Acute hyperinsulinemia inhibits intramyocellular triglyceride synthesis in high-fat-fed obese rats

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Abstract Hyperinsulinemia is common in obesity, but whether it plays a role in intramyocellular triglyceride (imcTG) buildup is unknown. In this study, hyperinsulinemic-euglycemic clamp experiments were performed in overnight-fasted lean and high-fat-fed obese rats, awake, to determine the effect of insulin on imcTG synthesis (incorporation of [14C]glycerol, [14C]glucose, and [3H]oleate). Insulin infusion at 25 (low insulin) and 100 (high insulin) pmol/kg/min increased plasma insulin by 5- and 16-fold, respectively, whereas plasma and intramyocellular glycerol, FFAs, triglycerides, and glucose levels were maintained at their basal levels by co-infusion of exogenous glycerol, FFAs, triglycerides, and glucose. ImcTG pool size was not affected by insulin (P > 0.05). The high insulin suppressed incorporation of fatty acid into imcTG-glycerol in all three muscles (P < 0.01–P < 0.001) in gastrocnemius and tibialis anterior, but only the high insulin suppressed it in soleus (P < 0.05). The low insulin suppressed glucose incorporation into imcTG-glycerol in all three muscles (P = 0.01–P < 0.01). However, the low insulin did not affect (P > 0.05) and the high insulin suppressed (P < 0.05–P < 0.01) fatty acid incorporation into imcTG in all three muscles. Insulin also suppressed glycerol incorporation in lean rats (P < 0.01–P < 0.04). On the other hand, imcTG pool size was not affected by insulin (P > 0.05). These observations suggest that acute hyperinsulinemia inhibits imcTG synthesis and thus does not appear to promote imcTG accumulation via the synthetic pathway, at least in the short term.—Guo, Z.K., L. Zhou, and M. D. Jensen. Acute hyperinsulinemia inhibits intramyocellular triglyceride synthesis in high-fat-fed obese rats. J. Lipid Res. 2006, 47: 2640–2646.

Supplementary key words muscle • triglycerides • insulin • obesity

The content of intramyocellular triglycerides (imcTG) in skeletal muscle of obese subjects is abnormally high, and this imcTG excess is significantly correlated with muscle insulin resistance (1–3). However, the pathways responsible for the lipid accumulation have not been identified. Some observers have suggested that decreased imcTG lipolysis and oxidation contribute to excess lipid accumulation in skeletal muscle (4). Other reports have suggested that lipid oxidation in obesity is increased instead (5). Therefore, whether and how lipid oxidation plays a role in imcTG accumulation in obesity remains a subject of debate. By comparison, there has been much less discussion on the role of imcTG synthesis in the lipid abnormality, even though synthesis is the initial step of imcTG metabolism. We recently found that imcTG synthesis in skeletal muscle is greatly accelerated in rats after 3 months on a high-fat diet, and this increase persists to at least 12 months of age on the diet (6). The finding indicates that the synthetic limb of imcTG metabolism probably participates in the abnormal lipid accumulation, at least in this animal obesity model. The accelerated imcTG synthesis, however, is not attributable to direct dietary fat effects, as one might suspect, but is instead due to a phenotype effect of obesity (6).

Because hyperinsulinemia is a characteristic of human obesity and type 2 diabetes, and because insulin is an anabolic hormone, conceptually, hyperinsulinemia may promote imcTG synthesis and thus accumulation. For example, in vitro insulin was found to stimulate imcTG synthesis (7–9). Thus, clarifying a potential role of hyperinsulinemia in imcTG accumulation is important. Currently, however, limited information on this topic is available only from in vitro studies, and in vivo data are scarce. Therefore, in vivo studies are needed. The high-fat-fed obese rat is a useful model for this purpose, because it is mildly insulin resistant without apparent hyperinsulinemia. Thus, the effects of insulin on imcTG synthesis can be manipulated and examined. The present study was designed to test the hypothesis that hyperinsulinemia stimulates imcTG synthesis and promotes its accumulation in this obesity model. To gain more-global insights into skeletal muscle lipid metabolism, three muscle types were studied: red gastrocnemius (FOG), soleus (SO), and tibialis anterior (FG) (10).

