

## PPAR $\gamma$ agonist-induced alterations in $\Delta$ 6-desaturase and stearoyl-CoA desaturase 1: Role of MEK/ERK1/2 pathway

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**Supported by** A Grant from the Drug Applied Research Center of Tabriz University of Medical Sciences, to Darabi M, Research Projects numbers 89/102 and 90/73

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Received: January 5, 2012 Revised: October 21, 2012

Accepted: October 26, 2012

Published online: April 27, 2013

agonist-induced alterations in  $\Delta$ 6-desaturase ( $\Delta$ 6D) and stearoyl-CoA desaturase 1 (SCD1) in hepatocellular carcinoma cell line HepG2.

**METHODS:** HepG2 cells cultured in RPMI-1640 were exposed to the commonly used ERK1/2 pathway inhibitor PD98059 and PPAR $\gamma$  agonist, pioglitazone. Total RNA was isolated and reverse transcribed from treated cells. Changes in gene expression and metabolites ratio, as activity index for  $\Delta$ 6D and SCD1, were then determined using reverse transcription-polymerase chain reaction and gas liquid chromatography, respectively.

**RESULTS:** The expression of both  $\Delta$ 6D ( $P = 0.03$ ) and SCD1 ( $P = 0.01$ ) increased following PD98059 treatment, with a higher impact on SCD1 (24.5% vs 62.5%). Although pioglitazone increased the mRNA level ( $1.47 \pm 0.10$  vs  $0.88 \pm 0.02$ ,  $P = 0.006$ ) and activity index ( $1.40 \pm 0.07$  vs  $0.79 \pm 0.11$ ,  $P < 0.001$ ) of  $\Delta$ 6D, no such changes have been observed for SCD1 activity index in pioglitazone-treated cells. SCD1 gene expression (+26.4%,  $P = 0.041$ ) and activity index (+52.8%,  $P = 0.035$ ) were significantly increased by MEK inhibition in the presence of pioglitazone, as compared with pioglitazone alone and control cells. However, the response of  $\Delta$ 6D expression and activity index to pioglitazone was unaffected by incubation with PD98059.

**CONCLUSION:** PPAR $\gamma$  and ERK1/2 signaling pathway affect differentially and may have inhibitory crosstalk effects on the genes expression of  $\Delta$ 6D and SCD1, and subsequently on their enzymatic activities.

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**Key words:** Pioglitazone; PD98059;  $\Delta$ 6-desaturase; Stearoyl-CoA desaturase; HepG2 cells

### Abstract

**AIM:** To investigate the effect of MEK/ERK1/2 pathway on peroxisome proliferator-activated receptors (PPAR $\gamma$ )

Saliani N, Darabi M, Yousefi B, Baradaran B, Khaniani MS, Darabi M, Shaaker M, Mehdizadeh A, Naji T, Hashemi M. PPAR $\gamma$  agonist-induced alterations in  $\Delta 6$ -desaturase and stearoyl-CoA desaturase 1: Role of MEK/ERK1/2 pathway. *World J Hepatol* 2013; 5(4): 220-225 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v5/i4/220.htm> DOI: <http://dx.doi.org/10.4254/wjh.v5.i4.220>

## INTRODUCTION

Fatty acid desaturation is a lipid modification process that is critically important in multiple biological functions, such as cell membrane fluidity, signal transduction, differentiation, inflammatory responses and brain development<sup>[1,2]</sup>. Both the  $\Delta 6$ -desaturase ( $\Delta 6D$ ) and stearoyl-CoA desaturase 1 (SCD1) are two important regulatory enzymes in hepatic *de novo* fatty acid synthesis. Activities of these enzymes provide essential precursors for structural cell components and bioactive metabolites such as prostaglandins<sup>[3]</sup>. Altered levels of both SCD1 activity<sup>[4-6]</sup> and  $\Delta 6D$  activity<sup>[7]</sup> have been reported in various human diseases.

