Free fatty acids increase basal hepatic glucose production and induce hepatic insulin resistance at different sites

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Lam, Tony K. T., Gérald van de Werve, and Adria Giacca. Free fatty acids increase basal hepatic glucose production and induce hepatic insulin resistance at different sites. Am J Physiol Endocrinol Metab 284: E281–E290, 2003; 10.1152/ajpendo.00332.2002.—To investigate the sites of the free fatty acid (FFA) effects to increase basal hepatic glucose production and to impair hepatic insulin action, we performed 2-h and 7-h Intralipid + heparin (IH) and saline infusions in the basal fasting state and during hyperinsulinemic clamps in overnight-fasted rats. We measured endogenous glucose production (EGP), total glucose output (TGO), the flux through glucose-6-phosphatase), glucose cycling (GC), index of flux through glucokinase = TGO – EGP), hepatic glucose 6-phosphate (G-6-P) content, and hepatic glucose-6-phosphatase and glucokinase activities. Plasma FFA levels were elevated about threefold by IH. In the basal state, IH increased TGO, in vivo glucose-6-phosphatase activity (TGO/G-6-P), and EGP (P < 0.001). During the clamp compared with the basal experiments, 2-h insulin infusion increased GC and in vivo glucokinase activity (GC/TGO; P < 0.05) and suppressed EGP (P < 0.05) but failed to significantly affect TGO and in vivo glucose-6-phosphatase activity. IH decreased the ability of insulin to increase GC and in vivo glucokinase activity (P < 0.01), and at 7 h, it also decreased the ability of insulin to suppress EGP (P < 0.001). G-6-P content was comparable in all groups. In vivo glucose-6-phosphatase and glucokinase activities did not correspond to their in vitro activities as determined in liver tissue, suggesting that stable changes in enzyme activity were not responsible for the FFA effects. The data suggest that, in overnight-fasted rats, FFA increased basal EGP and induced hepatic insulin resistance at different sites. 1) FFA increased basal EGP through an increase in TGO and in vivo glucose-6-phosphatase activity, presumably due to a stimulatory allosteric effect of fatty acyl-CoA on glucose-6-phosphatase. 2) FFA induced hepatic insulin resistance (decreased the ability of insulin to suppress EGP) through an impairment of insulin’s ability to increase GC and in vivo glucokinase activity, presumably due to an inhibitory allosteric effect of fatty acyl-CoA on glucokinase and/or an impairment in glucokinase translocation.

glucose-6-phosphatase; glucokinase; total glucose output; glucose cycling

THE ASSOCIATION among obesity, insulin resistance, and type 2 diabetes mellitus is well documented (see Refs. 8, 24, 31, and 34 for review). Free fatty acids (FFA) have been implicated as an important causative link among obesity, insulin resistance, and type 2 diabetes mellitus. An elevation of plasma FFA has been shown to impair insulin action and to be a risk factor for the development of type 2 diabetes (40).

In overnight-fasted (liver glycogen-depleted) rats, we have demonstrated that elevation of FFA, achieved by Intralipid + heparin (IH) infusion, increases endogenous glucose production (EGP) in the basal state and during hyperinsulinemic clamps and, after 7 h, induces hepatic insulin resistance (28). With the use of the overnight-fasted rat as our experimental model, we wished to study the effects of FFA on insulin resistance under conditions (stimulated gluconeogenesis and glycogen depletion) that are relevant to type 2 diabetes. The increase in basal EGP induced by FFA in our model was probably due to the fact that, after overnight fasting, glycogenolysis is limited by glycogen depletion (7, 17, 30) and may not further decrease to provide autoregulation of basal EGP in the presence of FFA-stimulated gluconeogenesis. We have also suggested that FFA induces hepatic insulin resistance through hepatic PKC-6 translocation (28). However, to this date, the sites of FFA action on basal EGP and on the suppression of EGP remain unclear.

A regulatory site that is potentially responsible for the FFA-induced increase in basal EGP and hepatic insulin resistance is the final common pathway of glucose production and uptake involving glucose-6-phosphatase (G-6-Pase) and glucokinase. G-6-Pase and glucokinase are the enzymes that catalyze the dephosphorylation of glucose 6-phosphate (G-6-P) (G-6-P → glucose) and the phosphorylation of glucose (glucose → G-6-P), respectively. Previous studies in rats have shown that a high-fat diet decreased hepatic glucokinase (39) and decreased (36) or did not change (39) G-6-Pase activity. However, a high-fat diet decreased the ability of insulin to suppress G-6-Pase activity (39). Total glucose output (TGO), which is the flux through G-6-Pase, and glucose cycling (GC), which is an index of flux through glucokinase, were not measured in these studies.

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At the molecular level, IH infusion increased G-6-Pase expression (33), and fatty acyl-CoA esters allosterically stimulated or inhibited G-6-Pase activity in vitro (37). A high-fat diet was found to inhibit glucokinase activity through allosteric binding in vitro (49). However, the physiological impact of these effects on EGP, TGO, and GC induced by fatty acids is not known.

