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Insulin and oleic acid increase PPARγ2 expression in cultured mouse hepatocytes ☆

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Abstract

Hepatic PPARγ expression is increased in several animal models of diabetes and obesity, and liver-specific overexpression of PPARγ induces liver steatosis. The aim of this study was to investigate the regulation of PPARγ expression in primary mouse hepatocytes. PPARγ2, but not PPARγ1, was up-regulated by insulin and to a lesser extent by oleic acid. Insulin increased transcription of the PPARγ2 gene via phosphatidylinositol 3-kinase activation. The PPARγ agonist, rosiglitazone, increased PPARγ2 expression, but not PPARγ1, only in the presence of insulin. Also aP2 mRNA expression was induced by rosiglitazone to a higher degree in the presence of insulin, while acyl-CoA oxidase was increased independently of insulin. In summary, PPARγ2 is increased in hepatocytes by oleic acid and insulin. These results may help to understand the regulation of PPARγ expression in liver, which possibly plays a role in the development of liver steatosis.

Keywords: Primary hepatocytes; PPARγ; PPARγ2; Insulin; Oleic acid; PI3-kinase; Rosiglitazone; Liver steatosis

Peroxisome proliferator-activated receptor γ (PPARγ) is a ligand-activated transcription factor that plays a key role in adipocyte differentiation [1,2]. Natural ligands for PPARγ are fatty acids [3,4], 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 [3,5,6], and nitrolinoleic acid [7]. Synthetic ligands, used in clinic to treat type 2 diabetes patients, are the insulin sensitizing thiazolidinediones (TZD) [1].

PPARγ is present as two isoforms, PPARγ1 and PPARγ2, that are generated by alternate promoter usage and splicing, resulting in 30 additional amino acids in the N-terminal of the PPARγ2 protein [8–10]. PPARγ2 is expressed mainly in adipocytes, whereas PPARγ1 is ubiquitously expressed [10–14]. We and others have shown that hepatic PPARγ2 expression is increased in *ob*/*ob* mice [15–17], lipoatrophic A-ZIP/F-1 mice [18], and in PPARα null mice [19]. Furthermore, a number of studies have demonstrated elevated levels of PPARγ (isoform not specified) in the liver of other models of diabetes [20–22]. The signals that increase the PPARγ expression in the liver of diabetic and obese mice models are unknown.

The physiological role of hepatic PPARγ expression has been studied in several models. In PPARα null mice, liver specific PPARγ1 overexpression resulted in increased mRNA expression of adipogenic and lipogenic genes, and liver steatosis [23]. In addition, PPARγ2 overexpression in AML 12 hepatocytes gave rise to increased de novo triacylglycerol synthesis and lipid accumulation [24]. In *ob*/*ob* mice [25] and in lipoatrophic A-ZIP/F-1 mice [18], liver specific PPARγ disruption was shown to alleviate liver steatosis. Together these results indicate a significant role for PPARγ in the development of fatty liver. Apart from the effects on liver triglycerides, hepatic PPARγ has been

☆ *Abbreviations:* PPAR, peroxisome proliferator-activated receptor; PI3-kinase, phosphatidylinositol 3-kinase; CREB, cAMP responsive element binding protein; TZD, thiazolidinedione(s); 36B4, acidic ribosomal phosphoprotein P0; ACO, acyl-CoA oxidase; PPRE, PPAR response element.

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shown to be important for clearance of plasma triglycerides [18,25].

In human, mouse, and rat adipose tissue, PPARγ expression has been shown to be regulated by nutritional signals [13,14,26]. In mouse, both isoforms were decreased by fasting, whereas high-fat diet resulted in increased PPARγ2 levels in adipose tissue [13]. Diabetic insulin-deficient mice have been shown to have decreased PPARγ levels in adipose tissue that were restored by insulin replacement [13]. Also in isolated human adipocytes, PPARγ expression was increased by insulin an effect that was potentiated by corticosteroids [14,27]. The PPARγ expression in mouse liver has been shown to be up-regulated by high-fat diet [13], cholesterol [28,29], and *trans*-10, *cis*-12-conjugated linoleic acid [30]. Moreover, PPARγ1 expression in mouse liver was repressed by cAMP-responsive element binding protein (CREB) [31].

