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Research Article

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Effects of Free Fatty Acid Availability, Glucagon Excess, and Insulin Deficiency on Ketone Body Production in Postabsorptive Man

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ABSTRACT The present studies were undertaken to assess the relative effects of free fatty acid (FFA) availability, glucagon excess, and insulin deficiency on ketone body (KB) production in man. To determine whether an increase in FFA availability would augment KB production in the absence of insulin deficiency and glucagon excess, plasma insulin and glucagon were maintained at basal concentrations by infusion of somatostatin and exogenous insulin and glucagon, and plasma FFA were increased from 0.32 ± 0.06 to 1.4 ± 0.1 mM by a 2.5-h-infusion of a triglyceride emulsion plus heparin. KB production increased fivefold from 2.2 ± 0.4 to $11.4 \pm 1.2 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, $P < 0.001$. To determine whether insulin deficiency would further augment KB production, analogous experiments were performed but the replacement infusion of insulin was stopped. Despite a greater increase in plasma FFA (from 0.26 ± 0.04 to 1.95 ± 0.3 mM), KB production increased (from 1.5 ± 0.3 to $11.1 \pm 1.8 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) to the same extent as in the absence of insulin deficiency. To determine whether hyperglucagonemia would augment KB production beyond that accompanying an increase in plasma FFA and, if so, whether this required insulin deficiency, similar experiments were performed in which the glucagon infusion rate was increased to produce plasma glucagon concentrations of 450–550 pg/ml with and without maintenance of the basal insulin infusion. When basal plasma insulin concentrations were maintained, hyperglucagonemia did not further increase KB production; however, when the basal insulin infusion was discontinued, hyperglucagonemia increased KB production significantly, whereas no change was observed in saline control experiments. These studies indicate that, in man, FFA availability is a major

determinant of rates of KB production; insulin does not appear to influence ketogenesis rates by a direct hepatic effect, and glucagon can further augment KB production when FFA concentrations are increased, but only in the setting of insulin deficiency.

INTRODUCTION

In recent years considerable emphasis has been placed on the importance of glucagon and insulin in the regulation of ketogenesis (1). Diabetic ketoacidosis is characterized by hyperglucagonemia and absolute or relative insulinopenia (2). In vitro experiments (3–9) and studies performed in animals (10, 11) indicate that glucagon can augment hepatic conversion of free fatty acids (FFA)¹ into ketone bodies. However, a direct effect of insulin (6–8, 12) and the relative importance of FFA availability on ketogenesis (1, 9, 13–15) remain controversial. Moreover, although several studies in man which examined changes in circulating ketone body concentrations rather than changes in ketone body production rates have indicated that glucagon may directly affect hepatic ketone body production (16–19), these studies were confounded by changes in circulating FFA or insulin concentrations. Finally, a recent study, in which ketone body production rates were determined, has suggested that physiologic hyperglucagonemia does not affect ketogenesis in man (20).

In the present studies we examined the relative effects of FFA availability, hyperglucagonemia, and insulin deficiency on ketone body production in man. Of these factors, our results indicate that substrate availability, as reflected by plasma FFA concentra-

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¹ Abbreviations used in this paper: AcAc, acetoacetate; FFA, free fatty acid; HPLC, high performance liquid chromatography; βOHB , β -hydroxybutyrate.

tions, is the major determinant of ketone body production, and that hyperglucagonemia can augment the ketogenic response of the liver by an increase in FFA availability, but only when accompanied by insulin deficiency. Finally, insulin primarily influences ketone body production indirectly, i.e., by altering FFA availability since it appears to have little or no direct effect on ketogenesis other than modulating the hepatic response to a ketogenic hormone such as glucagon.

METHODS

Subjects. Informed consent was obtained from 32 normal volunteers, ages 20–32, all of whom were within 10% of their ideal body weight (Metropolitan Life Insurance Co. tables) and had no family history of diabetes mellitus.