The expressions of both enzymes are coordinately regulated and efficiently induced by the addition of thiazolidinediones (TZDs). TZDs are known as agonists of the gamma isoform of the peroxisome proliferator-activated receptors (PPAR $\gamma$ ), a family of nuclear receptors regulating the expression of genes involved in fatty acid metabolism. Indeed, functional PPAR response elements in the promoter region of the  $\Delta 6D$  and SCD1 have been identified<sup>[8,9]</sup>.

Pioglitazone, a member of the TZDs family, is widely used as an antidiabetic agent with glucose-lowering and lipid modifying effects in non-insulin-dependent diabetes mellitus<sup>[10]</sup>. Despite the increasing clinical use, the mechanisms by which pioglitazone exerts its effects are yet relatively unknown.

Alternatively, signaling pathways might modulate the activity of PPAR $\gamma$  to regulate cellular fatty acid desaturation events. We have recently shown that fatty acid content of HepG2 cells is susceptible to inhibition of MEK/ERK1/2 pathway<sup>[11]</sup>. Exposure of cells to the ERK1/2 pathway inhibitor induced an increase in monounsaturated fatty acids (MUFA) and the fatty acid desaturation index. Consistent with these findings, the data of Mauvoisin *et al*<sup>[12]</sup> show that SCD1 expression level is modulated *via* the ERK1/2 signaling. PPAR $\gamma$  agonists and ERK1/2 kinases may also interact in a complex manner with one another. It has been shown that pioglitazone activates ERK1/2 pathway<sup>[13]</sup>. ERK1/2, on the other hand, modulates PPAR $\gamma$  activation by altering protein phosphorylation and gene expression<sup>[14]</sup>. Thus, it is possible that ERK1/2 signaling affects  $\Delta 6D$  and SCD1 expression by altering PPAR $\gamma$  activity.

Based on the importance of PPAR $\gamma$  activity and ERK1/2 signaling in the regulation of cellular lipid, we

tested the effects of pioglitazone and ERK1/2 signaling pathway blockade by PD98059 on  $\Delta 6D$  and SCD1 expression in HepG2 human hepatic cell line.

## MATERIALS AND METHODS

### Materials

Cell culture materials, media, FBS and standard fatty acid methyl esters were obtained from Sigma Chemical Company (St. Louis, MO, United States). Pioglitazone and PD98059 were purchased from Cayman Chemical (Ann Arbor, MI, United States). HepG2 cell line was obtained from the Pasteur Institute Culture Collection in Tehran. The TRIzol reagent for RNA isolation was purchased from Invitrogen (Carlsbad, CA, United States). AccuPower RT PreMix for the first-strand cDNA synthesis was purchased from Bioneer (Daejeon, South Korea). All other chemicals used were of analytical grade.

### Cell culture

HepG2 cells were grown in RPMI1640 containing 10% FBS, L-glutamine (2 mmol/L), penicillin (100 units/mL), and streptomycin (100  $\mu$ g/mL) at 37 °C, 5% CO<sub>2</sub>. The cells were seeded at a density of  $2.5 \times 10^5$ /well in a 6-well plate. After allowing the cells to attach overnight, the medium was replaced with fresh medium containing  $\pm$  pioglitazone (20  $\mu$ mol/L) and PD98059 (20  $\mu$ mol/L). Following 48 h incubation, culture medium was removed; the cell monolayer was washed, and collected for gene expression study and cellular fatty acid measurement.

