. Protein was extracted from tissues according to Garcia and Phillips [18]. The extraction solution contained the following: 1 M (Tris mol L^{-1}), 0.1 M EDTA (mol L^{-1}), 1 M sodium chloride (NaCl), 0.1% protease inhibitor cocktail; 0.5% sodium azide (NaN_3). A 500 μL extraction solution plus 50 mg tissue was incubated overnight at 5°C under agitation. Subsequent centrifugation at 13,000 rmp for 10 min was performed to obtain total protein, which was spectrophotometrically determined by Bradford's assay [19] (Hewlett Packard Agilent 8453 UV-visible (595 nm)) with an albumin standard curve.

2.5. Extraction of 2,4-TZD from Tissues. Extractions of 2,4-TZD from tissue samples (250 mg) were carried out using 500 μL of 20% KOH in methanol at 65°C for 45 min; then, 2 mL of diethyl ether were added and two washings of an equal volume of water were performed to remove KOH. The upper phase was evaporated under nitrogen atmosphere; residues were dissolved in the mobile phase of ammonium acetate-acetonitrile and transferred into vials [20].

2.6. Extraction of 2,4-TZD from a Concentrate. To ensure the presence of 2,4-TZD in the concentrate feed, we conducted a high-performance liquid chromatography (HPLC) analysis. Five grams were mixed with 7.5 mL of extraction solution (hexane : acetone : alcohol : toluene, 10 : 7 : 6 : 7) in a 25 mL volumetric flask, hand shaken for 1 min, and under conditions of darkness overnight (16 h). Subsequently, KOH (0.5 mL) at 40% was added, shaken, filled with Na_2SO_4 at 10%, and maintained again in the dark for 1 h. The extract (7 mL) was placed into Falcon tubes for further nitrogen

evaporation. The residue was dissolved in the mobile phase of ammonium acetate-acetonitrile and was transferred in vials to be analyzed by HPLC [20].

2.7. Determination of 2,4-TZD by High-Performance Liquid Chromatographic Method (HPLC). Chromatography for separation and determination of 2,4-TZD was carried out on an HPLC 1046A (Hewlett Packard) with a fluorescence detector. Separation and determination were performed utilizing a 5 μm C18 column (250 × 4.6 mm) (Phenomenex, Torrance, CA, USA) [21]. A reference standard of 2,4-TZD was used (Sigma). The mobile phase consisted of ammonium acetate (Fisher Scientific Company, Fairlawn, NJ, USA) 0.01 M in acetonitrile HPLC grade reagent (J.T. Baker, SOLUSORB; Mallinckrodt Baker, Inc., Paris, KY, USA), and the pH was adjusted to 8.0 at a ratio of 65 : 35 v/v, as described by Muxlow et al. [22]. A stock solution of 2,4-TZD was prepared at a concentration of 1 mg/mL of the mobile phase as diluent. Plasma samples (500 μL) were prepared according to He et al. [23]; these were diluted with 500 μL of acetonitrile and the mixture was agitated in vortex for 3 min and centrifuged at 13,000 rpm for 10 min. The upper phase was evaporated under nitrogen atmosphere. This was reconstituted with the mobile phase and transferred onto a conical insert in a vial with insert (100 μL) (amber autosampler vials; Agilent Technologies, Santa Clara, CA, USA). Chromatographic separations were performed at room temperature at a flow rate of 0.7 mL/min with a fluorescence detector at a 269 nm wavelength. Injections were made by a duplicate with 50 μL of sample.

2.8. Quantification of PPAR by Real-Time PCR. In order to quantify PPAR expression in liver, muscle, and SC adipose tissue, cDNA was isolated from the total RNA using oligo $(dT)_{12–18}$ (Sigma) primer and SuperScript II Reverse transcriptase (RT) (Invitrogen) following the manufacturer's instructions. PPARα primers were the following: forward 5′-AGCCTCTGGCTACCACTACG, reverse 5′-CATCCCAACTGAAAGGCACT-3′; PPARδ forward 5′-GGTGACCCTGCTCAAGTACG-3′, reverse 5′-ACTTGACGGCAAACTCGAAC-3′; PPARγ forward 5′-CCATCATGAAGTGTGACGTTG-3′, reverse 5′-ACAGAGTACTTGCGCTCAGGA-3′-PPIA was employed as a housekeeping gene: forward 5′-AGCACTGGGGAGAAAGGATT-3′, reverse 5′-AGCCACTCAGTCTTGGCAGT-3′.

Samples were analyzed in a LightCycler (1.5 instrument; Roche Diagnostics, Basel, Switzerland); a LightCycler FastStart DNA Master Sybr Green I (Roche) was used as well as Capillary Formulation (Sigma). PCR conditions for *PPARα* and *PPIA* genes were as follows: initial denaturation, 95°C/10 min; second denaturation, 95°C/10 sec; annealing, 56°C/10 sec; amplification, 72°C/10 sec for 55 cycles. PPARδ conditions were as follows: denaturation, 95°C/10 min; second denaturation, 95°C/10 sec; annealing, 56°C/10 sec; amplification 72°C/8 sec, for 55 cycles, and for PPARγ, denaturation, 95°C/10 min; second denaturation, 95°C/10 sec; annealing, 58°C/10 sec; amplification, 72°C/8 sec, for 55 cycles. Samples were analyzed by the duplicate; to determine a relative expression of the target genes, they were compared with the reference gene employing the $2^{-\Delta Ct}$ method.

3. Statistical Analysis

Animals were sorted by weight in a completely randomized design with two treatments (Control C versus 2,4-TZD, T) and four replicates. Carcass yield, *LM* rib-eye area, and fat thickness were compared with Duncan's test ($P < 0.05$); DWG, days on feed, and initial and final liveweight were tested by a linear regression analysis. Synthesis and cellular size were analyzed with the least squares means (LS MEANS) procedure. All data were analyzed with SAS system statistical software package [24].

4. Results

4.1. Growth Performance. No differences were found ($P > 0.05$) between treatments in DWG, days on feed, initial and final liveweight, carcass yield, *LM* rib-eye area, or fat thickness (Table 1).

4.2. Carcass Quality. Carcass evaluation was according to NAMPA [17] procedures. Fat and meat color, carcass morphology, and marbling data showed no differences between treatments. Fat coloration observed in both treatments, according to the Pantone colorimetric system, was 7499, which represents cream coloration. Meat color was reported as 1805C in both treatments; according to quality standards in meat coloration, this tonality is reported in selected and standard carcasses. Marbling in *LM* resulted in nongrade carcasses, which indicated no traces of fat in either treatments (Table 1).

4.3. Total DNA, RNA, and Protein in Tissues. To observe the effect of 2,4-TZD on metabolically related tissues, cellular synthesis and size were measured. Cellular synthesis was estimated with RNA/DNA ratios, and cellular size with DNA/protein ratios (results are shown in Table 2).

No differences in hepatic cellular synthesis ($P = 0.135$) and size ($P = 0.090$) were exhibited between treatments. Muscle cell synthesis was lower ($P < 0.05$) in animals treated with 2,4-TDZ, contrary to cell size, which was higher as compared with (C) animals.

Visceral adipose tissue (omentum and perirenal) and SC tissue were sampled to compare adipose depots. Omentum only showed differences ($P < 0.001$) in cell synthesis between (T) and (C). Perirenal samples ($P < 0.05$) had lower cell synthesis in (T) (Table 2), while (T) animals were the largest in size. SC tissue resulted in higher synthesis ($P < 0.0001$) in treatment than in control tissues, contrary to cell size ($P < 0.01$), which was smaller for (T).

4.4. Concentration of 2,4-TZD in Blood, Tissues, and Supplement. No peak was detected in the blood of either treatments when compared with the internal standard, demonstrating the absence of compounds related with 2,4-TZD

Table 1: Productive parameters in Limousin bulls with 2,4-Thiazolidinedione (TZD) (T) versus Control (C) animals.

