

Liver lipid molecules induce PEPCK-C gene transcription and attenuate insulin action

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Abstract

Cytosolic phosphoenolpyruvate carboxykinase (PEPCK-C) plays key roles in gluconeogenesis, glyceroneogenesis, and cataplerosis. Experiments were designed to examine the effects of endogenous lipid molecules from rat livers on the expression of PEPCK-C gene in primary rat hepatocytes. The lipid extracts prepared from livers of Zucker fatty, lean, and Wistar rats induced the expression levels of PEPCK-C transcripts. Insulin-mediated reduction of PEPCK-C gene expression was attenuated by the same treatment. The lipid extracts induced the relative luciferase activity of reporter gene constructs that contain a 2.2-kb 5' promoter fragment of PEPCK-C gene, but not the construct that contains only the 3' untranslated region (UTR) of its mRNA. The estimated half life of PEPCK-C transcripts in the presence of the lipid extract is the same as that in the absence of it. My results demonstrate for the first time that endogenous lipid molecules induce PEPCK-C gene transcription and attenuate insulin action in liver.

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Liver plays a key role in controlling lipid and glucose homeostasis. Profound changes of hepatic lipid and glucose metabolism are usually associated with metabolic abnormalities, such as obesity and diabetes [1], and are attributed to alterations of the gene expression in liver [2,3]. PEPCK-C which converts oxaloacetate into phosphoenolpyruvate in the presence of GTP has been considered as the rate limiting enzyme for hepatic gluconeogenesis [4]. Mice with homozygous deletion of PEPCK-C gene died after birth with severe hypoglycemia and profound changes in lipid and amino acid metabolism prior to the death. Although mice with liver-specific deletion of PEPCK-C gene maintained euglycemia after a 24-h fasting, their lipid metabolism was impaired [5,6]. In intact mouse liver, PEPCK-C flux correlated tightly with tricarboxylic acid cycle flux [7]. Given its role in gluconeogenesis, glyceroneogenesis, and cataplerosis, it is reasonable to hypothesize that hepatic PEPCK-C may be

regulated by endogenous metabolites in multiple metabolic pathways [8–10].

Because allosteric regulation of PEPCK-C activity has not been reported in mammalian cells, its activity is primarily controlled by regulation of its gene expression in different physiologic conditions [8,11]. In the liver, fasting, diabetes, carbohydrate-free, and high fat diets increase, whereas re-feeding, insulin treatment, and high carbohydrate diets decrease its expression. Glucagon, glucocorticoids, thyroid hormone, and retinoic acid induce hepatic PEPCK-C gene expression, while insulin and glucose inhibit it [11]. Multiple regulatory elements mediating its responses to hormones and nutrients have been identified at the 5' promoter region [8,11]. The transcription factors and co-activators that interact with these elements and regulate PEPCK-C gene transcription have been proposed or identified [8,11,12]. Some of them play key roles in controlling hepatic lipid metabolism. Over-expression of the active form of sterol regulatory element-binding protein 1c (SREBP-1c) stimulated lipogenesis and abolished PEPCK-C gene expression in cultured rat hepatocytes [13,14].

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In liver of rodents fed with T0901317, a synthetic agonist for liver X receptor (LXR) activation, the expression of lipogenic genes was induced and that of PEPCK-C gene was suppressed [15,16]. These results imply that signals mediating changes of hepatic lipid metabolism may regulate PEPCK-C gene expression.

In this study, lipid molecules were extracted from rat livers and their effects on PEPCK-C gene expression were measured in primary rat hepatocytes. To this end, I report for the first time that lipid mediators in hepatic lipid extracts induce transcription of PEPCK-C gene and attenuate insulin-mediated reduction of its expression.

Materials and methods

Reagents. α -Amanitin, bovine insulin, dexamethasone, and 3, 3', 5-triiodo-L-thyronine were obtained from Sigma (Saint Louis, MO). Lipofectin transfection reagent and medium 199 were obtained from Invitrogen Corporation (Carlsbad, CA). Dulbecco's PBS and fetal bovine serum were obtained from Mediatech (Herndon, VA). Sixty millimeter rat tail collagen I-coated dishes were obtained from BD Biosciences (Bedford, MA). Petroleum ether, *n*-heptane, isopropanol, ethanol, and potassium hydroxide were obtained from Fisher Scientific (Pittsburgh, PA).

