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M. WIERZBICKI[1], A. CHABOWSKI, M. ZENDZIAN-PIOTROWSKA, J. GORSKI

DIFFERENTIAL EFFECTS OF *IN VIVO* PPAR α AND γ ACTIVATION ON FATTY ACID TRANSPORT PROTEINS EXPRESSION AND LIPID CONTENT IN RAT LIVER

[1]Department of Physiology, Medical University of Bialystok, 15-089 Bialystok, Poland

Peroxisome proliferator-activated receptors (PPAR`s) serve as lipid sensors and when activated modify gene expression of proteins highly involved in the regulation of fatty acid metabolism. Recently, the accumulation of lipids in liver was shown to be depended on the excessive protein-mediated transmembrane transport of long chain fatty acids (LCFAs). The aim of the present study was to determine the *in vivo* effects of PPARα and γ activation at two levels: 1) on the expression of fatty acid transporters, 2) on the content and fatty acids saturation status of lipids in rats liver. PPARα agonist (WY 14,643) treatment upregulated the liver expression of FAT/CD36 (+20%, $p<0.05$) and did not significantly affect the content of FABPpm and FATP-1. Accordingly there was a significant increase in the content of phospholipid (+12%, $p<0.05$), diacylglycerol (+65%, $p<0.05$) and triacylglycerol (+46%, $p<0.05$) fractions followed PPARα activation. In contrast, pioglitazone (PPARγ agonist) had no effect on the content of fatty acid transporters (FAT/CD36, FABPpm and FATP-1) as well as the content of liver lipid fractions with the exception for triacylglycerols, which have been reduced significantly (-89%, $p<0.05$). These findings suggest that *in vivo* PPARα and PPARγ activation exert different effects on both the expression of fatty acid transporters and lipid content in rat's liver.

Keywords: *Peroxisome proliferator-activated receptors, FAT/CD36, FABPpm, FATP-1, lipids, liver*

INTRODUCTION

Long chain fatty acids (LCFAs) on their way from the microvascular compartment to the hepatocytes, first have to pass the capillary endothelium. As outlined in more details elsewhere, in the liver, unlike other tissues such as skeletal muscles or cardiac myocytes, the interendothelial clefts are abundantly present and allow the passage of the albumin-LCFA complexes (1). LCFAs are also present in the portal vein as a compound of chylomicrones, from which are released, and then traffic through the capillary endothelial cells *via* simple diffusion, according to their's concentration gradient (2). However, recent years provided a considerable evidence to support the existence of a saturable, protein-mediated transport process for LCFAs into many tissues, including adipocytes (3, 4), intestine (5, 6), kidney (7), myocytes (8) and liver (9-11). Although controversy still exists as to the role of proteins in facilitating fatty acid uptake into hepatocytes, a recent study by Koonen *at el.* strongly supports the involvement of protein-mediated LCFA transport into the liver cells (12). Currently, at the transcriptional level, several proteins involved in facilitated fatty acid transport have been identified in the liver, including fatty acid translocase (FAT/CD36), plasma membrane associated fatty acid binding protein (FABPpm) and fatty acid transport proteins 1 and 5 (FATP1 and 5) (9-11). Among these, a key role for FAT/CD36 in the hepatic fatty acid transport had been proposed (12) as this appears to be a true for other metabolically

important tissues, such as cardiac and skeletal myocytes (13-15). Further, the role of CD36 in protein mediated fatty acid uptake in liver was strengthening by studies with CD36 deficient mice that had shown liver insulin resistance (12). In a series of studies, in liver, second fatty acid transporter, namely FABPpm was identified by Stremmel and co-workers (16-18) and subsequently, it was also shown that increased expression of FABPpm in the mouse liver is a regulatory mechanism to control fatty acid uptake (19). However, studies of amino acid sequences showed FABPpm to be identical to mitochondrial aspartate aminotransferase (mAspAT) (20), which may complicate the interpretation of the results obtained from the hepatocytes, as mAspAT is highly abundant in liver cells (21). Some debate still exists regarding the role of the third group of putative transport proteins present in liver, specifically, fatty acid transport proteins (FATPs). Concerns are rising because FATPs share considerable sequence homology and domain organization with acyl-CoA synthetases (17, 18). As opposed to facilitating fatty acid transport per se the main function of FATPs might be the long chain fatty acid activation being coupled to fatty acid metabolism (22-24).

