Transcription of the mitochondrial citrate carrier gene: Role of SREBP-1, upregulation by insulin and downregulation by PUFA

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Abstract

In this study we investigated the transcriptional role of the sterol regulatory element (SRE) present in the promoter of the mitochondrial citrate carrier (CIC). We show that wild-type (but not mutated) CIC SRE cloned in front of the luciferase promoter confers transcriptional activation of the gene reporter. We also demonstrate that insulin activates, and polyunsaturated fatty acids (PUFA) inhibit, the gene reporter activity driven by the CIC promoter containing wild-type (but not mutated) SRE. Finally, both insulin treatment and overexpression of SRE binding protein (SREBP-1) increase the CIC transcript and protein levels, whereas PUFA have an opposite effect. These results show that SRE/SREBP-1 play a role in the transcriptional regulation of CIC by insulin and PUFA.
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Mature SREBPs are helix–loop–helix leucine zipper transcription factors that bind to SREs and/or to E-boxes of several gene promoters leading to activation of their target genes [1]. These factors are encoded by two genes, SREBP-1 and SREBP-2. SREBP-1 upregulates the transcription of some lipogenic genes, such as fatty acid synthase (FAS), acetyl CoA carboxylase, and ATP-citrate lyase (see [2] for a review), whereas SREBP-2 regulates the transcription of some sterol biosynthesis genes [2].

The mitochondrial citrate carrier (CIC), also known as the tricarboxylate carrier, is an integral protein of the mitochondrial inner membrane that exports citrate from mitochondria to the cytosol (see [3,4] for reviews). In the

cytosol, citrate is cleaved by ATP-citrate lyase to oxaloacetate and acetyl-CoA, which is essential for fatty acid and sterol biosynthesis. Oxaloacetate produced by citrate lyase is reduced to malate, which in turn is converted to pyruvate via malic enzyme with production of cytosolic NADPH (also necessary for fatty acid and sterol synthesis).

In early studies it was found that streptozotocin-treated diabetic rats exhibited a decrease in the activity of hepatic CIC [5], and this decrease was adjusted after insulin administration [6]. More recently, it was shown that starvation reduces CIC activity in rat liver mitochondria [7] and, based on half-life measurements of hepatic CIC mRNA from fed and starved rats, it was proposed that CIC expression is regulated at the posttranscriptional level [8]. Furthermore, a reduction of CIC activity, in parallel with CIC mRNA abundance, was demonstrated in the liver of rats fed with a diet enriched with PUFA [9,10]. However, nothing is known about the molecular mechanisms of the transcriptional control of the CIC gene.

In this study we show that the CIC gene promoter contains an active SRE site and that overexpression

of SREBP-1 in HepG2 cells significantly increases SRE-driven LUC transgene activity as well as CIC transcript and CIC protein levels. Moreover, direct evidence is provided that insulin upregulates and PUFA downregulate CIC gene transcription via the SRE site. To our knowledge, this is the first study to investigate the role of SRE/SREBP-1 in the transcriptional regulation of the CIC gene.

Materials and methods

Construction of plasmids. The human CIC gene promoter (chromosome 22q11.2, Cosmid Clone 79h12) from −1785 to −20 bp, with or without the mutations in SRE reported below, was amplified by PCR and cloned into the pGL3 basic-LUC vector (Promega) upstream of the LUC gene coding sequence. For heterologous promoter expression, a three-fold repeat wtSRE (5′-AGGCTGTGGGGTGCTTGGACAC-3′) or mutSRE (5′-AGGCTGTGTTTGGCTTGGACAC-3′) was cloned into the pGL3 promoter-LUC vector (Promega) upstream of the SV40 basal promoter. The SREBP-1a expression vector (pcDNA3-SREBP-1) was obtained by cloning the cDNA of the mature form of SREBP-1a [11] into the pcDNA3 vector (Invitrogen).