Protocols. Subjects were studied in the Mayo Clinic Clinical Research Center in the postabsorptive state (i.e., after an overnight [13–14 h] fast). A 19-gauge scalp vein needle was placed retrogradely in a hand vein, and the hand was placed in a heated (50°–55°C) box for sampling or arterialized venous blood as previously described (21, 22). An 18-gauge infusion catheter was placed in a forearm vein of the contralateral arm and kept patent by an infusion of 0.45% NaCl (20 ml/h). 3 h before initiation of experiments, secretion of insulin, glucagon, and growth hormone was suppressed by infusion of synthetic somatostatin (500 µg/h, kindly provided by R. Guillemin and N. Ling, Salk Institute, San Diego, CA); and human growth hormone (National Pituitary Agency, lot 12, 8 ng·kg⁻¹·min⁻¹), glucagon (Eli Lilly & Co., Indianapolis, IN, 0.4 ng·kg⁻¹·min⁻¹), and crystalline pork insulin (Eli Lilly & Co., 0.15 mU·kg⁻¹·min⁻¹) were infused at rates chosen to approximate basal plasma concentrations. The insulin infusion rate was adjusted in each subject to maintain euglycemia. A primed (~0.1 µCi/kg) continuous (~0.002 µCi·kg⁻¹·min⁻¹) infusion of [³-¹⁴C]p(-)-β-hydroxybutyrate (New England Nuclear, Cambridge, MA) was begun, and 2 h were allowed for isotope equilibration.

Experiments began at 0 time with an infusion of a 10% triglyceride emulsion (Intralipid, 1.5 ml/min for 2.5–5 h, Cutter Laboratories, Berkeley, CA) and hourly intravenous injections of heparin (40 U/kg, Upjohn Co., Kalamazoo, MI) to increase plasma FFA concentrations. Three groups of experiments were performed: in the first group, the insulin infusion was discontinued at time 0 in six subjects (three male, three female) and was maintained in another six subjects to assess the effects of an increase in FFA availability on ketone body production with and without insulin deficiency. In the second group of experiments, the insulin infusion was maintained in six subjects (four female, two male), and the infusion of glucagon was increased after 150 min to 3.4 ng·kg⁻¹·min⁻¹ for 90 min to determine whether hyperglucagonemia would further augment ketone body production in the absence of insulin deficiency. In the third group of experiments, 14 subjects were studied in a similar fashion except that, at time zero, the basal infusions of both insulin and glucagon were discontinued to create a combined deficiency of these hormones, and at 150 min either glucagon (3 ng·kg⁻¹·min⁻¹, n = 8, 5 females and 3 males) or saline (4 females and 2 males) for 150 min to assess the effect of hyperglucagonemia during insulin deficiency.

Analytical and statistical analyses. Arterialized venous blood was sampled at 15-min intervals for determination of plasma glucose (YSI Glucose Analyzer, Yellow Springs, OH),

insulin (23), glucagon (24), growth hormone (25), FFA (26), acetoacetate (AcAc) (27), and β-hydroxybutyrate (βOHB) (27).

Radioactivity in plasma AcAc and βOHB was determined as follows: 2.0 ml of plasma to which ~3,000 dpm of L-[4,5-³H]isoleucine (New England Nuclear) had been added as an internal standard was deproteinized using ultrafiltration cones (CF-25, Amicon Corp., Danvers, MA). The cones were placed in conical supports (Amicon Corp.), which in turn were placed within 29 × 103-mm centrifuge tubes so that their tips entered a 4-ml screw top vial (Kimble Div., Owens-Illinois, Inc., Toledo, OH) at the bottom of the tube. Samples were centrifuged at 1,000 g for 90 min at 4°C; following a rinse with 2 ml distilled water, a second 90-min centrifugation was performed. The resultant ultrafiltrate was frozen, lyophilized in a chamber maintained at 4°C as previously described (28), resuspended in 0.5 ml NaH₂PO₄ buffer (0.5 M, pH 2.1) and subjected to reverse-phase high performance liquid chromatography (HPLC, Varian model 5060 [Varian Associates, Inc., Palo Alto, CA] with a fixed (214 nM) Waters model 441/UV detector [Waters Associates, Milford, MA]) on a 0.11 × 25-cm, 10-µmC₁₈ silica column (Alltech Associates, Deerfield, IL), eluted with 0.1 M NaH₂PO₄ buffer (pH 2.5) at a flow rate of 2.0 ml/min.

