Time of Day and Glucose Tolerance Status Affect Serum Short-Chain Fatty Acid Concentrations in Humans

Thomas M.S. Wolever, Robert G. Josse, Lawrence A. Leiter, and Jean-Louis Chiasson

Short-chain fatty acids (SCFA) are derived from endogenous (metabolism of fat, carbohydrate, and amino acids) and exogenous (colonic fermentation) sources. To see how time of day and glucose tolerance status influenced serum SCFA concentrations, we determined serum SCFA throughout the day in 22 subjects with impaired glucose tolerance (IGT) and 10 young and eight middle-aged normal controls. On 1 day, insulin sensitivity was assessed as the steady-state plasma glucose (SSPG) level achieved during intravenous infusion of glucose insulin, and somatostatin. On another day, plasma glucose and insulin and serum SCFA levels were measured 12 times over 12 hours with subjects eating a standard diet. SSPG in young controls (5.5 ± 1.1 mmol/L) was less than in middle-aged controls (9.3 ± 1.6 mmol/L), which in turn was less than in IGT subjects (13.7 ± 0.6 mmol/L; $P < .01$). Mean plasma glucose in IGT subjects was greater than in normal controls, and mean plasma insulin in IGT subjects was higher than in young controls but similar to the levels in middle-aged controls. Mean 12-hour serum acetate in young controls (143 ± 13 μmol/L) was greater than in middle-aged controls (104 ± 11 μmol/L) and IGT subjects (113 ± 5 μmol/L; $P < .05$). Mean 12-hour serum propionate in young controls (3.8 ± 0.5 μmol/L) was less than in IGT subjects (5.4 ± 0.3 μmol/L; $P < .01$), with middle-aged controls being intermediate (4.6 ± 0.3 μmol/L). Both young (1.6 ± 0.3 μmol/L) and middle-aged (1.0 ± 0.2) controls had lower mean butyrate than IGT subjects (3.1 ± 0.4 μmol/L; $P < .05$). Levels of all three SCFA varied significantly during the day, tending to decrease after breakfast and increase transiently after lunch and dinner. It is concluded that both time of day and glucose tolerance status affect serum SCFA levels in nondiabetic humans. The results suggest that serum acetate is derived primarily from colonic fermentation, serum butyrate primarily from endogenous fatty acid metabolism, and serum propionate from both exogenous and endogenous sources.

THE SHORT-CHAIN FATTY ACIDS (SCFA), acetic, propionic, and butyric acids, are the major products of bacterial fermentation of the carbohydrate that enters the colon.[1] SCFA have important local effects in the colon such as promoting fluid and electrolyte absorption[2,3] and possibly influencing colonic carcinogenesis.[4] Although butyrate is taken up primarily by colonic mucosal cells,[5,6] acetate and propionate are rapidly absorbed[7,8] and may influence carbohydrate and lipid metabolism.[9-11]

It is generally considered that colonic fermentation is the major source of acetate.[12,13] However, presumably, the rate of colonic fermentation varies throughout the day because the rate of entry of glucose into the colon varies, with peaks just after consumption of meals.[14] Under conditions of increased fat oxidation such as prolonged starvation[12] and diabetes,[15] endogenous acetate production predominates. In addition, propionate, and to a lesser extent acetate, is produced endogenously from the metabolism of branched-chain amino acids and methionine.[16,17] It has been estimated that in subjects with inborn errors of propionate metabolism, amino acid catabolism accounts for approximately 50% of propionate production and colonic fermentation about 22%.[18] Modest increases in plasma insulin markedly suppress proteolysis and cause branched-chain amino acids to be taken up by muscle.[19] Thus, after carbohydrate-containing test meals, plasma insulin increases and branched-chain amino acids decrease.[20-22] However, subjects with insulin resistance have a smaller decrease in plasma branched-chain amino acids after an oral glucose load than normal subjects.[23] This suggests that impaired glucose tolerance (IGT) results in abnormal amino acid metabolism and possibly in increased endogenous production of acetic and propionic acids. Thus, the relationship between serum SCFA concentrations and colonic fermentation may not hold in subjects with insulin resistance. Therefore, the purpose of this study was to determine the effect of time of day and IGT on serum SCFA concentrations.

