Five-Hour Fatty Acid Elevation Increases Muscle Lipids and Impairs Glycogen Synthesis in the Rat

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Insulin-mediated muscle glycogen synthesis is impaired after several weeks of high-fat feeding in rats, but not by short-term (2-hour) nonesterified fatty acids (NEFA) elevation induced by intravenous triglyceride/heparin infusion (TG/H). We examined whether a longer TG/H infusion induces defective glycogen synthesis. Five-hour hyperinsulinemic (700 pmol/L) euglycemic clamps with either TG/H or saline infusion were performed. TG/H-infused rats developed insulin resistance, but only after 2 to 3 hours. Red gastrocnemius glycogen synthesis rate decreased by 50% ($P < .01$ *v* saline) associated with decreased glycogen synthase activity (GSa; assessed at several glucose-6-phosphate [G-6-P] levels; two-way ANOVA, $P = .02$) and increased muscle TG and total long-chain acyl coenzyme A (LCAC) content (twofold; $P < .05$ *v* saline). Thus a 3- to 5-hour NEFA elevation in the rat produced significant impairment of insulin-stimulated muscle glycogen synthesis, associated with muscle lipid accumulation. These effects were similar to those observed after several weeks of fat feeding. The 5-hour TG/H-infused rat is a useful model for studying lipid-induced muscle insulin resistance.
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AN INCREASED AVAILABILITY of nonesterified-fatty acids (NEFA) has been implicated in the pathogenesis of non–insulin-dependent diabetes mellitus (NIDDM).[1] In humans, short-term increases of NEFA levels by a triglyceride/heparin (TG/H) infusion during an euglycemic hyperinsulinemic clamp has produced inconsistent effects on glycogen synthase activity (GSa) or nonoxidative glucose disposal, with some studies showing an inhibitory effect,[2-5] while others do not.[6,7]

In rats, studies with a 2-hour TG/H infusion during a hyperinsulinemic euglycemic clamp have also demonstrated that acute NEFA elevation produces variable effects on GSa, net glycogen synthesis, or glycogen mass in skeletal muscle; two studies reported a positive effect[8,9] and one reported a mixed effect with inhibition of GSa at maximal insulin stimulation, but increased glycogen deposition at physiological insulin levels.[10] These studies did not demonstrate any substantial impairment of insulin action at the whole-body level over a 2-hour period. Overall, there is an apparent anomaly between the majority of human and rat studies.

Another method of increasing the availability of fatty acids is via high-fat feeding. High-fat feeding of rats for 3 weeks results in reduced insulin-mediated muscle glucose uptake and glycogen synthesis, associated with an increased muscle triglyceride content.[11,12] We postulate that a prolonged intravenous TG/H infusion in rats might produce metabolic effects more substantial than those seen after 2 hours[8-10] and similar to those seen after several weeks of high-fat feeding in rats.[11,12] As far as we are aware, no studies using a lipid infusion in rats have been continued significantly past 2 hours to examine whether a defect in glycogen synthesis could become obvious, as in human studies.[13]

It has been postulated that an increase in long-chain acyl coenzyme A (LCAC) may inhibit GSa, and this notion has some support from two previous in vitro studies; one demonstrated inhibition of rat liver glycogen synthase by palmitoyl-coenzyme A,[14] while the other demonstrated inhibition of human skeletal muscle glycogen synthase by palmitate.[15] We report here the effects of a 5-hour TG/H infusion on net glycogen synthesis and GSa, and their relationship in vivo to LCAC content of skeletal muscle.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (body weight, 350 to 400 g; Table 1) were used in this study. The animals had free access to water and standard laboratory chow (Allied Feeds, Rhodes, Australia) and were subject to controlled temperature (22 ± 1°C) and lighting (lights on 6 AM to 6 PM). All surgical and experimental procedures performed were approved by the Garvan Institute of Medical Research/St. Vincent's Hospital Animal Experimentation Ethics Committee, and were in accordance with National Health and Medical Research Council of Australia guidelines for the use of animals in research.