With this system, AcAc, βOHB, and isoleucine elute at 5.1, 6.4, and 6.7 min, respectively (Fig. 1). Glucose elutes with the void volume (1.1 min). Alanine, glutamate, and lactate, all of which may become labeled with ¹⁴C during ¹⁴C ketone body infusion (28), elute between 1.5 and 3.0 min. The column is flushed with 2.0 ml methanol between runs. Isoleucine was chosen as an internal standard since it nearly cochromatographs with βOHB but elutes at a distance from glucose (permitting simultaneous determination of [³H]glucose radioactivity if desired), and since its recovery from plasma was identical to βOHB, AcAc, and glucose. Labeled AcAc, βOHB, and glucose added to plasma were found to have identical retention times as the respective unlabeled compounds dissolved in buffer. Heparin and triglyceride emulsion, when added to plasma, did not affect recovery. The HPLC peaks corresponding to AcAc, βOHB, and isoleucine were collected in 20 ml liquid scintillation vials (Kimble Div., Owens-Illinois, Inc., Toledo, OH), suspended in 15 ml scintillation cocktail (Safety-Solve, Research Products International Corp., Mount Prospect, IL), and counted on a dual channel liquid scintillation spectrometer (Isocap 3001 Searle, G. D. & Co., Skokie, IL). Recovery using this procedure is ~60%, and minimum plasma radioactivity is ~80 cpm and ~500 cpm for AcAc and βOHB, respectively, at the chosen tracer infusion rates. Following correction for recovery and quench, total ketone body specific activity was calculated by dividing the sum of their concentrations in plasma. Rates of ketone body production were calculated using the equations of Steele et al. (29) as modified by Cherrington and Vranic (30). Use of these equations for determining ketone body turnover in vivo has previously been validated (28, 31). Data in text and figures are given as means ± SEM. Statistical evaluation was performed using Student's *t* test or analysis of variance (Anovar) where appropriate.

RESULTS

Effect of increased FFA availability on ketone body production with and without insulin deficiency (Fig. 1). Before increasing plasma FFA concentrations by infusions of the triglyceride emulsion and heparin,

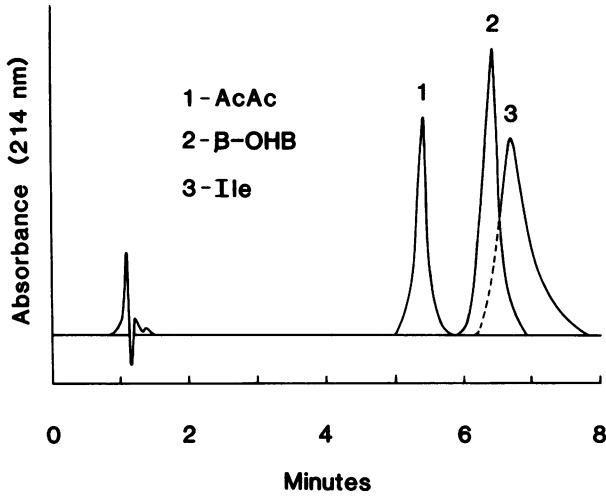


FIGURE 1 Representative elution profiles of AcAc, β OHB, and isoleucine (Ile) standards obtained by HPLC.

