In Vivo Effects of Hyperinsulinemia on Lipogenic Enzymes and Glucose Transporter Expression in Rat Liver and Adipose Tissues

Françoise Assimacopoulos-Jeannet, Sonia Brichard, Franck Rencurel, Isabelle Cusin, and Bernard Jeanrenaud

Chronic hyperinsulinemia with maintenance of euglycemia was imposed on normal rats for 4 days. In white adipose tissue, hyperinsulinemia resulted in a twofold increase in GLUT4 protein and mRNA and a sixfold to 15-fold increase in fatty acid synthase (FAS) and acetyl coenzyme A (CoA) carboxylase (ACC) activity, respectively. Lipogenic enzyme mRNA was also markedly increased (20- to 30-fold). This was specific for white adipose tissue and was not observed in brown adipose tissue. In the liver, hyperinsulinemia was accompanied by a threefold increase in glucokinase (GK) activity and mRNA and by a threefold to fivefold increase in lipogenic enzyme activities and mRNA. In agreement with the changes in lipogenic activities, lipogenesis was markedly increased in white adipose tissue and liver of hyperinsulinemic rats. The data strongly suggest that in the rat, insulin is a driving force leading to increased lipid synthesis in liver and white adipose tissue.

THE EXPRESSION and activity of lipogenic enzymes is controlled by nutritional and hormonal conditions. Glucose and fructose increase the activity and mRNA of lipogenic enzymes in the liver,[1] whereas fatty acids decrease it.[2,3] Hormones such as thyroid hormones[4] or insulin[4,5] also stimulate lipogenic enzyme activity or expression.

The role of insulin in lipogenic enzyme activities and mRNA has been studied in diabetic rats,[4,6] fasted-refed animals,[7] strains of obese hyperinsulinemic rodents,[8] and rats made obese by lesions of the ventromedial hypothalamus.[9] However in these animal models not only insulin but also counter-regulatory hormones, metabolic substrates, and food intake vary, making clear conclusions difficult.[10]

Hyperinsulinemia imposed on normal rats with or without correction of hypoglycemia mimics several of the effects observed in obese animals.[11,12] After 4 to 7 days of hyperinsulinemia, white adipose tissue shows an increased glucose uptake in response to insulin both in vivo[11] and in vitro,[13] an increased content of GLUT4 and GLUT4 mRNA,[12] as well as an increased activity of lipogenic enzymes.[13] The effect on lipogenic enzyme gene expression has not been investigated under these conditions. Moreover, the consequences on liver and brown adipose tissue have not yet been studied.

The present study examined the effect of chronic hyperinsulinemia with maintenance of euglycemia on the expression and activity of lipogenic enzymes in three insulin-responsive tissues: white and brown adipose tissues and liver. The effects on glucose transporters GLUT4 in the adipose tissue and GLUT2 and glucokinase (GK) in the liver were also studied.

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The data show that white adipose tissue responds to continuous hyperinsulinemia by increasing lipogenic enzymes and GLUT4 expression, whereas brown adipose tissue does not. This suggests a tissue specificity in the regulation by insulin of the expression of GLUT4 and lipogenic enzymes. In the liver, hyperinsulinemia results in an increased activity and mRNA of lipogenic enzymes, but to a lower extent than in white adipose tissue. All these changes contribute to the increased lipid synthesis measured in these tissues.

MATERIALS AND METHODS

Twelve-week-old normal female Zucker rats (Fa/?) weighing approximately 200 g were used throughout the study. They were housed in individual cages and fed ad libitum.

A long-term jugular catheter was implanted under pentobarbital (50 mg/kg intraperitoneally) anesthesia to allow for glucose or NaCl infusion. The catheter was routed subcutaneously and externalized between the shoulders. It was connected to an infusion device consisting of a swivel and an oscillating arm allowing the rat to move freely.[14] For 3 days the rats were allowed to recover and were infused with 0.9% NaCl (2 mL/24 h). After 3 days of recovery rats were anesthetized with ether, and osmotic minipumps (Alzet 2001, Alza, Palo Alto, CA) containing insulin (0.1 U/L Actrapid; Novo, Copenhagen, Denmark) were implanted intraperitoneally. The minipump delivered 1 μL/h. Glucose (250 mg/mL, in NaCl 0.45 mg/mL) was infused in rats receiving insulin to maintain euglycemia. NaCl (0.45 mg/mL) was infused at the same rate to control rats. One group of rats was anesthetized and sham-operated, but no infusion of NaCl was performed. Blood was collected from the tail vein as previously described[15] every morning between 9 and 10 AM for measurement of glucose by a glucose oxidase method (Glucose Analyzer, Beckman, Fullerton, CA), insulin,[16] free fatty acids (WAKO NEFA-C, Wako Chemical, Neuss, Germany), and triglycerides (Triglycerides enzymatiques BioMérieux, Marcy l'Etoile, France).

