Hormones and Nutrients Regulate Acetyl-CoA Carboxylase Promoter I in Rat Primary Hepatocytes

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Summary This study investigated the regulation of acetyl-CoA carboxylase (ACC) promoter activity by hormones and nutrients. Genomic clones including promoter I (PI) of the ACC gene were isolated and sequenced. ACC PI fragments (-1,049/+100 or -220/+21 bp) were subcloned into the pGL3-Basic vector that includes luciferase as a reporter gene. The ACC PI/luciferase chimeric plasmids were transfected into primary rat hepatocytes using lipofectin. Insulin treatment increased the activity of -1,049/+100 and -220/+21 ACC PI by 3.0- and 3.5-fold, respectively, compared to the control. The activity of both constructs was also increased by dexamethasone (Dex) and triiodothyronine (T3), with the greatest effects seen with all three hormones present. With -1,049/+100 or -220/+21 ACC PI, the addition of glucose increased luciferase activity compared to glucose-free control (p<0.05). On the other hand, polyunsaturated fatty acids (PUFA) reduced the activity of the -1,049/+100 ACC PI construct, with eicosapentaenoic acid and docosahexaenoic acid showing the greatest effect (about 70% of the control). However, the addition of PUFA to the culture media did not affect the activity of -220/+21 ACC PI. Therefore, insulin, Dex, T3, glucose, and PUFA regulate ACC gene expression, at least in part, through the PI promoter.

Key Words acetyl-CoA carboxylase, gene expression, promoter, hormone, nutrition

When the intake of dietary carbohydrates exceeds the immediate energy needs of the animal, excess carbohydrates are converted to triacylglycerols, which can be used for energy during periods of fasting. The mammalian liver and adipose tissue are primarily responsible for the conversion of excess dietary carbohydrates to triacylglycerides, a process known as lipogenesis.

Acetyl-CoA carboxylase (ACC) plays a pivotal role in regulating this conversion of carbohydrates to fat. Acetyl-CoA carboxylase (EC 6.4.1.2) catalyzes the ATP-dependent carboxylation of acetyl-CoA to malonyl-CoA. This is the first committed step of fatty acid biosynthesis and represents an important control point for intermediary metabolism. There are two ACC genes, ACC1 and ACC2. ACC2 is the major isoform expressed in heart and skeletal muscle; however, comparatively little is known about ACC2 regulation (1). ACC1 is the principal isoform expressed in tissues that exhibit high rates of fatty acid synthesis such as liver, adipose tissue, and mammary glands. ACC1 is subject to both short-term (enzyme activity) and long-term (enzyme synthesis) regulation by hormones and nutrients in a tissue-specific manner. Short-term regulation of ACC1 is a result of covalent modifications and allosteric inhibition (2, 3). Long-term regulation involves the interaction of multiple hormonal and dietary factors that inhibit or amplify ACC1 gene expression (1). For example, 48 h food deprivation reduced the levels of ACC mRNA by 50-85% in rat liver and adipose tissue; refeeding a high carbohydrate diet after food deprivation increased ACC mRNA expression 20-fold compared with the 48 h starved animals (4).

The ACC1 and ACC2 genes are transcribed from ACC promoter I (PI) and promoter II (PII) (5). Studies on the physiological regulation of these two promoters in the rat indicate that PII is fairly uniformly active in all tissues and does not respond to dietary changes (6). The PI promoter is active predominantly in adipose and liver, and its utilization is markedly elevated in response to meals, thereby inducing lipogenesis (6, 7). Hence, the 20-fold increase in ACC mRNA in livers of fasted rats that have been refed a high carbohydrate diet (4) is largely a consequence of PI promoter induction. However, only limited information on the regulatory mechanisms controlling ACC gene expression is currently available (8-10).

The major signal controlling ACC gene expression has been reported to be glucose (11, 15). Although an intrinsic repressor element has been described (1), the role of this element in the regulation of the PI promoter is unknown. In this study, we examined the effects of...
hormones and nutrients such as insulin, thyroid hormone (T3), dexamethasone (Dex), glucose and polyunsaturated fatty acids (PUFA) on two different ACC PI constructs, which provides a basis for further research into the identification of the regulatory mechanisms operating through ACC PI.

