Role of Hyperglucagonemia in Catabolism Associated with Type 1 Diabetes

Effects on Leucine Metabolism and the Resting Metabolic Rate

Michael R. Charlton and K. Sreekumaran Nair

The catabolic state of poorly controlled type 1 diabetes has largely been attributed to insulin deficiency. However, the role of hyperglucagonemia, which occurs concomitantly with insulin deficiency, has not been fully investigated. We studied the effects of hyperglucagonemia during insulin deprivation on energy expenditure (using indirect calorimetry) and protein metabolism (using L-[1-^{13}C,^{15}N]leucine and L-[1-^{13}C]leucine as tracers) in 12 type 1 diabetic subjects. Five protocols were used: insulin treatment, insulin deprivation, insulin deprivation with suppression of endogenous glucagon with somatostatin (SRIH) and growth hormone replacement, insulin deprivation with endogenous glucagon suppression with SRIH (no growth hormone replacement), and insulin deprivation with SRIH and a high level of glucagon replacement (no growth hormone replacement). It was observed that leucine oxidation and the resting metabolic rate (RMR) were significantly lower during insulin treatment and insulin deprivation with concomitant SRIH infusion (lowering glucagon) than during insulin deprivation alone. Replacement of glucagon at a high level during SRIH infusion in the insulin-deprived state increased leucine oxidation and the RMR. Hyperglucagonemia was also associated with a trend for decreased protein synthesis. Hyperglucagonemia did not affect leucine transamination. Insulin replacement decreased leucine flux and oxidation. Leucine oxidation (R^2 = 0.79) and the RMR (R^2 = 0.81) were seen, by multiple regression analysis, to correlate with glucagon levels and not with other hormones. We conclude that while insulin deficiency increases protein breakdown, hyperglucagonemia is primarily responsible for the increased leucine oxidation and RMR seen during insulin deprivation. *Diabetes* 47:1748–1756, 1998

From the Endocrine Research Unit, the Mayo Clinic and Foundation, Rochester, Minnesota.

Address correspondence and reprint requests to Michael Charlton, MD, Eisenberg 3-G, the Mayo Clinic and Foundation, 200 First St. S.W., Rochester, MN 55905.

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BCAA, branched-chain amino acid; BOHB, β-hydroxybutyrate; GC/MS, gas chromatograph/mass spectrometer; Ei, isotopic enrichment of [^{15}N]leucine; Ep, [^{15}N]KIC enrichment at plateau; i, infusion rate; KIC, ketoisocaproic acid; Q_L, leucine flux; Q_N, leucine nitrogen flux; RMR, resting metabolic rate; SRIH, somatostatin; X_N, reamination of KIC; X_O, deamination of leucine.

An association between diabetes and protein catabolism has been known to humans for a millennia (1). Sir William Osler, almost a 100 years ago, described the disease in terms of "progressive emaciation," involving massive urinary losses of both glucose and urea in diabetic patients (2). Insulin treatment not only improves control of glucose levels but also has a profound effect on protein metabolism, correcting increased urinary losses of nitrogen and increasing whole-body protein accretion, as indicated by whole-body potassium measurements (3–6). It remains to be clearly defined whether insulin deficiency, per se, is the cause of the catabolic state that occurs in poorly controlled type 1 diabetes. During insulin deprivation, type 1 diabetic patients are not only insulin deficient but also have elevated glucagon levels (7–10). Although hyperglucogonemia undoubtedly contributes to the hyperglycemic state in type 1 diabetes, the role of glucagon in the increased catabolism associated with type 1 diabetes remains controversial (11). There is considerable indirect evidence to suggest a catabolic role for glucagon in protein metabolism in humans. Hyperglucagonemia occurs in a variety of catabolic conditions, including trauma, burns, sepsis, cirrhosis, glucagonoma, and the postoperative state (12–16). Patients with glucagonoma, in whom glucose metabolism is only minimally disturbed, have a marked reduction in free intracellular branched-chain amino acids (BCAAs) in liver and muscle (17). It has been postulated that accelerated oxidation of BCAAs is a cause of their decreased intracellular concentrations. Previous studies in healthy volunteers demonstrated that high glucagon levels during insulin deficiency are associated with a substantial increase in leucine oxidation (7,11). Although increased protein breakdown also occurred during insulin deficiency, increased leucine oxidation occurred only with concurrent hyperglucagonemia. More recently, glucagon was shown to play a pivotal role in the disposal of amino acids during an amino acid load and to exert an overall protein catabolic effect (18).