Lipogenesis In Vivo

Three hours after food withdrawal, the rats were injected intraperitoneally with 2 mCi ^3H_2O in 0.2 mL 0.9% NaCl.[17] Glucose infusion to insulin-treated rats was continued during lipogenesis measurement to maintain euglycemia. Tail vein blood was collected three times for measurement of glycemia and ^3H_2O specific activity. After 60 minutes, rats were killed with pentobarbital and tissues were removed for the measurement of ^3H fatty acid levels. Total lipids were extracted in chloroform:methanol 2:1[18] and saponified, and fatty acids were extracted with petroleum ether and

counted. Rates of fatty acid synthesis were calculated as microgram-atoms of ^3H incorporated per hour from the radioactivity in the fatty acid fraction divided by the specific activity of plasma water.

Enzyme Activity and Cyclic Adenosine Monophosphate Measurements

The maximal activity of fatty acid synthase (FAS) was determined at 37°C using the spectrophotometric assay of Linn.[19] Total acetyl coenzyme A (CoA) carboxylase (ACC) activity was determined in a 100,000x*g* supernatant passed through a Sephadex G-25 (Pharmacia, Uppsala, Sweden) column. ACC activity was measured at 37°C with 10 mmol/L citrate as in the study by Majerus et al.[20] GK activity was determined at 37°C as in the study by Newgard et al.[21] The hexokinase activity, measured at 0.5 mmol/L glucose was subtracted from the activity measured at 100 mmol/L glucose. The liver cyclic adenosine monophosphate (cAMP) level was measured with the cAMP [^{3} H] assay (Amersham International, Amersham, UK).

Preparation of Crude Membrane and Immunoblotting

Total membranes (ie, plasma and microsomal membranes) were prepared from brown adipose tissue according to the method of Le Marchand-Brustel et al,[22] except that 100 μmol/L phenylmethylsulfonylfluoride and 5 μg/mL aprotinin were added to the homogenization medium. The same protocol was used to isolate total membranes prepared from adipocytes obtained from periovarian adipose tissue isolated by collagenase digestion.[23] Liver plasma membranes were prepared as described in Thorens et al[24] for measurements of GLUT2 levels. Membrane proteins were solubilized in Laemmli buffer, submitted to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis,[25] and electrophoretically transferred to Immobilon membranes (Immobilon-P, Millipore, Bedford, MA). Rainbow protein markers were used as molecular weight standards. The efficiency of transfer was verified by Coomassie blue staining of the gels. Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline, pH 7.6, for 1 hour at room temperature, washed, and incubated with the polyclonal antibody R820[26] directed against GLUT4 protein for another hour (1:500 dilution in Tris-buffered saline; Biogenesis, Bournemouth, UK), or in the case of liver they were incubated with a polyclonal antibody to GLUT2.[24] The blocked filters were incubated with ^{125}I-protein A (Amersham), washed, and submitted to autoradiography. Finally, they were quantified by determination of the radioactivity content of excised membrane pieces corresponding to the glucose transporter.

RNA Extraction and Northern Blot Analysis

Tissues were homogenized in guanidium isothiocyanate buffer and extracted with phenol-chloroform, and total RNA was precipitated with ethanol as previously described.[27] The amount of total RNA was estimated by measurement of optical density at 260 nm.

Total glyoxylated RNA (10 to 20 μg) was electrophoresed on 1.5% (GLUT4) or 1% (GK, ACC, FAS) agarose gel, transferred to Hybond-N membrane (Amersham), and cross-linked by UV irradiation. Blots were hybridized to random primed labeled GLUT4,[26] β-actin, ACC,[28] FAS,[29] or GK[30] cDNA, under high-stringency conditions. Probes were labeled with ^{32}P using the multiprime labeling system kit (Amersham) according to the recommendations of the providers. Filters were autoradiographed, and optical densities were quantified by scanning densitometry.

