Effects of FFA on insulin-stimulated glucose fluxes and muscle glycogen synthase activity in rats

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Park, J. O., Chul-Hee Kim, Sung K. Hong, Kyo I. Suh, and Ki-Up Lee. Effects of FFA on insulin-stimulated glucose fluxes and muscle glycogen synthase activity in rats. Am. J. Physiol. 275 (Endocrinol. Metab. 38): E338–E344, 1998.—To examine effects of free fatty acids (FFA) on insulin-stimulated glucose fluxes, euglycemic hyperinsulinemic (86 pmol·kg-1·min-1) clamps were performed for 5 h in conscious rats with (n = 8) or without (n = 8) lipid-heparin infusion. Glucose infusion rate required to maintain euglycemia was not different between the two groups during the first 2 h of clamps but became significantly lower with lipid-heparin infusion in the 3rd h and thereafter. Whole body glycolysis was significantly lower at 5 h. Thus increased availability of FFA significantly reduced whole body glucose oxidation, but compensatory increase in muscle glycogen synthase activity occurred. On the basis of these data, we postulated that increased availability of FFA suppresses whole body glucose oxidation and a compensatory increase in muscle glycogen synthase activity occurs.

RESEARCH DESIGN AND METHODS

Animals

Male Sprague-Dawley rats weighing 300–350 g were used for the study. The age and weight of the animals used in the various experiments were very similar (Table 1). Animals were prepared for glucose clamp experiments according to the method of Buchanan et al. (3) as previously described (22). At least 4 days before the clamp studies, animals were placed in individual cages and were in tail restraint, which was required for access to tail blood vessels during glucose clamp experiments. For this, the distal one-third of the animal’s tail was drawn through a hole at the bottom of the cage and

THE CONCEPT OF SUBSTRATE competition between glucose and free fatty acids (FFA) as oxidative fuel sources in muscle (glucose-fatty acid cycle hypothesis) was introduced more than 30 years ago by Randle et al. (26). Since then, many investigators have examined the effect of FFA on whole body and/or skeletal muscle glucose metabolism (7, 9, 21). However, it is still controversial whether increased plasma FFA levels are responsible for insulin resistance observed in diabetes and obesity.

Many studies in humans have suggested that reduction in glycogen synthase (GS) activity and glycogen synthesis in skeletal muscle is the main cause of insulin resistance in non-insulin-dependent diabetes and obesity (6, 31). Although it is now generally accepted that increased provision of FFA suppresses whole body glucose oxidation (2, 7, 9, 14), the effect of FFA on glycogen synthesis or GS activity is not established. Previous studies have reported decreased (1, 2, 7, 14), increased (11, 15, 20), or unchanged (12, 28, 32) insulin-stimulated glycogen synthesis or GS activity with elevated plasma FFA levels. The cause of this discrepancy is presently unknown.

Several recent studies have demonstrated that the effects of FFA on insulin-stimulated glucose metabolism are time dependent (2, 28). Increased plasma FFA by lipid-heparin infusion replaced glucose as fuel for oxidation within 1 h, but glucose uptake did not decrease until 2–4 h of lipid infusion (2). In a recent study, Kim et al. (16) found a similar pattern of changes in insulin-stimulated glucose fluxes in high-fat-fed rats; insulin-stimulated glycolysis was suppressed within 2 days of high-fat feeding, whereas insulin-stimulated glucose uptake decreased only after a prolonged period (>1 wk) of high-fat feeding. Insulin-stimulated glycogen synthesis increased significantly during the initial few days to compensate for the decrease in glycolysis but subsequently decreased as insulin resistance manifested. On the basis of these data, we postulated that suppression of glycolysis and a compensatory increase in glycogen synthesis would precede a decrease in insulin-stimulated glucose uptake in rats infused with lipid emulsion and heparin. In this study, we examined effects of intravenous infusion of lipid and heparin on insulin-stimulated glucose metabolism in the whole body and in skeletal muscle of conscious rats.
Table 1. Age, body weight, plasma insulin, and FFA levels during glucose clamps among different groups of animals

