Selective tonic inhibition of G-6-Pase catalytic subunit, but not G-6-P transporter, gene expression by insulin in vivo

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Hornbuckle, Lauri A., Dale S. Edgerton, Julio E. Ayala, Christina A. Svitek, James K. Oeser, Doess W. Neal, Sylvain Cardin, Alan D. Cherrington, and Richard M. O’Brien. Selective tonic inhibition of G-6-Pase catalytic subunit, but not G-6-P transporter, gene expression by insulin in vivo. Am J Physiol Endocrinol Metab 281: E713–E725, 2001.—The regulation of glucose-6-phosphatase (G-6-Pase) catalytic subunit and glucose 6-phosphate (G-6-P) transporter gene expression by insulin in conscious dogs in vivo and in tissue culture cells in situ were compared. In pancreatic-clamped, euglycemic conscious dogs, a 5-h period of hypoinsulinemia led to a marked increase in hepatic G-6-Pase catalytic subunit mRNA; however, G-6-P transporter mRNA was unchanged. In contrast, a 5-h period of hyperinsulinemia resulted in a suppression of both G-6-Pase catalytic subunit and G-6-P transporter gene expression. Similarly, insulin suppressed G-6-Pase catalytic subunit and G-6-P transporter gene expression in H4IIE cells. However, the magnitude of the insulin effect was much greater on G-6-Pase catalytic subunit gene expression and was manifested more rapidly. Furthermore, cAMP stimulated G-6-Pase catalytic subunit expression in H4IIE cells and in primary hepatocytes but had no effect on G-6-P transporter expression. These results suggest that the relative control strengths of the G-6-Pase catalytic subunit and G-6-P transporter in the G-6-Pase reaction are likely to vary depending on the in vivo environment.

glucose 6-phosphate; glucose-6-phosphatase; insulin; cyclic adenosine monophosphate; glucocorticoids; gene transcription

GLUCOSE-6-PHOSPHATASE (G-6-Pase) is a multicomponent system located in the endoplasmic reticulum (ER) that catalyzes the terminal step in glycogenolysis in the liver and gluconeogenesis in the liver and kidney (24, 48, 67). G-6-Pase activity has also been detected in the small intestine (54) and in pancreatic islets (3), although its role in these tissues is unclear. The kinetics of glucose 6-phosphate (G-6-P) hydrolysis by G-6-Pase are complex, and several models for the G-6-Pase system have been proposed, none of which fully accounts for all of the kinetic data (24, 48, 67). In one model, the active site of the G-6-Pase catalytic subunit is proposed to face the lumen of the ER, and the existence of various transporters is invoked for shuttling both substrate and product across the ER membrane (24, 48, 67). To date, only a G-6-P transporter has been identified; putative transporters for inorganic phosphate and glucose have not (24, 48, 67).

Mutations that inactivate the G-6-Pase catalytic subunit and G-6-P transporter genes give rise to glycogen storage disease (GSD) types 1a and 1b, respectively (13). The main characteristic of type 1 GSD is severe hypoglycemia in the postabsorptive state (13, 35, 42). In contrast, increased G-6-Pase activity is hypothesized to promote hyperglycemia in the postabsorptive state and contribute to the pathophysiology of diabetes (29). Thus, in both poorly controlled type 1 diabetics and in type 2 diabetics, the ability of insulin to repress hepatic glucose production (HGP) and stimulate peripheral glucose utilization is reduced as a consequence of insulin resistance (17, 18). In both type 1 (14) and type 2 diabetics (15), this elevation in HGP is due to an increased rate of gluconeogenesis rather than glycogenolysis. Several lines of evidence suggest that increased expression of both the G-6-Pase catalytic subunit and the G-6-P transporter contribute to this increase in HGP. Thus the expression of both genes is markedly elevated in diabetic animal models (5, 31, 39, 44, 46, 49). Similarly, overexpression of either the G-6-Pase catalytic subunit (59) or G-6-P transporter (2) in hepatocytes, with the use of recombinant adenovirus, was associated with enhanced rates of G-6-P hydrolysis as well as changes in glycogen metabolism. These observations (2, 59) suggest that the G-6-Pase catalytic subunit and the G-6-P transporter both contribute significantly to the overall control strength (53) of the G-6-Pase reaction and that both represent prime therapeutic targets for the treatment of the elevated HGP associated with diabetes.

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The increased hepatic expression of the G-6-Pase catalytic subunit and the G-6-P transporter genes in diabetic animal models could be caused by changes in the concentrations of a number of factors including insulin, glucagon, glucose, and glucocorticoids. Studies in conscious rats (46, 47), primary hepatocytes (4, 12, 45), and FAO hepatoma cells (4, 37) have shown that glucose stimulates G-6-Pase catalytic subunit gene expression. In all three systems, insulin inhibits basal G-6-Pase catalytic subunit gene expression and overrides the stimulatory effect of glucose (5, 37, 47). Studies in primary hepatocytes (5, 12) and hepatoma cells (5, 37, 56) have shown that insulin also overrides the stimulatory effects of cAMP and glucocorticoids. The regulation of G-6-P transporter gene expression has so far been studied only in the HepG2 cell line (39, 40). Glucose and glucocorticoids stimulated, whereas insulin inhibited, G-6-P transporter gene expression (39, 40). Li et al. (39) initially reported that, in HepG2 cells, cAMP stimulated G-6-P transporter gene expression, but more recently, Li and van de Werve reported that cAMP had no effect (40).

