Free fatty acid-induced hepatic insulin resistance: a potential role for protein kinase C-δ

TONY K. T. LAM,1 HIDENORI YOSHII,1 C. ANDREW HABER,1 ELENA BOGDANOVIC,1 LORETTA LAM,1 I. GEORGE FANTUS,1,2 AND ADRIA GIACCA1,2

Departments of 1Physiology and 2Medicine, University of Toronto, Toronto M5S 1A8, Canada

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Lam, Tony K. T., Hidenori Yoshii, C. Andrew Haber, Elena Bogdanovic, Loretta Lam, I. George Fantus, and Adria Giacca. Free fatty acid-induced hepatic insulin resistance: a potential role for protein kinase C-δ. Am J Physiol Endocrinol Metab 283: E682–E691, 2002; 10.1152/ajpendo.00038.2002.—The mechanisms of the impairment in hepatic glucose metabolism induced by free fatty acids (FFAs) and the importance of FFA oxidation in these mechanisms remain unclear. FFA-induced peripheral insulin resistance has been linked to membrane translocation of novel protein kinase C (PKC) isoforms, but the role of PKC in hepatic insulin resistance has not been assessed. To investigate the biochemical pathways that are induced by FFA in the liver and their relation to glucose metabolism in vivo, we determined endogenous glucose production (EGP), the hepatic content of citrate (product of acetyl-CoA derived from FFA oxidation and oxaloacetate), and hepatic PKC isoform translocation after 2 and 7 h Intralipid + heparin (IH) or SAL in rats. Experiments were performed in the basal state and during hyperinsulinemic clamps (insulin infusion rate, 5 mU·kg⁻¹·min⁻¹). IH increased EGP in the basal state (P < 0.001) and during hyperinsulinemia (P < 0.001) at 2 and 7 h. Also, 7-h infusion of IH induced resistance to the suppressive effect of insulin on EGP (P < 0.05). Glycerol infusion (resulting in plasma glycerol levels similar to IH infusion) did not have any effect on EGP. IH increased hepatic citrate content by twofold, independent of the insulin levels and the duration of IH infusion. IH induced hepatic PKC-δ translocation from the cytosolic to membrane fraction in all groups. PKC-δ translocation was greater at 7 compared with 2 h (P < 0.05). In conclusion, 1) increased FFA oxidation may contribute to the FFA-induced increase in EGP in the basal state and during hyperinsulinemia but is not associated with FFA-induced hepatic insulin resistance, and 2) the progressive insulin resistance induced by FFA in the liver is associated with a progressive increase in hepatic PKC-δ translocation.

hepatic glucose production; free fatty acid oxidation; hyperinsulinemic-euglycemic clamp

THE ASSOCIATION between obesity, insulin resistance, and type 2 diabetes mellitus is well documented (for review, see Refs. 4, 22, 25, 28, and 32). Free fatty acids (FFA) have been implicated as an important causative link in this association. 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 (38). A number of groups have investigated the mechanisms that underlie the FFA-induced impairment of glucose metabolism in muscle (5, 15, 19, 20, 23, 37, 39, 43, 46), but little is known about the mechanisms of the FFA-induced impairment of glucose metabolism in the liver (30, 37).

In the liver, FFAs increase gluconeogenesis both in vitro and in vivo (4, 11, 54), and a large number of studies have demonstrated that Intralipid + heparin (IH) increases endogenous glucose production (EGP) during euglycemic clamps (3, 5, 29, 42, 45, 47, 51). However, the time course of this increase in EGP is not known. Intralipid has a high content of free glycerol, and both glycerol and FFA are released from the triglycerides of Intralipid. In most studies, because glycerol was not infused as a control, it cannot be excluded that the effect of IH on EGP was due, at least in part, to the effect of glycerol, as glycerol is a gluconeogenic precursor.