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Age (wk)</th>
<th>Body Weight (g)</th>
<th>Plasma Insulin (nm)</th>
<th>Plasma FFA (nm)</th>
</tr>
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<tbody>
<tr>
<td>Experiment A</td>
<td></td>
<td></td>
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<tr>
<td>Intralipid</td>
<td>8</td>
<td>8.5 ± 0.2</td>
<td>324 ± 3.7</td>
<td>2.0 ± 0.15</td>
<td>1.4 ± 0.12*</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>8.5 ± 0.2</td>
<td>323 ± 3.5</td>
<td>2.1 ± 0.15</td>
<td>0.2 ± 0.04</td>
</tr>
</tbody>
</table>

| Experiment B   |    |          |                 |                    |                 |
| 1-h Clamp      |    |          |                 |                    |                 |
| Intralipid     | 8  | 8.5 ± 0.2| 323 ± 3.4       | 1.8 ± 0.10         | 1.3 ± 0.11*     |
| Control        | 8  | 8.5 ± 0.2| 325 ± 3.6       | 1.8 ± 0.10         | 0.4 ± 0.06      |
| 2-h Clamp      |    |          |                 |                    |                 |
| Intralipid     | 8  | 8.4 ± 0.2| 322 ± 3.8       | 1.8 ± 0.15         | 1.3 ± 0.13*     |
| Control        | 8  | 8.5 ± 0.2| 324 ± 3.6       | 1.9 ± 0.15         | 0.4 ± 0.05      |
| 3-h Clamp      |    |          |                 |                    |                 |
| Intralipid     | 8  | 8.5 ± 0.2| 323 ± 3.5       | 1.9 ± 0.13         | 1.4 ± 0.13*     |
| Control        | 8  | 8.6 ± 0.2| 324 ± 3.7       | 2.0 ± 0.15         | 0.3 ± 0.04      |
| 5-h Clamp      |    |          |                 |                    |                 |
| Intralipid     | 8  | 8.5 ± 0.2| 324 ± 3.6       | 2.0 ± 0.13         | 1.4 ± 0.12*     |
| Control        | 8  | 8.4 ± 0.2| 323 ± 3.4       | 2.0 ± 0.4          | 0.3 ± 0.05      |

Data are means ± SE; n = no. of rats. FFA, free fatty acids. *P < 0.001 vs. control.

secured there with a rubber stopper. This arrangement allowed animals to freely move about and did not restrict access to food or water.

Catheterization

Five hours before the clamp experiments, food was removed from the cage, and each animal underwent a placement of catheters (PE-10, Intramedic, Clay Adams, Parsippany, NJ) into two tail veins and a tail artery for the purpose of infusion and blood sampling, respectively. Catheters were placed percutaneously during local anesthesia with lidocaine while animals were briefly restrained in a towel. Animals were returned to their cages after catheter placement, with tails secured as described above. Patency of arterial catheter was maintained by a slow (0.015 ml/min) infusion of saline.

Euglycemic Hyperinsulinemic Clamps

Experiment A: effects of lipid-heparin infusion on plasma FFA and glucose infusion rate. Euglycemic hyperinsulinemic clamps were conducted for 5 h with (n = 8) or without (n = 8) an infusion of heparin (40 U/h with 10 U as a priming bolus) and triglyceride emulsion (Intralipid; 10% wt/vol; 1.2 ml/h) to raise plasma FFA levels. Human insulin (Velosulin, Novo-Nordisk, Gentofte, Denmark) was infused at a rate of 86 pmol·kg⁻¹·min⁻¹ starting at time 0. Blood samples were taken for glucose measurement at 10-min intervals, and 25% dextrose was infused at variable rates to clamp plasma glucose at basal levels. Blood samples for the measurements of insulin and FFA were obtained at time 0 and at 1, 2, 3, and 5 h. FFA samples were collected in prechilled tubes containing EDTA and Paraaxon (dithiethyl p-nitrophenyl phosphate, Sigma; a lipoprotein lipase inhibitor, 0.275 mg/ml of blood), immediately centrifuged, and stored at −70°C until analysis.