To gain more insight into the mechanism, whereby PPARγ expression is induced in liver, we sought to identify factors that directly affect the PPARγ expression in primary mouse hepatocytes. A secondary aim was to study the effect of rosiglitazone at conditions where PPARγ expression was induced.

Materials and methods

Animals. C57BL/6 mice were from Taconic Europe (Ry, Denmark). The animals were maintained under standardized conditions of temperature (21–22 °C) and humidity (40–60%), with light from 06.00 h to 18.00 h for at least 1 week before the experiments. The study protocol was approved by the Ethics Committee of Göteborg University. All experiments were conducted in accordance with accepted standards of humane animal care.

Primary hepatocyte cultures. Mouse hepatocytes were obtained by non-recirculating collagenase perfusion through the portal vein of male C57BL/6 mice (between 10 and 16 weeks of age) as described [32]. In brief, the cells were seeded at 100,000 cells/cm^2 in dishes (Falcon, Plymouth, UK) coated with laminin-rich Matrigel (BD Biosciences, Bedford, MA). The cells were cultured during the first 16–19 h in Williams' E medium with Glutamax (Invitrogen, Carlsbad, CA) supplemented as described [32]. The cells were then treated up to 72 h with 500 μM oleic acid (Sigma, St. Louis, MO), 30 nM insulin (Actrapid, Novo Nordisk A/S, Denmark) or 28 mM glucose (Sigma) in Williams' E medium with Glutamax supplemented with 0.75% albumin (Sigma), penicillin (50,000 IU/L), streptomycin (50 mg/L; Life Technologies), 0.28 mM sodium ascorbate (Sigma), and 0.1 μM sodium selenite (Sigma). In some experiments, primary mouse hepatocytes were exposed to 1 or 10 μM rosiglitazone (Medicinal Chemistry, AstraZeneca R&D, Mölndal, Sweden) or 10 μM Wy14,643 (Chemsyn Science Laboratories, Lenaxa, KS), each dissolved in dimethylsulfoxide (DMSO, final concentration 0.15% v/v) and diluted in medium supplemented as above with or without 30 nM insulin. In experiments with actinomycin D (5 μg/ml, dissolved in DMSO, final concentration 0.15%), cells were plated overnight before addition of either control medium or medium containing 30 nM insulin. After 7 h incubation, actinomycin D was added to control cells and insulin incubated cells, and incubation proceeded for 6 h before cells were collected for RNA preparation. The kinase inhibitors used were: Herbimycin A (2 μM), LY294002 (30 μM), and U0126 (10 μM) (all from Sigma), all dissolved in DMSO, final concentration 0.15%. After plating overnight, cells were incubated in control medium containing the inhibitors above for 30 min, before addition of control medium or insulin (final concn, 30 nM). After 13 h incubation, cells were collected for RNA preparation.

Quantitative real-time PCR analysis. Total RNA of cultured primary hepatocytes was isolated with TriReagent (Sigma, St. Louis, MO) according to the manufacturer's protocol and the concentration of RNA was determined spectrophotometrically at 260 nm. To remove contaminating DNA, total RNA was treated with DNA free (Ambion, Austin, TX) before retrotranscribed using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA). Quantitative real-time PCR analyses were performed on ABI PRISM 7700 Sequence Detection System (96 wells) or ABI PRISM 7900HT Sequence Detection System (384 wells) (Applied Biosystems) using SYBR Green detection chemistry. All samples were analyzed in triplicate. To exclude that the amplification-associated fluorescence was associated with residual genomic DNA and/or from the formation of primer dimers, controls without reverse transcriptase or DNA template were analyzed. RT-PCR products were analyzed by electrophoresis in ethidium bromide-stained agarose gel to check that a single amplicon of the expected size was obtained. The expression data were normalized to the endogenous control acidic ribosomal phosphoprotein P0 (36B4). The expression of 36B4 was not influenced by the various treatments in this study. Specific primers for each gene (Table 1) were designed using Primer Express software (Applied Biosystems).

