Modulation of carbohydrate response element-binding protein gene expression in 3T3-L1 adipocytes and rat adipose tissue

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He, Zhibin, Tao Jiang, Zhuowei Wang, Moshe Levi, and Jinping Li. Modulation of carbohydrate response element-binding protein gene expression in 3T3-L1 adipocytes and rat adipose tissue. Am J Physiol Endocrinol Metab 287: E424–E430, 2004. First published April 20, 2004; 10.1152/ajpendo.00568.2003.—Carbohydrate response element-binding protein (ChREBP) is a rat homolog of human Williams-Beuren syndrome region 14 and a member of the basic helix-loop-helix leucine zipper transcription factor family. Its activation was found to be inducible by carbohydrate in the liver of rats fed a high-sucrose diet. ChREBP is able to bind to the carbohydrate response element on the promoter of L-type pyruvate kinase and initiate the gene transcription. The detailed expression profile and transcriptional regulation of the ChREBP gene in adipocytes have not been characterized. In the present study, we provide evidence showing that 1) the ChREBP gene is expressed in differentiated 3T3-L1 adipocytes and rat adipose tissue; 2) insulin, glucose, and the antidiabetic agent troglitazone can significantly upregulate the expression of ChREBP in 3T3-L1 adipocytes, whereas free fatty acids suppress its expression in this cell type; 3) fasting followed by refeeding with a high-carbohydrate diet resulted in a 10-fold increase of ChREBP mRNA level in rat adipose tissue; and 4) ChREBP expression in adipose tissue is not significantly affected by the diabetic state. Taken together, the results we present are consistent with the idea that ChREBP is an important modulator of adipocyte biology and that its expression in adipose tissue is subject to combined regulation by glucose and insulin in vivo. The induction of ChREBP activity may serve as a novel pharmacological pathway for troglitazone-mediated hypoglycemic effects in vivo.

Adipogenesis; lipogenesis

WILLIAMS-BEUREN SYNDROME region 14 (Wschr 14) was first identified as a transcription factor bearing basic helix-loop-helix leucine zipper (bHLHZip) structure (3). This gene is among at least 14 deleted genes in patients with Williams-Beuren syndrome, which is characterized by various clinical symptoms, including mental retardation, heart abnormalities, unique personality profile, growth retardation, and hypercalce-mia (17). Even though a disorder of glucose metabolism has not been documented in the patients with this unique syndrome, Uyeda et al. (22) recently demonstrated that a rat homolog of the Wschr14 gene, the carbohydrate response element-binding protein (ChREBP), is activated in response to high-carbohydrate feeding and subsequently initiates the gene transcription of L-type pyruvate kinase (L-PK) in the liver of rats (24). They further demonstrated that the activation of ChREBP is mediated by the glucose molecule xylulose 5-phosphate. This metabolite activates protein phosphatase 2A (PP2A), which dephosphorylates Ser196 and Thr668 and subsequently activates ChREBP. The activated ChREBP translocates into the nucleus and binds to the carbohydrate response element within the promoter of the L-PK gene (7, 8). In addition, ChREBP is also proposed to be involved in the transcriptional regulation of a certain set of lipogenic enzymes in liver (21, 22). Nevertheless, to date, except L-PK, no other critical lipogenic enzyme has been identified to be under the direct control of ChREBP. The mechanism underlying the regulation of ChREBP gene expression remains unknown.

Adipose tissue is one of the most important organs for lipid and glucose metabolism. Because adipocyte function is constantly under rigorous regulation by food intake and dietary nutrients, we speculate that ChREBP is likely playing some functional roles in the adipose tissue. Thus we investigated the regulation profile of the ChREBP gene in 3T3-L1 preadipocyte adipogenesis and the effects of common nutritional factors, including glucose, free fatty acids, insulin, and the peroxisome proliferator-activated receptor-γ (PPARγ) agonist troglitazone. We further extended the gene expression regulation analysis to primary adipose tissue from rats with fasting/refeeding and streptozotocin-induced diabetes. Our aim was to determine whether ChREBP is involved in metabolic regulation in this important cell type.