Rats and primary hepatocytes. Male Sprague–Dawley, Zucker lean and fatty rats (8–15 weeks of age) were purchased from Harlan Breeders (Indianapolis, IN). All experimental procedures were approved by the Institutional Animal Care and Use Committee. All the guidelines for the use and care of laboratory animals were followed.

Hepatocytes were isolated from non-fasted male Sprague–Dawley rats (250–300 g of body weight) and seeded as described [17]. After 3–4 h, the attached cells were washed once with 4 ml of PBS, and incubated in medium A (Medium 199 supplemented with 100 nM dexamethasone, 100 nM T₃, 100 U/ml sodium penicillin, and 100 μ g/ml streptomycin sulfate) with 1 nM insulin for overnight before treatments as described in each experiment.

Lipid extraction. Livers of ad libitum fed rats were collected, weighed, washed, minced by razor blades, and homogenized in *n*-heptane at 1 to 10 ratio (1 g to 10 ml, w/v) using Polytron homogenizer at 6000 rpm for 1 min. The homogenate was kept in glass bottle filled with nitrogen (N₂) and stirred for 48 h at room temperature (RT). After that, the homogenate was allowed to stand for at least 30 min. The supernatant was removed and dried under N₂. The dried lipids were weighed and stored under N₂ at –20 °C until being used. For saponification, 150 mg of total lipids was mixed with 10 ml of 0.5 M KOH in ethanol (1 volume of 5 M KOH + 9 volume of ethanol) and incubated at 65 °C for 40 min. The non-saponifiable matters were extracted twice with 15 ml of petroleum ether (PE), dried and reconstituted in 100% ethanol at 40 mg/ml. The non-saponifiable matters extracted by PE were designated as the lipid extracts in this paper.

Treatments of the lipid extracts and α -amanitin. Primary rat hepatocytes were washed once with PBS and treated with 0.2% ethanol as vehicle control or 80 μ g/ml lipid extracts in the presence or absence of insulin in medium A at 37 °C and 5% CO₂ for 6 h. For α -amanitin experiments, the remaining cells that have been only treated with the lipid extract for 6 h were washed once with 3 ml PBS and incubated in medium A containing 3 μ M α -amanitin with or without the lipid extract. Total RNAs were isolated at 0.5, 1, 2, 4, and 6 h, and subjected to RT-PCR analysis.

cDNA synthesis and quantitative real time PCR (RT-PCR). All the reagents and equipment sets were from Applied Biosystems unless described otherwise. Total RNA was isolated using the RNA STAT 60 reagent (TEL-TEST, Inc, Friendswood, TX). DNA contamination was removed using the DNA-free™ kit. First strand cDNA was synthesized from 2 μ g of DNA-free RNA with random hexamer primers using cDNA synthesis kit. The RT-PCR primer sequences were designed using Primer Express software and will be provided upon request. Each reaction con-

tains, in a final volume of 14 μ l, cDNA from 14 ng of reverse transcribed total RNA, 2.33 pmol primers, and 7 μ l of 2 \times SYBR Green PCR Master Mix. Triplicate PCRs were carried out in 96-well plates using the 7300 Real Time PCR System. The conditions are 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The relative amounts of all mRNAs except for Fig. 4B and C were calculated using the comparative C_T method as described [18] with 36B4 as the invariant control. In the α -amanitin experiment, the relative amounts of 36B4 (Fig. 4B) and PEPCK-C (Fig. 4C) transcripts were calculated using the following formula, relative amounts of mRNA = $2^{(C_{T-timeX} - C_{T-time0})}$. Here, C_{T-timeX} is the C_T number at one experimental time point, and C_{T-time0} is the C_T number at time 0, the time that α -amanitin was added. The levels of 36B4 and PEPCK-C transcripts at time 0 were arbitrarily assigned as 100%.

Reporter gene constructs and assay. Standard protocols (Molecular Cloning) were followed in all recombinant DNA engineering procedures. A 2.2-kb promoter fragment (PEPCK-2.2k) was cloned and inserted into pGL3-basic reporter gene vector (Promega) to obtain plasmid I. For plasmid II, a 622 bp 3' UTR fragment was cloned and inserted into pGL3-Promoter (Promega) reporter gene vector to replace the SV40 late poly (A) signal sequence. For plasmid III, the 2.2 kb promoter fragment of PEPCK-C gene was inserted into plasmid II to replace the SV40 promoter. The primer sequences will be provided upon request. The methods for plasmid transfection using Lipofectin transfection reagent, and dual luciferase assay have been described elsewhere [17].