Randle et al. (41) have shown that FFAs compete with glucose for substrate oxidation (this has been termed the glucose-fatty acid cycle) in isolated rat heart muscle and diaphragm. With regard to the mechanism of the FFA effect on hepatic glucose metabolism, a Randle-like mechanism has been invoked to explain the FFA-induced stimulation of gluconeogenesis, i.e., acetyl-CoA derived from FFA oxidation allosterically activates pyruvate carboxylase, and NADH, also a product of FFA oxidation, is used for the formation of glyceraldehyde 3-phosphate from 1,3-bisphosphoglycerate. Additionally, citrate-induced inhibition of phosphofructokinase-1 was observed in the perfused rat liver and in isolated hepatocytes exposed to FFA (31, 35, 52). However, the finding that hepatic insulin resistance in high-fat-fed rats was not ameliorated by etomoxir (37), an inhibitor of fatty acid oxidation, suggests that other mechanisms are likely involved. FFA-induced protein kinase C (PKC) activation has been investigated as a potential mechanism responsible for the FFA-induced insulin resistance in muscle (19); however, the role of PKC in FFA-induced hepatic insulin resistance is not known. It has been observed...
that in hepatocytes, oleic acid promotes translocation of PKC from the cytosol to the plasma membrane (14).

The present study was performed 1) to examine the time course of the effect of FFA on EGP and 2) to determine whether the postulated FFA-induced increase in EGP is associated with evidence of increased FFA oxidation and/or PKC activation.

**RESEARCH DESIGN AND METHODS**

**Animal models.** Normal female Wistar rats (Charles River, St-Constant, 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 rats. The latter are a convenient model of high-fat diet-induced diabetes (13). The rats were housed in the Univ. of Toronto Dept. of Comparative Medicine. They were exposed to a 12:12-h light-dark cycle and were fed rat chow (Purina no. 5001, 4.5% fat; Ralston Purina, St. Louis, MO) and water ad libitum. The Animal Care Committee of the Univ. of Toronto approved all procedures.

**Surgical procedures.** After 3–5 days of adaptation to the facility, rats were anesthetized with ketamine-xylazine-acetromazine (100:0:1:0.5 mg/ml, 1 μg body wt), and indwelling catheters were inserted into the right internal jugular vein for infusions and the left carotid artery for sampling. Polyethylene catheters (PE-50; Clay Adams, Becton Dickinson, Sparks, MD), each extended with a segment of Silastic tubing (length of 3 cm, internal diameter of 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 3- to 4-day period of postsurgery recovery before experiments.

**Experimental design.** The rats were fasted overnight 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 a saline (SAL; equivalence)-treated control. Both IH- and SAL-treated rats were randomly assigned to different protocols that varied in the duration of IH/SAL infusion and in the conditions of the experimental determinations. The duration of IH and SAL was 2 and 7 h, and experimental determinations were made in the basal fasting state and during hyperinsulinemic-euglycemic clamp.

For the 2-h IH basal and the 2-h SAL basal protocols, IH or SAL was infused intravenously for 2 h through the jugular catheter together with [6-3H]glucose (20 μCi, bolus + 0.4 μCi/min infusion) to assess the metabolic clearance rate (MCR) of glucose and EGP. The 2-h SAL clamp and the 2-h IH clamp protocols were similar to the 2-h SAL basal and the 2-h IH basal protocols, with the addition of an intravenous infusion of insulin (5 mU·kg⁻¹·min⁻¹), resulting in plasma insulin levels in the postprandial range. 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 determination (every 5 min). The glucose infusate was radiolabeled with 15 μCi/g [6-3H]glucose to avoid variability in plasma glucose specific activity due to changes in the rate of the cold glucose infusate.

For the 7-h IH basal and 7-h SAL basal protocols, IH or SAL was infused throughout the experiments. [6-3H]glucose was given during the last 2 h of the experiments. The 7-h SAL clamp and 7-h IH clamp protocols were similar to the 7-h IH basal and 7-h SAL basal protocols, with the additional infusion of insulin (5 mU·kg⁻¹·min⁻¹) and radiolabeled glucose (15 μCi/g) during the last 2 h of the experiments.