Experimental Protocol

A detailed description of surgical procedures and preparation of animals has been given previously.[16] In brief, about 1 week before the day of study, cannulae were inserted into the right jugular vein and left carotid artery and exteriorized at the back of the neck, under ketamine hydrochloride (90 mg/kg) and xylazine (10 mg/kg) anesthesia. Only those rats that reached or exceeded their preoperation weight were subsequently studied.

All animals were deprived of food for 5 hours before the study. At time 0, a 5-hour hyperinsulinemic euglycemic clamp was commenced. Hyperinsulinemia was achieved with a constant infusion of human insulin (0.25 U/kg/h) and euglycemia was maintained with a variable-rate 35% (wt/vol) dextrose infusion, which was adjusted every 10 to 15 minutes. Also at time 0, a constant infusion at 10 μCi/h of ^{3}H-6-glucose (Amersham Australia, Sydney, Australia) was commenced. Animals were randomly allocated to receive either an infusion of 0.6 mL/h Intralipid (20% TG; Travenol, Sydney, Australia) combined with heparin at 40 U/h (TG/H-infused group, n = 6) or normal saline (saline-infused group, n = 6) also commencing at time 0.

An initial blood sample of 600 μL was collected at time 0 for estimation of plasma insulin, NEFA, and TG levels. Similar samples were taken at time 180 and 300 minutes. From 60 minutes onwards, 200 μL of blood was collected every 15 minutes for plasma glucose and [^{3}H]glucose determination. All withdrawn red blood cells were returned

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Table 1. Basal Body Weight, and Basal and Clamp Plasma Levels of Glucose, Insulin, NEFAs and TGs

Variable	Saline-Infused Group		Lipid-Infused Group	
	Basal	Clamp	Basal	Clamp
Body weight (g)	366 ± 2		370 ± 5	
Plasma glucose (mmol/L)	7.6 ± 0.1	7.5 ± 0.1	7.6 ± 0.1	7.6 ± 0.1
Plasma insulin (pmol/L)	220 ± 25	650 ± 60	235 ± 45	780 ± 90
Plasma NEFA (mmol/L)	0.66 ± 0.11	0.33 ± 0.06	0.67 ± 0.15	2.19 ± 0.38†
Plasma TG (mmol/L)	1.12 ± 0.11	0.62 ± 0.13	1.23 ± 0.22	1.07 ± 0.16*

NOTE. Clamp plasma glucose levels are the average of the levels over the 300-minute infusion period, whereas the clamp insulin, NEFA, and TG levels are the average of the 180- and 300-minutes levels. n = 6 in each group with data expressed as the mean ± SE.

*$P < .05$ and †$P < .01$ *v* clamp period of the saline-infused group.

to the animal after resuspension in 200 μL of 0.9% saline. Hematocrit was determined at the start and at the end of each experiment.

At the end of the clamp period, the rats were killed with a lethal dose of pentobarbitone sodium (Nembutal; Abbott Laboratories, Sydney, Australia). Red portions of the gastrocnemius and quadriceps muscles, plus liver were rapidly removed and freeze-clamped in liquid nitrogen and then stored at −70°C for subsequent biochemical assays.

Analytical Procedures

Whole-body glucose turnover. For the determination of tritiated plasma glucose, each plasma sample was initially deproteinized with $ZnSO_4$ and $Ba(OH)_2$, then dried to remove tritiated water before counting of radioactivity. Steele's non–steady-state equations were used to calculate glucose disappearance (Rd), and hepatic glucose output (HGO) was calculated as rate of glucose appearance (Ra) minus the glucose infusion rate (GIR).[17]

Tissue-specific analyses. The net rate of glycogen synthesis in red skeletal muscle and liver was calculated according to the method of Rossetti and Giacarri (net rate of glycogen synthesis = dpm of tritium in glycogen per gram of tissue/area under the curve for glucose-specific activity).[18] Tissue glycogen levels were determined as described by Handel.[19] In vitro GSa was determined in the absence and presence of five glucose-6-phosphate (G-6-P) concentrations (0.1, 0.5, 1, 5, and 10 mmol/L) over 5- and 10-minute periods according to the method of Thomas et al[20] and Nuttall and Gannon.[21] Fractional velocity of GSa was calculated as the rate of incorporation of labeled uridine diphosphate (UDP)-^{14}C-glucose into glycogen at 0.1 mmol/L G-6-P divided by the rate at 10 mmol/L and expressed as a percentage.