The aim of this study was to compare the regulation of G-6-Pase catalytic subunit and G-6-P transporter gene expression in parallel, in vivo, and in situ. Because of the design of the in vivo experiments, it was possible to distinguish between the effects of insulin and glucose and to ascertain whether a reduction in the basal insulin concentration, in the presence of euglycemia, affected the expression of either gene. The in situ experiments were designed to compare the magnitude of the effects of insulin, cAMP, and glucocorticoids on the expression of both genes in the H4IIE cell line. The results demonstrated that basal insulin selectively repressed G-6-Pase catalytic subunit gene expression in vivo. In cell culture, the magnitude of the effect of insulin was much greater on G-6-Pase catalytic subunit than on G-6-P transporter gene expression. Moreover, cAMP selectively stimulated only G-6-Pase catalytic subunit gene expression. These results suggest that the relative control strengths of the G-6-Pase catalytic subunit and G-6-P transporter in the G-6-Pase reaction are likely to vary depending on the in vivo environment.

METHODS

Materials

\[ \alpha^{32P}]dATP (>3,000 Ci/mmol), \[\gamma^{32P}]ATP (>5,000 Ci/mmol), \] and \[\alpha^{32P}]UTP (>3,000 Ci/mmol) were obtained from Amersham. For tissue culture studies, 8-(4-chlorophenylthio)-cAMP (8-CPT-cAMP), dexamethasone 21-phosphate, and bovine insulin were purchased from Boehringer Mannheim, Sigma Chemical, and Collaborative Bioproducts, respectively. Rat genomic DNA was purchased from Clontech. Dog genomic DNA was isolated from dog liver by means of the DNAzol genomic DNA isolation reagent (Molecular Research Center) according to the manufacturer’s instructions.

Animal Care and Surgical Procedures

Experiments were conducted on 20 conscious mongrel dogs (23–29 kg) of either sex that had been fed a meat and Chow diet (34% protein, 46% carbohydrate, 14.5% fat, and 5.5% fiber, based on dry weight; Kal-Kan beef dinner, Vernon, CA) and Purina Lab Canine Diet no. 5006 (Purina Mills, St. Louis, MO) once daily. The surgical facility met the standards published by the American Association for the Accreditation of Laboratory Animal Care, and the protocols were approved by the Vanderbilt University Medical Center Animal Care Committee.

Each dog underwent a laparotomy that was performed under general anesthesia (15 mg/kg pentothal sodium pre-surgery and 1% isoflurane inhalation anesthetic during surgery) 2 wk before the experiment. By use of standard sterile techniques that have been described previously (1), Silastic infusion catheters (0.03 in. ID; Dow Corning, Midland, MI) were placed into a splenic and a jejunal vein for intraportal infusions. Catheters (0.04 in. ID) for blood sampling were placed into the portal and hepatic veins and the femoral artery, as described previously (20). The catheters were filled with a saline solution that contained heparin (200 U/ml; Abbott Laboratories, North Chicago, IL), and their free ends were knotted. The position of the catheter tips was confirmed upon autopsy at the end of each experiment.

Only dogs that exhibited a leukocyte count <18,000/mm\(^3\), a hematocrit >34%, normal stools, and consumption of their daily food ration were used for the study. On the day of the experiment, after an 18-h fast, the catheters and flow probe leads were taken out under local anesthesia (2% lidocaine; Abbott Laboratories). The contents of each catheter were aspirated and then flushed with saline. The intraportal catheters (splenic and jejunal) were used for the infusion of insulin (Eli Lilly, Indianapolis, IN) and glucagon (Eli Lilly). Angiocaths (Deseret Medical, Becton-Dickinson, Sandy, UT) were inserted percutaneously into a leg vein for infusion of somatostatin (Bachem, Torrance, CA) and peripheral glucose (50% dextrose, Baxter Healthcare, Deerfield, IL). Animals were allowed to rest quietly in a Pavlov harness for 30 min before the experiments started.

Animal Experimental Procedures

Each experiment \((n = 4)\) consisted of an equilibration period \((-120 \text{ to } -40 \text{ min})\), a basal period \((-40 \text{ to } 0 \text{ min})\), and an experimental period \((0 \text{ to } 300 \text{ min})\). At \(-120 \text{ min}\), a constant infusion of somatostatin \((0.8 \mu \text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1})\) was started in a peripheral vein to inhibit endogenous pancreatic hormone secretion. At the same time, constant intraportal glucagon \((0.5 \text{ ng} \cdot \text{kg}^{-1} \cdot \text{min}^{-1})\) and insulin \((300 \mu \text{U} \cdot \text{kg}^{-1} \cdot \text{min}^{-1})\) infusions were started to replace basal secretion of these hormones. Also at \(-120 \text{ min}\), a glycogen phosphorylase inhibitor \((\text{BAY R 3401; } 10 \text{ mg/kg in a } 0.5\% \text{ methyl cellulose-water solution})\) was given orally or intragastrically (if a stomach catheter had been implanted during surgery) \((10, 61)\). Shortly thereafter, variable glucose infusion was begun to maintain euglycemia in the presence of the glycogen phosphorylase inhibitor. \(^{[3]H}\)Glucose was infused throughout to assess glucose production. The in vivo metabolic data from these and additional experiments are presented in depth in a series of papers to be published elsewhere. There were five experimental protocols.