Experiment B: effects of lipid-heparin infusion on insulin-stimulated whole body glucose fluxes and muscle glycogen synthesis. To investigate changes in whole body glucose fluxes during lipid-heparin infusion, additional clamps were performed for 1, 2, 3, or 5 h with or without lipid-heparin infusion (n = 8 each). Insulin was infused at a rate of 86 pmol·kg⁻¹·min⁻¹. A primed (20 µCi) and continuous (0.2 µCi/min) infusion of [3-3H]glucose (NEN, Boston, MA) was initiated at −120 min and continued throughout the experiments. Blood samples for the determination of [3H]glucose and [3H]water specific activity were obtained every 10 min during the final 40 min of each 1-, 2-, 3-, or 5-h clamp. Blood samples for the measurements of insulin and FFA were obtained at the end of each 1-, 2-, 3-, or 5-h clamp.

At the end of each clamp (1, 2, 3, or 5 h), rats were anesthetized with pentobarbital sodium (50 mg/kg; intraperitoneal injection), and gastrocnemius muscles were frozen in situ by aluminum tongs precooled in liquid nitrogen. The frozen muscle samples were kept at −70°C until analysis.

Analytic Procedure

Plasma glucose was measured by the glucose oxidase method using a glucose analyzer (Beckman Instruments, Palo Alto, CA). Plasma FFA was measured by enzymatic assay using a kit from Eiken Chemical (Tokyo, Japan). Plasma insulin was measured by radioimmunoassay using kits for rat (basal insulin; Linco, St. Charles, MO) and human insulin (damp insulin; Dainabott, Tokyo, J. P). Plasma [3H]glucose radioactivity was measured in duplicate by deproteinizing plasma samples with BaOH₂ and ZnSO₄, drying to eliminate tritiated water, and counting for [3H] in a liquid scintillation spectrophotometer (Beckman). The plasma concentration of [3H]water was determined by the difference between [3H] counts with and without drying.

Skeletal muscle glucose 6-phosphate (G-6-P) was determined by an enzymatic assay as described by Michal (23). Because muscle G-6-P concentration may be sensitive to plasma glucose, glucose infusion was continued during the muscle sampling procedures to prevent any significant perturbation of plasma glucose concentration. Care was also taken to prevent G-6-P concentration from rising because of glycogenolysis during the procedures. Frozen muscles were crushed in liquid nitrogen and homogenized with 6% perchloric acid at 0°C.

Incorporation of [3H]glucose into muscle glycogen was measured to estimate de novo glycogen synthesis during the glucose clamps (29). Muscle samples were digested in 30% KOH at 100°C for 30 min and then incubated in 60% ethanol and 0.3% lithium bromide for 30 min at 0°C. The precipitates were washed twice with 60% ethanol and digested with amyloglucosidase (4). The digested solution was mixed with scintillation solution, and the radioactivity was measured on a liquid scintillation spectrophotometer. The amount of [3H] in muscle glycogen was expressed as disintegrations per minute per gram of tissue wet weight. The rate of [3H]glycogen accumulation in muscle was estimated by the increment of [3H] radioactivity in each time period between muscle sampling.

GS activity was measured according to the method of Golden et al. (10) with modifications. We used a superficial part of gastrocnemius muscle, which mainly consists of white muscle fibers, for the determination of GS activity, since we previously observed that superficial parts of gastrocnemius have higher GS activities than deep parts of the muscle (25). GS activity was expressed as the ratio of the activity in the absence of G-6-P to the activity at 10 mM G-6-P (GSi; GS independent of G-6-P (13)) or as the ratio of the activity at 0.1 mM G-6-P to the activity at 10 mM G-6-P (the fractional velocity of GS activity) (18). GSi and the fractional velocity of GS activity are indicators of the active form of GS and are believed to represent GS activity in vivo.

Isotopic Determination of Glucose Fluxes

Rates of total glucose appearance and whole body glucose uptake (Rₜ) were determined as the ratio of the [3H]glucose accumulation in muscle and the [3H] glucose appearance in plasma. The rate of glucose turnover was estimated as the product of Rₜ and the plasma glucose concentration.
infusion rate (dpm/min) to the specific activity of plasma glucose (dpm/mol) during the final 40 min of the clamps (30). Whole body glycolysis was calculated from the rate of increase in plasma $^3$H$_2$O concentration during the final 40 min of the clamps as previously described (29). The rate of increase in plasma $^3$H$_2$O was determined by linear regression of the measurements at 10-min intervals during the final 40 min of the clamps. Whole body glycogen synthesis was estimated by subtracting whole body glycolysis from whole body glucose uptake, with the assumption that glycolysis and glycogen synthesis account for the majority of insulin-stimulated glucose uptake (29).