In summary, no studies, to our knowledge, have investigated the time course effects of lipid infusion on EGP, TGO, GC, G-6-Pase, and glucokinase activity in the same experiments in vivo. Thus this study was designed to examine whether 1) an elevation of FFA obtained by IH infusion increases basal EGP and induces hepatic insulin resistance (evaluated by comparison of EGP between basal and clamp experiments) through alterations in TGO and/or GC, and 2) these alterations are progressive over time and associated with stable changes in G-6-Pase and glucokinase activity. Stable enzymatic changes, which are determined by the in vitro enzymatic activity assay, refer to the expression levels of the enzyme and any stable enzyme changes (i.e., covalent changes) affecting specific activity. Thus noncovalent allosteric and translocation effects on the enzyme would not be detected by the in vitro assay.

RESEARCH DESIGN AND METHODS

Animal models. Normal female Wistar rats (Charles River, QC, Canada), weighing 250–300 g, were used for experiments. Female Wistar rats were used to allow for future comparison of the effects of FFA with those on female Zucker diabetic fatty, or ZDF, rats, which are a convenient model of high-fat diet-induced diabetes (12). The rats were housed in the University of Toronto’s Department of Comparative Medicine. They were exposed to a 12:12-h light-dark cycle and were fed rat chow (Purina 5001, 4.5% fat; Ralston Purina, St. Louis, MO) and water ad libitum. The animal care committee of the University of Toronto approved all procedures.

Surgical procedures. After 3–5 days of adaptation to the facility, rats were anesthetized with ketamine-xylazine-acepromazine (100:1:0.5 mg/ml, 1 µl/g body wt), and indwelling catheters were inserted into the right internal jugular vein for infusion and the left carotid artery for sampling. Polyethylene catheters (PE-50; Cay Adams, Boston, MA), each extended with a segment of Silastic tubing (length 3 cm, ID 0.02 in.; Dow Corning, Midland, MI), were used. The venous catheter was extended to the level of the right atrium, and the arterial catheter was advanced to the level of the aortic arch. Both catheters were tunneled subcutaneously and exteriorized. The catheters were filled with a mixture of 60% polyvinylpyrrolidone and heparin (1,000 U/ml) to maintain patency and were closed at the end with a metal pin. The rats were allowed a minimum period of 3–4 days of postsurgery recovery before experiments.

Experimental design. The rats were overnight fasted and randomized to two groups, one of which received IH infusion (20% Intralipid + 20 U/ml heparin, 5.5 µl/min), while the other group was an (equimolar) saline (SAL)-treated control. Both IH- and SAL-treated rats were randomly assigned to different protocols, which varied in the duration of IH/SAL infusion and in the condition of the experimental determinations. The duration of IH or SAL infusion was 2 or 7 h, and experimental determinations were made in the basal fasting state or under conditions of a hyperinsulinemic euglycemic clamp.

For the basal protocols, IH or SAL was infused intravenously for 2 or 7 h through the jugular catheter. A 1:1 mixture of HPLC-purified [2-3H]- and [6-3H]glucose (40 µCi, bolus + 0.8 µCi/min, infusion) was infused through the jugular catheter for 2 h (in the 2-h protocols) or for the last 2 h of the 7-h experiments (in the 7-h protocols) to assess EGP, TGO, and GC. The clamp protocols were similar to basal protocols, but with the addition of an intravenous infusion of insulin resulting in plasma insulin levels in the postprandial range, which was given for 2 h (in the 2-h protocols) or for the last 2 h of the 7-h experiments (in the 7-h protocols). The rate of insulin infusion was 5 mU·kg⁻¹·min⁻¹ for the 2- or 7-h SAL clamp experiments, 4 mU·kg⁻¹·min⁻¹ for the 2-h IH clamp experiments, and 2.5 mU·kg⁻¹·min⁻¹ for the 7-h IH clamp experiments. Intravenous insulin infusion for IH clamp groups was given at a lower rate than for the SAL clamp groups to match the insulin levels between the groups. We have shown previously that, if the same insulin infusion is given at the same rate (5 mU·kg⁻¹·min⁻¹), IH infusion increases plasma insulin levels compared with SAL during hyperinsulinemic clamps (28). This is due to the impairing effect of IH on insulin clearance (51). To match the insulin levels with the corresponding SAL groups, the insulin infusion rate for the 7-h IH clamp (2.5 mU·kg⁻¹·min⁻¹) was lower than that for the 2-h IH clamp (4 mU·kg⁻¹·min⁻¹), because the increase in insulin levels induced by IH during hyperinsulinemic clamp is progressive over time (28).

To maintain euglycemia during insulin infusion, a variable infusion of 20% glucose was given intravenously through the jugular catheter and adjusted according to frequent glycemic determinations (every 5 min). The glucose infusion was radiolabeled with 15% Cig of [2-3H]- and [6-3H]glucose to avoid variability in plasma glucose specific activity due to changes in the rate of the cold glucose infuse. Steady-state conditions for [6-3H]- and [2-3H]glucose specific activities were achieved in the last 30 min of the 2-h and 7-h infusion experiments. The coefficient of variation for [6-3H]glucose specific activities was 7% (basal and clamp) and for [2-3H]glucose was 9% (basal) and 6.5% (clamp). Despite addition of the tracer to the glucose infuse, plasma glucose specific activity declined by ~50% in the clamp vs. basal experiments. As expected, this decrease did not affect calculation of glucose turnover in our steady-state model (unpublished data from our laboratory showing the same EGP results when specific activity was maintained constant in the basal state and during clamps). The EGP and metabolite results of the SAL experiments and some of the IH experiments have already been reported (28).