Statistical analyses. Values are expressed as means ± SE. Comparisons between groups were made by Kruskal–Wallis test followed by Mann–Whitney *U* test. A value of $p < 0.05$ was considered statistically significant.

Results

Effects of high glucose, oleic acid, and insulin on PPARγ mRNA expression in primary mouse hepatocytes

The hepatic expression of PPARγ has previously been shown to be increased in several mice models of obesity and diabetes [15–18,20–22]. In this study, we aimed to identify factors that directly up-regulate the PPARγ expression in isolated mouse hepatocytes. We studied the effects of 3 days of incubation with oleic acid (500 μM), insulin (30 nM), and high concentration of glucose (28 mM) on hepatic PPARγ1 and PPARγ2 mRNA expression. PPARγ1 expression was not significantly influenced by any of the treatments (Fig. 1A), whereas the PPARγ2 expression was increased both by insulin and to a lesser

Table 1
Primers used for quantitative real-time PCR analyses

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
PPARγ1	GCGGCTGAGAAATCACGTTC	GAATATCAGTGGTTCACCGCTTC
PPARγ2	AACTCTGGGAGATTCTCCTGTTGA	GAAGTGCTCATAGGCAGTGCAT
Albumin	GATCGCCCATCGGTATAATGA	GGCAATCAGGACTAGGCCTTT
aP2	TTCGATGAAATCACCGCAGA	AGGGCCCCGCCATCT
ACO	CAGCAGGAGAAATGGATGCA	GGGCGTAGGTGCCAATTATCT
36B4	GAGGAATCAGATGAGGATATGGGA	AAGCAGGCTGACTTGGTTGC

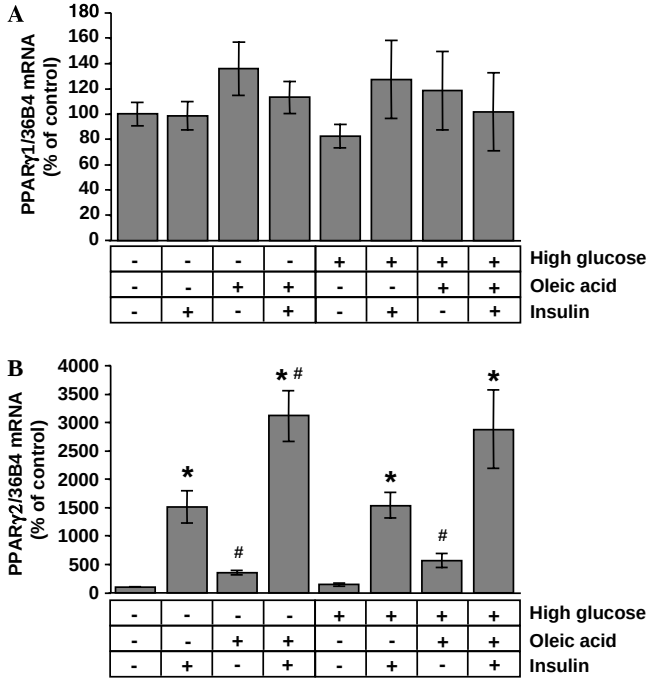

Fig. 1. Effects of insulin on PPARγ1 (A) and PPARγ2 (B) mRNA expression in primary mouse hepatocytes. Hepatocytes were isolated from C57BL/6 mice by liver perfusion and cultured for three days with or without high glucose (28 mM), oleic acid (500 μM) or insulin (30 nM). mRNA expressions were quantified by quantitative real-time PCR. Values are means ± SE, based on three independent liver perfusions with two culture dishes in each group. $^{*}p < 0.05$, insulin vs respective control group, $^{\#}p < 0.05$, oleic acid vs respective control group. Kruskal–Wallis test, followed by Mann–Whitney *U* test.