It has been demonstrated that during insulin deprivation, there is not only an increase in leucine flux (an indicator of protein breakdown) and leucine oxidation, but also a substantial increase in the rate of transamination of leucine to its ketoacid, ketoisocaproic acid (KIC) (3). This increased transamination rate is believed to be a means of providing amino groups for the production of alanine and glutamine. The

cause of this increased leucine transamination is either insulin deficiency or increased glucagon levels during insulin deprivation (3). In this study, we investigated the effect of normal and of elevated glucagon levels on leucine transamination during insulin deprivation in type 1 diabetic patients.

Together, these experimental data and the association of hyperglucagonemia and protein catabolism in other clinical states strongly suggest a catabolic role for glucagon. The goal of our present study was to elucidate the effects of glucagon, independent of any change in insulin, on leucine metabolism and the resting metabolic rate (RMR) in poorly controlled type 1 diabetic patients. Somatostatin, which causes a suppression of glucagon secretion, allows us to measure leucine metabolism and the RMR *1*) during insulin treatment (during which glucagon levels are low), *2*) during insulin deficiency with concomitant hyperglucagonemia, and *3*) during insulin deficiency with lower glucagon levels. This permits us to test the hypothesis that the increase in leucine oxidation and the RMR previously demonstrated in type 1 diabetic patients is attributable to hyperglucagonemia, rather than insulin deficiency, per se.

RESEARCH DESIGN AND METHODS

Subjects. We studied 12 volunteers (7 men, 5 women) with type 1 diabetes who were C-peptide negative. Mean age was 28.3 years (range 21–39). Mean height ± SE was 165 ± 4.5 cm, and mean weight was 67.8 ± 5.9 kg. The mean time since the diagnosis of type 1 diabetes was 10.4 years (range 6–19). All volunteers were within 10% of ideal body weight for height and were screened by detailed history, physical examination, and biochemical profile (urine and blood) for any other overt illness or complication from diabetes. Subjects taking any regular medications and subjects with chronic renal insufficiency, hypertension, retinopathy, neuropathy, or vascular complications of diabetes were excluded.

C-peptide levels were drawn after a standard protein meal to document endogenous insulin deficiency in all subjects. Volunteers with an insulin requirement of <35 U/day or >80 U/day were excluded from the study. Informed written consent was obtained after detailed explanation of the protocol, which had been approved by the institutional Human Investigation Committees of the University of Vermont and the Mayo Clinic and Foundation.

Materials. L-[1-^{13}C,^{15}N]leucine, L-[1-^{13}C]leucine, and sodium (^{13}C)bicarbonate were obtained from Cambridge Isotopes (Cambridge, MA). Chemical, isotopic, and optical purity of the L-[1-^{13}C,^{15}N]leucine and L-[1-^{13}C]leucine were confirmed before use. Solutions of the isotopes were prepared under sterile precautions and were tested for pyrogens and sterility before use.

Study design. All subjects were placed on a weight-maintaining diet for the duration of the study. For 3 days before each study, the volunteers were placed on 6-h regimens of regular insulin at the exclusion of all longer acting forms to avoid a carry-over effect from long-acting insulin during the study period. Subjects were admitted to the Clinical Research Center 1 day before each study. Five study protocols were carried out in two phases, which were designed to determine the following:

1. Whether the increased leucine oxidation and the RMR that occur during insulin deprivation are normalized by inhibition of glucagon secretion by somatostatin;
2. Whether the reduction of leucine oxidation and RMR during insulin deprivation by SRIH can be reversed by the infusion of exogenous glucagon;
3. Whether hyperglucagonemia during insulin deficiency stimulates leucine transamination.

Six subjects underwent three protocols as part of phase 1, and six other subjects underwent two different protocols as part of phase 2. All studies in the same subject were separated by at least 3 weeks. Studies in women were always carried out in the luteal phase of the menstrual cycle, as determined by history. The order in which participants underwent the protocols was assigned in a random fashion. All studies were carried out after an overnight fast.

