The effect of an acute elevation of NEFA concentrations on glucagon-stimulated hepatic glucose output

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Submitted 27 January 2006; accepted in final form 30 March 2006

A REDUCED DECREASE in endogenous hepatic glucose output (HGO) is a main cause of the postprandial hyperglycemia observed in type 2 diabetics (20, 36, 45, 59, 64). In healthy controls, HGO is decreased by 50% after a meal (3), whereas in individuals with type 2 diabetes, this inhibition is impaired (45). The reduced decrease in HGO in type 2 diabetes depends not only on the first phase of insulin secretion (40), but also on the plasma concentrations of glucagon (20, 59, 64) and nonesterified fatty acids (NEFA) (36, 54). Glucagon and NEFA concentrations normally fall during the postprandial period, but in individuals with type 2 diabetes they remain higher than in nondiabetic controls (20, 54, 55, 59). It is well established that both glucagon and NEFA are essential regulators of HGO. However, it is unclear how glucagon and NEFA interact to acutely regulate HGO.

Glucagon-stimulated HGO occurs in a rapid, but time-dependent, manner and results from a direct action of the hormone on the liver (8, 9, 26, 27, 41), resulting primarily in an increase in glycogenolysis and to a lesser extent in a modest and slower rise in gluconeogenesis (8). The increase in HGO caused by glucagon occurs after activation of the glucagon receptor which, being a heterotrimeric Gs-coupled receptor, triggers a rise in intracellular adenosine 3′,5′-cyclic monophosphate (cAMP) (23).

Glucagon can stimulate both transcription and activation of enzymes involved in hepatic gluconeogenesis in vitro (23, 35, 50, 51). In fact, glucagon has been shown to increase hepatic gluconeogenic efficiency in vivo (42, 62). However, the increase in gluconeogenesis contributed minimally to total HGO. The modest increase in hepatic gluconeogenesis may be explained by the fact that glucagon has no effect on gluconeogenic substrate release from muscle and little or no effect on lipolysis in adipose tissue (18, 42). Thus any enhancement of gluconeogenic flux would transiently increase gluconeogenesis. However, eventually plasma gluconeogenic substrate concentrations would fall, and the gluconeogenic contribution to HGO would return toward its basal rate.

Studies in animals (17, 19, 34) and humans (33, 66) have shown that increasing the availability of NEFA (11, 15, 24, 65) increases gluconeogenesis. Although it is controversial as to whether NEFA increases total HGO, it is clear from human (2, 6, 52) and dog (10, 11, 60) experiments that an elevation in plasma NEFA stimulates hepatic gluconeogenesis. Studies in the conscious dog (11) showed that increases in plasma NEFA and glycerol (GLYC) stimulated hepatic gluconeogenesis and inhibited hepatic glycogenolysis without a change in total HGO. The authors also showed that a rise in plasma NEFA significantly limited epinephrine’s glycogenolytic effect and augmented its gluconeogenic action on the liver in vivo (10).

Like glucagon, epinephrine has also been shown to increase HGO in a rapid, time-dependent manner but with a decreased sensitivity on a molar basis compared with glucagon (7, 56, 57, 61). In contrast to glucagon, the stimulatory effect of epinephrine on HGO has been shown to arise from direct action on the liver and indirect actions on extrahepatic tissues. Chu et al. (12) have shown that the direct action of the hormone on the liver results in an increase in glycogenolysis (13, 14) through the

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activation of β2-adrenergic receptors. This effect has been shown to be a cAMP-mediated process (23, 63). The indirect actions of epinephrine on muscle and adipose tissue increase hepatic gluconeogenic substrate (alanine, lactate, glycerol) and NEFA load, which in turn results in an increase in hepatic gluconeogenesis.

Because the direct actions of both glucagon and epinephrine on HGO are mediated by cAMP, we hypothesize that elevated NEFA will alter glucagon-stimulated HGO as it does epinephrine action at the liver. Therefore, the aim of the present study was to determine the effects of physiological increments in glucagon and NEFA on contributions of gluconeogenesis and glucose metabolism in the presence of basal insulin and matched glycemia in the conscious dog.

MATERIALS AND METHODS

Animal Care

Experiments were conducted on 27 18-h-fasted conscious mongrel dogs (19–30 kg) of either gender that had been fed a standard diet of meat (Pedigree canned beef; Pedigree, Vernon, CA) and chow (Laboratory Canine Diet no. 5006; PMI Nutrition International, Brentwood, MO) once daily. The diet totaled 33% protein, 12% fat, 49.5% carbohydrates, and 5% fiber based on dry weight. Only dogs that had a good appetite, a leukocyte count <18,000/mm³, a hematocrit >35%, and normal stools were used for studies. The animal housing and surgical facilities met Association for Assessment and Accreditation of Laboratory Animal Care International Standards. Protocols were approved by the Vanderbilt University Medical Center Animal Care Committee.