### RT/PCR analysis

Total RNA was purified with TRIzol reagent. One  $\mu$ g of RNA was reverse-transcribed using the AccuPower cDNA kit as per the manufacturer's instructions. Semiquantitation of the cDNA was performed by RT/PCR using the primer-dropping method according to a previous report<sup>[15]</sup>. Each reverse transcript (*i.e.*,  $\Delta 6D$  and SCD1 cDNA) was amplified with  $\beta$ -actin as an internal control. Under standard conditions of PCR, all the test transcripts were amplified within their log linear phase. The primers used were: D9D: F (5'-CATAATTCCCGACGTGGCTTT-3'), R (5'-AGGTTTGTAGTACCTCCTCTGGAACA-3') (150 bp); D6DF: (5'-CTGCCAACTGGTGGGAATCATC-3'), R (5'-ACAAACACGTGCAGCATGTTC-3') (94 bp),  $\beta$ -actin: F (5'-TGGACTTCGAGCAAGAGATG-3'), R (5'-GAAGGAAGGCTGGAAGAGTG-3') (137 bp). For comparing the amount of PCR product between samples, a gel digitizing software, UVItec (version 11.01), was used for estimating the intensity of each band on the gel. Each experiment was repeated four times. The coefficients of variation (CV) were about 5%-8%.

### Fatty acid analysis

Fatty acid methyl esters were extracted and analyzed for fatty acid composition, as described previously by us<sup>[16]</sup>. Briefly, fatty acid methyl ester derivatives formed from

**Table 1** Effect of pioglitazone on the fatty acid composition of HepG2 human hepatic cells

	Control	Pioglitazone	Pioglitazone + PD98059
14:0 (myristic acid)	2.01 $\pm$ 0.42	2.03 $\pm$ 0.35	1.77 $\pm$ 0.17
16:0 (palmitic acid)	23.12 $\pm$ 1.24 <sup>a</sup>	23.26 $\pm$ 1.11 <sup>a</sup>	19.84 $\pm$ 1.21 <sup>b</sup>
16:1 (palmitoleic acid)	7.44 $\pm$ 1.11	9.60 $\pm$ 1.55	8.52 $\pm$ 1.45
18:0 (stearic acid)	10.44 $\pm$ 1.29	11.12 $\pm$ 1.37	8.78 $\pm$ 1.14
18:1n 9 (oleic acid)	38.72 $\pm$ 1.43 <sup>a</sup>	37.33 $\pm$ 0.80 <sup>a</sup>	44.94 $\pm$ 1.08 <sup>b</sup>
18:2n 6 (linoleic acid)	9.09 $\pm$ 0.27 <sup>a</sup>	6.18 $\pm$ 0.68 <sup>b</sup>	6.09 $\pm$ 0.36 <sup>b</sup>
18:3n 6 (linolenic acid)	0.89 $\pm$ 0.13	0.72 $\pm$ 0.12	0.61 $\pm$ 0.12
20:4n 6 (arachidonic acid)	7.19 $\pm$ 0.79	8.62 $\pm$ 0.58	8.85 $\pm$ 0.85
20:5n 3 (eicosapentaenoic acid)	0.42 $\pm$ 0.13	0.47 $\pm$ 0.21	0.14 $\pm$ 0.10
22:6n 3 (docosahexaenoic acid)	0.68 $\pm$ 0.14	0.67 $\pm$ 0.10	0.46 $\pm$ 0.11
Saturated fatty acids	35.58 $\pm$ 1.29 <sup>a</sup>	36.41 $\pm$ 2.18 <sup>a</sup>	30.39 $\pm$ 2.05 <sup>b</sup>
Monounsaturated fatty acids	46.16 $\pm$ 1.75 <sup>a</sup>	46.93 $\pm$ 2.24 <sup>a</sup>	53.46 $\pm$ 1.53 <sup>b</sup>
Polyunsaturated fatty acids	18.27 $\pm$ 0.61	16.66 $\pm$ 1.44	16.15 $\pm$ 1.22

Cells were incubated with pioglitazone (20  $\mu$ mol/L) and PD98059 (20  $\mu$ mol/L) for 48 h. Lipid extracts were prepared and analyzed by gas liquid chromatography for a comprehensive fatty acid profile. The mean  $\pm$  SD of 3 independent experiments done in duplicate are given. <sup>a</sup> $P$  < 0.05 and <sup>b</sup> $P$  < 0.01 (Tukey's test, <sup>a</sup> $\alpha$  = 0.05). Detection limit was 0.05% of the total area.

isolated cellular lipids were separated on a 60 mm  $\times$  0.25 mm Teknokroma TR-CN100 column using a Buck Scientific model 610 gas chromatograph equipped with a split injector and a flame ionization detector. Helium was used as the carrier gas. The oven temperature program was 170–210  $^{\circ}$ C, 1  $^{\circ}$ C/min, and then isothermal for 45 min. Tridecanoic acid (13:0) was used as internal standard. Peak retention times were identified by injecting known standards.