basal (mean values from -45 to 0 min) plasma glucose, insulin, glucagon, and growth hormone averaged 94 ± 5 mg/dl, 16 ± 1 μ U/ml, 138 ± 24 pg/ml, and 3.0 ± 0.4 ng/ml, respectively. During administration of the triglyceride emulsion and heparin, plasma FFA increased from 0.32 ± 0.06 to 1.40 ± 0.1 mM ($P < 0.001$), ketone body production increased progressively from 2.2 ± 0.4 to 11.4 ± 1.2 μ mol \cdot kg $^{-1}$ \cdot min $^{-1}$ at 150 min ($P < 0.001$) and plasma ketone body concentration increased from 0.19 ± 0.04 to 1.37 ± 0.2 mM ($P < 0.001$). Plasma glucose (98 ± 4 mg/dl), insulin (18 ± 2 μ U/ml), glucagon (137 ± 26 pg/ml), and growth hormone (3.7 ± 0.6 ng/ml) did not change. Thus, an increase in FFA availability in the absence of insulin deficiency or glucagon excess caused a fivefold increase in ketone body production.

Effect of increased FFA availability on ketone body production with insulin deficiency (Fig. 2). Basal plasma glucose (96 ± 5 mg/dl), insulin (16 ± 2 μ U/ml), glucagon (177 ± 20), and growth hormone (1.9 ± 0.4)

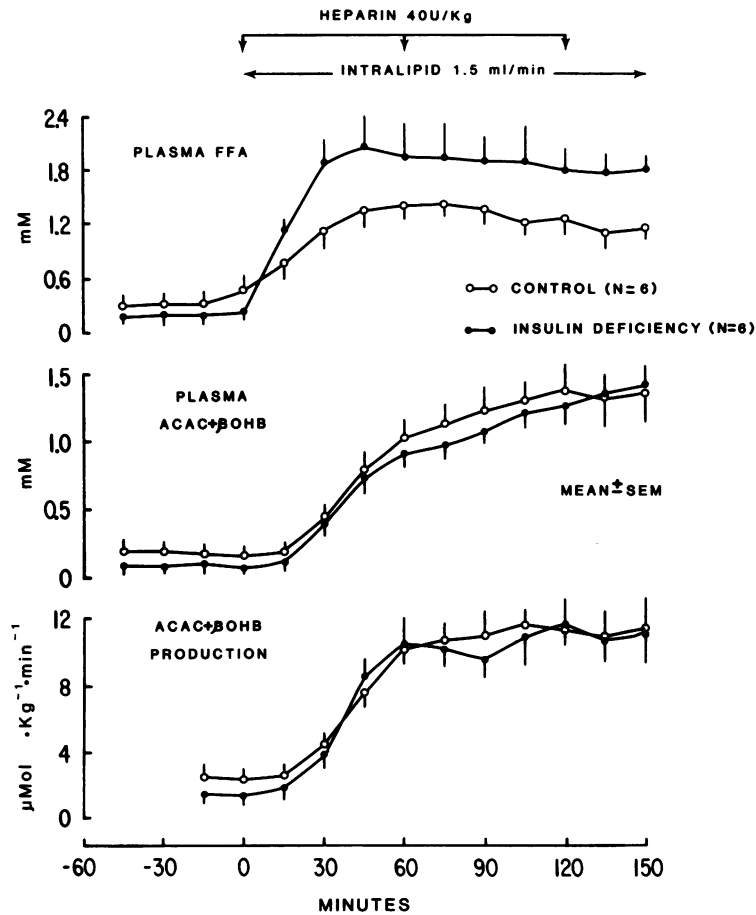


FIGURE 2 Plasma FFA and total ketone body (AcAc plus β OHB) concentrations and rates of ketone body production following elevation of plasma FFA by administration of a triglyceride emulsion plus heparin with and without insulin deficiency.