The liver is a key metabolic tissue involved in fatty acid utilization and so the tight balance between hepatic fatty acid uptake (protein-mediated or simple diffusion), lipogenesis, lipolysis and fatty acid oxidation must be under precise regulation (25). Peroxisome proliferator activated receptors (PPARs) are ligand-activated transcription factors highly involved in maintaining hepatic lipid homeostasis. Currently, three distinct PPAR isoforms have been identified (PPARα, β/δ and γ), which, in general, govern the expression of genes participating in regulating either fatty acid transport or FA oxidation or lipogenesis (26, 27). Specifically, PPARα had been shown to be present in the liver (at relatively high amounts compared to PPARγ) and several studies indicated that increased activity of this nuclear receptor is associated with enhanced expression of genes involved in hepatic mitochondrial biogenesis and fatty acid oxidation (26, 28, 29). In contrast to the liver cells, the third PPAR isoform, PPARγ is the predominant PPAR type expressed in adipocytes and stimulation of this nuclear receptor results in a stimulation of adipogenesis and lipogenesis (30, 31) and very little is known regarding it`s function in liver.

Given that, it is of particular interest to examine whether *in vivo* activation of PPAR (α and γ) is associated with the changes in the hepatic expression of fatty acid transporters: FAT/CD36, FABPpm and FATP-1. Furthermore, since PPAR activity may be a key factor regulating LCFA flux and subsequent LCFA utilization in the liver, we prompted to investigate the content and fatty acid profile in different lipid fractions in this tissue.

MATERIALS AND METHODS

Male Wistar rats (200-250g) were housed in approved animal holding facilities (at 22°C±2, on a reverse light-dark cycle, with unrestricted access to water and standard laboratory rat chow). This study was approved by the local ethics committee on animal care.

The animals were randomly divided into 3 groups: 1) control (receiving only 0.5 % methylcellulose by an oral gavage, n=10), 2) treated daily for two weeks with a selective PPARα agonist, WY 14,643 (Cayman Chemicals) in a dose of 3 mg/kg of body weight (n=10), 3) treated daily for two weeks with a selective PPARγ agonist, pioglitazone ("Actos", Lilly) in a dose of 3 mg/kg of body weight (n=10). The drugs (WY 14,643 and pioglitazone) were suspended in 0.5 % methylcellulose and administrated by an oral gavage. The animals were anaesthetized by intraperitoneal injection of pentobarbital in a dose of 80 mg/kg of body weight. Samples of the liver were excised, cleaned of blood and immediately freeze-clamped with aluminum tongs precooled in liquid nitrogen and then stored at -80 °C until further analysis.

Lipid analyses

The liver was pulverized in an aluminium mortar precooled in liquid nitrogen. The powder was transferred to a glass tube and lipids were extracted using the Bligh and Dyer method (32). The fractions of total phosholipids (PL), triacylglycerols (TAG), diacylglycerols (DAG) and free fatty acids (FFA) were separated by thin-layer chromatography (TLC) according to van der Vusse *et al*. (33). Individual fatty acid methyl esters were identified and quantified according to the retention times of standards by gas liquid chromatography (Hewlett-Packard 5890 Series II gas chromatograph, with a Varian CP-SIL capillary column [50mx0.25mm internal diameter] and flame-ionization detector [FID] (Agilent Technologies, CA, USA). Total free fatty acid (FFA), diacylglycerol (DAG), phospholipid (PL) and triacylglycerol (TAG) content was estimated as the sum of the particular fatty acid species of the assessed fraction and it was expressed in nanomoles per gram of the tissue. We have also calculated the following indices of fatty acid profile of each lipid fractions examined: saturated fatty acids (SAT), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA).

Protein analyses

Routine Western blotting procedures were used to detect proteins as described previously (34, 35). FAT/CD36 and FATP-1 were detected using commercially available antibody (ab 36977, Abcam EU; I-20, Santa Cruz, CA, respectively) and FABPpm with FABPpm antisera (a gift from Dr Calles-Escandon J). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). The total protein expression of fatty acid transporters was determined in crude membranes of the liver. Proteins were separated using 10% SDS-polyacrylamide gel electrophoresis. Membranes were immunoblotted with primary antibodies. Protein content was determined with bicinchoninic acid method with BSA serving as a protein standard. Signals obtained by Western blotting were quantified by densitometry (Biorad, Poland). The protein expression (Optical Density Arbitrary Units) in control was set to 100 and other experimental groups were expressed relative to the control. Equal protein concentrations were loaded in each lane as also confirmed by Ponceau S staining.

