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ORIGINAL ARTICLE

Tissue Specific Effects of Dietary Carbohydrates and Obesity on ChREBPα and ChREBPβ Expression

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Abstract Carbohydrate response element binding protein (ChREBP) regulates insulin-independent *de novo* lipogenesis. Recently, a novel ChREBPβ isoform was identified. The purpose of the current study was to define the effect of dietary carbohydrates (CHO) and obesity on the transcriptional activity of ChREBP isoforms and their respective target genes. Mice were subjected to fasting-refeeding of high-CHO diets. In all three CHO-refeeding groups, mice failed to induce ChREBPα, yet ChREBPβ increased 10- to 20-fold. High-fat fed mice increased hepatic ChREBPβ mRNA expression compared to chow-fed along with increased protein expression. To better assess the independent effect of fructose on ChREBPα/β activity, HepG2 cells were treated with fructose ± a fructose-1,6-bisphosphatase inhibitor to suppress gluconeogenesis. Fructose treatment in the absence of gluconeogenesis resulted in increased ChREBP activity. To confirm the existence of ChREBPβ in human tissue, primary hepatocytes were incubated with high-glucose and the expression of ChREBPα and -β was determined. As with the animal models, glucose induced ChREBPβ expression while ChREBPα was decreased. Taken together, ChREBPβ is more responsive to changes in dietary CHO availability than the -α isoform. Diet-induced obesity increases basal expression of ChREBPβ, which may increase the risk of developing hepatic steatosis, and fructose-induced activation is independent of gluconeogenesis.

Keywords *De novo* lipogenesis · Fasting-refeeding · Fatty liver · Fructose · Gluconeogenesis · Obesity

Abbreviations

ChREBP	Carbohydrate response element binding protein
CHO	Carbohydrates
DNL	*De novo* lipogenesis
TG	Triglycerides
NAFLD	Non-alcoholic fatty liver disease
LID	Low-glucose inhibitory domain
GNG	Gluconeogenesis
IPGTT	Intraperitoneal glucose tolerance tests
RNAP2	RNA polymerase II
PCR	Polymerase chain reaction
FBPase-1	Fructose-1,6-bisphosphatase
DMEM	Dulbecco's modified Eagle's medium
RPMI	Roswell Park Memorial Institute medium
cDNA	Complementary DNA
SEM	Standard error of the mean
L-PK	Liver pyruvate kinase
SCD-1	Stearoyl-CoA desaturase-1
FOXO1	Forkhead box protein O1
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator-1α

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PEPCK	Phosphoenolpyruvate carboxykinase
G6Pase	Glucose-6-phosphatase
mRNA	Messenger RNA
LG	Low glucose
HG	High glucose
LF	Low fructose
HF	High fructose
ACCα	Acetyl-CoA carboxylase-α
FASN	Fatty acid synthase
TXNIP	Thioredoxin-interacting protein
KHK	Ketohexokinase
HFD	High-fat diet
UnT	Untreated
X5P	Xylulose-5-phosphate

Introduction

Carbohydrate response element-binding protein (ChREBP) is a transcription factor known to regulate *de novo* lipogenesis (DNL). Evidence has shown that ChREBP is largely glucose responsive [1, 2] and is activated independent of insulin [3], although it can be activated by additional carbohydrates [4]. Increased expression of ChREBP in the liver has been correlated with both obesity and hepatic steatosis [5, 6]. The persistent elevation of ChREBP expression under non-fed conditions is thought to increase conversion of carbohydrates (CHO) into triglycerides (TG) even when plasma glucose levels remain in the normal range. Since ChREBP plays such a prominent role in DNL, increased expression in the liver may result in the development of non-alcoholic fatty liver disease (NAFLD) by channeling excess CHO into TG.

Recently, a novel isoform of ChREBP was discovered, identified as ChREBPβ and unlike ChREBPα, it is considered to be active under low-glucose conditions. One of the primary means of regulation of the classical ChREBPα isoform is via the low-glucose inhibitory domain (LID) in which glucose binds to the LID and sequesters it in the cytosol. Due to the use of an alternative exon 1 b and a new translation start site, the LID is absent from ChREBPβ and is thus likely to be active under low glucose conditions. It is thought that induction of ChREBPβ mRNA is the result of high-glucose induced activation of ChREBPα that in turn transactivates the ChREBPβ promoter from a distant exon 1 b–10 Kb upstream from the translation start site that is spliced into exon 1 and utilizing a new ATG start codon [7].

The relative effect of glucose, fructose, or sucrose on ChREBPβ expression in the liver and other CHO-responsive tissues was tested to gauge the effects of ChREBP isoform expression. With fructose feeding, we found a noticeable elevation in gluconeogenesis (GNG), which could indirectly modulate ChREBP transcriptional activity. Therefore, we tested the effects of fructose *in vitro* while preventing its ability to be converted into glucose. Additionally, given the fact that the -β isoform lacks the LID, we tested the effect of obesity on chronic ChREBPβ expression in an effort to better understand its role in obesity-induced DNL in liver. Results showed that with the exception of the skeletal muscle, ChREBPβ is not expressed in any tissue after a prolonged fast by chow-fed mice but becomes expressed when mice are refed CHO. Fructose, independent of its gluconeogenic capacity, increased ChREBPβ expression in a cell culture model. Lastly, obese mice had significantly higher ChREBPβ expression in the liver compared to chow-fed mice. The results from the present investigation suggest that ChREBPβ, and not ChREBPα, may be responsible for CHO-induced lipogenesis in the liver and other CHO-responsive tissues.