Phase 1 experiments

Common protocol for protocols I–III. On the morning of each study day, an 18-gauge venous catheter was placed in a retrograde fashion in a dorsal hand vein. The hand with this catheter was then rested in a "hot box" that circulated air at a temperature of 60°C to arterialize venous blood (19). Arterialized venous blood accurately reflects labeled leucine and KIC concentrations in arterial blood (20). A second 18-gauge catheter was placed in the contralateral forearm for infusions. Once baseline blood and expired air samples were obtained, bolus doses of sodium (^{13}C)bicarbonate (1.5 μmol/kg) and L-[1-^{13}C]leucine (7.5 μmol/kg) were given to prime the respective pools to achieve early isotopic plateaus (21,22). Immediately after the administration of the priming dose, a continuous infusion of L-[1-^{13}C]leucine was started at a rate of 7.5 μmol · kg^{-1} · h^{-1} and continued for 480 min.

Arterialized venous blood samples for isotope analysis were drawn at 0 min and 120 min and then every 30 min until the end of the study at 480 min. Expired air for isotope analysis was collected at the same intervals as blood isotopes. Hormones and substrates were measured at 0, 120, 360, and 480 min.

Whole-body indirect calorimetry was carried out for a period of 45 min, starting at 240 min to measure CO_2 production and oxygen consumption. The indirect calorimetry results from the final 30 min of this period were used for calculations of leucine oxidation and RMR.

Protocol I: Insulin deprivation. On the day of admission, no insulin was administered after 6:00 P.M. for the remainder of this study. At 7:00 P.M. on the evening of admission, an infusion of normal saline was commenced at a rate of 1 ml · kg^{-1} · h^{-1}, instead of insulin. This infusion was continued throughout the study. Water intake was encouraged and blood glucose measurements were obtained hourly.

Protocol II: Insulin treatment. At 7:00 P.M. on the evening of admission, an intravenous infusion of regular insulin was started and adjusted at half-hour intervals to maintain blood glucose levels at between 4.4 and 5.6 mmol/l. This infusion was adjusted every 15 min between 8:00 A.M. and the end of the study to maintain the blood glucose level in this range.

Protocol III: Insulin deprivation + somatostatin (administered for the suppression of endogenous glucagon production). As in protocol I, no insulin was administered after 6:00 P.M. on the day of admission for the remainder of this study. The rest of the protocol was the same as protocol I, except for infusion of somatostatin. At 8:00 A.M. in the morning after admission, an infusion of somatostatin (Stilamin; Serono Laboratories, Randolph, MA) was initiated at a rate of 500 μg/h and continued at this rate for the 8-h duration of the study.

Recombinant human growth hormone (Genentech, San Francisco, CA) was also infused at a rate of 0.5 μg · kg^{-1} · h^{-1} simultaneously and continued throughout the study to prevent the fall in circulating growth hormone levels.

Phase 2 experiments. When the phase 1 experiments had been completed and the results analyzed, a second phase of experiments was designed and carried out (protocols IV-V). Six type 1 diabetic patients participated in the second phase of experiments, consisting of two studies separated by 8 weeks. In addition to the parameters measured in the first phase of experiments, the leucine transamination rate was also measured to determine whether glucagon affects leucine transamination. The order in which the participants underwent the protocols was assigned in a random fashion.

Common protocol for protocols IV–V. Once baseline blood and expired air samples were obtained, bolus doses of sodium (^{13}C)bicarbonate (1.5 μmol/kg) and L-[1-^{13}C,^{15}N]leucine (7.5 μmol/kg) were given. Immediately after the administration of the priming dose, a continuous infusion of L-[1-^{13}C,^{15}N]leucine was started at a rate of 7.5 μmol · kg^{-1} · h^{-1} and continued for 360 min. L-[1-^{13}C,^{15}N]leucine was chosen as a tracer because leucine is an essential amino acid and its carboxyl group (1-C) is not synthesized in humans. The addition of the ^{15}N label allows the rates of transamination of leucine to be determined.

Arterialized venous blood samples for isotope analysis were drawn at 0, 100, 110, 120, 240, 340, 350, and 360 min. Expired air for isotope analysis was collected at the same intervals as blood isotopes. Hormones and substrates were measured at 0, 120, 330, and 360 min.

Whole-body indirect calorimetry was carried out for two periods of 45 min starting at 55 and 255 min to measure the RMR, CO_2 production, and oxygen consumption.