Abbreviations: FG, fast-twitch glycolytic; FOG, fast-twitch oxidative-glycolytic; G3P, glycerol 3-phosphate; imcTG, intramyocellular triglyceride; SA, specific activity; SO, slow-twitch oxidative.

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MATERIALS AND METHODS

Animals

Male Sprague Dawley rats (100 g body weight) were purchased from Harlan Sprague Dawley (Indianapolis, IN). Three days after delivery, they were switched to a high-fat pellet diet (55% calories from lard; Dytes Inc., Bethlehem, PA) (obese) or continued to feed on a standard rat chow (lean), ad libitum, with free access to drinking water. The animals were kept in a room with 12 hr light/12 hr dark cycles and stable temperature and humidity. After 4 months, they were studied awake after an overnight fast (16 h). At the time of the study, the body weight of the obese rats was 20–25% greater than that of the lean rats. The study protocols were approved by the Mayo institutional animal care and use committee.

Study design

After an overnight fast, the rats were catheterized in a tail artery using procedures established previously (11) for glucose monitoring and blood collection. Briefly, while the rats were restrained in a plastic tube, the catheterization site was anesthetized with 2% lidocaine subcutaneously and peri-arterially. No general anesthetics were used. A flexible plastic catheter (2.5 × 0.8 mm) was then surgically inserted into the ventral tail artery 3 cm distal to the tail base. The skin incision (1 cm long) was closed by surgical sutures, and the long-lasting local anesthetic bupivacaine was topically applied to the site. The rats were then transferred to spacious metabolic cages (1 cubic foot), with the catheter and the catheterized tail entirely exposed to the exterior and retained using a rubber-cushioned metal clamp proximal to the catheter. The clamp was engineered such that tail blood flows were not affected. The rats were allowed to rest in the cages, where they could move laterally and upward, groom, and drink water; but they usually stayed calm and relaxed. Regression of stress level was assessed by monitoring blood glucose using a glucose monitor (Accu-Chek; Boehringer Mannheim). When glucose returned to basal level (Fig. 1), experiments were initiated by inserting a flexible plastic i.v. infusion line, instantaneously by venipuncture, into a lateral tail vein, with a 25 G needle; the line was secured by taping. Then the following study protocols were started immediately.

Main study protocol

The study protocol for determining imcTG synthesis started with the infusion of a porcine insulin solution at 0 (saline) or 25 pmol/kg/min (n = 8 each; body weight 513 ± 13 g and 539 ± 17 g, respectively). The insulin infusion rate was designed to achieve an increase of 5-fold in plasma level to mimic the pathophysiologic hyperinsulinemia seen in obesity. Simultaneously, glycerol, FFAs (from soy oil, as a solution at 60 mmol/l in 5% human albumin, pH 8–9), and triglycerides [10% Intralipid (Fresnius Kabi Clayton, Clayton, NC) without heparin] were co-infused at previously determined rates (Fig. 2) required to maintain their levels without major changes. Blood glucose was maintained at basal level by varying infusion rates. When a steady state was achieved (≥30 min after the initiation of the insulin/substrate infusions), as indicated by stable blood glucose from two consecutive measurements, the tracer infusions were begun. [U-14C]glycerol and [9,10-3H]oleate were constantly infused at 0.1 and 0.2, respectively, μCi/kg/min for 120 min without priming. All infusions of insulin, substrates, and tracers were via the single i.v. line and were driven by two mini infusion pumps (Harvard Apparatus), undisturbed except for glucose, which was adjusted every 15 min, when necessary, based on real-time blood glucose levels. The metabolic conditions thus achieved are stable substrates with insulin as the predominant variable. At the final 20, 10, and 0 min, blood samples of 1 ml were collected and the plasma separated on site and saved in liquid nitrogen. After the...

Fig. 1. The time course of blood glucose in rats after the arterial catheterization until the end of the hyperinsulinemic-euglycemic clamp. The catheterization increased blood glucose dramatically, followed by rapid regression, returning to baseline while resting in the metabolic cage (this dynamic phase is shown for obese rats only). At −20 min (arrow), infusions of insulin, FFA, glycerol, and Intralipid were started. When two consecutive measurements indicated blood glucose to be within 10% of baseline value, the infusions of tracers (constant rates) and glucose (varying rate) were started (0 min). At this point, plasma FFA, glycerol, and TG were also stabilized until the end of the experiment (Fig 3). There were no statistically significant differences in blood glucose between lean and obese rats during the tracer infusion.