Because the insulin levels were higher in the 7-h IH clamp protocol than in the 2-h IH or 7-h SAL clamp protocol (see RESULTS), with the same insulin infusion rate, an additional set of experiments (7-h IH clamp (1/2 Ins)) was performed with insulin infused at one-half the original rate (2.5 mU·kg⁻¹·min⁻¹) to match the plasma insulin levels. In this way, the effects of FFAs on EGP could be more accurately compared.

In addition, to control for possible effects on EGP of the glycerol elevation generated by IH, a set of glycerol infusion experiments (5 mg·kg⁻¹·min⁻¹) was done 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 (7-h glycerol basal and 7-h glycerol clamp protocols), as gluconeogenesis from glycerol increases with increased duration of fasting.

Blood samples for glucose, insulin, FFA, C-peptide, triglyceride, glycerol, and [6-3H]glucose specific activity were taken during 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, and the liver was freeze-clamped with precooled aluminum tongs within 45 s of anesthetic injection while infusions were maintained through the jugular vein.

**Laboratory methods.** Plasma glucose was measured with a Beckman Glucose Analyzer II (Beckman, Fullerton, CA). Plasma radioactivity from [6-3H]glucose was determined after deproteinization with Ba(OH)₂ and ZnSO₄, passage through ion exchange columns, and subsequent evaporation. Aliquots of the [6-3H]glucose and of the tritiated glucose infusate were assayed together with the plasma samples. The intra-assay coefficient of variation was 2.5%, and the interassay coefficient of variation was 6.5%. Insulin and C-peptide levels in plasma were determined by radioimmunoassays (RIAs) by using kits specific for rat insulin (but with 100% cross-reactivity with porcine insulin used for infusion) and C-peptide from Linco Research (St. Charles, MO). The coefficients of variation were <5 and 10.5% for insulin and C-peptide, respectively. Plasma FFA levels were measured using a colorimetric kit from Wako Industrials (Osaka, Japan). Plasma triglyceride levels and glycerol levels were also measured using colorimetric kits from Boehringer Mannheim (Mannheim, Germany).

**Hepatic content of citrate.** Hepatic content of citrate was measured as an indicator of FFA oxidation in the presence of a source of pyruvate (precursor of oxaloacetate). Citrate is the product of acetyl-CoA (derived from β-oxidation of fatty acids) and oxaloacetate, the production of which from pyruvate is increased by the allosteric effect of acetyl-CoA on pyruvate carboxylase. Citrate accumulates when Krebs cycle enzyme activity is slowed down by NADH (from substrate oxidation). For citrate assay, liver samples were snap-frozen in liquid nitrogen at the end of the experiment and stored at ~70°C. The samples were homogenized with 13% perchloric acid, precipitated proteins were removed by centrifugation, and the supernatants were neutralized by adding 2 N KH₂CO₃. The precipitated potassium perchlorate salt was eliminated by centrifugation, and the resulting supernatants were stored at ~20°C and subsequently used for citrate
measurements. Citrate was assayed by coupling the citrate lyase/malate dehydrogenase reactions according to Williamson and Corkey (53). Total cellular proteins were measured by the Bradford assay from BioRad (Mississauga, ON, Canada) after dissolution of the trichloroacetic acid-precipitated protein pellets in 1 N NaOH.