concentrations were not significantly different from corresponding values in the above experiments. Following discontinuation of the insulin infusion and administration of the triglyceride emulsion and heparin, plasma insulin decreased to $5 \pm 1 \mu\text{U/ml}$ ($P < 0.05$) within 30 min and remained at that value thereafter; plasma glucagon ($161 \pm 90 \text{ pg/ml}$) and growth hormone ($2.1 \pm 0.3 \text{ mg/ml}$) did not change, plasma glucose increased progressively to $179 \pm 13 \text{ mg/dl}$ at 150 min ($P < 0.001$), and plasma FFA increased from 0.26 ± 0.04 to $1.95 \pm 0.3 \text{ mM}$, a value significantly greater than was observed without insulin deficiency ($P < 0.05$). Despite this greater increase in plasma FFA, ketone body production and plasma ketone body concentration both increased to a similar extent as was observed without

insulin deficiency (from 1.5 ± 0.3 to $11.1 \pm 1.8 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ and from 0.12 ± 0.01 to $1.42 \pm 0.2 \text{ mM}$, respectively). Thus, insulin deficiency in the absence of glucagon excess did not increase ketone body production above that observed with a mere increase in FFA availability.

Effect of hyperglucagonemia and increased FFA availability on ketone body production in the absence of insulin deficiency (Fig. 3). Base-line plasma glucose, insulin, glucagon, growth hormone, FFA, and ketone body concentrations averaged $96 \pm 4 \text{ mg/dl}$, $16 \pm 2 \mu\text{U/ml}$, $154 \pm 28 \text{ pg/ml}$, $1.9 \pm 0.1 \text{ ng/ml}$, $0.29 \pm 0.07 \text{ mM}$, and $0.16 \pm 0.03 \text{ mM}$, respectively; ketone body production was $2.0 \pm 0.04 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$. After administration of the triglyceride emulsion and heparin,

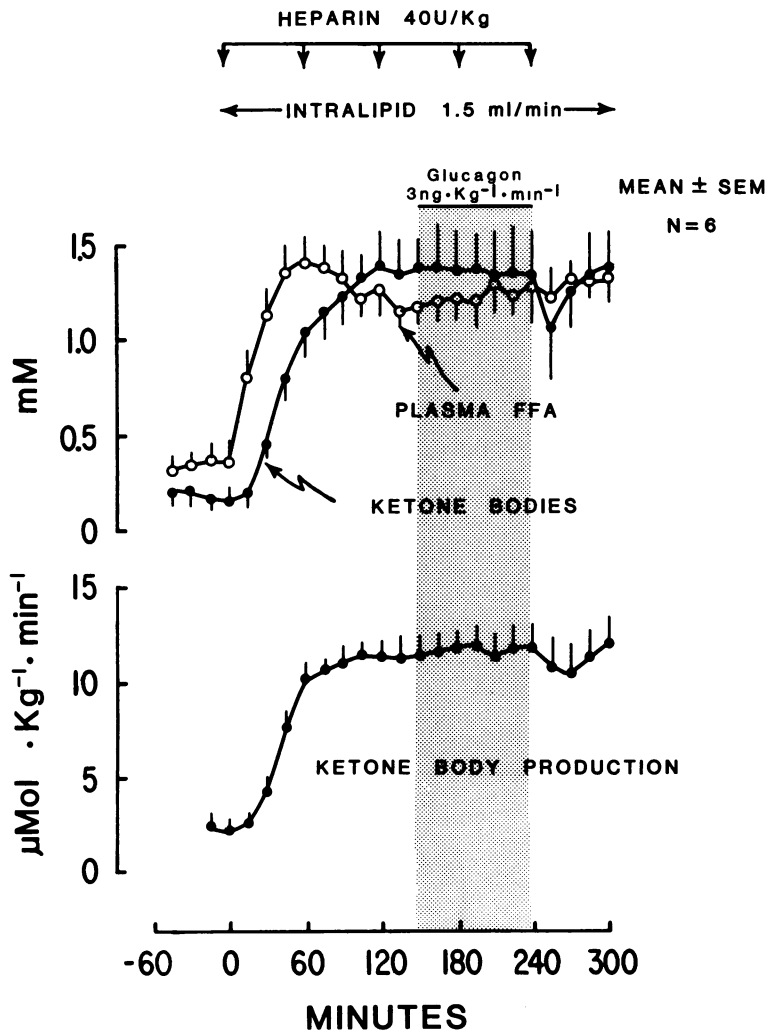


FIGURE 3 Effects of glucagon infusion (shaded area— $3 \text{ ng} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) on plasma FFA and total ketone body (AcAc and βOHB) concentrations and rates of ketone body production in the absence of insulin deficiency.