Protocol 1: low insulin, basal glucose \((n = 4)\): beginning at 0 min, the intraportal insulin infusion was stopped, resulting in almost complete insulin deficiency within the 1st h of the experimental period (data not shown); glucose was infused as needed to maintain euglycemia.
Protocol 2: basal insulin, basal glucose (n = 4): no change was made in hormone infusion rates; glucose was infused as needed to maintain euglycemia.

Protocol 3: high insulin, basal glucose (n = 4): beginning at 0 min, the plasma glucose level was raised to 220 mg/dl (~12 mmol/l) by an increase in the glucose infusion rate; no change was made in hormone infusion rates.

Protocol 4: basal insulin, high glucose (n = 4): beginning at 0 min, the plasma glucose level was raised to 220 mg/dl (~12 mmol/l) by an increase in the glucose infusion rate; no change was made in hormone infusion rates.

Protocol 5: high insulin, high glucose (n = 4): beginning at 0 min, the intraportal insulin infusion rate was increased to (10–15%) in hematocrit occurred with this procedure.

Immediately after the final sampling time, each animal was anesthetized with pentobarbital sodium. It was then removed from the Pavlov harness while the hormones and glucose continued to be infused. A midline laparotomy incision was made, the liver was exposed, and clamps cooled in liquid nitrogen were used to simultaneously freeze sections of two hepatic lobes in situ. The hepatic tissue was then immediately cut free, placed in liquid nitrogen, and stored at −70°C. Approximately 2 min elapsed between the time of anesthesia and the time of tissue sampling. All animals were then euthanized.

Animal Analytical Procedures

Immediately after each blood sample was drawn, the blood was centrifuged to obtain plasma. Four 10-μl aliquots of plasma were immediately analyzed for glucose by the glucose oxidase method with a Beckman glucose analyzer (Beckman Instruments, Fullerton, CA). A 1-ml aliquot of plasma received 50 μl of 100,000 kallikrein inhibitor units/ml Trasylol (FBA Pharmaceuticals, New York, NY) and was stored for analysis of immunoreactive glucagon. The remainder of the plasma was used for analysis of insulin. All samples were kept in an ice bath during the experiment and were then stored at −70°C until the assays were performed. Immunoreactive insulin was measured using a double-antibody radioimmunoassay (50). Immunoreactive glucagon was measured using a modification of the double-antibody insulin method (50).

Isolation of Dog and Rat G-6-P Transporter Genomic DNA Fragments

With the use of dog genomic DNA as the template, a fragment equivalent to exon 3 of the human G-6-P transporter gene (27) was generated using PCR in conjunction with the following primers: 5′-GGAAATCCCGGATCCAAA-CATCAGCAGGTTC-3′ and 5′-CCCAACGTTGACCATCTCAGTTGGCACCPTTGGTG-3′ (EcoRI and HindIII cloning sites underlined). With rat genomic DNA as the template, the equivalent fragment of the highly homologous rat G-6-P transporter gene (41) was generated using PCR in conjunction with the following primers: 5′-GGAAATTCGGGATCCAAACAATCACGGACCTGC-3′ and 5′-CCCAACGTTGACCATCTCAGTTGGCACCPTTGGTG-3′ (EcoRI and HindIII cloning sites underlined). The isolated PCR fragments were digested with EcoRI and HindIII and ligated into the EcoRI- and HindIII-digested pGEM7 vector (Promega) and then sequenced using the United States Biochemical (USB) sequenase kit. Within the rat G-6-P transporter fragment, the sequence TTCTGTCTG differed from the published TTCTGTCT sequence at a single base (underlined). This altered sequence is equivalent to that in the mouse G-6-P transporter gene (41) and was generated in two independent PCR reactions. The nucleotide sequence of the dog G-6-P transporter fragment, minus primer sequence, has been submitted to GenBank with the accession number AF302774. These plasmids were linearized with HindIII such that in vitro transcription using T7 polymerase generated 254-bp antisense RNA probes.

Isolation of Rat and Dog G-6-Pase Catalytic Subunit Genomic DNA Fragments

Fragments of the rat and dog G-6-Pase catalytic subunit genes were generated using rat or dog genomic DNA, respectively, as the templates in PCR reactions with the following primers: 5′-GGAAATTCAGAGGTTGACCATCTGCC-3′ and 5′-CCCAACGTTGACCATCTCAGTTGGCACCPTTGGTG-3′ (EcoRI and HindIII cloning sites underlined). These data are contained in a series of papers to be published elsewhere. Arterial plasma was centrifuged to obtain plasma. Four 10-μl aliquots of plasma were immediately analyzed for glucose by the glucose oxidase method with a Beckman glucose analyzer (Beckman Instruments, Fullerton, CA). A 1-ml aliquot of plasma received 50 μl of 100,000 kallikrein inhibitor units/ml Trasylol (FBA Pharmaceuticals, New York, NY) and was stored for analysis of immunoreactive glucagon. The remainder of the plasma was used for analysis of insulin. All samples were kept in an ice bath during the experiment and were then stored at −70°C until the assays were performed. Immunoreactive insulin was measured using a double-antibody radioimmunoassay (50). Immunoreactive glucagon was measured using a modification of the double-antibody insulin method (50).

