

# Chronic Hyperglycemia Enhances PEPCK Gene Expression and Hepatocellular Glucose Production Via Elevated Liver Activating Protein/Liver Inhibitory Protein Ratio

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Acute hyperglycemia normally suppresses hepatic glucose production (HGP) and gluconeogenic gene expression. Conversely, chronic hyperglycemia is accompanied by progressive increases in basal HGP and is a major contributor to hyperglycemia in both type 1 and type 2 diabetes by mechanisms that are poorly understood. The aim of this study was to investigate the molecular mechanisms whereby hyperglycemia contributes to excessive gluconeogenesis in Fao hepatoma cells. Increasing glucose from 5 to 20 mmol/l resulted in loss of glucose inhibition of PEPCK gene expression after 12 h. Furthermore, 24 h of incubation with 20 mmol/l glucose increased cAMP-stimulated PEPCK mRNA by ~40% ( $P < 0.05$ ) and similarly increased glucose production. Although total CCAAT/enhancer-binding protein  $\beta$  (C/EBP $\beta$ ) protein levels were suppressed, 20 mmol/l glucose increased the liver activating protein (LAP; an active isoform of C/EBP $\beta$ )/liver inhibitory protein (LIP; an inhibitory isoform of C/EBP $\beta$ ) ratio significantly. Chromatin immunoprecipitation studies of the endogenous PEPCK gene demonstrated an increased association of LAP with the cAMP response element of the promoter. Using transient transfection to manipulate the LAP/LIP ratio, we also demonstrate a direct relationship between this ratio and PEPCK promoter activity. An increased LAP/LIP ratio not only enhanced cAMP- and dexamethasone-induced PEPCK gene expression but also impaired the repressive effect of insulin. These results demonstrate that sustained hyperglycemia diminishes the inhibitory effect of glucose and insulin on PEPCK expression and enhances hormone-stimulated PEPCK gene expression and hepatocellular glucose production. Because prolonged hyperglycemia increases the LAP/LIP ratio and can

potentiate hormone induction of PEPCK transcription, our results suggest that a hyperglycemia-driven increased LAP/LIP ratio may be a critical molecular event in the pathogenesis of increased HGP in diabetes. *Diabetes* 54:976–984, 2005

**T**he liver plays a critical role in blood glucose homeostasis by maintaining a balance between the uptake and storage of glucose via glycogenesis and the release of glucose via glycogenolysis and gluconeogenesis (1,2). During periods of fasting, the liver can produce glucose by breaking down glycogen and by de novo synthesis of glucose from noncarbohydrate precursors such as lactate, pyruvate, glycerol, and alanine (gluconeogenesis). The rate of gluconeogenesis is controlled principally by the activities of several key enzymes, including PEPCK, fructose-1,6-bisphosphatase, and glucose-6-phosphatase (1,2). The genes encoding these enzymes are tightly controlled at the transcriptional level via the interaction of several hormones, principally glucagon, glucocorticoids, and insulin. In addition to insulin, it has been reported that glucose can directly suppress PEPCK gene expression and gluconeogenesis in the liver or liver cells (3–5). Acute elevations in circulating glucose normally suppress hepatic glucose output and gluconeogenic gene expression (1,2). However, continuous hyperglycemia interferes with this suppressive effect through mechanisms that are poorly understood.

Recently, two animal studies demonstrated a direct linkage between sustained hyperglycemia and increased hepatic glucose production (HGP) (6,7). Kim et al. (6) reported that muscle-specific GLUT4 gene-deficient mice develop a diabetes phenotype including reduced insulin suppression of HGP. Normalization of glucose by phloridzin treatment, which inhibits renal glucose reabsorption, restored HGP, suggesting that increased HGP was secondary to hyperglycemia in this model (6). A recent clinical study also showed that chronic hyperglycemia contributes to the impaired effectiveness of glucose in regulating glucose fluxes in type 2 diabetes and hence to worsening of the overall metabolic condition (8). This study also revealed that even short-term normalization of plasma glucose can improve the suppressive effect of glucose on HGP. Together, these studies indicate that prolonged hyperglycemia may directly provoke inappropriate HGP by mechanisms that are poorly understood.

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ACh, acetylated histone; CBP, CREB-binding protein; C/EBP, CCAAT/enhancer-binding protein; ChIP, chromatin immunoprecipitation; CREB, cAMP response element-binding protein; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HGP, hepatic glucose production; LAP, liver activating protein; LIP, liver inhibitory protein; Pol II, RNA polymerase II.

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PEPCK is the first committed step in gluconeogenesis in hepatic and renal cells. The balance between synthesis and degradation determines the total PEPCK activity because no allosteric modifiers of PEPCK have been described (4). The regulation of PEPCK expression is primarily at the level of gene transcription. Gene knockout studies suggest that CCAAT/enhancer-binding protein (C/EBP) $\alpha$ , C/EBP $\beta$ , cAMP response element-binding protein (CREB), and more recently forkhead box O1 and peroxisome proliferator-activated receptor- $\gamma$  coactivator 1 are involved in regulation of PEPCK gene expression (9–11). Of these transcription factors, only C/EBP $\beta$  has been shown to be modulated by glucose (12). C/EBP $\beta$  binds to multiple sites on the PEPCK promoter and contributes to hormonal regulation by insulin, glucagon, and glucocorticoids (13,14). There are three alternative C/EBP $\beta$  translation products from a single C/EBP $\beta$  mRNA: the 38- and 34-kDa forms of C/EBP $\beta$ , also known as liver activating protein (LAP), and the 20-kDa form of C/EBP $\beta$ , known as liver inhibitory protein (LIP) (15). LIP possesses only the DNA-binding and leucine zipper domains (15). Heterodimerization of LIP with full-length C/EBP $\beta$  attenuates transcriptional activity, suggesting a dominant-negative mechanism of transcriptional regulation (15). Duong et al. (13) have shown that insulin represses glucocorticoid-induced PEPCK expression within 30 min by increasing LIP expression, which disrupts the association of the CREB-binding protein (CBP) and RNA polymerase II (Pol II).