All data are expressed as mean ± SEM. Statistical difference between groups was tested with analyses of variance and appropriate post-hoc tests, or with a Student t-test. Statistical significance was set at $P<0.05$.

RESULTS

General features of the experimental groups

No differences were observed in the whole body weight and the weight of the liver in between animals from control and experimental groups (data not shown). The administration of pioglitazone decreased serum free fatty acid concentration and stimulation of PPAR α (WY 14,643) had no effect on serum FFA (*Table 1*). Treatment with WY 14,643 or pioglitazone did not alter protein expression of PPARα or PPARγ or PGC1α in the liver, although a trend for an increase in PPARα expression has been observed after WY 14,643 administration (data not shown).

Effects of PPARα and PPARγ activation on fatty acid transporter expression (FAT/CD36, FABPpm, FATP-1) in the liver

There was a small, but significant, increase in the expression of FAT/CD36 (+20%, $p<0.05$ *Fig. 1A*) after the administration of WY 14,643. Similarly, pioglitazone treatment resulted also in a small increase in the expression of FAT/CD36, however it did not reach significance level (+8%, $p=0.09$, *Fig. 1*A). Compared to the untreated animals, there was no change in FABPpm and FATP-1 protein expressions in both experimental groups (*Fig 1B*, *C*).

Effects of PPARα and PPARγ activation on lipid content and FA profile in the liver

Liver content of free fatty acids fraction was not affected by either PPARα or PPARγ activation, although pioglitazone treatment, resulted in the reduction of the content of saturated fatty acids (-14%, $p<0.05$) with concomitant increase in monounsaturated FA species (+105%, $p<0.05$) in this lipid fraction (*Table 2*).

Administration of PPARα agonist (WY 14,643) increased the content of liver phospholipids (+ 12%, $p<0.05$), with parallel increases in the saturated (+16%, $p<0.05$) and monounsaturated FA species (+38%, $p<0.05$). In contrast, no significant changes were observed for PPARγ stimulation (pioglitazone) in the content and composition of phospholipid fraction (*Table 3*).

For both treatments (with WY 14,643 or pioglitazone) we observed significant increases in the content of DAG fraction in the liver (+65% and +12%, $p<0.05$, respectively). However, only PPARα agonist induced also changes in the saturation profile of FA species (SAT: +66%; MUFA: +105% n3PUFA:

Table 1. Effects of PPAR agonists on FFA serum content and FA profile (nmol/ml ±SD; n=10)

	Control	PPARα agonist	PPARγ agonist
SAT	**90,05±16,05**	**76,25±4,92**	**51,15±6,11 ***
MUFA	**63,28±10,42**	**66,02±8,13**	**35,66±4,56 ***
PUFA	**44,91±16,29**	**56,61±21,95**	**23,60±6,04 ***
n3 PUFA	**4,58±0,68**	**2,30±0,70 ***	**3,55±0,87**
n6 PUFA	**40,33±16,4**	**54,31±22,23**	**20,04±5,28 ***
TOTAL	**198,25±31,70**	**198,88±20,55**	**110,40±16,11 ***

SAT – saturated fatty acids; MUFA- monounsaturated fatty acids; PUFA- polyunsaturated fatty acids;
* - $p<0.05$ vs control