To control for the possible effects on EGP, TGO, and GC of the glycerol elevation generated by IH, a set of glycerol infusion experiments (5 mg·kg⁻¹·min⁻¹) was performed that matched the plasma glycerol levels obtained in the 7-h IH basal and 7-h IH clamp experiments. We chose to study the 7-h condition because gluconeogenesis from glycerol is much lower than that from glucose. The rate of insulin infusion is given at the same rate (5 mU·kg⁻¹·min⁻¹), and the increase in insulin levels induced by IH during hyperinsulinemic clamp is progressive over time (28).

Blood samples for FFA, triglyceride, glycerol, glucose, C-peptide, insulin, and [2-3H]- and [6-3H]glucose specific activities were taken in the last 30 min (every 10 min) of each experiment. The total blood volume withdrawn was ~3.3 ml during the basal experiments and ~3.8 ml during the clamp experiments. After plasma separation, red blood cells diluted 1:1 in heparinized saline (4 U/ml) were reinfused into the rats. At the end of the experiments, the rats were anesthetized with intra-arterial pentobarbital sodium, and the liver
was freeze-clamped with precooled aluminum tongs within 45 s of anesthetic injection while infusions were maintained through the jugular vein. The liver samples were assayed for G-6-P content and in vitro G-6-Pase and glucokinase activity. 

**Laboratory methods.** Plasma glucose was measured on a Beckman Glucose Analyzer II (Beckman, Fullerton, CA). Separation of [2-3H]- and [6-3H]glucose was achieved by dimedone precipitation (15). In brief, plasma radioactivity from [2-3H]- and [6-3H]glucose was determined after deproteinization with Ba(OH)₂ and ZnSO₄, passage through ion exchange columns, and subsequent evaporation. The radioactivity of [6-3H]glucose was determined in an aliquot of the eluate as described by Dunn et al. (14). In all rats studied, the recovery for [6-3H]glucose by the dimedone procedure was quite reproducible and ~95%. The radioactivity of [2-3H]glucose was calculated as the difference between the total radioactivity and that of [6-3H]glucose (corrected for recovery). Aliquots of the infusate tracer mixture and of the radiolabeled glucose infusate were run in parallel with the plasma samples through the dimedone procedure. The intra-assay coefficient of variation was 2.5%, and the interassay coefficients varied 6.5%. Buffer [2-3H]-glucose was centrifuged at 10,000 g at 4°C, and plasma were determined by radioimmunoassays with kits specific for rat insulin (with 100% cross-reactivity with porcine insulin used for infusions) and C-peptide (Linco Research, St. Charles, MO). The interassay coefficients of variation were <9 and 10.5% from insulin and C-peptide, respectively. Plasma FFA levels were measured using a colorimetric kit from Wako Industrial (Osaka, Japan). Plasma triglyceride levels and glycerol levels were also measured using colorimetric kits from Boehringer Mannheim (Mannheim, Germany). Hepatic G-6-P levels were determined by a spectrophotometric method as previously described (37a).

**In vitro G-6-Pase activity.** The G-6-Pase activity assay allows for determination of the in vitro G-6-Pase activity (expression of G-6-Pase + stable enzyme changes affecting specific activity). Thus noncovalent allosteric effects on G-6-Pase would not be detected by this assay. The liver (~100 mg) was homogenized by hand-held homogenizer (20 times) in 50 mM Tris·HCl at 4°C, pH 7.1. The homogenate was then passed through a 23-gauge needle syringe (5 times) to ensure that the sample was well mixed. G-6-Pase activity (Vₘₐₓ) was determined in homogenates of liver samples with 50 mM [U-14C]G-6-P at 30°C, as described by Kitcher et al. (26) and van de Werve (50). One unit of enzyme activity is defined as the amount of enzyme protein that is required to synthesize 1 mole of [U-14C]glucose from 1 mole of [U-14C]G-6-P in the conditions of the assay. The Bio-Rad protein assay method was used to measure protein concentration.

**In vitro glucokinase activity.** The glucokinase activity assay allows for determination of the in vitro glucokinase activity (expression level of glucokinase + stable enzyme changes affecting specific activity). Thus noncovalent allosteric effects on glucokinase and the effects on glucokinase translocation would not be detected by this assay. Glucokinase activity was determined by the method described by Bontemps et al. (9) with ~100 mg of liver tissue. The liver was homogenized by hand-held homogenizer (20 times) in buffer A (in mM: 50 triethanolamine, 5 MgCl₂, 1 DTT, and 5 EDTA, pH adjusted to 7.5). The homogenate was then passed through a 23-gauge needle syringe (5 times), to ensure that the sample was well mixed, and was subsequently centrifuged at 10,000 g at 4°C. The supernatant was saved for the activity assay. The spectrophotometric assay of glucose-phosphorylating activity was performed at two glucose concentrations: at 100 mM (measures all hexokinases including glucokinase) and at 0.5 mM (measures only the low-Kₘ hexokinases). The difference between the two assays gives glucokinase activity. One unit is the amount of enzyme that catalyzes the formation of 1 μmol of substrate/min, in the conditions of the assay. The Bio-Rad protein assay method was used to measure protein concentration.