Materials and Methods

Animals, Diets, and Treatments

All animal experiments were approved by the Texas Tech University Institutional Animal Care and Use Committee. Male C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME), fed *ad libitum*, group caged, and kept on a 12-h light/dark cycle. For experiments involving diet-induced obesity, mice were fed either 5P14—Prolab® RMH 2500 standard rodent chow (LabDiet, St. Louis, MO) or a high-fat custom research diet (TD.08500, Harlan Laboratories, Madison, WI) at 8 weeks of age. Non-fasted mouse body weights were measured weekly at the same approximate time of day. Fasting-refeeding, which is designed to robustly induce *de novo* lipogenic gene expression [8–11], was used with chow-fed 10-week old male mice that were either fasted for 24 h before sacrifice, or fasted for 24 h then fed a very low-fat, very high-CHO diet (Harlan Laboratories, Madison, WI) for 12 h, then sacrificed. The composition of the diets is provided in Table 1.

Plasma Triglyceride and Glucose Measurements

Intraperitoneal glucose tolerance tests (IPGTT) and subsequent blood glucose measurements were performed as previously described [12]. Blood was collected via a tail nick at baseline and 20, 40, 80, and 120 min post-injection. Plasma from terminal blood collected by cardiac exsanguination was analyzed to quantify plasma TG levels via using the L-type triglyceride M kit (Wako Chemicals USA, Inc., Richmond, VA).

Table 1 Experimental diets

Diet	Purpose	Cal per gram	Cal from CHO (%)	Cal from fat (%)	Cal from pro (%)
Chow	Control diet	3.0	58.0	13.5	28.5
High-fat diet	Diet-induced obesity	5.1	21.3	60.3	18.4
High-glucose diet	Fasting-refeeding	3.6	77.2	3.0	19.8
High-fructose diet	Fasting-refeeding	3.6	77.4	3.0	19.6
High-sucrose diet	Fasting-refeeding	3.6	77.4	3.0	19.6

Semi-quantitative RT-PCR and Quantitative RT-PCR

To detect expression of genes, semi-quantitative RT-PCR was performed. For changes in gene expression, quantitative RT-PCR was performed, as previously described [12]. A list of primers used is located in Table 2. The housekeeping gene used for quantitative RT-PCR for HepG2 cells and mouse tissues was RNA polymerase II (RNAP2).

Western Blotting

Protein extraction of tissues and cells involved NP40 lysis buffer with triton X-100 and protease inhibitor cocktail (AMRESCO, LLC, Solon, OH). Protein content was determined via Bradford assay. The ChREBP (P-13) and actin (I-19) were from Santa Cruz Biotechnology, Inc. (Dallas, TX).

HepG2 Cell Culture

HepG2 ells were cultured in DMEM supplemented with 10 % FBS and 1 % pen/strep (Caisson Laboratories Inc, North Logan, UT). To inhibit GNG, cells were treated with 10 μM FBPase-1 inhibitor (5-chloro-2-(*N*-(2,5-dichlorobenzenesulfonamido))-benzoxazole) (Santa Cruz Biotechnology, Inc. Dallas, TX) with DMSO used as a vehicle. Treatments lasted 24 h in low-glucose media (5.5 mM) with either 5.5 or 25 mM fructose, with 25 mM glucose serving as a positive control. Parallel experiments using 400 nM N^8-(cyclopropylmethyl)-N^4-(2-(methylthio) phenyl)-2-(1-piperazinyl)-pyrimido[5,4-d]pyrimidine-4,8-diamine (EMD Millipore, Billerica, MA) were conducted to block ketohexokinase activity.

Human Primary Hepatocytes

Primary human hepatocytes were isolated using collagenase-based perfusion of liver fragments obtained by resection of specimens far away from the tumor margin during biopsy as previously described [13]. Human liver samples used for hepatocyte isolation were obtained from patients undergoing operations for therapeutic purposes at the Service of Digestive Tract Surgery, University of Alberta. Ethical approval was obtained from the University of Alberta's Faculty of Medicine Research Ethics Board and informed consent was obtained from all patients that participated in the study. Isolated primary hepatocytes were plated in 60 mm collagen-coated dishes (BD Biosciences) at a concentration of 1.5 million cells per dish containing 2.5 mL of cell culture medium. The cells were incubated in modified RPMI-1640 culture medium (GIBCO) containing 10 % fetal bovine serum (GIBCO) for 2 h to allow the cells to adhere to the plate. After this period the medium was replaced DMEM (GIBCO) that contained either high (25 mM) or low (5 mM) glucose and incubated for 24 h. The cells were quickly washed with saline, after which 1 mL of Trizol (Invitrogen) was added directly to the cells. A portion of cells that were not plated were rinsed with saline and treated with Trizol, then frozen at −80 °C until further analysis. All incubations were conducted at 37 °C in humidified air containing 5 % CO_2 and viability was tested using trypan blue exclusion. cDNA was synthesized using Superscript II (Invitrogen) reverse transcriptase and a mix of Oligo(dT) (Invitrogen) and Random Hexamers (Invitrogen). Semi-quantitative PCR and qPCR reactions were conducted on a StepOnePlus qPCR machine from Applied Biosystems using Power SYBR green master mix from Applied Biosystems using primers provided in Table 2. Semi-quantitative PCR reactions were run on a 1.5 % agarose gel in TAE buffer for 1.5 h, stained with GelRed (Thermo) for 10 min, and visualized using a ChemiDoc (Biorad) camera with ImageLab software.

Statistical Analyses

Analysis of statistics was performed using R statistical software in package version 3.0.3 from the R-project. Results are given as means ± SEM with a minimum of three replicates per group unless otherwise indicated. Differences between groups were analyzed via Student's *t* test or pairwise differences when comparing control groups *versus* treatment groups. Differences between groups were considered significant at $p < 0.05$.