Tissue TG levels were determined by thin-layer chromatography and Coomassie blue staining according to the method of Nakamura and Handa.[22] Red skeletal muscle and liver LCAC content were measured as previously described.[23] Essentially, this method involves extraction and purification of LCAC from tissues and injection into a Novapak C18 reverse-phase high-performance liquid chromatography (HPLC) column (Waters Millipore, Milford, MA). Individual LCAC species were identified according to their respective retention times under UV detection. Peaks corresponding to palmitoyl (16:0), palmitoleoyl (16:1), linolenoyl (18:3), linoleoyl (18:2), oleoyl (18:1), and stearoyl (18:0) coenzyme A were identified and individually quantitated with respect to a known amount of internal standard (lauryl coenzyme A 12:0).

Other analytical methods. Plasma glucose levels were analyzed by the glucose oxidase method (Yellow Springs Instruments, Yellow Springs, OH). Plasma insulin was measured by radioimmunoassay (Linco Research, St Charles, MO). Plasma NEFA levels were determined by an acyl-coenzyme A oxidase-based colorimetric kit (Wako Pure Chemicals Industries, Osaka, Japan) and plasma TGs were measured by Triglyceride (INT), Procedure no. 336 (Sigma Diagnostics, St Louis, MO).

Data analyses. Results are expressed as the mean ± SE. Comparisons between groups were made using Student's *t* test for unpaired data. Regression analyses were performed to determine if significant changes occurred with time over the 5-hour study in each group. Two-way ANOVA was used to examine the group effect on GSa (Macintosh Statview SE + Graphics program; Abacus Concepts, Brain Power, Berkeley, CA).

RESULTS

Average plasma glucose levels for the duration of the clamp were similar in both saline- and TG/H-infused groups. The average of the 180- and 300-minute plasma insulin levels was also similar between the two groups, while the average of the 180- and 300-minute plasma NEFA and TG levels were elevated in the TG/H-infused group compared with the control group (Table 1). There was no significant difference in hematocrit between the initial and final level in either group or between groups (44% ± 1% *v* 43% ± 1% and 44% ± 1% *v* 43% ± 1% for saline- and TG/H-infused groups, respectively).

Systemic Glucose Fluxes

Compared with the saline-infused group, the TG/H-infused group demonstrated a marked reduction in GIR evident from 150 minutes onwards, a reduction in Rd from 180 minutes onwards, and an elevation of HGO from 180 to 270 minutes (Fig 1). These data suggest that insulin resistance in the TG/H-infused group began on a whole-body scale at about 150 minutes after TG/H infusion; this was accounted for by approximately equal magnitude of decrease in insulin sensitivity in the periphery and the liver.

Interestingly, after 120 minutes, there was a small but significant progressive increase in Rd of the saline group (slope = 0.01 mg/kg/min^2, $P < .05$), which was also seen with HGO of both the saline- and TG/H-infused groups (slope = 0.03 and 0.05 mg/kg/min^2, respectively; $P < .05$ for both).