**Calculations.** Glucose turnover [rate of appearance (Ra) of glucose determined with [6-3H]glucose] was calculated using steady-state formulas (49), taking into account the extra tracer infused with the glucose infusate (16). In the basal state, the total glucose Ra corresponds to the EGP. During the clamps, EGP was calculated by subtracting the exogenous glucose infusion rate from the Ra measured with [6-3H]glucose. TGO was determined by subtracting the rates of glucose infusion from the tracer-determining Ra measured with [2-3H]glucose. GC was calculated as the difference between TGO and EGP. In vivo G-6-Pase activity = TGO/hepatic G-6-P content, and under conditions of net glucose production, in vivo glucokinase activity = GC/hepatic glucose content, and under conditions of net glucose production, in vivo glucokinase activity = GC/hepatic glucose content.
Table 1. Plasma levels of FFA, triglycerides, glycerol, glucose, C-peptide, and insulin

<table>
<thead>
<tr>
<th></th>
<th>2-h SAL</th>
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<th>2-h IH</th>
<th></th>
<th>7-h SAL</th>
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<th>7-h IH</th>
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<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>Clamp</td>
<td>Basal</td>
<td>Clamp</td>
<td>Basal</td>
<td>Clamp</td>
<td>Basal</td>
<td>Clamp</td>
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<tr>
<td></td>
<td>(n = 7)</td>
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<td>(n = 7)</td>
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<td>(n = 7)</td>
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<td>(n = 7)</td>
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</tr>
<tr>
<td>FFA, meq/l</td>
<td>0.50 ± 0.07</td>
<td>0.20 ± 0.02</td>
<td>1.79 ± 0.23*</td>
<td>1.03 ± 0.15*</td>
<td>0.48 ± 0.04</td>
<td>0.17 ± 0.05</td>
<td>1.31 ± 0.13*</td>
<td>1.19 ± 0.12*</td>
</tr>
<tr>
<td>Triglycerides, mM</td>
<td>0.22 ± 0.03</td>
<td>0.05 ± 0.02</td>
<td>1.08 ± 0.15*</td>
<td>0.70 ± 0.11*</td>
<td>0.24 ± 0.03</td>
<td>0.05 ± 0.01</td>
<td>0.83 ± 0.22*</td>
<td>0.73 ± 0.04*</td>
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<tr>
<td>Glycerol, μM</td>
<td>233 ± 26</td>
<td>58 ± 10</td>
<td>811 ± 67*</td>
<td>511 ± 45*</td>
<td>135 ± 25</td>
<td>94 ± 26</td>
<td>717 ± 72*</td>
<td>529 ± 26*</td>
</tr>
<tr>
<td>Glucose, mM</td>
<td>6.5 ± 0.3</td>
<td>6.6 ± 0.2</td>
<td>7.4 ± 0.3*</td>
<td>6.7 ± 0.2</td>
<td>6.7 ± 0.3</td>
<td>6.2 ± 0.1</td>
<td>7.3 ± 0.2*</td>
<td>6.7 ± 0.4</td>
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<tr>
<td>C-peptide, nM</td>
<td>0.23 ± 0.03</td>
<td>0.027 ± 0.004</td>
<td>0.85 ± 0.16*</td>
<td>0.028 ± 0.003</td>
<td>0.28 ± 0.05</td>
<td>0.030 ± 0.003</td>
<td>0.60 ± 0.13*</td>
<td>0.080 ± 0.047</td>
</tr>
<tr>
<td>Insulin, pM</td>
<td>55 ± 6</td>
<td>527 ± 61</td>
<td>95 ± 7*</td>
<td>541 ± 54</td>
<td>89 ± 8</td>
<td>560 ± 67</td>
<td>147 ± 16*</td>
<td>615 ± 35</td>
</tr>
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</table>

Data are means ± SE and refer to the last 30 min of each experiment. Basal, basal fasting state; Clamp, hyperinsulinemic euglycemic clamp. SAL, saline; IH, intralipid + heparin; FFA, free fatty acid. *P < 0.001, IH vs. SAL.

(P = 0.07). During hyperinsulinemic clamps, IH increased EGP at 2 h (P < 0.001) and 7 h (P < 0.001; Fig. 1). Two-hour insulin infusion in SAL experiments suppressed EGP by ~55% (P < 0.05). Two-hour IH infusion tended to decrease the insulin-induced suppression of EGP (Basal vs. Clamp), but the effect was not significant (P = 0.13). Seven-hour IH markedly decreased the ability of insulin to suppress EGP (P < 0.05; Fig. 1), indicating hepatic insulin resistance.