Variables	Control	2,4-TZD
Initial weight (kg)	317 ± 45.5	308 ± 48.2
Final weight (kg)	517 ± 13.3	516 ± 11.8
Days at feedlot	136 ± 34.5	152 ± 41.8
Daily weight gain (DWG) (kg)	1.479 ± 0.2	1.407 ± 0.1
Liveweight (kg)	515 ± 13.9	514 ± 11.1
Carcass weight (kg)	324 ± 8.6	317 ± 9.2
Carcass yield (%)	63 ± 1.3	62 ± 1.3
Fat thickness (cm)	0.3 ± 0.2	0.4 ± 0.2
Longissimus dorsi muscle (LM) rib-eye area (cm^2)	104 ± 29.4	114 ± 20.5

No differences were found ($P > 0.05$).

Table 2: Cellular synthesis and size in treated 2,4-Thiazolidinedione (TZD) (T), and Control (C) animals' tissues.

Tissues	Cellular synthesis RNA : DNA ratios			Cellular size DNA : protein ratios		
	C	T	*P*	C	T	*P*
Muscle	20.5	5.8	*	0.012	0.019	*
Adipose (omentum)	30.9	11.6	***	0.016	0.017	NS
Adipose (perirenal)	32.5	18.5	*	0.011	0.016	*
Adipose (SC)	14.8	59.0	***	0.014	0.006	**

NS: not significant; C: Control; T: Thiazolidinedione-(TZD-) treated; SC: Subcutaneous. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

metabolism, as was expected since plasmatic clearance in humans treated with TZDs occurs after 3-4 hours of administration [12]. The 2,4-TZD residue in hepatic tissue was significantly ($P < 0.0001$) different between the treatments (Table 3). No peaks regarding the presence of 2,4-TZD residue in (T) muscle sample analysis appeared from HPLC analyses.

Table 3: Comparisons of chromatography residues in liver tissue.

Treatment	Concentration*	MSE**
Control (C)	Not detectable	$2.64E{-}8$
2,4-TZD (T)	$8.48E{-}7$	$2.64E{-}8$

*Liver concentration (2,4-Thiazolidinedione (TZD) mg/g tissue); **MSE: medium standard error.

4.5. PPAR Expression in Tissues. Expressions of PPAR in liver (Figure 1) were significantly different between treatments ($P < 0.01$); all PPAR exhibited greater expression in Control (C) animals than in treated (T) ones. In the case of muscle, only PPARα was different ($P < 0.05$) between treatments (Figure 2). There was no difference in PPARγ between treatments ($P > 0.1$) (Figure 3).

5. Discussion

5.1. Growth Performance. Thiazolidinediones are widely used antidiabetic drugs with proven efficacy mainly as surrogate markers of diabetes management. However, the latter may not always translate into benefits in clinical outcomes. In humans, common side effects associated with TZD include an average weight gain of 3-4 kg over the first 6 months of TZD treatment [25], and the rate of weight gain decreases after the first 6–12 months [26]; however, in this work, no differences between treatments in DWG nor weight at slaughter was observed; this could be due to differences between the weight of the animals and the time they were in the experiment; moreover with a higher population of bulls we probably would be able to see an increase in DWG. Concerning DM intake, no differences were found between treatments ($P > 0.1$); however, other authors have reported increases during peripartum and postpartum periods in dairy cows with the use of 2,4-TZD [14, 27].

5.2. Carcass Quality. Although our data did not show significant differences between treatments, this could relate in some manner to the breed's characteristics. Limousin cattle are known to be late maturing as compared with Angus and Hereford [28], which means that our bulls might have not reached an adequate weight. Differences in maturing weight have been related with carcass yield, meat quality, and marbling; moreover, Limousins are categorized as low producers, when it comes to fat deposition [10].

5.3. Cellular Synthesis and Size. The glucose-lowering action of TZD is attributed to its agonistic action on peroxisome proliferator-activated receptor gamma (PPARγ), a nuclear receptor that is expressed predominantly in adipose tissue and that regulates adipogenesis. In muscle and liver, which

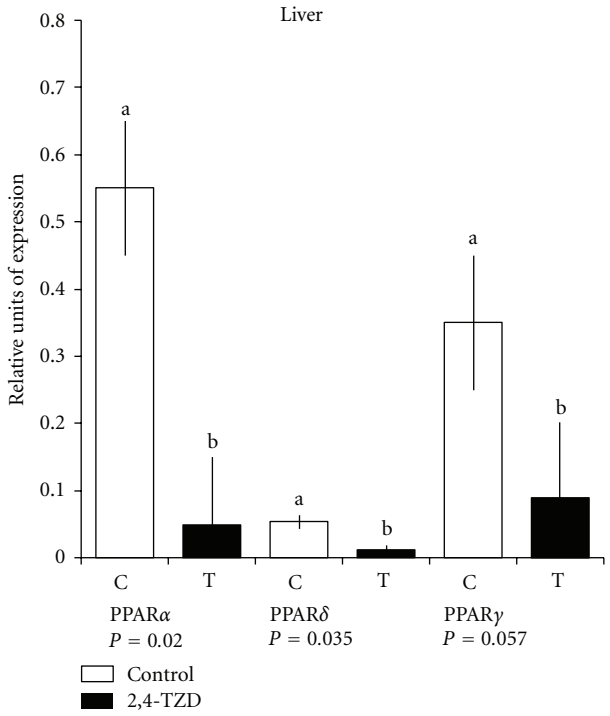

Figure 1: Quantitative polymerase chain reaction (qPCR) of peroxisome proliferation-activated receptor (PPAR)α, -δ, and -γ in the liver. Columns show relative expression of PPAR (α, δ, and γ) in Control (C) and Thiazolidinedione (TZD) (T) groups. [a,b]Different lowercase letters indicate a significant difference.

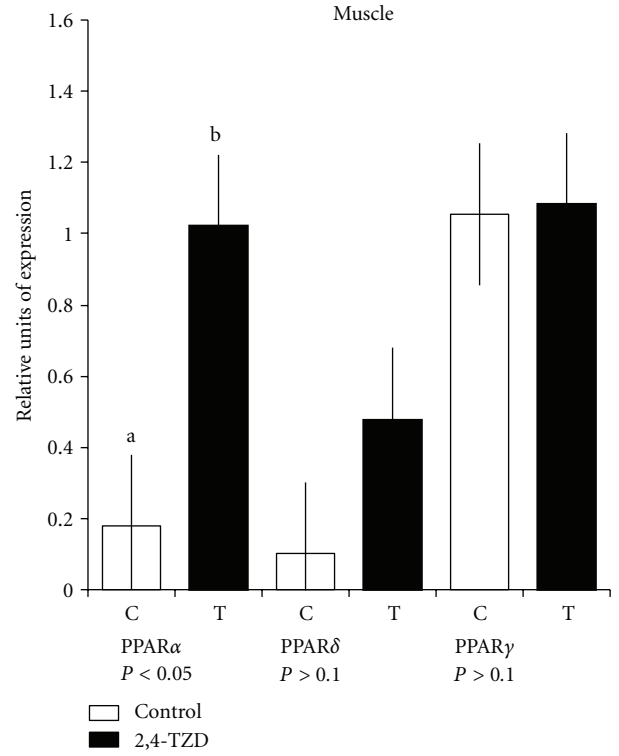

Figure 2: Quantitative polymerase chain reaction (qPCR) of peroxisome proliferation-activated receptors (PPAR)α, -δ, and -γ in the muscle. Columns show relative expression of PPAR (α, δ, and γ) in Control (C) and Thiazolidinedione (TDZ) (T) groups. [a,b]Different lowercase letters indicate a significant difference.