Table 2 Primers list

Type	Name	Fwd/rev	5′–3′ primer sequences
HepG2	ACCα	Forward	5′-TACCATCAGGTAGCCGTGCAGTTT-3′
		Reverse	5′-GCTCAGGGTTGGCATTGTGGATTT-3′
HepG2	ChREBPα	Forward	5′-CATCCACAGCGGTCACTTCATGG-3′
		Reverse	5′-CACTTGTGGTATTCCCGCATCACC-3′
HepG2	ChREBPβ	Forward	5′-CTCTGCAGGTCGAGCGGATTC-3′
		Reverse	5′-CACTTGTGGTATTCCCGCATCACC-3′
HepG2	FASN	Forward	5′-ACGTCACGGACATGGAGCACAACA-3′
		Reverse	5′-ATGGTACTTGGCCTTGGGTGTGTA-3′
HepG2	RNAP2	Forward	5′-ACCACGTCATCTCCTTTGATGGCT-3′
		Reverse	5′-TTCTCTGCATCAAGCAGGAGGTCA-3′
HepG2	TXNIP	Forward	5′-TCAAGTTCATGCCACCACCGACTTA-3′
		Reverse	5′-GCCTGCTGACCACCTCCTACATTA-3′
Human hepatocytes	ACC	Forward	5′-GATCGCTGGAGAATCCTCAT-3′
		Reverse	5′-GACAAGGTAAGCCCCAATCC-3′
Human hepatocytes	ChREBPα	Forward	5′-CATCCACAGCGGTCACTTCATGG-3′
		Reverse	5′-CACTTGTGGTATTCCCGCATCACC-3′
Human hepatocytes	ChREBPβ	Forward	5′-CTCTGCAGGTCGAGCGGATTC-3′
		Reverse	5′-CACTTGTGGTATTCCCGCATCACC-3′
Human hepatocytes	FASN	Forward	5′-CATCGGCTCCACCAAGTC-3′
		Reverse	5′-GCTATGGAAGTGCAGGTTGG-3′
Human hepatocytes	TXNIP	Forward	5′-AACATCCCTGATACCCCAGA-3′
		Reverse	5′-TCTCCAATCGGTGATCTTCA-3′
Mouse	ChREBPα	Forward	5′-ACAGCGGACACTTCATGGTGTCTT-3′
		Reverse	5′-TATTCGCGCATCACCACCTCGAT-3′
Mouse	ChREBPβ	Forward	5′-AGACCCGAGGTCCCAGGAT-3′
		Reverse	5′-TATTCGCGCATCACCACCTCGAT-3′
Mouse	FOXO1	Forward	5′-GCGTGCCCTACTTCAAGGATAAGG-3′
		Reverse	5′-CTGGATTGAGCATCCACCAAGAACTC-3′
Mouse	G6Pase	Forward	5′-ACAGCAACAGCTCCGTGCCTATAA-3′
		Reverse	5′-CAAACACCGGAATCCATACGTTGGC-3′
Mouse	L-PK	Forward	5′-TCGAGAACCATGAAGGCGTGAAGA-3′
		Reverse	5′-TCTCTGCTGGGATCTCAATGCCAA-3′
Mouse	PEPCK	Forward	5′-GTAGGAGCAGCCATGAGATCTGAGG-3′
		Reverse	5′-GCCGAAGTTGTAGCCGAAGAAGG-3′
Mouse	PGC-1α	Forward	5′-AGCACTCAGAACCATGCAGCAAAC-3′
		Reverse	5′-TTTGGTGTGAGGAGGGTCATCGTT-3′
Mouse	RNAP2	Forward	5′-GCACCATGTCATCTCCTTTGATGGTT-3′
		Reverse	5′-TCTCAGCATCAAGCAGGAGGTCAA-3′
Mouse	SCD-1	Forward	5′-GAGGCGAGCAACTGACTATCATCATG-3′
		Reverse	5′-GCACCGTCTTCACCTTCTCTCG-3′

Results

ChREBPβ is Expressed in Extrahepatic Tissues After High Carbohydrate Refeeding

Present literature has only identified gene expression of ChREBPβ in adipose tissue and liver of mice and humans but not in other CHO-responsive tissues [7, 14, 15]. Therefore, we tested the role of different CHO-refed conditions on the expression of ChREBP isoforms from additional CHO-responsive tissues, such as red gastrocnemius and kidney, as well as a side-by-side comparison of epididymal and subcutaneous fat (Fig. 1). ChREBPβ was not expressed during prolonged fasting in any tissues

assessed or in the red gastrocnemius during refeeding. ChREBPβ expression was found in the subcutaneous fat, epididymal fat, and kidneys of refed mice with all three CHO sources. ChREBPα mRNA was expressed in all tissues analyzed in both fasted and refed states. These results indicate ChREBPβ expression changes with CHO flux, while the expression of ChREBPα does not appear to change during the fasting or postprandial states assessed.

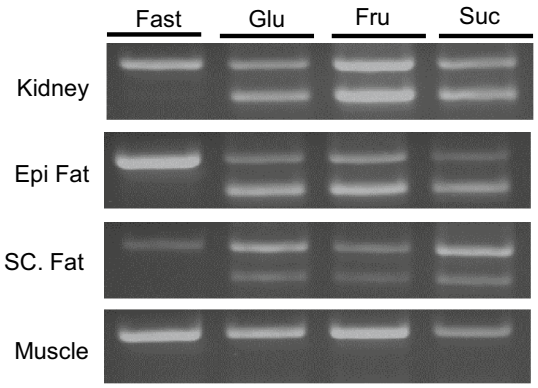

Fig. 1 Refeeding carbohydrates induces ChREBPβ expression in multiple tissues. Tissues from mice fasted for 24 h (Fast) and mice fasted for 24 h then refed glucose (Glu), fructose (Fru), or sucrose (Suc) for 12 h were collected and analyzed for ChREBPα and -β expression via semi-quantitative RT-PCR. In kidney (K), epididymal fat (E), subcutaneous fat (S), and red gastrocnemius muscle (M) fasted animals displayed ChREBPα (larger base pair amplicon) and in refed groups ChREBPβ (smaller base pair amplicon) appeared in all tissues except muscle. Figure is representative of $n = 5$ animals per group