Protocol IV: Insulin deprivation + somatostatin. No insulin was administered after 6:00 P.M. on the day of admission for the remainder of this study. At 8:00 A.M. the following morning, a primed continuous infusion of L-[1-^{13}C,^{15}N]leucine was initiated. At 2-h after initiation of the primed continuous infusion of L-[1-^{13}C,^{15}N]leucine (0–120 min), an SRIH infusion was started at a rate of 500 μg/h. This was continued for the remainder of the study (120–360 min). The growth hormone was not replaced.

Protocol V: Insulin deprivation + somatostatin + high level of glucagon replacement. The study design was exactly the same as for protocol IV except that 2-h after initiation of the primed continuous infusion of L-[1-^{13}C,^{15}N]leucine (0–120 min), a somatostatin infusion was started at a rate of 500 μg/h together with a concomitant infusion of glucagon at a high rate of replacement (3 ng · kg^{-1} · min^{-1}). This was continued for the remainder of the study (120–360 min). The growth hormone was not replaced.

Analysis of samples

Isotopes

Plasma [^{13}C]leucine, [^{13}C]KIC, and [^{15}N,^{13}C]leucine isotope enrichment. The enrichment of levels of [^{13}C]leucine and [^{15}N,^{13}C]leucine were determined using

an HP5988 gas chromatograph/mass spectrometer (GC/MS; Hewlett-Packard, Palo Alto, CA) by multiple ion monitoring under positive ion methane chemical ionization conditions. Fragment ions from the heptofluorobutyryl ester derivative were monitored at m/z 283/282 and m/z 384/385/386 to determine the ^{15}N- and mono/di-labeled species, respectively (23,24). The [^{13}C]KIC enrichment was then determined as its quinoxalinol-TMS derivative under electron ionization conditions using an HP5971A GC/MS by monitoring ions 233/232 (23,25).

Enrichment of $^{13}CO_2$ in expired air. Expired air samples were collected in a modified Foley catheter bag and transferred into a 20-ml evacuated test tube. The isotopic enrichment of $^{13}CO_2$ in expired air was measured with an automated isotope ratio mass spectrometer as previously described (23,26).

Hormones and substrates. Plasma glucose concentrations were measured with an auto-analyzer (Beckman Instruments, Fullerton, CA). Glucagon, free insulin, growth hormone, and C-peptide concentrations were measured by radioimmunoassay using a commercial kit (Diagnostic Products, Los Angeles, CA). Cortisol was also measured using radioimmunoassay kit (Cortisol Kit; Diagnostic Products). Free insulin was measured after precipitation with polyethylene glycol (27). Plasma amino acid concentrations were measured by high-performance liquid chromatography with fluorometric detection with precolumn *O*-phtaldehyde derivatization (28). Glycerol was measured using the free glycerol assay kit, and β-hydroxybutyrate (BOHB) was measured using the BOHB assay kit (Sigma Chemical, St. Louis, MO). Free fatty acids were measured using an in vitro enzymatic calorimetric kit (Wako Chemicals, Neuss, Germany). Plasma concentrations of epinephrine and norepinephrine were measured by radioenzymatic assay (29).

Calculations. For phase 1 experiments, all inpatient calculations of leucine kinetics were made from values obtained during the isotopic plateau of the last 2 h. For phase 2 experiments (protocols IV and V) calculations of leucine kinetics were carried between 90 and 120 min (baseline) and again between 330 and 360 min (intervention period). Leucine oxidation was calculated from plateau values of $^{13}CO_2$ production rate and using plasma [^{13}C]KIC enrichment as the precursor pool (21). The production rate of $^{13}CO_2$ was calculated from $^{13}CO_2$ enrichment and CO_2 production rate, as previously described (21,22). Leucine flux (Q_L) was calculated as follows: $Q_L = i(Ei/Ep - 1)$ (micromoles per kilograms per hour), where i is the infusion rate (micromoles per kilograms per hour); Ei is the isotopic enrichment of [^{13}C]leucine in the infusate; and Ep is [^{13}C]KIC enrichment at plateau. Leucine deamination and reamination were calculated using the following formula: $Q_N - Q_C = X_N = X_O$, where Q_N is leucine nitrogen flux ($Q_N = i(Ei/Ep - 1)$ (micromoles per kilograms per hour); i is the infusion rate (micromoles per kilograms per hour); Ei is the isotopic enrichment of [^{15}N]leucine in the infusate; Ep is [^{15}N]KIC enrichment at plateau; X_O is deamination of leucine; and X_N is reamination of KIC (23).

