Meal Frequency Influences Circulating Hormone Levels But Not Lipogenesis Rates in Humans

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To determine whether human lipogenesis is influenced by the frequency of meal consumption, 12 subjects were divided into two groups and fed isocaloric nutritionally adequate liquid diets over 3 days, either as three larger diurnal (n = 6) or as six small, evenly spaced (n = 6) meals per day. On day 2 (08:00 h) of each diet period, 0.7 g deuterium (D) oxide/kg body water was administered and blood was collected every 4 hours over 48 hours for measurement of plasma insulin and glucose-dependent insulinotropic polypeptide (GIP) levels. At each time point, the incorporation of D into plasma triglyceride fatty acid (TG-FA) was also determined by isotope ratio mass spectrometry after TG-FA extraction and combustion/reduction. Insulin and GIP levels were elevated over daytime periods in subjects fed three versus six meals per day. Contribution of de novo synthesis to total TG-FA production was not significantly different for days 2 and 3 in subjects consuming three (6.56% ± 1.32% and 6.64% ± 2.08%, respectively) and six (7.67% ± 2.29% and 7.88% ± 1.46%, respectively) meals per day. Net TG-FA synthesis rates over days 2 and 3 were 1.47 ± 0.33 and 1.55 ± 0.53 g/d, respectively, for subjects fed three meals per day, and 1.64 ± 0.47 and 1.69 ± 0.30 g/d for subjects fed six meals per day. These findings suggest that consuming fewer but larger daily meals is not accompanied by increases in TG-FA synthesis, despite the observation of hormonal peaks.

HUMANS CLEARLY POSSESS the capacity to convert energy precursors to fat, since lipogenic enzymes are present in adipose[1] and liver[2] tissue. Lipogenesis, as indicated by respiratory gas-exchange ratios in excess of unity, has been demonstrated in humans during glucose infusion[3] and consumption of high-carbohydrate diets.[4] However, net human lipogenesis rates in many situations are minimal. Using mass isotopomer distribution analysis, Hellerstein et al[5] reported that lipogenesis was quantitatively unimportant in subjects administered intravenous glucose or fed oral liquid or mixed-food breakfasts. Similarly, in healthy males consuming nutritionally adequate liquid diets as six evenly spaced meals per day, de novo lipogenesis was minor as measured by deuterium (D) incorporation from body water.[6] Conditions such as massive prolonged carbohydrate overfeeding[7] or infusion[8] are required to evoke quantitatively significant lipogenesis rates in humans. These findings support the earlier notion that under routine dietary intakes, fat and carbohydrate fluxes exist largely independently in humans.[9]

Although de novo lipogenesis may be relatively minor in humans, dietary factors influencing fatty acid synthesis in vivo remain to be fully understood. Frequency of meal consumption is one such dietary factor that has been shown to influence other aspects of lipid metabolism, including circulating cholesterol levels,[10] hormone levels and cholesterol synthetic rates.[11] Consumption of fewer, larger meals is associated with elevated peak insulin and glucose-dependent insulinotropic polypeptide (GIP) concentrations,[10,11] with the former hormone having been shown to stimulate lipogenesis in vitro.[12] However, whether meal frequency influences the conversion of energy substrates to fats in humans has not been investigated. The purpose of the present experiment was to examine circulating hormone levels and triglyceride fatty acid (TG-FA) synthesis in subjects consuming normal caloric intakes fed as three versus six meals each day. It was hypothesized that the pattern of consumption of daily nutrients does not alter lipogenesis rates through changes in circulating insulin and GIP levels in humans consuming weight-maintenance diets.

SUBJECTS AND METHODS

Subjects and Diets

Twelve male volunteers reporting no history of diabetes or lipid disorders were divided randomly into two equal groups consuming either three or six meals per day. The ages of subjects consuming three and six meals per day were 28.0 ± 1.9 and 31.6 ± 1.6 years (mean ± SEM), respectively. Body weights and heights were 73.2 ± 4.0 kg and 1.79 ± 0.04 m for subjects consuming three meals per day and 72.0 ± 3.1 kg and 1.77 ± 0.03 m for those consuming six meals per day, respectively. Screening plasma triglyceride[13] and total cholesterol[14] levels were within normal limits (normal ranges: total cholesterol, 2.6 to 5.4 mmol/L; total triglyceride, 0.11 to 2.1 mmol/L). All procedures were approved by the Ethical Review Committee of the University of British Columbia. Subjects provided informed consent before the investigation.