High Hepatic ChREBPβ Expression Occurs in the Liver in Response to High Carbohydrate Refeeding

Semi-quantitative RT-PCR was also used to detect ChREBP isoform expression in the livers of mice after fasting-refeeding (Fig. 2a). While ChREBPβ did not appear to be expressed after fasting, ChREBPα was detected in the livers of fasted mice. Both isoforms, however, were shown to be expressed when mice were refed carbohydrates. Changes in ChREBP isoform gene expression were then assessed via qRT-PCR (Fig. 2a, b). Refeeding CHO decreased ChREBPα gene expression, which was not expected. Under the same conditions, ChREBPβ mRNA expression increased with all three CHO sources. These results imply that hepatic ChREBPβ expression is robustly induced following high-CHO refeeding.

High Carbohydrate Refeeding Increases *de novo* Lipogenic Gene Expression in the Liver

Fasting-refeeding was used to induce *de novo* lipogenic gene expression (Fig. 3a, b). All refed groups showed a significant increase in the ChREBP target gene liver pyruvate kinase (L-PK) indicating that ChREBP transcriptional activity was likely increased. The lipogenic target stearoyl-CoA desaturase-1 (SCD-1), another known target gene of ChREBP [16, 17], was also increased by refeeding CHO. Finally, plasma TG levels were also assessed in fasted and refed mice (Fig. 3c), with elevations in TG concentrations observed with refeeding. From these results along with data from Fig. 2, it appears that CHO-refeeding suppressed ChREBPα and increased ChREBPβ along with known

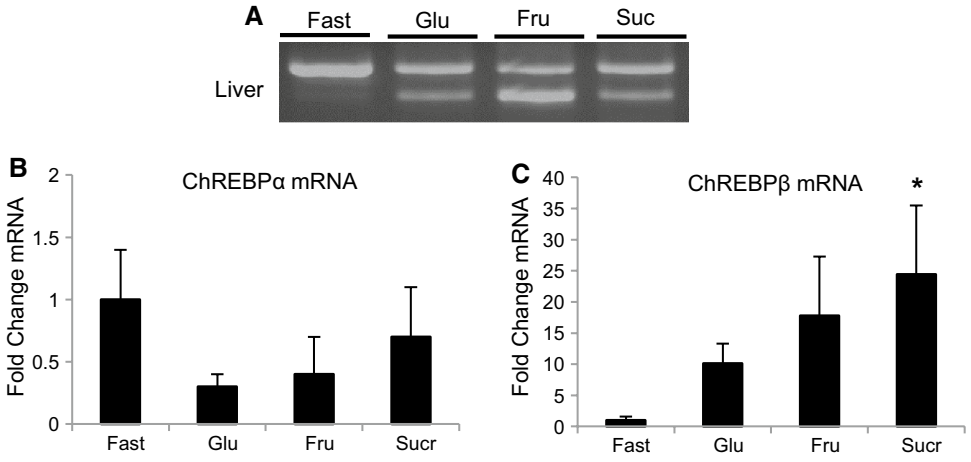

Fig. 2 Carbohydrate refeeding increases ChREBPβ and not ChREBPα expression in the liver. **a** Mice refed high-glucose (Glu),—fructose (Fru), or—sucrose (Suc) diets expressed both ChREBPα (larger base pair amplicon) and ChREBPβ (smaller base pair amplicon) in the liver as assessed by semi-quantitative RT-PCR, while ChREBPβ did not appear to be expressed in fasted mice. **b** Refed mice did not increase ChREBPα gene expression in the liver when measured by qRT-PCR. **c** ChREBPβ expression however was increased in all refed groups ($n = 5$ animals/group; $^*p < 0.05$ *vs* fasted group)

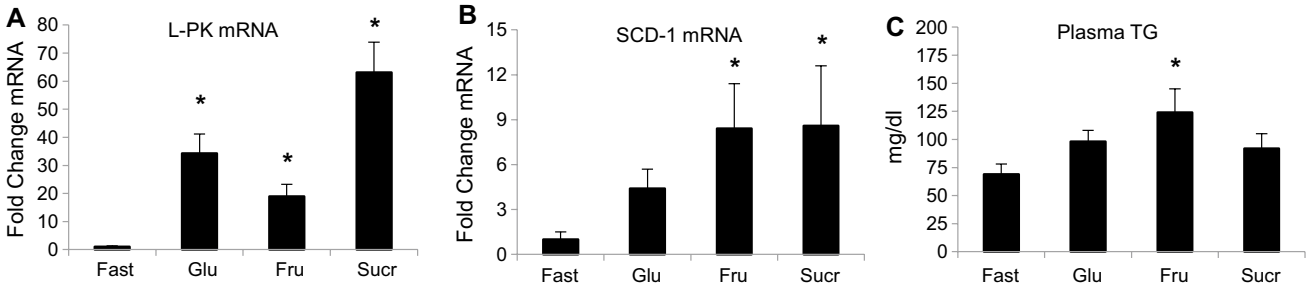

Fig. 3 Refeeding carbohydrates increases hepatic *de novo* lipogenic gene expression and increases plasma triglycerides. The expression of ChREBP target genes liver pyruvate kinase (L-PK) (**a**) and stearoyl-CoA desaturase-1 (SCD-1) (**b**) increased in all three refed groups. All three refed groups also increased plasma triglyceride (TG) levels (**c**) ($n = 5$ animals/group; $^*p < 0.05$ *vs* fasted group)