SUBJECTS AND METHODS

We studied three groups of volunteers: 22 had IGT according to World Health Organization criteria (plasma glucose concentration ≥7.8 and ≤11.1 mmol/L 2 hours after 75 g oral glucose), 10 were young normal subjects who were not screened with a glucose tolerance test, and eight were middle-aged spouses of the IGT subjects who had normal plasma glucose (<7.8 mmol/L) 2 hours after 75 g oral glucose. The procedures used were approved by ethics review boards at St. Michael's Hospital and the Hôtel-Dieu de Montréal.

Subjects came to the Clinical Nutrition Factor Modification Centre at St. Michael's Hospital or the Clinical Investigation Unit at the Hôtel-Dieu de Montréal in the morning after a 12-hour overnight fast for a 12-hour day profile test. An intravenous catheter was inserted into a forearm vein for blood sampling, and this was kept patent with normal saline. At about 8:00 AM, after a fasting blood sample had been obtained, subjects consumed a breakfast test meal consisting of 400 g liquid formula (Enrich; Ross Laboratories, Montreal, Quebec, Canada: 450 kcal, 55% of energy as carbohydrate (0% starch), 30.5% fat, 14.5% protein, and 5.7 g dietary fiber). Lunch consisting of cheese ravioli with crackers, fruit salad, pudding, and cookies (805 kcal, 64% of energy as carbohydrate (~64 g starch), 26% fat, 10% protein, and 9 g dietary fiber) was consumed 5 hours after the start of breakfast, and dinner consisting of a glazed chicken dinner, fruit, and cookies (700 kcal, 50% carbohydrate (~66 g starch), 23% fat, 26% protein, and 7 g dietary fiber) was consumed 5 hours after the start of lunch. Blood samples

From the Division of Endocrinology and Metabolism and Clinical Nutrition and Risk Factor Modification Centre, St. Michael's Hospital, and Department of Nutritional Sciences, University of Toronto, Toronto, Ontario; and Centre de Recherche, Hôtel-Dieu de Montréal, University of Montréal, Montréal, Quebéc, Canada.

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Address reprint requests to Thomas M.S. Wolever, MD, PhD, Department of Nutritional Sciences, University of Toronto, Toronto, Ontario, Canada M5S 3E2

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were obtained at 1, 1.5, 2, 4, and 5 hours after the liquid formula meal, 1, 2, 4, and 5 hours after lunch, and 1 and 2 hours after dinner.

Insulin sensitivity of all subjects was assessed using the insulin suppression test,[24] which consisted of a 3-hour intravenous infusion of 20% glucose (1.3 mL/min/m^2), regular human insulin (25 μU/min/m^2), and somatostatin (400 μg/h), with blood samples obtained every 30 minutes. The mean plasma glucose concentration achieved between 120 and 180 minutes, termed steady-state plasma glucose (SSPG), is a measure of insulin sensitivity that has been validated against the insulin clamp technique.[25] As SSPG increases, insulin sensitivity decreases. Dietary records were not available from the normal subjects. Thus, as an index of energy intake, resting energy expenditure (REE) was estimated using the Harris-Benedict equation.[26]

Plasma glucose level was measured using the hexokinase technique,[27] and plasma insulin was determined by radioimmunoassay using a commercial kit (Insulin RIA100; Pharmacia, Dorval, Quebec, Canada). Serum acetate, propionate, and butyrate levels were measured in quadruplicate by gas chromatography as previously described.[28] An 800-μL aliquot of serum was filtered through a micropartition system with a 30,000-dalton molecular weight cutoff point (MPS-1; Amicon, Danvers, MA) by rotation at 5,000 rpm at 4°C for 70 minutes. The filters were washed four times before use to remove glycerine, which interfered with the propionate and butyrate peaks. The protein-free filtrate was stored at −20°C before vacuum distillation using the procedure described by Tollinger et al.[29] Distillation was performed using a 225-μL sample of protein-free serum to which was added a 25-μL internal standard solution consisting of 1.1 mmol/L methyl butyric acid and 110 mmol/L ^{13}C-formic acid (Sigma Chemical, St Louis, MO) to prevent ghosting in the injector sleeve and to reduce the pH in the sample to approximately 3 to ensure complete SCFA recovery. An HP 7673 automatic sampler (Hewlett Packard, Mississauga, Ontario, Canada) was used to inject 1-μL aliquots of sample into an HP 5890 Series II gas chromatograph equipped with a split-splitless inlet, a J&W DB-FFAP fused silica capillary column (30 m × 0.25 mm × 0.25-μm film; Alltech, Deerfield, IL), and a flame ionization detector. The oven temperature was 80°C until 0.1 minute after injection, after which it was increased by 15°C/min to 165°C and held for 1 minute. The carrier gas was pure helium at a flow rate of 1 mL/min, and the detector was supplied with helium at 30 mL/min, hydrogen at 30 mL/min, and air at 350 mL/min.