are the quantitatively most important tissues for insulin-dependent glucose homeostasis, TZD-induced insulin sensitization appears to occur associated with PPARγ-mediated changes in lipid handling and signal output from adipose tissue [29]. While such a fat-mediated mode of TZD action is undisputed, the evidence accumulates that the pharmacology of TZD could be driven not only by PPARγ activation, but also by PPARγ-independent and nongenomic effects on mitochondria [30]. In this work, the lower muscle synthesis shown in (C) compared with the greater size of (T) might be due to 2,4-TZD action-related insulin stimuli, resulting in muscular glucose and fatty acids uptake from blood; this could indicate that cells were in a positive metabolic state. Therefore, (T) maintained cellular synthesis, while cellular protein degradation was lower compared with (C), and this was reflected in a larger size. Redistributing adipose tissue to visceral organs by inducing adipogenesis of smaller lipid droplets, which are more insulin sensitive, is one of the main important effects of TZD [31]; in humans and rodents, TZD increases the SC adipocyte cell surface [32]. Cell synthesis in omentum and perirenal was different between treatments, being higher for (C). It is well known that visceral adipose tissue cells are larger in size because of the union of lipid compared with SC droplets. Explanation of a larger size in (T) could be due to 2,4-TZD action resulting in accumulation of lipid droplets. Even though cell size did not exhibit differences in omental tissue; in perirenal tissue, (T) was

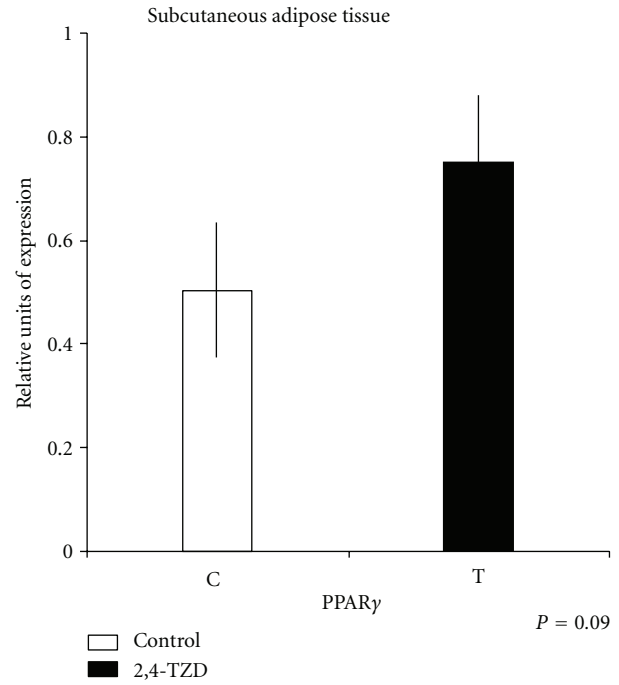

Figure 3: Quantitative polymerase chain reaction (qPCR) of peroxisome proliferation-activated receptor (PPAR)γ in subcutaneous (SC) adipose tissue. Columns show relative expression of PPARγ in Control (C) and Thiazolidinedione (TDZ) (T) groups.

higher than (C), which could mean that certain expression factors, such as interleukin (IL)-6, resistin, and PPARγ are higher in visceral fat compared with SC fat, in which the expression comprises adipsin, leptin, and adiponectin [33, 34].

5.4. 2,4-TZD Presence in Blood, Liver, and Muscle. 2,4-TZD concentrations were measured in blood, liver, and muscle in order to verify the presence of residues. As mentioned previously, blood samples were collected at the 28-day intervals. There was no evidence of TZD in blood (T). In contrast with other studies, TZD is supposed to have a 3-4 h elimination period [35], which explains its absence if samples were taken at around >12 h after the last meal when 2,4-TZD was administered. Hepatic samples from (T) presented significant differences from those of (C) (Table 3). Liver is the main route in which TZD is metabolized by cytochrome P450 enzymes and isoenzymes (CYP2C8 and CYP2C9) [36]. Liver injuries in terms of toxicity due to TZD doses (or toxicity during TZD treatment) are uncertain; troglitazone has been withdrawn from the market due to the mitochondrial hepatotoxicity [37]. In addition, rosiglitazone is metabolized by liver, and 64% and 23% of metabolites are excreted by feces and urine, respectively [12].

Muscle samples did not show the presence of TZD nor did its metabolites, but further research is required on hepatic and muscular pharmacokinetics and its correlation with doses, administration, and animal species.

5.5. PPAR Expression. In liver, lower expression of PPAR in (T) is consistent with that reported in the literature [38, 39]. TZD is markedly effective in reducing liver fat content by 30%–50% and in sensitizing the liver to insulin. This reduces the amount of endo- and exogenous insulin required to inhibit hepatic glucose production [40]. Studies with murine hepatic tissue have reported low expression of PPARγ when pioglitazone has been utilized in a chronic treatment [41], similar to our results. On the other hand, PPARα expression in tissues with high beta-oxidation activity, such as hepatic tissue, is frequently found.

Studies of PPAR expression in tissues have proved that when there is the severe insulin resistance in the muscular tissue, this could result in abnormal absence of the PPAR transcription factor [42]. Our results showed that PPAR possesses higher expression in (T) than in (C) in muscle. In the case of PPARα in muscle, there is an evidence that the myogenic differentiation implicated in the process of mitochondrial biogenesis is governed by PPAR and in the coactivator PGC1-α in which they mediate OXPHEN (oxidative phenotype), which is the capacity of the muscle's activity, substrate, and metabolism implied in fiber type and established during myogenesis, repair, or hypertrophy of muscle [43].

PPARδ participates in the development, induction, and maintaining of type I fibers, suggesting the conversion of some muscle fibers from type II to oxidative fibers, by inducing coactivator PGC-1α expression in skeletal muscle. Moreover, expression of PPARδ has been related with genes involved in the regulation of lipid and glucose metabolism in human skeletal muscle [44]. The increase of PPARδ in (T) is in agreement with other studies, in which unsaturated fatty acids have increased coactivator PGC-1α in differentiated myotubes, and it could be possible that PPARδ ligands in muscle could be fatty acids and some of their metabolites [45]. On the other hand, some studies propose that PGC-1α levels in murines are inversely correlated with IM fatty acid levels, but, in this case, the animal's metabolism varies among species [45].

In muscle, PPARγ, as well as PPARδ, has been related with adipogenesis and is thought to contribute to transdifferentiation into adipocyte-like cells [46]. In agreement with the higher PPARγ expression in SC tissue, cell synthesis in the 2,4-TZD treatment was as expected [47]. Moreover, studies in Holstein transition cows suggested a higher expression of PPARγ in adipose tissue biopsies performed on the final day of treatment with 2,4-TZD [48]. First, SC adipose cells are more sensitive to the effect of the dietary nutrients [49]. SC tissue is well known to possess better cell differentiation than omentum; therefore, it is more receptive to the effect of TZD than other tissues [1, 50]. Moreover, evaluations of different subcompartments of SC and abdominal adipose tissues have observed posttranscriptional difference levels of adiponectin secretion and that the action of 2,4-TZD in SC fat could be related with abdominal adipose tissue changes [51]. It could be expected that animals that are not genetically predisposed to form marbling would deposit fat in SC rather than IM [52]. These could explain the effect of 2,4-TZD on (T) animals, concerning which we can discuss that SC depots were accumulating fat, and, probably, if we would increase the treatment length, we could have reached some marbling. In contrast, if we were to have used another breed, such as Angus, treatment with 2,4-TZD might have resulted in a larger amount of intramuscular fat. In addition, in our study, steers were also treated with trenbolone acetate and 17β-estradiol, since it has been proved that anabolic compounds increase protein synthesis, involving insulin-like growth factor (IGF)-I and decreasing protein degradation. On the other hand, anabolic compounds are known to promote reduction in body fat. Some studies have revealed that these compounds act by diminishing and blocking PPARγ and C/EBPα [53]. Therefore, and according to our study's results, 2,4-TZD would contribute to the improvement of meat quality by its interaction with the anabolics to promote an increment in muscle and adipose tissue accretion.