Statistics. Data are presented as means \pm SD and compared by unpaired Student's *t* test. *P* < 0.05 was considered statistically significant.

Results

Lipid extracts from livers of Zucker fatty rats induce PEPCK-C gene expression

Fig. 1A shows the relative levels of PEPCK-C transcripts in primary hepatocytes treated with vehicle or the lipid extracts from Zucker Fatty rat livers in the absence or presence of 100 nM insulin. The lipid extract treatment resulted in a 5.7 ± 3.0 -fold induction of PEPCK-C transcripts in the absence of insulin. Insulin reduced the levels of PEPCK-C transcripts to 0.07 ± 0.03 - and 0.5 ± 0.4 -fold of the control value in the absence or presence of the liver lipid extracts, respectively. The levels of insulin receptor transcripts did not change under these conditions as shown

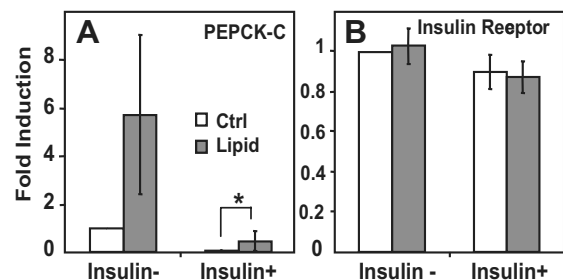


Fig. 1. The lipid extracts from Zucker fatty rats induce the levels of PEPCK-C (A), but not insulin receptor (B) transcripts. Primary hepatocytes were treated with either vehicle control or the lipid extracts in the absence or presence of 100 nM insulin. The levels of PEPCK-C (A) and insulin receptor (B) transcripts were measured by RT-PCR. The gene expression level in control group without insulin was arbitrarily assigned as 1. Results represent the means \pm SD of five independent experiments (**P* < 0.04).

in Fig. 1B. These results indicated that a lipid mediator in the lipid extracts from Zucker fatty rat livers induces the expression levels of PEPCK-C transcripts. Although insulin still suppressed it, the content of PEPCK-C transcripts has been maintained at a significantly higher level in the presence of the lipid extract (0.5 vs 0.07, $P < 0.04$). It suggests an attenuation of insulin action in the presence of the lipid extracts.

The lipid extracts from livers of Zucker lean rats attenuated insulin-mediated reduction of PEPCK-C gene expression

It is important to determine whether the lipid mediator activities exist in livers of lean rats and cause attenuation of insulin action in lower concentrations of insulin. A batch of the lipid extract was prepared from livers of Zucker lean rats and its effects were measured in the presence of increasing concentrations of insulin. Fig. 2A shows that the lipid extract from lean rat livers induced the expression levels of PEPCK-C transcripts by 5.8 ± 2.8 -fold. Fig. 2B shows that insulin at 0.1, 1, 10, and 100 nM reduced them to $33.0 \pm 0.8\%$, $5.8 \pm 3.5\%$, $3.9 \pm 2.6\%$, and $3.8 \pm 3.8\%$ of the control level in the absence of the lipid extract, respectively. In the presence of the lipid extract, the same treatments only reduced them to $58.2 \pm 7.2\%$ ($P < 0.01$), $32.7 \pm 2.0\%$ ($P < 0.001$), $21.6 \pm 6.5\%$ ($P < 0.02$), and $14.5 \pm 2.6\%$ ($P < 0.03$), respectively. At each concentration of insulin, the remaining level of PEPCK-C transcripts was significantly higher in the presence than that in the absence of the lipid extract. To achieve 67% reduction, it required 0.1 or 1 nM insulin in the absence or presence of the lipid extract, respectively, indicating a tenfold reduction of insu-