The cDNA probes were kindly provided by Dr D. James for GLUT4, Dr A. Goodridge for FAS (pFAS 18 cDNA for rat liver FAS mRNA), Dr K-H. Kim for ACC (p181-6 cDNA for rat mammary gland ACC mRNA), and Dr P. Iynedjian for rat liver GK. The antibody against GLUT2 was a gift from Dr B. Thorens.

Statistical Analysis

Results are expressed as the mean ± SE. Statistical analysis was performed using Student's *t* test for unpaired data.

RESULTS

Characteristics of Animals

The effect of long-term infusion of NaCl or insulin and glucose on weight gain, plasma glucose, insulin, nonesterified fatty acids (NEFA), and triglycerides is shown in Table 1. The group of rats infused with insulin gained significantly more weight than the control groups during the 4 days of insulin and glucose administration. Insulin was maintained at levels fivefold higher than those of control rats (Table 1, Fig 1). Food intake was lower in the hyperinsulinemic group (65 ± 3 and 49 ± 3 g/4 d, $P < .005$, control *v* hyperinsulinemic group, respectively), but total caloric intake was higher in the hyperinsulinemic group (260 ± 9 and 320 ± 10 kcal/4 d, $P < .005$, control *v* hyperinsulinemic group, respectively). To evaluate a possible effect of NaCl infusion on the parameters measured in the control group, an additional group of control rats without a catheter and NaCl infusion was studied. Table 1 shows that the two groups of controls did not differ except for a slight but significant difference in the level of NEFA. Similarly, catheterization and saline infusion did not affect the activity of any of the enzymes measured in the liver (Fig 3) and adipose tissue (data not shown). Therefore, hyperinsulinemic animals infused with glucose were compared with saline-infused rats. Figure 1A and B shows insulinemia and glycemia measured in control and hyperinsulinemic animals during 4 days of insulin administration. Glycemia was maintained at the level of controls. This was obtained by infusing increasing amounts of glucose (Fig 1C).

Table 1. Body Weight Gain and Plasma Glucose, Insulin, Free Fatty Acid, and Triglyceride Levels in Hyperinsulinemic, Control Saline-Infused, and Control Noninfused Rats

	Body Weight Gain (g/4 d)	Glucose (mmol/L)	Insulin (pmol/L)	Free Fatty Acids (mmol/L)	Triglyceride (mg/dL)
Control infused	3.0 ± 0.7	5.7 ± 0.2	318 ± 60	0.52 ± 0.06	60 ± 4
Hyperinsulinemic	13.8 ± 4.3*	6.0 ± 0.7	1,720 ± 198†	0.43 ± 0.06	64 ± 15
Control noninfused	3.8 ± 1.8	5.9 ± 0.2	250 ± 27	0.75 ± 0.05*	70 ± 5

NOTE. Results are the mean ± SEM of four rats per group. Values for day 4 are shown.

*$P < .05$.

†$P < .0005$.