Data analysis. Data analysis was carried out with the SAS software package (SAS, Cary, NC). Comparisons of phase 1 (protocols I-III) and phase 2 (protocols IV–V) were carried out separately. Analysis of variance (ANOVA) was used to compare treatment effects and covariance with hormone levels. Following a significant treatment effect, individual contrasts were computed to compare individual treatment means. Regression analyses were performed to determine which of the hormonal changes were best correlated to the differences in leucine oxidation or the RMR. All results are means ± SE.

RESULTS

Hormones and substrates

Phase 1 (protocols I–III). The mean plateau glucagon level during protocol I (insulin deprivation) was 42% higher than during protocol II (insulin treatment) ($P < 0.01$) and was 26% higher than during protocol III (insulin deprivation + SRIH infusion) ($P < 0.04$) (Table 1). Plateau-free insulin levels were significantly higher during insulin treatment than during insulin deprivation or insulin deprivation + SRIH infusion ($P < 0.0001$).

The plateau values of other hormones and substrates are given in Table 1. There were no significant differences in the mean plateau levels of cortisol, epinephrine, or norepinephrine between insulin deprivation, insulin treatment, and insulin deprivation + SRIH infusion. Mean plateau growth hormone levels were higher ($P < 0.05$) during protocol I than during protocols II or III.

The mean plateau glucose concentration was lower ($P < 0.001$) during insulin treatment (protocol II) than during protocols I (insulin deprivation) or III (insulin deprivation + SRIH infusion). Furthermore, the mean plateau glucose level was lower during protocol III (insulin deprivation + SRIH infusion) than during protocol II (insulin deprivation) ($P < 0.01$).

Plasma levels of BOHB, glycerol, and free fatty acids all were lower ($P < 0.001$) during insulin treatment than during insulin deprivation or insulin deprivation + SRIH infusion (Table 2).

Phase 2 (protocols IV–V). The mean glucagon levels were similar during the baseline periods of protocols IV and V. The mean intervention value (i.e., during SRIH) of glucagon in protocol IV was 20% lower than the mean level of glucagon seen during the baseline period of this protocol ($P < 0.016$). The mean glucagon level during the intervention period of protocol V (i.e., during SRIH + high level of replacement glucagon infusion) was significantly higher than the level of glucagon seen during the baseline period of protocol V ($P < 0.015$) and the intervention period of protocol IV ($P < 0.007$).

Free insulin levels were similar during the baseline and intervention periods of both protocols IV and V. Mean baseline values of glucose were similar in protocols IV and V. However, the mean glucose concentration was lower ($P < 0.001$) during the intervention period of protocol IV (12.2 ± 0.05 mmol/l) than during the intervention period of protocol V (20.3 ± 1.6 mmol/l) in which glucagon was infused at 3 ng · kg^{-1} · h^{-1}.

TABLE 1
Mean plasma values of hormones and substrates for phase 1 experiments

	Protocol I (Insulin deprivation)	Protocol II (Insulin treatment)	Protocol III (Insulin deprivation + SRIH)
Glucagon (ng/l)	115 ± 12	67 ± 6*	85 ± 6*†
Insulin (pmol/ml)	35 ± 1	87 ± 16*	35 ± 1*
Growth hormone (µg/l)	5 ± 1	3.5 ± 1	3.5 ± 1
Cortisol (nmol/l)	440 ± 80	300 ± 50	390 ± 50
Epinephrine (pmol/l)	190 ± 40	230 ± 40	220 ± 50
Norepinephrine (nmol/l)	310 ± 50	210 ± 40	210 ± 30
Glucose (mmol/l)	19 ± 2	4.9 ± 0.04*	10 ± 2*†
Glycerol (mmol/l)	1.70 ± 0.30	0.60 ± 0.20*	1.75 ± 0.30†
BOHB (µmol/l)	3,170 ± 400	680 ± 95*	3,270 ± 450†
Free fatty acids (g/l)	1.7 ± 0.5	0.73 ± 0.2*	1.7 ± 0.4†

Data are means ± SE. Insulin levels refer to free insulin. *A value significantly lower than that obtained during protocol I; †a value significantly higher than that obtained during protocol II.