6. Conclusions

The results show a positive effect of 2,4-TZD on muscle metabolism and PPARα expression, which suggests increased fatty acid oxidation, thus the improvement in lipid metabolism. In adipose tissue, it appears that the effect of 2,4-TZD, combined with the anabolic used produced inhibition of PPARγ expression in visceral adipose tissues (omentum and perirenal) according to cellular synthesis ratios. To our knowledge, this is the first study that considers the use of a TZD to improve beef production; further studies are required to provide information about dose, breed, and time

of use, before its use of these in animal production could be considered.

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Review Article

Regulation of Genes Involved in Carnitine Homeostasis by PPARα across Different Species (Rat, Mouse, Pig, Cattle, Chicken, and Human)

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Recent studies in rodents convincingly demonstrated that PPARα is a key regulator of genes involved in carnitine homeostasis, which serves as a reasonable explanation for the phenomenon that energy deprivation and fibrate treatment, both of which cause activation of hepatic PPARα, causes a strong increase of hepatic carnitine concentration in rats. The present paper aimed to comprehensively analyse available data from genetic and animal studies with mice, rats, pigs, cows, and laying hens and from human studies in order to compare the regulation of genes involved in carnitine homeostasis by PPARα across different species. Overall, our comparative analysis indicates that the role of PPARα as a regulator of carnitine homeostasis is well conserved across different species. However, despite demonstrating a well-conserved role of PPARα as a key regulator of carnitine homeostasis in general, our comprehensive analysis shows that this assumption particularly applies to the regulation by PPARα of carnitine uptake which is obviously highly conserved across species, whereas regulation by PPARα of carnitine biosynthesis appears less well conserved across species.

1. Introduction

Peroxisome proliferator-activated receptor α (PPARα) is considered a master transcriptional regulator of lipid metabolism and energy homeostasis [1], because typical genes regulated by PPARα are involved in all aspects of fatty acid catabolism (cellular fatty acid uptake, activation of fatty acids, intracellular fatty acid transport, import of fatty acids into the mitochondria, and mitochondrial and peroxisomal fatty acid β-oxidation), ketogenesis, as well as gluconeogenesis [2]. PPARα-dependent gene transcription is initiated when a ligand, for example, fatty acids which are released from white adipose tissue during energy deprivation and taken up into tissues during this state, or exogenous ligands such as fibrates (WY-14,643, clofibrate, fenofibrate, bezafibrate, and gemfibrozil), binds to the ligand-binding domain of this transcription factor. Mechanistic details of gene regulation by PPARα and tissue distribution of PPARα has been extensively described in the literature, wherefore the reader is referred to the literature with regard to this [3]. Interestingly, earlier studies repeatedly reported that energy deprivation or treatment of rats with fibrates causes a marked, up to 5-fold elevation of the hepatic concentration of carnitine [4–7]. The molecular mechanisms underlying this phenomenon, however, have not been resolved from these studies. It was not until about twenty years later that activation of hepatic PPARα, which is common to energy deprivation and fibrate treatment, was shown to cause an increase in the expression of genes involved in carnitine uptake and biosynthesis in liver cells [8] serving as a reasonable explanation for the abovementioned phenomenon. In subsequent studies it was shown that elevation of hepatic carnitine concentration in response to fasting, or fibrates occurs only in wild-type mice but not in transgenic mice lacking a functional PPARα protein strengthening the assumption that PPARα is a critical regulator of carnitine homeostasis [9, 10].

Using more sophisticated molecular biological techniques it could be convincingly demonstrated that the mouse genes encoding the carnitine transporter novel organic cation transporter 2 (OCTN2/SLC22A5) and two enzymes of the carnitine biosynthetic pathway, γ-butyrobetaine dioxygenase (BBOX1) and 4-trimethylaminobutyraldehyde dehydrogenase (ALDH9A1), are direct PPARα target genes as evidenced by the identification of functional PPRE within the regulatory region of the respective genes [11–13].

It is well established that PPARα activators exert distinct species-specific actions [14–17]. In rodents, like mice and rats, administration of PPARα activators leads to a marked peroxisomal enzyme induction, peroxisome proliferation, and even hepatocarcinogenesis [18, 19]. In contrast, PPARα activators cannot induce peroxisome proliferation and hepatocarcinogenesis and the induction of peroxisomal metabolism pathways is much less pronounced in human hepatocytes and livers from nonhuman primates [17]. This distinct response of the peroxisomes to PPARα activators is responsible for the classification of different species into proliferating (mice, rats) and nonproliferating ones (humans, monkeys, guinea pigs). Several factors are considered to account for the marked difference in the response to PPARα activators between different species: expression level of PPARα, degree of conservation and functionality of the PPRE in the regulatory region of target genes, and lack or overexpression of transcriptional coregulators [17]. Apart from these marked differential effects of PPARα activators on peroxisome proliferation between proliferating and nonproliferating species, a comparative analysis of gene regulation by PPARα between mouse and human revealed that at least the role of PPARα as a master regulator of hepatic lipid catabolism is well conserved [20]. It may be therefore expected that regulation of carnitine homeostasis, which is intrinsically linked to fatty acid catabolism because the transport of fatty acids from the cytosol into the mitochondrial matrix for subsequent fatty acid β-oxidation is carnitine-dependent, is also a well conserved function of PPARα. However, despite its well conserved role as an important regulator of lipid catabolism in general, the specific genes under control of PPARα within each lipid metabolic pathway were shown to differ at least between humans and mice [20]. Thus, whether PPARα can be considered as a critical regulator of genes involved in carnitine homeostasis across different species requires thorough analysis of the effect of PPARα activation on carnitine homeostasis in each individual species and cannot be predicted for one species by simply transferring observations obtained in mice or rats. In light of the abovementioned species specificities with regard to the response to PPARα activation the present paper aims to (1) briefly describe current knowledge about the genes involved in the regulation of carnitine homeostasis and (2) to comprehensively analyse available data from genetic and animal studies with mice, rats, pigs, cows, and laying hens and from human studies in order to compare the regulation of genes involved in carnitine homeostasis by PPARα across different species.

2. Regulation of Carnitine Homeostasis

Carnitine is a water soluble quaternary amine (3-hydroxy-4-N,N,N-trimethylaminobutyric acid) which is essential for normal function of all tissues. The primary function of carnitine is to facilitate the translocation of activated long-chain fatty acids from the cytosol into the mitochondrial matrix, a process called carnitine shuttle, for subsequent fatty acid β-oxidation. In mammals, carnitine is considered a conditionally essential nutrient because it is synthesized by the organism but most is taken up from the diet [21]. Food of animal origin, such as meat and dairy products, containing high carnitine levels, makes the greatest contribution to total carnitine uptake. In contrast, the intake of food of plant origin is negligible for dietary carnitine uptake due to its very low carnitine levels [22]. Thus, dietary uptake of carnitine in strict vegetarians is very low and has been estimated to be less than 0.02 mg per kg body weight and day [23], whereas dietary carnitine uptake through an omnivorous diet provides approximately 0.3–1.9 mg carnitine per kg body weight and day. Nonetheless, plasma carnitine levels in vegetarians are only 15–30% lower than those in nonvegetarians, being yet within the normal physiological range (25–50 μmol/L), because vegetarians have a more efficient renal reabsorption of carnitine (urinary total carnitine excretion was 55% less in vegetarians than in nonvegetarians [24]) and a greater rate of endogenous carnitine biosynthesis [25, 26]. In healthy vegetarians, carnitine deficiency (plasma carnitine concentration $< 25\,\mu$mol/L [26]) may develop only if certain micronutrients, such as vitamin C, vitamin B_6, and iron, which are required as co-factors for carnitine biosynthesis are not provided from the diet in sufficient amounts. The tubular reabsorption of carnitine in the kidney, where >95% of filtered free carnitine is reabsorbed when plasma free carnitine concentration is within the normal range, is of great importance for maintaining normal plasma carnitine levels. This is evidenced by the fact that patients with inborn or acquired defects in this tubular carnitine reabsorption process develop primary systemic carnitine deficiency with markedly reduced serum carnitine levels (0–5 μmol/L) because most of the filtered carnitine is lost in the urine [27]. If plasma carnitine concentration exceeds the normal range (supraphysiologic levels) due to the uptake of high dosages of carnitine (e.g., oral or i.v. supplementation), the excess carnitine is rapidly eliminated due to saturation of the tubular reabsorption mechanism [26, 28]. This explains the fact that the ability to maintain supra-physiologic plasma carnitine concentrations is limited [29, 30]. The skeletal muscle contains the majority of the total body carnitine [31], and, like the myocardium, is dependent on the active uptake of carnitine from plasma against a strong concentration gradient (from 25–50 μmol/L in plasma to about 4000 μmol/L in skeletal muscle) [32]. Due to this large endogenous carnitine pool, a single intravenous dose of carnitine or short-term oral supplementation with carnitine at high doses (4–6 g/day) has little or no impact on the muscle carnitine content [33–35].