### Statistical analysis

Data presented are the mean  $\pm$  SD of 3 or 4 separate experiments done in duplicate. Calculation of significance between groups was done according to analysis of variance (ANOVA) with *post hoc* Tukey's tests for multiple comparisons, and a  $P$  < 0.05 was considered statistically significant.

## RESULTS

To determine the effect of ERK1/2 MAPK pathway on  $\Delta$ 6D and SCD1, HepG2 cells were treated with PD98059. PD98059 significantly increased the expression levels of both  $\Delta$ 6D ( $P$  = 0.03) and SCD1 ( $P$  = 0.01). Our data also revealed that ERK1/2 deprivation had a higher impact on SCD1 expression than on  $\Delta$ 6D expression (24.5% *vs* 62.5%; Figure 1).

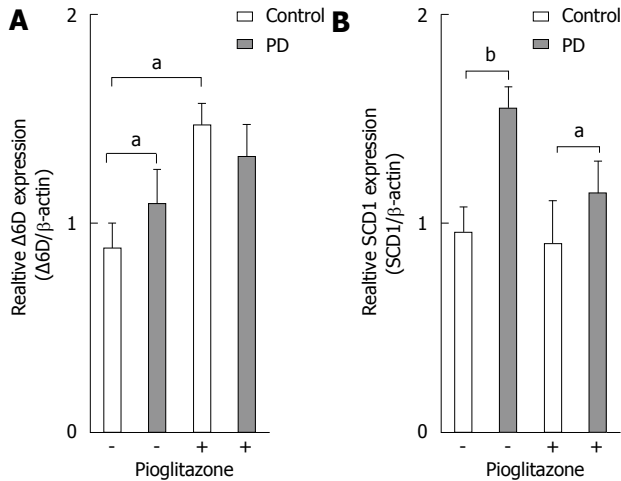
To determine the effect of PPAR $\gamma$  stimulation on  $\Delta$ 6D and SCD1 expression, HepG2 cells were treated with pioglitazone, a PPAR $\gamma$  agonist.  $\Delta$ 6D showed significant increase in mRNA level (1.47  $\pm$  0.10 *vs* 0.88  $\pm$  0.02,  $P$  = 0.006), whereas SCD1 expression did not significantly change ( $P$  = 0.47; Figure 1). We next determined the effect of the PPAR $\gamma$  agonist on fatty acid composition of HepG2 cells (Table 1). The ratios of arachidonic acid (20:4n-6)/linoleic acid (18:2n-6) and oleic acid (18:1n-9)/stearic acid (18:0) were calculated as indices of  $\Delta$ 6 fatty acid desaturase and SCD1 activity, respectively. Incubation with pioglitazone reduced 18:2n-6 levels ( $P$

= 0.001) and increased  $\Delta$ 6D activity index (1.40  $\pm$  0.07 *vs* 0.79  $\pm$  0.11;  $P$  < 0.001). However, no such change has been observed for SCD1 activity index in pioglitazone-treated cells (Figure 2).