Skeletal Muscle and Liver Glycogen, TG, and LCAC Content

Compared with the saline-infused group, glycogen content in the TG/H-infused group was significantly reduced ($P < .05$) in red gastrocnemius at the conclusion of the 300-minute infusion period (Fig 2). Glycogen content in the liver was similar for the saline- and TG/H-infused groups (42.0 ± 6.0 *v* 47.9 ± 12.5 μmol/g of wet weight, respectively). Compared with the saline group, TG content was increased in the TG/H-infused group in red gastrocnemius muscle (Fig 2). Also compared with the saline group, an approximately twofold increase in LCAC content was found in the TG/H-infused group in red quadriceps muscle (Fig 2) and in the liver (34.1 ± 6.0 *v* 65.8 ± 8.5 nmol/g of wet weight, respectively; $P < .01$). Of the six individual LCAC species, in the Intralipid-infused group, linoleoyl and oleoyl coenzyme A were significantly increased in the liver and red quadriceps muscle ($P < .01$), as well as palmitoyl coenzyme A in red quadriceps ($P < .05$). The Intralipid infusion contains largely soya oil, of which 72.3% of the fatty acids is

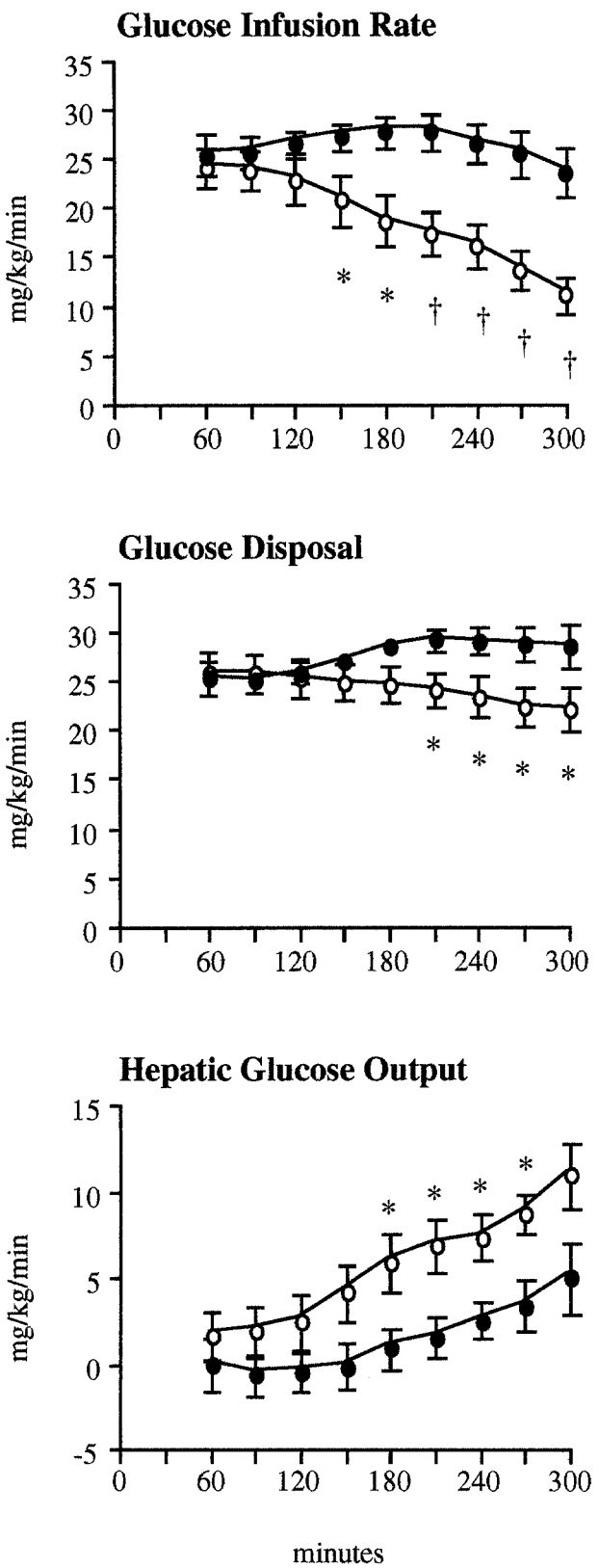

Fig 1. Whole-body GIR, Rd, and HGO. Data for saline-infused group (●; n = 6) and TG/H-infused group (○; n = 6) are expressed as the mean ± SE. *$P < .05$ and †$P < .01$ *v* saline group.