Each protein-free serum sample was distilled in duplicate, and each distilled sample was injected in duplicate. SCFA concentrations were determined from the ratio of the height of each SCFA peak to the height of the internal standard peak using a standard curve included in each assay. The mean ± SD concentrations of acetate, propionate, and butyrate in pooled serum (27 duplicate injections in 20 consecutive assays) were 176 ± 61, 5.7 ± 3.0, and 2.1 ± 1.5 μmol/L, respectively, and for serum spiked with 200:20:20 μmol/L acetate:propionate:butyrate, the concentrations were 367 ± 100 (coefficient of variation, 27%), 26.6 ± 4.6 (17%), and 23.2 ± 3.5 (15%) μmol/L.

Results are expressed as the mean ± SEM. Statistical analysis was made by ANOVA with a nested design examining for the effects of time, subject, group, and time × group interaction. Individual means were compared using the Newman-Kuels technique to adjust for multiple comparisons.[30] Differences were considered significant at P less than .05.

RESULTS

The young and middle-aged controls and IGT subjects were matched with respect to sex distribution (Table 1). There was no significant difference in body weight between the three groups, but young controls were significantly taller than middle-aged controls and IGT subjects. The young controls were lean and had a mean body mass index (BMI) that was significantly less than in the other groups. Middle-aged controls and IGT subjects tended to be obese with a similar mean BMI (Table 1). During the intravenous glucose/insulin/somatostatin infusion, the mean SSPG of young controls was significantly less than that of middle-aged controls, which in turn was significantly less than in IGT subjects (Table 1).

Table 1. Subject Characteristics

Characteristic	Group			$F_{(2,37)}$*
	YNOR	MNOR	IGT	
Sex ratio (males:females)	6:4	4:4	15:7	0.86
Age (yr)	30 ± 2^a	45 ± 3^b	56 ± 2^c	37.76§
Height (cm)	175 ± 10^a	162 ± 9^b	164 ± 9^b	6.28‡
Weight (kg)	74.9 ± 4.7	77.4 ± 3.2	84.0 ± 2.9	1.87
BMI (kg/m^2)	24.2 ± 1.0^a	29.8 ± 1.8^b	31.2 ± 0.9^b	9.33§
REE (kcal)	1,700 ± 101	1,545 ± 54	1,601 ± 50	0.96
SSPG (mmol/L)	5.5 ± 1.1^a	9.3 ± 1.6^b	13.7 ± 0.6^c	20.75§
Glucose (mmol/L)	4.4 ± 0.1^a	4.4 ± 0.2^a	5.2 ± 0.2^b	7.36§
Insulin (pmol/L)	59 ± 8^a	117 ± 20^b	134 ± 12^b	7.88§
Acetate (μmol/L)	148 ± 11	113 ± 14	131 ± 9	1.89
Propionate (μmol/L)	4.5 ± 0.6^a	5.0 ± 0.4^a	6.6 ± 0.3^b	7.37§
Butyrate (μmol/L)	2.2 ± 0.4^{ab}	2.0 ± 0.4^a	3.9 ± 0.5^b	4.40†

NOTE. Values are the mean ± SEM. Means in the same row with a different letter superscript are significantly different ($P < .05$). Glucose and insulin represent concentrations in fasting plasma; acetate, propionate, and butyrate are concentrations in fasting serum.