Cell culture and transient transfection. Human hepatocytes (Cambrex) were maintained in hepatocyte culture medium (Cambrex) following the manufacturer's instructions. HepG2 cells (Sigma) were grown in high glucose DMEM (Dulbecco's modified Eagle's medium) containing 10% (v/v) heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U penicillin and 100 μg/ml streptomycin at 37 °C in 5% CO_2. Transient transfection was performed as reported [12] using 0.5 μg of pGL3 basic-LUC vector containing the −1785/−20 region of the CIC gene promoter or 0.5 μg of pGL3 promoter-LUC vector containing the CIC gene SRE site alone, and 10 ng of pRL-CMV (Promega) to normalize the extent of transfection [13]. Transfected cells were assayed for LUC activity using the Dual-Luciferase® Reporter Assay System (Promega). Where indicated, HepG2 cells or human hepatocytes were treated for 4 h with insulin (100 nM) starting 24 h after they had been depleted of serum, or with a fatty acid (60 μM) that had been complexed with 300 μM fatty acid-free bovine serum albumin by stirring for 1 h at 37 °C. LY-294002 (50 μM; Alexis) was added 2 min before insulin. For overexpression of SREBP-1, HepG2 cells were transfected using 0.5 μg of pcDNA3-SREBP-1 vector.

Other methods. Electrophoretic mobility shift assays (EMSA) were performed as described in Sambrook et al. [14]. The double-stranded oligonucleotide probes were 5'-end labeled using T4 polynucleotide kinase and [γ^{32}P]-ATP at 37 °C for 30 min. The gels were dried and images acquired by phosphoimager (Bio-Rad). Total RNA was extracted from 1×10^6 HepG2 cells treated as indicated, and reverse-transcription was performed as reported [12]. Real-time PCR was carried out as described previously [15]. Assay-on-demand for human CIC (Catalog No. Hs00761590_m1) and human actin (Catalog No. Hs99999903_m1) were purchased from Applied Biosystems. The CIC transcript levels were normalized against the expression levels of actin. For Western blot analysis, proteins were electroblotted onto PVDF membranes (Roche) subsequently treated with anti-CIC (specific for the C-terminus of the human mitochondrial CIC [16]), anti-SREBP-1 (Santa Cruz), anti-FAS (Sigma) or anti-β-actin (BioLegend) antibodies. The immunoreaction was detected by the ECL plus system (Amersham).

Results

The CIC gene promoter contains a functional SRE

A computer search of a vertebrate transfactor database (http://www.cbrc.jp/research/db/TFSEARCH.html) with the −5550/−20 region of the CIC gene revealed the presence of an SRE motif at −1696/−1686, which shares 86%

identical nucleotides with the canonical SRE [2]. In contrast, no E-box was found in the CIC gene promoter.

The binding activity of the CIC promoter SRE was investigated by EMSA experiments using nuclear extracts of HepG2 cells and a labeled probe from −1700 to −1679 bp encompassing the SRE region. A band shift was observed with the SRE probe alone (Fig. 1, lane 2). This band disappeared by competition with unlabeled probe (wtSRE), whereas it was unaffected by the presence of mutated probe (mutSRE) (Fig. 1, lanes 3–4). A supershift was obtained by pre-incubating HepG2 nuclear extracts with an anti-SREBP-1 antibody (Fig. 1, lane 5). These results show that the CIC gene promoter contains a functional SRE site.

The CIC SRE is involved in the transcriptional regulation of the CIC gene

To investigate the promoter activity of the CIC SRE in vivo, we generated wtSRE- and mutSRE-driven LUC gene reporter constructs. HepG2 cells were transfected with either of these constructs (or with the empty pGL3 promoter-LUC vector) in the presence or absence of pcDNA3-SREBP-1 expression vector; the relative LUC activity of each reporter gene construct was measured. LUC activity was strongly enhanced in cells transfected with the pGL3 promoter-LUC vector harbouring wtSRE (W) and overexpressing SREBP-1 (Fig. 2A). By contrast,

	1	2	3	4	5
HepG2 NE	-	+	+	+	+
probe (-1700/-1679)	+	+	+	+	+
competitor (wtSRE)	-	-	+	-	-
competitor (mutSRE)	-	-	-	+	-
anti–SREBP-1	-	-	-	-	+