Surgical Procedures

Each dog underwent a laparotomy performed under general anesthesia (15 mg/kg pentothal sodium presurgery and 2.5% isoflurane during surgery) 14–16 days before the experiment to implant catheters and transonic flow probes into or around the appropriate vessels. Silastic catheters (0.03 in. internal diameter; Dow Corning, Midland, MI) were placed into jejunal and splenic veins for the intraportal infusion of pancreatic hormones. Catheters (0.04 in. id) for blood sampling were placed into the left hepatic vein, the hepatic portal vein, and left femoral artery as previously described (61). All catheters were filled with heparinized saline (200 U/ml; Abbott Laboratories, North Chicago, IL), and their free ends were knotted prior to closure of the skin. Transonic flow probes (Transonic Systems, Ithaca, NY) were placed around the hepatic artery and portal vein to determine hepatic blood flow as described elsewhere (13). The transonic leads and catheters were placed in a subcutaneous pocket prior to closure of the abdominal skin.

On the day of the experiment, the catheters and transonic probes were externalized under local anesthesia (2% lidocaine; Abbott Laboratories). The contents of each catheter were aspirated and the catheters flushed with saline. The intraportal catheters (splenic and jejunal) were used for the infusion of glucagon and insulin. Angiocaths were inserted percutaneously into the cephalic and saphenous veins for the infusion of [3-3H]glucose plus indocyanine green (ICG; Sigma, St. Louis, MO), as well as the infusion of somatostatin, glucagon, glucose and Intralipid plus heparin infusions. Each animal was allowed to rest quietly in a Pavlov harness for 30 min before the start of the experiment.

Experimental Procedures

First study. Each experiment consisted of a tracer and dye equilibration period (~140 to 40 min), a basal period (~40 to 0 min), and an experimental period (0–195 min). At ~140 min, a priming dose of [3-3H]glucose (33.3 μCi) was given, and a constant infusion of [3-3H]glucose (0.35 μCi/min) began to allow the assessment of hepatic glucose production. Constant infusions of indocyanine green (0.077 mg/min), to assess hepatic blood flow, and somatostatin (0.8 μg·min⁻¹·kg⁻¹), to inhibit endogenous insulin and glucagon secretion, were also started at ~140 min. A constant intraportal infusion of glucagon (0.55 ng·min⁻¹·kg⁻¹) was given (t = ~140 min) to replace basal endogenous glucagon secretion. Endogenous insulin was replaced intraportally at a variable rate beginning at ~140 min. The plasma glucose concentration was monitored every 5 min, and euglycemia was maintained by adjusting the rate of insulin infusion. Once the plasma glucose concentration had been stabilized at euglycemia for 30 min, basal sampling was started (~40 min), and the infusion rate of insulin remained unchanged thereafter. The average insulin infusion rate was 234 ± 23 μU·min⁻¹·kg⁻¹.

The first study included five protocols.

PROTOCOL 1: GGN, N = 6. At 15 min, the intraportal glucagon infusion was increased from 0.55 ng·min⁻¹·kg⁻¹ to three times the basal rate (1.65 ng·min⁻¹·kg⁻¹).

PROTOCOL 2: HG, N = 5. To control for the effects of hyperglycemia on HGO, a hyperglycemic (HG) clamp was performed. Twenty percent dextrose was infused via a leg vein to clamp the arterial glucose at the concentrations seen in the groups receiving three times basal glucose.

PROTOCOL 3: NEFA + GGN, N = 7. A constant Intralipid (0.02 ml·min⁻¹·kg⁻¹, 20% fat emulsion; Baxter Healthcare, Deerfield, IL) plus heparin (0.5 U·min⁻¹·kg⁻¹) infusion was started at 0 min via a leg vein. After a NEFA/glycerol equilibration period (15 min), the intraportal glucagon infusion was increased from 0.55 to 1.65 ng·min⁻¹·kg⁻¹, as seen in protocol 1.

PROTOCOL 4: GLYC + GGN, N = 5. To control for the rise in glycerol, a constant glycerol infusion (via a leg vein) of 0.95 mg·min⁻¹·kg⁻¹ was started at 0 min. After a glycerol equilibration period (15 min), the intraportal glucagon infusion was increased from 0.55 to 1.65 ng·min⁻¹·kg⁻¹, as seen in protocol 1.

PROTOCOL 5: NEFA + HG, N = 5. To control for the increase in NEFA, glycerol, and glucose, a constant 20% Intralipid (0.02 ml·min⁻¹·kg⁻¹) plus heparin (0.5 U·min⁻¹·kg⁻¹) infusion was started at 0 min in the presence of a hyperglycemic clamp. Twenty percent dextrose was infused via a leg vein to clamp the arterial glucose at the concentrations seen in the groups receiving three times basal glucagon.

The glucagon infusion rate was increased by 7% every hour in groups 1, 3, and 4 to compensate for aggregation occurring in the infusion syringe.

Arterial blood samples were taken every 10 min during the basal period and every 15 min during the experimental period. In the NEFA + HG and HG groups, arterial blood samples were also taken every 5 min to monitor glucose concentrations. The total blood volume withdrawn did not exceed 20% of the dog’s total blood volume and each volume of blood was replaced with two volumes of saline.

Second study. The equilibration (~140 to ~40 min) and basal periods (~40 to 0 min) in the second study were identical to those in the first study (described above). The average insulin infusion rate for this study was 264 ± 28 μU·min⁻¹·kg⁻¹. Not only was the experimental period (0–45 min) shortened to 45 min, but the NEFA/glycerol equilibration period was extended by 15 min and the glucagon and Intralipid/heparin infusion rates were increased by 33 and 50%, respectively, compared with the first study.

The second study included only two protocols.