Abbreviations: YNOR, young normal controls; MNOR, middle-aged normal controls.

*From ANOVA except for sex ratio, where the value is chi-squared: †$P < .05$; ‡$P < .01$; §$P < .001$; otherwise, not significant ($P > .05$).

The mean fasting plasma glucose of young controls was the same as that of middle-aged controls and significantly less than that of IGT subjects (Table 1). By ANOVA, there were significant effects of time of day ($F_{(11,406)} = 69.68$, $P < .0001$) and subject group ($F_{(2,37)} = 13.57$, $P < .0001$) on plasma glucose concentrations throughout the day (Fig 1). In addition, there was a significant time × group interaction ($F_{(22,406)} = 5.65$, $P < .0001$) indicating that the changes in plasma glucose with time were significantly different in the different groups. The mean 12-hour plasma glucose of young controls (4.9 ± 0.2 mmol/L) was not significantly different from that of middle-aged controls (5.1 ± 0.2 mmol/L), with both of these values being significantly less than in IGT subjects (6.2 ± 0.9 mmol/L; $P < .01$). Fasting plasma insulin of young controls was significantly lower than that of middle-aged controls and subjects with IGT, with the latter two groups having similar values (Table 1). ANOVA showed significant effects of time of day ($F_{(11,406)} = 95.36$, $P < .0001$), subject group ($F_{(2,37)} = 12.80$, $P = .0002$), and time × group interaction ($F_{(22,406)} = 7.23$, $P < .0001$) on plasma insulin concentrations throughout the day (Fig 1). The mean 12-hour plasma insulin in young controls (220 ± 26 pmol/L) was significantly less than in middle-aged controls (534 ± 85 pmol/L) and subjects with IGT (661 ± 58 pmol/L), with the difference between the latter two groups not reaching statistical significance.

Fasting serum acetate did not differ significantly in the three groups (Table 1). Fasting propionate and butyrate concentrations were significantly higher in subjects with IGT than in

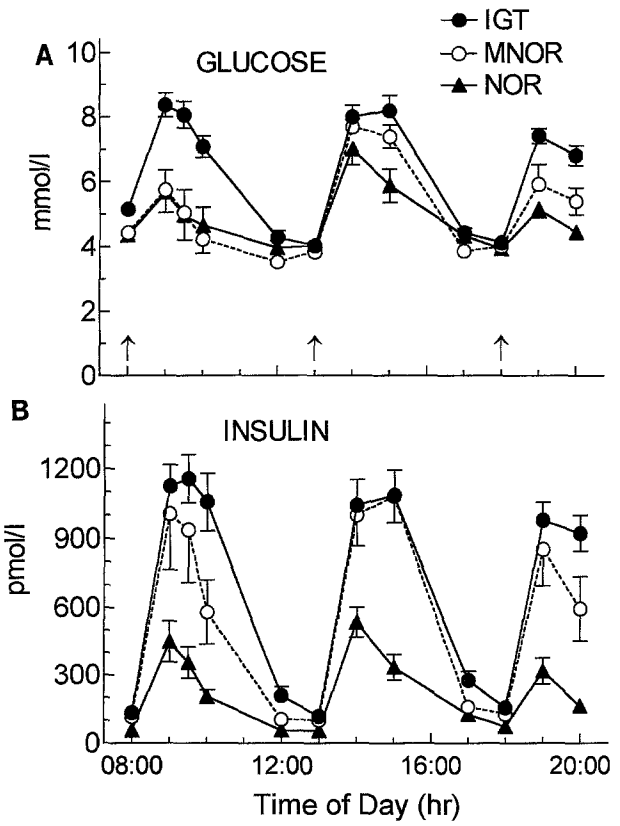

Fig 1. Fasting and postprandial plasma glucose (A) and insulin (B) in young (▲) and middle-aged (○) normal controls and subjects with IGT (●). Arrows indicate times at which consumption of test meals started (08:00, 13:00, and 18:00 hr). Values are the mean ± SEM. For clarity, error bars are not shown if they are smaller than the symbol or overlap other bars or symbols.