For 3 days prior to study, subjects in each group were requested not to consume alcohol or caffeine. The study was conducted over 72 hours in a metabolic unit, during which time subjects were fed a nutritionally complete liquid diet (Ensure Plus, Ross Laboratories, Montreal, Quebec, Canada). This diet contained 14.7%, 53.3%, and 32% kcal as protein, carbohydrate, and fat, respectively. Caloric intakes for each subject were calculated to maintain weight balance using a predictive equation for resting energy expenditure,[15] adjusted for activity level. The diet was provided either as three meals per day eaten at 08:00, 13:00, and 18:00 h or as six meals per day at 08:00 h and at 4-hour intervals thereafter. This diet was consumed under supervision as the sole source of nutrients over the 72-hour period.

At 07:30 h on day 2 of the diet, a baseline blood sample (28 mL) was taken. Subjects then consumed 0.7 g deuterium (D) oxide/kg estimated body water, and sequential blood samples (12 mL) were

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collected every 4 hours over the following 48 hours. Additional D_2O doses (0.7 D_2O/kg water consumed) were administered, either as separate beverages (three meals per day) or mixed with the evenly spaced liquid meals (six meals per day) to maintain body water D enrichment at plateau.

Analytical Methods

Blood samples were centrifuged at 1,500 *g* at 4°C immediately following collection. Plasma was separated, aliquoted, and frozen at −10°C. Plasma was then analyzed for insulin[16] and GIP[17] levels at each of the 13 time points. Additional plasma aliquots were used to determine D enrichment in body water and TG-FA as previously described.[6,18] Plasma sample aliquots (1.5 mL) were combined in triplicate with methanol and hexane:chloroform (4:1 vol/vol), centrifuged, and mechanically shaken for 20 minutes. H_2O was added to the mixture before shaking again, and the upper solvent layers were removed. These extraction steps were repeated, and solvent layers were combined. Extracted lipid components were then separated by thin-layer chromatography (Whatman, Clifton, NJ) using a solvent system of petroleum ether:ethyl ether:acetic acid (135:15:1.5). Triglyceride bands were identified using a co-chromatographed standard (Supelco, Bellefonte, PA), eluted from scraped silica three times using hexane:chloroform:ether (5:2:1), and dried under N_2 gas. Dried TG-FA samples were transferred in chloroform to 18-cm × 6-mm Pyrex (Corning Glass Works, Corning, NY) combustion tubes containing 500 mg cupric oxide and a silver wire catalyst. Tubes were flame-sealed at less than 50 mm Hg pressure after thorough solvent evaporation.

Tubes containing the triglyceride samples were combusted at 520°C over 4 hours. The water produced was vacuum-distilled into Pyrex 24-cm × 6-mm tubes containing 60 mg zinc reagent (Biogeochemical Laboratories, Indiana University, Bloomington, IN). In addition, 2 μL undiluted plasma from 0-hour time points and 2 μL diluted (6:1) plasma from 36- and 48-hour time points post–initial dose were vacuum-distilled into zinc-containing Pyrex tubes. Post-dose 36- and 48-hour samples were diluted with tap water of known isotopic composition to bring deuterium enrichments within the normal analytical range. TG-FA and plasma water samples were reduced to hydrogen gas at 520°C for 30 minutes. D enrichments were measured by differential isotope ratio mass spectrometry (VG Micromass 903D, Cheshire, England) using H^{3+} compensation.

Calculation of De Novo Lipogenesis Rate

A model similar to that used presently has been described.[6] The plasma TG-FA pool is considered a single compartment with inputs from chylomicrons and very-low-density lipoproteins (VLDL). Output from the pool is via tissue uptake. The uptake of D into plasma TG-FA is expected to increase exponentially to reach a maximum enrichment as determined by the following equation:

$$\text{del}(‰)\text{TG-FA max} = \text{del}(‰)_{\text{plasma}} \times 0.477, \qquad \text{Eq 1}$$

where del is D enrichment in parts per thousand ((‰) relative to Standard Mean Ocean Water. The correction factor 0.477 takes into account the ratio of 0.87 g-atoms ^3H per g-atom carbon incorporated into adipose fatty acids reported by Jungas [19] and carbon atoms of glycerol in a hypothetical triglyceride containing three monounsaturated 17-carbon fatty acids.[6]