Fig. 1. Specificity of protein binding to the SRE motif of the CIC gene promoter. The 5′-end labeled DNA probe (wtSRE) from −1700 to −1679 bp of the CIC gene was incubated with 10 μg protein of HepG2 cell nuclear extracts (HepG2 NE) (lane 2). Where indicated, unlabeled wtSRE or mutSRE was added in 100-fold molar excess (lanes 3 and 4, respectively). For the super-shift (lane 5), 2 μg of anti-SREBP-1 antibody were used.
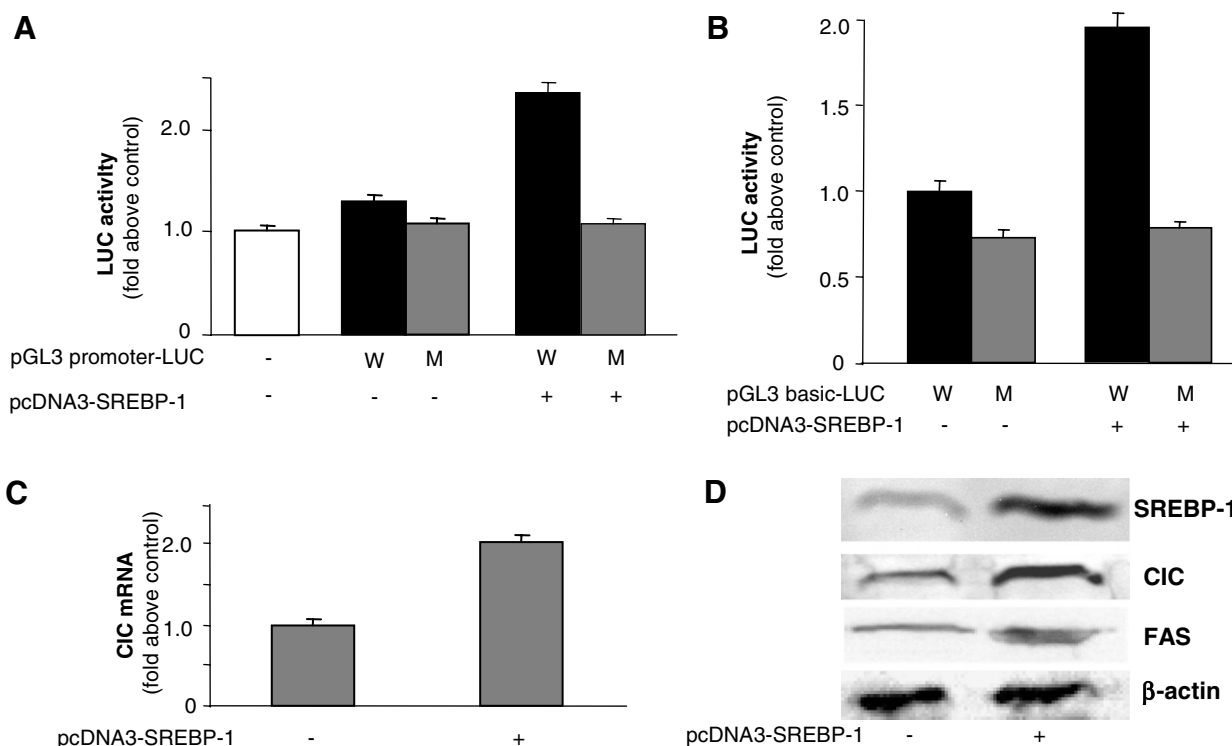
A

B

C

D

Fig. 2. Effect of SREBP-1 overexpression on CIC gene SRE promoter activity and on CIC content both at the transcript and protein levels. (A) HepG2 cells, co-transfected with pcDNA3-SREBP-1 (+) or empty pcDNA3 (−) and with pGL3 promoter-LUC vector containing a three-fold repeat wtSRE (W) or mutSRE (M), were assayed for LUC expression activity. (B) HepG2 cells co-transfected with pcDNA3-SREBP-1 (+) or empty pcDNA3 (−) and pGL3 basic-LUC vector, containing the −1785/−20 region of the CIC gene with (M) or without (W) mutations in the SRE site, were assayed for LUC expression activity. (C) Total RNA extracted from HepG2 cells transfected with pcDNA3-SREBP-1 (+) or empty pcDNA3 (−) was used to quantify the CIC mRNA by real-time PCR. (D) SREBP-1, CIC, FAS and β-actin of HepG2 cells transfected with pcDNA3-SREBP-1 (+) or empty pcDNA3 (−) were immunodecorated with specific antibodies. In (A), (B), and (C) means ± SD of three duplicate independent experiments are shown; all the differences between samples and relative controls were significant ($P \ll 0.05$, one-way ANOVA), including those between cells transfected with the pGL3 promoter-LUC vector containing a three-fold repeat wtSRE or mutSRE.