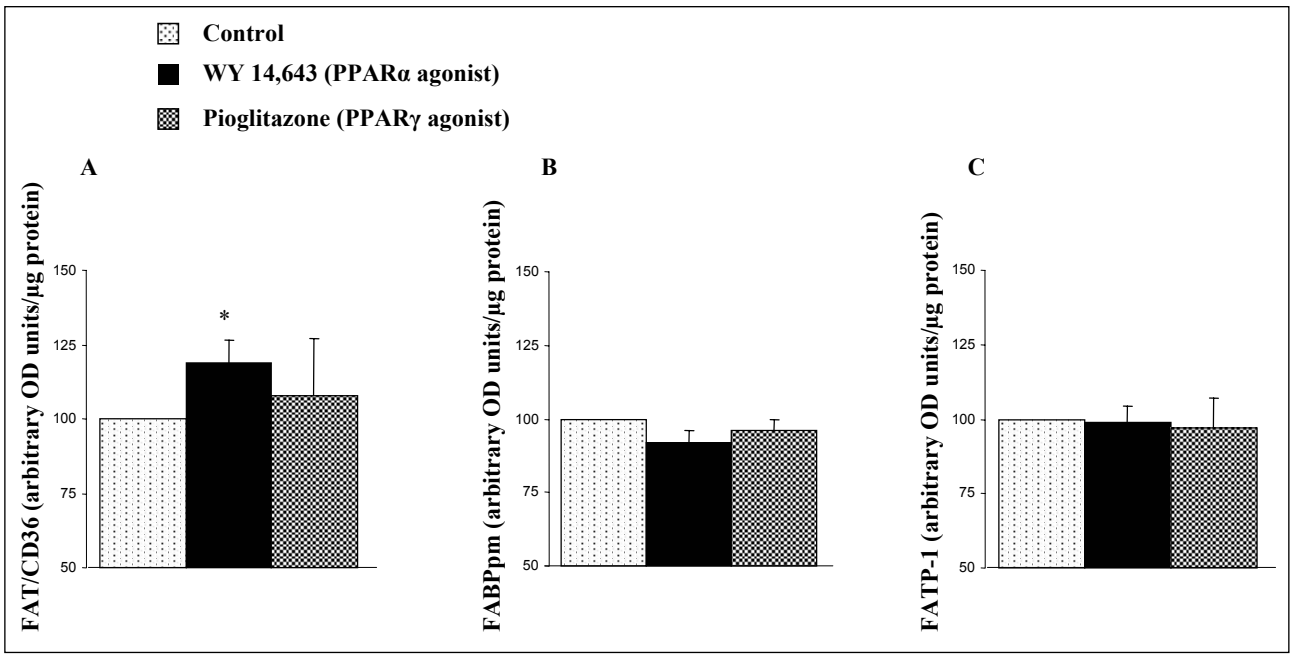

Fig. 1. Effects of PPARα and γ activation on the liver expression of (A) FAT/CD36, (B) FABPpm and (C) FATP-1. Total liver homogenates were prepared as described in Materials and Methods. Data are based on 10 independent determinations for each liver (Optical Density Arbitrary Units ± SD).
*P<0.05, control vs treatment

Table 2. Effects of PPAR agonists on FA`s content and composition in free fatty aids fraction in rat liver (nmol/g ± SD; n=10)

	Control	PPARα agonist	PPARγ agonist
SAT	**166,28±13,40**	**174,04±11,34**	**143,24±6,30***
MUFA	**17,49±2,02**	**20,09±4,23**	**37,45±6,91 ***
PUFA	**27,33±2,73**	**19,65±3,04**	**20,85±3,60**
n3 PUFA	**16,68±2,46**	**7,48±1,60 a**	**5,75±1,00**
n6 PUFA	**10,65±0,98**	**12,17±2,73**	**15,10±3,35**
TOTAL	**211,10±13,01**	**213,78±10,80**	**201,55±6,60**

SAT – saturated fatty acids; MUFA- monounsaturated fatty acids; PUFA- polyunsaturated fatty acids;
* - p<0.05 vs control

Table 3. Effects of PPAR agonists on FA`s content and composition in phospholipid fraction in rat liver (nmol/g ± SD; n=10)

	Control	PPARα agonist	PPARγ agonist
SAT	**22779,01±818,77**	**26489,69±1638,87 ***	**24274,80±369,70**
MUFA	**3163,05±223,51**	**4382,64±741,60 ***	**3499,54±116,21**
PUFA	**22930,92±389,64**	**23622,41±1646,76**	**23472,23±600,06**
n3 PUFA	**5882,85±367,02**	**4042,05±659,24**	**4794,08±293,31**
n6 PUFA	**17048,06±323,49**	**19580,36±1228,06**	**18678,15±493,65**
TOTAL	**48872,97±970,85**	**54494,74±3121,9 ***	**51246,57±764,11**

SAT – saturated fatty acids; MUFA- monounsaturated fatty acids; PUFA- polyunsaturated fatty acids;
* - p<0.05 vs control

+61%, p<0.05, respectively) as pioglitazone had no effect on the composition of DAG-FA's (*Table 4*).

Interestingly, opposite effects of PPARα and PPARγ activation were observed with respect to the content and composition of TAG lipid fraction. Specifically, WY 14,643 treatment induced significant increase in TAG lipid pool (+46%, p<0.05) with parallel increments in the saturated and monounsaturated FA species (SAT: +92%; MUFA: +76% with reduction in n-3PUFA: -68%, p<0.05), whereas pioglitazone (PPARγ agonist) reduced the liver fraction of TAG (-89%, p<0.05) with parallel changes in the saturation status of TAG-FA`s (*Table 5*).

