A Biotin Analog Inhibits Acetyl-CoA Carboxylase Activity and Adipogenesis*

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Keith L. Levert, Grover L. Waldrop, and Jacqueline M. Stephens‡

From the Division of Biochemistry and Molecular Biology, Louisiana State University, Baton Rouge, Louisiana 70803

Acetyl-CoA carboxylase catalyzes the first committed step in the synthesis of long chain fatty acids. In this study, we observed that treatment of 3T3-L1 cells with biotin chloroacetylated at the 1′ nitrogen reduced the enzymatic activity of cytosolic acetyl-CoA carboxylase and concomitantly inhibited the differentiation of 3T3-L1 cells in a dose-dependent manner. Treatment with chloroacetylated biotin blocked the induction of PPARγ, STAT1, and STAT5A expression that normally occurs with adipogenesis. Moreover, addition of chloroacetylated biotin inhibited lipid accumulation, as judged by Oil Red O staining. Our results support recent studies that indicate that acetyl-CoA carboxylase may be a suitable target for an anti-obesity therapeutic.

Obesity is characterized by an increase in the number and size of adipocytes (1). During the course of adipogenesis the activities of several lipogenic enzymes such as acetyl-CoA carboxylase, fatty acid synthase, and ATP citrate lyase are increased (2). The up-regulation of these enzymes suggests they could be targets for anti-obesity agents. For example, it has been shown recently that mice treated with inhibitors of fatty acid synthase resulted in decreased food intake and weight loss (3). The hypothesis that acetyl-CoA carboxylase could be a target for anti-obesity agents was strengthened by a recent study demonstrating that treatment of 3T3-L1 cells with CABI treatment inhibits the adipocyte differentiation of 3T3-L1 cells. Acetyl-CoA carboxylase is a biotin-dependent enzyme that catalyzes the following two-step mechanism.

\[
\text{Enzyme-biotin} + \text{HCO}_3^- + \text{MgATP} \rightarrow \text{enzyme-biotin-CO}_2^+ + \text{ADP} + \text{Pi}
\]

**REACTION 1**

\[
\text{Enzyme-biotin-CO}_2^- + \text{Acetyl-CoA} \rightarrow \text{malonyl-CoA} + \text{enzyme-biotin}
\]

**REACTION 2**

The first half-reaction is carried out by the biotin carboxylase component of acetyl-CoA carboxylase and involves the ATP-dependent carboxylation of biotin with bicarbonate serving as the source of CO₂. The carboxyl group is transferred from biotin to acetyl-CoA to form malonyl-CoA in the second half-reaction, which is catalyzed by carboxyltransferase. For both half-reactions, biotin remains covalently linked to the enzyme through an amide bond to a specific lysine residue of the biotin carboxyl carrier protein and is designated as enzyme-biotin in the reaction scheme. Mammalian acetyl-CoA carboxylase contains all three components on a single polypeptide (5).

We have recently synthesized a bisubstrate analog inhibitor of the carboxyltransferase component of bacterial acetyl-CoA carboxylase (Fig. 1) (6). Since human acetyl-CoA carboxylase is now a target for anti-obesity drugs, the question arose as to whether the bisubstrate analog we synthesized would inhibit mammalian acetyl-CoA carboxylase and in turn act to reduce lipid accumulation. Unfortunately, the bisubstrate analog contains the nucleotide ADP and therefore is not permeable to the cell membrane. However, the precursor to the analog, a chloroacetylated biotin derivative (CABI)号楼，is sufficiently hydrophobic to diffuse across the cell membrane. The results of this study clearly demonstrate that treatment of 3T3-L1 cells with CABI inhibits the activity of acetyl-CoA carboxylase. Moreover, we have shown that CABI treatment inhibits the adipocyte differentiation of 3T3-L1 cells by blocking the induction of PPARγ and other adipocyte transcription factors.