Net TG-FA synthesis rates were calculated as the increment of theoretical maximum enrichment obtained in the plasma TG-FA pool. It was assumed that the relative plateau obtained over 12 to 48 hours indicates semi–steady state. Under such conditions, no appearance of label would indicate zero TG-FA synthesis. Conversely, attainment of the maximum plateau would indicate that all TG-FA entering the pool had been newly synthesized. The percentage of theoretical maximum obtained, multiplied by an estimate of daily hepatic VLDL TG-FA (300 mg/kg body weight)[20-22] production rate, is used to yield a value for net TG-FA synthesis. In this study it was assumed that (1) in the six-meal per day group chylomicron TG-FA from the previous meal had been cleared from the circulation at the time of blood sampling 4 hours later, and (2) in the three-meal per day group chylomicron TG-FA resulting from the evening meal would be largely removed from the circulation by midnight. Thus, TG-FA in plasma taken over the final three time points on each day was used for the calculation of de novo synthesis of VLDL TG-FA.

RESULTS

Subject groups were demographically similar. Energy intakes were 2,704 ± 231 and 2,848 ± 92 kcal/d, and screening plasma triglyceride levels were 0.85 ± 0.15 and 0.85 ± 0.06 mmol/L, respectively. Subjects were observed to have consumed entirely all liquid test meals and reported no gastrointestinal discomfort.

Plasma insulin levels are shown in Fig 1 for subjects consuming three and six meals per day. For most of the test period, insulin concentrations remained below 10 μU/mL in subjects consuming six meals per day. Values in subjects consuming three meals per day were significantly elevated ($P < .05$) over those in subjects eating six meals per day during periods coincident with meal ingestion. GIP level profiles were similar to those of insulin, with higher ($P < .05$) GIP levels occurring in subjects consuming three meals per day during the period when meals were consumed (Fig 2).

Uptake rates of body water D into plasma total TG-FA in subjects consuming three and six meals per day are presented for individual subjects in Fig 3A and B, respectively. The most rapid D incorporation occurred in each group during the initial 12-hour period, with comparatively minor net changes observed subsequently until study completion. This plateau was better defined in subjects consuming the evenly spaced meals. In each group, one subject's incorporation profile differed markedly from those of the others.

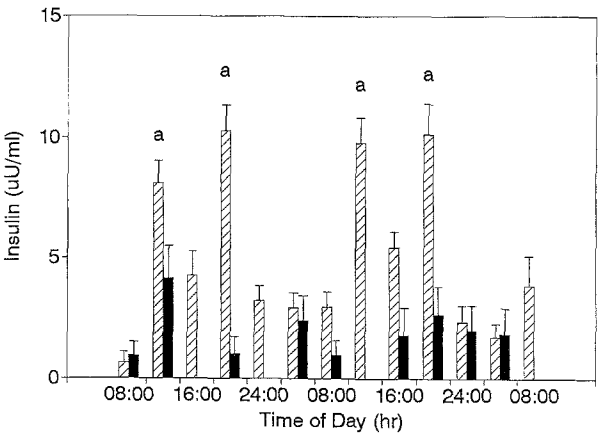

Fig 1. Plasma insulin level profiles over the 48-hour test period for subjects consuming three (▨) and six (■) meals per day. Mean ± SEM. The lower detection limit of the assay was 2 μU/mL, and thus, values below this concentration were taken as zero. $^aP < .05$, $^bP < .001$: *v* six meals per day.

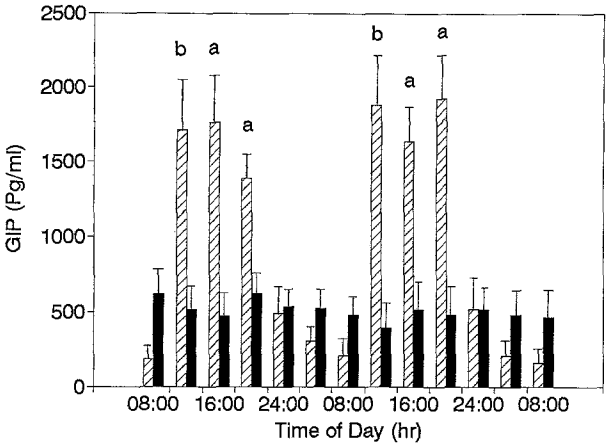

Fig 2. GIP level profiles over the 48-hour test period for subjects consuming three (▨) and six (■) meals per day. Mean ± SEM. [a]$P < .05$, [b]$P < .001$: *v* six meals per day.

Figure 4 shows the mean D uptake responses of subjects within each group. There were no significant intergroup differences in rates of incorporation at 24 or 48 hours after D-dosing.