Comparison of control with the combined drug condition showed a significant increase in the expression of both  $\Delta$ 6D ( $P$  = 0.02) and SCD1 ( $P$  = 0.04). The expression of  $\Delta$ 6D increased in comparison to the condition which was just treated with PD98059 ( $P$  = 0.032), but comparable to pioglitazone alone. The expression of SCD1 was more than the situation treated with pioglitazone alone (1.15  $\pm$  0.15 *vs* 0.91  $\pm$  0.20,  $P$  = 0.041, Figure 1). Consistent with data from gene expression analyses, MEK inhibition induced a significant increase in SCD1 activity index (+52.82%,  $P$  = 0.035), compared with pioglitazone-treated and control cells. These changes were coupled with significant alteration in fatty acid composition, including increased percentage of MUFA ( $P$  = 0.012) and reduced saturated fatty acids (SFA;  $P$  = 0.018). In addition, the response of  $\Delta$ 6D activity index to pioglitazone was unaffected by incubation with PD98059 when compared to cells incubated with pioglitazone alone (Figure 2).

## DISCUSSION

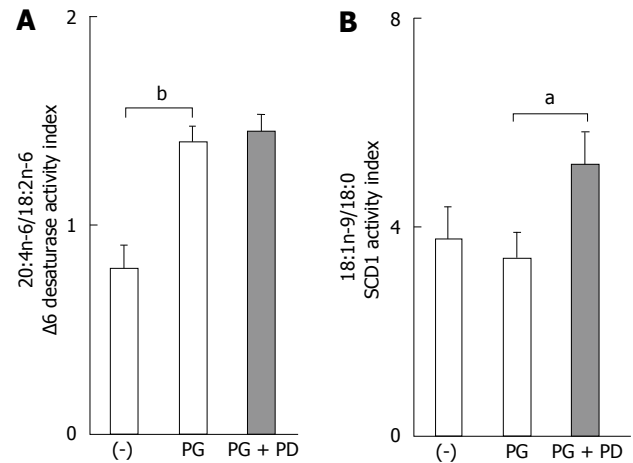
The expression of  $\Delta$ 6D and SCD1 is regulated by complex environmental and hormonal factors<sup>[17,18]</sup>. Activities of these enzymes can affect several hepatic metabolic processes, such as glucose metabolism and membrane permeability, through modulation of cellular fat content. On the other hand, altered lipid content of hepatic cells makes a major contribution in the rate of *de novo* lipogenesis and inducing steatosis<sup>[19]</sup>. Abnormal lipid uptake or *de novo* lipogenesis has been reported in various types of hepatic disorders, which is characterized by increased production of bioactive lipids and an inflammatory response<sup>[20–22]</sup>. In a previous study, we have demonstrated that the fat composition of hepatocellular carcinoma



**Figure 1** Effect of pioglitazone and MEK inhibition on mRNA expression of  $\Delta 6$  desaturase and stearoyl-CoA desaturase. HepG2 cells were incubated for 48 h  $\pm$  20  $\mu$ mol/L pioglitazone with or without 20  $\mu$ mol/L PD98059 (PD) as indicated. Cell lysates were prepared and analyzed by reverse transcription-polymerase chain reaction for expression levels of  $\Delta 6$  desaturase ( $\Delta 6$ D) and stearoyl-CoA desaturase (SCD1). Expression of  $\Delta 6$ D (A) and SCD1 (B) in each lysate were quantified and normalized to the amount of  $\beta$ -actin. The mean  $\pm$  SD of four independent experiments are given.  $^aP < 0.05$  and  $^bP < 0.01$ , Student's *t* test, respectively.

cell line HepG2 was affected by MEK/ERK1/2 signaling. We concluded that MEK/ERK1/2 kinase signaling serves to coordinate fatty acid metabolism in HepG2 cells. In the current study, the expression levels of  $\Delta 6$ D and SCD1 were increased in the presence of MEK inhibitor. These findings are consistent with our earlier report indicating increased 18:1n-9/18:0 and 20:4n-6/18:2n-6 ratios as indices of desaturase activity after inhibition of ERK1/2 signaling. Thus, these enzymatic activities may be under inhibitory control of ERK1/2 signaling. Presumptively, ERK1/2 modulates desaturases expression by implying several molecular mechanisms, including enhanced affinity of transcription factors<sup>[12]</sup>, gene suppression through direct interaction with DNA<sup>[23]</sup>, modulation of transcription factors<sup>[24]</sup>, and MAPK-dependent attenuation of PPAR $\gamma$  transcriptional activity<sup>[14,25]</sup>. Camp *et al.*<sup>[14]</sup> reported that activation of ERK1/2 in adipocytes abrogates both ligand-independent and ligand-dependent activities of PPAR $\gamma$ . Taken together, the regulation of desaturases expression by key transcription factors such as PPAR $\gamma$  could be modulated by ERK1/2 cascade.