middle-aged controls, with values in the latter group being no different from those in young controls (Table 1). ANOVA showed a significant effect of time of day on serum acetate ($F_{(11,406)} = 14.77$, $P < .0001$), propionate ($F_{(11,406)} = 16.59$, $P < .0001$), and butyrate ($F_{(11,406)} = 3.70$, $P = .004$) (Fig 2). Also, there was a significant effect of group for acetate ($F_{(2,37)} = 4.51$, $P = .023$), propionate ($F_{(2,37)} = 6.56$, $P = .0058$), and butyrate ($F_{(2,37)} = 6.54$, $P = .0059$). The mean 12-hour acetate in young controls (143 ± 13 μmol/L) was greater than in middle-aged controls (103 ± 11 μmol/L) and subjects with IGT (112 ± 5 μmol/L ($P < .05$; Fig 2). Mean 12-hour propionate in young controls (3.7 ± 0.5 μmol/L) was less than in IGT subjects (5.3 ± 0.2 μmol/L, $P < .01$), with the mean for middle-aged controls (4.6 ± 0.3 μmol/L) not being significantly different from either of the other groups. Mean 12-hour butyrate in IGT subjects (3.1 ± 0.4 μmol/L) was greater than in middle-aged (0.9 ± 0.2 μmol/L, $P < .01$) and young controls (1.5 ± 0.3 μmol/L, $P < .05$). By ANOVA, there was no significant time × group interaction for butyrate ($F_{(22,406)} = 0.47$, $P = .98$), but the interaction was significant for propionate ($F_{(22,406)} = 1.81$, $P = .014$) and approached significance for acetate ($F_{(22,406)} = 1.48$, $P = .077$).

As expected, there were significant interrelationships between BMI, SSPG, fasting plasma glucose, and fasting plasma insulin. BMI was positively related to fasting plasma glucose ($r = .352$, $P = .026$), insulin ($r = .741$, $P < .0001$), and SSPG (Fig 3). Fasting serum acetate was not significantly related to BMI, SSPG, fasting plasma glucose, fasting plasma insulin, or REE. Fasting serum propionate was positively related to BMI ($r = .425$, $P = .006$), SSPG, and fasting plasma glucose ($r = .476$, $P = .002$), but not to fasting insulin or REE. Fasting serum butyrate was positively related to SSPG and fasting