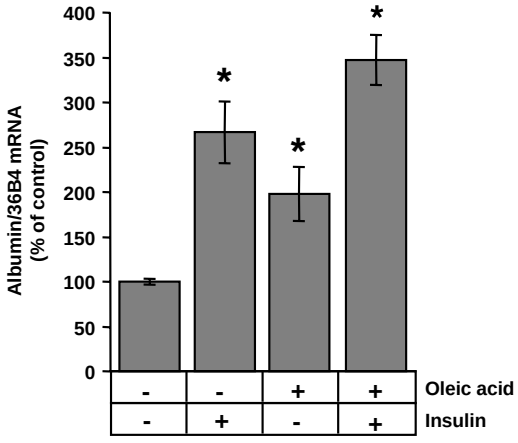

Fig. 2. Effects of insulin on albumin mRNA expression in primary mouse hepatocytes. Hepatocytes were isolated from C57BL/6 mice by liver perfusion, and cultured for three days in medium containing oleic acid (500 μM) and/or insulin (30 nM). mRNA expressions were quantified by quantitative real-time PCR. Values are means ± SE, based on three independent liver perfusions with two culture dishes in each group. $^{*}p < 0.05$, insulin and/or oleic acid vs control. Kruskal–Wallis test, followed by Mann–Whitney *U* test.

extent by oleic acid (Fig. 1B). Insulin and oleic acid in combination resulted in an additive effect on PPARγ2 mRNA expression. Increased concentration of glucose (11 mM vs 28 mM) did not affect the PPARγ2 expression. Furthermore, the PPARγ expression level was not affected by 1 nM dexamethasone nor was the effect of insulin or oleic acid influenced by the addition of dexamethasone (data not shown).

Effects of insulin and oleic acid on albumin expression

To explore that the cells kept the hepatic phenotype after exposure to insulin and/or oleic acid, mRNA expression of albumin was measured. The albumin expression increased by incubation with insulin and/or oleic acid (Fig. 2), indicating that the cells did not de-differentiate by the treatment.

Dose–response and time-course of the effects of insulin on PPARγ2 mRNA expression

PPARγ2 mRNA expression was determined in primary mouse hepatocytes after three days of exposure to different concentrations of insulin. Insulin dose-dependently increased the expression of PPARγ2, reaching a maximum level at 30 nM (Fig. 3A). Next, we determined the time-course of the effect of insulin on PPARγ2 mRNA expression by incubating the cells with 30 nM insulin for 6, 24, 48, and 72 h. The PPARγ2 expression was not significantly affected after 6 h, while 24 h exposure resulted in increased PPARγ2 expression that was stable up to 72 h exposure (Fig. 3B). We also measured the PPARγ2 expression in freshly isolated mouse hepatocytes. The expression in freshly isolated cells was comparable to the levels in hepatocytes exposed to 3–30 nM insulin (not shown).

Effects of insulin on transcription of the PPARγ2 gene

PPARγ2 mRNA was induced between 6 and 24 h of incubation with insulin (as shown in Fig. 3B). Therefore, we incubated primary mouse hepatocytes with insulin for 13 h and the transcriptional inhibitor actinomycin D for the last 6 h. Addition of actinomycin D abolished the increase in PPARγ2 expression induced by insulin, while it had no effect on control cells (Fig. 4). This finding indicates that insulin increases PPARγ2 mRNA expression via activation of transcription.