Isolation of Dog Cyclophilin A cDNA Fragment

A fragment of the dog cyclophilin A gene was generated for use as a hormonally unresponsive internal control in ribonuclease protection assays (see Ribonuclease Protection Assay). Total dog liver RNA was isolated using the TRI reagent (Molecular Research Center) according to the manufacturer’s instructions. This RNA was used as the template for generating cDNA by use of an oligo d(T)16 primer (Perkin Elmer) and avian myeloblastosis virus reverse transcriptase (Promega) under reaction conditions described in the GeneAmp RNA PCR kit (Perkin Elmer). The cDNA was then used as

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the template in a PCR reaction in conjunction with the following primers: 5′-GGAATTCATGGTCAACCCCACCAGTTCTCTC-3′ and 5′-GGAATTCCTTACACCTGAGCTACGAGGAATG-3′ (EcoRI cloning sites underlined). The isolated PCR fragment, predicted to contain the entire open reading frame of the dog cyclophilin A gene, on the basis of comparison with the highly homologous mouse, rat, and human cyclophilin A genes (16, 32, 34), was digested with EcoRI and ligated into the EcoRI-digested pGEM7 vector (Promega) and then sequenced using the USB sequenase kit. The nucleotide sequence of the dog cyclophilin A cDNA, minus primer sequence, has been submitted to GenBank with the accession number AF243140. The dog cyclophilin A plasmid was digested with BglII and EcoRI to isolate the dog cyclophilin A sequence between +489 and +556, relative to the translation start site. This fragment was ligated into an EcoRI- and BamHI-digested pGEM7 vector that was then linearized using SaeI. In vitro transcription of this truncated, linearized plasmid by use of T7 polymerase generated a 121-bp antisense RNA probe.

RNA Isolation

H4IIE cells were cultured as previously described (64). After a 16-h period of serum starvation, H4IIE cells were incubated in fresh, serum-free DMEM supplemented with various combinations of 8-CPT-cAMP (100 μM), dexamethasone (500 nM), and insulin (100 nM), as indicated in the figure legends. Total RNA was then isolated at the times indicated in the figure legends by cesium chloride centrifugation as previously described (21). Rat primary hepatocytes were purchased from In Vitro Technologies and were cultured as recommended by the supplier. After a 5-h period of serum starvation, cells were incubated for 3 h in fresh, serum-free DMEM supplemented with or without 8-CPT-cAMP (100 μM). Total RNA was then isolated using the TRI reagent according to the manufacturer’s instructions. The TRI reagent was also used to isolate total RNA from dog liver samples, again according to the manufacturer’s instructions.

Ribonuclease Protection Assay

[α-32P]UTP labeled antisense dog and rat G-6-P transporter, dog and rat G-6-Pase catalytic subunit, and dog cyclophilin A probes were generated using the plasmids described above and the MAXiScript T7 kit (Ambion) according to the manufacturer’s instructions. Similarly, a linearized plasmid containing a fragment of the rat cyclophilin A gene was purchased from Ambion and was used to generate a 165-bp antisense probe. Ribonuclease protection assays were performed using 10 μg of total dog liver, H4IIE, or rat primary hepatocyte RNA and the RPA III kit (Ambion), again according to the manufacturer’s instructions, except that the combined RNA and probe precipitate was dissolved in 1 μl of water before the addition of 10 μl of hybridization buffer. After RNase A/T1 digestion, RNA products were resolved on 5% polyacrylamide-urea-TBE (89 mM Tris, 89 mM boric acid, and 2 mM EDTA) gels, and sizes were estimated by comparison with coelectrophoresed DNA sequencing reactions. The sizes of the rat G-6-Pase catalytic subunit, rat G-6-P transporter, and rat cyclophilin A products were close to the calculated sizes of 190, 201, and 103 bp, respectively. The rat G-6-P transporter product appeared as a doublet, probably because the EcoRI site used in the cloning of the PCR product is partially homologous to the gene sequence. The sizes of the dog G-6-Pase catalytic subunit, G-6-P transporter, and cyclophilin A products were close to the calculated sizes of 256, 201, and 68 bp, respectively. Data were quantitated through the use of a Packard Instant Imager.

Primer Extension Analysis

A 26-bp primer (5′-TAGCGAGTCGACTGAGATCCCAAAGT-3′), complementary to exon 1 of the rat G-6-Pase catalytic subunit gene (5), was synthesized for the analysis of G-6-Pase catalytic subunit gene expression in H4IIE cells. A 30-bp primer (5′-ATGTCGAAGACACGTGGGTTTGCATTAG-3′), complementary to exon 1 of the mouse, rat, and human cyclophilin A genes (16, 32, 34), was synthesized for use as a nonhydrolytically responsive internal control. For the determination of the dog liver G-6-Pase catalytic subunit gene transcription start site, a 27-bp primer (5′-GAGTCCTGTAATTTCACCTGGAGGTAG-3′), complementary to exon 1 of the dog G-6-Pase catalytic subunit gene (see Isolation of Rat and Dog G-6-Pase Catalytic Subunit Genomic DNA Fragments), was synthesized. After gel purification, these primers were 5′ end labeled with [γ-32P]ATP to a specific activity of ~2 Ci/μmol (55). The labeled primers (~3 × 105 cpm) were then annealed to 50 μg of either total H4IIE RNA or total dog liver RNA for 1 h at 60°C, and then primer extension was performed as previously described (23). Extension products were visualized by electrophoresis on polyacrylamide-urea-TBE gels (23). The sizes of the extension products were calculated by comparison with a DNA sequencing ladder. The rat G-6-Pase catalytic subunit primer gave an extension product of 130 bp, 2 bp greater than previously reported (5). This places the rat G-6-Pase catalytic subunit transcription start site closer to that mapped for the mouse and human genes (38, 60). With H4IIE RNA, the cyclophilin A primer gave a major extension product of 71 bp, as predicted (16), and a minor extension product of 72 bp. The dog G-6-Pase catalytic subunit primer gave an extension product of 154 bp, indicative of a transcription start site similar to that of other species (Fig. 3). Data were quantitated through the use of a Packard Instant Imager.