MATERIALS AND METHODS

Animal preparation. Ten-week-old Sprague-Dawley male rats were used in the animal experiments (Charles River). The use of laboratory rats complied with National Institute of Health guidelines and was approved by the animal care facility at the Denver Veterans Affairs Medical Center. The rats were maintained in a room on a 12:12-h light-dark cycle and kept on Teklad diets from Harlan Teklad Premier Diets (Madison, WI). For the streptozotocin (STZ) experiments, rats were treated with STZ (Sigma) as previously described (5). STZ-induced diabetes was confirmed by measuring blood glucose level. For the fasting/refeeding experiment, 12 rats were separated into three groups (n = 4 each): 1) the ad libitum group had free access to standard diet chow; 2) fasting rats were deprived from chow for 48 h; and 3) the fed rats were fasted for 36 h and then were fed high-carbohydrate chow (60% sucrose by calories, D02022703, Research Diets, New Brunswick, NJ) for 12 h before they were killed. The fat tissue from the ad libitum group was harvested at 10 AM. The epididymal fat pads were removed and weighed, frozen in liquid nitrogen, and stored at −72°C until further use.

Cell culture and treatment. The 3T3-L1 cells were purchased from the American Type Culture Collection. The cells were maintained in

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Table 1. Primers employed

<table>
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<th>Gene</th>
<th>GenBank Accession No.</th>
<th>Forward Primers</th>
<th>Reverse Primers</th>
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mPPARγ, mouse peroxisome proliferator-activated receptor-γ; mInsig, mouse insulin-induced gene; mSREBP-1c, mouse sterol regulatory element-binding protein 1c; maP2, mouse adipocyte fatty acid-binding protein 2; mChREBP, mouse carbohydrate response element-binding protein; m36B4, mouse 36B4; rACC, rat acetyl-CoA carboxylase; rFAS, rat fatty acid synthase.

DMEM containing 10% FBS and penicillin-streptomycin at 37°C and 5% CO2. The differentiation of preadipocytes was carried out in the DMEM containing insulin-dexamethasone-IBMX, as previously described (9). In specified experiments, indicated amounts of BSA-conjugated free fatty acids (oleate-palmitate, 2:1) or troglitazone were added to the medium. 3T3-L1 adipocytes were harvested for RNA analysis by day 8 postdifferentiation. For the insulin stimulation experiment, 3T3-L1 adipocytes were serum starved for 20 h in medium containing either 25 mM or 5 mM glucose, and then 100 nM insulin was added into the culture medium. RNA was isolated 12 h after insulin stimulation.

RNA isolation, Northern blot, and real time PCR. Total RNA was isolated from rat epididymal fat pads or from cultured 3T3-L1 cells by use of TRIzol (Invitrogen). cDNA was synthesized with reverse transcript reagents (Applied Biosystems). ChREBP gene expression in adipose tissue was carried out using the standard Northern blot method. Total RNA (20 μg) from rat adipose or liver tissue was loaded in each lane. The cDNA probes of ChREBP and β-actin were cloned by RT-PCR into the pcDNA2.1 vector (Invitrogen) and were purified by restrictive digest and gel purification. The filter was blotted with 32P-labeled cDNA probes and exposed. The mRNA level was quantified by using Bio-Rad iCycler Real Time PCR system. Briefly, total reaction mix (25 μl) contains 20 ng of reverse-transcribed cDNA, 100 nM forward/reverse primers, and 1× iQ Supermix (Bio-Rad). 36B4 was used as internal control, and the amount of RNA was calculated by the comparative Ct method, as recommended by the manufacturer. All of the data were calculated from triplicate reactions. The sequences used are indicated in Table 1.

Statistical analysis. The results were expressed as means ± SE. The statistical significance of differences was assessed by Student’s t-test.

RESULTS

Induction of ChREBP mRNA expression during 3T3-L1 preadipocyte differentiation and the opposite effect of glucose and free fatty acid. To explore the potential roles of ChREBP in adipocyte biology, we applied the in vitro adipogenesis