IH increased basal TGO at both 2 h (P < 0.001) and 7 h (P < 0.001; Fig. 2). The effect of IH on TGO was greater after 7-h than after 2-h infusion (P < 0.001; Fig. 2). During hyperinsulinemic clamps, IH increased TGO at 2 h (P < 0.001) and 7 h (P < 0.001; Fig. 2). Two-hour insulin infusion tended to suppress TGO, but the effect was not significant in either SAL or IH experiments (Fig. 2).

IH did not affect basal GC after 2-h infusion but tended to increase basal GC after 7-h infusion (7-h SAL = 1.4 ± 0.7 μmol·kg⁻¹·min⁻¹, 7-h IH = 6.9 ± 3.7; SAL vs. IH P = 0.10; Fig. 3). During the clamps, 2-h insulin infusion increased GC (P < 0.05; Fig. 3). IH infusion decreased insulin’s ability to increase GC after 2 h (P < 0.01) and 7 h (P < 0.01; Fig. 3).

The rats infused with glycerol to match the glycerol levels obtained with IH had EGP, TGO, and GC values similar to those of the SAL-treated rats (Table 2). As a result, the effects on EGP, TGO, and GC induced by IH infusion can be attributed mainly to FFA. The EGP data have already been reported (28).

Because EGP = TGO – GC, IH increased basal EGP through increased TGO (flux through G-6-Pase), because IH did not decrease basal GC. During the clamps, insulin decreased EGP mainly through an increase in GC. IH induced hepatic insulin resistance (decreased the ability of insulin to suppress EGP) by impairing the ability of insulin to increase GC. TGO did not play an important role in IH-induced hepatic insulin resistance because, in the current experimental model, insulin failed to suppress TGO.

Hepatic G-6-P levels tended to be slightly lower than in 5-h-fasted rats (32) and were comparable in all
groups (Table 3), although 7-h IH infusion tended to decrease G-6-P content in both the basal \( (P = 0.35) \) and clamp \( (P = 0.12) \) conditions (Table 3). As expected, hepatic G-6-Pase activity was higher in the current overnight-fasted model than in the 5-h-fasted model (32). During the basal state, there was a slight decrease in in vitro G-6-Pase activity after 2-h IH \( (P = 0.14; \text{Table 3}) \). This effect became significant after 7-h IH (Table 3). During the hyperinsulinemic clamps, insulin and IH had no effect on in vitro G-6-Pase

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**Fig. 2.** Total glucose output (TGO). Data are means ± SE and refer to the last 30 min of each experiment. *\( P < 0.001 \), IH vs. SAL; *\( P < 0.001 \), effect of IH was greater in 7-h Basal than in 2-h Basal.

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**Fig. 3.** Glucose cycling (GC). Data are means ± SE and refer to the last 30 min of each experiment. *\( P < 0.05 \), Clamp vs. Basal. *\( P < 0.01 \), hepatic insulin action (Basal vs. Clamp) on GC was impaired by IH.
activity (Table 3). Also as expected, hepatic glucokinase activity was lower in the current overnight-fasted model than in the 5-h-fasted model (32). Although 2-h insulin tended to increase in vitro glucokinase activity, the effect was not significant \( (P = 0.19; \text{Table 3}) \). In vitro glucokinase activity was not affected by 2-h IH infusion either in basal or clamp experiments (Table 3). However, in vitro glucokinase activity was suppressed by 7-h IH infusion in both basal \( (P < 0.05) \) and clamp \( (P < 0.05) \) experiments (Table 3). Thus in vitro activities of G-6-Pase and glucokinase did not correspond to the in vivo determined flux through the two enzymes. Next, in vivo activities of the two enzymes were calculated as reported in Research Design and Methods.

Similar to TGO, IH increased basal in vivo G-6-Pase activity (TGO/G-6-P) at 2 h \( (P < 0.001) \) and 7 h \( (P < 0.001; \text{Table 4}) \). The effect was greater at 7 h than at 2 h \( (P < 0.001) \). Also similar to TGO, during the hyperinsulinemic clamps, insulin clamp experiments did not have significant effects on in vivo G-6-Pase activity. Similar to GC, IH did not affect basal in vivo glucokinase activity (GC/TGO) after 2 h but had a tendency to increase in vivo glucokinase after 7-h infusion \( (P = 0.085; \text{Table 4}) \). Also similar to GC, during the hyperinsulinemic clamps, insulin infusion increased in vivo glucokinase activity after 2-h \( (P < 0.05) \) and 7-h \( (P < 0.05) \) SAL infusion (Table 4). IH impaired the ability of insulin to stimulate in vivo glucokinase activity at 2 h \( (P < 0.01) \) and 7 h \( (P < 0.01; \text{Table 4}) \).

**DISCUSSION**

In this study, we have demonstrated that, in overnight-fasted rats, short-term elevation of FFA 1) increases basal EGP through an increase in TGO (flux through G-6-Pase) and 2) induces hepatic insulin resistance through an impairment of insulin stimulation of GC (index of flux through glucokinase). IH elevated basal plasma FFA to levels that were above the physiological range but within the FFA elevation seen in uncontrolled diabetes. The FFA levels in the clamps were lower than the basal FFA levels, a situation consistent with the antilipolytic and FFA reesterification effects of insulin (10). The metabolic conditions of overnight fasting in rats are associated with stimulated gluconeogenesis and glycogen depletion, and thus they are similar to those observed in diabetes.