Table 4. Effects of PPAR agonists on FA`s content and composition in diacylglicerols fraction in rat liver (nmol/g ± SD; n=10).

	Control	PPARα agonist	PPARγ agonist
SAT	**217,57±10,66**	**361,89±28,09 ***	**263,18±7,38**
MUFA	**98,24±3,99**	**201,46±20,50 ***	**110,44±7,04**
PUFA	**154,63±9,17**	**213,62±29,53**	**151,95±3,31**
n3 PUFA	**31,64±9,66**	**50,14±11,36 ***	**26,83±1,53**
n6 PUFA	**122,99±7,08**	**163,48±30,06**	**125,12±2,51**
TOTAL	**470,45±12,24**	**776,96±43,14 ***	**525,57±10,09 ***

SAT – saturated fatty acids; MUFA- monounsaturated fatty acids; PUFA- polyunsaturated fatty acids;
* - $p<0.05$ vs control

Table 5. Effects of PPAR agonists on FA`s content and composition in triacylglycerols fraction in rat liver (nmol/g ± SD; n=10)

	Control	PPARα agonist	PPARγ agonist
SAT	**6795,89±803,03**	**13088,47±1516,27 ***	**918,29±82,92 ***
MUFA	**5394,37±964,42**	**9513,79±731,70 ***	**591,34±48,53 ***
PUFA	**4872,66±269,88**	**2364,28±469,17**	**380,59±21,35 ***
n3 PUFA	**983,80±158,86**	**314,41±77,56 ***	**45,92±7,92 ***
n6 PUFA	**3888,86±227,65**	**2049,87±433,67**	**334,67±20,20 ***
TOTAL	**17062,92±1484,17**	**24966,54±1329,1 ***	**1890,22±86,80 ***

SAT – saturated fatty acids; MUFA- monounsaturated fatty acids; PUFA- polyunsaturated fatty acids;
* - $p<0.05$ vs control

DISCUSSION

In our study, chronic PPARγ activation (pioglitazone) significantly decreased serum free fatty acid concentration and stimulation with PPAR α agonist (WY 14,643) had no effect on serum FFA. Previously, rosiglitazone (another PPARγ activator) has been found to reduce serum fatty acid concentration in obese Zucker rats (36), but not in obese mice (37). It has been also reported that treatment with WY 14,643 results in either no change in circulating fatty acids (38) or reduction in NEFAs (Non Estrificated Fatty Acids) (39, 40).

The present study revealed profound effects of chronic, *in vivo*, PPARα and/or PPARγ activation on the hepatic a) expression of fatty acid transporters, whereas other studies measured only transcriptional mRNA (41) and b) lipid content and FA profile in different lipid fractions. This is the first report showing (at the protein level) the induction of hepatic FAT/CD36 expression followed by chronic PPARα activation. Although, the role of FAT/CD36 in participating in fatty acid transport across the plasma membranes has been widely studied in skeletal muscles, heart and adipocytes (13-15), little is known about its function in liver. The first link suggesting a possible role of PPARα activation in the regulation of liver FAT/CD36 has been provided by studies showing FAT/CD36 mRNA upregulation after PPARα activation (41). Based on the present study and others (12), it appears that, the liver expression of FAT/CD36 is highly regulatable, which might not be surprising as it is well recognized that the FAT/CD36 gene contains a peroxisome-proliferator response element (PPRE) in it`s 5 non-coding regions (42). In contrast to PPARα activation, our work demonstrated lack of changes in the hepatic expression of FAT/CD36 followed by chronic PPARγ stimulation, which is quite surprising since Zhou *et al*. showed upregulation of FAT/CD36 transcripts after either PPARα or PPARγ activation (43). However, this discrepancy may be related to the post-transcriptional changes of FAT/CD36 after PPAR stimulation since mature CD36 protein is highly glicolysated and mRNA levels are not always well matched with functional protein expression (44). Possibly, there might be a specific response of hepatic FAT/CD36 protein expression to different PPAR agonists (WY14,643 vs pioglitazone) as occurs for other protein transporters such as FATPs (45). Data from liver cell lines, provided evidence for such a specific regulation of fatty acid transport proteins associated with only PPARα activation (by fibrates) as no change in the liver expression of FATPs after PPARγ stimulation (by BRL 49653) was observed (45). Nonetheless, cited study did not distinguish FATP isoforms that had been upregulated. Apparently, others suggested that FATP-5 is the only functional FATP in liver (46,47) and had shown in FATP-5 null mice to be involved in hepatic lipid metabolism (48). In present work, FATP-1 expression in liver remained not alter with either PPARα or PPARγ stimulation. Similarly to the lack of changes in the expression of FATP-1, protein