PROTOCOL A: GGN, N = 3. At 30 min, the intraportal glucagon infusion was increased from 0.55 ng·min⁻¹·kg⁻¹ to four times the basal rate (2.2 ng·min⁻¹·kg⁻¹).

PROTOCOL B: NEFA + GGN, N = 3. A constant Intralipid (0.03 ml·min⁻¹·kg⁻¹, 20% fat emulsion; Baxter Healthcare) plus heparin...
(0.75 U-min⁻¹·kg⁻¹) infusion was started at 0 min via a leg vein. After a NEFA/glycerol equilibration period (30 min), the intraportal glucagon infusion was increased from 0.55 to 2.2 ng·min⁻¹·kg⁻¹, as seen in protocol A.

Arterial blood samples were taken every 10 min during the basal period, every 10 min during the NEFA/glycerol equilibration (0–30 min), and every 5 min after the glucagon infusion was increased to four times the basal rate (30–45 min). The total blood volume withdrawn did not exceed 20% of the dog’s total blood volume, and each volume of blood was replaced with two volumes of saline.

Immediately following the final blood sample, each animal was anesthetized with pentobarbital sodium. The animal was then removed from the harness while the hormones, glucose, and Intra Lipid/heparin continued to be infused. A midline laparotomy incision was made, and clamps cooled in liquid nitrogen were used to freeze sections of left and right central and left lateral lobes of the liver in situ. The hepatic tissue was then cut free, placed in liquid nitrogen, and stored at −70°C. Approximately 2 min elapsed between the time of anesthesia and the time of tissue clamping. All animals were then euthanized.

Sample Collecting and Processing

Plasma glucose, plasma [¹H]glucose, blood glycerol, alanine, β-hydroxybutyrate (β-OHB), acetoacetate, lactate, glutamine, glutamate, glycine, serine, threonine, and plasma NEFA were determined as described previously (13, 60). Assays used for the assessment of blood metabolites and plasma NEFA were adapted to the Multiprobe Integrated Fusion Gripper (PerkinElmer, Shelton, CT). The concentrations of plasma insulin, glucagon, cortisol, and blood catecholamines (epinephrine and norepinephrine) were also determined as described elsewhere (13, 60). Transonic flow probes and ICG were used to measure total hepatic blood flow (32, 39). Because Intralipid interferes with the measurement of ICG in plasma, the net hepatic balance and fractional extraction of substrates were calculated using transonic-determined flow. The net hepatic balance and fractional extraction of substrates were calculated using the ICG method in two dogs within the HG group due to transonic flow probe failure.

Hepatic cAMP concentrations were assessed by a modified high-performance liquid chromatography method developed by Ally and Park (1). Liver samples (~500 mg) were homogenized in ice-cold 0.4 M perchloric acid containing 0.5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid. After 1 min on ice, the acid extract was centrifuged at 2,500 l·min⁻¹·g⁻¹ for 10 min at 4°C. The supernatant, neutralized with ~360 μl of 0.5 M K₂CO₃ to pH 6.8, was centrifuged at 4°C for 5 min to precipitate insoluble KClO₄. The mobile phase consisted of elution buffer A (90:10, 100 mM potassium phosphate, pH 6.0, adjusted with K₂PO₄-methanol) and buffer B (80:20, 100 mM potassium phosphate, pH 6.0, adjusted with K₂PO₄-methanol). The chromatographic separation was carried out via autoinjection of 200 μl of sample onto an LC18-T column (25 cm × 4.6 mm ID, 5 μm particle size; Supelco, Bellefonte, PA) protected with a C₁₈ guard column (Supelco). Elution occurred at a flow rate of 0.8 ml·min⁻¹ and a column temperature of 30°C. Detection of cAMP was achieved with UV detection at 254 nm (0.1 AUFS) and a retention time of ~20.0 min.

Basal hepatic cAMP concentrations were assessed from 18-h-fasted dogs with basal glucagon and insulin concentrations.

Calculations. The net hepatic balance of glucose, lactate, glycero, β-OHB, alanine, other gluconeogenic amino acids, and NEFA was calculated using arteriovenous difference methods, as previously described (13). Total glucose production and utilization were calculated using a two-compartment model with parameters established for the dog (21).

In the present studies, we estimated net hepatic gluconeogenic (NHNG) flux and net hepatic glycolysis (NHGLY). The hepatic gluconeogenic flux from circulating gluconeogenic precursors was calculated using previously described methods (22). Briefly, the net hepatic gluconeogenic precursor (alanine, glutamate, glutamine, glycero, glycine, lactate, serine, and threonine) balance was measured. The net hepatic balance of pyruvate was assumed to be 10% of net hepatic lactate balance. The hepatic gluconeogenic flux rate to glucose 6-phosphate (G-6-P) sum of net hepatic uptake of gluconeogenic precursors was calculated by dividing the uptake rate by two to account for the incorporation of the C-3 precursors into the C-6 glucose molecule. NHNG flux was determined by subtracting the summed net hepatic gluconeogenic precursor output rates (when such occurred) and hepatic glucose oxidation from gluconeogenic flux to G-6-P. In the present study, glucose oxidation was assumed to be 0.2 mg·min⁻¹·kg⁻¹ in all groups (13, 28). Our laboratory (46) has found glucose oxidation to vary minimally under hyperglycemic, hyperlipidemic conditions. All parameters were expressed in glucose equivalents. When NHGFLUX is positive, there is net flux to G-6-P, whereas a negative number indicates net flux to pyruvate or net glycolysis.