Fig. 1. Changes of carbohydrate response element-binding protein (ChREBP) expression during 3T3-L1 preadipocyte differentiation. A: time course of induction of ChREBP mRNA expression with 25 mM glucose (●) or 5 mM glucose (○) in the culture medium. B: adipocyte fatty acid-binding protein (aP2) expression in cells cultured in 25 mM glucose (●) or 5 mM glucose (○). C: 3T3-L1 adipocyte ChREBP expression in cells cultured in DMEM with insulin (Ins)/dexamethasone (dex)/IBMX and in the absence (open bar) or presence of 1 mM free fatty acids (solid bar). D: dose response of inhibition of ChREBP gene expression by free fatty acids (FFA) vs. that of stearoyl-CoA desaturase (SCD)-1. Values are means ± SE from 2 independent experiments; n = 5.
A significant amount of ChREBP mRNA was detectable, but aP2 expression was not expected (Fig. 1). At a lower glucose concentration (5 mM) no established adipocyte maturation marker, was also induced as shown in Fig. 1A. Meanwhile, adipocyte fatty acid-binding protein 2 (aP2), an expected adipocyte differentiation marker, was also induced as shown in Fig. 1B. This indicates that 3T3-L1 adipocyte differentiation is not significantly affected by the lower glucose level we used, but rather that lipogenesis might be blunted as we have reported before (9). On the contrary, in the presence of 1 mM free fatty acids, the induction of the ChREBP gene in response to adipocyte differentiation was largely inhibited, as there was a 90% decrease when compared with controls (P < 0.05; Fig. 1, C and D), with a coincidental 30% reduction in the level of mRNA for stearoyl-coenzyme A desaturase 1 (SCD-1). These data suggest that glucose and free fatty acids exert opposite effects on the expression of ChREBP gene in 3T3-L1 adipocytes, with induction by glucose and inhibition by free fatty acids, respectively.

Induction of ChREBP mRNA expression in 3T3-L1 cells by troglitazone. Thiazolidinediones (TZDs) are among the major antidiabetic agents that are widely used for the clinical treatment of diabetes. The possible pharmacological mechanism accounting for their antidiabetic effects is that TZDs stimulate lipogenesis in adipose tissue. The newly formed adipocytes are more sensitive to insulin and take up more glucose (11, 13). Therefore, TZD-treated adipocytes undergo active lipogenesis by utilizing glucose as a substrate. This process requires the involvement of a number of lipogenic enzymes. To gain insight into whether ChREBP is possibly linked to the diabetic state, we tested the effect of troglitazone on the expression of ChREBP, because troglitazone can stimulate lipogenesis and glucose uptake by itself. Surprisingly, we found that troglitazone induced ChREBP expression in this cell type in a dose-dependent fashion. As shown in Fig. 2A, 10–50 μM troglitazone treatment induced a three- to sixfold increase of ChREBP mRNA level when compared with the cells treated with insulin-dexamethasone-IBMX alone (P < 0.05). To examine whether this effect was due to a general induction of gene expression, we checked three other adipogenic genes at the same time. As shown in Fig. 2B, PPAR-γ and aP2 mRNA levels were also higher than those in controls, as reported previously (2, 12), but sterol regulatory element-binding protein 1c (SREBP-1c) levels remained the same in the cells either treated or not with troglitazone. However, troglitazone failed to stimulate the ChREBP gene activation in maturely differentiated 3T3-L1 adipocytes (data not shown).

Augmentation of ChREBP mRNA expression by insulin in 3T3-L1 adipocytes. Previous results suggest that glucose stimulates ChREBP activation in vivo and in vitro (8, 24). Physiologically, increased input of glucose is always accompanied by enhanced secretion of insulin from the pancreatic β-cells. This led us to speculate that insulin may play a role in the regulation of ChREBP at the transcriptional level, the post-translational level, or both. We found that the level of ChREBP mRNA was significantly induced in 3T3-L1 adipocytes after 12 h of insulin treatment (~3-fold increase in insulin-treated adipocytes compared with controls, as shown in Fig. 3A, P 0.05). Meanwhile, insulin-induced gene 1 (insig-1) mRNA was also induced 2.8-fold in the same set of cells, as expected (Fig. 3B, P < 0.05). However, the stimulation of ChREBP gene expression by insulin was detectable in the cells cultured in the medium containing 25 mM glucose, but not in that with 5 mM glucose.
than in the ad libitum-fed or fasted controls (Fig. 4). Simultaneously, liver ChREBP mRNA was induced 30-fold ($P < 0.05$, data not shown). To further explore the relationship between insulin and ChREBP expression in vivo, we induced diabetes in rats by STZ. We found that the mRNA level of ChREBP in adipose tissues from the insulin-deficient diabetic rats was not significantly different from that of normal controls ($P > 0.05$; Fig. 5A) despite the fact that their blood glucose levels were elevated 2.5- to 4-fold compared with controls. Meanwhile, FAS, ACC, and Insig 1 genes were all significantly downregulated in the adipose tissue from diabetic rats (Fig. 5, B–D). The physiological significance of Insig 1 gene downregulation in these diabetic rats remains to be determined.