Statistical Analysis

The animal study data were analyzed for differences from the control group values. Statistical comparisons were calculated using an unpaired Student’s t-test. The RNA study data were analyzed for differences from the control group values that were normalized to 1. Statistical comparisons were calculated using a paired Student’s t-test. The level of significance was P < 0.05 (two-sided test).

RESULTS

Plasma Glucose, Insulin, and Glucagon Levels

To investigate the effects of insulin and glucose on G-6-Pase catalytic subunit and G-6-P transporter gene expression in conscious dogs in vivo, five protocols were utilized in which these parameters were selectively manipulated (Fig. 1). The plasma glucose, insulin, and glucagon levels in the five experimental groups were as follows.

Control group: basal insulin, basal glucose. No change was made in hormone infusion rates after the equilibration period (Fig. 1). Glucose was infused as needed to maintain euglycemia in the presence of the phosphorylase inhibitor. The arterial plasma insulin, glucagon, and glucose levels remained basal and unchanged throughout the course of the study (data not shown).

Low-insulin group: low insulin, basal glucose. Beginning at 0 min, the intraportal insulin infusion was stopped, resulting in complete insulin deficiency (Fig. 1).

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Glucose was infused as needed to maintain euglycemia. The arterial plasma insulin level decreased from 4.8 ± 0.4 mU/ml (29 ± 3 pmol/l) during the basal period to 0.6 ± 0.1 mU/ml (4.1 ± 1 pmol/l) during the last 4 h of the study (P<0.05) (Fig. 2). The arterial plasma glucagon level remained unchanged, and the arterial plasma glucose level rose slightly from 106 ± 2 mg/dl (5.9 ± 0.1 mmol/l) during the basal period to 125 ± 6 mg/dl (7.0 ± 0.3 mmol/l) during the last 4 h of the study (P<0.05) (Fig. 2).

High-insulin group: high insulin, basal glucose. Beginning at 0 min, the intraportal insulin infusion rate was increased to 1,200 mU·kg⁻¹·min⁻¹ (7,200 pmol·kg⁻¹·min⁻¹), resulting in hyperinsulinemia (Fig. 1). Glucose was infused as needed to maintain euglycemia. The arterial plasma insulin level increased from 5 ± 0.9 mU/ml (30 ± 6 pmol/l) during the basal period to 21.8 ± 1.6 mU/ml (131 ± 6 pmol/l) during the last 4 h of the study (P<0.05), whereas the arterial plasma glucagon and glucose levels remained unchanged (Fig. 2).

High-glucose group: basal insulin, high glucose. Beginning at 0 min, the plasma glucose level was raised to 220 mg/dl (~12 mmol/l) by an increase in the glucose infusion rate (Fig. 1). No change was made in the hormone infusion rates. The arterial plasma insulin and glucagon levels remained almost unchanged throughout the study, whereas the arterial plasma glucose level rose from 107 ± 1 mg/dl (5.9 ± 0.1 mmol/l) during the basal period to 224 ± 2 mg/dl (12.4 ± 0.1 mmol/l) during the last 4 h of the study (P<0.05) (Fig. 2).

Combined high-insulin and high-glucose group: high insulin, high glucose. Beginning at 0 min, the intraportal insulin infusion rate was increased to 1,200 mU·kg⁻¹·min⁻¹ (7,200 pmol·kg⁻¹·min⁻¹), and the plasma glucose level was raised to ~220 mg/dl (~12 mmol/l) by an increase in the glucose infusion rate (Fig. 1). The arterial plasma insulin level rose from 4.6 ± 0.7 mU/ml (28 ± 4 pmol/l) during the basal period to 16.0 ± 1.7 mU/ml (96 ± 10 pmol/l) during the last 4 h of the study (P<0.05) (Fig. 2). The arterial plasma glucagon level remained unchanged, and the arterial plasma glucose level rose from 107 ± 1 mg/dl (5.9 ± 0.1 mmol/l) during the basal period to 218 ± 2 mg/dl (12.1 ± 0.1 mmol/l) during the last 4 h of the study (P<0.05) (Fig. 2).

Selective Regulation of G-6-Pase Catalytic Subunit and G-6-P Transporter Gene Expression In Vivo

Liver samples were collected at the end of the 5-h experimental period (Fig. 1; n = 4 dogs/group), and total RNA was isolated. G-6-Pase catalytic subunit and G-6-P transporter gene expression was then quantifi-
tated using the ribonuclease protection assay (RPA). RPA probes representing fragments of the dog G-6-Pase catalytic subunit, G-6-P transporter, and cyclophilin A genes were all generated using PCR in conjunction with primers representing conserved sequences in the mouse, rat, and human genes. The dog G-6-Pase catalytic subunit gene fragment spanned the region between the proximal promoter and exon 1 and differed from the published sequence (36) in the vicinity of the transcription start site. The published dog sequence in this region (36) also differs from that in the mouse, rat, human, and pig G-6-Pase catalytic subunit genes, whereas the sequence that we isolated is homologous to that in these other species (Fig. 3A). Therefore, the 5' end of the published dog sequence is presumably a non-G-6-Pase catalytic subunit sequence and instead represents a cloning artifact or linker sequence. A primer extension analysis was used to demonstrate that the G-6-Pase catalytic subunit transcription start site is indeed similar to that in other species (Fig. 3B), thus confirming that the dog exon 1 sequence that we isolated represents the 5' end of the gene.