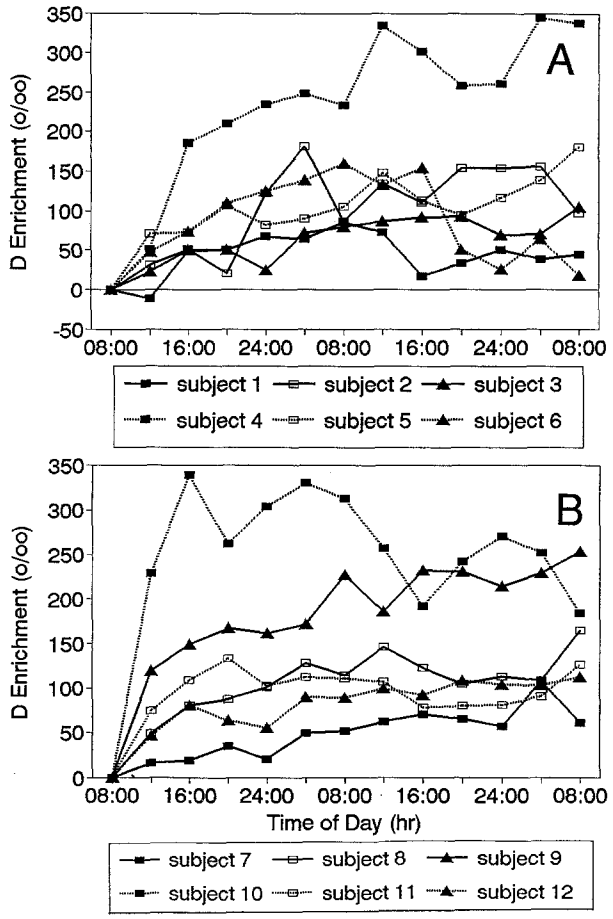

Fig 3. Individual incorporation rates of body water D into plasma total TG-FA in subjects consuming (A) three meals per day and (B) six meals per day.

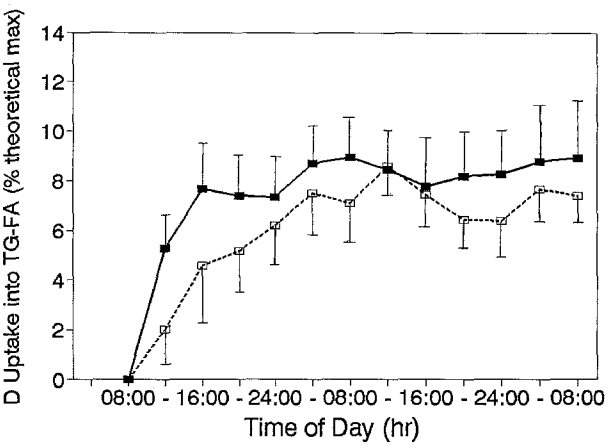

Fig 4. Mean D incorporation responses of subjects over the 48-hour test period for subjects consuming (□) three and (■) six meals per day.

Plasma water D enrichments over the TG-FA measurement period were similar between subjects consuming three (3,694 ± 144 ‰) and six (3,536 ±127 ‰) meals each day. These water enrichments were used in equation 1 to derive values for the theoretical maximum D incorporation (X + Y) used in Fig 5. Mean D incorporation rates in subjects on each diet are also presented (X). There were no significant differences between groups in calculated total VLDL TG-FA production (Table 1). The percent contribution values of de novo synthesis to total production [(X/X + Y) · 100] were similar for days 1 and 2 in subjects consuming three (6.56% ± 1.32% and 6.64% ± 2.08%, respectively) and six (7.67% ± 2.29% and 7.88% ± 1.46%, respectively) meals per day. Calculated net TG-FA synthesis rates over days 1 and 2 were 1.47 ± 0.33 and 1.55 ± 0.53 g/d, respectively, for subjects fed three meals per day and 1.64 ± 0.47 and 1.69 ± 0.30 g/d for subjects fed six meals per day.