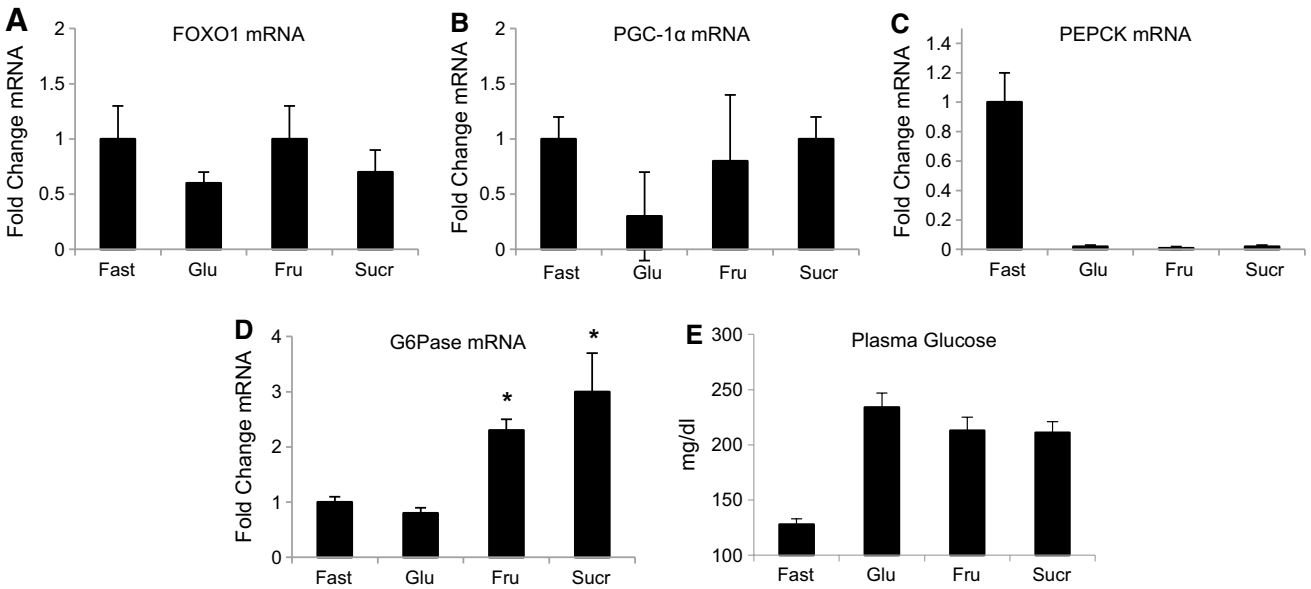

Fig. 4 Fructose and sucrose refeeding increases gluconeogenesis. In addition to lipogenesis, ChREBP activity increases gluconeogenesis (GNG). Mice refed high-CHO diets did not increase FOX01 (**a**), PGC-1α (**b**), or PEPCK (**c**) expression. The terminal GNG enzyme glucose-6-phosphatase (G6P) was unaffected by Glu refeeding yet increased with Fru and Suc (**d**). Plasma blood glucose levels were higher in all refed groups confirming elevated GNG activity (**e**) ($n = 5$ animals/group; $^*p < 0.05$ *vs* fasted group)

ChREBP responsive genes and TG production. This suggests that the -β isoform may be responsible for mediating CHO-induced lipogenesis in a variety of tissues rather than the -α isoform as has been suggested [7].

Fructose and Sucrose Refeeding Stimulates Gluconeogenesis

Gluconeogenic genes were assessed because they are also targets of ChREBP and it is speculated that refeeding diets high in fructose would induce GNG (Fig. 4a–d). No significant difference was observed between treatment groups and the fasted group for peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) or forkhead box protein O1 (FOXO1). A significant decrease in phosphoenolpyruvate carboxykinase (PEPCK) was observed in the refeeding groups. Refeeding fructose and sucrose was shown to significantly increase glucose-6-phosphatase (G6Pase) gene expression, while glucose refeeding lowered its expression. Plasma glucose levels were also assessed in the fasted and refed mice (Fig. 4e). As expected, refeeding CHO resulted in significantly higher plasma glucose concentrations. The decrease in PEPCK was expected given that it is primarily involved in amino acid-mediated GNG. Similarly, glucose refed mice would not be expected to increase G6Pase whereas the fructose and sucrose refed mice would likely require it to release glucose from fructose metabolism. As such, the increase in G6Pase may be important for fructose metabolism in the livers of the mice that were refed fructose or sucrose.