**Materials and Methods**

**Cloning and sequencing of the rat ACC PI promoter.** A PI clone was selected from a rat genomic library by Genome Systems (St. Louis, MO), using primers recognizing exon 1 of the ACC PI gene (upstream, 5’ TTTGAC-CAGGGCAAAGGGACT 3’; downstream, 5’ GGAGGC-CACTTGCTCTTTGA 3’). The 8.5 kb clone was digested with BamHI and the fragments shotgun ligated into BamHI digested and dephosphorylated pBluescript vector (Stratagene, La Jolla, CA). After transformation into NovaBlue competent cells (Novagen, Madison, WI, USA), plasmid DNA was extracted from insert-containing colonies. A single clone that overlapped with exon 1 was selected by Southern hybridization, using a [32P]-labeled oligonucleotide probe generated with the above primers.

The clone was sequenced from both directions using repeated thermal cycle sequencing in the presence of BigDye terminators (ABI Prism, Perkin-Elmer, Foster City, CA) using an ABI Prism 377 DNA Sequencer at 37°C in an atmosphere of 5% CO2 in air with 95% humidity. The ACC PI promoter fragment (-1,049/+100) corresponding to -1,049 to +100 bp containing SacI and XhoI restriction sites at the 5’- and 3’-ends, was cleaved and inserted into the pGL3 basic vector (Promega, Madison, WI, USA). In addition, a smaller fragment (-220/+21) was derived from this clone in the laboratory of Dr. Towle (15) and returned to us for use in these studies.

**Primary hepatocyte culture.** Male Sprague-Dawley rats (200–250 g) were maintained individually in stainless steel wire mesh cages on a 12/12 h light/dark cycle at 20–22°C and given free access to rodent laboratory chow (Purina Mills, St. Louis, MO). Primary hepatocytes were isolated by a collagenase perfusion method (13). Before plating, cells were judged to be greater than 85% viable using the trypan blue exclusion method (13). Cells were suspended at a density of approx. 1.2×10^6 cells per 3mL culture media (Willard E media, without fatty acids, supplemented with 300 μmol/L of various PUFA in the presence of 0.1 μmol/L insulin, 1 μmol/L Dex, 5 μmol/L triiodothyronine (T3), and 25 mmol/L glucose.

Before experiments, non-esterified fatty acids (Sigma) were dissolved in 95% ethanol (v/v) (14). Fatty acids (1.11 mmol/L) were combined with 75 g/BSA by stirring for 1 h at 37°C. Butylated hydroxytoluene (BHT) (0.1%) and 20 μmol/L α-tocopherol dissolved in DMSO, were added to the fatty acid-albumin stock solution to minimize oxidation. The fatty acids used in the present study included oleic acid (OA), C18:1(n-9); γ-linolenic acid (GLA), C18:3(n-6); arachidonic acid (AA), C20:4 (n-6); docosahexaenoic acid (DHA), C22:6 (n-3); and eicosapentaenoic acid (EPA), C20:5(n-3).

**Luciferase activity and protein assay.** After 48 h, treated cells were collected, resuspended in 400 μL luciferase lysis buffer (Promega), and incubated for 10 min at room temperature (15). For centrifugation for 2 min at 500 x g, supernatants (50 μL) were incubated for 5 s with 100 μL luciferase assay buffer including luciferin (0.2 mmol/mL). The luciferase activity was determined using a luminometer (TD 20/20, Promega) and protein concentration was measured by the Bradford method (16). The ACC PI activity was expressed as luciferase activity (Relative Light Unit: RLU) measured per microgram of protein. The experiments were repeated three times with triplicate samples for each treatment.

**Statistical analysis.** One-way analysis of variance (ANOVA) was used to test for differences among experimental groups. Tukey’s multiple range test was employed to examine differences between individual treatment conditions. Differences were considered significant at p<0.05.