The effects of insulin and glucose on G-6-Pase catalytic subunit and G-6-P transporter RNA levels were quantitated relative to the level of cyclophilin A RNA in the same samples. Expression of the latter is not responsive to hormones/metabolites, such that it serves as an internal control for variations in RNA loading. Figure 4 shows that, under euglycemic conditions, hyperinsulinemia represses both G-6-Pase catalytic subunit and G-6-P transporter gene expression. In the presence of hyperglycemia, insulin reduced G-6-Pase catalytic subunit gene expression but appeared to have no effect on G-6-P transporter gene expression (Fig. 4). In contrast, a 5-h period of hypoinsulinemia resulted in a marked stimulation of G-6-Pase catalytic subunit gene expression but appeared to have no effect on G-6-P transporter gene expression (Fig. 4). This result suggests that the expression of the former is tonically suppressed by a basal concentration of insulin. The hyperglycemia (2-fold over basal; Fig. 2)
investigated in these studies was insufficient to induce expression of either gene in the presence of a basal concentration of insulin (Fig. 4).

Selective Regulation of G-6-Pase Catalytic Subunit and G-6-P Transporter Gene Expression In Situ

To complement the in vivo conscious dog studies, the regulation of G-6-Pase catalytic subunit and G-6-P transporter gene expression by insulin, dexamethasone, and cAMP was compared in the rat H4IIE hepatoma cell line. Cells were serum starved overnight and then incubated with various combinations of insulin, 8-CPT-cAMP, and dexamethasone for 3 h before isolation of total RNA. G-6-Pase catalytic subunit gene expression was quantitated using the primer extension assay, whereas G-6-P transporter expression was barely detectable with this assay (data not shown). Therefore, the more sensitive ribonuclease protection assay was used to quantitate G-6-P transporter gene expression. In all experiments, expression of the cyclophilin A gene was again used as a nonhormonally responsive internal control.

Figure 5 shows that both 8-CPT-cAMP and dexamethasone stimulated G-6-Pase catalytic subunit gene expression in H4IIE cells and that insulin caused a marked inhibition of both basal gene expression and the stimulatory effects of 8-CPT-cAMP and dexamethasone. By contrast, although dexamethasone also stimulated G-6-P transporter expression in H4IIE cells, 8-CPT-cAMP had no effect. In addition, although insulin blocked this stimulatory effect of dexamethasone on G-6-P transporter gene expression, it only slightly repressed (~25%) basal expression after the 3-h incubation (Fig. 5). A greater repression of basal G-6-P transporter gene expression was seen at longer insulin treatment times (Fig. 6). However, the magnitude of the insulin effect on basal G-6-Pase catalytic subunit gene expression was clearly greater than that on basal G-6-P transporter gene expression at all time points examined (Fig. 6). Moreover, the time course of insulin action on G-6-Pase catalytic subunit gene expression was more rapid (Fig. 6).

Given the conflicting reports on the effect of cAMP on G-6-P transporter gene expression (39, 40), the regulation of G-6-Pase catalytic subunit and G-6-P transporter gene expression by cAMP was also compared in rat primary hepatocytes by use of the ribonuclease protection assay (Fig. 7). cAMP stimulated G-6-Pase catalytic subunit gene expression approximately nine-fold in primary hepatocytes but had no effect on G-6-P transporter gene expression (Fig. 7). Thus, in both rat H4IIE cells and primary hepatocytes, cAMP stimulates G-6-Pase catalytic subunit but not G-6-P transporter gene expression.

DISCUSSION

The translocation of G-6-P across the ER membrane rather than G-6-P hydrolysis by the G-6-Pase catalytic subunit was originally proposed to be the major control point in the G-6-Pase reaction (7). However, Seoane et al. (59) and Trinh et al. (66) subsequently showed, in both primary hepatocytes and liver, respectively, that overexpression of the G-6-Pase catalytic subunit was sufficient to cause an increased rate of hepatic glucose
production. These data suggested that the reaction catalyzed by the G-6-Pase catalytic subunit may, in fact, be a major control point in the G-6-Pase reaction. More recently, An et al. (2) have shown, again using adenoviral technology, that overexpression of the G-6-Pase catalytic subunit in hepatocytes also results in enhanced rates of G-6-P hydrolysis, albeit less than those achieved by G-6-Pase catalytic subunit overexpression. Therefore, these experiments suggest that both the G-6-Pase catalytic subunit and the G-6-P transporter contribute significantly to the overall rate of the G-6-Pase reaction. The data presented here suggest that

Fig. 5. Multihormonal regulation of G-6-Pase catalytic subunit and G-6-P transporter gene expression in the rat H4IIE hepatoma cell line. After 16-h serum starvation, H4IE cells were placed in fresh, serum-free medium in the absence (control; C) or presence of various combinations of 500 nM dexamethasone (D), 100 μM 8-(4-chlorophenylthio) (CPT)-cAMP (A) or 100 nM insulin (I). Three hours later, total RNA was isolated, and G-6-Pase catalytic subunit mRNA and G-6-P transporter mRNA were then quantified, as described in Methods, by primer extension or ribonuclease protection, respectively. A: representative autoradiographs; B: means ± SE of 3 experiments, each performed with independent RNA preparations. Data are expressed as the ratio of G-6-Pase catalytic subunit mRNA or G-6-P transporter mRNA to cyclophilin A relative to that in control cells. *P < 0.05 vs. control.