3. Genes Encoding Proteins Involved in Carnitine Homeostasis

3.1. Carnitine Biosynthesis. The carnitine biosynthesis pathway consists of a cascade of four distinct enzymatic reactions through which 6-N-trimethyllysine (TML), which is the substrate for carnitine biosynthesis, is converted stepwise into carnitine. TML is the product of lysosomal and proteasomal degradation of proteins containing N-methylated lysines, such as calmodulin, myosin, actin, and histones [21]. In the first enzymatic step, TML is hydroxylated by the enzyme TML dioxygenase (encoded by TMLHE) to yield 3-hydroxy-TML (HTML). Subsequently, the second enzyme, called HTML aldolase (encoded by HTMLA), catalyzes an aldolytic cleavage of HTML, which results in the formation of 4-trimethylaminobutyraldehyde (TMABA). The third enzyme, called TMABA dehydrogenase (encoded by ALDH9A1), catalyzes the dehydrogenation of TMABA to 4-N-trimethylaminobutyrate or γ-butyrobetaine (BB). In the final biosynthetic step, BB is hydroxylated by BB dioxygenase (encoded by BBOX1) to form carnitine [21]. In all mammals, a significant BBD activity is found in the liver [36], and in some species such as in humans, pigs, cats, cows, hamsters, rabbits, or Rhesus monkeys also in the kidney [36, 37]. Other tissues than liver and kidney have either no or only a very low activity of BBD [36, 37], and are therefore highly dependent on active carnitine uptake from blood. The BB, which is formed in extrahepatic tissues, is excreted and transported via the circulation to the liver, where it is converted into carnitine [36].

3.2. Carnitine Uptake. Tissues which are incapable of providing carnitine via endogenous biosynthesis, such as skeletal muscle and myocardium, are highly dependent on the uptake of carnitine from the circulation. This transport across the plasma membrane against a high concentration gradient (in skeletal muscle > 100-fold) is mediated by the novel organic cation transporters (OCTNs) which belong to the solute carrier 22A family [38, 39]. The OCTN2 isoform, which is sodium-dependent and high-affinity, is considered the physiologically most important one due to its wide tissue expression [40, 41]. This transporter represents the molecular basis for the tubular reabsorption process of carnitine in the kidney and is therefore fundamental for maintaining normal carnitine levels in serum. As mentioned above, defects in the renal reabsorption process of carnitine due to a mutation in the OCTN2 gene are causative for severe carnitine deficiency in such patients [42]. In the small intestine, OCTN2 also plays a key role for the absorption of carnitine from the diet [43]. This is based on the observation that in mice with a genetic defect in the OCTN2 gene oral bioavailability of carnitine was reduced by approximately 50% [44].

The OCTN1 isoform is considered to contribute less to carnitine transport than OCTN2 due to its low carnitine transport activity. OCTN1 is localized in the mitochondrial membrane in close proximity to CPT I, the rate-limiting enzyme for carnitine-dependent fatty acid oxidation. Due to this localization, OCTN1 has been proposed to operate on the mitochondrial influx and efflux of carnitine and acylcarnitine esters indicating that OCTN1 is mainly involved in maintaining intracellular carnitine homeostasis [45]. Another OCTN isoform, namely, OCTN3, has been suggested to play a role for carnitine uptake into testis and to also mediate renal reabsorption of carnitine [41].

4. Evidence for a Role of PPARα in Regulating Genes Involved in Carnitine Homeostasis in Different Species

4.1. Rat. Based on earlier reports that energy deprivation or treatment with fibrates, both of which induce activation of hepatic PPARα, causes a marked elevation of the hepatic carnitine concentration [4–7], we have recently tested the hypothesis that PPARα activation is responsible for this phenomenon. Indeed, we demonstrated for the first time that PPARα activators strongly increase transcript levels of OCTN2 in rat liver and cultivated rat hepatocytes [8]. Moreover, we found that the increase in OCTN2 mRNA abundance in response to treatment with PPARα activators was accompanied by an elevation of the carnitine concentration in rat liver and cultivated rat hepatocytes [8]. These findings provided the first evidence that PPARα plays a role in regulating carnitine homeostasis through stimulating OCTN2-mediated carnitine uptake from blood into the liver. In subsequent studies with rats, we found that treatment with PPARα activators increases also OCTN2 transcript levels in small intestine [46, 47], and improves intestinal carnitine absorption [47]. Thus, these observations confirmed our assumption that PPARα is an important regulator of carnitine uptake and that upregulation of OCTN2 in small intestine may contribute to the elevation of hepatic carnitine concentration in response to PPARα activators through increasing carnitine availability from the diet. A further study in rats revealed that energy deprivation, which is a physiologic state of PPARα activation, also results in increases of OCTN2 transcript levels and carnitine concentration in the liver [48]. Since administration of oxidized fats causes a strong activation of PPARα in rats [49–51] due to the high content of hydroxylated fatty acids and cyclic fatty acid monomers, both of which are ligands of PPARα, we also investigated whether feeding of oxidized fats causes similar effects on carnitine homeostasis as energy deprivation and fibrate treatment [52]. Indeed, we observed that administration of oxidized fat for 6 d causes an elevation of OCTN2 transcript levels in liver and small intestine and increases hepatic carnitine concentration of rats indicating that carnitine homeostasis is regulated also by nutritive PPARα activators.

Since the results from these experiments suggested that OCTN2 might be a direct target gene of PPARα, we performed in silico analysis of the rat OCTN2 promoter which revealed several putative PPRE upstream of the transcription start site [46]. Using reporter gene and gel mobility shift assays, Maeda et al. [53] recently identified one functional PPRE in the rat OCTN2 promoter confirming our

assumption that the rat OCTN2 gene is a direct PPARα target gene. However, in comparison to the marked induction of OCTN2 mRNA by fibrates and fasting [8, 46, 48] the weak stimulation of rat OCTN2 promoter activity reported from Maeda et al. [53] suggested that a more potent PPRE, located in other regulatory regions than the proximal promoter, might be responsible for OCTN2 upregulation in response to PPARα activation.

Although previous studies in rats indicated that the clofibrate-induced increase in hepatic carnitine concentration is due to an increase in the rate of hepatic carnitine synthesis [6, 7], results from analysis of gene expression of enzymes of the carnitine biosynthesis pathway in rats do not point towards a role for PPARα in regulating genes involved in carnitine biosynthesis in rats. All of the abovementioned studies in rats did not show any increase in the transcript levels of ALDH9A1 and BBOX1 in the liver in response to fibrate treatment, fasting, or administration of oxidized fat. This indicates that at least the rat genes encoding ALDH9A1 and BBOX1 are not transcriptionally regulated by PPARα, despite the fact that several conserved PPRE were identified in the proximal promoter of the rat BBOX1 gene using NUBIScan software [9]. However, studies in rats demonstrated that both clofibrate and fasting increase the concentration of the carnitine precursor TML in the liver [46, 48, 54]. Since carnitine biosynthesis starts with the enzymatic conversion of TML, the availability of TML has been considered to be rate limiting for carnitine biosynthesis [55]. In fact, TML is subsequently converted into BB, which itself is rapidly further converted into carnitine due to the large capacity of the liver to convert BB into carnitine [56]. Thus, it is possible that carnitine biosynthesis is stimulated by PPARα activation, an effect that is not mediated by increasing expression of genes encoding enzymes of the carnitine biosynthesis pathway but rather by stimulating lysosomal and proteasomal degradation of proteins which leads to the release of TML [57, 58]. The observation that both clofibrate and fasting stimulate proteolysis [59] is supportive for this assumption.