Short-term exposure of liver cells to high glucose suppresses PEPCK gene expression (3). However, the molecular mechanisms for glucose-suppressive effects on the PEPCK promoter have not been identified. Unlike several of the lipogenic genes, the PEPCK promoter does not contain a carbohydrate response element. Thus, the transcription factors involved in PEPCK gene regulation under high-glucose conditions are unknown. C/EBP $\beta$  is a key regulator of PEPCK and gluconeogenesis, as demonstrated by gene knockout mouse studies of diabetes, fasting, and responsiveness to insulin (13,16,17). Therefore, the current studies were conducted to determine the role of C/EBP $\beta$  in glucose regulation of PEPCK gene expression.

Our results demonstrate that prolonged exposure of Fao cells to hyperglycemia diminishes glucose inhibition and augments cAMP stimulation of PEPCK gene expression. Prolonged high-glucose treatment also increases hormone-stimulated HGP in liver cells, while increasing the LAP/LIP ratio. Chromatin immunoprecipitation (ChIP) assays of the PEPCK promoter demonstrated increased association of LAP under high-glucose conditions. Transient transfection analysis demonstrated a direct relationship between the LAP/LIP ratio and PEPCK promoter activity, and a selective increase in the LAP/LIP ratio enhanced cAMP- and dexamethasone-induced PEPCK expression. Together, these data demonstrate that chronic hyperglycemia characteristic of the diabetic state increases the LAP/LIP ratio in Fao cells, which may be part of the pathogenic mechanism(s) for increased HGP in diabetes.

## RESEARCH DESIGN AND METHODS

**Cell culture.** Fao cells, derived from the H4IIE hepatoma cell line, possess a complete gluconeogenic enzyme system that allows the cells to survive and grow in low-glucose (5.5 mmol/l) or glucose-free (0.0 mmol/l) medium. Fao

cells are usually maintained in a high-glucose medium (25 mmol/l). Before any procedures in the current study, cells were incubated in Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Herndon, VA) with 5.5 mmol/l glucose and 10% fetal bovine serum (FBS) for more than 2 months, which allowed the cells to adapt to growth in a medium with "normal" concentrations of glucose. The medium was changed every day to maintain the glucose supply. Before any testing, the medium was replaced with the same medium containing a normal glucose concentration without FBS and was incubated overnight. Glucose treatment protocols were performed using DMEM and various concentrations of glucose, as indicated in RESULTS. Mannitol was used to control for any potential osmotic effects.

**Glucose production assay.** Glucose production from Fao cells was measured as described by Duong et al. (13) using a colorimetric glucose oxidase assay (Sigma, St. Louis, MO). Briefly, after 24 h of treatment with different glucose concentrations, the cells were washed three times with PBS. Then the cells were incubated for 6 h at 37°C, 5% CO<sub>2</sub>, in glucose production buffer (glucose-free DMEM, pH 7.4, containing 20 mmol/l sodium lactate, 1 mmol/l sodium pyruvate, and 15 mmol/l HEPES, without phenol red). The glucose assays were conducted in duplicate and the intra-assay coefficient of variation was <5%.

**Nuclear protein isolation and Western blot analysis.** Fao cell nuclear protein extracts were isolated as described previously (18). Protein concentration was measured using the Bio-Rad protein assay system (Bio-Rad, Hercules, CA). Nuclear protein extract (30  $\mu$ g) was separated by 10% acrylamide SDS-PAGE gel, and proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad) with a Mini Trans Blot cell from Bio-Rad. After blocking with 5% fat-free milk, the membrane was blotted with 1:1,000 dilution of rabbit polyclonal antibody raised against the COOH-terminal of human C/EBP $\beta$  (Santa Cruz Biotechnology, Santa Cruz, CA), which cross-reacts with both LAP and LIP, anti-C/EBP $\alpha$  (Santa Cruz Biotechnology), or anti-CREB antibody (Cell Signaling Technology, Beverly, MA). After washing, the membrane was blotted with a 1:10,000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG antibody (Bio-Rad). The peroxidase activity was visualized with ECL-Plus from Amersham Pharmacia Biotech. The specific bands were quantitated using a GEL-DOC density scanner and Quantity One software (Bio-Rad).

**RNA extraction and Northern blot analysis.** Total RNA was prepared from plated Fao cells with Trizol reagent following the manufacturer's protocol (Invitrogen, Carlsbad, PA). RNA was denatured, separated by agarose gel, and transferred to a positively charged nylon membrane (Roche Molecular Biochemicals, Indianapolis, IN). The cDNA fragment of rodent C/EBP $\beta$ , PEPCK, and GAPDH was labeled with [ $\alpha$ -<sup>32</sup>P]dATP using a Prime-It II Random Primer Labeling kit (Stratagene, Cedar Creek, TX). The membrane was blotted with labeled probes, and radioactive signals were quantified and analyzed with PhosphorImager and Imagequant software (Molecular Dynamics, Sunnyvale, CA).

**Plasmid constructs.** A 562-bp fragment of the 5'-flanking region of the mouse PEPCK gene, extending from -490 to +73 bp, was generated by PCR using mouse genomic DNA as a template. The PCR product was ligated into the pA3Luc vector at the *Kpn*I and *Hind*III sites (pA3-PEPCK-Luc). The inserted DNA fragment was verified by restriction analysis and sequencing. The full-length mouse C/EBP $\beta$  cDNA was cloned and inserted into the pcDNA3.1 vector, which expresses both LAP and LIP. The LAP expression plasmid (pcDNA-LAP) was generated by mutation of the LIP translation start codon using a QuickChange Site-directed Mutagenesis kit (Stratagene, La Jolla, CA). The sequences of mutagenic primers were as follows: 5'-CGACGCGCCCGc ATcGCGGCCGTTTC-3' and 5'-GGAAACCGCCCGcATcGCGGCCGCGTC G-3'. The LIP expression plasmid was created by inserting the full-length mouse LIP cDNA into pcDNA3.1 vector at *Bam*HI and *Eco*RI sites. The PEPCK promoter-luciferase gene reporter construct and pcDNA-LAP or pcDNA-LIP plasmids were cotransfected into Fao cells using the Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's protocol. A pCMV- $\beta$ -galactosidase plasmid was cotransfected for correction of transfection efficiency (Clontech Laboratories, Palo Alto, CA). The luciferase activity measurement has been described previously (19).