plasma FFA increased to 1.2 ± 0.4 mM; ketone body production increased to $10.8 \pm 0.9 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, and plasma ketone body concentration increased to 1.4 ± 0.3 mM. Plasma glucose (97 ± 3 mg/dl), insulin ($17 \pm 2 \mu\text{U/ml}$), glucagon (96 ± 20 pg/ml), and growth hormone (2.1 ± 0.1 mg/ml) were unchanged. When the glucagon infusion rate was increased, plasma glucagon increased to 440 ± 52 pg/ml, and plasma glucose increased progressively to 156 ± 10 mg/dl ($P < 0.001$); plasma insulin ($19 \pm 3 \mu\text{U/ml}$), growth hormone (2.5 ± 0.3 ng/ml), FFA (1.30 ± 0.1 mM), ketone bodies (1.35 ± 0.12 mM), and ketone body production did not significantly change. Thus, superimposition of hyperglucagonemia upon increased FFA availability did not augment ketone production when plasma insulin was maintained at basal concentrations.

Effect of hyperglucagonemia and increased FFA availability on ketone body production in the presence of insulin deficiency (Fig. 4). In these experiments, the basal insulin and glucagon infusions were stopped when the administration of the triglyceride emulsion and heparin was started. 2.5 h later either glucagon or saline was infused for 2.5 h. Plasma glucose (88 ± 6 vs. 85 ± 3 mg/dl), insulin (5.2 ± 0.5 vs. $5.3 \pm 0.5 \mu\text{U/ml}$), glucagon (71 ± 26 vs. 70 ± 16 pg/ml), growth hormone (2.9 ± 0.4 vs. 3.1 ± 0.2 ng/ml), FFA (2.2 ± 0.3 vs. 2.3 ± 0.3 mM), ketone bodies (1.8 ± 0.2 vs. 2.1 ± 0.2 mM), and rates of ketone body production (11.9 ± 1.2 vs. $12.4 \pm 1.0 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) were not

significantly different before the beginning saline or glucagon infusions, respectively.

Plasma FFA remained relatively constant during infusion of saline, and although a slight decrease in plasma FFA was observed during the glucagon infusion, this decrease was not significant. During infusion of glucagon ketone body production increased significantly (Anovar, $P < 0.05$) but did not change during infusion of saline. The changes in rates of ketone body production during infusion of glucagon was significantly greater (threefold) than that observed during infusion of saline by linear trend analysis ($P < 0.05$) (32). Moreover, maximum ketone body production rates were greater during glucagon infusion than those during saline infusion (17.1 ± 1.1 vs. $13.7 \pm 0.9 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, $P < 0.05$). Plasma ketone body concentrations increased during infusion of glucagon to 2.91 ± 0.35 mM, which was significantly greater than the ketone body concentration observed at the end of the saline infusion (2.11 ± 0.25 mM, $P < 0.05$).

During infusion of glucagon, plasma glucagon and glucose concentrations increased to 490 ± 60 pg/ml and 212 ± 15 mg/dl, respectively ($P < 0.001$), whereas during infusion of saline plasma glucose and glucagon concentrations were unchanged. During glucagon infusion, plasma insulin increased slightly to $8.5 \pm 1.0 \mu\text{U/ml}$ ($P < 0.05$), but remained unchanged during infusion of saline. Plasma growth hormone did not change in either study.