PPAR- $\gamma$  has been shown to be critically important in multiple biological functions<sup>[26]</sup>. TZDs, high-affinity synthetic PPAR $\gamma$  agonists, mediate the transcription of PPAR $\gamma$  dependant genes by binding to PPARs as a ligand<sup>[27]</sup>. Herein, we have shown that treatment of HepG2 cells with pioglitazone, a PPAR $\gamma$  agonist, increased both  $\Delta 6$ D mRNA expression and  $\Delta 6$ D activity index whereas had no effect on SCD1. These results led us to speculate that an additional mechanism was at work. Remarkably, it has been illustrated that PPAR $\gamma$  agonists not only can function in a PPAR $\gamma$  dependent



**Figure 2** Effect of pioglitazone on derived fatty acid indices of HepG2 human hepatic cells. A:  $\Delta 6$  desaturase activity index (20:4n 6/18:2n 6); B: Stearoyl-CoA desaturase 1 (SCD1) activity index (18:1n 9/18:0). Cells were incubated with pioglitazone (PG) (20  $\mu$ mol/L) and PD98059 (PD; 20  $\mu$ mol/L) for 48 h. Data are mean  $\pm$  SD,  $n = 3$ .  $^aP < 0.05$  and  $^bP < 0.01$ , Tukey's test,  $\alpha = 0.05$ . 18:0, stearic acid; 18:1n 9, oleic acid; 18:2n 6, linoleic acid; 20:4n 6, arachidonic acid.

manner but also are capable of activating ERK1/2 pathway independently of PPAR $\gamma$ <sup>[28]</sup>. Accordingly, Kempná *et al.*<sup>[13]</sup> demonstrated that pioglitazone activates ERK1/2 MAPK pathway in NCI-H295R cells. Presumably, no changes in SCD1 expression could be attributed to equal and opposite effects of pioglitazone *via* PPAR $\gamma$  dependent and PPAR $\gamma$  independent mechanisms. In accordance with our results, the administration of pioglitazone to Zucker obese rats did not affect the mRNA level of SCD1<sup>[29]</sup>. However, treatment of rats fed a high-sucrose diet with TZDs decreased significantly the hepatic SCD1 mRNA expression<sup>[30]</sup>. In this context, TZDs have also been reported to significantly reduce  $\Delta 6$ D mRNA level<sup>[31,32]</sup>. These contradictory findings might be due to differences in applying TZDs, types of cell lines, tissues and animal models<sup>[33,34]</sup>.

Cotreatment of HepG2 cells with pioglitazone and ERK1/2 inhibitor PD98059 resulted in enhanced rather than additive or synergistic expression. It is of particular interest that activated ERK1/2 MAPK pathway *via* pioglitazone in a PPAR $\gamma$  independent manner could also occur without phosphorylation of upstream MEK<sup>[13]</sup>. So, this underscores pioglitazone ability in activation of ERK1/2 MAPK pathway through other additional pathways. Accordingly, in presence of both PD98059 and pioglitazone the ERK1/2, which may be activated independently of MEK, could prevent synergistic increase of SCD1 expression, and subsequently SCD1 activity. Studies in humans have reported that increased expression of SCD1 may protect against insulin resistance<sup>[35,36]</sup>. The fact that a combination treatment using the PPAR $\gamma$  agonist pioglitazone and the MEK/ERK1/2 inhibitor was more efficient at inducing SCD1 than pioglitazone alone suggests that TZDs along with MEK/ERK1/2 inhibition may be therapeutically beneficial for insulin



resistance related to type 2 diabetic patients.