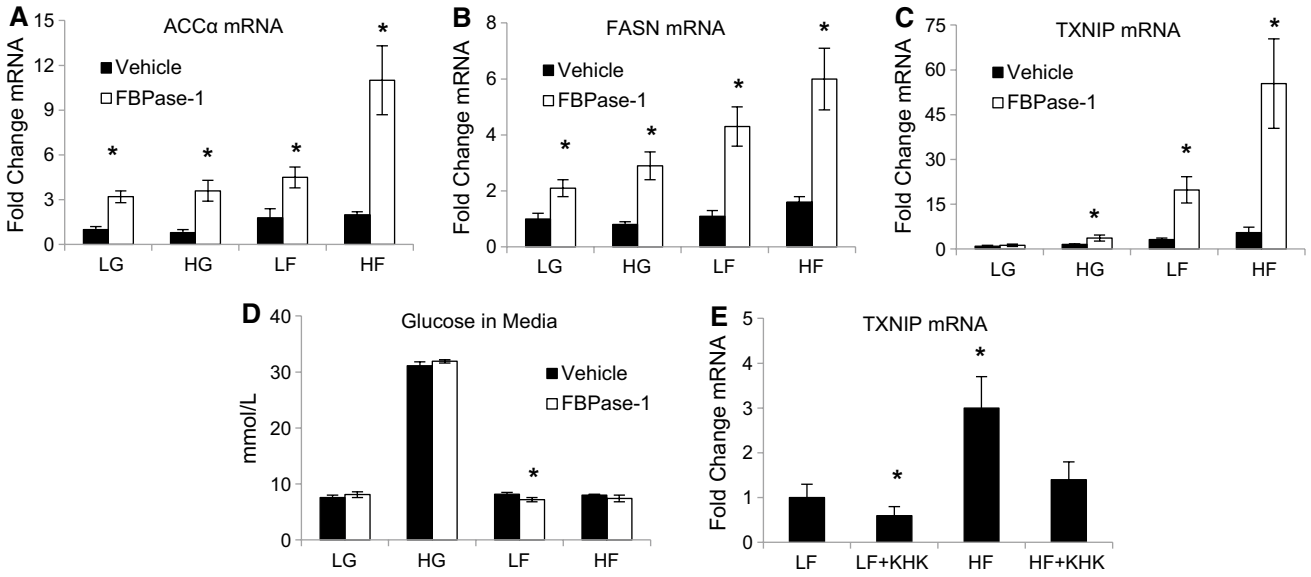

Fig. 5 Gluconeogenesis is dispensable for fructose-induced ChREBP activity. HepG2 cells were treated with low glucose (LG) (negative control), high glucose (HG) (positive control), low fructose (LF) or high fructose (HF) in the presence (vehicle) or absence (FBPase-1) of fructose 1,6-bisphosphatase activity. Despite FBPase-1 inhibition, ChREBP target genes including ACCα (**a**), FASN (**b**), and TXNIP (**c**) were increased with LF and HF treatments. Glucose content of media was measured to confirm inhibition of GNG (**d**). Initial phosphorylation of fructose into fructose-1-phosphate was blocked using a ketohexokinase inhibitor (KHK). When fructose metabolism is inhibited, ChREBP activity is completely repressed (**e**) ($n = 5$–6 replicates/treatment; $^*p < 0.05$ *vs* LG in **a**–**c**, between groups in **d**, *vs* LF in **e**)

Given the fact that fructose and sucrose fed mice increased G6Pase mRNA, it is possible that fructose is being converted to glucose and mediating the activation of ChREBP. Therefore, to eliminate this possible mechanism, an FBPase-1 inhibitor was used to suppress conversion of fructose-1,6-bisphosphate into fructose-6-phosphate (Supplemental Fig. 1). We treated HepG2 liver-like cells with low glucose (LG), high glucose (HG), low fructose (LF), or high fructose (HF) for 24 h in the presence or absence of FBPase-1 activity. Only HF treated cells displayed increased lipogenic activity in the presence of FBPase-1 (Fig. 5a, b). However, when GNG activity was blocked, all four treatments resulted in increased lipogenesis reflected by increases in ACCα and FASN mRNA. The ChREBP target gene TXNIP (Fig. 5c) was used as a specific marker for altered ChREBP activity and we observed significant increases compared to LG treated cells among HG, and HF indicating that HG and HF both increase ChREBP transcriptional activity. When GNG activity was blocked, LG treated cells displayed no change in TXNIP expression while TXNIP expression was increased in HG, LF, and HF treatments. Changes in glucose content of the media was used to confirm GNG activity (Fig. 5d). As expected, inhibition of FBPase-1 lowered glucose in media of cells in the fructose treatment groups. To ensure specificity of fructose metabolism, we also used a ketohexokinase (KHK) inhibitor to block fructose conversion into fructose-1-phosphate, thereby preventing commitment into the cell's metabolic pathways. HF treated cells increased TXNIP expression with KHK-mediated inhibition of fructose metabolism reducing ChREBP activity in both LF and HF treated groups (Fig. 5e). Taken together, these data indicate that fructose, independent of its gluconeogenic capacity, activates ChREBP transcriptional activity. Furthermore, these data suggest that GNG may in fact suppress DNL since FBPase-1 inhibition increases lipogenic gene expression in all groups.

Obesity Increases ChREBPβ Expression

Obesity is associated with increased ChREBP expression and CHO-mediated fatty liver and we sought to examine the effect of high-fat diet (HFD) induced obesity on ChREBPα and -β expression. Eight-week old male mice were either fed a chow or HFD for 13 weeks. HFD-fed mice were heavier than chow-fed mice (Fig. 6a) and displayed impaired glucose tolerance (Fig. 6b) confirming the validity of our obesity model. Mice were fasted for 4 h prior to collecting mRNA to assess ChREBP isoform expression in carbohydrate-responsive tissues (Fig. 6c–g). ChREBPα was higher in the kidneys of HFD-fed mice and lower in white adipose tissue and red gastrocnemius with no observed differences in ChREBPβ expression in the kidneys or adipose between groups. In the liver, ChREBPα