PKC isoform translocation. The translocation of the 2,3-diacylglycerol (DAG)-sensitive isoforms of PKC from cytosol to membrane reflects their activation and was assessed by comparing immunoblots of the cytosolic and membrane-associated fractions. Initial studies were performed in which we examined the expression of the PKC isoforms-α, -β, -δ, -ε, and -θ using the following antibodies: polyclonal antibody specific for PKC-α, -β, -δ, and -θ from Sigma and -θ from Santa Cruz Biotechnology (Santa Cruz, CA). All isoforms were detected in the rat liver. In preliminary experiments, we found that only PKC-δ showed evidence of membrane translocation induced by IH. Therefore, we concentrated our studies on PKC-δ. Liver samples (150 mg) were homogenized by a handheld glass homogenizer in 20 mM Tris-HCl, pH 7.5; 10 mM EGTA; 2 mM EDTA; 1 mM NaHCO3; 5 mM MgCl2; 1 mM Na2VO4; 1 mM NaF; 1 μg/mL aprotinin, leupeptin, and pepstatin; 0.1 mM phenylmethylsulfonyl fluoride; and 1 μM microcystin. The homogenates were centrifuged at 100,000g for 1 h at 4°C, and the supernatants were retained as the cytosolic fraction. The pellet was resuspended in buffer B (buffer A + 1% Triton X-100), homogenized by passing through a 23-gauge needle three times, incubated for 15 min on ice, and centrifuged at 100,000g for 1 h at 4°C. The supernatant provided the solubilized membrane fraction. The purity of the cytosolic and membrane fractions was assessed by assaying glucose-6-phosphate dehydrogenase (Sigma) and 5′-nucleotidase activities (Sigma), respectively. The results showed that the index of purity of both fractions was >90%. The protein concentration in all samples was determined by the detergent-compatible modified Lowry microassay (BioRad), using serum albumin as the standard. Fifty micrograms of protein in all samples were mixed with equal volumes of 3× sample-loading buffer (6.86 M urea, 4.29% SDS, 300 mM dithiothreitol, and 43 mM Tris-HCl, pH 6.8) and left at room temperature for 30 min. The mixture was then vortexed and subjected to SDS-PAGE (10% polyacrylamide). After electrophoretic separation, proteins were transferred to Immobilon-P membranes. The membranes were then incubated for 1 h at 4°C in Tris-buffered saline-Tween (TBST) containing 5% nonfat dried milk, pH 7.4. After the blocking step, membranes were washed in rinsing solution (TBST, pH 7.4) and then incubated overnight at a concentration of 1:1,000 with an affinity-purified polyclonal antibody specific for PKC-δ (Sigma). After washing, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham, Baie d’Urfe, Quebec). The membranes were then washed several times with TBST and developed with the use of enhanced chemiluminescence (ECL; Zymed Laboratories, San Francisco, CA). The bands obtained from immunoblotting were quantified by scanning laser densitometry.

Calculations. Glucose turnover (rate of appearance 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 (17). In the basal state, the total rate of glucose appearance corresponds to the EGP. During the clamps, EGP was calculated by subtracting the exogenous glucose infusion rate from the total rate of glucose appearance. At steady state, glucose disappearance corresponds to the rate of glucose appearance, and at euglycemia, glucose disappearance corresponds to tissue glucose utilization, because renal glucose clearance is zero. MCR of glucose is defined as glucose utilization divided by the plasma glucose levels. Data are presented as average values of the samples that were taken in the last 30 min of the experiment.

Statistical analysis. One-way analysis of variance for repeated measures was used to compare differences between treatments (SAL vs. IH). Two-way analysis of variance with interaction was used to compare differences between the effects of IH at 2 and 7 h, using treatment (SAL vs. IH) and duration of infusion (2 vs. 7 h) as independent variables in both the basal and the clamp groups. Two-way analysis of variance with interaction was used to compare differences between the effects of IH in basal and clamp groups, using treatment (SAL vs. IH) and experimental conditions (basal vs. clamp) as independent variables at both 2 and 7 h. Statistical calculations were performed using SAS software (Statistical Analysis System, Cary, NC). Significance was accepted at P < 0.05.

RESULTS

IH elevated plasma FFA levels by approximately three- to fourfold in all groups (P < 0.001; Table 1), and the levels of FFAs were lower during the hyperinsulinemic clamps than during the basal fasting state, as expected. The triglyceride and glycerol levels were also elevated by IH and were lower during the clamps than during the basal experiments (Table 1). Plasma glucose levels were higher with IH vs. SAL infusion in the basal experiments but were maintained at ~6.5 mM during the hyperinsulinemic clamps (Table 1). IH significantly increased plasma C-peptide levels in the basal experiments (Table 1), suggesting that FFAs

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**Table 1. Plasma levels of FFA, triglycerides, glycerol, glucose, and C-peptide**