NHFGLY was estimated by subtracting NHNGFLUX from net hepatic glycolytic flux. A positive value therefore indicates net glycogen breakdown, whereas a negative number represents net glycogen synthesis.

Statistical analysis. Data are expressed as means ± SE. Statistical comparisons were made by two-way ANOVA with repeated-measures design run on SigmaStat (SPSS Science, Chicago, IL). Post hoc analysis was performed with Tukey’s test. Statistical significance was accepted at P < 0.05.

RESULTS

Hormone Concentrations and Hepatic Blood Flow

Arterial plasma concentrations of insulin remained basal in all groups throughout the study (Tables 1 and 2). During the basal period, arterial and portal vein plasma glucagon concentrations were ~45 ± 2 and 60 ± 2 pg/ml, respectively (data not shown). During the experimental period, glucagon infusion at three times the basal rate (1.65 ng·min⁻¹·kg⁻¹) caused
increases of ~40 and 100 pg/ml in arterial and portal vein plasma glucagon concentrations, respectively (all \( P < 0.05 \); Fig. 1 and Table 2). In the HG and NEFA + HG groups, arterial and portal vein plasma glucagon concentrations remained basal and stable during the experimental period (Fig. 1). Arterial plasma cortisol, arterial plasma epinephrine and norepinephrine, and hepatic blood flow remained basal in all groups throughout the study (data not shown).

**Arterial Concentrations and Net Hepatic Uptakes of Plasma NEFA and Blood GLYC**

Peripheral infusions of Intralipid and heparin increased the arterial plasma NEFA concentrations from ~545 to ~1,650 \( \mu \text{mol/l} \) by the end of the study (\( P < 0.05 \); Fig. 2). The arterial plasma NEFA concentrations remained unchanged in the GLYC + GGN group (Table 2), GGN, and HG groups (Fig. 2). Net hepatic uptake of NEFA increased from ~2 to ~4.5 \( \mu \text{mol min}^{-1} \text{kg}^{-1} \) by the last hour in the NEFA + GGN and NEFA + HG groups (\( P < 0.05 \); Fig. 2). Net hepatic uptake of NEFA remained basal and unchanged in the GLYC + GGN group (Table 2), GGN, and HG groups (Fig. 2).

Peripheral infusions of Intralipid and heparin increased the arterial blood glycerol concentrations from ~70 to ~200 \( \mu \text{mol/l} \) by the end of the study in the NEFA + GGN and NEFA + HG groups (\( P < 0.05 \); Fig. 2). In the GLYC + GGN group, a peripheral glycerol infusion of 0.95 \( \text{mg min}^{-1} \text{kg}^{-1} \) increased the arterial blood glycerol concentration from 74 ± 9 to 195 ± 21 \( \mu \text{mol/l} \) (\( P < 0.05 \); Table 2). The arterial blood glycerol concentrations remained basal and unchanged in the GGN and HG groups (Fig. 2). Net hepatic glycerol uptake increased from ~1.5 to ~3.5 \( \mu \text{mol min}^{-1} \text{kg}^{-1} \) in the NEFA + GGN, GLYC + GGN, and NEFA + HG groups (all \( P < 0.05 \); Fig. 2 and Table 2). Net hepatic uptake of glycerol remained basal and unchanged in the GGN and HG groups (Fig. 2).

**Glucose Metabolism**

In all five groups, plasma glucose concentrations rose from just over 100 to ~200 mg/dl (Fig. 3 and Table 2). In response to a portal infusion of glucagon (1.65 \( \text{ng min}^{-1} \text{kg}^{-1} \)), net hepatic glucose output (NHGO) increased by 5.4 ± 1.6 and 4.8 ± 1.0 \( \text{mg min}^{-1} \text{kg}^{-1} \) both \( P < 0.05 \) [not significant (NS) between groups] in GGN and GLYC + GGN groups, respectively, by 15 min of glucagon infusion (minute 30; Fig. 3 and Table 2). In the presence of Intralipid and heparin infusions, glucagon-stimulated NHGO only increased by 3.2 ± 1.1 \( \text{mg min}^{-1} \text{kg}^{-1} \) (\( P < 0.05 \) vs. GGN and GLYC + GGN groups; Fig. 3). Hyperglycemia alone (HG) and peripheral infusions of Intralipid and heparin in the presence hyperglycemia (NEFA + HG) resulted in a decrease in NHGO from 1.6 ± 0.3 to ~0.4 ± 0.5 and 2.2 ± 0.2 to 0.2 ± 0.7 \( \text{mg min}^{-1} \text{kg}^{-1} \), respectively, by the last hour of the experimental period (Fig. 3). Changes in tracer-determined endogenous glucose production (\( R_{e} \)) paralleled the changes in NHGO (Tables 2 and 3). Elevation in circulating NEFA blunted glucagon-stimulated endogenous glucose production by 50% (\( \Delta 2.2 \text{mg min}^{-1} \text{kg}^{-1} \), \( P < 0.05 \), GGN – HG vs. NEFA + GGN – NEFA + HG at minute 30; Table 3).