**Basal state.** FFA increased TGO, as previously shown in isolated hepatocytes (23), and this effect increased over time. The increase in TGO was responsible for the increase in EGP because FFA did not affect basal GC significantly, as previously shown by Clore et al. in humans (11). TGO is defined as the content of G-6-P times in vivo G-6-Pase activity. Thus the increase of TGO in the basal state could be attributed to an increase in G-6-P content and/or in vivo G-6-Pase activity. The liver G-6-P content, in our experiments, was comparable in all groups, although 7-h IH infusion tended to decrease G-6-P content. This was consistent with previous studies in which palmitate (6) and oleate (23) were found to have no significant effects on G-6-P level in hepatocytes isolated from 24-h-fasted rats. The lack of significant effects of FFA on G-6-P level suggested that the increase in TGO induced by FFA was due largely to increased in vivo G-6-Pase activity. In fact, we have calculated the in vivo G-6-Pase activity, and IH was found to increase in vivo G-6-Pase activity at 2 h and at 7 h, and the effect was progressive over time.

Contrary to the in vivo G-6-Pase activity, the in vitro-determined activity of G-6-Pase activity was not increased by FFA in our model, which underlies the discrepancy between the in vivo and in vitro enzymatic measurements. The G-6-Pase assay that was used in the present study allows for determination of the in vitro glucokinase activities.

### Table 2. **Effect of glycerol infusion on EGP, TGO, and GC**

<table>
<thead>
<tr>
<th>Activity</th>
<th>7-h SAL</th>
<th>7-h IH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glycerol, μM</strong></td>
<td>Basal (n = 7)</td>
<td>Clamp (n = 5)</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Glycerol, μM</td>
<td>135 ± 35</td>
<td>94 ± 26</td>
</tr>
<tr>
<td>EGP, μmol·kg⁻¹·min⁻¹</td>
<td>44 ± 5</td>
<td>21 ± 5</td>
</tr>
<tr>
<td>TGO, μmol·kg⁻¹·min⁻¹</td>
<td>45 ± 5</td>
<td>41 ± 11</td>
</tr>
<tr>
<td>G6Pase activity (TGO/G-6-P)</td>
<td>1.5 ± 0.7</td>
<td>17 ± 8</td>
</tr>
</tbody>
</table>

Data are means ± SE. EGP, endogenous glucose production; TGO, total glucose output; GC, glucose cycling. *P < 0.001, IH or glycerol vs. SAL.

### Table 3. **Hepatic G-6-P content and in vitro hepatic G-6-Pase and glucokinase activities**

<table>
<thead>
<tr>
<th>Activity</th>
<th>2-h SAL</th>
<th>2-h IH</th>
<th>7-h SAL</th>
<th>7-h IH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal (n = 7)</td>
<td>Clamp (n = 6)</td>
<td>Basal (n = 7)</td>
<td>Clamp (n = 6)</td>
</tr>
<tr>
<td>G-6-P, nmol/mg protein</td>
<td>0.75 ± 0.09</td>
<td>1.07 ± 0.11</td>
<td>0.83 ± 0.01</td>
<td>1.28 ± 0.32</td>
</tr>
<tr>
<td>In vitro G-6-Pase activity, mU/mg protein</td>
<td>43 ± 7</td>
<td>37 ± 4</td>
<td>30 ± 2</td>
<td>57 ± 18</td>
</tr>
<tr>
<td>In vitro glucokinase activity, mU/mg protein</td>
<td>1.4 ± 0.2</td>
<td>2.0 ± 0.7</td>
<td>2.5 ± 0.7</td>
<td>1.6 ± 0.3</td>
</tr>
</tbody>
</table>

Data are means ± SE. G-6-P, glucose 6-phosphate; G-6-Pase, glucose-6-phosphatase. *P < 0.05, IH vs. SAL.

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Such effect became significant G-6-Pase changes affecting specific sites of G-6-Pase activity. These changes could have been responsible for the induction of TGO.

Preferential stimulation of G-6-Pase increased G-6-Pase mRNA and protein levels. The effect of FFA on in vitro G-6-Pase activity (37), could have contributed to the induction of TGO.

In vivo G-6-Pase activity, μmol·kg⁻¹·min⁻¹ per μmol/mg protein

<table>
<thead>
<tr>
<th></th>
<th>Basal (n = 7)</th>
<th>Clamp (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-h SAL</td>
<td>64 ± 7</td>
<td>46 ± 9</td>
</tr>
<tr>
<td>2-h IH</td>
<td>82 ± 9*</td>
<td>61 ± 14</td>
</tr>
<tr>
<td>7-h SAL</td>
<td>69 ± 20</td>
<td>39 ± 11</td>
</tr>
<tr>
<td>7-h IH</td>
<td>140 ± 20†</td>
<td>113 ± 10‡</td>
</tr>
</tbody>
</table>

Data are means ± SE. *P < 0.001, IH vs. SAL. †P < 0.001, effect of IH was greater in 7-h Basal vs. 2-h Basal. §P < 0.05, Clamp vs. Basal. §P < 0.01, hepatic insulin action on in vivo glucokinase activity was impaired by IH.

