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Inhibition of MEK/ERK1/2 Signaling Affects the Fatty Acid Composition of HepG2 Human Hepatic Cell Line

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ABSTRACT

Introduction: The extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase pathway, also known as the MEK/ERK1/2 kinase cascade, has recently been implicated in the regulation of lipid metabolism and fatty liver disease. However, its functional effect on cellular fatty acid composition is unknown. Herein, we examined the effect of a pharmacological inhibitor of MEK, the upstream kinase activator of ERK1/2, on fatty acid composition of hepatocellular carcinoma cell line HepG2. ***Methods:*** HepG2 cells cultured in RPMI-1640 were exposed to the commonly used ERK1/2 pathway inhibitor PD98059 and were investigated with respect to fatty acid composition by gas-liquid chromatography. ***Results:*** Exposure of cells to the ERK1/2 pathway inhibitor induced an increase in monounsaturated fatty acids and the fatty acid desaturation index and a decrease in polyunsaturated fatty acid content. Specifically, we showed a significant increase of oleic acid (18:1n-9; +29%, P=0.003) and arachidonic acid (20:4n-6)/linoleic acid (18:2n-6) ratio (3.5-fold; P<0.001) in HepG2 cells. ***Conclusion:*** Cellular fatty acid composition of HepG2 cells appeared to be differentially regulated by ERK1/2 pathway, thus suggesting related metabolic pathways as potential mediators of the effects of ERK1/2 signaling on hepatic fatty acid composition.

Introduction

Cellular fatty acid composition is critically important in multiple biological functions including cell membrane fluidity, signal transduction, differentiation, inflammatory responses and brain development (Horrobin 1993, Ntambi and Miyazaki 2004). Fatty acids *de novo* synthesis and their metabolic conversion to other fatty acids are catalyzed by intracellular lipogenic enzymes such as fatty acid synthase, desaturases and elongases. These processes provide essential precursors for structural cell components and bioactive metabolites such as prostaglandins (Zaloga and Marik 2001). Therefore, a tight regulation of the fatty acid metabolism is a critical part of normal cell physiology.

The extracellular signal-regulated kinases 1 and 2 (ERK1/2) are members of the mitogen-activated protein kinase (MAPK) family, which is involved in many aspects of cellular regulation. ERK1/2 kinase signaling has been implicated in the regulation of cell growth and differentiation (Zvibel *et al* 2008).

Recent findings link ERK1/2 with fatty acid metabolism (Jump *et al* 2006). Based on the effect of ERK1/2 inhibitor PD98059, ERK1/2 kinase cascade has been suggested to be involved in cellular fatty acid uptake in skeletal muscle (Turcotte *et al* 2005). Interestingly, Mauvoisin *et al* (2010) showed that leptin modulated stearoyl-coA desaturase 1 (SCD1) expression via the ERK1/2 signaling pathway. SCD1 is considered a key enzyme for the regulation of hepatic lipid metabolism, as SCD1-deficient mice were shown to be protected against diet-induced fatty liver (Dobrzyn *et al* 2008). Despite evidence demonstrating the potential of ERK1/2 kinase signaling for lipid metabolism, there are no experimental results showing functional effect of ERK1/2 signaling on cellular fatty acid composition. In the present study, changes in the composition of cellular fatty acids have

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been reported by employing gas liquid chromatography (GLC) technique after *in vitro* incubation of HepG2 cells with ERK1/2 inhibitor PD98059.

Methods and Materials

Materials

Cell culture materials, media, FBS and standard fatty acid methyl esters were obtained from Sigma Chemical Company. PD98059 was purchased from Cayman Chemical. HepG2 cell line was obtained from the Pasteur Institute Culture Collection in Tehran. All other chemicals used were of analytical grade.

Cell culture

HepG2 cells were grown in RPMI1640 supplemented with 10% FBS, L-glutamine (2mM), penicillin (100 units/ml), and streptomycin (100µg/ml). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO2:air. The cells subcultured (1:6 ratio) every 5-6 days at a cell density of 5×10^6 cells/75-cm^2 flask. Cells were trypsinized and seeded in duplicate sets of 6-well plates at 1×10^6 cells/well. After allowing the cells to attach overnight, the medium was replaced with fresh medium containing either vehicle dimethyl sulphoxide (DMSO, 0.1%) or varying concentrations of PD98059 (10, 20, 40 µM). On day 2, treatment was repeated with fresh medium and reagents. Following 48 h incubation, culture medium was removed and the cell monolayer was washed three times with cold phosphate-buffered saline (PBS) and collected for cellular fatty acid measurement.