Fig. 6. Time course of inhibition of basal G-6-Pase catalytic subunit and G-6-P transporter gene expression by insulin in the rat H4IIE hepatoma cell line. After 16-h serum starvation, H4IIE cells were placed in fresh, serum-free medium in the absence or presence of 100 nM insulin. Total RNA was isolated at the indicated times, and G-6-Pase catalytic subunit mRNA and G-6-P transporter mRNA were then quantified, as described in Methods, using the ribonuclease protection assay. A: representative autoradiographs; B: means ± SE of 3–5 experiments, each performed with independent RNA preparations. Data are expressed as the ratio of G-6-Pase catalytic subunit mRNA or G-6-P transporter mRNA to cyclophilin A relative to that in control cells.
G-6-Pase catalytic subunit and G-6-P esterase are regulated by different hormonal conditions. An et al. found that overexpression of the G-6-Pase catalytic subunit, but not G-6-P transporter, results in changes in gene expression that vary under different hormonal conditions. The lack of a stimulatory effect of glucose on G-6-Pase catalytic subunit and G-6-P transporter gene expression, but more recently, Li and van de Werve (40) reported that cAMP had no effect, although an explanation for the difference between their initial results and later results was not provided. Our results indicate that cAMP selectively stimulates G-6-Pase catalytic subunit, but not G-6-P transporter, gene expression in rat primary hepatocytes (Fig. 7). The observations that insulin tonically suppresses G-6-Pase catalytic subunit, and not G-6-P transporter, gene expression in vivo (Fig. 4) and that, in tissue culture, the inhibitory effect of insulin on their expression varies in magnitude (Fig. 5) and time course (Fig. 6) may indicate that insulin regulates the expression of these two genes through distinct mechanisms. Multiple distinct promoter elements, referred to as insulin response sequences (IRSs), have been identified in various genes that mediate the action of insulin on gene transcription (51). Which, if any, of these IRSs mediates the action of insulin on G-6-P transporter gene expression remains to be determined. Moreover, it is possible that the effects of insulin on basal and glucocorticoid-stimulated G-6-P transporter gene expression are mediated through different promoter elements. Thus the inhibitory effects of insulin on basal, glucocorticoid-, and cAMP-stimulated phosphoenolpyruvate carboxykinase (PEPCK) gene expression may all be mediated through distinct mechanisms (51, 69, 70). The suppression of basal G-6-Pase catalytic subunit gene expression by insulin is mediated through the same T/G(A)TTT(T/G)(G/T) IRS motif that mediates the inhibitory effect of insulin on glucocorticoid-stimulated PEPCK gene expression (9, 52, 63, 64).

G-6-Pase catalytic subunit gene expression is subject to greater hormonal regulation than G-6-P transporter gene expression (Figs. 4–8). Thus basal insulin selectively suppressed G-6-Pase catalytic subunit, but not G-6-P transporter, gene expression in vivo (Fig. 4), and the magnitude and time course of the inhibitory effect of insulin on G-6-Pase catalytic subunit gene expression is greater and quicker than on G-6-P transporter gene expression in H4IIE cells (Figs. 5 and 6). Finally, cAMP stimulated G-6-Pase catalytic subunit, but not G-6-P transporter, gene expression (Figs. 5 and 7). The results imply that the relative control strength of the G-6-Pase catalytic subunit and G-6-P transporter in determining the overall rate of the G-6-Pase reaction will vary under different hormonal conditions. Interestingly, An et al. found that overexpression of the G-6-Pase catalytic subunit and G-6-P transporter had different qualitative effects on hepatic glucose metabolism (2). Thus overexpression of both proteins in hepatocytes reduced the rate of glycogen synthesis, whereas overexpression of the G-6-Pase catalytic subunit, but not the G-6-P transporter, also reduced the glycolytic rate (2). This observation suggests that changes in the ratio of these two proteins in response to hormones will affect more than just the rate of G-6-P hydrolysis and will, instead, have multiple effects on hepatic glucose metabolism.
that glucose stimulates G-6-Pase catalytic subunit gene expression, whereas studies in HepG2 cells have shown that glucose stimulates G-6-P transporter gene expression (39). We hypothesize that the twofold increase in glucose concentration examined in our studies was insufficient to overcome the inhibitory effect of the basal insulin concentration. Furthermore, the studies investigating the effect of glucose on G-6-Pase catalytic subunit gene expression in primary hepatocytes (4, 12, 45) and FAO hepatoma cells (4, 37) were conducted in the absence of insulin. In contrast, the previously reported stimulation of G-6-Pase catalytic subunit gene expression by glucose in vivo in conscious rats did occur in the presence of a basal insulin concentration (47). However, the concentration of glucose used in that study (∼17 mmol/l) (47) was significantly greater than the concentration used here (220 mg/dl, ∼12 mmol/l; Fig. 2). It is also possible that there are differences in the regulation of dog and rat G-6-Pase catalytic subunit gene expression by glucose. Indeed, the action of glucose is complex in that it regulates both G-6-Pase catalytic subunit gene transcription and mRNA stability (45). Finally, the dog studies described here were initially designed for a different purpose, namely to investigate the regulation of gluconeogenesis by glucose and insulin in vivo in the absence of the effects of these regulators on glycogenolysis and the attendant changes in glycolytic flux. This was achieved by using a glycogen phosphorylase inhibitor (BAY R 3401) (10) to block glycogenolysis (Fig. 1). Thus a particular concern in these studies is that chronic suppression of glycogen phosphorylase may have affected hepatic G-6-P levels. Because G-6-P and/or its metabolic by-products in the pentose phosphate shunt have been implicated in regulating the expression of a variety of genes encoding enzymes of carbohydrate and lipid metabolism (25, 68), it is possible that the use of the phosphorylase inhibitor may help explain the lack of an effect of hyperglycemia on G-6-Pase catalytic subunit gene expression in the dog. In addition, as with any such study, the possibility exists that this purportedly specific phosphorylase inhibitor may have affected other hepatic enzymes and indirectly affected glucose-regulated gene expression.