4.2. Mouse. According to convincing data from studies with rats that PPARα plays a role in the regulation of carnitine homeostasis, studies with PPARα knockout and corresponding wild-type mice have been conducted [9, 10]. van Vlies et al. [9] were the first demonstrating that PPARα regulates gene expression of OCTN2 in the liver of mice as evidenced by the observation that upregulation of OCTN2 in response to fasting or WY-14,643 treatment occurs only in wild-type but not in PPARα knockout mice. Using the same mice genotypes, Koch et al. [10] largely confirmed these findings from van Vlies et al. [9] but additionally demonstrated that PPARα activators cause OCTN2 upregulation also in kidney and small intestine. Studies from both groups showed that the elevation of hepatic carnitine concentration in response to PPARα activation occurs only in wild-type mice [9, 10], which provided further evidence that PPARα is a critical player for regulating carnitine homeostasis. Noteworthy, these studies revealed also upregulation of genes encoding the carnitine biosynthetic enzymes ALDH9A1 and BBOX1 in the liver of wild-type but not PPARα knockout mice indicating that genes involved in carnitine biosynthesis are regulated by PPARα in mice, which is in contrast to the rat.

Further indication for the PPARα dependency of regulation of the mouse genes encoding OCTN2, ALDH9A1, and BBOX1 is provided by the observation that hepatic mRNA, and protein levels of OCTN2, ALDH9A1, and BBOX1 are decreased in obese mice compared to lean mice [60], because high fat diet-induced obesity was reported to disrupt hepatic PPARα function and to impair PPARα dependent gene transcription [61, 62]. Noteworthy, this study showed that the reduced hepatic expression levels of OCTN2, ALDH9A1, and BBOX1 were partially restored to expression levels of lean mice in a subgroup of the obese mice which were regularly exercised on a motorized treadmill (35 min, 5 x/wk, 10 wk). Since endurance exercise causes activation of PPARα, these data suggest that endurance exercise was able to restore at least in part the obesity-induced disruption of PPARα function and thereby contributed to the elevated gene expression of OCTN2, ALDH9A1, and BBOX1.

Besides direct transcriptional regulation of genes involved in carnitine homeostasis by PPARα, evidence has been provided that PPARα might influence the availability of requisite biosynthetic precursors—through the abovementioned stimulatory effect of PPARα activation on proteolysis—and enzymatic cofactors required for carnitine synthesis. In this context a study from Makowski et al. [63] is worth mentioning which reported that PPARα knockout mice display markedly lower levels of methionine, which serves as a methyl donor during posttranslational assembly of methylated proteins, and α-ketoglutarate, which is a cofactor of TMLHE and BBOX1, in plasma and tissues, respectively, than wild-type mice.

Recent molecular biological studies by our own group revealed that the mouse genes encoding OCTN2, BBOX1, and ALDH9A1 are direct PPARα target genes [11–13], which is in line with the abovementioned observations from studies with PPARα knockout mice [9, 10]. Direct regulation of these genes by PPARα was evidenced by the identification of one functional PPRE each in the regulatory region of these genes. The functional PPREs were shown to be located in either the proximal promoter (BBOX1 and ALDH9A1; [12, 13]) or the first intron (OCTN2; [11]). Taken together, these findings confirm that PPARα plays a key role in the regulation of carnitine homeostasis in the mouse by controlling genes involved in carnitine synthesis and carnitine uptake.

4.3. Pig. The abovementioned observations in rodents cannot be directly applied for humans, because of marked differences in the response to PPARα activators between rodents and humans [17, 18]. In contrast to rodents, pigs have a low expression of PPARα in the liver and the response to PPARα activators (induction of peroxisomal metabolism pathways, peroxisome proliferation) is very weak, wherefore pigs like humans and nonhuman primates belong to the nonproliferating species. A recent study from our group showed that PPARα mRNA levels in the liver are comparable between pig and human [64], which suggests that the pig is a suitable model for humans to study the effects of

PPARα activation. Activation of PPARα in liver and other tissues of pigs have been already demonstrated in response to clofibrate, oxidized fat as well as fasting [65–67]. In order to study whether carnitine homeostasis is regulated by PPARα also in pigs we performed two experiments in which pigs were either treated with clofibrate or fasted for 24 h. Treatment with clofibrate caused an upregulation of OCTN2 in liver, skeletal muscle, and small intestine, and increased carnitine concentrations in liver and skeletal muscle [68]. Upregulation of OCTN2 in the liver and elevated carnitine concentrations in liver and kidney were also found in pigs which were fasted for a period of 24 h [67]. In the latter study, fasting was also shown to increase BBOX1 mRNA level and BBOX1 activity in liver and kidney [67]. Thus, these observations from studies with pigs indicate that carnitine homeostasis in pigs is also regulated by PPARα, even though the extent of upregulation of OCTN2 and BBOX1 is lower in pigs than in rodents. The latter may be explained by the lower tissue expression level of PPARα in pigs than in rodents but also by species differences in the availability of transcriptional coregulators. In this context it is worth mentioning that a large number of PPAR related coregulators, such as CBP/p300, SRC-1-3, PGC-1α, PGC-1β, PRIP, PRIC285, CARM1, and PIMT, have been described to influence PPAR target gene transcription and that their relative availability in a given tissue is at least partially responsible for the tissue specific expression of target genes and the responsiveness of PPAR isotypes to specific ligands [69].

4.4. Cattle. In contrast to the large body of literature with regard to the regulation of carnitine homeostasis by PPARα in rodents, only limited information is available on the regulation of PPARα activity and its role for carnitine homeostasis in cattle liver. Apart from demonstrating that PPARα is functional in cattle liver [70] and long-chain fatty acids are able to activate PPARα in bovine cells [71, 72], it was shown that the negative energy balance occurring in early lactating dairy cows is associated with an upregulation of several established PPARα target genes in nonruminants in the liver being indicative of PPARα activation during early lactation [73–76]. Based on previous observations that hepatic carnitine concentration in dairy cows is increasing during the transition from late pregnancy to early lactation [77, 78], we have recently investigated whether hepatic genes of carnitine synthesis and uptake of carnitine are upregulated during early lactation in dairy cows [79]. As expected and in accordance with results from a recent study [73], our study showed that the negative energy balance occurring at early lactation was associated with elevated plasma levels of free fatty acids and increased transcript levels of established PPARα target genes in nonruminants [79], which is indicative of activation of hepatic PPARα in early lactating cows. In line with our hypothesis, our study showed that the transition from late pregnancy (3 wk prepartum) to early lactation leads to an upregulation of various genes involved in carnitine synthesis (ALDH9A1, TMLHE, BBOX1) and carnitine uptake (OCTN2) in the liver of cows at 1 wk postpartum [79]; transcript levels of TMLHE, ALDH9A1, BBOX1, and OCTN2 were 10-, 6-, 1.8-, and 13-fold, respectively, higher in the liver of dairy cows at 1 wk postpartum than at 3 wk prepartum. In addition, concentration of carnitine in the liver was increased from 3 wk prepartum to 1 wk postpartum. In contrast, from 1 wk to 5 and 14 wk postpartum transcript levels of TMLHE, ALDH9A1, BBOX1, and OCTN2 and hepatic carnitine concentrations were declining [79]. Thus, it is likely that the observed changes in the expression of these genes account for the alterations of hepatic carnitine concentration during the transition period and the lactation cycle. Noteworthy, we also found that plasma concentrations of free fatty acids and hepatic carnitine concentrations at 1 wk, 5 wk, and 14 wk postpartum were positively correlated. Although it remains to be established that the bovine genes encoding TMLHE, ALDH9A1, BBOX1, and OCTN2 are direct PPARα target genes, the positive correlations between plasma free fatty acids, which are endogenous activators of PPARα, and hepatic carnitine concentrations during lactation are supportive for a role of PPARα in the regulation of carnitine homeostasis in cattle. Besides these data from pregnant and lactating cows which provide indirect evidence for a PPARα-dependency of carnitine homeostasis in cattle, unpublished data from our own group from cell culture experiments provide stronger evidence for a role for PPARα in regulating genes involved in carnitine homeostasis in cattle. We found that treatment of bovine kidney cells with a PPARα agonist increases transcript and protein levels of OCTN2. Whether the bovine BBOX1 gene is also regulated by PPARα cannot be answered with certainty because BBOX1 is not expressed in this bovine kidney cell line (unpublished observation).