content of FABPpm remained constant alongside with *in vivo* PPARγ stimulation as only a trend for the induction of the FABPpm expression after PPARα activation was observed. This may reflect the hypothesis suggesting that FABPpm and FATP-1 play rather a minor role in LCFA transmembrane transport, as it has been implicated for other tissues (i.e. cardiac myocytes and myocytes) (35, 49, 50).

In present study we observed different effects of PPARα vs PPARγ activation especially in the liver content of triacylglycerol (+46% vs -89%, respectively) and diacyloglycerol fractions (+65% vs +12%, respectively). Several studies have dealt with alternations in fatty acid metabolism in liver after PPAR activation showing contradictory results. It was reported that, WY 14,643 treatment results in an accumulation of liver TAG, most probably due to decreased synthesis of VLDL (Very Low Density Lipoproteins) (51). In this study an accumulation of TAG was noticed despite the enhanced rates of LCFA oxidation. Interestingly, in the process of TAG accumulation in the liver a regulatory role for FAT/CD36 had also been shown (12,43). Our present study underscores the fact that, susceptibility to increased intracellular fatty acid transport and subsequent lipid accumulation is to a large extent determined by an increase in the expression of FAT/CD36 (13-15). Similarly, others had also shown that genetic or pharmacologic activation of nuclear receptors such as LXR (Liver X Receptor) or PXR (Pregnane X Receptor) results in the induction of both the expression of FAT/CD36 and TAG accumulation in liver (43). However, lipid accumulation in the liver after PPARα activation might be a surprising finding since a number of studies indicted that PPARα regulates the expression of genes encoding enzymes involved in FA oxidation (26, 28, 29). Gourdriaan *et al*. reported also that CD36 deficient mice display an increased accumulation of lipids in the liver, but this was probably due to increased serum NEFA concentration and rather CD36-independent hepatic LCFA uptake (52). Importantly in present study along with increased accumulation of TAG (and FAT/CD36 upregulation) we observed also the accumulation of PL and DAG fractions in liver followed by PPARα activation. These lipids fractions (TAG, DAG and PL) were also enriched in saturated FA species, which may suggest that, whereas the skeletal muscles become more insulin-sensitive after PPARα activation (53, 54), the liver may reveal insulin-resistance.

In contrast, different effects were observed with *in vivo* PPARγ stimulation. First we confirmed previous observations showing the reduction in plasma NEFA concentration (55, 56), but we also observed a reduction in liver TAG content. Likely explanation would involve the fact that the availability of circulating LCFAs represents a major precursor for TAG synthesis in liver (57). If so, possibly, *in vivo* PPARγ treatment targets mainly adipocytes which abundantly express PPARγ receptors (as in liver cells PPARα is the predominant PPAR isotype) and because PPARγ stimulation elicits the differentiation of adipocytes (53), increases FA uptake into adipocytes and promotes their storage as TAGs (58) the reduced plasma NEFA levels are observed along with PPARγ stimulation, which in turn might be the reason for a decrease in hepatic TAG fraction. The other mechanism that might be responsible for observed reduction of TAG fraction in the liver cells possibly involves activation of HSL (Hormone Sensitive Lipase) as recently Deng T *et al.* had shown increase in hepatic HSL mRNA after PPARγ activation (58). However, controversy still exists regarding the abundance and the activity of liver HSL and likely other lipases are also involved in this process (i.e. DAGT1) (59).

In present study we have provided data regarding the effects of PPAR α and γ stimulation on the expression of fatty acid transporters and lipid profiles in the liver. We have demonstrated the upregulation of FAT/CD36 with parallel increases in the content of phospholipid, diacylglycerol and triacylglycerol lipid fractions but only followed PPARα activation. In contrast, PPARγ stimulation had no effect on either fatty acid transporters expression or the content of lipids in the liver, but reduced triacylglycerols content.

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Author's address: A. Chabowski, M.D., PhD, Department of Physiology, Medical University of Bialystok, Mickiewicza 2C street, 15-089 Bialystok, Poland; Phone: (085) 7485588; Fax: (085) 7485585; email: adrian@amb.edu.pl