<table>
<thead>
<tr>
<th></th>
<th>2-h SAL (n = 7)</th>
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<th>2-h IH (n = 6)</th>
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<th>7-h SAL (n = 5)</th>
<th></th>
<th>7-h IH (n = 6)</th>
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<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>0.89 ± 0.18*</td>
<td>0.48 ± 0.04</td>
<td>0.17 ± 0.05</td>
<td>1.31 ± 0.13*</td>
<td>0.63 ± 0.05*</td>
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<tr>
<td>Triglycerides, mM</td>
<td>0.22 ± 0.11</td>
<td>0.05 ± 0.02</td>
<td>1.1 ± 0.2*</td>
<td>0.61 ± 0.14*</td>
<td>0.24 ± 0.03</td>
<td>0.04 ± 0.009</td>
<td>0.82 ± 0.22*</td>
<td>0.33 ± 0.06*</td>
</tr>
<tr>
<td>Glycerol, μM</td>
<td>233 ± 26</td>
<td>58 ± 10</td>
<td>811 ± 67*</td>
<td>501 ± 47*</td>
<td>135 ± 25</td>
<td>94 ± 26</td>
<td>717 ± 72*</td>
<td>585 ± 31*</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>6.4 ± 0.3</td>
<td>6.7 ± 0.3</td>
<td>6.2 ± 0.1</td>
<td>7.3 ± 0.2*</td>
<td>6.6 ± 0.2</td>
</tr>
<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>0.026 ± 0.004</td>
<td>0.28 ± 0.05</td>
<td>0.030 ± 0.003</td>
<td>0.60 ± 0.13*</td>
<td>0.053 ± 0.016</td>
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</table>

Data are means ± SE and refer to the last 30 min of each experiment. IH, Intralipid + heparin; SAL, saline; basal, basal fasting state; clamp, hyperinsulinemic-euglycemic clamp (insulin infusion rate, 5 mU·kg⁻¹·min⁻¹); clamp (½ Ins), hyperinsulinemic-euglycemic clamp (insulin infusion rate, 2.5 mU·kg⁻¹·min⁻¹); FFA, free fatty acid. ∗P < 0.001: IH vs. SAL.

AJP-Endocrinol Metab • VOL 283 • OCTOBER 2002 • www.ajpendo.org
increased endogenous insulin secretion. During the clamps, C-peptide levels were very low, and IH infusion did not increase C-peptide levels (Table 1), indicating that insulin secretion was almost completely suppressed by exogenous hyperinsulinemia with both SAL and IH infusion.

IH significantly increased plasma insulin levels in the basal experiments (Fig. 1), consistent with the increase in C-peptide. Of interest, insulin levels were also higher with IH in the clamp experiments despite the suppressed C-peptide, consistent with a decrease in insulin clearance as we have previously shown (51). This effect of IH was greater in the 7-h IH clamp vs. 2-h IH clamp group (P < 0.001; Fig. 1). When exogenous insulin infusion was given at one-half the original rate (2.5 mU·kg⁻¹·min⁻¹) in the 7-h IH clamp (1/2 Ins) group, the plasma insulin levels were matched with those observed in the 2-h IH clamp or 7-h SAL clamp group.

IH significantly decreased glucose infusion rate (Ginf) at 2 and 7 h (Table 2). The effect of IH vs. the corresponding SAL experiments was greater at 7 vs. 2 h (P < 0.001; Table 2), indicating that IH induced whole body insulin resistance in a time-dependent fashion. In basal steady-state conditions, the rate of appearance of glucose (EGP) is equal to the rate of disappearance of glucose (glucose utilization; GU). IH was found to increase basal EGP = GU after 2 and 7 h of infusion (P < 0.001; Table 2). During the clamps, IH decreased GU after 2 h, and the effect of IH vs. the corresponding SAL experiments was significantly greater (P < 0.01) after 7-h infusions (Table 2). Glucose MCR is equal to GU divided by plasma glucose. IH did not have significant effects on glucose MCR in the basal state, presumably because of the increased endogenous insulin levels (Fig. 2). IH decreased the insulin stimulation of glucose MCR during the clamps vs. the corresponding SAL experiments, and the effect was greater after 7 h than after 2 h (P < 0.001), indicating a time-dependent augmentation of FFA-induced peripheral insulin resistance (Fig. 2).