In vitro G-6-Pase activity (expression of G-6-Pase + stable enzyme changes affecting specific activity) and does not detect allosteric effects on G-6-Pase. This suggests that an increased in vivo G-6-Pase activity through the allosteric stimulation of fatty acyl-CoA on G-6-Pase, as previously shown in vitro (37), could have contributed to the induction of TGO.

Massillon et al. (33) showed that IH infusion increased G-6-Pase mRNA and protein levels. The effect of insulin was not addressed in that study (33), because basal insulin levels were maintained with somatostatin and insulin infusion. This stimulation of G-6-Pase expression could have been responsible for the increased in vivo G-6-Pase activity induced by IH in the current study. However, in the present study (with no pancreatic clamp), IH not only failed to increase in vitro G-6-Pase activity (expression of G-6-Pase + stable G-6-Pase changes affecting specific activity), but tended to decrease in vitro G-6-Pase activity at 2 h. Such effect became significant after 7-h IH infusion. In basal experiments with no pancreatic clamp (similar to the current study), Oakes et al. (39) and Garg et al. (19) showed that a high-fat diet did not affect in vitro G-6-Pase activity (19). High-fat diet increased insulin levels in the study of Oakes et al., and the insulin levels were not measured in the study by Garg et al. Thus, in the current study and that by Oakes et al., the slight but prolonged elevation of basal insulin induced by IH and high-fat diet could have prevented detection of increased in vitro G-6-Pase activity, since insulin suppresses G-6-Pase expression (3, 29). Alternatively, or in addition, IH, despite increasing G-6-Pase expression, may decrease in vitro G-6-Pase activity, as suggested by the tendency of IH to decrease G-6-Pase activity at 2 h and by the results of other studies (36, 41). Notably, there is a discrepancy between previous findings of the effect of FFA on in vitro-determined G-6-Pase expression and activity.

IH did not affect in vitro glucokinase activity (expression level of glucokinase plus stable enzyme changes affecting specific activity) after 2 h but significantly decreased glucokinase activity after 7 h. The inhibitory effects of IH on in vitro glucokinase activity is in accordance with previous studies in which high-fat diet was found to decrease glucokinase mRNA and activity at weaning in rats (22) and inhibit glucokinase gene expression in lean rats (25). However, the IH-induced decrease in in vitro glucokinase activity did not correspond to a decrease in in vivo glucokinase activity or GC. The fact that GC or in vivo glucokinase activity did not decrease indicates that 1) the rate-limiting step for in vivo glucokinase activity is not glucokinase expression level, and 2) IH increased basal EGP at the site of G-6-Pase and not glucokinase.

Because IH increased plasma glucose and insulin levels and basal EGP, it is likely that hepatic insulin resistance may have contributed to the FFA-induced increase in EGP in the basal state, as suggested by our recent findings of FFA-induced PKC-δ translocation (28). We did not evaluate hepatic insulin resistance at basal insulin levels; therefore, we cannot quantify the contribution of insulin resistance to the increase of basal EGP or exclude that FFA elevated in vivo G-6-Pase activity by impairing noncovalent suppressive effects of basal insulin on G-6-Pase (47), in addition to having a direct allosteric effect on the enzyme. Both hepatic insulin resistance and the direct allosteric effect of FFA on G-6-Pase might have contributed to the lack of autoregulation of basal EGP in our model, in which gluconeogenesis was driven by increased hepatic FFA oxidation, as suggested by our findings of increased hepatic citrate content (28).

**Hyperinsulinemic clamp.** Previous studies in dogs and humans have shown that, during hyperinsulinemic euglycemic clamps, there is still net hepatic glucose production under a condition of ~50% (as in the present study), or even greater suppression of EGP (1, 13, 46). Thus under a condition of net hepatic glucose production (basal state and low-dose hyperinsulinemic euglycemic clamp, as in the present study), glucokinase is the rate-limiting factor for GC, as suggested by previous studies (15, 38).

EGP was suppressed through an insulin-induced increase in GC and in vivo glucokinase activity in the SAL infusion groups. Previous studies in humans and dogs (4, 20, 42) have shown that 2-h or 3-h hyperinsulinemic euglycemic clamps did not affect or modestly increased GC when expressed in absolute terms but increased GC when expressed as a percentage of TGO (defined as in vivo glucokinase activity in our study). In 5-h fasted rats, 2-h insulin infusion (~400 pM) did not appear to increase GC above basal levels (43, 44). Furthermore, a 2-h insulin infusion at a high rate (~2,300 pM) even decreased GC (3). The difference in observations may be due to a difference in duration of fasting. In overnight-fasted rats, the in vivo glucokinase...
nase activity may be more sensitive to insulin because of liver glycogen depletion. For example, it is well known that, after exercise, the sensitivity of liver glucose uptake to insulin is increased, possibly due to glycogen depletion (27). Thus, in the current study, GC (index of flux through glucokinase) may have become the rate-limiting site for insulin-induced suppression of EGP. On the other hand, in 5-h-fasted rats in which liver glycogen is not depleted, 2-h insulin infusion (~2,300 pM) suppresses EGP through a decrease in TGO (3). The fact that TGO is a rate-limiting site for insulin-induced EGP suppression in 5-h-fasted rats was also supported by a study conducted by Oakes et al. (39), in which 2-h insulin infusion (~550 pM) decreased G-6-Pase activity.