Statistical Analysis

Data are presented as means ± SE. Statistical analysis was performed using the PC-SAS program (SAS Institute, Cary, NC). The significance of differences between the two experimental groups was assessed by ANOVA with repeated measures (experiment A) and by two-way ANOVA with the use of group (with 2 levels) and time (with 4 levels: 1st, 2nd, 3rd, and 5th h) as independent factors (experiment B). To test whether the differences between groups were time dependently different, we first checked the significance of interaction effects between group and time. If it turned out to be significant at the 5% level, then various contrasts for the detection of differential between-group effects by time were examined. To handle multiplicity (the total number was 6) in time-pair comparisons, Bonferroni adjustment for significance level was adopted.

RESULTS

Experiment A: Effects of Lipid-Heparin Infusion on Plasma FFA and Glucose Infusion Rate

Plasma insulin was raised to ~2.1 nM, and plasma glucose was maintained at levels (~6.8 mM) similar to basal levels in both groups (Fig. 1, A and B). Plasma glucose and insulin concentrations were not different between the two groups. Plasma FFA levels decreased from 0.45 ± 0.03 mM in the basal state to 0.15 ± 0.03 mM during the control clamps. In contrast, plasma FFA levels increased threefold (from 0.43 ± 0.03 to 1.53 ± 0.04 mM) during the clamps with lipid-heparin infusion ($P < 0.001$ vs. control clamp; Fig. 1C).

Glucose infusion rate (GIR) was almost identical between the two groups during the initial 2 h of the clamps (Fig. 1D). In the control clamps, GIR further increased to reach peak level at 3 h after the start of insulin infusion. In contrast, in the clamps with lipid-heparin infusion, GIR began to decrease at 2 h, resulting in significantly lower rates compared with the control clamps thereafter ($P < 0.01$).

Experiment B: Effects of Lipid-Heparin Infusion on Insulin-Stimulated Whole Body Glucose Fluxes and Muscle Glycogen Synthesis

Insulin-stimulated whole body glucose fluxes. Plasma insulin levels were raised to similar levels at the end of each 1-, 2-, 3-, or 5-h clamp (Table 1). There was no statistical difference among these values for each clamp and between the control and treatment groups. Plasma FFA levels at the end of the clamps with lipid-heparin infusion were all significantly higher than those in the control clamps ($P < 0.001$).

Similar to the changes of GIR, $R_d$ (measured during the final 40 min of each clamp) was almost identical between the two groups in the 1st and 2nd h of clamps (Fig. 2A). In contrast, $R_d$ was 40% lower with lipid-heparin infusion in the 3rd and 5th h ($P < 0.01$). Two-way ANOVA analysis revealed that contrasts of 1st vs. 3rd h, 1st vs. 5th h, 2nd vs. 3rd h, and 2nd vs. 5th h were significant ($P < 0.001$ each), which means that the lipid-heparin group had significantly lower mean $R_d$. 

Fig. 1. Plasma glucose (A), insulin (B), and free fatty acid (FFA; C) concentrations and glucose infusion rate (D) during hyperinsulinemic euglycemic clamps in rats with (●) or without (○) lipid-heparin infusion. Data are means ± SE. *$P < 0.01$ and **$P < 0.001$ vs. control.
levels at later hours (4th and 5th h) compared with early hours (1st and 2nd h).

Whole body glycolysis was significantly lower with lipid-heparin infusion in all of the time periods, i.e., 1st, 2nd, 3rd, and 5th h of the clamps (Fig. 2B). In contrast, whole body glycogen synthesis was higher with lipid-heparin infusion in the 1st h (72 ± 4 vs. 60 ± 2 µmol·kg⁻¹·min⁻¹, P < 0.05) and 2nd h (124 ± 4 vs. 104 ± 2 µmol·kg⁻¹·min⁻¹, P < 0.01) but lower in the 5th h (69 ± 4 vs. 112 ± 5 µmol·kg⁻¹·min⁻¹, P < 0.01; Fig. 2C). The differences between groups were significant between 1st and 2nd h vs. 5th h (P < 0.001), and 3rd h vs. 5th h (P < 0.01).