IH increased EGP = GU in the basal fasting state at both 2 and 7 h as reported above (Table 2 and Fig. 3). There was a tendency for the effect of IH on basal EGP

<table>
<thead>
<tr>
<th>Table 2. Ginf and GU</th>
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<tr>
<td><strong>Ginf, μmol/kg·min</strong></td>
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<tr>
<td><strong>2h-SAL</strong></td>
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<tr>
<td>Basal</td>
</tr>
<tr>
<td>0</td>
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<tr>
<td>42 ± 3</td>
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Data are means ± SE and refer to the last 30 min of each experiment. Ginf, glucose infusion rate; GU, glucose utilization. *P < 0.001: IH vs. SAL. †P < 0.001: the effect of IH vs. the corresponding SAL experiments was greater at 7 than 2 h. ‡P < 0.001: 7-h IH clamp (1/2 Ins) vs. 2-h IH clamp.
to be greater after 7 h than after 2 h ($P = 0.07$). During the hyperinsulinemic clamps, IH increased EGP at 2 and 7 h (Fig. 3; 2-h SAL = 21 ± 3, 2-h IH = 46 ± 5, 7-h SAL = 21 ± 5, 7-h IH = 48 ± 5 μmol/kg$^{-1}$·min$^{-1}$; SAL vs. IH, $P < 0.001$). To assess the effect of insulin on EGP, the suppression of EGP between basal and clamp conditions was compared in the SAL and IH protocols. IH tended to decrease the ability of insulin to suppress EGP at 2 h from 50 to 30% and at 7 h from 54 to 28%. These impairments did not reach statistical signifi-
Liver insulin resistance and PKC

cance but were observed despite the higher levels of circulating insulin in the IH group. When the insulin levels were matched between 7-h SAL and 7-h IH clamp (1/2 Ins), IH markedly decreased the ability of insulin to suppress EGP from ~54 to ~6% (P < 0.05; Fig. 3), indicating hepatic insulin resistance. Furthermore, at matched insulin levels between 7-h IH clamp (1/2 Ins) and 2-h IH clamp, the insulin-induced suppression of EGP was markedly lower with 7-h than with 2-h IH infusion, indicating that hepatic insulin resistance was progressive over time.

To exclude possible effects of glycerol on EGP during IH infusion, glycerol was infused for 7 h (5 mg kg⁻¹ min⁻¹), resulting in plasma glycerol levels similar to 7-h IH in the basal state (Table 3) and even higher than that observed in 7-h IH during the clamps (Table 3). The glycerol infusion had no effect on EGP compared with SAL infusion either in the basal fasting state or during the hyperinsulinemic-euglycemic clamp (Table 3). These findings suggest that the effects of IH on EGP are due largely, if not entirely, to FFAs rather than glycerol.

To investigate the mechanisms responsible for FFA-induced impairment in hepatic glucose metabolism, we measured citrate content (to indirectly assess FFA oxidation) as well as PKC-δ isoform translocation. IH increased citrate content by approximately twofold (P < 0.05) in all groups (Table 4), and the elevation was independent of the insulin levels and the duration of IH infusion, being the same in the basal and clamp experiments and at 2 and 7 h. IH induced hepatic PKC-δ translocation from the cytosolic to the membrane fraction in all groups (Figs. 4 and 5). The degree of PKC-δ translocation was not different between basal and clamp conditions (Figs. 4 and 5). Thus the data from the basal and clamp studies were combined, and PKC-δ translocation induced by IH was found to be greater at 7 h (membrane-to-cytosolic ratio: 4.22 ± 0.22) vs. 2 h (membrane-to-cytosolic ratio: 3.01 ± 0.16) (P < 0.05). Hepatic PKC-δ translocation and citrate content were not measured in the IH clamp experiments with exogenous insulin infused at 2.5 mU·kg⁻¹·min⁻¹ because their levels were not found to be dependent on insulin levels. As stated in Research Design and Methods, we focused on PKC-δ because we found no consistent translocation of hepatic PKC-α, -β, -ε, and -θ in response to IH in preliminary studies. Figure 6 shows some of these preliminary studies indicating that 7-h IH infusion did not induce hepatic PKC-θ or PKC-α translocation.