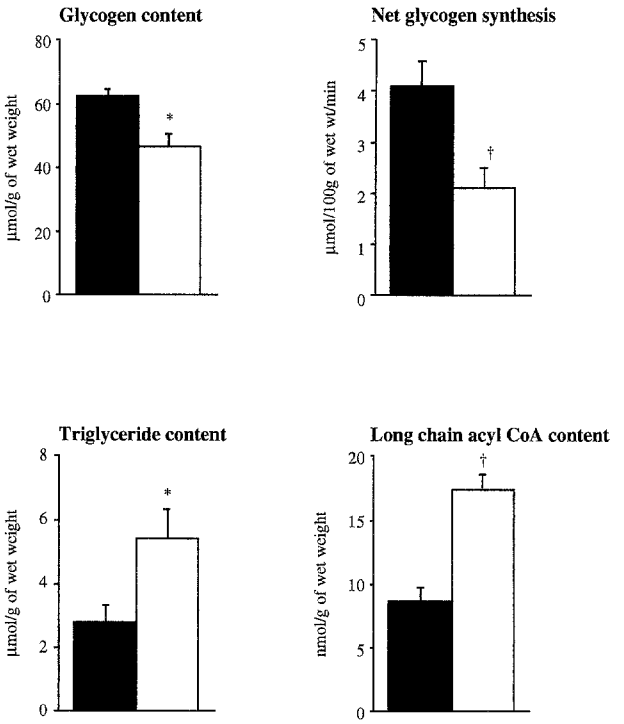

Fig 2. Glycogen content, net rate of glycogen synthesis, and TG content of red gastrocnemius muscle, and LCAC content of red quadriceps muscle. Data for saline-infused group (■; n = 6) and TG/H-infused group (□; n = 6) are expressed as the mean ± SE. *$P < .05$ and †$P < .01$ *v* saline group.

linoleate, 13.4% is oleate, 6.2% is palmitate, 2.4% is stearate, and 0.3% is linolenate.[24] Thus the increase in LCAC species in the liver and red quadriceps muscle reflects the composition of the fatty acids in the Intralipid infusion.

Net Rate of Tritiated Glucose Incorporation Into Glycogen and GSa

Consistent with the glycogen content changes at the conclusion of the clamp, the net rate of tritiated glucose incorporation into glycogen was significantly ($P < .01$) reduced in red gastrocnemius muscle (Fig 2), but not in the liver (0.9 ± 0.1 *v* 1.0 ± 0.1 µmol/100g wet weight/min, respectively, for saline- and TG/H-infused groups). The in vitro activity of glycogen synthase in red gastrocnemius muscle excised at the conclusion of the 300-minute infusions was significantly lower in the TG/H-infused group at 0.1, 0.5, and 10mmol/L G-6-P stimulation ($P < .05$; Fig 3). GSa was significantly lower in the TG/H-infused group ($P = .02$, two-way ANOVA) taken over all the G-6-P levels. There was no significant difference between the fractional velocity of the saline- and TG/H-infused groups (57% ± 3% *v* 54% ± 4%, respectively).

DISCUSSION

The main focus in this study was the effect of a TG/H infusion on glycogen synthesis and GSa. In red gastrocnemius muscle, the increase in lipid supply produced by a TG/H infusion reduced the net rate of glycogen synthesis in vivo and reduced GSa in vitro at several G-6-P levels. These findings are