**DISCUSSION**

ChREBP, a newly discovered transcription factor, plays a critical role in glucose-induced liver L-PK gene transcription by directly binding to the carbohydrate-responsive element on its promoter, as reviewed in Ref. 22. Although the pattern of activation of ChREBP has been carefully characterized, the transcriptional regulation of its gene has remained obscure. In the present study, we focused on an adipocyte cell line and rat adipose tissue in an attempt to investigate the potential roles of ChREBP in adipocyte physiology. We demonstrated that ChREBP is present in rat adipose tissue as well. We revealed that adipocyte ChREBP is induced during 3T3-L1 preadipocyte adipogenesis and that the expression of its gene is modulated dynamically by various important factors, including glucose, free fatty acids, insulin, the antidiabetic agent troglitazone, and nutrient status. The robust up- or downregulation of ChREBP gene expression in adipocytes by those important physiological or pharmacological modulators strongly suggest the potential functional role that ChREBP may play in adipocyte metabolism and in the physiological and pathophysiological conditions associated with glucose homeostasis, diabetes, and obesity.

The finding that ChREBP is expressed in the adipocyte after the onset of differentiation implies that ChREBP is most likely involved in fuel metabolism of the mature adipocyte. A fairly established paradigm suggests that most, if not all, of the important transcription factors controlling adipocyte differentiation or lipogenesis undergo transcriptional and posttranslational regulation by insulin and nutrients. For example, SREBP-1c and PPARγ are almost exclusively expressed in the mature adipocyte rather than in the preadipocyte, as are their target genes, SCD-1 and FAS (16, 18–20). Of particular interest is that the activation of ChREBP gene transcription in adipocytes depends on the presence of a large amount of glucose in the medium. As shown in Fig. 1, there is little induction of ChREBP mRNA in well-differentiated 3T3-L1 adipocytes cultured in the medium containing low glucose (5 mM glucose), suggesting that ChREBP is not necessary for 3T3-L1 preadipocyte differentiation. Instead, it becomes required once glucose is in great excess. Interestingly, in the presence of high glucose plus 1 mM free fatty acids, the ChREBP gene is largely inhibited, down to $<10\%$ of that on 25 mM glucose culture. This implies that the cells can delicately sense their nutritional status and decrease glucose-induced lipogenesis when free fatty acids are abundant. The stimulatory effect of glucose on ChREBP gene expression is
consistent with the reported result observed on INS-1 (insulin-secreting) cells by Wang and Wollheim (23). The mechanism underlying the suppression of ChREBP by free fatty acids needs further investigation.

Previous reports suggest that PP2A seems to be critical in the acute activation of ChREBP by dephosphorylation of Ser\textsuperscript{196}/Thr\textsuperscript{666} residues (7, 8, 22). However, the regulatory role of PP2A on the activation of the ChREBP gene has not been studied. Shimizu et al. (14) reported recently that the insulin-sensitive protein tyrosine phosphatase 1B stimulates liver SREBP-1a and SREBP-1c gene expressions via enhancing PP2A activity. It remains to be determined whether a similar regulatory mechanism also applies to ChREBP gene expression. It is worthwhile to mention that, under normal physiological conditions, increased carbohydrate intake is always accompanied by a rapid increase of insulin secretion in both rodents and humans. Several lines of evidence support our assumption that the activation of ChREBP and its gene transcription in vivo are most likely subject to control by insulin or some other hormones, in addition to glucose. In addition to its ability to stimulate PP2A activation (10), an increased level of insulin may result in 1) the direct stimulation of ChREBP gene

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**Fig. 4. ChREBP expression in rat adipose tissue.**