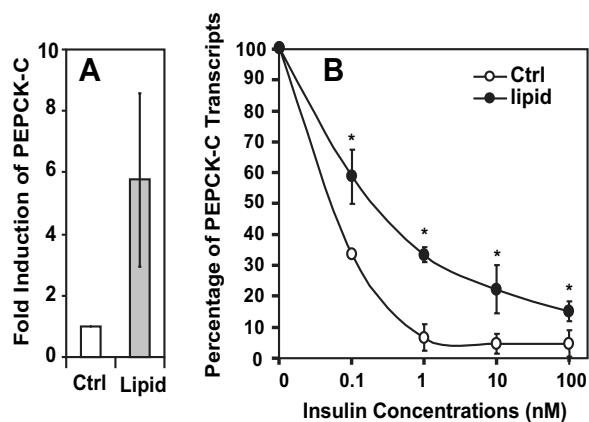


Fig. 2. The lipid extract from Zucker lean rats induces the level (A) and attenuates the insulin-mediated reduction of PEPCK-C gene expression (B). Primary hepatocytes were treated with either vehicle control or the lipid extract in the absence or presence of different concentrations of insulin as indicated. The levels of PEPCK-C transcripts were analyzed by RT-PCR. The expression level of PEPCK-C transcripts in the vehicle control group was arbitrarily assigned as 1 in (A). The transcript levels of the vehicle control or lipid extract group without insulin was arbitrarily assigned as 100% in (B). Results represent the means \pm SD of three independent experiments ($*P < 0.01$, 0.001, 0.02, and 0.03 for 0.1, 1, 10, and 100 nM insulin, respectively).

lin action. These results demonstrated that the lipid extract from lean rat livers induce the PEPCK-C gene expression and causes attenuation of insulin action. This phenomenon has been repeatedly observed using several batches of the lipid extracts (data not shown).

The lipid extract induces transcription of PEPCK-C gene in primary rat hepatocytes

It is important to determine whether the lipid extract caused elevation of synthesis, stability or both, of PEPCK-C transcripts. Reporter gene assay has been used to determine the roles of 5' promoter region of PEPCK-C gene [19] and 3' UTR of its mRNA [20] in controlling cellular levels of its transcripts. Fig. 3A shows the reporter gene constructs I, II, and III generated for this purpose. The construct I contains PEPCK-2.2k, *Firefly* luciferase cDNA and SV40 late poly(A) signal. The *Firefly* luciferase activity derived from it will be correlated with the activation of the promoter. The construct II contains SV40 promoter, *Firefly* luciferase cDNA and a 622-bp 3' UTR. The *Firefly* luciferase activity derived from it will reflect the stability of the 3' UTR of rat PEPCK-C mRNA. The construct III contains PEPCK-2.2k, *Firefly* luciferase cDNA and the 3' UTR. The *Firefly* luciferase activity derived from it will be determined by the activation of the promoter and the stability of the 3' UTR. As shown in Fig. 3B, for plasmid I, the lipid extract induced its activation by 2.3 ± 0.6 -fold. Insulin significantly reduced the relative luciferase

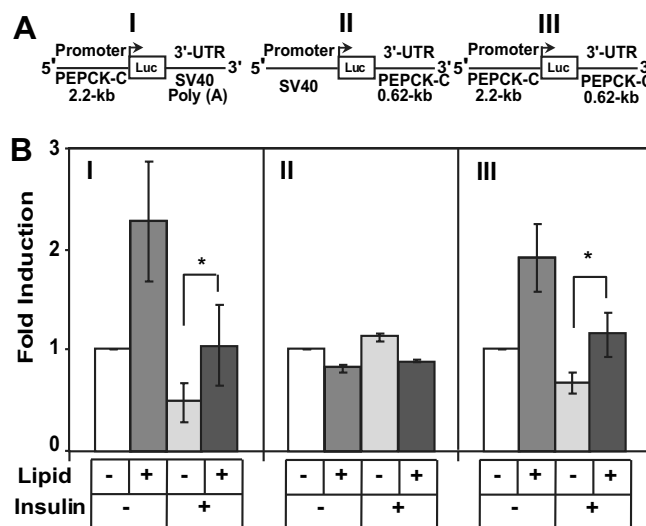


Fig. 3. The lipid extract induces transcription of PEPCK-C gene, but not 3' UTR of its mRNA. (A) Schematic of luciferase reporter gene constructs I, II, and III. (B) Relative luciferase activities of PEPCK-C reporter gene constructs I, II, and III in hepatocytes treated with the lipid extracts in the absence or presence of 1 nM insulin. The normalized luciferase activity of each construct in the vehicle control group in the absence of insulin was arbitrarily assigned a value of 1. The fold induction was calculated as the ratio of normalized luciferase activity in the presence of the lipid extract, insulin, or lipid extract + insulin to that of the control group. Each value represents the means \pm SD of three independent transfection experiments each assayed in duplicate ($*P < 0.04$).