**ChIP assay.** The association of C/EBP $\beta$  protein with the endogenous PEPCK promoter was measured by ChIP assay as described previously (13,20). Fao cells were cross-linked with 1% formaldehyde (Sigma) in serum-free DMEM at RT for 5–10 min. Glycine was added at a final concentration of 125 mmol/l to stop the cross-link reaction. Cells were rinsed with ice-cold PBS. The pelleted cells were resuspended in SDS lysis buffer (1% SDS, 10 mmol/l EDTA, 50 mmol/l Tris-HCl, pH 8.0) and incubated on ice for 10 min. The chromatin was sheared by sonicating on ice for 10-s pulses at a setting of 6 (output of 4–5 watts), yielding chromatin fragments of 100–600 bp in size. Samples were centrifuged at 14,000 rpm for 10 min at 4°C to remove detritus, and the supernatant was divided into aliquots for subsequent 10-fold dilution in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mmol/l EDTA, pH 8, 16.7

mmol/l Tris-HCl, pH 8.0). Chromatin complexes were immunoprecipitated for 12–18 h at 4°C while rotating with amounts (5 µg) of primary anti-C/EBPβ, Pol II, CBP, acetylated histone (AcH)<sub>3</sub>, AcH<sub>4</sub>, histone deacetylase 1, or LAP antibody, or without antibody to provide negative controls. LAP-specific antibody was generated and provided by Dr. Linda Sealy (Vanderbilt University Medical Center), against the COOH-terminus of rat C/EBPβ (13). Immune complexes were collected with 40 µl protein A agarose, while rotating for 3 h at 4°C, followed by centrifugation at 1,000g for 1 min at 4°C. The pellets were washed with the following buffers in succession: low salt, high salt, LiCl wash buffer, and twice with TE (1 mmol/l EDTA, 10 mmol/l Tris-Cl, pH 7.5) buffer. Chromatin complexes were eluted from the beads in three consecutive 30-min rotating incubations with 200 µl elution buffer (1% SDS, 0.1 mol/l NaHCO<sub>3</sub>) at room temperature. To reverse cross-linking and digest RNA present in the samples, NaCl (200 mmol/l final concentration) and RNase mixture (Ambion, Austin, TX) was added, and the samples were incubated at 65°C for >6 h. The proteins were digested by incubating at 45°C for 90 min after the addition of the following at their final concentrations: 10 mmol/l EDTA, 40 mmol/l Tris-HCl, pH 6.5, and 50 µg/ml proteinase K. DNA was isolated and purified with the Quick PCR Purification kit (Qiagen, Valencia, CA).

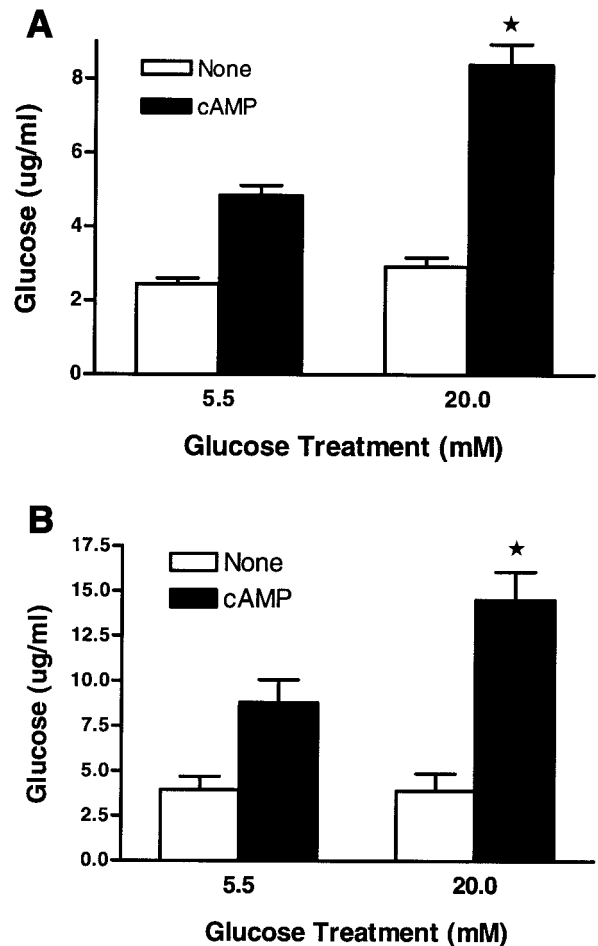
**PCR for ChIP assay.** The PEPCK promoter-specific PCR primers were designed with Jellyfish software. These included primers specific for the PEPCK promoter (5'-GAGGCCTCCCAACATTCAT-3', 5'-CTCAGAGCGTCTC GCCGGA-3') across the cAMP response element. Two percent of the immunoprecipitate (DNA) was used as template for the PCR, with 1 nmol/l of each primer, 200 µmol/l dNTP mix, PCR buffer, and 2.5 units *Taq* DNA polymerase (total 50 µl). After 36 cycles of amplification in a thermocycler, PCR products were run on a 2% agarose gel, visualized by ethidium bromide staining, and analyzed using Quantity One software.

**Data analysis.** Data are expressed as means ± SE. Statistical analysis was performed using the Student's *t* test or ANOVA analyses and was followed by a contrast test with Tukey or Dunnett error protection. Differences were considered significant at *P* < 0.05.