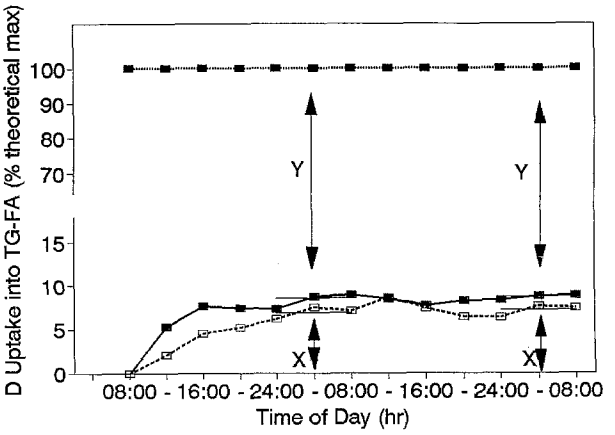

Fig 5. D incorporation response in relation to theoretical maximum for subjects consuming (□) three and (■) six meals per day. X, mean D incorporation rates of subjects on each diet. (X + Y), theoretical maximum D incorporation calculated from plasma water enrichments. [(X/X + Y) · 100], percent contribution of de novo synthesis to total VLDL TG-FA production.

Table 1. Calculation of TG-FA Synthesis Rates Over Days 2 and 3 in Subjects Consuming Three and Six Meals Per Day

	Three Meals Per Day, Subject No.						
	1	2	3	4	5	6	Mean ± SEM
X (‰)							
Day 2	72.2	130.1	59.1	238.6	92.5	141.2	122.3 ± 26.7
Day 3	41.8	135.3	81.1	314.6	145.1	36.0	125.7 ± 42.1
X + Y ‰	1986	1893	2051	2053	1593	1652	1,871 ± 83
X/X + Y (%)							
Day 2	3.63	6.87	2.88	11.62	5.80	8.55	6.56 ± 1.32
Day 3	2.10	7.15	3.95	15.33	9.11	2.18	6.64 ± 2.08
Hepatic VLDL TG-FA production (g/d)	18.4	23.6	20.5	23.9	25.9	19.5	22.0 ± 1.19
TG-FA synthesized (g/d)							
Day 2	0.67	1.62	0.59	2.77	1.50	1.67	1.47 ± 0.33
Day 3	0.39	1.69	0.81	3.66	2.36	0.43	1.55 ± 0.53
	Six Meals Per Day, Subject No.						
	7	8	9	10	11	12	
X (‰)							
Day 2	40.9	114.8	186.9	316.1	109.0	78.2	141.0 ± 40.2
Day 3	75.3	129.0	232.2	236.0	99.7	107.0	146.5 ± 28.6
X + Y (‰)	1802	1862	2154	1731	1787	1737	1,846 ± 65
X/X + Y (%)							
Day 2	2.27	6.16	8.67	18.3	6.09	4.50	7.67 ± 2.29
Day 3	4.18	6.93	10.8	13.6	5.58	6.16	7.88 ± 1.46
Hepatic VLDL TG-FA production (g/d)	18.9	23.2	19.9	21.0	21.3	25.2	21.6 ± 0.93
TG-FA synthesized (g/d)							
Day 2	0.43	1.43	1.73	3.83	1.30	1.14	1.64 ± 0.47
Day 3	0.79	1.61	2.15	2.86	1.19	1.55	1.69 ± 0.30

DISCUSSION

Although human fat synthesis has been previously measured using respiratory gas-exchange and other indirect techniques, direct approaches have been limited due to a lack of methods suitable for use in humans. Using the incorporation rate of D into VLDL TG-FA as a determinant of synthesis, we report that TG-FA synthesis is a relatively minor pathway of fat metabolism in subjects consuming diets at energy levels commensurate with caloric needs. Moreover, at constant daily caloric intake the number of meals consumed per day was not an influencing factor in the extent of fat synthesis, despite significant changes in hormone profiles. The present findings are in agreement with the low lipogenesis capacity in humans reported previously,[5] where fatty acid synthesis assessed using mass isotopomer distribution analysis in subjects fed various breakfast meals remained below 500 mg/d regardless of the type of meal consumed. However, they are in contrast to results of other feeding studies that suggest an inverse relation between the number of meals consumed per day and lipogenesis in humans and animals.[23-25] Bray[23] showed that rapid ingestion of nutrients resulted in an increased uptake of labeled carbon from glycerol and pyruvate, suggesting that body fat accretion is linked to consumption of larger, fewer meals each day. Similarly, a review of earlier literature[24] suggests that excessive weight gain and high circulating glucose levels are more common in individuals with infrequent eating habits as compared with those consuming more numerous smaller meals. Overall, more recent studies[5,6] suggest that whole-body carbohydrate and fat balances are largely under distinct control and that intake, and thus utilization for energy, is regulated separately for each of these two macronutients.[9]