Effects of inhibitors of insulin signaling on PPARγ2 mRNA

Next, we addressed the question which pathway in the insulin signaling cascade might be involved in the stimulatory effect of insulin on PPARγ2 mRNA transcription. Mouse hepatocytes were incubated with herbimycin A (tyrosine kinase inhibitor), LY294002 (PI3-kinase inhibitor) or U0126 (MAP kinase kinase inhibitor) and insulin for 13 h. As expected, the tyrosine inhibitor Herbimycin A was shown to inhibit the expression of the PPARγ2 gene (Fig. 5). The effect of insulin

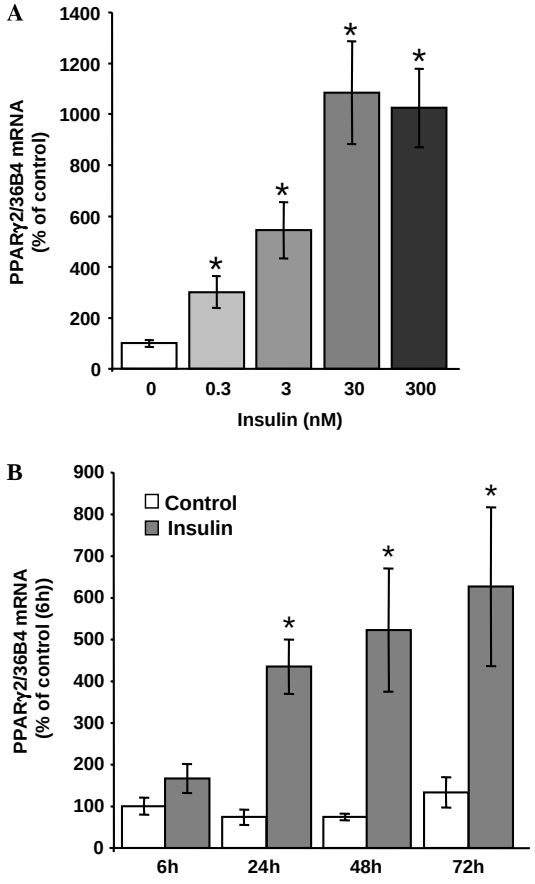

Fig. 3. Dose–response (A) and time-course (B) of the effect of insulin on PPARγ2 mRNA expression in primary mouse hepatocytes. Hepatocytes were isolated from C57BL/6 mice by liver perfusion as described in Materials and methods. (A) Hepatocytes were cultured for three days in the presence of insulin concentrations indicated in the figure. (B) After plating overnight, hepatocytes were incubated in medium containing 30 nM insulin. Cells were collected for RNA preparation at the time points indicated in the figure. PPARγ2 mRNA expression was quantified by quantitative real-time PCR. Values are means ± SE, based on two independent liver perfusions with two culture dishes in each group. *$p < 0.05$, control vs insulin. Kruskal–Wallis test, followed by Mann–Whitney U test.

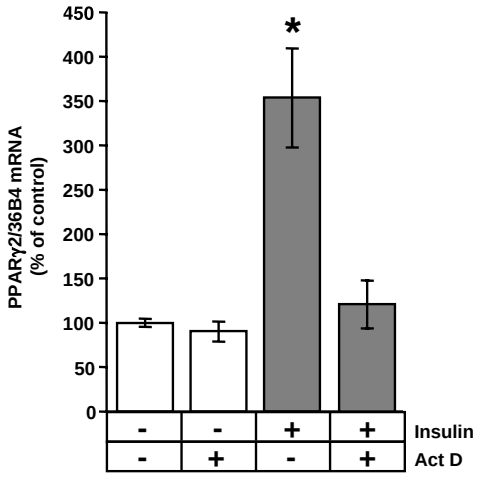

Fig. 4. Effect of actinomycin D on PPARγ2 mRNA expression in primary mouse hepatocytes incubated with insulin. Hepatocytes were isolated from C57BL/6 mice by liver perfusion. After plating overnight, cells were incubated with 30 nM insulin for 13 h. Actinomycin D (5 μg/ml) was added to the cell cultures during the last 6 h of the insulin incubation. PPARγ2 mRNA levels were measured by quantitative real-time PCR. Values are means ± SE, based on two independent liver perfusions with three culture dishes in each group. *$p < 0.05$, Kruskal–Wallis test followed by comparison between the control group and the other groups using Mann–Whitney U test.