4.5. Chicken. Like in mammals, PPARα has been shown to be highly expressed in chicken liver and to play an important role for the homeostasis of energy and lipid metabolism during fasting [80]. In addition, a high homology of avian PPARα with mouse, rat, and human PPARα [81, 82] and a similar expression pattern of PPARα in tissues between chicken and rodents as well as humans has been reported [81, 82]. Moreover, a recent study demonstrated that PPARα in the liver of laying hens can be strongly activated by the administration of clofibrate as evidenced from elevated transcript levels of classical PPARα target genes [83]. In order to study the regulation of carnitine homeostasis by PPARα in laying hens, we have recently performed a study with laying hens which were fed diets supplemented without (control) or with clofibrate [84]. Interestingly, this study revealed that treatment with clofibrate increased carnitine concentration not only in the liver but also in the whole egg, yolk, and albumen. On the molecular level, activation of PPARα in the liver of clofibrate-treated hens could be demonstrated by elevated transcript levels of classical PPARα target genes. In addition, this study demonstrated that OCTN2 but not genes encoding enzymes of carnitine biosynthesis in the liver are upregulated by clofibrate in the liver of laying hens [84], which indicates that increased carnitine concentrations in the liver of hens treated with clofibrate might be due to stimulation of OCTN2-mediated carnitine uptake from plasma into liver cells. Thus, the findings from this study

suggested that PPARα has an essential role in the regulation of carnitine homeostasis in hens like in mammalian species. Unlike in mice and pigs, however, PPARα in laying hens appears to play a role only for regulating OCTN2-mediated carnitine uptake but not carnitine biosynthesis. In a further study, it has been investigated whether carnitine homeostasis in laying hens can be also influenced by the administration of nutritive PPARα activators [85]. This study however failed to demonstrate an influence of either fish oil or conjugated linoleic acid (CLA) on carnitine homeostasis in laying hens. The lack of effect of nutritive PPARα agonists on carnitine homeostasis, however, is not a contradiction to the abovementioned study but rather reflects the fact that activation of PPARα by both fish oil and CLA in this study was negligible, which itself is likely due to the low binding affinity of n-3 PUFA and CLA isomers when compared to the synthetic PPARα activator clofibrate.

4.6. Human. In contrast to extensive research on the regulation of carnitine homeostasis by PPARα in animals, only few studies with limited significance are available to evaluate whether PPARα regulates carnitine homeostasis in humans as well. One important reason for the limited significance of human studies is that, with few exceptions, most of them used plasma samples only, which is not appropriate for evaluating changes in carnitine homeostasis. To our knowledge only one study is available in the literature analyzing the change in the urinary profile of carnitine and its derivates in healthy adults in response to starvation [86], which is the physiological state of PPARα activation. According to this study, 48 h starvation caused a slight decrease in the urinary excretion of free carnitine and a marked increase in that of acetyl carnitine. Albeit being speculative, the reduced urinary excretion of free carnitine in the starved subjects may be indicative of a PPARα-induced increase in the tubular reabsorption of carnitine in the kidney which is possibly mediated by an upregulation of OCTN2. In another study with human subjects, from which skeletal muscle biopsies were taken, no change in skeletal muscle carnitine levels were found in patients under starvation conditions [87]. This finding however does not argue against the hypothesis that PPARα is a regulator of carnitine homeostasis also in humans because the carnitine concentration in skeletal muscle, which is the main storage site for carnitine in the body, is expected to change only slightly even if OCTN2 is upregulated by PPARα activation. Supportive of this assumption is the observation that concentrations of total carnitine in skeletal muscle also did not change in rats and pigs which were starved for 24 h [48, 67]. Further indications with regard to the regulation of carnitine homeostasis by PPARα in humans may be expected to be obtained from clinical studies dealing with pharmacological PPARα agonists (i.e., fibrates). However, according to our literature research no clinical studies investigating the efficacy of different fibrates (gemfibrozil, bezafibrate, fenofibrate, etiofibrate, ciprofibrate) for blood lipid modifying purposes were found that also reported on either plasma or urinary carnitine levels.

5. Evidence for a Role of Other PPAR Isotypes in Regulating Genes Involved in Carnitine Homeostasis

Besides PPARα, two other PPAR isotypes, PPARγ, which is expressed in two different full-length translated isoforms (PPARγ1, PPARγ2), and PPARδ, exist in mammals and birds. The distribution pattern and expression levels of the PPARs show great differences between tissues. Whereas PPARα is highly expressed in tissues with high rates of fatty acid oxidation (liver, kidney, myocardium, skeletal muscle), PPARγ1 is poorly expressed in these tissues. Both PPARα and PPARγ1 are found in cells of the immune system and the vessel wall and in epithelial cells. The adipocyte-specific PPARγ2 isoform is exclusively and highly expressed in adipose tissue. PPARδ is ubiquitously expressed and the predominant PPAR isotype in skeletal muscle. To our knowledge only one study has been published investigating the role of other PPAR isotypes than PPARα on genes involved in either carnitine uptake or carnitine biosynthesis [88]. According to this study the expression of OCTN2 in the colon is upregulated by PPARγ in humans and mice and thereby contributes to local and systemic carnitine homeostasis. Whether PPARγ is also a transcriptional regulator of genes encoding enzymes of the carnitine biosynthesis pathway has not been investigated in this study. In addition, the role of PPARδ in regulating genes involved in carnitine homeostasis has not been addressed so far. However, PPARδ has similar and partially overlapping functions as PPARα, in particular with regard to fatty acid catabolism [89]. For instance, genes encoding proteins of the carnitine shuttle system, such as carnitine-palmitoyltransferase I [90] and carnitine-acylcanitine translocase [91], were shown to be regulated by both PPARα and PPARδ. Thus, it would be not unlikely that PPARδ is also a transcriptional regulator of OCTN2 and genes of the carnitine biosynthesis pathway. This, however, remains to be shown in future studies.