*Panel A*: Northern blot of ChREBP (or β-actin as control) in primary rat adipose and liver tissue. Each lane contains 20 μg of total RNA. *Panel B*: Changes in ChREBP, SREBP-1c, fatty acid synthase (FAS), and acetyl-CoA carboxylase (ACC) mRNA expression in adipose tissue from the ad libitum, fasted, or fasted/refed rats. Values are means ± SE; n = 4 in each group. *P < 0.05.
transcription, as supported by our observation showing that insulin directly increases the ChREBP mRNA expression in 3T3-L1 adipocytes (Fig. 3); and 2) the elevation of intracellular glucose from stimulated glucose uptake and, subsequently, activation of ChREBP gene transcription, as supported by our finding that an elevated level of glucose (25 mM) in medium could result in significant upregulation of the ChREBP gene (Fig. 1A). Both of these pathways might contribute to enhance the expression of the ChREBP gene in fat cells by insulin. Furthermore, our data in STZ-diabetic rats, showing that there is no significant induction of adipocyte ChREBP mRNA level in the diabetic animals, also argue against the hypothesis that increased glucose level alone is sufficient to induce ChREBP gene expression, given the fact that those diabetic rats have a 2.5- to 4-fold higher blood glucose level, but an extremely low insulin level compared with the controls. Thus we favor the idea that insulin would be required for glucose to control the ChREBP gene expression in vivo, at least via the steps of facilitation of glucose uptake and subsequent metabolism. However, further studies are required to determine the exact mechanism of insulin-mediated ChREBP expression regulation and the exact interplay between glucose and insulin that occurs to modulate the level of ChREBP.

In any case, the fact that ChREBP mRNA level remains unchanged in the fasting condition suggests the possibility that, instead of being actively involved in basal glucose metabolism, ChREBP may be reserved as a backup system in the body to accommodate a sudden influx of a large amount of carbohydrate. This assertion is supported by our observation of a 10-fold increase in the level of ChREBP mRNA in the adipose tissue in response to refeeding a high-carbohydrate diet, a treatment that would provide a dramatic influx of available carbohydrate to the animals. A similar effect also exists in some other tissues, such as the liver (up to 30-fold increase vs. the fasting condition; J. Li, unpublished data).

Dentin et al. (4) have reported that glucose and insulin stimulate ChREBP gene expression in the mouse hepatocyte. Moreover, they also demonstrated that glucose phosphorylation by glucokinase seems to be required for the stimulatory effect on ChREBP and lipogenic enzyme gene expression. These researchers showed that knocking out glucokinase attenuates the glucose/insulin-induced elevation of ChREBP and the coincidental induction of L-PK, ACC, FAS, and Spot 14 gene expression.

By serving as the ligand for PPARγ activation, TZDs are able to directly stimulate glucose uptake in fat cell and muscle (13, 15) in addition to their ability to promote adipogenesis. Both of these mechanisms might account for the antidiabetic effect of TZDs (1, 6, 11, 16). One of the most intriguing findings in the current study is that troglitazone could induce
ChREBP gene expression during 3T3-L1 preadipocyte differentiation. This is a very important observation, when we consider that adipose tissue is the major target for TZD's actions. In the present study, we demonstrated that, in 3T3-L1 preadipocytes, troglitazone induces a simultaneous increase of ChREBP and PPARγ mRNA. It seems to be likely that, in addition to stimulation of in situ adipogenesis via activation of PPARγ, troglitazone also stimulates ChREBP gene expression, thereby promoting lipogenesis in newly formed adipocytes through the production of lipogenic enzymes. All of those effects would favor augmented glucose utilization by adipose tissue during troglitazone treatment. Thus the induction of ChREBP may serve as a novel pharmacological pathway for troglitazone-mediated hypoglycemic effects in vivo. Indeed, we recently observed that there is a 5- to 10-fold increase of ChREBP mRNA in adipose tissue from normal rats treated with rosiglitazone for 2 mo (J. Li, unpublished data). However, the question concerning whether troglitazone directly activates ChREBP gene expression through direct binding and activation of its promotor, or indirectly activates the ChREBP gene via PPARγ activation or stimulation of glucose transport, remains unresolved at this stage.

In summary, in the work presented here we demonstrate that the transcription factor ChREBP is exclusively expressed in the 3T3-L1 adipocyte rather than the 3T3 preadipocyte. Adipocyte ChREBP expression is subject to control by various important factors, including glucose, free fatty acids, insulin, and the PPARγ agonist troglitazone, as well as feeding status. Our data support the hypothesis that insulin and glucose are working synergistically in vivo to regulate ChREBP gene expression in adipose tissue. The working project toward the complete elucidation of promoter structure of the ChREBP gene is currently underway in our laboratory.

ACKNOWLEDGMENTS

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GRANTS

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REFERENCES

7. Kabashima T, Kawaguchi T, Wadzinski BE, and Uyeda K. Xylose