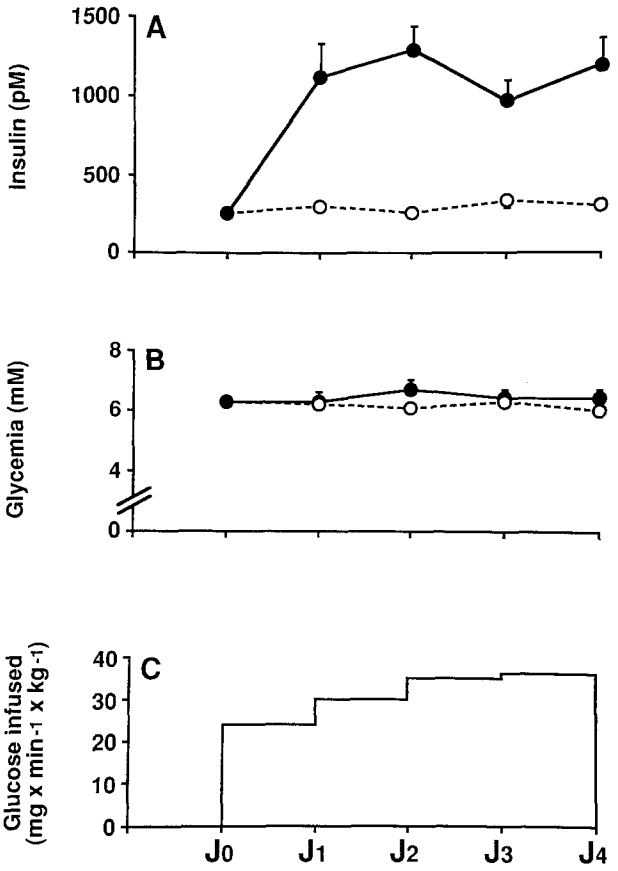

Fig 1. (A) Insulinemia and (B) glycemia in (○) control rats and (●) euglycemic hyperinsulinemic rats. (C) Rate of glucose infusion in euglycemic hyperinsulinemic rats. Values are the mean ± SE of 10 animals per group.

Lipogenesis, Lipogenic Activities, and mRNA, GLUT4, and GLUT4 mRNA in Control and Hyperinsulinemic Rats

Lipogenesis was measured in control and euglycemic hyperinsulinemic rats after 4 days of insulin administration. The measurements were performed 3 hours after food removal, and glucose infusion was continued to maintain euglycemia in the insulin-treated group. Lipogenesis was assessed by the incorporation of ^3H from ^3H_2O into hepatic and white adipose tissue fatty acids. Lipogenesis increased fivefold in the liver and 20-fold in adipose tissue of insulin-treated rats (Fig 2).

In the liver, the activities of glucokinase GK, ACC, and FAS were markedly increased by insulin (Fig 3). No significant changes in GLUT2 were measured (data not shown). The abundance of mRNA encoding for GK, ACC, and FAS was assessed by Northern blot analysis. The yield of total RNA was similar in the two groups: 4.21 ± 0.12 and 4.11 ± 0.10 μg/mg for control and insulin-treated groups, respectively. Quantification of the autoradiographic signals showed that insulin administration markedly increased GK, ACC, and FAS mRNAs (Fig 5A). For FAS mRNA, the two mRNA species known to be transcribed via alternative polyadenylation sites were equally increased by insulin administration. cAMP levels were not decreased and were even slightly increased in livers from insulin-treated rats (0.76 ± 0.04 and 0.97 ± 0.07 pmol/mg wet weight, $P < .02$, control *v* insulin-treated rats, respectively).

In white adipose tissue, a twofold increase in GLUT4 was measured in crude membranes prepared from isolated adipocytes in the insulin-treated group (Fig 4). This was specific for white adipose tissue and was not measured in brown adipose tissue (Fig 4). Similarly, the 10-fold increase in lipogenic enzyme activities for ACC and FAS measured in white adipose tissue was not observed in brown adipose tissue (Fig 4). Consistent with the changes measured for the protein, GLUT4 mRNA increased markedly in white (Fig 5B) but not brown adipose tissue (data not shown). The mRNA of the lipogenic enzymes FAS and ACC increased 35- and 25-fold, respectively, in white adipose tissue of hyperinsulinemic rats (Fig 5B). As in the liver, the yield of total RNA was unchanged by insulin administration: total RNA was 0.27 ± 0.05 and 0.30 ± 0.07 μg/g for control and insulin-treated groups, respectively.

DISCUSSION

The present study shows that chronic hyperinsulinemia imposed on normal rats increases GK and lipogenic enzyme expression in liver and GLUT4 and lipogenic enzyme expression in white but not brown adipose tissue.

Most in vivo studies involving insulin and its role in the regulation of gene expression have used fasted animals refed with carbohydrate or lipid diets[7] or diabetic animals treated with insulin.[4,6] In these experiments, enzyme activi-