**Results and Discussion**

ACC catalyzes the rate limiting step in de novo fatty acid biosynthesis: the carboxylation of acetyl-CoA to malonyl-CoA. ACC gene expression is regulated in a tissue-specific manner by numerous nutritional and hormonal factors (1, 4). This study evaluated the effects of some of these factors on the regulation of the ACC gene PI in primary hepatocytes. Fukuda et al. (17) reported that insulin increased ACC mRNA by 5.5-fold above the control values in primary hepatocytes cultured without any other hormones. They also reported that with insulin plus Dex, and with insulin plus T3, ACC mRNA was increased by 8.5- and 6-fold, respectively; and that combining insulin, T3 and Dex increased ACC mRNA by 10-fold. These results indicate that insulin, Dex and T3 additively activate ACC gene expression.
Fig. 1. Effects of insulin, Dex, and T3 on ACC PI promoter activity in primary rat hepatocytes. Primary hepatocytes were transfected with the ACC PI/luciferase reporter vectors shown. Cells were cultured for 48 h in serum-free, hormone-free culture media with or without hormones at the concentrations listed below. I, 0.1 μmol/L insulin; D, 1 μmol/L Dex; T, 5 μmol/L T3. Luciferase activities were normalized for transfection efficiency by dividing relative light units by β-galactosidase activity expressed from a cotransfected pCMVβ plasmid. The results were expressed as fold induction over control. Triplicate samples were included in each experiment, and results are shown as mean ± SD.

In our experiments, the luciferase activity was not significantly changed by any of the hormones in the cells that were transfected with pGL3-Basic vector without promoter. Insulin treatment significantly increased activity of -1,049/+100 and -220/+21 ACC PI by 3.0- and 3.5-fold, respectively, compared to the control (p<0.05) (Fig. 1). Our results suggested that the increase of ACC mRNA by insulin is due to the activation of ACC promoter I and that an insulin response element may reside in the first -220 bp of ACC PI. Dex treatment significantly increased activity of -1,049/+100 and -220/+21 ACC PI by 4.3- and 5.2-fold, respectively, compared to the control (p<0.05) (Fig. 1). Treatments with Dex also potentiated the action of insulin on both ACC PI activity -1,049/+100 and -220/+21, which was consistent with the observation by Fukuda et al. (17) that Dex increased ACC mRNA induction by insulin. Travers and Barber (18) also reported that Dex potentiated the action of insulin on ACC PI transcript abundance in ovine adipose tissue. When treated with insulin alone for 72 h, the ovine adipose tissue ACC transcripts were increased 2-fold over controls, but when combined with Dex there was approximately a 4.5-fold increase (18). Dex also enhanced the effect of insulin on fatty acid synthase promoter activity, which is regulated in a similar manner to ACC (17). The effect of Dex on fatty acid synthase (FAS) expression was mediated by DNA sequences in its promoter region (19). Therefore, it can be postulated that the effect of Dex on ACC gene expression is mediated by DNA sequences in the first -220 bp of ACC PI.

However, O'Callaghan et al. (15) reported insulin did not increase ACC PI activity. The difference between the two experiments was the addition of Dex, which might enhance the insulin response in our results. T3 significantly increased activity of -1,049/+100 and -220/+21 ACC PI by 3.5- and 2.5-fold, respectively, compared to the control (p<0.05). The effects of Dex and T3, both separately and together, appeared additive to those of insulin on ACC PI activation. The activity of -1,049/+100 and -220/+21 ACC PI was also significantly increased by Dex and T3 by 4.2- and 3.6-fold, respectively (p<0.05) (Fig. 1). In general, the pattern of response seen with the shorter (-220/+21) fragment was very similar to that seen with the longer (-1,049/+100) construct, with the combination of all three hormones resulting in the greatest reporter gene expression. Zhang et al. (20) reported that ACC transcription was increased approximately 7-fold by T3 in chick embryo hepatocyte cultures. However, the fold changes seen with T3 in our experiment are relatively modest compared with those seen in ACC gene expression with T3 in chick embryo hepatocyte cultures (20) or in vivo (21, 22) suggesting the possible differences between avian and mammalian systems or additional distant response elements in ACC PI. The glucose responsiveness of the ACC PI promoter was investigated by transfection of the -1,049/+100 or -220/+21 ACC PI with luciferase constructs into primary hepatocytes and growing the cells in 0, 5, or 25 mmol/L glucose. The level of reporter gene activity was not significantly changed by glucose concentration in the cells that were transfected with pGL3-Basic vector without the promoter (data not shown). Treatment with 5 or
25 mmol/L glucose increased significantly the activity of ACC PI (−1,049/+100) by 4.0- or 7.0-fold, respectively compared to glucose-free control (p<0.05). The level of ACC PI (−220/+21) activity was also increased by 2.8- and 7.4-fold in 5 and 25 mmol/L glucose concentrations, respectively (Fig. 2).