**Table 2. Plasma hormone concentrations, arterial plasma concentrations and net hepatic uptake of NEFA, arterial blood concentrations, and hepatic balances of glycerol and lactate, glucose metabolism, and net hepatic GLY and GNG flux for the GLYC + GGN group during basal and experimental periods in the presence of a pancreatic clamp in conscious 18-h-fasted dogs**

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<tr>
<th>Basal Period, min</th>
<th>Experimental Period, min</th>
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<tr>
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<td>30</td>
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<tr>
<td><strong>Glucose metabolism</strong></td>
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<tr>
<td>Arterial plasma glucose, mg/dl</td>
<td>232 ± 22</td>
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<tr>
<td>Net hepatic glucose output, mg/min kg⁻¹</td>
<td>4.8 ± 0.08</td>
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<tr>
<td>Net hepatic GNG flux, mg/min kg⁻¹</td>
<td>4.6 ± 0.5</td>
</tr>
<tr>
<td><strong>Net hepatic glycerol balance</strong></td>
<td>1.9 ± 0.4</td>
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</table>

Values are means ± SE. GLYC, glyc erol; GLYC, glycogenolysis; GNG, gluconeogenesis. Basal period values are an average of samples taken at ~40 and 0 min. *\( P < 0.05 \) vs. respective basal concentrations; †\( P < 0.05 \) NEFA + GGN, GGN, n = 6; NEFA + GGN, n = 7; HG, n = 5; NEFA + HG, n = 5.
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After the first 75 min of the experimental period, three times basal glucagon caused an initial modest increase in arterial blood lactate concentrations due to an rise in net hepatic lactate output (NHLO; Fig. 5 and Table 2). In the presence of hyperglycemia (HG), arterial blood lactate

Net Hepatic Glycerogenolysis and Gluconeogenesis

In response to a portal infusion of glucagon (1.65 ng·min⁻¹·kg⁻¹), NHGLY had increased by 5.4 ± 1.6 and 5.7 ± 1.4 mg·min⁻¹·kg⁻¹in the GGN and GLYC + GGN groups, respectively, 15 min after initiation of the glucagon infusion (minute 30, both P < 0.05; Fig. 4 and Table 2). In the NEFA + GGN group, NHGLY had increased by only 3.2 ± 1.1 mg·min⁻¹·kg⁻¹ by 15 min (minute 30, P < 0.05 vs. GGN and GLYC + GGN groups; Fig. 4). In the HG and NEFA + HG groups, NHGLY decreased from 2.2 ± 0.5 to −0.03 ± 0.4 and 3.0 ± 0.5 to 0.08 ± 0.4 mg·min⁻¹·kg⁻¹ (last 30 min of the experimental period; Fig. 4).

NHGNG flux had fallen by 0.9 ± 0.2, 0.7 ± 0.4, and 0.8 ± 0.4 mg·min⁻¹·kg⁻¹ 15 min after the start of glucagon infusion (minute 30) in the GGN, NEFA + GGN, and GLYC + GGN groups, respectively (Fig. 4). By the last 30 min of the study, NHGNG flux had returned to basal rates in the GGN and GLYC + GGN groups (Fig. 4 and Table 2). However, in the NEFA + GGN and NEFA + HG groups, peripheral infusions of Intralipid and heparin resulted in an increase in NHGNG flux of 0.8 ± 0.2 and 0.8 ± 0.3 mg·min⁻¹·kg⁻¹, respectively, by the end of the study (P < 0.05; Fig. 4). NHGNG flux fell slightly in response to hyperglycemia alone (NS; Fig. 4).

Arterial Blood Concentrations and Net Hepatic Balances of β-OHB and Acetoacetate

Peripheral infusions of Intralipid and heparin tended to cause a rise in arterial blood β-OHB concentrations (~20 to 27 μmol/l, last hour), even though there was no significant in-crease in net hepatic β-OHB output (NS; data not shown). In the absence of elevated NEFA, there was no significant change in arterial concentrations or net hepatic output of β-OHB (data not shown). The arterial blood concentrations of acetoacetate remained unchanged, regardless of treatment, despite the tendency for net hepatic output of acetoacetate to increase over time in the GGN, NEFA + GGN, and NEFA + HG groups (~Δ0.5 μmol·min⁻¹·kg⁻¹) and decrease in the GLYC + GGN and HG groups (~Δ−0.2 μmol·min⁻¹·kg⁻¹, NS; data not shown).

Arterial Blood Concentrations and Net Hepatic Outputs of Lactate

Fig. 1. Arterial and portal vein concentrations of plasma glucagon (GGN). Change in arterial (A) and portal vein (B) glucagon concentrations during basal (−40 to 0 min) and experimental (0–195 min) periods in the presence of a pancreatic clamp. A break was set in the x-axis between 0 and 16 min. Protocols were performed in conscious 18-h-fasted dogs. Values are means ± SE. *P < 0.05 vs. respective basal concentrations for both groups receiving 3 times basal glucagon. NEFA, nonesterified fatty acids; HG, hyperglycemic.

Fig. 2. Arterial plasma NEFA and blood glycerol concentrations and net hepatic uptakes. Arterial plasma NEFA concentrations (A), net hepatic NEFA uptake (B), arterial blood glycerol concentrations (C), and net hepatic glycerol uptake (D) during basal (~40 to 0 min) and experimental (0–195 min) periods in the presence of a pancreatic clamp. Protocols were performed in conscious 18-h-fasted dogs. Values are means ± SE. *P < 0.05 vs. respective basal concentrations for both groups receiving 20% Intralipid plus heparin infusions (NEFA + GGN and NEFA + HG).
Glutamate and total amino acid fractional extractions increased by 30% in response to three times basal glucagon (\(P < 0.05\)). By the end of the study, individual (excluding glutamine and glutamate) and total amino acid fractional extractions remained constant throughout the study.