no increase was observed in cells transfected with mutSRE-LUC vector (M), with or without the pcDNA3-SREBP-1 expression vector (Fig. 2A). In another set of experiments, HepG2 cells were co-transfected with pGL3 basic-LUC vector, containing the CIC gene promoter from −1785 to −20 bp with or without mutations in the SRE site, and pcDNA3-SREBP-1 expression vector. In this case as well (i.e. with the CIC gene promoter cloned in front of the LUC coding sequence), LUC activity was markedly enhanced in cells co-expressing SREBP-1 and the pGL3 basic-LUC construct containing wtSRE in the CIC gene promoter (Fig. 2B). It is noteworthy that the presence of mutSRE diminished the gene reporter activity significantly even in the absence of pcDNA3-SREBP-1 expression vector (Fig. 2B). Consistent with the results of Figs. 2A and B showing the ability of wtSRE to respond to SREBP-1 and activate transcription, Figs. 2C and D show that both CIC transcript and CIC protein levels were higher in cells transfected with the pcDNA3-SREBP-1 expression vector than in cells transfected with the empty vector. An increase of FAS, used as positive control, was also observed in cells transfected with pcDNA3-SREBP-1. All together, these

results provide evidence for direct involvement of SREBP-1 in the regulation of the of CIC gene expression.

Insulin activates the CIC promoter-driven gene reporter via SRE

To test the effect of insulin on CIC gene transcription, HepG2 cells transfected with the pGL3 basic-LUC vector harbouring the CIC gene promoter, with or without mutations in SRE, were treated with insulin in the presence or absence of LY-294002, a specific inhibitor of PI3 kinase that blocks the insulin-induced phosphorylation pathway [17]. As shown in Fig. 3A, insulin treatment increased the gene reporter expression activity in cells transfected with the construct containing wtSRE; this increase was abolished by the presence of LY-294002. In contrast, insulin had no effect in HepG2 cells transfected with the construct containing mutSRE. The effect of insulin on CIC mRNA was tested on both HepG2 cells and human hepatocytes. In both cell lines (Figs. 3B and C) insulin increased the amount of CIC transcript, and LY-294002 abolished this increase. The CIC protein level also confirmed the
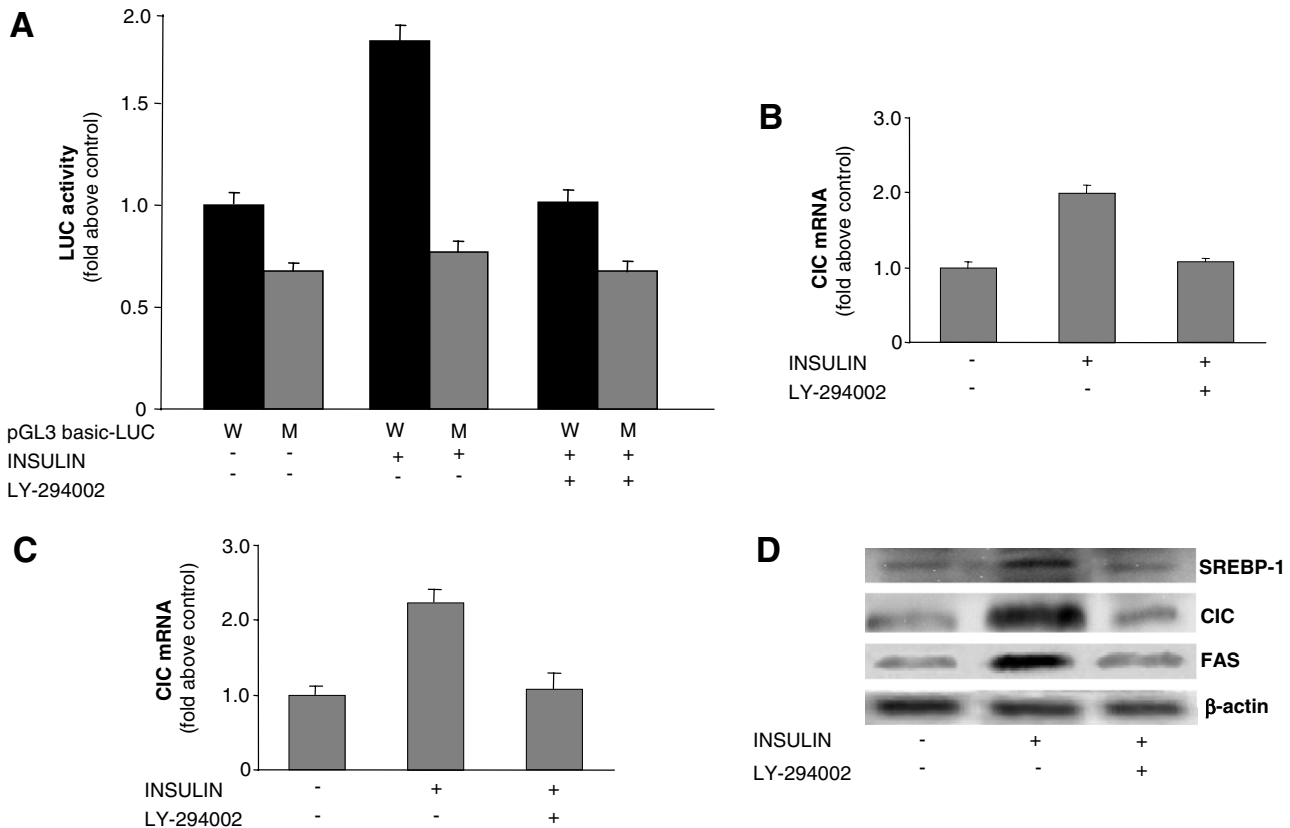
Fig. 3. SRE-mediated effect of insulin on CIC gene promoter activity. HepG2 cells were incubated with and without 100 nM insulin for 4 h, in the presence or absence of 50 μM of LY-294002. (A) HepG2 cells transfected with pGL3 basic-LUC vector, containing the −1785/−20 region of the CIC gene with (grey bars) or without (black bars) mutations in the SRE site, were incubated as above and assayed for LUC expression activity. (B) Total RNA of HepG2 cells and (C) human hepatocytes, incubated as above, was used to quantify the CIC mRNA by real-time PCR. (D) SREBP-1, CIC, FAS and β-actin of HepG2 cells, incubated as above, were immunodecorated with specific antibodies. In (A), (B), and (C) means ± SD of three duplicate independent experiments are shown; all the differences between samples and relative controls were significant ($P \ll 0.05$, one-way ANOVA).