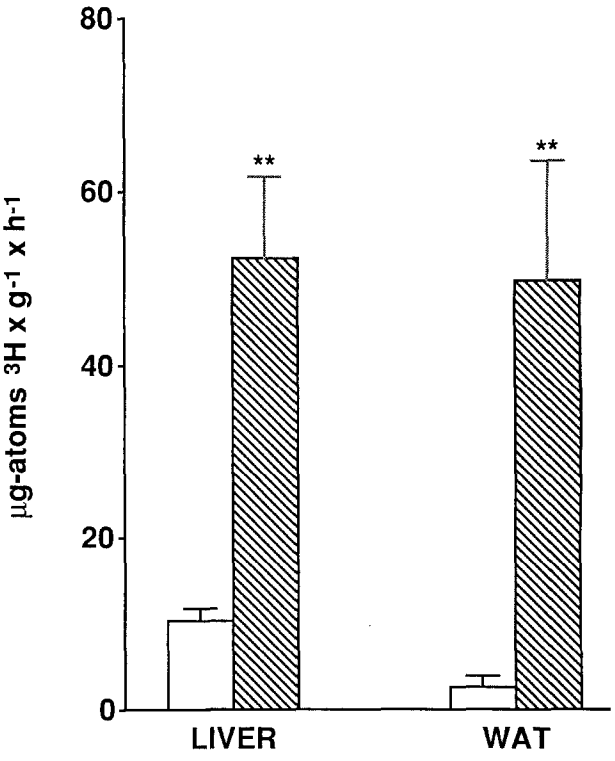

Fig 2. Lipogenesis in liver and periovarian white adipose tissue (WAT) in (□) control and (▨) euglycemic hyperinsulinemic rats. Values are the mean ± SE of four to five animals per group. **$P < .005$.

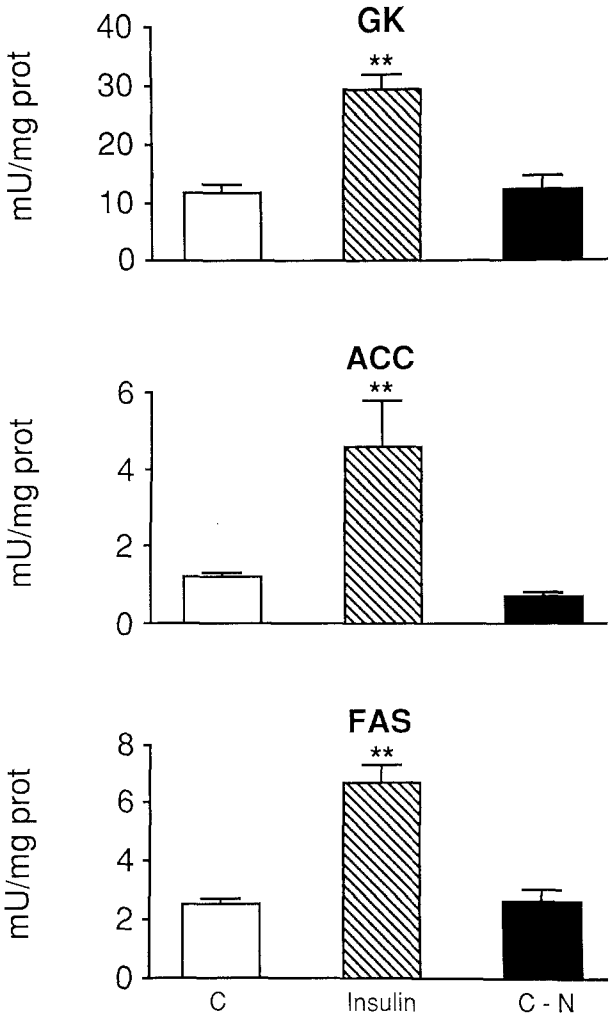

Fig 3. Activities of GK, ACC, and FAS in the liver of control rats (C), euglycemic hyperinsulinemic rats (insulin), and control rats not infused with NaCl (C-N). Values are the mean ± SE of four animals per group. **$P < .005$.

ties and mRNA were first decreased, and then restoration to normal values was studied. Not only insulin but also counterregulatory hormones and substrate concentration varied with time. The present study uses normal fed rats that are exposed to constant high insulin concentrations while glycemia is kept constant by glucose infusion. NaCl-infused rats behave like control noninfused rats. Increasing amounts of glucose have to be infused to maintain glycemia (Fig 1C). This may reflect increased glucose utilization both by adipose tissue[11,13] and by the liver (the present data). With the K_m of GK for glucose being approximately 5 mmol/L, a threefold increase in this enzyme activity results in an increased glucose phosphorylation by the liver at euglycemia. No change in the hepatic glucose transporter GLUT2 is observed. This is compatible with data obtained in short-term studies in which GLUT2 mRNA is transiently decreased by insulin without a change in the protein.[31] The increased activity of ACC and FAS is likely to reflect an increased protein mass. Indeed, FAS activity closely parallels the enzyme content.[32] ACC activity is known to be allosterically regulated, but the values obtained in the presence of citrate reflect the enzyme quantity.[33] Similarly, GK activity closely parallels the GK protein.[34] Hyperinsulinemia results in a threefold to fivefold increase in hepatic GK and lipogenic enzyme activities, and this resulted in a fivefold to sixfold increase lipogenesis.