**EXPERIMENTAL PROCEDURES**

**Materials—** Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Invitrogen. Bovine and fetal bovine serum (FBS) were obtained from Sigma and Invitrogen, respectively. PPARγ was a mouse monoclonal antibody from Santa Cruz Biotechnology. STAT antibodies were monoclonal IgGs purchased from Transduction Laboratories or polyclonal IgGs from Santa Cruz Biotechnology. Streptavidin linked to horseradish peroxidase (HRP) was from Pierce. HPLC was performed using a Waters HPLC system equipped with a Waters 996 photodiode array detector. Analytical HPLC was used a Discovery C-18 column (15 cm × 4 mm, 5 μm) purchased from Supelco. All other reagents were from Sigma or Aldrich.

**Cell Culture—** Murine 3T3-L1 preadipocytes were plated and grown to 2 days postconfluence in DMEM with 10% bovine serum. Medium was changed every 48 h. Cells were induced to differentiate by changing the medium to DMEM containing 10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine (MIX), 1 μM dexamethasone (DEX), and 1.7 μM insulin. After 48 h this medium was replaced with DMEM supplemented with 1 The abbreviations used are: CABI, a chloroacetylated biotin derivative; PPAR, peroxisome proliferator-activated receptor; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; STAT, signal transducers and activators of transcription; HRP, horseradish peroxidase; HPLC, high performance liquid chromatography; MIX, 3-isobutyl-1-methylxanthine; DEX, dexamethasone.

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‡ To whom correspondence should be addressed: Dept. of Biological Sciences, Rm. 202 Life Sciences Bldg., Louisiana State University, Baton Rouge, LA 70803. Tel.: 225-578-1749; Fax: 225-578-2597; E-mail: jsteph1@lsu.edu.
10% FBS, and cells were maintained in this medium until utilized for experimentation. CABI was dissolved in Me2SO and added to the cell culture medium at a 1 to 1000 dilution. Vehicle additions were performed in every experiment.

Synthesis of Chloroacetylated Biotin—Synthesis of chloroacetylated biotin was as described previously (7).

Preparation of Whole Cell Extracts—Monolayers of 3T3-L1 adipocytes were rinsed with phosphate-buffered saline and then harvested in a nondenaturing buffer containing 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.5% Nonidet P-40, 1 μM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 50 μM trypsin inhibitor, 10 μM leupeptin, and 2 mM sodium vanadate for Western blot analysis. Samples were extracted for 30 min on ice and centrifuged at 10,000 × g at 4 °C for 15 min. Supernatants containing whole cell extracts were analyzed for protein content using a BCA kit (Pierce) according to the manufacturer’s instructions. This procedure was modified to prepare extracts for enzymatic analysis. The nondenaturing buffer contained 150 mM KCl, instead of NaCl, and there was no Triton X-100, Nonidet P-40, or any protease or phosphatase inhibitors included in the buffer. The cell monolayers were scraped in this buffer and were homogenized with 16 strokes in a Dounce homogenizer. The homogenates were centrifuged at 10,000 × g for 5 min, and the supernatants were saved as cytosolic extract and used to assay acetyl-CoA carboxylase activity.

Gel Electrophoresis and Immunoblotting—Proteins were separated in 5, 7.5, or 12% polyacrylamide (acrylamide from National Diagnostics) gels containing sodium dodecyl sulfate (SDS) according to Laemmli (8) and transferred to nitrocellulose (Bio-Rad) in 25 mM Tris, 192 mM gels containing sodium dodecyl sulfate (SDS) according to Laemmli (8) in 5, 7.5, or 12% polyacrylamide (acrylamide from National Diagnostics) carboxylase activity.