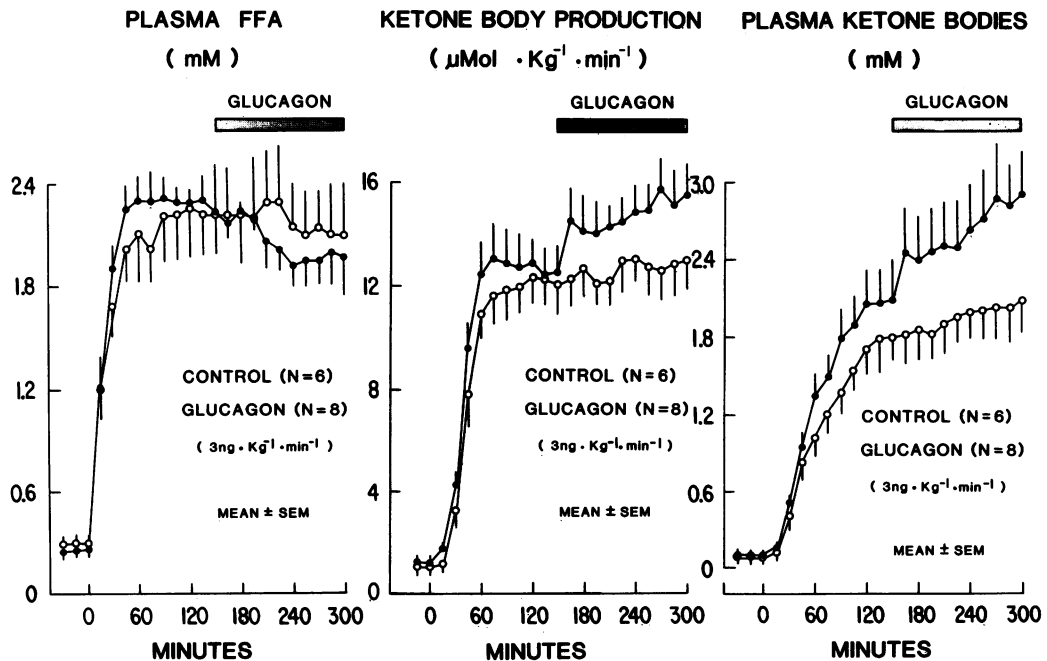


FIGURE 4 Effects of hyperglucagonemia on plasma FFA concentrations and total ketone body (AcAc and βOHB) rates of production and concentrations during insulin deficiency.

DISCUSSION

The present studies indicate that an increase in plasma FFA to levels seen in diabetic ketoacidosis (19) can augment ketone body production in postabsorptive normal man in the absence of insulin deficiency or hyperglucagonemia. These observations are consistent with previous *in vitro* studies demonstrating that an increase in FFA concentration per se can increase ketogenesis (9, 13–15, 33). In previous *in vivo* studies (11, 18, 34), increases in circulating FFA were accompanied by increases in circulating ketone body concentrations. However, only one study examined the effect of FFA availability on rates of ketone body production (11); in that study, which was performed in dogs, an increase in plasma FFA comparable to that produced in the present study resulted in only a 50% increase in ketone body production. The greater (four- to fivefold) increase observed in the present study in man suggests that there may be a species difference in the response of the liver. Furthermore, since the rates observed in the present study are about one-half to two-thirds the rates seen in diabetic ketoacidosis (19), our results suggest that the increase in FFA availability in diabetic ketoacidosis could account for a substantial proportion of the augmented ketogenesis seen in this condition.

When insulin deficiency was induced during an increase in FFA availability in the present study, there was no increase in ketone body production above that observed when FFA availability was increased without insulin deficiency, in spite of higher plasma FFA levels. These results indicate that, at least in the absence of a hormonal stimulus for ketogenesis, insulin may have no direct effect on ketone body production. Such a conclusion is consistent with *in vitro* studies in which no effect of insulin was found on hepatic ketone body production stimulated only by increased FFA availability (6, 12). These results further suggest that, although increased FFA availability clearly augments ketone body production rates, a V_{\max} for ketogenesis may be achieved at relatively low (1–1.5 mM) FFA concentrations, as suggested by previous *in vitro* studies (9, 15, 35). Nevertheless, since insulin deficiency *in vivo* accelerates lipolysis and thus augments FFA availability, insulin must, via this indirect action, be considered an important modulator of ketogenesis. Finally, a direct effect of pharmacologic levels of insulin on ketogenesis is not excluded by the present study.