activity to 0.5 ± 0.2 - and 1.1 ± 0.4 -fold of the control value ($P < 0.04$) in the absence or presence of the lipid extract, respectively. The reduction caused by insulin in the absence of the lipid extract (from 1 to 0.5) is similar to that in the presence of it (from 2.3 to 1.1). For plasmid II, the relative luciferase activity was not affected by the lipid extract, insulin, or both. For plasmid III, the lipid extract induced its relative luciferase activity by 1.9 ± 0.3 -fold. Insulin reduced the activity to 0.7 ± 0.1 - and 1.2 ± 0.2 -fold ($P < 0.04$) of the control value in the absence or presence of the lipid extract, respectively. The reduction caused by insulin in the presence of the lipid extract (from 1 to 0.67) is similar to that in the absence of it (from 1.9 to 1.2). These results demonstrated that the lipid extract induces the levels of PEPCK-C gene expression through the activation of its promoter, but not the stability of its mRNA.

The lipid extract does not affect the decay of PEPCK-C transcripts

The stability of PEPCK-C mRNA was measured in the presence of α -amanitin, a specific inhibitor of RNA polymerase II complex [21]. Fig. 4A illustrates the schematic of the experimental design as described in the experimental

procedures. As shown in Fig. 4B, the lipid extract from Wistar rat livers increased the levels of PEPCK-C transcripts by 3.5 ± 0.6 -fold. 1 nM insulin reduced them to 0.07 ± 0.01 - and 0.7 ± 0.4 -fold of the control value in the absence and presence of the lipid extract, respectively, reflecting a tenfold difference (0.7 vs 0.07, $P < 0.04$). The 80% (from 3.5 to 0.7) reduction by insulin in the presence of the lipid extract is smaller than 93% reduction (from 1 to 0.07) in the absence of it, demonstrating attenuation of insulin action. These results demonstrated that the lipid mediator activities exist in livers of Wistar rats.

As shown in Fig. 4C, the contents of 36B4 transcripts started to drop 6 h after α -amanitin treatment and exhibited no difference between the vehicle control and lipid extract groups. The estimated half life of 36B4 transcripts is more than 4 h. Fig. 4D shows the decay of PEPCK-C transcripts in the absence or presence of the lipid extract. At 0.5, 1, 2, 4, and 6 h after α -amanitin treatment, the contents of PEPCK-C transcripts in the control group dropped to $84 \pm 6\%$, $64 \pm 10\%$, $32 \pm 8\%$, $20 \pm 8\%$, and $12 \pm 4\%$ of that at time 0, respectively. At the same time points, their amounts in the presence of the lipid extract dropped to $87 \pm 5\%$, $63 \pm 10\%$, $37 \pm 9\%$, $22 \pm 6\%$, and $11 \pm 3\%$ of that at time 0, respectively. The decay rates for both groups are almost identical. The estimated half life of PEPCK-C