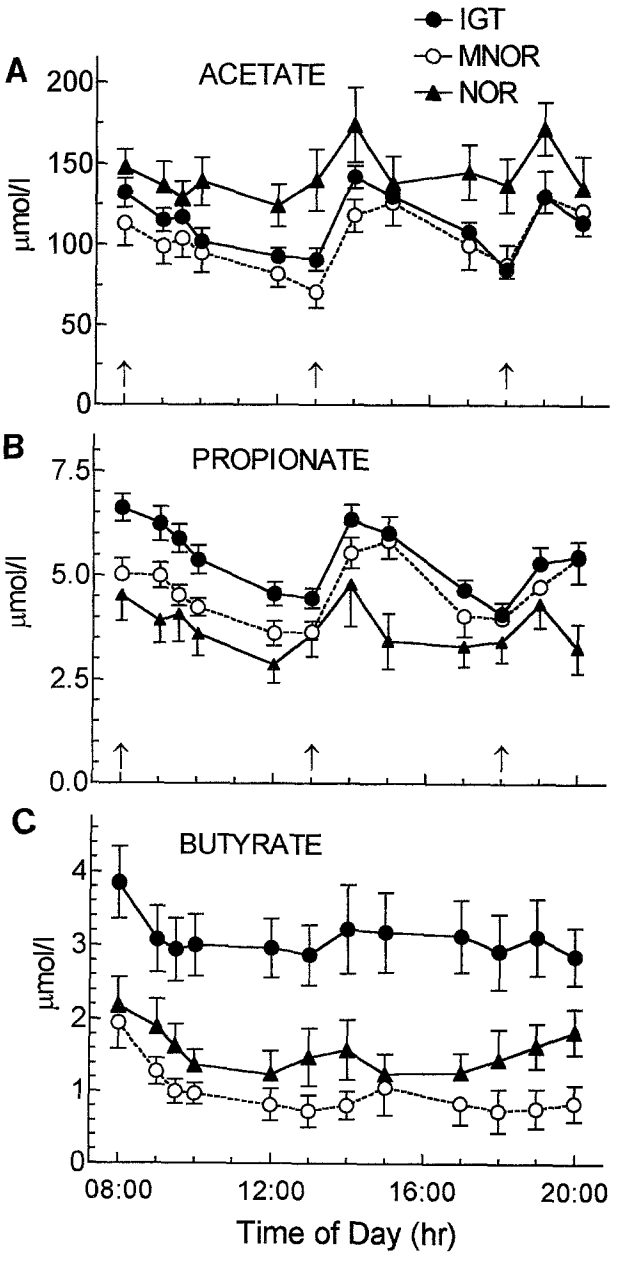

Fig 2. Fasting and postprandial serum acetate (A), propionate (B), and butyrate (C) in young (▲) and middle-aged (○) normal controls and subjects with IGT (●). Arrows indicate times at which consumption of test meals started (08:00, 13:00, and 18:00 hr). Values are the mean ± SEM. For clarity, error bars are not shown if they are smaller than the symbol or overlap other bars or symbols.

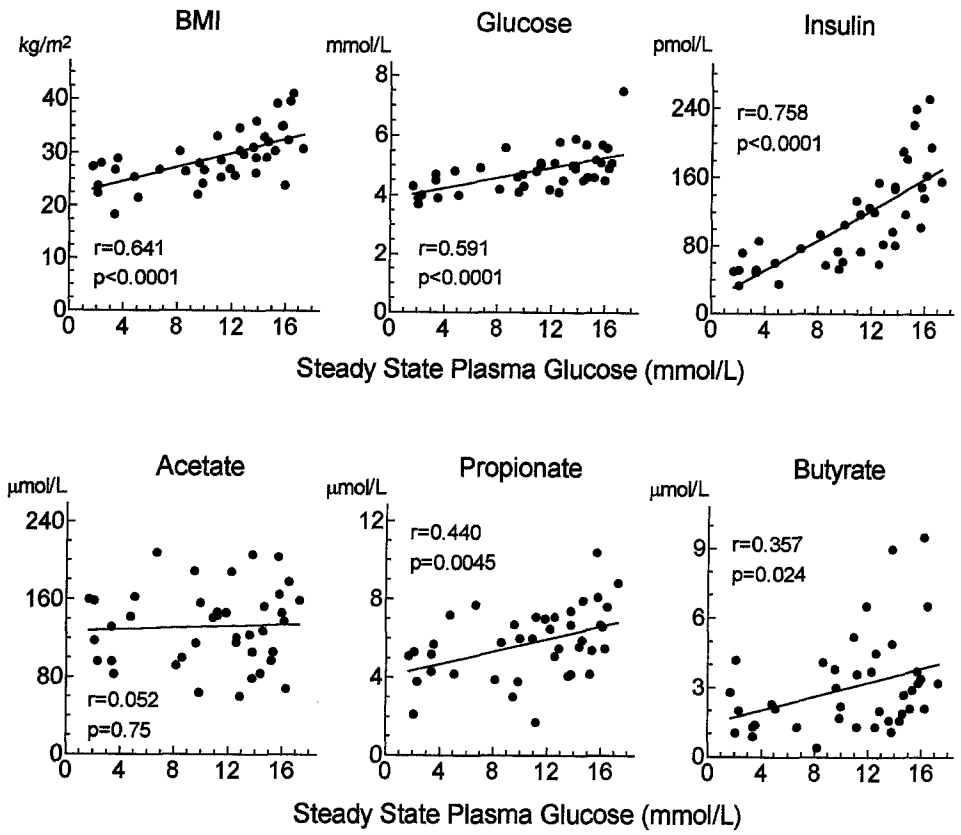

Fig 3. Correlations between SSPG (a measure of insulin sensitivity) and BMI, fasting plasma glucose, insulin, acetate, propionate, and butyrate in all 40 subjects. Regression lines are indicated.

plasma glucose ($r = .372$, $P = .018$), but not to insulin, BMI, height, or REE.