LY-294002–sensitive activation by insulin of the CIC gene expression (Fig. 3D).

PUFA suppress the CIC promoter-driven gene reporter via SRE

It is known that administration of fish oil (n-3 PUFA) to rats significantly decreases CIC mRNA and protein, whereas diets rich in monounsaturated (olive oil) or saturated (beef tallow) fatty acids have pratically no effect [10]. Fig. 4A shows that LUC activity driven by the CIC promoter containing wtSRE (black columns) was reduced when HepG2 cells were treated with PUFA (n-3 docosahexaenoic acid or n-6 arachidonic acid), but not when the cells were treated with either oleic (monounsaturated) or palmitic (saturated) acid. Conversely, gene reporter activity driven by the CIC promoter containing mutSRE was not affected by treatment with either PUFA or oleic and palmitic acids (Fig. 4A). CIC mRNA also was reduced in HepG2 cells treated with PUFA and unaffected in cells treated with saturated or monounsaturated fatty acids (Fig. 4B). Similar effects were observed using human hepatocytes instead of HepG2 cells (Fig. 4C). In addition, the endogenous levels of both

CIC and SREBP-1 were decreased by PUFA but not by oleic and palmitic acids (Fig. 4D).

Discussion

The experimental data presented in this study show that the SRE site present in the human CIC gene promoter at −1696/−1686 is an activation domain that regulates the transcription of the CIC gene. Among the reported results, the following supporting evidence can be mentioned. In cells overexpressing SREBP-1, both the wild-type (but not the mutated) SRE alone and the CIC gene promoter containing wild-type (but not mutated) SRE strongly enhance gene reporter expression activity. The role of SRE in the regulation of CIC gene transcription is also supported by the increase in the levels of both CIC transcript and protein induced by overexpression of SREBP-1. It is noteworthy that the SRE sequence is present in the promoters of the other mitochondrial CIC genes sequenced. For example, *M. musculus*, *C. elegans* and *S. cerevisiae* CIC gene promoters exhibit SRE motifs at −4189/−4179, −690/−680 and −1460/−1450, respectively, which are 89–90% identical to the canonical SRE [2].