6. Conclusions

Comparison of data from genetic and animal studies with mice, rats, pigs, cows, and laying hens and from human studies on the regulation of genes involved in carnitine homeostasis by PPARα suggests that carnitine homeostasis, which is intrinsically linked with lipid catabolism, is well conserved across different species. This confirms recent observations from genome-wide comparative analysis of gene regulation by PPARα between mouse and human demonstrating that at least the role of PPARα as a master regulator of hepatic lipid catabolism is well conserved [20]. However, despite demonstrating a well conserved role of PPARα as a key regulator of carnitine homeostasis in general, our comprehensive analysis shows that this assumption particularly applies to the regulation of genes involved in carnitine uptake (OCTN2) by PPARα which is obviously highly conserved across species. The highly conserved regulation of OCTN2 by PPARα is possibly explained by the fact that the sequence of the functional PPRE identified in the mouse

	cons. PPRE	TGACCTtTGACCT ACTGGAaACTGGA	
H. sapiens	OCTN2-PPREint1 gt	AGGAGTTATGTGCCCTtTCACCTACTTATATGT	ag 2261–2293
M. musculus	OCTN2-PPREint1 gt	AAGAGTTATATGCCCTtTCACCTACTTACAGGT	ag 1840–1872
R. norvegicus	OCTN2-PPREint1 gt	GAGAGTTATATGCCCTtTCACCTACTTACAGGT	ag 2009–2041
S. scrofa	OCTN2-PPREint1 gt	ACGAGTTGTGTGCCCTtTCACCTACTTACAGGT	ag 2319–2351
B. taurus	OCTN2-PPREint1 gt	AAGAGCTCTGTGCCCTtTCACCTACTTCCAGGT	ag 1707–1739
G. gallus	OCTN2-PPREint1 ge	GAAGCTTACCTGAACTtTGCACTGCAGTGCACT	ag 2143–2175
		* * ** **** ** * *	

Figure 1: Sequence alignment of the functional PPRE in the intron 1 of human, mouse, rat, pig, cattle, and chicken OCTN2. The PPRE, which is comprised of two hexanucleotides separated by a single nucleotide, termed direct repeat 1, is underlined. Matching nucleotides are shown by asterisks. Chromosomal localization, accession number of cDNA, and genomic DNA sequences from Genbank of NCBI are: hOCTN2 chr.5, AF057164 cDNA, AC118464 genomic DNA; mOCTN2 chr.11, BC031118 cDNA, AL596182 genomic DNA; rOCTN2 chr.10, NM_019269 cDNA, AC120085 genomic DNA; sOCTN2 chr.2, AK393575/AK394838/FS677719 cDNA, CU372899 genomic DNA; cOCTN2 chr.7, NM_001046502 cDNA, AC149665 genomic DNA; chOCTN2 chr.13, NM_001045828 cDNA, JH374679 genomic DNA.

	cons. PPRE	AGGTCAaAGGTCA TCCAGTtTCCAGT	
H. sapiens	BBOX1-PPREint2 gt	TACTCTCACCAGAACAaAGGTCCCAGCGTCAAT	ag 13752−13784
M. musculus	BBOX1-PPREpro	TACTCTAATCAGAACAaAGGTCCCGGCATGGGG	−65−−101
R. norvegicus	BBOX1-PPREint1 gt	TGCTCTAAGCAGAACAaAGGTCCCAGCGTTGGT	ag 1307–1339
B. taurus	BBOX1-PPREint1 gt	TACTCTCATCAGAACAaAGGTCCCAGCACAGAT	ag 9997–10029
G. gallus	BBOX1-PPREint1 gt	TAAATGCTCTGGAATGaAGGTCAACCTTAAAAA	ag 2002–2034
		* *** ******	

Figure 2: Sequence alignment of the functional PPRE in the promoters, intron 1 and intron 2, respectively, of human, mouse, rat, pig, cattle, and chicken BBOX1. The PPRE, which is comprised of two hexanucleotides separated by a single nucleotide, termed direct repeat 1, is underlined. Matching nucleotides are shown by asterisks. The BBOX1-PPRE for *S. scrofa* is not shown due to gaps in the first and second intron. Chromosomal localization, accession number of cDNA, and genomic DNA sequences from Genbank of NCBI are: hBBOX1 chr.11, NM_003986 cDNA, AC015756 genomic DNA; mBBOX1 chr.2, NM_130452 cDNA, AL691416 genomic DNA; rBBOX1 chr.3, NM_022629/FQ210746 cDNA, AABR03024937 genomic DNA; cBBOX1 chr.15, NM_001101881 cDNA, genomic DNA; sBBOX1 chr.2, AK393528/AK391112 cDNA. CU694591 genomic DNA; chBBOX1 Chr.5, BX936048 cDNA, JH374511 genomic DNA.

	cons. PPRE	TGACCTtTGACCT ACTGGAaACTGGA	
H. sapiens	ALDH9A1-PPRE	CCTGACACTTTTTCCTgTGGCCTTTGCTCTTCG	−1029−−1068
M. musculus	ALDH9A1-PPRE	TGGAACTGGAAGACCTtTGGCCTAGATAATTAC	−122−−154
R. norvegicus	ALDH9A1-PPRE	GAGGGCTAGAAGCCCTcTGGCCTAGCGGAGAGC	−381−−413
S. scrofa	ALDH9A1-PPRE	ATCGGAGCTGTAGCCAcTGGCCTATGCCAGAGC	−3153−−3185
B. taurus	ALDH9A1-PPRE	CTTTTCTCCTTTGCCTtTCGCCTCTCTTCTTTT	−4778−−4810
G. gallus	ALDH9A1-PPRE	CAAATGCTGCTGCCCTaAGCCCTCAGGCAATAA	−1050−−1082
		** ***	

Figure 3: Sequence alignment of the functional PPRE in the promoter of human, mouse, rat, pig, cattle, and chicken ALDH9A1. The PPRE, which is comprised of two hexanucleotides separated by a single nucleotide, termed direct repeat 1, is underlined. Matching nucleotides are shown by asterisks. Chromosomal localization, accession number of cDNA, and genomic DNA sequences from Genbank of NCBI are: hALDH9A1 chr.1, AK392520 cDNA, AL451074, genomic DNA; mALDH9A1 chr.1, NM_019993, cDNA, AC113970 genomic DNA; rALDH9A1 chr.13, NM_022273 cDNA, AABR06075994 genomic DNA; sALDH9A1 chr.4, AK392520 cDNA, CU468388 genomic DNA; cALDH9A1 chr.3, BC105335 cDNA, AAFC03093575 genomic DNA; chALDH9A1 Chr.8, BU460904 cDNA, JH374592 genomic DNA.

OCTN2 gene is completely identical (100%) between mouse, rat, pig, cattle, and even human (Figure 1). The comparison of studies in pigs with studies in mice and rats, however, shows that the upregulation of OCTN2 in the liver by PPARα activation is clearly stronger in rodents than in pigs, which is in line with the view that nonproliferating species (pig, human, nonhuman primates) generally show a weaker response to PPARα activation than proliferating species (mice, rats). In contrast, regulation of genes involved in carnitine biosynthesis (BBOX1, ALDH9A1) by PPARα appears less well conserved across species, which is demonstrated by the fact that PPARα activation causes upregulation of genes involved in carnitine biosynthesis in mice, pigs and cattle but not in rats and chicken. The reasons underlying these species specificities cannot be simply explained by differences in the PPARα expression level between species because mice and rats, for instance, exhibit comparably high hepatic PPARα expression levels. In the case of BBOX1 differences in the nucleotide sequence of the functional PPRE of the BBOX1 gene between mouse and rat also cannot explain this species specificity because this PPRE shares a complete (100%) sequence identity between mouse and rat and even human and cattle (Figure 2). One factor that may account for the species specificity regarding BBOX1 regulation by PPARα is the different location of the translation start site of the BBOX1 gene between mouse (translation start site in the first exon) and rat (translation start site in the second exon). In addition, a species specific expression pattern of transcriptional coregulators in the liver may be causative for the different regulation of BBOX1 by PPARα between mouse and rat. By contrast, a small discrepancy in the sequence of the functional PPRE of the ALDH9A1 promoter between mouse and rat (one nucleotide in the proximal half site of the PPRE is different) could explain the species specificity regarding ALDH9A1 regulation by PPARα (Figure 3). Nevertheless, multiple factors may be responsible for the different regulation of carnitine biosynthesis by PPARα across different species and, therefore, further research is required to unravel the underlying reasons. Overall, our comparative analysis indicates that PPARα is not only a master transcriptional regulator of fatty acid catabolism, ketogenesis, and gluconeogenesis but also of carnitine homeostasis—a role which is well conserved across species.

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