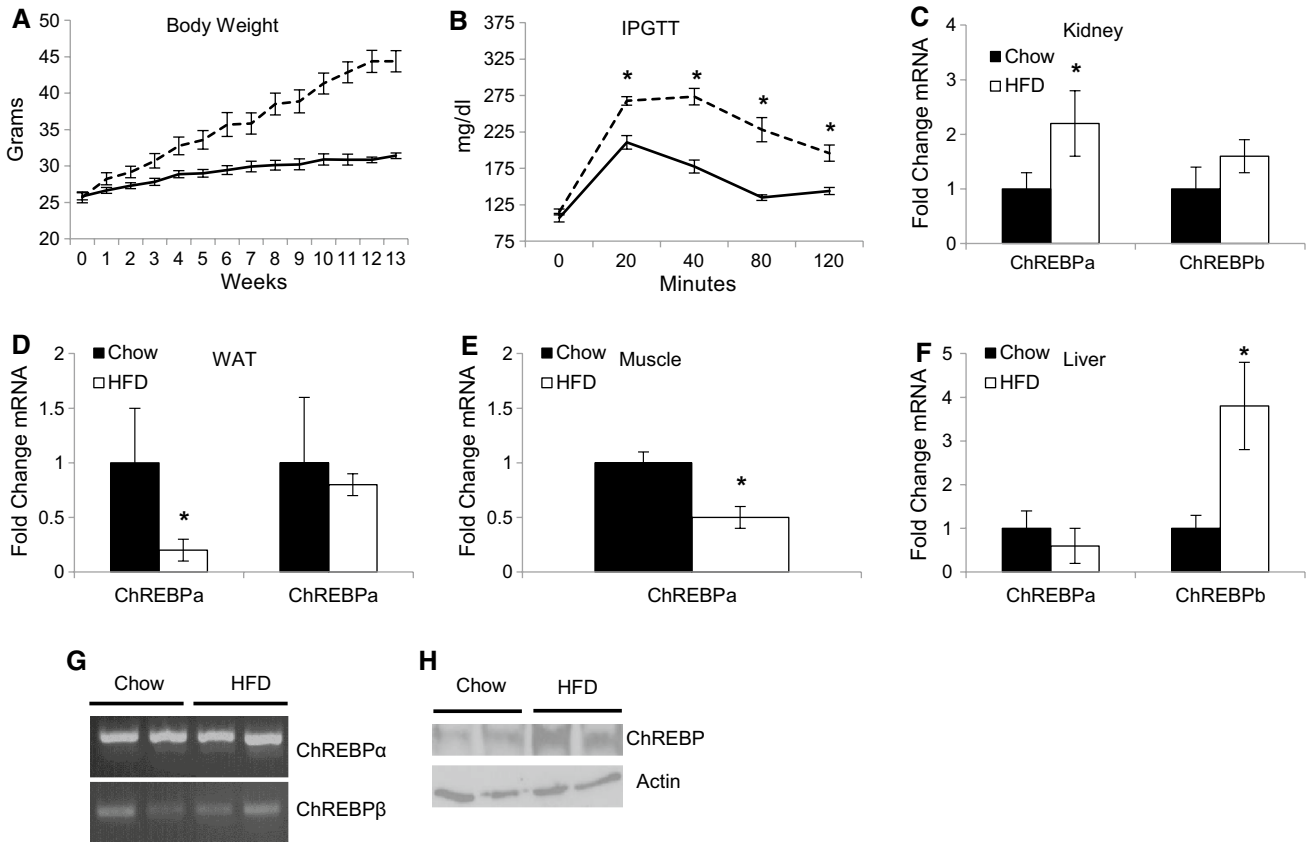

Fig. 6 ChREBPβ expression is increased in livers of obese fasted mice. Obesity (**a**) and glucose intolerance (**b**) were induced in high-fat fed mice. Expression of ChREBPα and -β isoforms was determined in kidneys (**c**), subcutaneous white adipose tissue (WAT) (**d**), red gastrocnemius muscle (**e**), and liver (**f**). The -α isoform was increased in kidney and repressed or unchanged in all other tissues and ChREBPβ was increased in liver of high-fat fed (HFD) mice. Detection of ChREBPα andChREBPβ after a 4-h fast in the livers of chow-fed and HFD mice via semi-quantitative RT-PCR (**g**). Protein expression measured by western blot showed higher total ChREBP levels in obese mice compared to lean mice (**h**). Since ChREBPα was unchanged, it is likely that the increase in protein is due to the -β isoform ($n = 5$ animals/group; $^*p < 0.05$ *vs* fasted group)

was unaffected by HFD, however ChREBPβ was significantly higher compared to chow-fed mice based on qRT-PCR. Lastly, ChREBP(pan) protein expression was examined in the livers of mice fed HFD or chow by western blot. ChREBP protein expression appeared higher in high-fat fed mice (Fig. 6h) but we were unable to distinguish between isoforms via western blot. Since the livers of high-fat fed mice had no change in ChREBPα mRNA expression, it is logical to conclude that the increase in ChREBP protein is likely due to increased -β isoform expression.

High-Glucose Treatment Increases ChREBPβ and Target Gene Expression in Human Hepatocytes

Our studies on dietary CHO's and obesity were conducted in mice, and while informative, they may be irrelevant with respect to human ChREBP expression. That is to say, if ChREBPβ is strictly a murine phenomenon, it has little utility toward understanding the mechanism of human obesity. Therefore, we sought to determine whether glucose treatment induced ChREBPβ expression in human liver. To do so, primary human hepatocytes were isolated and cultured as described above, then treated with serum free low-glucose (5.5 mM) or high-glucose (25 mM) containing DMEM for 24 h. Semi-quantitative RT-PCR was performed to gauge mRNA abundance between the -α and -β isoforms of ChREBP (Fig. 7a). Results show the ChREBPβ is expressed in human primary hepatocytes (untreated, UnT) and it appears to increase with a high-glucose treatment while ChREBPα appears to decrease. These results are in accordance with our fasting-refeeding experiments in mice that showed refeeding CHO resulting in a decrease in ChREBPα, but ChREBPβ increasing. High glucose treatment also significantly increased mRNA expression of the ChREBP target genes ACC, FASN, and TXNIP (Fig. 7b–d). In light of the above studies, it appears that ChREBPβ responds acutely to changes in dietary CHO availability and that obesity increases its expression in the liver. This is