Cells were collected into methanol: chloroform and their lipids were extracted according to Bligh and Dyer (BLIGH and DYER 1959). Insoluble material was removed by centrifugation. The supernatant was transferred in a glass vial, and the lipids were esterified with methanol during catalysis with acetyl chloride (Lepage and Roy 1986). Fatty acid methyl esters were extracted and analyzed for fatty acid composition, as described in our previous study (Noori *et al* 2009). Briefly, fatty acid methyl ester derivatives formed by isolated cellular lipids were separated on a 60×0.25 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 ˚C, 1˚C /min. then isothermal continued 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±SD of 3 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 value <0.05 was considered statistically significant.

Results

Table 1 shows the level of fatty acids measured by GLC method in HepG2 cells exposed to PD98059 (0-40µM) for 48h. Oleic acid (18:1n-9) was the major cellular fatty acid, followed by palmitic acid (16:0) and stearic acid (18:0).

Table 1. Effect of different doses of PD98059 on fatty acid composition of HepG2 human hepatic cells

% of total fatty acids	PD98059 (µM)			
	0	10	20	40
14:0 (myristic acid)	02.04±0.58	01.83±0.27	01.84±0.19	01.34±0.57
16:0 (palmitic acid)	22.88±1.73	25.28±2.53	25.45±3.07	25.07±2.23
16:1 (palmitoleic acid)	07.54±1.81	10.80±1.81	09.45±1.26	10.05±1.86
18:0 (stearic acid)	10.30±1.21 [a]	10.43±0.94 [a]	07.24±1.41 [a,b]	07.02±1.29 [b]
18:1n-9 (oleic acid)	38.79±1.64 [a]	37.19±2.73 [a,b]	49.94±4.22 [c]	50.04±4.29 [c]
18:2n-6 (linoleic acid)	09.09±1.09 [a]	06.72±1.29 [a,b]	01.44±0.36 [c]	01.53±0.56 [c]
18:3n-6 (linolenic acid)	01.07±0.36 [a]	00.70±0.24 [a,b]	00.25±0.10 [b]	00.34±0.25 [b]
20:4n-6 (arachidonic acid)	07.13±1.54 [a]	05.98±1.60 [a,b]	03.56±0.55 [b]	04.00±0.90 [b]
20:5n-3 (eicosapentaenoic acid)	00.42±0.15	00.46±0.19	00.38±0.22	00.20±0.15
22:6n-3 (docosahexaenoic acid)	00.74±0.13	00.62±0.19	00.47±0.24	00.40±0.13
Saturated fatty acids	35.21±2.37	37.54±3.33	34.52±3.58	33.44±3.36
Monounsaturated fatty acids	46.33±0.21 [a]	47.98±1.61 [a,b]	59.39±3.50 [c]	60.09±4.65 [c]
Polyunsaturated fatty acids	18.46±2.23 [a]	14.48±2.89 [a,b]	06.09±0.74 [c]	06.47±1.54 [c]

Cells were incubated with PD98059 (0-40 µM) for 48h. Lipid extracts were prepared and analyzed by gas liquid chromatography for a comprehensive fatty acid profile. The mean values±SD of 3 independent experiments done in duplicate are given. Values with different superscripts are significantly different (Tukey's test, alpha=0.05). Detection limit was 0.05% of the total area.

Incubation of HepG2 cells in PD98059 not only caused significant alterations in fatty acid composition, but also resulted in considerable changes in the ratio of specific classes of fatty acids, which are regarded as indices of desaturation/elongation (Fig. 1). In addition, the results of the post hoc analyses showed significant correlations between the extent of cellular fatty acid modification and dose of MEK inhibitor treatment.