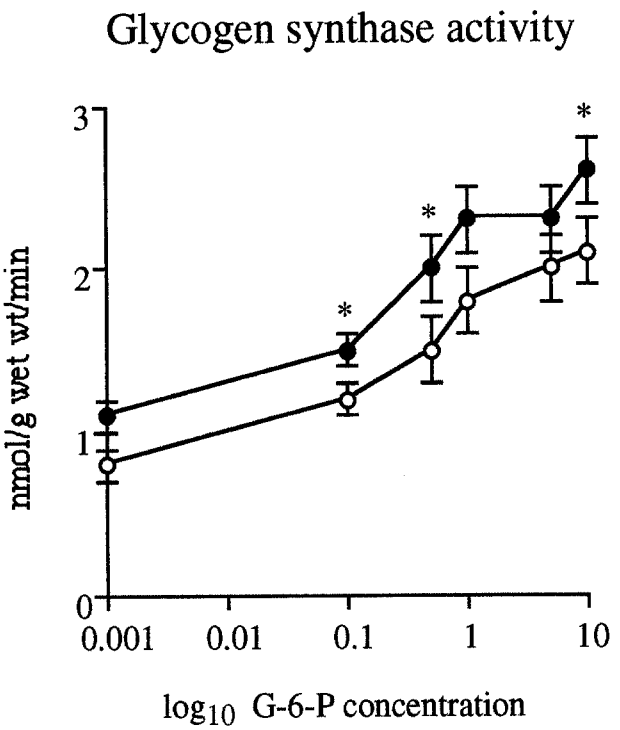

Fig 3. In vitro GSa (average of 5 and 10 minute assays) in absence or presence of increasing G-6-P concentrations. Data for saline-infused group (●; n = 6) and TG/H-infused group (○; n = 6) are expressed as the mean ± SE. *$P < .05$.

in agreement with several human studies[2,3,5] that demonstrated an inhibition of GSa with TG/H infusion, and suggest that previously reported studies in the rat,[8-10] including one from our group,[7] were not continued long enough to allow the development of a defect in insulin-mediated glycogen synthesis.

It has been suggested that the flux through glycogen synthase is regulated through the proximal steps (glucose transport/phosphorylation) via G-6-P levels in both human and rat muscle.[25] In the study by Boden et al,[3] at the time glycogen synthase was inhibited, G-6-P levels were elevated and these elevated levels would ordinarily allosterically stimulate GSa via stimulation of synthase phosphorylase.[26] Hence, the decreased rate of glycogen synthesis could not be entirely attributable to a decrease in glucose flux through glucose transport and/or phosphorylation. Similarly, in our study, after 5 hours of TG/H infusion, GSa was not able to be stimulated to the level of activity found in the saline-infused group at several G-6-P concentrations in vitro, and especially at G-6-P concentrations that would be expected to occur during a hyperinsulinemic euglycemic clamp in rats.[3,18] This, taken together with the finding of reduced rate of net glycogen synthesis in vivo, suggests that glycogen synthase was inhibited irrespective of the prevailing G-6-P concentration. An increased glycogen content may inhibit glycogen synthesis by negative feedback inhibition[27]; however, in our study, the decrease in glycogen synthesis was associated with reduced glycogen content, suggesting another mechanism, possibly occurring through accumulation of one or more fatty acid metabolic products within the cell. The notion that fat accumulation in the cell is a major factor in the etiology of insulin resistance of obesity and NIDDM has been considered previously and could be due to an accumulation of TG or LCAC.[1,28-30]

An elevated TG concentration within muscle has been associated with insulin resistance in high-fat–fed rats,[11,12] and also in people with NIDDM.[31] However, elevated TG levels are also found in exercise-trained muscles,[32] where there is enhanced insulin sensitivity. So, an elevated TG content per se may not necessarily cause insulin resistance in skeletal muscle. TGs are stored as neutral fat within muscle, whereas LCACs, the activated form of fatty acids, are one of the high-energy molecules within cells. Relevant to the insulin resistance of high-fat feeding in rats, elevated concentrations of LCACs in muscle and liver have correlated positively with fasting plasma insulin levels.[33] LCACs can be derived from intracellular TG, produced as an intermediate in de novo lipid synthesis, or derived from extracellular TG and NEFAs. Glucose metabolic enzymes influenced by cytosolic or mitochondrial LCACs include glucokinase, glycogen synthase, and pyruvate dehydrogenase.[14,34,35] The effect of LCACs on enzymes occurs at levels below the critical level of micelle formation (which is ≃ 30 to 60 μmol/L for palmitoyl coenzyme A).[35] That is, enzyme inhibition can occur at levels of LCAC below those that would have nonspecific effects through detergent action.