Accumulation of [³H]glycogen in gastrocnemius muscle. The radioactivity of [³H] in muscle glycogen was significantly higher with lipid-heparin infusion at 1 and 2 h (35,284 ± 2,450 vs. 9,924 ± 540 and 97,420 ± 3,520 vs. 39,499 ± 2,706 dpm/g wet wt; P < 0.05, respectively). The radioactivity of [³H] in muscle glyco-
rate of accumulation of $[^{3}H]$glycogen at early hours (1st and 2nd h) compared with later hours (4th and 5th h) ($P < 0.001$). These data are consistent with the data of whole body glycogen synthesis rate (Fig. 2C).

Skeletal muscle G-6-P and GS activity. G-6-P concentrations in gastrocnemius muscle taken at 1, 2, 3, and 5 h of clamps were all significantly higher with lipid-heparin infusion than those in the control group ($P < 0.05$ at 1 h, $P < 0.01$ at 2, 3, and 5 h; Fig. 3A). GSI ratio and the fractional velocity of GS activity were not altered at 1, 2, and 3 h but were significantly lower at 5 h with lipid-heparin infusion (GSI ratio: $0.26 \pm 0.03$ vs. $0.32 \pm 0.06$; fractional velocity: $0.37 \pm 0.04$ vs. $0.44 \pm 0.07$; $P < 0.05$ for both) (Fig. 3, B and C). The differences in GSI ratio and fractional velocity between groups were significant between 1 and 2 h vs. 5 h ($P < 0.01$), respectively.

**DISCUSSION**

In agreement with a previous study in humans (2), the present study showed that the GIR required to maintain euglycemia was not different between the lipid-heparin group and the control group during the first 2 h of the clamps but became significantly lower with lipid-heparin infusion in the 3rd h and thereafter. Additional experiments to investigate the changes in whole body glucose fluxes during lipid-heparin infusion disclosed that FFA had multiple effects on glucose metabolism that were time dependent (2, 17, 28). Although increased provision of FFA by intravenous lipid-heparin infusion rapidly (within 1 h) suppressed whole body glycolysis, insulin-stimulated whole body glucose uptake did not change until 3 h of lipid-heparin infusion. Of note in our study is that during an early period of lipid-heparin infusion, compensatory increase in skeletal muscle glycogen synthesis in association with accumulation of G-6-P counterbalanced the decrease in glycolysis, resulting in the lack of FFA effect on glucose uptake.

Profound inhibition of insulin-stimulated glycolysis with elevated plasma FFA levels is consistent with the glucose-fatty acid cycle (26). According to this hypothesis, elevated FFA oxidation increases intracellular acetyl-CoA and citrate concentrations. Acetyl-CoA suppresses glucose oxidation by inhibiting the activity of pyruvate dehydrogenase, the entry point of pyruvate into oxidative metabolism. Citrate decreases glycolysis by inhibiting phosphofructokinase (24), a key enzyme for glycolysis. Many studies in vivo confirmed the inhibition of glucose oxidation by elevated plasma FFA (2, 7, 9, 14). However, other studies have shown that inhibition of glucose oxidation by FFA does not necessarily lead to a decrease in glucose uptake. One explanation for this phenomenon would be shunting of glucose carbons from oxidation to production of lactate or alanine (14, 34). However, our data showing a reduced rate of whole body glycolysis, assessed by measuring $[^{3}H]$water production from $[^{3-}^{3}H]$glucose, suggest that glycolysis is also suppressed by FFA at a step(s) proximal to triose isomerization between dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, presumably the reaction catalyzed by phosphofructokinase (15).