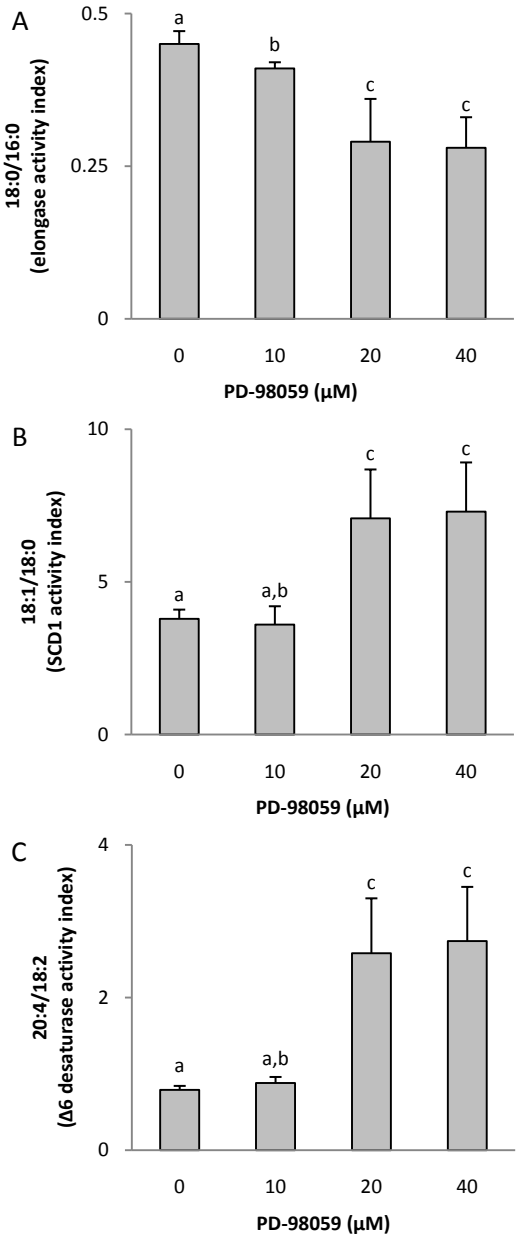

Fig. 1. Effects of different doses of PD98059 on derived fatty acid indices of HepG2 human hepatic cells. A: elongase activity index (18:0/16:0); B: SCD1 activity index (18:1n-9/18:0); C: Δ6 activity index (20:4n-6/18:2n-6). Data are means±SD, n=3. Bars with different superscripts are significantly different (Tukey's test, alpha=0.05). SCD1 (stearoyl-coA desaturase 1); 16:0, palmitic acid; 18:0, stearic acid; 18:1n-9, oleic acid; 18:2n-6, linoleic acid; 20:4n-6, arachidonic acid.

In general, these changes were reflected by increased percentage of monounsaturated fatty acids (MUFA, P=0.001) and reduced polyunsaturated fatty acids (PUFA, P<0.001). Specifically, there was a significant increase of 18:1n-9 (+29%, P=0.003) and a significant decrease of linoleic acid (18:2n-6; -65%, P<0.001) in HepG2 cells following ERK1/2 inhibitor treatment at 40μM. The ratio of 18:0/16:0 was calculated as an elongase activity index. The 18:1n-9/18:0 and arachidonic acid (20:4n-6)/18:2n-6 ratios were calculated as indices of SCD1 and Δ6 fatty acid desaturase activity, respectively. The value of 18:0/16:0 ratio was significantly lower (P=0.002), but 18:1n-9/18:0 (P=0.007) and 20:4n-6/18:2n-6 (P=0.002) ratios were higher in the presence of PD98059 (Fig. 1). The latter effect was a result of greater decrease in the percentage of 18:2n-6 than that in the 20:4n-6.

Discussion

Fatty acid composition of cellular lipids can modulate several metabolic processes, e.g. glucose metabolism and membrane permeability, which take place in hepatic cells. On the other hand, altered lipid content of hepatic cells makes a major contribution in the rate of *de novo* lipogenesis and inducing steatosis (Pachikian *et al* 2011). Abnormal lipid uptake or *de novo* lipogenesis has been reported in various types of hepatic disorders, with consequent increasing production of bioactive lipids and an inflammatory response (Carter-Kent *et al* 2008, Musso *et al* 2009, Arendt *et al* 2009).

Given the potential importance of MEK/ERK1/2 signaling in lipid metabolism, we tested the hypothesis that the fat composition of hepatocellular carcinoma cell line HepG2 would be affected by MEK/ERK1/2 signaling. The results of this study showed that in the presence of the commonly used MEK1/2 inhibitor PD98059, there were higher proportions of 18:1n-9, as the major MUFA and lower proportions of PUFA in the HepG2 cells. The change in fatty acid composition of the cells in response to the ERK1/2 inhibition confirms that metabolic activities such as rate of endogenous synthesis of fatty acids may be influenced by ERK1/2 kinase signaling.

In our experiments, the levels of MUFA were increased in the presence of MEK1/2 inhibitor, suggesting a potential link between dysfunction of MEK/ERK1/2 signaling and an increased MUFA content in hepatic cells. Indeed, suppressed MEK/ERK1/2 signaling pathway has been associated with fatty liver disease (Wang *et al* 2010), which is characterized by hepatic 18:1n-9 accumulation (Ricchi *et al* 2009, Puri *et al* 2007). However, possible protective effects from 18:1n-9 have also been found against oxidative stress. Duval *et al* (2002) showed that 18:1n-9 contributes in enhancement of cellular antioxidant capability against

oxidative stress through epidermal growth factor receptor (EGFR)-dependent activation of glutathione peroxidase. Thus, MEK/ERK1/2 signaling may be functionally important in the development of hepatic lipid abnormalities and would be rational therapeutic target.