**Arterial Blood Concentrations, Net Hepatic Balances, and Fractional Extraction of Gluconeogenic Amino Acids**

Portal infusion of glucagon (1.65 ng·min\(^{-1}·kg\(^{-1}\)) had no effect on arterial blood concentrations, net hepatic balances, or fractional extractions of individual or total amino acids by 30 min after the initiation of glucagon infusion (data not shown). By the end of the study, individual (excluding glutamine and glutamate) and total amino acid fractional extraction increased in response to three times basal glucagon (\(P < 0.05\), data not shown). This, in turn, resulted in a fall in arterial blood concentrations and some decrease in net hepatic uptake of the gluconeogenic amino acids (NS; data not shown). However, an elevation of NEFA concentrations did not alter glucagon’s effect on amino acid fractional extraction at any time point throughout the experiment (data not shown). In the HG and NEFA + HG groups, individual and total amino acid arterial blood concentrations, net hepatic balances, and fractional extractions remained constant throughout the study.

**Hepatic cAMP Concentrations at the Peak of Glucagon Action**

To determine whether elevated plasma NEFA inhibit the rise in cAMP caused by glucagon in vivo, we repeated the GGN and NEFA + GGN protocols (n = 3/group) and terminated the

**Tracer-determined endogenous glucose Ra during basal and experimental periods in the presence of a pancreatic clamp in conscious 18-h-fasted dogs**

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<th>Experimental Period, min</th>
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<tr>
<td>(R_a) mg·kg(^{-1}·min(^{-1})</td>
<td>40 to 0</td>
<td>30</td>
</tr>
<tr>
<td>GGN</td>
<td>2.8±0.1</td>
<td>6.5±0.7*</td>
</tr>
<tr>
<td>NEFA + GGN</td>
<td>2.5±0.2</td>
<td>5.5±1.0*</td>
</tr>
<tr>
<td>HG</td>
<td>2.4±0.2</td>
<td>2.0±0.3</td>
</tr>
<tr>
<td>NEFA + HG</td>
<td>2.4±0.2</td>
<td>3.2±0.4</td>
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Values are means ± SE. \(R_a\) production. Basal period values are an average of samples taken at 40 and 0 min. *\(P < 0.05\) vs. respective basal concentrations. GGN, n = 6; NEFA + GGN, n = 7; HG, n = 5; NEFA + HG, n = 5.
EFFECT OF NEFA ON GLUCAGON ACTION

Arterial plasma NEFA (570 ± 36 μmol/l) and blood glycerol (55 ± 7 μmol/l) remained basal in both groups (data not shown). Intralipid/heparin infusion rates were increased by 33 and 50%, glycerol equilibration period by 15 min, and the glucagon and lactate output (D) during basal (~40 to 0 min) and experimental (0–45 min) periods in the presence of a pancreatic clamp. Protocols were performed in conscious 18-h-fasted dogs. Values are means ± SE. *P < 0.05 vs. respective basal concentrations.

In the NEFA + GGN group, peripheral infusions of Intralipid and heparin increased the arterial plasma NEFA and blood glycerol concentrations from 419 ± 51 to 1,482 ± 241 and 42 ± 9 to 252 ± 47 μmol/l, respectively (P < 0.05). Arterial glucose concentrations (mg/dl) rose from 106 ± 4 to 125 ± 2 in the GGN group and from 108 ± 2 to 135 ± 7 in the NEFA + GGN group (P < 0.05).

In response to a portal infusion of GGN (2.2 ng·min⁻¹·kg⁻¹), NHGO increased from 1.9 ± 0.2 (0–30 min) to 10.1 ± 1.8 (minute 45) ng·min⁻¹·kg⁻¹ (P < 0.05 vs. basal; Fig. 6). However, in the NEFA + GGN group, NHNG flux did not change in response to glucagon (Fig. 6).

In the GGN group, there was a slight increase in arterial lactate concentrations from 721 ± 254 to 1,103 ± 247 μmol/l due to a dramatic rise in NHLO (μmol·min⁻¹·kg⁻¹) from 9.7 ± 4 to 31 ± 8 (P < 0.05; Fig. 6). Elevated NEFA and glycerol completely inhibited glucagon's increase in NHLO (Fig. 6). Hepatic cAMP concentrations (ng/mg of tissue) increased from a basal concentration of 96 ± 17 to 178 ± 9 and 176 ± 24 in the GGN and NEFA + GGN groups, respectively (n = 3/group; data not shown).

**DISCUSSION**

The present study determined the effects of physiological increments in glucagon and NEFA on HGO in the presence of basal insulin and matched glycemia in the conscious dog.
Glucagon was elevated to concentrations approximately one-half those needed for the hormone’s maximal effect on HGO (62) so that we would be able to detect any change caused by NEFA. Intralipid and heparin were infused at rates needed to achieve NEFA concentrations identical to those seen in our previous studies in which we assessed the ability of a simulated rise in lipolysis to alter epinephrine’s hepatic glycogenolytic and gluconeogenic effects (61, 62). Because the direct actions of both glucagon and epinephrine on HGO are mediated by cAMP, we hypothesize that elevated NEFA will alter glucagon-stimulated HGO as it does epinephrine action at the liver. Although the results from the present studies showed that an increase in NEFA did in fact limit the initial increase in glucagon-stimulated HGO by blunting glycogenolysis, the elevation of NEFA did not increase gluconeogenic flux.