Enzyme Assays—The activity of acetyl-CoA carboxylase from 3T3-L1 cell lysates was determined using a fixed time assay. Assays were performed by measuring the loss in acetyl-CoA and/or the production of malonyl-CoA at 5 min intervals for 20 min, using reverse phase HPLC. The rate of conversion of acetyl-CoA to malonyl-CoA was found to be linear for 20 min, and velocities were calculated by linear regression for other samples. Reactions were initiated by transferring 50 μl of lysate to the reaction mixture (final volume 200 μl) and incubated for 5–20 min at 25 °C. Reactions were terminated by addition of 50 μl 10% perchloric acid. Following termination of the reaction the samples were centrifuged (3 min, 10,000 × g) and analyzed by HPLC. A mobile phase of 10 mM KH2PO4, pH 6.7 (solvent A), and MeOH (solvent B) was used. The flow rate was 1.0 ml/min, and the gradient was as follows: hold at 100% solvent A for 1 min followed by a linear gradient to 30% solvent B over the next 5 min, then hold at 30% solvent B for 5 min. Using this method the retention times were 7.5 and 9.0 min for malonyl-CoA and acetyl-CoA, respectively.

RESULTS

To test the ability of CABI to reduce acetyl-CoA carboxylase activity, confluent 3T3-L1 preadipocytes were treated for 4 h with 10 μM CABI, and whole cell extracts were prepared and immediately used to measure acetyl-CoA carboxylase activity by analytical reverse phase HPLC. As shown in Fig. 2A, the activity of acetyl-CoA carboxylase from cells treated with CABI was 0.30 nmol of malonyl-CoA/min·mg, and cells treated with Me2SO had an activity of 1.40 nmol/min·mg. Thus, treatment of preadipocytes with CABI resulted in a 79% reduction in acetyl-CoA carboxylase activity. These samples were also analyzed for the expression of acetyl-CoA carboxylase using streptavidin HRP (Fig. 2B). These results clearly demonstrate that the reduced activity of acetyl-CoA carboxylase is not due to altered expression levels of the enzyme.

Since treatment of 3T3-L1 preadipocytes with CABI reduced acetyl-CoA carboxylase activity, we examined the effects of CABI on the adipogenesis of these cells. At 2 days postconfluence, the 3T3-L1 cells were exposed to the normal differentiation mixture (FBS, MIX, DEX, and insulin) for 48 h in the presence or absence of various doses of CABI. After 48 h, the cells were maintained in 10% FBS in DMEM. A vehicle addition of Me2SO was also performed. CABI or Me2SO was added to the cells in a 1 to 1000 dilution into the cell culture medium every 24 h. The isolation of whole cell extracts and Oil Red O staining was performed 1 week following treatment with the induction mixture. Adipogenesis was assessed by examining the expression of several transcription factors, PPARγ and STATs 1 and 5A, which are highly induced during adipogenesis (9), and by examining lipid accumulation, as judged by Oil Red O staining. Optimal differentiation, as judged by PPARγ expression and Oil red O staining, was achieved when 3T3-L1 cells were exposed to the normal differentiation mixture containing FBS, MIX, DEX, and insulin, which resulted in 100% adipocyte conversion as reported previously (10). However, we observed a dose-dependent inhibition of differentiation in the

16348

Pharmalogical Inhibition of Acetyl-CoA Carboxylase

FIG. 1. Structures of CABI-CoA and CABI.

FIG. 2. CABI treatment of 3T3-L1 preadipocytes results in decreased acetyl-CoA carboxylase activity, but not protein levels, in isolated cell extracts. A, whole cell extracts were prepared from 3T3-L1 preadipocytes following a 4-h treatment with either Me2SO (DMSO) or 10 μM CABI dissolved in Me2SO. Acetyl-CoA carboxylase activity was measured by analytical reverse phase HPLC. B, the whole cell extracts used to measure acetyl-CoA carboxylase activity were used to examine acetyl-CoA carboxylase protein levels. One-hundred μg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and incubated for 4 h with 2 μg/ml streptavidin HRP. Results were visualized by detecting HRP with enhanced chemiluminescence (Pierce).
presence of CABI. Exposure of differentiating adipocytes to 17 or 8 μM CABI blocked the induction of PPARγ expression (Fig. 3) and lipid accumulation as judged by Oil Red O staining (Fig. 4). Parallel with the 100% conversion, STATs 1 and 5A were highly expressed, and the induction in expression of these two transcription factors was also inhibited in a dose-dependent manner by CABI treatment. The specificity of this treatment is demonstrated by examining the expression of STAT3, a protein whose expression is not substantially regulated during differentiation. As shown in Fig. 3, the expression of STAT3 was unaffected by CABI treatment and is shown as a loading control.