Discussion

In this study, the effects of FFA on hepatic glucose metabolism and the biochemical mechanisms that underlie these effects were examined. 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, which is consistent with the antilipolytic and FFA reesterification effects of insulin (7). IH increased plasma insulin levels in all groups because of increased insulin secretion in the basal state and a decreased insulin clearance during the clamp. The FFA-induced impairment in insulin clearance has been demonstrated in previous in vivo studies (8, 21, 34, 51). The mechanism for this impairment is likely a decrease in hepatocyte insulin binding, which our in vitro studies suggest is caused in part by FFA-induced PKC activation (10). In the present study, there was a progressive impairment of insulin clearance over time, which, interestingly, paralleled the increase in hepatic PKC-δ translocation.

It is well established that FFAs induce peripheral insulin resistance by an inhibition of insulin-stimulated glucose uptake in skeletal muscle. During the clamps, IH decreased GU and MCR in a time-dependent fashion, indicating marked insulin resistance. This time-dependent augmentation of peripheral insulin resistance is consistent with previous findings (20, 43). The suggested mechanism is a decrease in insulin receptor substrate-1 (IRS-1)-associated phosphatidylinserine.
inositol 3-kinase activity, which is mediated by PKC-θ activation in muscle (15, 19).

In the basal state, IH increased EGP after 2- and 7-h infusions despite increased insulin and glucose concentrations. Previous studies in humans revealed that IH increased gluconeogenesis but did not have any effects on EGP under basal conditions (6, 44). However, when endogenous insulin secretion was inhibited by somatostatin and exogenous insulin was given to maintain insulin at basal levels, IH increased EGP in some studies (6) but not in others (44). IH was also found to increase EGP during insulinopenia achieved by somatostatin infusion without insulin replacement (1, 16). Taken together, these studies suggest that in humans, the stimulatory effect of IH on EGP in the basal state is counteracted by an increase in insulin secretion. Furthermore, there may be autoregulation of basal EGP independent of an increase in basal insulin (44).

In 5-h-fasted rats, the lowering of plasma FFA levels with the use of acipimox did not affect basal EGP (27). However, it has recently been demonstrated by Bergeron et al. (2) that in 12-h-fasted rats, a fourfold elevation of FFAs achieved by IH infusion increases basal EGP (although not significantly) by ~25% despite an increase in plasma insulin concentration. Also, Song et al. (48) have recently shown that in overnight-fasted rats, high-fat diet increases basal EGP in the presence of elevated plasma insulin levels. The difference between the 5-h-fasted and overnight-fasted rats may be due to the fact that after overnight fasting, glycogenolysis is limited by glycogen depletion (27, 48) and may not further decrease to provide autoregulation of basal EGP in the presence of FFA-stimulated

Fig. 4. Representative immunoblots of protein kinase C (PKC)-δ translocation. C, cytosolic fraction; M, membrane fraction. PKC-δ translocation was not measured in clamp (1/2 Ins) because it was found not to be dependent on insulin.

Fig. 5. Graphical representation of the density of the immunoblots of PKC-δ. Mem/Cyt ratio, membrane-to-cytosolic ratio. 2-h SAL basal, n = 4; 2-h SAL clamp, n = 4; 2-h IH basal, n = 5; 2-h IH clamp, n = 5; 7-h SAL basal, n = 5; 7-h SAL clamp, n = 5; 7-h IH basal, n = 5; 7-h IH clamp, n = 5. PKC-δ translocation was not measured in clamp (1/2 Ins) because it was found not to be dependent on insulin. *P < 0.05: IH vs. SAL.
LIVER INSULIN RESISTANCE AND PKC

Suppressed EGP under hyperinsulinemic clamp conditions. However, an increase in FFA oxidation does not appear to explain the progressive decrease of insulin’s ability to suppress EGP induced by IH infusion, since the change in hepatic citrate content was independent of the duration of IH infusion. This suggests that, similar to peripheral tissues, other mechanisms are responsible for the FFA-induced insulin resistance in the liver.