BAY R 3401 has been demonstrated to effectively reduce glycogen phosphorylase activities and thus suppress glycogenolysis in hepatocytes (10), in perfused intact liver (10), and in the whole animal (11, 22, 61). The active metabolite of BAY R 3401 reduces glycogenolysis both by facilitating inactivation, through dephosphorylation, and by allosterically inhibiting glycogen phosphorylase a (10). In experiments in the dog in which euglycemia was maintained by exogenous glucose infusion after administration of BAY R 3401, net hepatic glucose output decreased by 1.5 (61) and 2.4 (22) mg·kg⁻¹·min⁻¹ compared with the untreated basal state. This effect was entirely due to an effect on net hepatic glycogen breakdown, which decreased from 1.5 mg·kg⁻¹·min⁻¹ during the basal state to −1.3 mg·kg⁻¹·min⁻¹ 180 min after BAY R 3401 administration (22). Gluconeogenic precursor uptake by the liver and hepatic gluconeogenic flux were not inhibited (22, 61). Furthermore, when euglycemia was maintained during drug treatment, there was no change in plasma insulin, glucagon, cortisol, norepinephrine, or epinephrine levels or in hepatic blood flow (22, 61). Arterial plasma lactate levels declined, however, and net hepatic lactate uptake tended to increase, presumably because of a decrease in intracellular pyruvate levels (22, 61). There was no effect of the drug on the metabolism (level or net hepatic balance) of nonesterified free fatty acids or ketone bodies or on the gluconeogenic precursors glycerol, alanine, glutamine, glycine, or threonine (22, 61). These data strongly suggest that BAY R 3401 reduces glycogenolytic output by its direct inhibitory actions on glycogen phosphorylase and that it acts without any other significant metabolic alterations. In addition, it should be noted that, in the present study, apart from the effects of the drug on hepatic glycogenolysis, the control group exhibited no other metabolic consequences of drug treatment. Furthermore, because all groups were treated with BAY R 3401, the various changes in hormone and/or glucose levels were all on the same background of phosphorylase inhibition. It should be remembered, however, that the effects of changes in plasma insulin and glucose were investigated under circumstances in which the confounding effects of these perturbations on glycogen breakdown were eliminated.

One interesting question arising from the in vivo dog studies (Fig. 4) is the nature of the hormone/metabolite that mediates the selective induction of G-6-Pase catalytic subunit gene expression in the hypoinsulinemic state. Basal glucagon appears to be a possible candidate, because it selectively stimulates G-6-Pase catalytic subunit gene expression (Figs. 5 and 7), whereas glucose (39) and glucocorticoids (Fig. 5) can stimulate both G-6-Pase catalytic subunit and G-6-P transporter gene expression. Alternatively, relief from the repression of basal G-6-Pase catalytic subunit gene expression by insulin, without a specific contribution from one of these stimulatory factors, might be sufficient. Either conclusion contrasts with those reached in related studies in diabetic animal models. Thus Arizmendi et al. (8) and Friedman et al. (26) investigated the mechanism responsible for the elevation of PEPCk gene expression caused by streptozotocin-induced diabetes. In this diabetic model, multiple experimental approaches demonstrated that the induction of PEPCk gene expression was caused by glucocorticoids (8, 26). In contrast, in a different diabetic model, namely partially pancreatectomized rats, Massilon et al. (46) showed that the increase in G-6-Pase catalytic subunit gene expression could be abolished by reducing the associated hyperglycemia through phlorizin treatment. The interpretation of this observation was potentially complicated by the fact that phlorizin not only lowers the blood glucose concentration by blocking the activity of the renal Na⁺-glucose cotransport (62), but also inhibits the hepatic G-6-P transporter (6). However, when glucose was infused to prevent the decrease in blood glucose concentration after phlorizin treat-
The decline in G-6-Pase catalytic subunit gene expression did not occur (46). Interestingly, the circulating insulin concentration in these partially pancreatectomized rats was reduced only ~50% (46), in contrast to our studies where, under hypoinsulinemic conditions, the circulating insulin concentration was reduced ~90% (Fig. 2). This difference may explain why, in partially pancreatectomized rats, restoration of euglycemia through phlorizin treatment was not associated with increased G-6-Pase catalytic subunit gene expression despite the hypoinsulinemia (46). The absence of elevated G-6-Pase catalytic subunit gene expression cannot be explained by a reduction in the plasma glucagon level, because it was unchanged after partial pancreatectomy (46).

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