## RESULTS

**Sustained high-glucose treatment increases glucose production and PEPCK gene expression in Fao cells.** Glucose production from gluconeogenic precursors was assayed in Fao cells and in primary hepatocytes by measuring the amount of glucose released into the medium, which initially contained gluconeogenic precursors but no glucose. In nonstimulated cells (no cAMP), glucose production was not significantly different in cells exposed to either low (5.5 mmol/l) or high (20 mmol/l) glucose for 24 h (Fig. 1A and B). In contrast, cAMP-stimulated glucose production was significantly increased by 45% in cells exposed to high glucose for 24 h. Thus, these data demonstrate that prolonged high-glucose treatment enhances cAMP-stimulated gluconeogenesis and glucose output. Glycogenolysis could contribute to HGP, but it is minor and unlikely to account for the increased glucose production because glycogen levels in these cells are not different and indeed are very low when exposed to 5 or 20 mmol/l glucose.

In a previous study, short-term exposure to high glucose suppressed PEPCK expression independent of insulin (3,5). Therefore, we investigated the time course of glucose regulation of PEPCK gene expression to determine whether this might account for our results with respect to glucose release. Consistent with a previous study (5), high glucose reduced PEPCK mRNA rapidly reaching a nadir after 2 h (Fig. 2). However, PEPCK mRNA levels rebounded and returned to initial levels after ~20 h (Fig. 2). The differences in PEPCK mRNA over time were not due to differences in the rate of glucose utilization, as this remained linear over the 24-h period (data not shown). Therefore, these data indicate that sustained high-glucose treatment diminishes the inhibitory effect of glucose on PEPCK gene expression in hepatocytes. In Fig. 3, Fao cells were treated with 5.5 or 20 mmol/l glucose for 24 h, the test



**FIG. 1.** Twenty-four-hour high-glucose treatment enhances cAMP-induced hepatocellular glucose production in Fao cells (A) and primary hepatocytes (B). Primary hepatocytes were prepared from rats as described by us previously (34). Fao cells and primary hepatocytes were incubated with DMEM with 5.5 or 20 mmol/l glucose for 24 h and were washed three times with PBS and subsequently cultured in serum and glucose-free medium supplemented with lactate and pyruvate with or without 100 µmol/l dibutyl-cAMP. Four hours after hormone treatment, the medium was collected for glucose measurement. The bars depict the mean ± SE of at least four independent assays. \**P* < 0.001 vs. 5.5 mmol/l glucose-treated cells.

media was changed to DMEM with 5.5 mmol/l glucose for both groups, and cells were incubated in the presence or absence of 100 µmol/l dibutyl-cAMP for 2 h. The cAMP-induced PEPCK mRNA levels in 20 mmol/l glucose-treated cells were significantly higher (by 50%) versus 5.5 mmol/l glucose-treated cells (*P* < 0.05). These data suggest that prolonged high-glucose treatment not only diminishes the suppressive effect of glucose on PEPCK gene expression but also enhances cAMP-induced PEPCK gene expression.

**High glucose decreases C/EBPβ expression but increases the LAP/LIP ratio.** C/EBPβ is an important accessory transcription factor in mediating hormonal regulation of PEPCK gene transcription (4) and has recently been shown to be rapidly induced by glucose deprivation in human HEPG2 hepatoma cells (12). Therefore, we investigated whether the fluctuation of glucose alters C/EBPβ expression in Fao cells. Cells were maintained in DMEM with 5.5 mmol/l glucose. After overnight serum-free incubation, the media were changed to the same media with 5.5 or 20 mmol/l glucose. After 24 h, nuclear

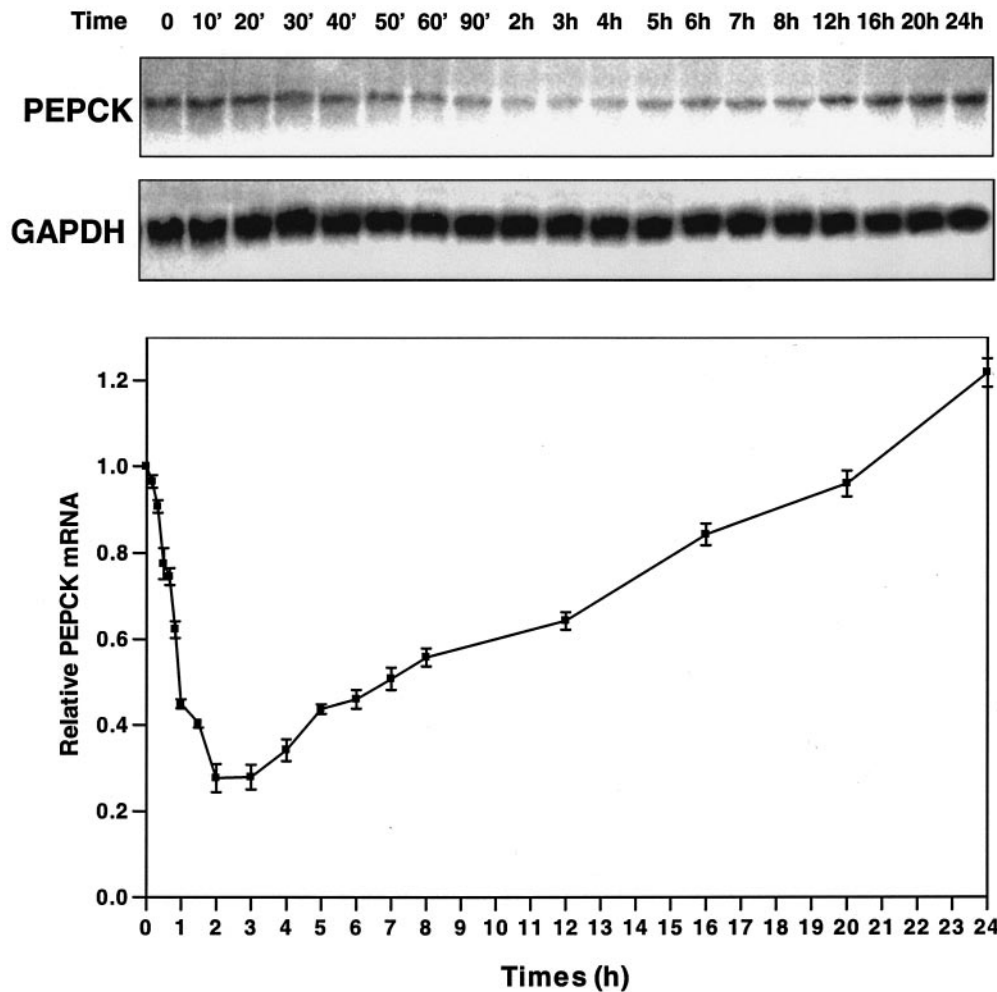
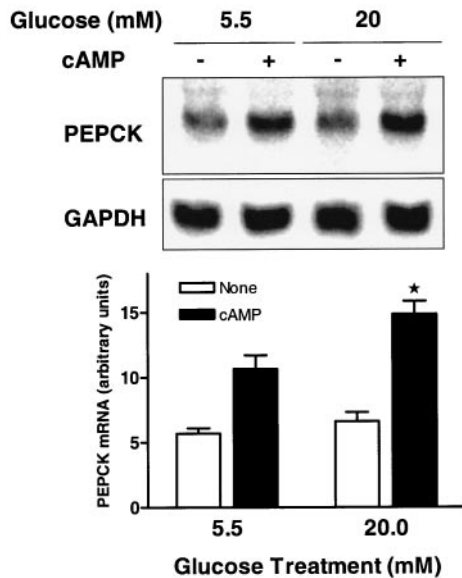