Fig. 2. Substrate co-infusion rates required for maintaining basal plasma levels during hyperinsulinemic-euglycemic clamp at increasing insulin in obese rats. The insulin infusions resulted in linear increases in plasma insulin ([insulin]), whereas substrate infusion rates increased curve-linearly. The data are from separate experiments but using the same protocol designed to determine substrate infusion rates at different insulin levels (n = 6 for each insulin level). The infusion rates of glycerol, fatty acids, and triglycerides (10% Intralipid, no heparin) used in the present studies were determined using this chart. Glucose infusion rates were determined in individual experiments of this study, and they are given here for reference purposes only. The corresponding substrate co-infusion rates were slightly lower (not shown), whereas that for glucose was significantly higher (15) in lean rats.
last blood collection, the tracer infusion was terminated and the animals were anesthetized instantaneously by pentobarbital injection via the i.v. line (i.e., 50 mg/kg). They were then immediately euthanized by bleeding to reduce blood in tissues. Red gastrocnemius, soleus, and tibialis anterior muscles were swiftly excised, cut into small pieces (10–20 mg), and saved in liquid nitrogen. The small muscle pieces were chilled to the core immediately, and thus their original metabolic state was preserved. With further extreme care during subsequent processing and analysis, as described below, changes in intramyocellular metabolites were kept to a minimum.

**Supplemental studies**

_Lean versus obese._ Obese and lean rats (n = 7 each, body weight ~550 g and 450 g, respectively) were studied using the same protocol but with insulin at 100 pmol/kg/min. Because of greater suppression of lipolysis by the high insulin, glycerol and fatty acids were co-infused at higher rates determined to maintain their basal plasma levels (Fig. 2).

_Glucose incorporation._ Two groups of four rats each (579 ± 21 g), with saline or insulin infusion at 25 pmol/kg/min, were studied using the same protocol except that [6-14C]glucose was the tracer (0.1 μCi/kg/min) to determine the effect of insulin on the incorporation of glycerol derived from glucose into imcTG-glycerol. Sampling and sample analyses were identical to those of the main study.

**Sample processing and analysis**

The frozen muscle samples, in small pieces (~20 mg), were individually thawed on ice and dissected under a stereo microscope (10–40×, Motic Microscopes, Hong Kong, China), still on ice, to visualize and remove adipocytes and small blood vessels that are usually associated with fat cells. Muscle fibers were gently manipulated to expose the interstitial space, followed by a rinse in cold saline to remove interstitial metabolites. On average, it took 2–5 min to complete the microdissection of a piece of muscle. The dissected muscles were immediately homogenized in chloroform-methanol (2:1) kept on ice, using an electric homogenizer. The homogenate was extracted for intracellular free glycerol and glucose (the upper phase) and total lipids (the lower phase) according to the method of Folch, Lees, and Sloane-Stanley (12). The upper phase was loaded onto a preconditioned resin, hydroxide form, 200–400 mesh). Free glycerol was eluted with hexane) mini ion exchange column (Bio-Rad; AG1-X8 resin, 20–40 mesh) according to the method of Folch, Lees, and Sloane-Stanley (12). The upper phase was loaded onto a preconditioned resin (with hexane) mini ion exchange column (Bio-Rad; AG1-X8 resin, 20–40 mesh). Free glycerol was eluted with hexane, followed by 1 N NaCl to elute glucose. The radioactivity resulting glycerol and fatty acid moieties were quantified enzymatically (13), and their SA was determined by liquid scintillation counting.

**Plasma**

The concentrations of FFA, triglycerides, and free glycerol were determined using the same enzymatic methods (13). An aliquot of plasma was treated with equal volumes of 3N ZnSO4 and 3N NaOH to precipitate the proteins. Plasma free glycerol was then separated by subjecting the diluted serum to the same ion exchange column. The dpm and SA of the isolated glycerol were determined by liquid scintillation counting. Plasma insulin concentration was determined using a kit (RI-13K) from Linco (St. Louis, MO). Muscle diglyceride acyltransferase (DGAT) activity was determined using [14C]palmitoyl CoA as a tracer.