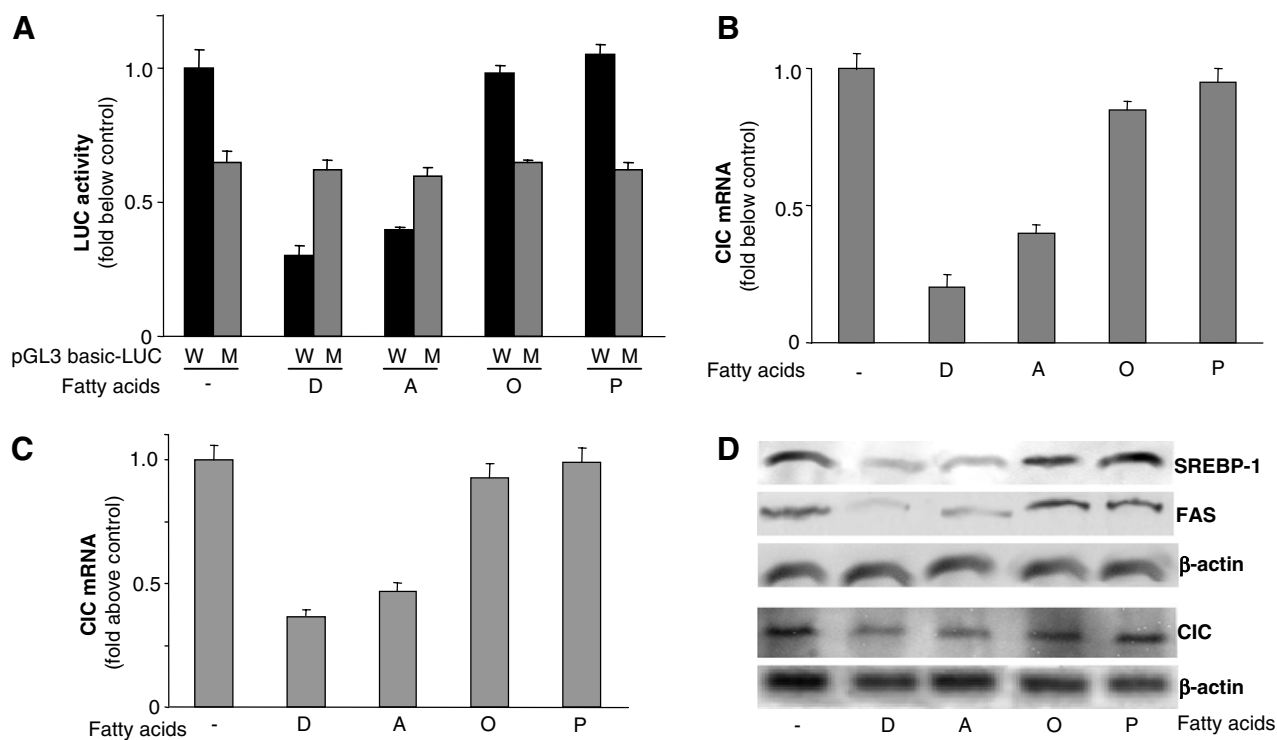
Fig. 4. SRE-mediated effect of PUFA on CIC gene promoter activity. HepG2 cells were incubated without (−) or with 60 μM docosahexaenoic acid (D), arachidonic acid (A), oleic acid (O) or palmitic acid (P) for 4 h. (A) HepG2 cells transfected with pGL3 basic-LUC vector, containing the −1785/−20 region of the CIC gene with (grey bars) or without (black bars) mutations in the SRE site, were incubated as above and assayed for LUC expression activity. (B) Total RNA of HepG2 cells and (C) human hepatocytes, incubated as above, was used to quantify the CIC mRNA by real-time PCR. (D) SREBP-1, CIC, FAS and β-actin of HepG2 cells, incubated as above, were immunodecorated with specific antibodies. In (A), (B), and (C) means ± SD of three duplicate independent experiments are shown.

Furthermore, it is interesting to note that SREBP-1 has also been found to be a positive transcriptional regulator of the cytosolic lipogenic enzymes [2], which work in sequence to CIC.

Our data also provide evidence that insulin enhances, and PUFA decrease, CIC gene promoter activity. The CIC transcriptional activation by insulin and suppression by PUFA are clearly mediated by the SRE/SREBP-1 regulatory system as their effects on CIC promoter-driven LUC activity are abolished by mutations in the SRE site of the CIC gene. These findings are in agreement with the observations, made in the context of the transcription of other enzymes, that insulin upregulates and PUFA downregulate SREBP-1 [1,2]. Furthermore, it is known that the level of SREBP-1 is decreased by starvation [18]. Therefore, our results provide a molecular basis and explain, at least in part, the changes in CIC activity or/and CIC level previously observed in diabetic rats before and after insulin administration [5,6], in rats fed with a PUFA-enriched diet [9,10], and in starved rats [7,8].

Acknowledgments

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