FIG. 2. Sustained high-glucose treatment diminishes the inhibitory effect of glucose on PEPCK expression. Fao cells were maintained in DMEM with 5.5 mmol/l glucose and 10% FBS. Twenty-four hours after cells reached confluence, the cells were incubated overnight in the same medium without FBS. Then the medium was replaced by the same medium with 20 mmol/l glucose. Total RNA was isolated at indicated time points, and PEPCK mRNA levels were analyzed by Northern blotting (20  $\mu$ g per lane). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were measured for internal controls. The time 0 sample was from cells treated with 5.5 mmol/l glucose before switching to high-glucose medium. Data are means  $\pm$  SE;  $n = 4$ .

protein was extracted and C/EBP $\beta$  was measured by Western blot using an antibody that cross-reacts with both LAP and LIP isoforms of C/EBP $\beta$ . Total C/EBP $\beta$  protein content was reduced in high-glucose-treated Fao cells (Fig. 4A). However, C/EBP $\alpha$  and CREB protein levels were not significantly different between treatments (Fig. 4A). C/EBP $\beta$  is a single gene, but several protein isoforms are produced by translation at different translation initiation sites. In Fao cells, two main isoforms can be found: LAP (43 kDa) and LIP (20 kDa). LIP contains only COOH-terminal dimerization and DNA binding domains but lacks a transactivation domain; thus, it has DNA binding activity but not transactivation potential. Therefore, LIP is considered to be a natural dominant-negative isoform of C/EBP $\beta$  (15). The LAP/LIP ratio has been reported to be more important than the total C/EBP $\beta$  protein level in controlling PEPCK gene transcription in response to insulin (13). To examine the effect of high-glucose treatment on the endogenous LAP/LIP ratio, the LAP and LIP protein levels were measured by Western blot and the LAP/LIP ratio was calculated. After 24 h of high glucose, the LAP/LIP ratio was significantly increased by nearly twofold, despite the

reduction of total C/EBP $\beta$  protein level (Fig. 4B,  $P < 0.001$ ).

**Effects of high-glucose treatment on association with the PEPCK promoter.** The reduction in C/EBP $\beta$  protein level after sustained hyperglycemia might lead to reduced availability of C/EBP $\beta$  to bind to DNA. However, the increase in the LAP/LIP ratio suggested there could be a shift in the proportion of LAP relative to LIP. We therefore investigated the effects of glucose on total C/EBP $\beta$  binding to the PEPCK promoter region using the ChIP assay. Incubation of Fao cells under either low or high glucose did not affect total C/EBP $\beta$  binding to the PEPCK promoter in the absence or presence of cAMP (Fig. 5). However, because the antibody we used recognizes both LAP and LIP, the results do not discriminate between LAP and LIP binding; therefore, a LAP-specific antibody was used to determine LAP-specific binding to the PEPCK promoter under high-glucose conditions. As shown in Fig. 6, high-glucose treatment increased LAP binding to the PEPCK promoter, whereas Pol II and CBP increased slightly, and AcH3 and binding were unchanged. Thus, the data indicate that the total C/EBP $\beta$  protein bound to the

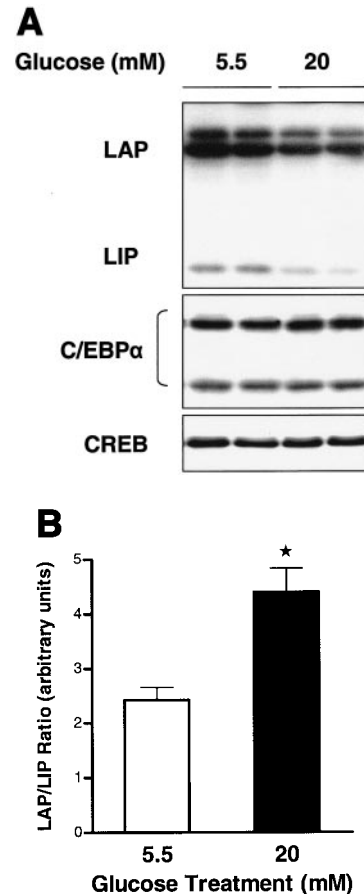


**FIG. 3.** Twenty-four-hour high-glucose treatment potentiates cAMP-induced PEPCK gene expression. Fao cells were maintained in DMEM with 5.5 mmol/l glucose and 10% FBS. The cells were incubated in the same medium without serum overnight and then treated with 5.5 or 20 mmol/l glucose. Twenty-four hours after the treatment, the medium was changed to serum-free DMEM with 5.5 mmol/l glucose, and 100  $\mu$ mol/l dibutyryl-cAMP was added to one group of cells to induce PEPCK expression. Two hours after the treatment with cAMP, total RNA was isolated as described in RESEARCH DESIGN AND METHODS. The PEPCK mRNA levels were measured using Northern blotting, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was tested for internal control. Data are means  $\pm$  SE;  $n = 5$ . \* $P < 0.05$  vs. 5.5 mmol/l glucose-treated cells with cAMP.

endogenous PEPCK promoter CRE site is similar; however, high-glucose treatment increased LAP association with the PEPCK promoter, which may be responsible for cAMP-induced higher PEPCK expression in the cells under high-glucose conditions.