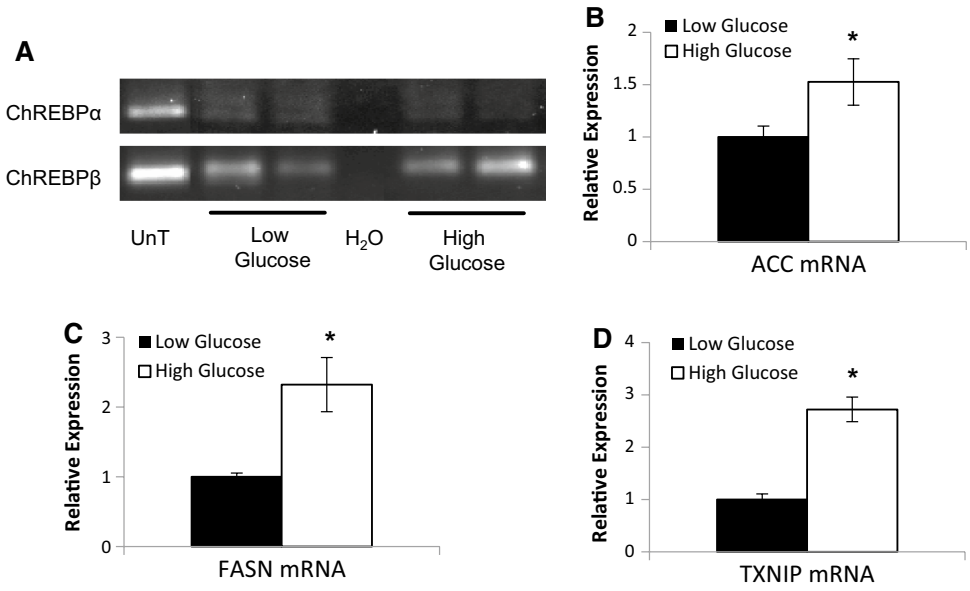

Fig. 7 ChREBPβ expression increases in response to high-glucose in primary human hepatocytes. Human primary hepatocytes were cultured then treated in DMEM containing either 5.5 or 25 mM glucose for 24 h. ChREBPα and ChREBPβ (**a**) are detectible with semi-quantitative RT-PCR in untreated (UnT) cells. ChREBPβ expression increased from low- to high-glucose treatment whereas the -α isoform appears to be unchanged. mRNA expression of the ChREBP target genes ACC (**b**), FASN (**c**), and TXNIP (**d**) were all significantly increased in high-glucose treated primary human hepatocytes

in agreement with previous studies that have shown human ChREBPβ expression increasing in the liver in the setting of obesity in addition to hepatic steatosis and insulin resistance [14, 15]. Given the fact that ChREBPβ is in fact expressed in the liver of humans, further examination of the transcriptional and metabolic impacts of this protein would provide insight into the mechanism of fatty liver disease and obesity-related co-morbidities.

Discussion

Prior to the identification of the ChREBPβ isoform, the mechanism of insulin-independent, glucose-mediated lipogenesis was attributed primarily to ChREBP (now referred to as ChREBPα). We were able to distinguish between each isoform among tissues and determine the pattern of expression under different dietary conditions that revealed a consistent effect of postprandial increases in -β expression with little or no change in ChREBPα. However, while -α responsiveness was not observed, it is likely that acute changes in its activity occurred in response to initial fluctuations in plasma and hepatic CHO levels. Previous studies have suggested that -α serves the role of transactivating the -β isoform; the latter of which provides the bulk of the lipogenic effect. Our results would support the claim that ChREBPα is acutely responsive to changes in CHO although further studies delineating a shorter time-course are warranted. Additionally, we did not directly measure lipogenesis via labeled carbon incorporation into lipids and the mRNA/protein measures are largely estimates of lipogenic activity. A more robust and direct assessment of lipogenic activity may provide more insight into the roles of ChREBPα and ChREBPβ.

ChREBPβ was not expressed in skeletal muscle of fasted or refed mice. While several reports have demonstrated the capacity for muscle to undergo lipogenesis and store intramuscular TG, it is not likely that CHO-mediated DNL plays a significant role in this tissue [18]. Alternatively, the ChREBPα/β (MondoB) paralog, MondoA, is highly expressed in skeletal muscle [19], and may act to regulate glucose-dependent transcription in this tissue [20]. MondoA expression was beyond the scope of our study but should be assessed in future work.

In order to better assess whether fructose was able to activate ChREBPα/β as fructose or whether it needed to be converted into glucose, we performed a series of cell culture experiments in which GNG was blocked at key regulated points. FBPase-1 inhibition resulted in significantly higher ChREBP target gene expression and suggests that preventing fructose from entering GNG results in more fructose to be used for TG synthesis. While it was not a direct question that was assessed, these data suggest that GNG may actually suppress lipogenesis. However, when viewed from the perspective of mass-action, it would seem logical to conclude that directing the flux of fructose metabolism away from glucose and glycogen synthesis would direct more carbon toward DNL. Another important point to consider is from GNG suppression via FBPase-1 inhibition regarding fructose is that more fructose is capable of entering the pentose phosphate pathway as glyceraldehyde-3-phosphate, which can then be used to synthesize xylulose-5-phosphate (X5P). ChREBP activation is thought to be X5P dependent [21]. Therefore, increased levels of X5P may be a plausible explanation regarding the significant increase in ChREBP target gene expression with FBPase-1 inhibition.

Increased ChREBPβ gene and ChREBP protein expression in obese mice may provide insight into the mechanism of CHO-mediated fatty liver disease. Since the β isoform lacks the LID, it can be active at lower glucose levels. While not constitutively active in the sense that it possesses posttranslational control mechanisms, its persistent expression during non-fed (4-h fasted) conditions and in the presence of elevated plasma glucose, as occurs with insulin resistance and glucose intolerance, could lead to a heightened DNL activity in the liver. It would be interesting to see how the difference between ChREBPα and ChREBPβ expression affect the development of fatty liver and steatohepatitis under chow-fed and high-CHO fed conditions.

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Compliance with Ethical Standards

Conflict of interest The authors have no conflicts of interest.

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