To our knowledge, this study is the first study to examine the combined effect of PPAR $\gamma$  agonist and ERK1/2 blockade on the gene expression and derived activity index of fatty acid desaturases. The regulatory effects were simultaneously analyzed by studying the expression and endogenous activity index of both  $\Delta$ 6D and SCD1, which made it possible to identify similarities and differences. It remained to be clarified what mechanism is involved in PPAR $\gamma$  and ERK1/2 MAPK crosstalk in the regulation of fatty acid desaturases in the liver cells.

In conclusion, our study showed that PPAR $\gamma$  and ERK1/2 MAPK signaling pathway affect the gene expression and activity of  $\Delta$ 6D and SCD1 in hepatic HepG2 cells. Furthermore, a possible inhibitory crosstalk between PPAR $\gamma$  and ERK1/2 MAPK signaling pathway may have different affects on  $\Delta$ 6D and SCD1 genes expression, and subsequently on their enzymatic activities.

## COMMENTS

### Background

The  $\Delta$ 6-desaturase ( $\Delta$ 6D) and stearoyl-CoA desaturase 1 (SCD1) are two important regulatory enzymes in hepatic *de novo* fatty acid synthesis. Altered levels of desaturases activity have been reported in various human diseases. Both enzymes are coordinately regulated by Pioglitazone, a drug that is widely used as an antidiabetic agent with glucose-lowering and lipid modifying effects. But up to now, the mechanisms by which pioglitazone exerts its effects are yet relatively unknown. Intracellular signaling pathways might modulate the effect of Pioglitazone on fatty acid metabolism.

### Research frontiers

Pioglitazone specifically stimulates peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ). In the area of hepatic lipid metabolism, the research hotspot was to clarify the integrated effect of pioglitazone and MEK/ERK1/2 pathway signaling in regulation of fatty acid desaturation.

### Innovations and breakthroughs

Previously, it has been shown that PPAR $\gamma$  agonists not only can function in a PPAR $\gamma$  dependent manner but also are capable of activating ERK1/2 pathway independently of PPAR $\gamma$ . Based on the importance of PPAR $\gamma$  activity and ERK1/2 signaling in the regulation of cellular lipid, the authors tested the effects of pioglitazone and ERK1/2 signaling pathway on  $\Delta$ 6D and SCD1 activity in a human hepatic cell line. The regulatory effects were simultaneously analyzed by studying the expression and endogenous activity index of both  $\Delta$ 6D and SCD1, which made it possible to identify similarities and differences.

### Applications

The study results suggest that a combination therapy using pioglitazone and MEK/ERK1/2 inhibition might improve the treatment efficiency of lipid disorders, in particular the changes in diabetes, obesity and atherosclerosis.

### Terminology

Desaturase: A fatty acid desaturase is an enzyme that removes two hydrogen atoms from a fatty acid, creating a carbon/carbon double bond.  $\Delta$ 9 desaturase, also known as stearoyl-CoA desaturase-1, produces oleic acid by desaturating stearic acid.  $\Delta$ 6D synthesizes highly unsaturated fatty acids such as eicosapentaenoic and arachidonic acid; MEK/ERK1/2 signaling pathway: The MEK/ERK1/2 pathway is a chain of proteins in the cell that communicates a signal from a receptor on the surface of the cell to the DNA in the nucleus of the cell. The pathway includes many proteins, including ERK and MEK; PPAR $\gamma$ , also known as the glitazone receptor, is a nuclear receptor which regulates fatty acid storage and glucose metabolism in human.

### Peer review

The present manuscript reports the modulatory effect of ERK/MEK1/2 pathway and PPAR $\gamma$  on  $\Delta$ 6D and SCD1 in hepatic HepG2 cells. The study is interesting and well written.

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