Our results showed that insulin-stimulated glycogen synthesis was significantly increased in the early phase (within 2 h) of lipid-heparin infusion. This was consistently demonstrated with two independent measurements: rate of whole body glycogen synthesis, calculated as the difference between whole body glucose uptake and glycolysis, and incorporation of $[^{3}H]$glucose into skeletal muscle glycogen. However, GS activity, assessed in vitro, was not different between the two groups in this early period. On the other hand, muscle G-6-P levels were significantly higher with lipid-heparin infusion. Because GS activity is dependent on the concentration of G-6-P, the increases in skeletal muscle and whole body glycogen synthesis appear to be due to increases in muscle G-6-P levels. Taken together, these results suggest that inhibition of glucose oxidation and glycolysis by FFA results in accumulation of G-6-P in skeletal muscle, which in turn leads to a stimulation of skeletal muscle glycogen synthesis. This compensatory increase in skeletal muscle glycogen synthesis in the early phase of lipid-heparin infusion appears to mask the effect of FFA on overall glucose metabolism.

In contrast to the effects in the early period, lipid-heparin infusion for a longer period (>2 h) induced a state of insulin resistance characterized by a decrease in both whole body glycolysis and glycogen synthesis. In agreement with previous studies (1, 2, 14), this was associated with a decrease in skeletal muscle GS activity. Delayed occurrence of these effects suggests that FFA do not inhibit GS activity directly but indirectly through secondary metabolic changes in the cells. The mechanism underlying FFA-induced inhibition of GS activity is presently not settled, but accumulation of certain metabolites, such as glycogen (8), long-chain acyl-CoA (33), or glucosamine pathway metabolites (5), has been implicated.

In the present study, muscle G-6-P levels were increased with lipid-heparin infusion throughout the hyperinsulinemic clamps. This may indicate that the impairments of glucose metabolism at sites distal to G-6-P (i.e., glycolysis or glycogen synthesis) are quantitatively more important than the impairments of more proximal fluxes such as glucose transport and phosphorylation. However, this study is limited in that FFA concentrations used in the present study were very high. Boden et al. (1) reported that, in humans, the mechanisms underlying the FFA effect to decrease insulin-stimulated glucose uptake were dependent on the concentration of FFA studied. An impairment of muscle GS activity was seen after 4–6 h of high FFA concentrations (~750 µM) and was associated with an increase in muscle G-6-P level. On the other hand, at lower FFA concentrations (~550 µM), a transport or phosphorylation defect, associated with a decrease in muscle G-6-P level, was the major defect and preceded changes in GS activity. Roden et al. (28) also indicated, on the basis of the nuclear magnetic resonance technology, that skeletal muscle G-6-P level was lower in
human subjects given lipid-heparin infusion during hyperinsulinemic euglycemic clamps, suggesting that FFA may induce insulin resistance by inhibiting glucose transport and/or phosphorylation. However, in that study, plasma FFA levels were raised to a concentration of 2 mM, higher than those in the present study and that of Boden et al. The reasons for the discrepancy among these studies are presently unclear.

Another limitation of our study is that we only examined the effects of FFA on maximal insulin-stimulated glucose fluxes. It is now well established that insulin actions on different tissues are quite different according to insulin levels (19, 27). Thus hepatic glucose production is completely suppressed at plasma insulin levels much lower than the level which stimulates peripheral glucose utilization maximally. Similarly, FFA actions on different tissues are dependent on insulin levels. Elevated plasma FFA levels lead to the suppression of peripheral glucose utilization at higher insulin levels but lead to the enhancement of hepatic glucose production at lower insulin levels (9, 21). Because the plasma insulin levels during the clamps were very high, we could not evaluate FFA actions on tissues other than skeletal muscle.

In conclusion, the present study clearly demonstrates time-dependent effects of FFA on insulin-stimulated glucose metabolism. Increased provision of FFA rapidly inhibited glucose oxidation and glycolysis in skeletal muscle and in the whole body. Consequent increase in intracellular G-6-P stimulated glycogen synthesis in skeletal muscle during the early phase of lipid-heparin infusion, and insulin-stimulated glucose uptake did not change significantly because of this compensatory increase in glycogen synthesis. On the other hand, insulin-stimulated glucose uptake was reduced in the later phase (>2 h) of lipid-heparin infusion, when glycogen synthesis was reduced in association with reduced GS activity.

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