**LAP/LIP ratio controls PEPCK promoter activity.** The above results indicate that high glucose suppresses total C/EBP $\beta$  protein levels but increases the LAP/LIP ratio in Fao cells. To verify the importance of the LAP/LIP ratio in controlling PEPCK gene transcription, we measured PEPCK promoter activity in response to different LAP/LIP ratios, which were achieved by manipulating the amounts of cotransfected LAP or LIP expression plasmids (Fig. 7). Because the expression of LAP and LIP was driven by the cytomegalovirus promoter in the expression plasmids, the expressed LAP or LIP protein level was closely correlated to the amount of transfected plasmid under current assay conditions (data not shown). Therefore, we used the pcDNA-LAP/pcDNA-LIP plasmid DNA ratio to represent the LAP/LIP ratio in transfected cells (Fig. 7), while keeping the total amount of LAP and LIP constant. When only pcDNA-LIP was cotransfected, the luciferase activities were suppressed 75% compared with the cells cotransfected with empty vector pcDNA ( $P < 0.001$ , Fig. 7). In contrast, increased LAP expression increased luciferase activity up to 25-fold ( $P < 0.0001$ ). There was a linear relationship between the pcDNA-LAP/pcDNA-LIP ratio and the reporter luciferase activity. Thus, these data demonstrate that the PEPCK promoter activity is precisely regulated by the LAP/LIP ratio.

**Increased LAP/LIP ratio potentiates cAMP- and dexamethasone-stimulated PEPCK gene expression.** In-



**FIG. 4.** High concentration of glucose decreases C/EBP $\beta$  protein level in Fao cells but increases LAP/LIP protein ratio. **A:** Fao cells were treated with the indicated glucose concentration for 24 h. The nuclear protein was extracted as described in RESEARCH DESIGN AND METHODS. Nuclear protein (40  $\mu$ g) was separated by 12% SDS-PAGE and immunoblotted with antibodies against C/EBP $\beta$ , C/EBP $\alpha$ , or CREB. These results are representative of at least eight independent experiments. **B:** Fao cells were treated, and nuclear extract was prepared as described above. The relative protein amount of LAP and LIP was measured by Western blot using an antibody that cross-reacts with both LAP and LIP. The LAP/LIP protein ratio was calculated based on the relative protein content. Data are means  $\pm$  SE;  $n = 6$ . \* $P < 0.001$  vs. 5.5 mmol/l glucose-treated cells.

creased LIP protein or a reduction of the LAP/LIP ratio inhibits the formation of transcription factor complexes on the PEPCK promoter region, thus inhibiting hormone-activated PEPCK gene transcription (13). Our above studies demonstrated that sustained high glucose increased the LAP/LIP ratio. To investigate whether an increased LAP/LIP ratio alters hormone regulation of PEPCK gene expression, Fao cells were studied in response to overexpression of LAP. As shown in Fig. 8, there was a slight increase in PEPCK mRNA when LAP was overexpressed in Fao cells in the absence of hormones or cAMP but was not statistically different ( $P > 0.05$ ). However, in the presence of cAMP, dexamethasone, or both, PEPCK mRNA levels were significantly higher in cells transfected with LAP (i.e., increased LAP/LIP ratio) versus vector alone (Fig. 8,  $P < 0.05$ ). Interestingly, insulin treatment reduced cAMP-induced PEPCK mRNA levels in both control and pcDNA-LAP-transfected Fao cells, but the PEPCK mRNA level was still significantly higher in pcDNA-LAP-transfected Fao cells ( $P < 0.05$ ). Together, therefore, these data indicate that increased LAP/LIP ratio enhances both

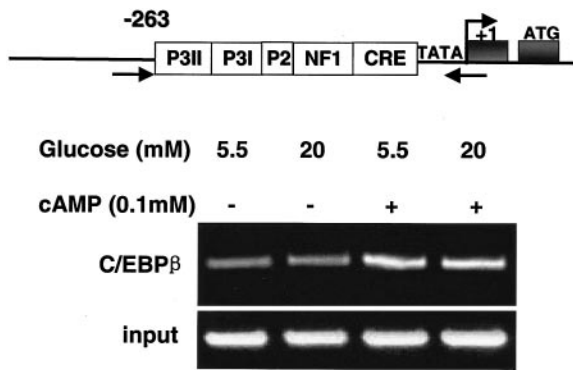


FIG. 5. Fluctuation of glucose in the medium does not change total binding of C/EBPβ on the PEPCK promoter region. The association of C/EBPβ with the endogenous PEPCK gene promoter was examined using the ChIP assay on Fao cells treated with the indicated glucose concentration and cAMP. The cells were treated with 5.5 or 20 mmol/l glucose for 24 h, and 100 μmol/l dibutylryl-cAMP was added to indicated cells and incubated for an additional 2 h, as described in RESEARCH DESIGN AND METHODS. Samples were analyzed by PCR using primers amplifying the PEPCK promoter sequence as shown. Immunoprecipitation was performed using anti-C/EBPβ or IgG antibodies. The results depicted are representative of three independent experiments with similar results.

cAMP- and dexamethasone-stimulated PEPCK gene transcription. Furthermore, the increased LAP/LIP ratio also appears to interfere with repression of PEPCK gene expression in response to insulin.

DISCUSSION

The liver is a critical organ in maintaining blood glucose concentration both via its ability to supply glucose to the circulation via glycogenolysis and gluconeogenesis in the postabsorptive state and remove glucose from the circulation after meal ingestion (1). These processes are coordinately regulated by hormones and nutrients (1,2).