In our study, FAS and ACC mRNAs were increased, respectively, threefold and fivefold by insulin in the liver. In contrast to our data, another study performed in rats acutely injected with insulin describes a stimulatory effect of the hormone in the liver of fasted but not fed rats.[6] In cultured hepatocytes, insulin by itself slightly increased ACC and FAS mRNAs, and this was potentiated by glucose.[7] In HepG2 cells, glucose (100 to 4,500 mg/L)

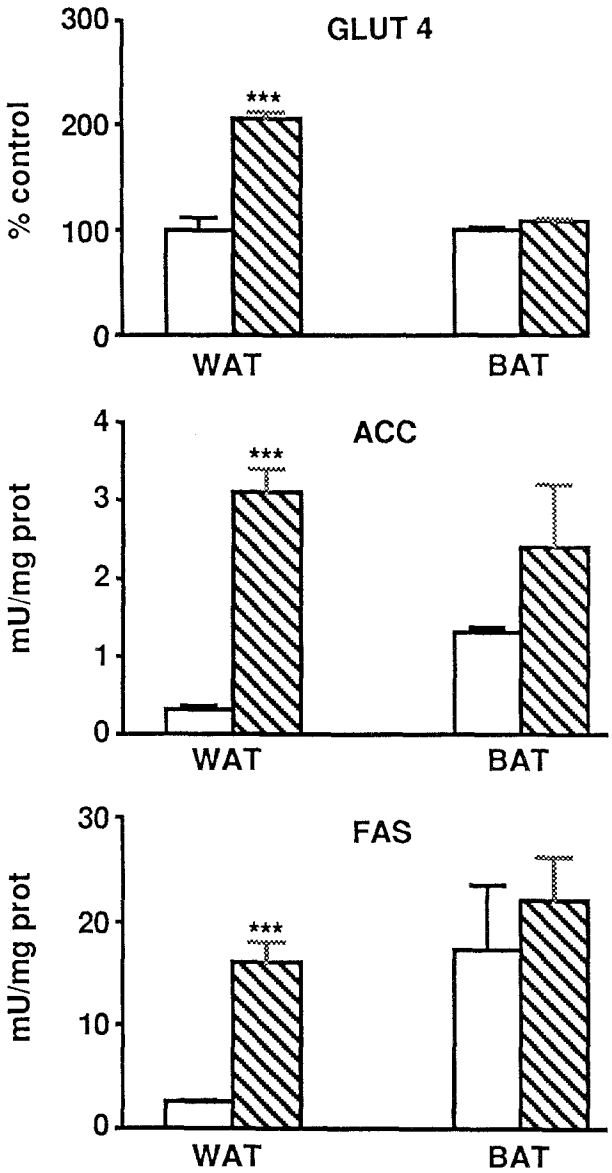

Fig 4. GLUT4 and activities of ACC and FAS in white (WAT) and brown (BAT) adipose tissues of (□) control and (▨) euglycemic hyperinsulinemic rats. Results of GLUT4 are expressed as % values measured in control animals. Values are the mean ± SE of four animals per group. **$P < .005$.