**DISCUSSION**

The novel observations in this study include the ability of CABI to diffuse into 3T3-L1 cells and reduce the activity of cellular acetyl-CoA carboxylase and to inhibit the adipogenesis of 3T3-L1 cells in a dose-dependent manner by blocking the induction of PPARγ expression. To account for these observations we suggest that upon entering the cell, CABI reacts with endogenous coenzyme A to form the bisubstrate analog CABI-CoA. Once formed the bisubstrate analog inhibits the cytosolic and/or mitochondrial isoforms of acetyl-CoA carboxylase. This hypothesis is supported by the observations that CABI had no effect on acetyl-CoA carboxylase activity in isolated cellular extracts (data not shown). However, the bisubstrate analog CABI-CoA was indeed able to inhibit acetyl-CoA carboxylase activity when added to isolated cellular extracts in vitro (data not shown). Moreover, there is precedent for enzyme inhibitors forming intracellularly by reaction of a precursor with a metabolite. Examples include finasteride, which inhibits 5α reductase (11), isoniazid, which inhibits the mycobacterial enzyme InhA (12), and a bisubstrate analog inhibitor of serotonin N-acetyltransferase (13). The first two examples are used clinically to treat benign prostatic hypertrophy and tuberculosis, respectively. Our attempts to detect CABI-CoA in extracts from CABI-treated 3T3-L1 cells have been unsuccessful using HPLC with absorption optics. However, studies are under way to detect the bisubstrate analog CABI-CoA in cellular extracts with more sensitive methods.

The observation that the expression of STAT3 was unaffected by CABI is very important, because it suggests that CABI is not acting as a nonspecific alkylating agent. Moreover, the fact that the level of acetyl-CoA carboxylase did not decrease with CABI treatment further indicates that CABI is not exerting a general toxic effect. It should be noted that the inhibition of differentiation by CABI was reversible. If the addition of CABI was not repeated every 24 h, the cells started to differentiate (data not shown).

We have recently determined the inhibition constant of the bisubstrate analog, CABI-CoA, for bacterial acetyl-CoA carboxylase is 23 μM (6). If the inhibition constant for the murine acetyl-CoA carboxylase in 3T3-L1 cells is similar to the bacterial enzyme, then it is tempting to speculate how an inhibitor with such a modest Ki value could result in such a significant biological effect. The answer begins by noting the importance of acetyl-CoA carboxylase for cell growth and the recent demonstration that a gene knock-out of cytosolic acetyl-CoA carboxylase is embryonic lethal (4). Our goal was not to abolish the activity of all cellular acetyl-CoA carboxylase and induce cell death. Instead, the objective was to attenuate the activity of acetyl-CoA carboxylase and prevent lipid accumulation. To this end, a molecule with a 23 μM Ki value would serve this purpose, while a molecule with a nm or lower Ki value would have greater cytotoxicity.

In summary, the results presented in this paper are the first demonstration of a link between a pharmacological modulation of cytosolic acetyl-CoA carboxylase and inhibition of adipogenesis. These studies support the gene knock-out experiments in mice,
which indicate that acetyl-CoA carboxylase is a very promising target for anti-obesity agents (4). It will be interesting to examine the effects of CABI on other types of cells in which acetyl-CoA carboxylase is up-regulated such as breast cancer cells (14). We are currently searching for alternative solvents for CABI and trying to synthesize a more soluble precursor so that these compounds can be tested in rodents.

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