In the present study, 2-h insulin infusion achieving insulin levels in the postprandial range did not significantly affect TGO, G-6-P content, in vitro G-6-Pase, and glucokinase activity, although there was a tendency for in vitro glucokinase activity to increase during the hyperinsulinemic clamps. This is in accord with previous studies, in which acute insulin infusion had no effect on in vitro G-6-Pase activity (18, 35, 36, 45) and glucokinase activity (36, 45). However, as discussed above, insulin infusion decreased G-6-Pase activity in a study conducted in 5-h-fasted rats (39). Also, in the high-dose insulin clamp study conducted by Barzilai and Rossetti (3), insulin infusion suppressed EGP through increased glucokinase activity, decreased G-6-Pase activity, and G-6-P content. The discrepancy in observations was probably due to 1) a difference in experimental model (5-h-fasted rats instead of overnight-fasted rats) and/or 2) a difference in insulin levels, as insulin levels were maintained at 2,300 pM in the study of Barzilai and Rossetti compared with ~550 pM in our study.

Two-hour and seven-hour FFA elevations increased EGP and TGO during the hyperinsulinemic clamps. FFA tended to decrease the insulin’s ability to suppress EGP (comparison between basal and clamp) after 2 h, and the effect became significant after 7-h infusion (hepatic insulin resistance). Insulin infusion did not have any significant effect on TGO in either SAL or IH experiments in our model.

Two-hour FFA elevation decreased insulin’s ability to stimulate GC (Basal vs. Clamp). Two-hour FFA elevation was also found to decrease the ability of insulin to increase in vivo glucokinase activity (GC/TGO). The in vitro glucokinase activity did not reflect the changes of in vivo glucokinase activity induced by 2-h elevation of FFA, indicating that an impairment in insulin-stimulated glucokinase translocation and/or allosteric inhibitory effect on glucokinase could be responsible for the FFA-induced impairment of insulin’s stimulation on GC. This is because translocation and allosteric effects on glucokinase are not detected in vitro glucokinase activity assays. Indeed, it is tempting to speculate that FFA decreased insulin’s stimulation on in vivo glucokinase activity through an impairment of insulin action on glucokinase translocation, because insulin potentiated glucose-induced glucokinase translocation (2), and palmitate partially counteracted glucose-induced glucokinase translocation in hepatocytes (2). The postulated effect of FFA on in vivo glucokinase activity could be direct and/or mediated through impairment of hepatic insulin signaling (possibly through PKC-δ) (28).

Similar to 2-h FFA elevation, 7-h FFA elevation decreased insulin’s ability to stimulate GC through an impairment in in vivo glucokinase activity. This led to hepatic insulin resistance (inability of insulin to suppress EGP). In addition to the possible translocation and allosteric effects on glucokinase, a decrease in glucokinase gene expression (reflected by decreased in vitro glucokinase activity) could be responsible for insulin’s decreased ability to stimulate in vivo glucokinase activity and suppress EGP after 7-h FFA elevation.

In the current study, we have shown that FFA induced hepatic insulin resistance at the level of glucokinase, not G-6-Pase, in overnight-fasted rats. This is in contrast with a previous, 5-h-fasted rat study (39), in which high-fat diet was shown to induce hepatic insulin resistance through an impairment of insulin’s ability to suppress G-6-Pase. The following three differences could explain such a discrepancy. 1) The first difference is in the duration of fasting. In the current model with overnight-fasted/glycogen-depleted rats, glucokinase (instead of G-6-Pase) was the regulatory site for insulin-induced suppression of EGP. Thus FFA induced hepatic insulin resistance at the level of glucokinase. In contrast, G-6-Pase (instead of glucokinase) was the regulatory site for insulin-induced suppression of EGP in 5-h-fasted rats (39). Thus G-6-Pase became the site where high-fat diet impaired hepatic insulin action. 2) The second difference is in duration of treatments. IH was infused for 2 and 7 h in the current model, and high-fat diet was maintained for 3 wk in the study by Oakes et al. (39), thus possibly allowing FFA effect on G-6-Pase gene expression to become manifested (39). 3) The third difference is in FFA levels. In the current study, FFA levels were elevated about threefold via IH infusion. This is in contrast to the study conducted by Oakes et al., in which fasting FFA levels were not significantly elevated.

In summary, we have shown that, in overnight-fasted rats, an elevation of FFA of <7-h duration increased basal EGP and induced hepatic insulin resistance at different sites. FFA increased basal EGP through an increase in TGO and in vivo G-6-Pase activity, presumably due to a stimulatory allosteric effect of fatty acyl-CoA on G-6-Pase. FFA induced hepatic insulin resistance through an impairment of insulin’s ability to stimulate GC and in vivo glucokinase activity. This impairment could be due to an allosteric inhibitory effect of fatty acyl-CoA on glucokinase and/or an impairment in glucokinase translocation.

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