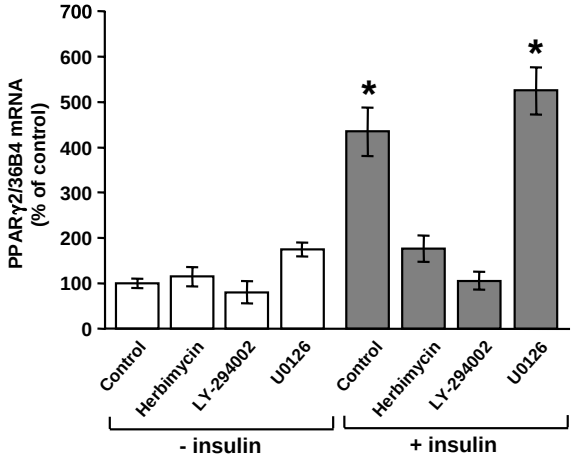

Fig. 5. Effects of kinase inhibitors on the stimulatory effect of insulin on PPARγ2 mRNA expression. Hepatocytes were isolated from C57BL/6 mice by liver perfusion. After plating overnight, cells were incubated with Herbimycin A (2 μM), LY294002 (30 μM) or U0126 (10 μM) for 30 min before the addition of control medium or insulin medium (final concn 30 nM). After 13 h incubation, cells were collected for RNA preparation. PPARγ2 mRNA levels were measured by quantitative real-time PCR. Values are means ± SE, based on three independent liver perfusions with three culture dishes in each group. *$p < 0.05$, Kruskal–Wallis test followed by comparison between the groups with the same kinase inhibitor treatment with and without insulin using Mann–Whitney U test.

on PPARγ2 mRNA expression was shown to be mediated by PI3-kinase activation and not via MAP kinase kinase since LY294002 and not U0126 blocked the stimulatory effect of insulin on PPARγ2 mRNA expression (Fig. 5).

Importance of insulin for effects of rosiglitazone and WY14,643 on gene expression

To investigate if insulin could influence the effect of rosiglitazone on gene expression in primary mouse hepatocytes, we exposed cells to rosiglitazone and 30 nM insulin for three days. The PPARα agonist Wy14,643 was also included in the experiment as positive control regarding effects on the expression of aP2 and acyl-CoA oxidase (ACO). In the presence of insulin, rosiglitazone (10 μM) increased the PPARγ2 expression 3-fold, whereas the expression was unaltered in the absence of insulin (Fig. 6A). Wy had no effect on PPARγ2 expression (data not shown). The PPARγ1 expression was not affected by rosiglitazone or Wy14,643 (data not shown). The expression of aP2 in primary hepatocytes was markedly