TABLE 2
Mean plasma values of hormones and substrates for phase 2 experiments

	Protocol IV		Protocol V	
	Baseline, insulin deprivation (0–120 min)	Intervention, insulin deprivation + SRIH (120–360 min)	Baseline, insulin deprivation (0–120 min)	Intervention, insulin deprivation + SRIH + high level of glucagon replacement (120–360 min)
Glucagon (ng/l)	105 ± 14	84 ± 14*	111 ± 11	225 ± 38†
Insulin (pmol/ml)	35 ± 1	35 ± 1	35 ± 1	35 ± 1
Growth hormone (µg/l)	4 ± 2.5	2.5 ± 2.0	5 ± 2.0	2.4 ± 1.5
Cortisol (nmol/l)	720 ± 110	410 ± 50	630 ± 60	720 ± 170
Epinephrine (pmol/l)	170 ± 40	180 ± 60	170 ± 30	230 ± 70
Norepinephrine (nmol/l)	230 ± 30	210 ± 20	200 ± 30	180 ± 20
Glucose (mmol/l)	17 ± 1.5	11 ± 2	16 ± 1.5	19 ± 2‡
Glycerol (mmol/l)	1.90 ± 0.30	1.40 ± 0.40	1.50 ± 0.25	1.90 ± 0.35
BOHB (µmol/l)	2,400 ± 290	3,460 ± 380	2,690 ± 380	2,880 ± 580
Free fatty acids (g/l)	2.0 ± 0.4	1.8 ± 0.3	1.7 ± 0.3	1.5 ± 0.1

Data are means ± SE. Insulin levels refer to free insulin. *The mean intervention value of glucagon in protocol IV was significantly lower than the mean level of glucagon seen during the baseline period of this protocol ($P < 0.016$); †the mean intervention value of glucagon in protocol V was significantly higher than the level of glucagon seen during the baseline period of protocol V ($P < 0.015$) and the intervention period of protocol IV ($P < 0.007$); ‡glucose values during the intervention period of protocol V were significantly higher than during the intervention period of protocol IV ($P < 0.001$).

The intervention period values of other hormones and substrates are given in Table 1. There were no significant differences between protocols IV and V in the mean baseline or intervention period levels of growth hormone, cortisol, epinephrine, or norepinephrine.

Plasma levels of BOHB, glycerol, and free fatty acids were all similar during the baseline and intervention periods of protocols IV and V (Table 2).

Amino acids

Phase 1 (protocols I–III). The branched chain amino acids (leucine, isoleucine, valine) were lower ($P < 0.05$) during insulin treatment than during either insulin deprivation alone or insulin deprivation + SRIH. Alanine, glycine, threonine, serine, lysine, and tyrosine were higher ($P < 0.05$) during insulin deprivation + SRIH infusion (with suppression of endogenous glucagon levels) than during either insulin deprivation alone and insulin treatment (Table 3).

Phase 2 (protocols IV and V). During the intervention period of protocol V (insulin deprivation with concomitant somatostatin clamp and high level of glucagon replacement), concentrations of alanine, serine, lysine, methionine, histidine, glycine threonine, phenylalanine, leucine, and isoleucine all were lower ($P < 0.05$) than during the intervention period of protocol IV.

Isotopes and leucine kinetics. [^{15}N,^{13}C]leucine, ^{13}C, and [^{13}C]KIC isotopic enrichment (atom percent excess) and $^{13}CO_2$ production were all stable during the periods used for kinetic calculations.

TABLE 3
Mean amino acid levels for all experiments

	Protocol I	Protocol II	Protocol III	Protocol IV		Protocol V	
				Baseline	Plateau	Baseline	Plateau
Serine	110 ± 12	129 ± 8	165 ± 13*	70 ± 10	88 ± 13†	64 ± 10	54 ± 5‡
Histidine	79 ± 4	76 ± 4	86 ± 5	48 ± 7	57 ± 7	41 ± 4	40 ± 5‡
Glycine	275 ± 17	349 ± 24	379 ± 16*	118 ± 9	136 ± 11	115 ± 11	93 ± 9‡
Threonine	146 ± 16	148 ± 11	200 ± 19*	53 ± 7	66 ± 11	46 ± 5	36 ± 4‡
Alanine	245 ± 19	252 ± 11	349 ± 29*	149 ± 8	208 ± 24†	134 ± 13	151 ± 15‡
Tyrosine	55 ± 3	48 ± 2	82 ± 3*	33 ± 6	33 ± 7	29 ± 5	27 ± 6
Valine	325 ± 31	175 ± 11	301 ± 21	200 ± 30	268 ± 26	193 ± 12	221 ± 14
Methionine	7 ± 1	11 ± 1	10 ± 2	24 ± 1	31 ± 3†	23 ± 2	21 ± 1‡
Phenylalanine	53 ± 2	48 ± 2	60 ± 3	40 ± 6	46 ± 6	36 ± 5	35 ± 6‡
Isoleucine	122 ± 12	44 ± 4	107 ± 8	103 ± 13	114 ± 9†	84 ± 6	90 ± 6
Leucine	255 ± 27	116 ± 7	235 ± 19	200 ± 24	245 ± 18†	168 ± 10	195 ± 13‡
Lysine	131 ± 5	150 ± 6	194 ± 9*	185 ± 15	218 ± 17†	163 ± 8	152 ± 12‡