In our study, the sum content of LCAC species in skeletal muscle (red quadriceps) was elevated approximately twofold in the TG/H-infused compared with the saline-infused group, and is comparable to the levels found in 10-week fat-fed rats.[33] How LCACs affect glycogen synthase is speculative. In a recent review, Prentki and Corkey[30] proposed several possible mechanisms through which LCACs may act: protein acylation, effects on genes encoding metabolic enzymes, effects on complex lipid formation, or effects on protein kinase C subtypes.

Wititsuwannakul and Kim[14] found that palmitoyl coenzyme A dissociated hepatic glycogen synthase from its active tetramer form into monomers that were unable to bind to the primer glycogen. However, it is not known whether similar mechanisms occur in muscle. It is interesting that high-fat feeding in addition to increasing LCAC content in muscle has been shown to alter protein kinase C isoenzymes ϵ and θ.[36] Furthermore, certain isoenzymes of protein kinase C have been shown to phosphorylate glycogen synthase[37] and multisite phosphorylation of glycogen synthase may be associated with decreased activation.[38]

In NIDDM, skeletal muscle GSa but not content is reduced.[39] Similarly, in our study, there was an inability to stimulate GSa in vitro in the TG/H group to the same extent as in the saline-infused group. Since the duration of TG/H infusion is only 5 hours, we consider it is unlikely that this is due to a decrease in glycogen synthase protein content; however, some form of noncompetitive inhibition might account for this, such as could occur with an increased state of glycogen synthase phosphorylation.

There was a significant increase in HGO with time in both groups, although this was more marked in the TG/H-infused group. This might be explained by nonsuppression of diurnal corticosterone release in rats during a hyperinsulinemic euglycemic clamp as previously shown by Koopmans et al,[40] or by some systematic stress that both groups of rats were possibly

exposed to. Findings of elevated HGO with elevated NEFA levels is consistent with another recent study in which NEFA levels and HGO were lowered by the thiazolidinedione BRL 49653,[41] and is also consistent with a recent report of an indirect effect of insulin on HGO acting via suppression of NEFA levels.[42] Another possible contributing factor to the increased HGO in the TG/H group could be the increased liberation of glycerol from intravascular triglyceride hydolysis.

The slight but significant upward trend in peripheral glucose disposal over the 5-hour hyperinsulinemic euglycemic clamp in the saline-infused group may be due to an increasing glucose uptake into white adipose tissue, as previously shown,[43] with an increasing incorporation of glucose into lipids with continued hyperinsulinemia.[44]

Thus, increased lipid availability, (elevated plasma NEFA and TG levels, and increased tissue TG and LCAC content) was associated with an inhibition of glycogen synthesis and decreased skeletal muscle GSa and increased HGO. The 5-hour TG/H infusion model of insulin resistance resembles an acute form of the several-week high-fat feeding model in rats with increased muscle content of TG and LCAC, as well as inhibition of insulin-mediated glycogen synthesis and GSa. With the TG/H infusion, peripheral and hepatic insulin resistance appeared to develop at about the same time, whereas with the high-fat–fed model, insulin resistance in the liver occurs earlier than in the periphery.[11] Possible reasons for this are the mode of delivery and the degree of fat elevation; intravenous delivery of lipid produces a more prolonged systemic elevation of lipid levels and may induce peripheral insulin resistance more readily. Overall, however, the 5-hour TG/H-infused rat is a suitable model to study the effects of increased fat availability on glucose metabolism; the relatively short time frame for development of insulin resistance may have advantages in determining potentially causative associations between lipid related metabolites and insulin-induced cellular metabolic changes.

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