**Calculations**

_Synthesis of imcTG is expressed as the rate of incorporation of intramyocellular free glycerol into imcTG._

glycerol incorporated (nmol/g wet weight/min)  
= _[^14C]dpm of imcTG-glycerol/g_  
÷ _SA of muscle free glycerol (dpm/nmol)/120._  

(Eq. 1)

The use of glycerol as a tracer was based on our previous finding that blood glycerol is actively incorporated into imcTG in fasted rats (14).

**Incorporation of glucose and fatty acids into imcTG**

The incorporation of intramyocellular glucose into imcTG-glycerol and nonesterified fatty acids (NEFAs) into imcTG (nmol/g wet weight/min) was calculated according to equations analogous to Equation 1, using muscle glucose SA or NEFA SA as the denominator, respectively.

**Statistics**

Values are mean ± SEM, unless otherwise indicated. Comparisons between hyperinsulinemia and control groups were made using unpaired _t_ test. Comparisons among three muscles or different time points used repeated measures analysis. Alpha of 0.05 was used as the cutoff for statistical significance.

**RESULTS**

**Overall metabolic profile**

During the clamp (25 pmol insulin/kg/min), plasma insulin concentration in obese rats increased to 1.0 ± 0.2 nmol/l from a baseline value of 0.2 ± 0.01 nmol/l ( _P_ < 0.001). On the other hand, plasma glycerol, FFAs, and triglycerides were kept stable during the clamp (Fig. 3). Therefore, insulin was the predominant, if not the sole, variable. In the supplemental studies with insulin infused at 100 pmol/kg/min, plasma insulin in obese rats increased to 3.3 ± 0.5 nmol/l, whereas glycerol, FFAs, and blood glucose remained unchanged. In the lean rats, plasma insulin, glycerol, and glucose were not different from levels in obese rats, but fatty acids were lower than those in obese rats (585 ± 78 vs. 694 ± 37 pmol/l; _P_ < 0.05).

**Intramyocellular metabolites**

The concentrations of free glycerol, nonesterified fatty acids, and imcTG in muscles of obese rats were not affected by insulin infusion at 25 pmol/kg/min (_P_ > 0.05) in the main study (Table 1), nor at 100 pmol/kg/min in the supplemental study (not shown).

**imcTG synthesis**

The rate of glycerol incorporation into imcTG of gastrocnemius (0.9 vs. 2.4 nmol/g/min; _P_ < 0.01) and tibialis
anterior (1.8 vs. 3.6 nmol/g/min; P < 0.01) decreased significantly compared with saline infusion in the initial study; in contrast, it increased by 34% in soleus (4.7 vs. 3.5 nmol/g/min; P < 0.05) (Fig. 4). At 100 pmol/kg/min, insulin decreased glycerol incorporation further to 0.54 ± 0.15, 0.53 ± 0.12 and 0.92 ± 0.3 nmol/g/min in gastrocnemius, tibialis anterior and soleus, respectively (all P < 0.01) in obese rats. In lean rats, glycerol incorporation into imcTG was 0.28 ± 0.08, 0.30 ± 0.10 and 0.48 ± 0.13 nmol/g/min in the basal state and was suppressed to 0.12 ± 0.05 (P = 0.01), 0.08 ± 0.02 (P < 0.01) and 0.32 ± 0.08 (P = 0.04) by the high insulin, for the same muscles, respectively.

Glucose incorporation

Table 2 shows that insulin infusion at 25 pmol/kg/min also resulted in marked suppression of glucose incorporation into imcTG by ~70% in gastrocnemius and tibialis anterior and ~40% in soleus.

Fatty acid incorporation

Figure 5 shows that although insulin infusion at the lower rate did not significantly affect the incorporation of NEFA into imcTG, at the high infusion rate, insulin significantly suppressed the incorporation of NEFA into imcTG by 50% (soleus) to 60% (gastrocnemius and tibialis anterior).