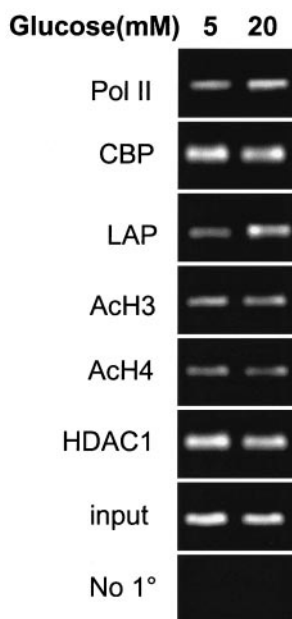


FIG. 6. High-glucose treatment increases LAP association with the PEPCK promoter. Fao cells were treated with 5.5 or 20 mmol/l glucose for 24 h. Protein binding on the PEPCK promoter was measured by ChIP assay, as described in RESEARCH DESIGN AND METHODS and in Fig. 5, using an LAP-specific antibody or antibodies against Pol II, CBP, AcH3, AcH4, or histone deacetylase 1 (HDAC1). The results depicted are representative of three independent experiments with similar results.

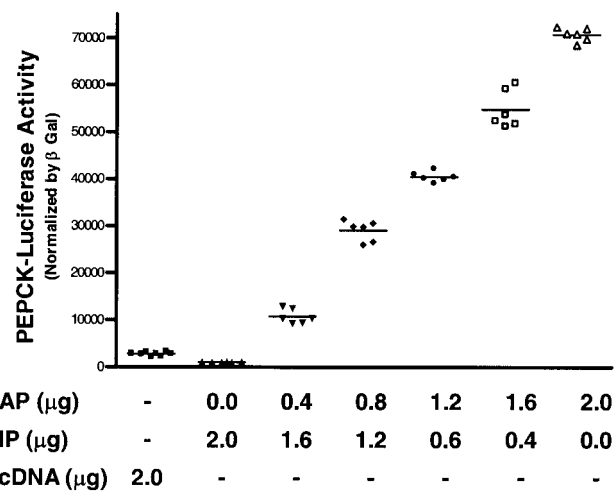


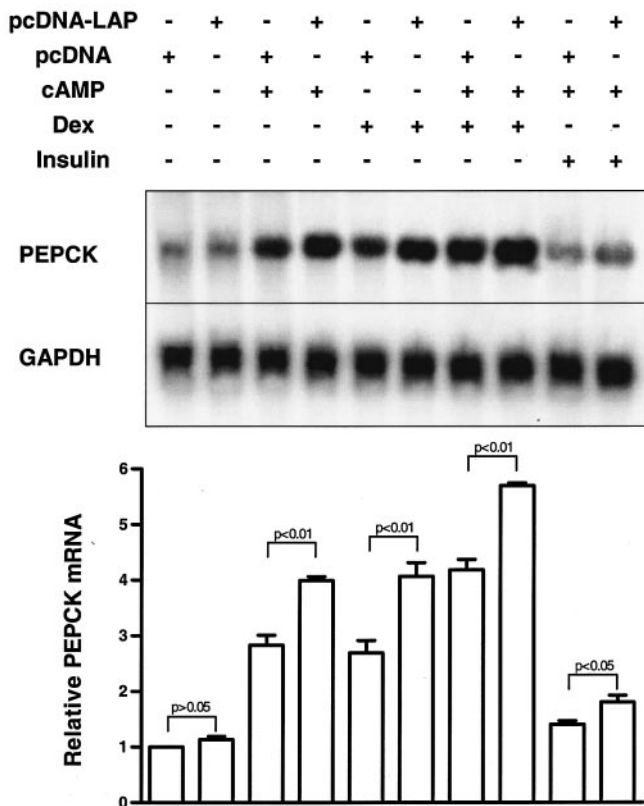
FIG. 7. LAP/LIP ratio controls PEPCK promoter activity in vivo. Fao cells were cultured in six-well plates. The cells were transfected with 0.5 μg pA3-PEPCK-Luc, 0.1 μg pCMV-β-galactosidase, and indicated amounts of pcDNA3.1, pcDNA3.1-LAP, or pcDNA3.1-LIP per well. Cells were lysed for luciferase assays 24 h after transfection, and the readings were normalized by β-galactosidase activity.

However, in both type 1 and type 2 diabetes, the gluconeogenic pathway is inappropriately active and supplies a relatively larger amount of glucose into the circulation, which contributes to both fasting and postprandial hyperglycemia (21–23). Despite a wealth of literature characterizing the molecular regulation of gluconeogenesis by hormones and nutrients, little is known regarding the specific molecular basis for inappropriate gluconeogenesis at the level of the PEPCK promoter. It is well known that sustained hyperglycemia impairs hepatic insulin sensitivity and results in reduced suppression of HGP by insulin (24). However, recent studies indicate that chronic hyperglycemia itself also plays an important role in the inappropriate gluconeogenesis, a phenomenon referred to clinically as “glucose toxicity” (6–8). The present study was undertaken to examine the molecular events that might account for this effect of hyperglycemia.

The regulation of PEPCK by hormones is largely at the transcription level, and most of the hormone response units in the PEPCK promoter region have been well characterized. Several transcription factors appear to be essential factors or important coactivators for regulation of PEPCK transactivation, including the C/EBPs, CREB, peroxisome proliferator-activated receptor-γ coactivator 1α, and forkhead box O1 (4,9,11,25,26). Of these, C/EBPβ is highly expressed in the liver and contributes to the transcriptional regulation by several gluconeogenic hormones and appears to be highly responsive to changes in glucose levels.