The method presently used was derived from the tritiated-water incorporation procedure developed in animals for fatty acid synthesis using in vitro tissue preparations[19] and organ perfusion systems,[26] as well as for cholesterol synthesis in vivo.[27] This method determines the fraction of TG-FA synthesized from the level of D incorporation relative to that of precursor. The primary features are that not only can the precursor D enrichment be held constant over time by continuous administration of D_2O, but also that it can be readily monitored in an accessible body water pool such as plasma or urine. D/hydrogen incorporation ratios for TG-FA have been derived in animals,[19] which we have applied in humans to calculate the TG-FA D enrichment maxima. The fractional TG-FA enrichment at plataeu, relative to this maximum, is taken as indicating the proportion of the plasma pool TG-FA originating from synthesis. In obtaining a value for net synthesis, the proportion of the plasma pool synthesized was multiplied by an estimate of total hepatic VLDL production. It was assumed that at the sampling times used, chylomicrons, with their plasma clearance half-life of approximately 5 minutes,[28] would be almost completely absent relative to VLDL particles, with a half-life of greater than 1 hour.[29] For this reason, recent estimates of the VLDL production rate were used as indices of plasma pool TG-FA input rates. For subjects fed the more frequent smaller meals, it was assumed that the majority of chylomicrons would have been cleared from the

circulation by the time that the subsequent blood sample was drawn. Nocturnal time points were used to estimate the D enrichment plateau in the case of subjects fed three larger meals each day also to ensure chylomicron clearance. A potential limitation to the present approach is the possibility that total TG-FA input rates vary with the feeding state or that chylomicron clearance may remain incomplete after 4 hours in the multiple-meal group; however, net daily delivery of dietary fat remained constant across treatment groups.

The present data suggest that in vivo human lipogenesis is not directly associated with circulating insulin and GIP levels. Insulin and GIP concentrations normally correlate well in humans,[30] since the release of both hormones is stimulated by carbohydrate ingestion[31] and reduced by increased meal frequency[11,12] or continuous versus bolus glucose ingestion.[32] The present lack of stimulation of lipogenesis by insulin is in contrast to the findings of in vitro studies where the addition of insulin to isolated hepatocytes from meal-fed rats enhanced ^3H incorporation from ^3H_2O into triacylglycerol.[12] Despite elevated levels of both insulin and GIP over the course of the daytime trial in the group consuming three meals per day, there was no detectable increase in TG-FA D incorporation. Perhaps for significant lipogenesis to occur both a favorable hormonal milieu and adequate substrate availability must exist. Acetyl units supplied through elevated glycolysis would provide the necessary substrate for the lipogenic pathway. In this study, it can be speculated that the three-meal-per-day regimen did not provide large enough acute substrate loads to evoke a measurable change in lipogenesis. A large excess in daily caloric intake has been found to be required to stimulate lipogenesis.[4,7] Thus, the balance of energy needs for oxidation versus storage may play a vital role as a regulator of lipogenesis. Under dietary conditions that promote fatty acid oxidation, such as fish-oil feeding, insulin's action in enhancing lipogenesis was shown to be depressed.[33] The present diet was high in refined carbohydrate as compared with typical Western intakes, which might have been expected to stimulate lipogenesis via stimulation of the insulin response. However, such an effect was not presently observed.

Within each feeding group, a single subject's D incorporation maximum exceeded other subjects' plateaus substantially. Neither fasting lipid levels nor reported disease history of these two individuals were remarkable in comparison to those of the overall group. However, it would appear that both of these subjects possessed a distinctly elevated synthesis capacity for TG-FA, possibly indicative of some inherent defect of lipogenesis. Indeed, enhanced TG-FA synthesis has been observed in type III hyperlipoproteinemia.[34] It cannot be excluded that this relatively simple D incorporation methodology may possess potential as a screening tool for latent disorders of triglyceride metabolism.

In summary, the present study has demonstrated that although consumption of three versus six meals per day was associated with an increase in peak levels of circulating insulin and GIP, meal frequency had no influence on D incorporation rates or calculated net synthesis into the plasma TG-FA compartment. It cannot be ruled out that the present study duration was too brief to produce a treatment effect on lipogenesis; however, Acheson et al[7] observed enhanced fat synthesis after only 1 day of overfeeding, suggesting a rapid response of lipogenesis to dietary modification. The present findings indicate that under normal dietary situations TG-FA synthesis is a relatively minor pathway, and that regulation of synthesis occurs independently of fluctuating hormone levels and meal frequency.

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