Specific activity of muscle free glycerol

Table 3 shows that during the clamp with low insulin, the SA of muscle free glycerol in gastrocnemius and tibialis anterior did not change, but it decreased in soleus (P < 0.05).

DGAT activity

To determine whether DGAT activity affected imcTG synthesis, its activity in gastrocnemius was determined. DGAT activity was not affected (P > 0.05) by the low or high insulin (6.2 ± 1.4 and 6.3 ± 2 nmol/g/min, respectively, vs. the basal value of 6.8 ± 0.9 nmol/g/min).

DISCUSSION

The obesity model

As we have previously reported, the high-fat-fed obesity model employed in the present study has modestly

## Table 1. Effect of insulin on intramyocellular metabolite concentrations in skeletal muscle of high-fat-fed obese rats

| Insulin infusion rate | Non-esterified fatty acids | Glycerol | imcTG |
|----------------------|----------------------------|----------|-------|
|                      | 0 (saline)                 | 25       |       |
|                      | Gn | So | TA | Gn | So | TA | Gn | So | TA |
| 0 (saline)           | 0.52 ± 0.06 | 1.19 ± 0.13 | 0.46 ± 0.05 | 0.41 ± 0.04 | 0.44 ± 0.04 | 0.37 ± 0.03 | 1.08 ± 0.13 | 3.1 ± 0.4 | 1.29 ± 0.10 |
| 25                   | 0.53 ± 0.04 | 1.05 ± 0.03 | 0.53 ± 0.05 | 0.49 ± 0.03 | 0.44 ± 0.05 | 0.49 ± 0.06 | 1.14 ± 0.19 | 3.5 ± 0.12 | 1.35 ± 0.18 |

Values are mean ± SEM (μmol/g wet muscle weight). The metabolites were extracted from red gastrocnemius (Gn), soleus (So) and tibialis anterior (TA) collected at the end of the clamp (see Materials and Methods). No differences were found for any metabolites between the basal state and clamp (P > 0.05). Insulin infusion rate, pmol/kg/min (at the infusion rates as shown, plasma concentrations were 0.2 ± 0.01 and 1.0 ± 0.2, respectively; n = 8 in each group).
TABLE 2. Effect of insulin infusion at 25 pmol/kg/min on the incorporation of glucose into imcTG in skeletal muscle of high-fat-fed obese rats

| Infusion     | Gastrocnemius | Soleus | Tibialis Anterior |
|--------------|---------------|--------|-------------------|
| Saline       | 7.80 ± 0.58   | 3.77 ± 0.43 | 12.30 ± 2.2       |
| Insulin      | 2.20 ± 0.35*  | 2.17 ± 0.08b | 4.04 ± 0.43b      |

Values are glucose incorporation (nmol/g/min) measured using [6-14C] glucose as a tracer (mean ± SEM, n = 4).

* Different from saline infusion at P < 0.01.

b Different from saline infusion at P = 0.01.

Fig. 5. Effects of insulin on the incorporation of fatty acids into imcTG of skeletal muscle in high-fat-fed obese rats as determined in the same hyperinsulinemic-euglycemic clamp experiments shown in Fig 4. The legend shows plasma insulin levels (nmol/l) resulting from insulin infusion at 0, 25, and 100 pmol/kg/min, respectively. ↑, *: Different from basal insulin (saline infusion) at P < 0.01 or P < 0.05, respectively. Open bar, plasma insulin at 0.2 nmol/l; closed bar, insulin at 1.0 nmol/l; dotted bar, insulin at 3.3 nmol/l. Error bars represent SEM.

TABLE 3. Specific activity of muscle free glycerol during hyperinsulinemic-euglycemic clamp in high-fat-fed obese rats

| Insulin Infusion (pmol/kg/min) | Gastrocnemius | Soleus | Tibialis Anterior |
|-------------------------------|---------------|--------|-------------------|
| 0 (saline)                    | 2.3 ± 0.3     | 3.1 ± 0.4 | 2.6 ± 0.3       |
| 25                            | 2.4 ± 0.2     | 2.1 ± 0.2* | 2.6 ± 0.2       |

Values are mean ± SEM (dpm/nmol). The muscle samples were collected at the end of 120 min tracer infusion, and free glycerol was isolated from Folch upper phase by ion exchange for determination of specific activity (see Materials and Methods). Insulin at the infusion rates as shown resulted in plasma concentrations of 0.2 ± 0.01 and 1.0 ± 0.2 nmol/l, respectively.