High-fat feeding has been shown to induce skeletal muscle insulin resistance in association with membrane translocation of PKC-θ and -ε (46), and, similarly, lipid infusion has been associated with membrane translocation of PKC-θ (19). Both PKC-θ and -ε are novel isoforms of PKC. In the present study, IH was found to induce the translocation of hepatic PKC-δ (also a novel PKC) from the cytosolic to the membrane fraction in all groups. This suggested that PKC-δ translocation could also have contributed to the increase in EGP induced by IH both in the basal state (at least at 7 h, when insulin resistance was observed) and during the clamps. Notably, the effect of PKC-δ translocation induced by IH was significantly greater at 7 vs. 2 h, which may be due to a progressive accumulation of long-chain fatty acyl-CoA and diacylglycerol activating this isoform (note that FFA oxidation did not appear to increase over time). In addition, the membrane-to-cytosol ratio of PKC-δ was similar in the basal state and the hyperinsulinemic clamps, indicating that in our model of 2-h continuous insulin infusion, contrary to other models (9), insulin does not activate this isoform of PKC in the liver. Thus PKC-δ translocation increased over time, independent of insulin levels. Under conditions of hyperinsulinemia, the progressive increase of PKC-δ translocation was associated with a progressive increase in EGP, suggesting a specific PKC-mediated impairment in hepatic insulin action, which was obviously more evident at high than at basal insulin levels.

Previous studies have reported that in obese hypertriglyceridemic diabetic rats, hepatic PKC activity is greater than in lean rats (40). Considine et al. (12) have shown that obese diabetic rats and obese subjects with type 2 diabetes have higher hepatic membrane-associated PKC-α, -ε, and -ζ than controls. Normalization of circulating glucose levels in obese diabetic rats did not result in reduction of hepatic membrane PKC content, suggesting that factors other than hyperglycemia were responsible for this finding. These factors may include elevated FFA and triglyceride levels, which were not measured in that study (12). In the same study, PKC-δ was not detected in human liver (using a different antibody from that used in our study) and was not assayed in rats. However, PKC-δ was immunodetected in a recent study in normal human liver with the use of a different antibody from ours (50) and from that used in the study of Considine et al. Also, PKC-δ mRNA was found to be expressed in human liver in another study (26). In the human hepatoma cell line HepG2, PKC-δ and -ε membrane translocation was found to mediate the downregulation of insulin action by glucose (36). Finally, Kellerer et al. (24) have found that PKC-δ has an inhibitory effect on tyrosine kinase activity of the insulin receptor in human embryonic kidney cells. Taken together, these data support the hypothesis that...
FFAs induce hepatic insulin resistance through the activation of PKC-δ.

Finally, it should be noted that although hepatic insulin resistance was evident when the insulin levels were matched in the 7-h experiment, suppression of EGP was not significantly impaired by IH when the same insulin infusion rate was used as in control experiments. This suggests that the concomitant elevation in insulin levels (due to the decrease in hepatic insulin clearance) maintains suppression of EGP relatively intact, although the rise in insulin is not sufficient to prevent a decrease in peripheral glucose uptake.

In conclusion, we have shown that FFAs increase EGP in the basal state and during hyperinsulinemic clamps. This effect was associated with at least two mechanisms, an increase in FFA oxidation and/or PKC-δ translocation. Furthermore, FFA induced a progressive impairment in insulin suppression of EGP (i.e., hepatic insulin resistance) in parallel with a progressive increase in PKC-δ translocation. Thus PKC-δ translocation may be relevant to the pathogenesis of hepatic insulin resistance in states associated with chronic FFA elevation such as obesity and type 2 diabetes.

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