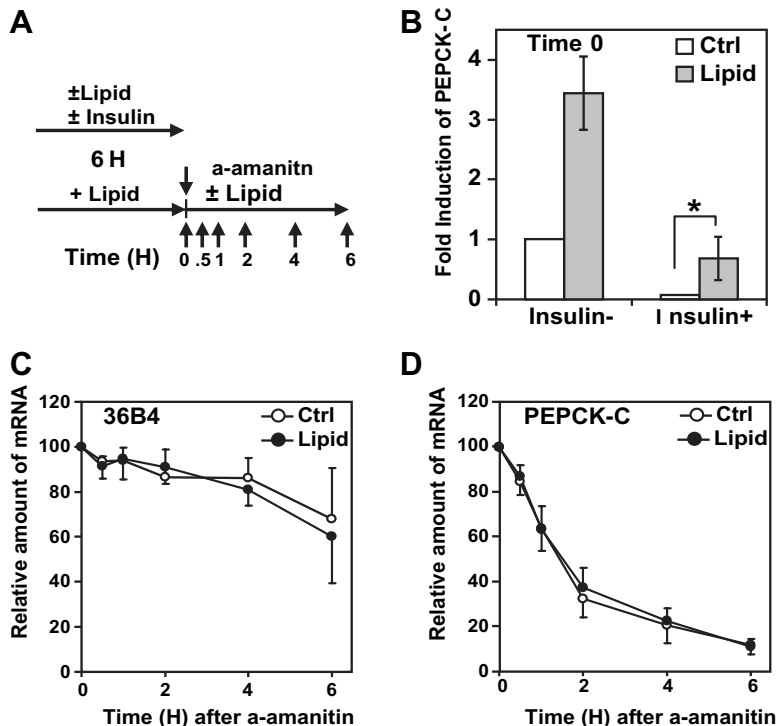


Fig. 4. The lipid mediator induces transcription of PEPCK-C gene in primary rat hepatocytes. (A) Schematic of experimental protocol. (B) Induction of PEPCK-C transcripts by the lipid extracts from Wistar rat livers. Primary rat hepatocytes were incubated in medium A containing either vehicle control or the lipid extract from Wistar rat livers without or with 1 nM insulin. Total RNAs were extracted and subjected to analysis by RT-PCR. Fold induction was calculated as in Fig. 1. (C and D). Relative levels of 36B4 (C) and PEPCK-C (D) transcripts of cells incubated with medium A containing 3 μ M α -amanitin in the absence or presence of the lipid extract. Total RNAs were extracted at 0.5, 1, 2, 4, and 6 h after treatment, and subjected to analysis by RT-PCR as described in "Materials and methods". The contents of 36B4 and PEPCK-C transcripts at time 0 were arbitrarily assigned as 100%. Each value represents the means \pm SD of three independent experiments (* $P < 0.01$).

transcripts is about 1.5 h for both groups, demonstrating no change of its stability after the lipid extract treatment. It supports the conclusion that the lipid mediator induces the transcription of PEPCK-C gene.

Discussion

My results demonstrate for the first time that a lipid mediator in the lipid extracts from rat livers induces transcription of PEPCK-C gene in primary rat hepatocytes. This novel approach and observation have not been published previously as far as I know. Because the activity of the lipid mediator has been observed in livers from different rat strains, the lipid mediator is probably a common metabolite in the liver. The existence of this lipid mediator in livers of other species and insulin resistant animals remains to be determined.

The identity of the lipid mediator remains to be determined. It could not be glucocorticoids since the medium A contains 100 nM dexamethasone. The unsaponifiable matters include cholesterol, oxysterols, and intermediates in cholesterol metabolic pathway [22]. Activation of the oxysterol receptor LXR reduced the PEPCK-C expression in rodent livers [15]. Insulin induces SREBP-1c transcription through activation of LXR at its promoter [17]. All these results would predict that oxysterols in the lipid extract will cause a reduction of the PEPCK-C gene expression, rather than the induction as I observed. Therefore, the active molecule is unlikely an activator of LXR. Alternatively, it might be an antagonist. However, the effects of fenofibrate ester, a LXR activation antagonist, on PEPCK-C gene expression have not been reported in the original observation [23].

Another novel observation is that the lipid extracts attenuated insulin-mediated reduction of PEPCK-C gene expression. Currently, it is not known whether the same lipid mediator induces the PEPCK-C gene transcription and attenuates insulin action. Additionally, whether the active molecule works directly on the PEPCK-C promoter or more upstream of insulin signaling cascade is another question. The fact that the attenuation has been observed only by RT-PCR, but not by reporter gene assays, implies that it acts directly on the promoter. The discrepancy may be caused by the lack of nucleosome structure in luciferase reporter plasmids used. Indeed, it has been shown that rapid, insulin-induced, histone demethylation at the PEPCK-C gene promoter plays a role in insulin-mediated reduction of PEPCK-C gene transcription [24]. Whether the lipid mediator interferes with this process deserves further study.

The activities of the endogenous lipid mediators indicate that the transcription of PEPCK-C gene can be coordinately regulated by both intracellular metabolites and extracellular signals. The levels of the active molecules and pathways responsible for their production may contribute to the role of liver in maintaining glucose and lipid homeostasis. The abnormal production of them may

reduce the responses of hepatocytes to extracellular hormonal and nutritional signals, and result in metabolic diseases, such as insulin resistance. Identification of these lipid mediators and their functional mechanisms will not only uncover the additional regulatory mechanisms of PEPCK-C gene transcription but also provide us with new tools to combat metabolic diseases.

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