In this study, when physiologic hyperglucagonemia was superimposed upon increased FFA availability without insulin deficiency, ketone body production did not increase. Similar results have been found in the dog and in man (1, 20, 36). In one study, splanchnic

ketone body production increased after glucagon infusion, but pharmacologic doses of glucagon were used (37). In our study hyperglucagonemia, when superimposed on increased FFA availability during insulin deficiency, significantly increased ketone body production. These observations are consistent with previous *in vitro* demonstrations of a direct enhancement of hepatic ketogenesis by glucagon (3–9) and are similar to the increases in ketone body production induced by hyperglucagonemia during somatostatin-induced insulin deficiency observed in the dog (10). The augmentation of ketone body production by glucagon in this study was small compared with the effects of infused FFA availability, however, and its physiologic role remains uncertain; nonetheless, it is quite possible that hyperglucagonemia is necessary for the achievement of maximal rates of ketone body production in diabetic ketoacidosis.

Our results appear to be at variance with the recent report of Sonnenberg et al. (20) in which no effect of glucagon was found on ketogenesis in insulin-deficient humans. The shorter duration of insulin deficiency and the lower glucagon infusion rate in that study may account for the failure to demonstrate a ketogenic effect of glucagon. It should be pointed out that in the present study during hyperglucagonemia and the resultant hyperglycemia, some breakthrough in the somatostatin-induced suppression of insulin secretion occurred and plasma FFA decreased slightly. For these reasons, as well as the fact that subjects were already 18 h postabsorptive and the fact that the present studies were short term, our results may underestimate the ketogenic potential of glucagon. It is quite possible that endogenous glucagon had already influenced ketogenesis in the control experiments (1), and, had the present study been performed 3–4 h after a meal, the effects of glucagon might have been even greater.

The fact that glucagon increased ketone body production in the present study only when insulin secretion was suppressed suggests that basal insulin secretion has a suppressive effect on the hepatic ketogenic response to glucagon in man. These observations are consistent with previous reports that insulin can antagonize the increase in ketone body production induced by glucagon *in vitro* (6–8). Why insulin does not appear to influence ketone body production augmented by merely increasing FFA availability, remains to be determined.

Although the present studies indicate that increases in both glucagon and FFA availability augment ketone body production in man, the exact contribution of each of these factors to the degree of ketone body production seen in diabetic ketoacidosis is unclear. In this condition rates of ketone body production as high as 25–30 $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ have been observed (19).

Based on the observations of the present study, increased FFA availability could account for perhaps half this rate. Glucagon and other potentially ketogenic hormones (e.g., catecholamines, growth hormone, cortisol) could account for the rest. It has been suggested that hyperglucagonemia is essential for maximal ketogenesis (1). This hypothesis is consistent with the observations of the present study as well as those studies in which glucagon-deficient diabetic patients developed less hyperketonemia than nonglucagon-deficient diabetic patients (16, 38). However, in studies using rat livers perfused in vitro, it has been reported that glucagon does not augment maximal rates of ketogenesis produced by ~2 mM oleate but does increase ketogenesis at submaximal FFA concentrations (9). Although this observation suggests that glucagon (and possibly other ketogenic hormones) may simply shift to dose-response curve for stimulation of ketone body production by FFA to the left without increasing maximal rates, it does not exclude the possibility that in vivo synergism between glucagon and other ketogenic hormones may increase otherwise maximal rates of ketogenesis. Before either of these two potential effects of glucagon on human ketogenesis can be established, it will be necessary to define the dose-response relationship between FFA availability and ketone body production and the influence of ketogenic hormones on that relationship in man.

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