The present studies confirmed previous findings that stimulation of HGO by glucagon primarily results from an initial rapid, potent, and time-dependent increase in glycolysis (8, 9, 26, 27, 41). In the first and second studies, the increments in portal glucagon concentrations increased NHGLY by 5.5 ± 1.6 and 7.2 ± 1.4 mg·min⁻¹·kg⁻¹, respectively, within 15 min (Figs. 4 and 6). However, in the first study, combined Intralipid and heparin infusion blunted glucagon’s initial increases in NHGO (40%) and NHGLY (40%; P < 0.05; Figs. 3 and 4). A trend for the inhibition of NHGO (20%) and NHGLY (30%) was also observed in the second study, but the small number of animals used prevented statistical significance from being achieved (n = 3/group; Fig. 6). In the presence of an isolated rise in glycerol equivalent to that seen with Intralipid (0.02 ml·min⁻¹·kg⁻¹) and heparin (0.5 U·min⁻¹·kg⁻¹), the rise in portal glucagon stimulated a similar increase in NHGLY (5.6 ± 1.4 mg·min⁻¹·kg⁻¹) by 15 min to that seen with glucagon administration alone in study one (Table 2 and Fig. 4). Thus the inhibition of glycolysis and, consequently, HGO seen in response to Intralipid and heparin must have been solely due to elevated plasma NEFA. As expected, hyperglycemia suppressed NHGLY and NHGO to approximately zero in the HG and NEFA + HG groups (Figs. 3 and 4). Therefore, these findings indicate that elevated NEFA inhibit the glycogenolytic effects of glucagon, as they do those of epinephrine. However, this effect was short-lived (1 h) compared with that of increased NEFA on epinephrine-induced net hepatic glycogenolysis, which lasted for a 2-h period (10).

Consistent with our earlier findings (8, 26, 27), glucagon administration resulted in an immediate but brief decrease in NHNG flux in the GGN and GLYC + GGN groups (Table 2 and Figs. 4 and 6). The initial decrease corresponds to and presumably results from the hormone’s rapid effect on NHGLY, in which some of the glucose produced by the breakdown of glycogen enters the glycolytic pathway and exits the liver as lactate (8, 9). In fact, the increase in net hepatic lactate output mirrored the initial increase in NHGLY in the GGN and GLYC + GGN groups (Table 2 and Figs. 4, 5, and 6). Over the first 75 min of the experimental period, Intralipid and heparin infusion showed a tendency to blunt the increase in net hepatic lactate output caused by glucagon (Fig. 5). Lack of statistical significance of this blunting effect was due to one outlier. If this particular dog was omitted, the initial change in net hepatic lactate output (μmol·min⁻¹·kg⁻¹) would be 3.8 ± 1.5 in the NEFA + GGN group, as opposed to 10.4 ± 1.1 in the GGN group (minute 30). In the second study, the fall in NHNG flux and rise in NHLO was completely inhibited in the NEFA + GGN group (P < 0.05, n = 3/group; Fig. 6). Thus the glucose and lactate data from both the first and second studies collectively indicate that elevated NEFA blunt glucagon-stimulated NHGO by inhibiting NHGLY.

Because overall NHNG flux did not change in the GGN group (Fig. 4), the present data are consistent with our previous findings that glucagon has little to no acute effect on hepatic gluconeogenesis. On the other hand, NHNG flux increased from −0.08 ± 0.4 to 0.67 ± 0.3 mg·min⁻¹·kg⁻¹ by the end of the study in the NEFA + GGN group (Fig. 4). Although an increase in NHNG flux was observed in this group, it cannot be interpreted as an augmentation of glucagon action because an identical increase in NHNG flux was observed in the NEFA + HG group (Fig. 4). In both Intralipid/heparin protocols, the almost threefold rise in blood glycerol concentrations (Fig. 2), giving rise to increases in net hepatic glycerol uptake, contributed to the increase in hepatic gluconeogenesis. However, the rate of NHNG flux in the GLYC + GGN group was identical to that seen with glucagon treatment alone. Arterial NHNG flux did not significantly change in the presence of hyperglycemia alone. It seems most likely, therefore, that the rise in hepatic gluconeogenesis was largely due to the increase in hepatic NEFA uptake. In contrast to the interaction of NEFA and epinephrine (10), elevated plasma NEFA did not augment glucagon’s ability to stimulate hepatic gluconeogenesis.

Elevated NEFA blunt the early response of hepatic gluconeogenesis to both glucagon and epinephrine. A rise in plasma NEFA has been shown to result in an increase in fatty acid metabolites (fatty acyl-CoA, diacylglycerol, ceramides) in liver and muscle (5), which have been suggested to activate protein kinase C (PKC) (25, 37, 58). Bouscarle et al. (4) have shown that activated PKC inhibits glucagon-induced cAMP formation in hepatocytes. Activated PKC can also phosphorylate and inactivate Gαs (29), resulting in a subsequent desensitization of the glucagon or β2-adrenergic receptor (49). Therefore, it could be hypothesized that an increase in fatty acid metabolites directly inhibit the glucagon- and epinephrine-signaling pathways via the activation of PKC, which would in turn inhibit adenylate cyclase, resulting in a decrease in cAMP. Our finding that hepatic cAMP content at the peak of glucagon-stimulated gluconeogenesis was not blunted by high concentrations of NEFA (data not shown) does not support this hypothesis.