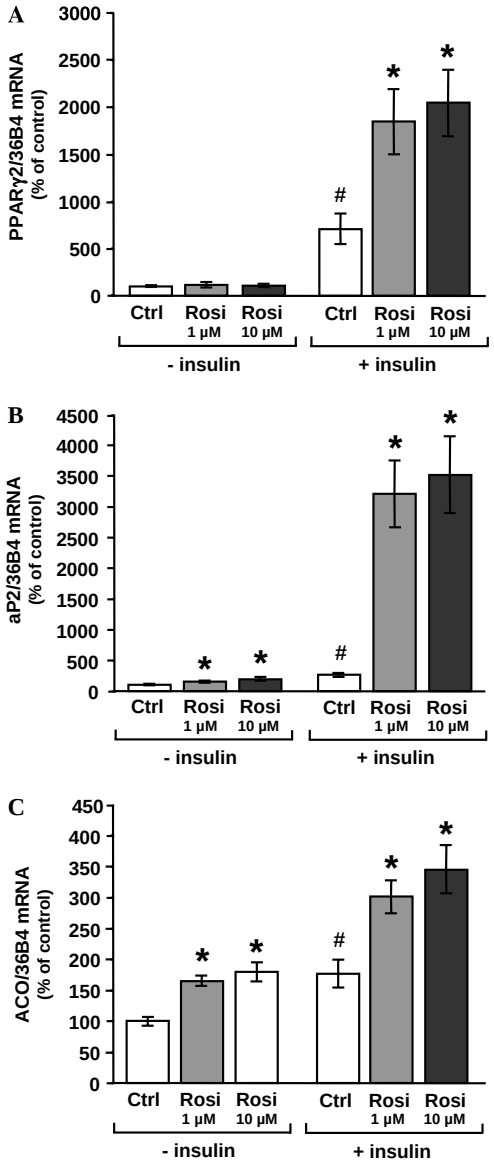

Fig. 6. Effects of rosiglitazone and insulin on PPARγ2 (A), aP2 (B), and ACO (C) mRNA expression in primary mouse hepatocytes. Hepatocytes were isolated from C57BL/6 mice by liver perfusion and cultured for three days in medium with 10 μM rosiglitazone ± 30 nM insulin. mRNA expressions were quantified by quantitative real-time PCR. Values are means ± SE, based on three independent liver perfusions with two culture dishes in each group. $^{*}p < 0.05$, control vs rosiglitazone without insulin or control vs rosiglitazone with insulin, $^{\#}p < 0.05$, control without insulin vs control with insulin. Kruskal–Wallis test, followed by Mann–Whitney *U* test.

increased by Wy14,643 both in the absence and presence of insulin, 60-fold and 30-fold, respectively (data not shown), whereas the increase in aP2 expression by rosiglitazone (10 μM) was more pronounced in the presence of insulin (13-fold) as compared to a 2-fold increase without insulin (Fig. 6B). The expression of ACO, an enzyme taking part in the peroxisomal fatty acid β-oxidation, was enhanced 20-fold by 10 μM Wy14,643 (data not shown) and 2-fold by rosiglitazone independently of insulin (Fig. 6C).

Discussion

Several studies have shown that diabetic and lipoatrophic mice have steatotic livers in parallel with an increased expression of PPARγ [15–18,20–22]. Liver-specific overexpression of PPARγ in mice showed that increased PPARγ level was followed by increased expression of lipogenic and adipogenic genes, and development of liver steatosis [23]. However, liver steatosis per se, induced by fasting or choline deficient diet, did not induce the PPARγ expression in mouse liver [23]. Thus, specific signals in the diabetic and insulin resistant state probably induce PPARγ that in turn could take part in the development of liver steatosis that characterizes these conditions. Interestingly, a recently published paper demonstrated that PPARγ expression was elevated in livers of patients with non-alcoholic fatty liver disease [33], a condition associated to the metabolic syndrome [34]. This finding indicates that increased hepatic PPARγ expression is not only associated with insulin resistance in mouse.

We addressed the question, which factors that might be of importance for the induction of PPARγ expression in liver. By using primary mouse hepatocytes, we show that insulin and oleic acid up-regulate the expression of the PPARγ2 isoform at the cellular level, whereas the expression of PPARγ1 is unaffected. The fact that only the PPARγ2 isoform is induced is in line with data from studies on *ob*/*ob* mice and lipoatrophic mice, which showed a specific increase in the hepatic expression of PPARγ2. In these models, the plasma insulin levels are markedly increased as compared to the control mice [15,22], which may explain the increased PPARγ2 expression. Moreover, dietary *trans*-10, *cis*-12 conjugated linoleic acid has been shown to induce the PPARγ expression and also in this animal model the insulin levels were markedly increased [30]. Insulin has previously been shown to induce the PPARγ expression in human adipose tissue, both in vivo and in vitro [27]. However, both isoforms were up-regulated in adipose tissue, which may indicate a tissue-specific regulation of the two isoforms.