The observed individual variations in the lipid fatty acid patterns could suggest an individual difference in fatty acid synthase, elongase and desaturase activities. The decreased ratios of 18:0/16:0, may suggest decreased activity of the enzyme involved in the biosynthesis of 18:0 from 16:0 i.e. elongase. Stearate is the preferred substrate of SCD1 which is converted to 18:1n-9. Similarly, increased 18:1n-9/18:0 and 20:4n-6/18:2n-6 ratios as indices of desaturase activity suggest that this enzymatic activity is under inhibitory control of ERK1/2 signaling. described findings are supported by the modulations of multiple lipogenic enzymes through MEK/ERK1/2 signaling including fatty acid synthase (Radenne *et al* 2008), desaturases (Mauvoisin *et al* 2010) and elongases (Green *et al* 2010). For instance, a study performed on HepG2 cells showed that leptin substantially diminishes SCD1 gene expression through a pathway involving ERK1/2 activation (Mauvoisin *et al* 2010). In many cell types including HepG2 cells, PUFA has been shown to inhibit desaturases genes' transcription which could be explained by their ability to activate MEK/ERK1/2 signaling pathway (Portolesi *et al* 2008). Furthermore, similar mechanism could favor T3 action in downregulating SCD1 expression (Radenne *et al* 2008). Presumptively, ERK1/2 modulates desaturases expression by implying several molecular mechanisms, including enhanced affinity of transcription factors (Mauvoisin *et al* 2010), gene suppression through direct interaction with DNA (Hu *et al* 2009) and modulation of transcription factor sterol-regulatory element binding protein-1c (SREBP1-c) (Wang *et al* 2006a). SREBP-1c is crucial in coordinating hepatic lipogenesis, as evidenced by lipid gene expression analyses of transgenic mice with selective deficiency of SREBP-1c (Liang *et al* 2002). SREBP-1c is direct target of ERK1/2 phosphorylation (Kotzka *et al* 1998). ERK-phosphorylation of SREBP-1c appears to destabilize it as MEK inhibitors markedly attenuates phosphorylated SREBP-1c and enhanced its transcriptional activity (Jump *et al* 2006). Fatty acid metabolism-related gene expression including desaturases was induced in transgenic mice overexpressing SREBP-1c (Wang *et al* 2006b). Taken together, the observed increase in desaturation indices could be attributed to the enhanced activity of SREBP-1c upon inhibition of ERK1/2 signaling pathway.

It could be argued that the decreased PUFA in the presence of ERK1/2 inhibitor might be due to an impaired elongase and desaturase activity. However, fatty acid biosynthesis is a multistep process that involves successive reactions catalyzed by several enzymes. It has been reported that the genes involved in the synthesis of MUFA and PUFA are regulated differentially by transcription factors (Horton *et al* 2003). Accordingly, ERK1/2 inhibition was shown in the present study to induce different effects on MUFA and PUFA levels. The decreased level of PUFA following ERK1/2 inhibition could be attributed to the suppressed expression of enzymes involved in PUFA synthesis. However, the perturbation in metabolism of fatty acids may be associated with lower cellular PUFA. This hypothesis is supported by the reduction in the hepatic 20:4n-6 and docosahexaenoic acid (22:6n-3) content when key enzymes involved in PUFA synthesis were induced in livers of C57BL/6 mice through adenoviral transduction (Wang *et al* 2008).

To our knowledge, this study is the first study to examine the effect of a pharmacological inhibitor of MEK, the upstream kinase activator of ERK1/2, on fatty acid composition of hepatic cells. We used hepatoma HepG2 cells which are well characterized human-derived cells for the studying human liver parenchymal cells *in vitro* (Jennen *et al* 2010, Furth *et al* 1992). It is worthy of a note that ERK1/2 pathway is modestly hyperactive in HepG2 cells (Tsai *et al* 2007), which may make their response being sensitive to inhibition by MEK inhibitor in nonstimulated culture condition. However, we observed dose dependent changes in fatty acid composition with ERK1/2 inhibitor. This is consistent with previous findings in which the type and extent of cellular response depends on the level of ERK1/2 activation (Zhu *et al* 2009). It remains to be determined whether ERK1/2 inhibition affects *in vivo* cellular fatty acid composition of the liver.

Conclusion

This study demonstrated that fatty acid content of HepG2 cells is susceptible to inhibition of ERK1/2. In the presence of MEK inhibitor PD98059, proportions of MUFA were high, while proportion of PUFA was lower compared to the absence of inhibitor. We conclude that MEK/ERK1/2 kinase signaling serves to coordinate fatty acid metabolism in HepG2 cells, thus suggesting related metabolic pathways (e.g., desaturation and elongation) as potential mediators in the association.

Ethical issues

Not applicable in this study.

Conflict of interests

Authors declare no conflict of interests.

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