NEFA have been shown to directly inhibit glucose-6-phosphatase (43, 44), which subsequently increases G-6-P, stimulates glycogen synthase and inhibits hepatic gluconeogenesis. By using liver samples from the first study, Hornbuckle et al. (30) have shown that NEFA did not alter mRNA expression of the catalytic subunit or transporter of glucose-6-phosphatase. However, these liver samples were collected at the end of the study, when NEFA had no effect on HGO. It remains possible that NEFA could directly inhibit glucose-6-phosphatase activity, thereby blunting the initial response to glucagon.

A more likely hypothesis is that elevated NEFA blunted the early rise in hepatic gluconeogenesis by inhibiting glycolysis. It has been proposed (31, 47, 53) that increased NEFA concentrations could raise intracellular citrate concentrations, leading to an inhibition of phosphofructokinase, thus at the same time limiting glycolysis and consequently increasing G-6-P which would in turn blunt NHGLY. Our present studies, particularly
the second of the two sets of experiments, show that a rise in NEFA blunted glucagon’s immediate, brief increase in NHLO, suggesting inhibition of net hepatic glycolysis. In response to hyperglycemia or glucagon- and epinephrine-stimulated glyco-
genolysis, an increase in NHLO occurs (9, 10, 22, 26, 27, 48, 61), most likely because some of the glucose taken up by the liver and produced by glycogen breakdown enters the glycolytic pathway and exits the liver as lactate, respectively. Intralipid and heparin infusions result in NHLO, consistent with inhibition of glycolysis or stimulation of gluconeogenesis (11). In agreement with this, hyperinsulinemia, which reduces plasma NEFA, results in an increase in NHLO (22, 60) that is not evident when the NEFA concentration is clamped (60). Additionally, in the present (Fig. 5) and previous studies (10, 11), an elevation of plasma NEFA decreased the ability of hyperglycemia to cause net hepatic lactate output. Although it has been suggested that elevated NEFA results in an increase in hepatic glucose metabolism, which then inhibits hepatic glyco-
genolysis (6, 11, 15, 16, 38, 52), these and other studies have shown that, by the time the rise in glycogenolysis was fully manifest (30 min), a small decrease had occurred in hepatic gluconeogenic flux (10, 26, 27).

Although NEFA have a similar effect on the initial action of both glucagon and epinephrine on HGO, NEFA temporally affect each hormone differently. With time, an elevation in plasma NEFA did not alter the overall rate of epinephrine-mediated HGO but enhanced the hormone’s ability to stimulate gluconeogenesis and inhibited its action on glycogenolysis (10). In contrast, by the end of the present study the rise in NEFA did not alter glucagon-stimulated glucose production by the liver or the mechanisms by which it was produced. Thus the autoregulation, which took place in the presence of epinephrine, did not occur in the presence of glucagon. One possible explanation for this lies in the fact that the potency of glucagon to stimulate glycogenolysis and thus force carbon down the glycolytic pathway is greater than that of epinephrine. This would override the ability of NEFA to inhibit glycolysis or fuel gluconeogenesis. When epinephrine is ad-
ministered peripherally, and therefore has both direct and indirect (i.e., stimulation of lipolysis) effects on the liver, hepatic lactate uptake occurs (14, 26, 27, 61), whereas NHLO takes place following portal administration of epinephrine (10, 13, 14).

In conclusion, these findings indicate that elevated NEFA inhibit the glycolgenolytic effect of glucagon, as they do the glycolgenolytic effect of epinephrine. However, this effect is short-lived compared with epinephrine. In contrast to epinephrine, an increase in NEFA does not augment glucagon-stimu-
lated hepatic gluconeogenesis. Although postprandial concentra-
tions of glucagon and NEFA are elevated in individuals with type 2 diabetes, our results suggests that their interaction alone does not potentiate HGO, nor does NEFA alter the mechanisms by which glucagon stimulates HGO.

ACKNOWLEDGMENTS

We thank Margaret Lautz, Jon Hastings, Wanda Snead, Eric Allen, Angela Penaloza, and Melanie Scott for technical support. We are grateful to Wanda Snead for valuable comments and careful review of the written method of the measurement hepatic cAMP concentrations. We are especially thankful to Genie Moore for valuable comments and careful review of this manuscript. This work was presented in part at the 62nd Annual Meeting of the American Diabetes Association, San Francisco, CA, June 2002.

GRANTS

This research was supported by a supplement (S1) to a National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) Grant, R37-DK-18243, and by an NIDDK grant for the Diabetes Research and Training Center at Vanderbilt University, SP60-AM20593.

DISCLOSURES

D. K. Sindelar is currently affiliated with the Endocrine Division of Eli Lilly and Company (Indianapolis, IN). C. A. Chang is currently affiliated with Clinical Pharmacologist in Hurley Consulting Associates (Chatham, NJ).

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