* P = 0.05 compared with the baseline value (n = 8/group).

uncontrolled lipolysis (data not shown). Thus, although nonphysiologic, the coinfusion strategy helped to establish the needed metabolic conditions required for evaluating the effects of hyperinsulinemia per se on imcTG synthesis with minimized confounding effects. In fact, the metabolic conditions so achieved resemble those seen in the insulin resistance state, in which insulin is unable to promptly suppress lipolysis (thus unchanged fatty acid and glycerol concentrations).

**imcTG synthesis**

Basal imcTG synthesis in high-fat-fed obese rats is already markedly accelerated, compared with their lean littermates (6). Because insulin is a broad anabolic hormone, we suspected that imcTG synthesis might be further enhanced by hyperinsulinemia. In other words, we hypothesized that insulin regulation of imcTG synthesis is preserved, at least to a certain degree, in this mild insulin resistance model. The results supported this by showing the responses of imcTG synthesis to acute hyperinsulinemia. However, contrary to our hypothesis, the insulin effect on imcTG synthesis, as measured by glycerol incorporation (especially for FOG and FG muscle), was inhibitory in a dose-dependent fashion. The observed pattern of muscle type dependence of the insulin effect echoed that for imcTG turnover, where insulin is inhibitory in FOG and FG but not in SO muscle (16). In both cases, however, imcTG pool size was not changed in any muscle type, suggesting a steady state of imcTG metabolism at the time of measurements. Overall, the data on fatty acid incorporation confirmed the glycerol data. A major difference was that the inhibition was not seen until the higher insulin infusion rate. This suggested that there are differences in responding to insulin by glycerol compared to fatty acid incorporation. To test whether insulin-inhibitory actions are unique in obesity, where imcTG is abnormally high and synthesis is accelerated (6), we repeated the same protocol in lean rats. At the high insulin, glycerol incorporation into imcTG was suppressed by 56, 32 and 75% of the baseline values in FOG, SO, and FG muscle, respectively. Thus, insulin is inhibitory in lean rats as well.

The study of imcTG metabolism has been limited, and therefore insulin effect is not well understood. Nonetheless, similar observations on insulin inhibition of glycerol incorporation into adipocyte triglycerides have been re-

elevated (~20%) plasma fatty acids and impaired (25%) insulin-stimulated glucose disposal, but plasma insulin level is within normal range (15). Therefore, the model is mildly insulin resistant without overt hyperinsulinemia. As such, it is a useful model for investigating the acute effect of hyperinsulinemia on imcTG metabolism.

**Metabolic homeostasis**

Both plasma glycerol and FFA fall acutely in response to insulin. This affects the kinetics of their incorporation into imcTG, thereby confounding the insulin effect on imcTG synthesis, the objective of the present study. Thus, it became necessary to prevent this from occurring by compensatory substrate co-infusion in addition to glucose infusion. The results indicated that the concentrations of plasma glycerol and fatty acids were successfully maintained during the clamp with insulin as the predominant, if not the sole, variable. Meanwhile, the intramyocellular concentrations of these metabolites were also kept stable, probably as a consequence of their stability in the plasma. The brief muscle microdissection before lipid extraction involved minimal autolipolysis, as shown by stable NEFA concentration with low variability, which is impossible with
ported previously (17). However, in perfused rat leg muscle, insulin did not affect palmitate incorporation into imcTG (18). Insulin appeared to promote imcTG accretion in rats fed olive oil, but the synthetic kinetics was not determined (19). Some reports have indicated that imcTG synthesis is stimulated by insulin independently only in isolated muscle preparations (8, 9), but this has not been confirmed in vivo. This seems to suggest that the differences in metabolic milieu between in vivo and in vitro conditions may, at least in part, explain the discrepancy.