DISCUSSION

The results show that fasting and postprandial SCFA concentrations were affected by glucose tolerance status, with high propionate and butyrate concentrations being associated with obesity and insulin resistance. In addition, serum acetate, propionate, and butyrate varied significantly with time throughout the day, with all three SCFA decreasing after breakfast and acetate and propionate peaking transiently after both lunch and dinner.

The results for plasma glucose and insulin are consistent with previous data[31] and are in line with current concepts about the development of insulin resistance and glucose intolerance.[32,33] The young controls represent insulin-sensitive individuals with relatively small and brief increases in postprandial glucose and insulin. With age and the development of obesity, insulin resistance increases, as observed in the middle-aged control group. In this situation, normal fasting and postprandial glucose concentrations are maintained at the expense of hyperinsulinemia. Prolonged hyperinsulinemia results in an exacerbation of insulin resistance,[34] as seen in IGT subjects in whom further increases in insulin secretion cannot be achieved, so fasting and postprandial plasma glucose levels increase. However, rather than examining glucose and insulin, our major purpose was to measure fasting and postprandial serum SCFA concentrations.

SCFA concentrations in the blood reflect the balance between rates of production and utilization. Endogenous sources of SCFA include fatty acid[35] and amino acid,[17] oxidation the rates of which vary diurnally with fasting and refeeding[12,36] and are also influenced by the degree of obesity and insulin sensitivity.[23,37-39] Fasting serum acetate is increased in diabetes,[15,40] but the present results show that serum acetate is not increased by obesity or IGT. This is consistent with the concept that in nondiabetic humans, colonic fermentation is the major source of blood acetate.[12,13] The effects of meal feeding and insulin resistance on serum butyrate have not been studied before, and the significant correlation between serum butyrate and SSPG suggests that, unlike acetate, endogenous production is the major source of serum butyrate. Propionate is produced endogenously from the catabolism of branched-chain amino acids, threonine, and methionine.[18] Obesity, which is associated with insulin resistance, increases leucine flux.[41] Thus, as insulin resistance increases, we would expect an increased rate of branched-chain amino acid catabolism and hence an increased rate of propionate production. This is consistent with the direct correlation between SSPG and fasting serum propionate. In addition, the decrease in serum propionate after the breakfast test meal could be due to the reduction in amino acid catabolism caused by the insulin-induced change from the fasted to the fed state that occurs after breakfast.[19,36]

It is generally agreed that under normal circumstances the major source of acetate in mammals is colonic fermentation of unabsorbed carbohydrate.[12,13] In addition, there is much evidence that changes in colonic acetate production are reflected in changes in serum acetate concentration. There is a dose-dependent increase in serum acetate after rectal infusion of sodium acetate.[42] Short-term fasting reduces serum acetate.[12] In addition, individuals with short-bowel syndrome who have had

their colon removed have significantly lower blood acetate concentrations (89 ± 25 μmol/L, n = 5) than short-bowel patients with an intact colon (145 ± 19 μmol/L, n = 7, $P < .05$) and subjects with a normal bowel (159 ± 31 μmol/L, n = 6, $P < .05$).[43] Increases in serum acetate in the postprandial state have been observed after adding the nonabsorbed, fermentable carbohydrates lactulose, pectin, or guar to a polysaccharide-free diet[44,45] and after adding lactulose or oat bran to a normal diet.[46,47] Thus, the peaks in serum acetate we observed after lunch and dinner are probably due to increased acetate production from colonic fermentation.