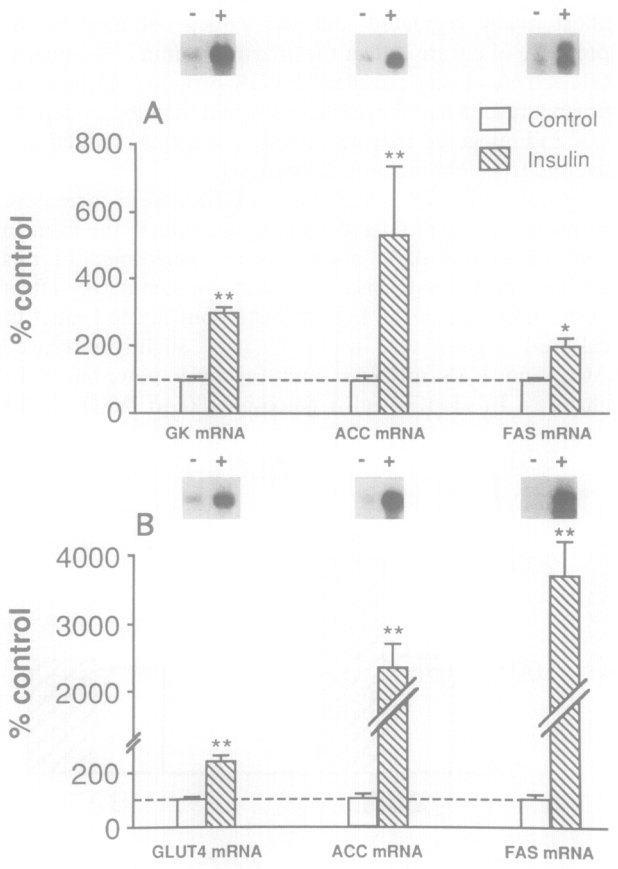

Fig 5. (A) Effect of hyperinsulinemia on GK, ACC, and FAS mRNAs in the liver of control and hyperinsulinemic rats. Above each column, one representative Northern blot is shown: (−) control; (+) hyperinsulinemic. (B) GLUT4, ACC, and FAS mRNAs in periovarian white adipose tissue of control and hyperinsulinemic rats. Values are the mean ± SE of four animals per group. *$P < .05$, **$P < .0005$.

increased FAS mRNA threefold to fivefold by increasing its stability.[35] This resulted in a similar increase in FAS activity. Although in our studies glycemia is maintained constant, an increase in GK activity could result in a higher level of metabolites of glucose, mediating their effect on mRNA stability.

One cannot totally rule out that insulin exerts in vivo its effect on lipogenic enzyme gene expression indirectly. First, ACC and FAS expression may be stimulated by a decrease in cAMP.[5,31,36] However, in our study cAMP levels were not reduced. Second, NEFA, especially the polyunsaturated ones, inhibit the expression of hepatic lipogenic enzymes.[2] Insulin could decrease an inhibitory tonus exerted by NEFA. However in the present study no significant reduction in plasma NEFA was observed.

The effect of insulin on expression of lipogenic enzymes is even larger in white adipose tissue, in which a twofold increase in GLUT4 expression is also measured. A similar increase in lipogenic enzyme and GLUT4 expression is measured in control and obese hyperinsulinemic fa/fa rats after weaning[8,37] and in normal rats after lesions of the ventromedial hypothalamus,[9] and is attributed to a large extent to the prevailing hyperinsulinemia. Young fa/fa rats, rats with lesions of the ventromedial hypothalamus, as well as rats receiving insulin for 8 days but in which glycemia was not corrected have adipocytes that show a higher incorporation of glucose into lipids in response to insulin,[8,9,11,13,37] as well as higher lipogenic enzyme activities.[8,13,37]

In white adipose tissue, the changes in lipogenic enzyme mRNA are attributable to an effect of insulin, although an indirect effect through increased glucose metabolism as a consequence of increased GLUT4 cannot be excluded. Indeed, in explants of adipose tissue, glucose has been shown to increase lipogenic enzyme mRNA through an increase in glucose-6-phosphate.[38] Another possibility is that the expression of the three genes is regulated in a coordinate manner by insulin at the transcriptional level. There is a considerable list of insulin-regulated genes.[10] Although great progress has been made in this field, the insulin-responsive sequences have been defined only in some genes.[10] This is not the case for GLUT4 or lipogenic enzymes.

The lack of effect of insulin on GLUT4 and lipogenic enzymes measured in brown adipose tissue indicates a different regulation in this tissue, likely dependent on sympathetic activity. Indeed, the expression of GLUT4 is increased in this tissue in response to sympathetic agonists.[39]

In summary, the present study demonstrates that insulin is one of the driving forces leading to increased lipid synthesis in rat liver and white but not brown adipose tissue, by acting on lipogenic enzymes, GK, and GLUT4 expression.

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