Because imcTG naturally undergoes lipolysis and esterification simultaneously (7, 20, 21), these two limbs of imcTG metabolism are mutually dependent (coupled). As such, changes in one probably propagate to the other. In fact, turnover, the rate at which imcTG is "renewed," is a measure of both hydrolysis and esterification. Therefore, fractional turnover essentially means that a certain amount (e.g., 1%/min in soleus; see Ref. 16) of imcTG is hydrolyzed and replenished so that its pool size remains unchanged. Therefore, the present data prompted us to propose that insulin probably affects substrate incorporation primarily via suppressing lipolysis (16). This slows down the normal recycling process (20), which, in turn, causes a secondary inhibition of glycerol/fatty acids incorporation. The stable imcTG pool size suggested that esterification and hydrolysis were approximately matched. For example, in gastrocnemius, the absolute insulin suppression of glycerol incorporation (180 nmol/g; Fig. 4) and hydrolysis (212 nmol/g; see Ref. 16) over the entire experiment was comparable. It is noted that the reduced glycerol incorporation was not caused by limited substrate availability, because glycerol 3-phosphate (G3P) was, in fact, increased, not decreased, in gastrocnemius and tibialis anterior (Table 4). This suggested that the G3P buildup was a result of reduced glycerol utilization, consistent with the glycerol incorporation data. Because both lipolysis (16) and synthesis (this study) were suppressed, imcTG-glycerol cycling must have been slowed down as a result. Whether this contributes to the excessive imcTG accumulation in obesity remains to be studied. Nonetheless, it seems clear that the lipolysis-synthesis coupling suggests that synthesis alone is of limited implication in imcTG accumulation. Conversely, lipolysis alone cannot predict pool size change (22).

In the present study, glycerol incorporation is quantified as the rate of tracer inflow into imcTG divided by the SA of muscle free glycerol (Equation 1). Because DGAT activity only affects the numerator, it is understandable that the enzyme activity may not match the results calculated using the equation, such as in the case of gastrocnemius. Therefore, measurement of imcTG synthesis using the equation has its limits, because it may appear discrepant under certain conditions. On the other hand, it has the advantage of taking both the product and precursor pools into account. For example, measuring isotope in the product pool alone may generate erroneous results. The results for soleus using Equation 1 may include some degree of error attributable to its slow isotope equilibration (14) due to its larger imcTG pool. By comparison, in the other two muscles, isotopic equilibration in the precursor pool reached steady state after 120 min of tracer infusion (14). The imcTG pool of soleus may have released a large quantity of less-labeled glycerol to render the SA of muscle free glycerol lower (Table 3). This could contribute to the distinctive pattern of glycerol incorporation and insulin effect observed for this muscle.

By using glycerol as the tracer to measure imcTG synthesis, the contribution of glucose carbons to imcTG is excluded. To assess whether this explains the observed insulin actions on imcTG-glycerol kinetics, the same protocol was repeated using [14C]glucose as the tracer. The results (Table 2) showed that incorporation of glucose into imcTG-glycerol was also suppressed by insulin. Therefore, although the true precursors for imcTG synthesis (G3P and acyl CoA) were not used in this study (Equation 1) due to technical infeasibility with radioactive tracers, the consistent data from all three tracers indicated that substrate incorporation into imcTG was indeed suppressed by insulin.

In summary, acute hyperinsulinemia inhibits imcTG synthesis without affecting imcTG pool size in high-fat-fed obese rats, and thus its role in promoting imcTG accumulation cannot be confirmed in the short term. However, the chronic effect of hyperinsulinemia on imcTG metabolism requires further investigation.

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### TABLE 4. Effect of insulin on concentration of muscle glycerol 3-phosphate in high-fat-fed obese rats

| Insulin Infusion Rate | Gastrocnemius | Soleus | Tibialis Anterior |
|-----------------------|---------------|--------|-------------------|
| 0                     | 67 ± 19       | 270 ± 58 | 33 ± 5           |
| 25                    | 125 ± 14      | 244 ± 26 | 112 ± 22         |
| P                     | 0.04          | 0.75    | 0.02              |

Values are mean ± SEM (nmol/g ww). Insulin infusion rate, pmol/kg/min (at the rates shown, plasma concentrations were 0.2 ± 0.01 and 1.0 ± 0.2 nmol/l, respectively).

P values: from comparisons between the two insulin infusion rates (n = 8/group).
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