It has been estimated that 10 to 60 g carbohydrate enters the colon per day.[1] About half of the carbohydrate entering the colon is from dietary fiber and half from undigested, or resistant, starch.[48,49] In addition, the glycoproteins of intestinal mucus provide a small amount of fermentable carbohydrate.[1] Even on a low-fiber, North American diet, enough carbohydrate enters the colon to provide 200 to 300 mmol SCFA daily.[50] The present results are consistent with studies in which ileal contents were aspirated from human subjects who consumed meals containing an unabsorbable marker. These studies showed that the rate of recovery of marker, glucose, and starch from the terminal ileum peaked 1 to 2 hours after consumption of lunch and dinner[14]; this corresponds to the peaks in serum acetate and propionate we found after lunch and dinner. Since the diet our subjects ate was not high in fiber and starch, only a limited amount of carbohydrate would be available for colonic fermentation. This and the fact that acetate is rapidly oxidized,[51] with a half-life in peripheral blood of about 10 to 15 minutes,[52] likely account for the rapid return of serum acetate and propionate to premeal levels. Interestingly, after breakfast, the rate of appearance of glucose and starch in the terminal ileum increases more slowly and to a lesser extent than after lunch and dinner.[14] Thus, the decrease in serum acetate and propionate after breakfast may be explained by the fact that the rate of delivery of substrate to the colon in the 4 hours after breakfast does not increase sufficiently to stimulate the rate of colonic SCFA production enough to overcome the decrease in endogenous SCFA production resulting from the reduction in fat and amino acid oxidation[38,53] that occurs after breakfast during the change from the fasted to the fed state. A decrease in serum acetate after a breakfast test meal has been noted previously[29]; however, temporary spikes of serum acetate after lunch and dinner have not been seen previously, presumably because subjects were either on a polysaccharide-free diet[44,45] or because blood samples were not taken frequently enough to detect them.[46,47]

A novel feature of this study was the measurement of serum propionate and butyrate levels throughout the day. Except for the decrease after breakfast, significant variation in serum butyrate during the day was not observed. Butyrate is thought to be taken up entirely by colonic mucosal cells,[5,6] so changes in colonic butyrate production would be expected to result in no changes or only small changes in peripheral blood concentrations, that could not be detected with the current butyrate analysis, which has a SD of approximately 1 μmol/L. However, significant differences in serum butyrate between subjects were large enough to be detected readily. In addition, the variability of serum propionate throughout the day was large enough to be detected, and it followed the same pattern as that of acetate, with temporary increases in serum propionate immediately after lunch and dinner. The significant interaction between time of day and subject group for serum propionate indicates that the changes in serum propionate throughout the day differed in different subjects. There was a larger and more prolonged increase in serum propionate after the lunch and dinner meals in middle-aged controls and IGT subjects than in younger controls. This seems most likely due to a higher rate of endogenous propionate production in the more insulin-resistant groups, but a difference in colonic fermentation cannot be ruled out by these data.

It has been suggested that propionate produced during colonic fermentation is virtually quantitatively removed by the liver.[54] However, we showed that infusion of sodium propionate into the human rectum results in an increase in serum propionate,[9] suggesting that changes in colonic propionate production are reflected in changes in serum propionate concentration. Nevertheless, there was evidence that the liver extracts more propionate than acetate from portal blood, as shown by the fact that the increase in serum acetate after rectal infusion of sodium acetate was about fourfold that seen after rectal infusion of an equimolar amount of sodium propionate.[9] Applying this ratio to the present results, where the increase in serum acetate after meals was 20 to 40 times greater than the increase in serum propionate, suggests that about five to 10 times more acetate than propionate was produced in the colon in these subjects on the test diets.

The relative proportion of acetate and propionate produced in vitro using fecal bacteria depends on the nature of the substrate,[55,56] the subject from whom the bacteria are harvested,[57,58] and the length of time of the fermentation reactions.[58,59] In general, there is a higher proportion of acetate to propionate produced from starch relative to fiber,[55,59] from rapidly digested relative to resistant starch,[59] and with shorter relative to longer periods of fermentation.[58,59] The majority of dietary carbohydrate consumed by the subjects in this study consisted of rapidly digested glucose polymers, and as speculated earlier, the postmeal spikes in serum acetate and propionate suggest that five to 10 times more acetate than propionate was produced. This is compatible with the 8.7 mean ratio of acetate to propionate produced during in vitro fermentation of cornstarch using fecal bacteria from four different subjects.[58]

It is concluded that both time of day and glucose tolerance status affect serum SCFA levels in nondiabetic humans. The results suggest that serum acetate is derived primarily from colonic fermentation, serum butyrate primarily from endogenous fatty acid metabolism, and serum propionate from both exogenous and endogenous sources.

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