There are several regions on the PEPCK promoter that bind C/EBPβ. Of these, the first region containing a CRE, which is ~60 bp 5' from the TATA box (14), has been shown to bind members of the leucine zipper family of transcription factors, including C/EBPβ, C/EBPα, and CREB (14). In region 2, there is a P3(I) site, which exclusively binds with C/EBP transcription factors. P3(I) is required for the liver-specific expression of the PEPCK gene (27). Deletion of this site from the PEPCK promoter decreases cAMP-stimulated PEPCK transcription by 60% (27). Region 3 contains the glucocorticoid response unit composed of two glucocorticoid regulatory elements and

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**FIG. 8.** Elevated LAP/LIP ratio enhances cAMP and dexamethasone (Dex)-stimulated PEPCK gene expression and diminishes insulin-induced repression. Fao cells were cultured in six-well plates in DMEM with 5.5 mmol/l glucose and 10% FBS. The cells were transfected with 0.3  $\mu$ g pcDNA3.1-LAP or pcDNA3.1 vector per well. Twenty-four hours after transfection, the medium was changed to serum-free DMEM. The cells were treated with 100  $\mu$ mol/l dibutyryl-cAMP, 500 nmol/l dexamethasone, or 10 nmol/l insulin. Two hours after the treatments, total RNA was collected, and PEPCK mRNA levels were tested by Northern blotting. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were also measured as internal control. Data are means  $\pm$  SE;  $n = 4$ .

three glucocorticoid accessory factor binding sites (28,29). It has been demonstrated that C/EBP $\beta$  mediates glucocorticoid-, glucagon-, and insulin-regulated PEPCK gene transcription through these units (4,9,13,14,26,29). Generally speaking, in response to hormonal stimulation, there is a change in protein level or location of specific transcription factors or coactivators and a change of binding of the transcription factor to the hormone response unit. For example, upon cAMP stimulation, there is a rapid (<30 min) increase in C/EBP $\beta$  protein and increased C/EBP $\beta$  binding at sites in the PEPCK promoter region, which increase PEPCK gene transcription. However, there are two main isoforms of C/EBP $\beta$  in hepatocytes: LAP and LIP. LIP is a functional dominant-negative isoform of C/EBP $\beta$ , which competes with LAP in regulating PEPCK gene expression (15). Thus, the LAP/LIP ratio may play an important role in controlling target gene transcription, independently of total transcription factor protein level. For example, insulin rapidly upregulates C/EBP $\beta$  expression; however, this increase in total C/EBP $\beta$  protein does not increase PEPCK gene transcription but instead results in suppression of glucocorticoid-mediated transactivation because LIP is preferentially induced compared with LAP (13). In the current study, we have further defined the

importance of the LAP/LIP ratio in controlling PEPCK gene transcription using a transient transfection system. Specifically, the data demonstrated a direct relationship between the LAP/LIP ratio and PEPCK promoter activity.

Over the past few years, several different models of PEPCK gene transcription have been proposed (13,25,30,31). Although none of these models affords the global mechanism defining regulation of PEPCK gene transcription in response to hormone stimulation, C/EBP $\beta$  has been suggested to be involved in several sites of action within the promoter affecting hormone sensitivity. Duong et al. (13) recently proposed a new model that characterized the role of the LAP/LIP ratio in mediating glucocorticoid-, insulin-, and cAMP-regulated PEPCK gene transcription. In this model, C/EBP $\beta$  constitutively binds to the PEPCK promoter region and functions to recruit activating factors in response to hormonal signals. Whereas basal transcription is maintained by recruitment of the Pol II holoenzyme, several factors are recruited in response to hormone stimulation. In the present study, sustained high-glucose treatment decreased total C/EBP $\beta$  protein level in hepatoma cells but increased the LAP/LIP ratio. Although the association of C/EBP $\beta$  with the PEPCK promoter was not altered by high-glucose treatment, LAP association appeared to increase after 24-h exposure to high glucose. We tried looking for CREB immunoprecipitation using ChIP, but unfortunately the commercially available CREB antibodies were very poor at immunoprecipitation, and thus we were unable to perform this experiment. We previously showed that diabetes (hyperglycemia) in mice does not change the levels of two other CRE-binding proteins, C/EBP $\alpha$  or CREB, or their binding to the PEPCK promoter using gel-shift assays of mouse liver nuclear extracts and PEPCK CRE (16). In the present report, we also saw no change in the levels of CREB or C/EBP $\alpha$  in Fao cells exposed to chronic high glucose, as shown in Fig. 4. Although we cannot rule out a possible additional effect of CREB on the cAMP response, our current data and previously published results lend credence to the idea that hyperglycemia has specific effects on LAP/LIP levels and functions, at least in part, to increase the PEPCK promoter's ability to interact with other proteins via cAMP to affect transcription. These data indicate that high glucose alone increases the in vivo occupancy of LAP on the PEPCK promoter but does not increase assembly of Pol II, CBP, or acetylated histone binding to the transcriptional machinery. Rather, the increase in LAP association under hyperglycemic conditions may serve as an important metabolic switch, allowing greater assembly of these factors in response to hormonal stimulation.

The physiological importance of these observations lies in the fact that patients with hyperglycemia not only have hepatic insulin resistance at the level of the insulin receptor but also experience hyperglucagonemia (32,33). Therefore, the present research suggests that an important manifestation of prolonged high glucose in liver may be an increase in the LAP/LIP ratio, which may further potentiate the effect of glucagon at the level of the PEPCK promoter, thereby contributing further to hyperglycemia in a vicious cycle. The present study focuses on mechanisms for chronic hyperglycemia. The molecular mechanism for acute inhibition of PEPCK gene expression by

high glucose is unknown. The effect could involve several transcription control regions of the PEPCK promoter, but these were not addressed by the studies.

In summary, the present study identifies a novel molecular mechanism for glucose regulation of the PEPCK gene and glucose production. Our data suggest that chronic hyperglycemia may induce a new steady-state level of the LAP/LIP proteins in addition to a rearrangement of the chromatin environment, or a greater recruitment of activating factors available to the DNA. Sustained hyperglycemia diminishes the ability of glucose to inhibit gluconeogenesis and PEPCK gene expression and augments hormone-induced PEPCK gene expression. The study further shows that although chronic hyperglycemia decreases C/EBP $\beta$  expression in liver cells, the increase in the LAP/LIP ratio potentiates hormone-induced PEPCK gene expression. Therefore, the current study, using an in vivo system, has identified an important molecular mechanism that mediates hyperglycemia-induced dysregulation of HGP at the level of the PEPCK promoter.

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