A recent study (15) identified a glucose-response element (−126/−102) in ACC PI, homologous to those previously described in other responsive genes, including L-type pyruvate kinase, S14 and fatty acid synthase. The transcription factor ChoRF (Carbohydrate Responsive Factor) that interacts with other conserved glucose-regulated elements, appeared likely to mediate this regulation. O’Callaghan et al. (15) showed strong increases of ACC PI promoter activity with glucose, when either −1,049/+100 or −220/+21 ACC PI constructs were tested. Our results also showed that glucose increased the activity of −1,049/+100 or −220/+21 ACC PI in the presence of insulin, Dex, and T3. However, we could see only a 2- to 2.5-fold increase going from low to high glucose, clearly less than that observed by O’Callaghan et al. (15). While the explanation for this difference is unclear, one difference between the two experiments was the addition of Dex, which might inhibit the glucose response in our results. In addition, concentrations of other hormones used differed and O’Callaghan et al. employed Matrigel to enhance the responsiveness of their transfected cells.

Fukuda et al. (17) reported that treatment with PUFA decreased ACC mRNA expression in rat primary hepatocytes. In our experiment, luciferase activity was not significantly changed by the various fatty acids in the cells that were transfected with pGL3-Basic vector without the promoter (data not shown). With −1,049/+100 ACC PI, the addition of unsaturated fatty acids to the primary hepatocyte culture media significantly decreased the activity of ACC PI compared to control (p<0.05) (Table 1). The greatest effects were seen with DHA and EPA, with about 70% reduction. AA, GLA, and OA reduced the activity of ACC PI by 52, 35, and 23%, respectively. However, the addition of unsaturated fatty acids to the culture media did not affect the activity of the shorter −220/+21 ACC PI construct (Table 1). Thus, it appears that the ACC promoter region required for PUFA suppression is located between −1,049 and −220.

Dietary PUFA are known to inhibit lipogenesis by suppressing the expression of lipogenic enzymes, such as FAS and ACC (23). The first 2.1 kb of the FAS promoter was sufficient for the suppression by PUFA in transgenic mice (24). Sterol response element binding protein-1 (SREBP-1) binds to the classic sterol response element (SRE) and/or to a palindrome CATG sequence which functions as an insulin response element (25).

Overexpression of SREBP-1c in liver is associated with high expression of lipogenic genes, and removal of the SREBP-1 gene results in low expression of lipogenic genes (26). It has been suggested that PUFA exert their effects on lipogenic genes by inhibiting expression of SREBP-1c (27, 28).

Recently, it was shown that the FAS promoter region required for PUFA suppression is located between −278 and −131, where SREBP functions (29). Moreover, the overexpression of SREBP-1c in liver prevented PUFA-mediated suppression of FAS gene expression (29). Compared to the SREBP-1c gene, much less is known about the regulation of ACC gene expression by PUFA (30).

From these results, it can be postulated that the expression of ACC, the rate-limiting enzyme in lipogenesis, is regulated by hormones, glucose, and PUFA at the transcriptional level through the PI promoter. The response elements for glucose, Dex, and T3 appear to be located in the first −220 bp of ACC PI, but the response element for PUFA appears to be located between −1,049 and −220 bp. Further research is required to identify the precise location of response elements and to identify the complex of proteins that mediate the regulation of ACC gene expression by these hormones and nutrients.

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