PPARγ2 has been described to be the adipocyte-specific isoform, whereas the PPARγ1 isoform is more ubiquitously expressed [10,11]. In mouse hepatocytes (C57BL/6) cultured without insulin and oleic acid for three days, the PPARγ2 expression was very low, approximately 1/50 of the PPARγ1 expression. However, in cells exposed to insulin and oleic acid, the expression of PPARγ2 increased to approximately 1/5 of the PPARγ1 expression (data not shown). In vivo studies have shown that PPARγ2 is the predominant form in livers of lipoatrophic mice [18] and in *ob*/*ob* mice [15], which might indicate that prolonged exposure to insulin and/or fatty acids or other factors in the in vivo situation is required for a more marked induction of the PPARγ2 gene.

Since only the PPARγ2 isoform was affected by insulin and oleic acid, a key question is whether there are functional differences between the PPARγ1 and PPARγ2 receptor. In one study, a ligand-independent activation domain in the N-terminal of PPARγ was identified, and reporter

activation of PPARγ2 was 5- to 10-fold greater than that of PPARγ1 [35]. In addition, using PPARγ null fibroblasts, PPARγ2 was shown to be a more potent inducer of adipogenesis at limiting ligand concentrations [36]. This difference in potency correlated with better binding of PPARγ2 to a specific coactivator complex [36]. Thus, when PPARγ2 is expressed at a level that is 1/5 of the PPARγ1 expression, the receptors are probably similarly important for PPARγ signaling. In addition, selective disruption of PPARγ2 in mice resulted in decreased levels of lipogenic and adipogenic genes, decreased adipose mass, and impaired insulin sensitivity [37]. These studies indicate that there are differences between the function of the PPARγ isoforms, which may imply a specific role of PPARγ2 in the liver.

We also show in this study that the stimulatory effect of insulin on PPARγ2 gene transcription is mediated via activation of the PI3-kinase pathway. This finding is consistent with the insulin-induced transcription of SREBP-1 in rat hepatocytes [38], which also has been shown to be mediated via activation of the PI3-kinase pathway [39–42]. Thus, insulin signaling in liver cells may lead to both increased PPARγ2 expression and increased levels of PPARγ ligands since SREBP-1 has been shown to control the production of endogenous ligands for PPARγ [43].

The importance of insulin for the effects of rosiglitazone on the expression of PPARγ2, aP2, and ACO in mouse hepatocytes was also investigated in this study. Similar to PPARγ2, the expression of aP2 and ACO was also found to be up-regulated by insulin without addition of rosiglitazone. This might be explained by the fact that PPARγ is activated by insulin in a ligand-independent manner [35], an increased PPARγ2 expression, and/or that SREBP-1 activation increases the amount of endogenous PPARγ ligand [43]. Rosiglitazone and insulin were shown to increase the PPARγ2 and aP2 expression synergistically, whereas the increase in aP2 expression by Wy14,643 exposure was independent of insulin. The expression of aP2 in 3T3-L1 cells has previously been shown to increase synergistically by TZD and insulin incubation [44]. The effect of rosiglitazone on ACO expression was unaffected by insulin. These data may suggest that regulation of lipogenic genes by PPARγ is insulin-dependent in contrast to the regulation of genes that are mainly activated by PPARα. PPAR response elements (PPREs) have been identified in the promoters of both aP2 [11] and ACO [45], but to the best of our knowledge a putative PPRE in the PPARγ2 promoter has not been identified.

To conclude, this is the first study that shows a direct effect of insulin and oleic acid on the regulation of PPARγ expression in hepatocytes. The finding that only the PPARγ2 isoform was induced by insulin and oleic acid helps to understand the observations that the hepatic PPARγ2 expression is increased in several in vivo models of obesity and insulin resistance. Future studies may reveal the mechanism behind the stimulatory effect on insulin on PPARγ2 expression, which might play an important role in the development of fatty liver.

Acknowledgments

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