Data are means ± SE of amino acid concentrations (milligrams per deciliter) during each of the five protocols. *A value significantly higher ($P < 0.05$) than protocol I; †a value significantly higher ($P < 0.04$) than during the baseline period of protocol IV; ‡a value significantly ($P < 0.05$) lower than the intervention period of protocol IV.

TABLE 4
Leucine kinetics and RMR values for phase 1 experiments

	Protocol I (Insulin deprivation)	Protocol II (Insulin treatment)	Protocol III (Insulin deprivation + SRIH)
RMR (calories/24 h)	2,100 ± 90	1,880 ± 60*	2,010 ± 50*
Respiratory quotient	0.74 ± 0.04	0.76 ± 0.06	0.75 ± 0.03
O_2 consumption (Vo_2)	271 ± 16	244 ± 12*	259 ± 13*
Leucine C flux (Q_c)	112 ± 7	94 ± 5*	105 ± 7
Leucine oxidation (E)	43 ± 3	21 ± 3*	27 ± 3*
Leucine → protein	69 ± 5	73 ± 5	78 ± 6

Data are means ± SE calculated during the final 120 min of each experiment. *A value significantly lower than during protocol I.

Phase 1 (protocols I–III). Leucine carbon flux (an index of protein breakdown) was 23% higher during protocol I (insulin deprivation) than during protocol II (insulin treatment) ($P < 0.001$) (Table 4). Leucine carbon flux during protocol III (insulin deprivation + SRIH) was similar to the rate seen during protocol I (insulin deprivation).

Leucine oxidation declined by >50% during insulin treatment when compared with the rate seen during insulin deprivation ($P < 0.0001$). In protocol III, in which SRIH was administered during insulin deprivation, the rate of leucine oxidation was also significantly lower (by 37%) than the rate seen during insulin deprivation alone (protocol I).

Regression analysis indicated that leucine oxidation correlated with glucagon levels ($R^2 = 0.79$, $P < 0.001$). When analysis of covariance (ANCOVA) was used to adjust leucine oxidation rates for differences in glucagon levels between treatments, the treatment differences nearly disappeared. Similar analysis of growth hormone, cortisol, epinephrine, and norepinephrine did not alter the treatment effect seen in these studies.

There was a trend for nonoxidative disposal of leucine, an indicator of protein synthesis, to be lower during insulin deprivation (69 ± 5 μmol · kg^{-1} · h^{-1}) than during insulin treatment (73 ± 5 μmol · kg^{-1} · h^{-1}) ($P < 0.2$) and insulin deprivation + SRIH infusion (78 ± 6 μmol · kg^{-1} · h^{-1}) ($P < 0.09$).

Phase 2 (protocols IV and V). Values for leucine kinetics during protocols IV and V are shown in Table 4. Leucine flux was similar during the baseline periods of protocols IV and V. During the intervention period of protocol IV, leucine flux decreased significantly (by 13%) when compared to the baseline period. In contrast, during the intervention period of protocol V, leucine flux decreased by 9%, a difference between protocols that did not reach statistical significance ($P < 0.21$) (Table 5 and Fig. 1).

Leucine oxidation was significantly lower during the intervention period of protocol IV (when glucagon levels were lowered by a somatostatin clamp) than during the baseline period of this protocol ($P < 0.02$). During the intervention period of protocol V, when glucagon was replaced at a high level, leucine oxidation was significantly higher than either the baseline period of protocol V ($P < 0.03$) and the intervention period of protocol IV ($P < 0.009$) (Table 5 and Fig. 1). Regression analysis demonstrated that leucine oxidation levels correlated with glucagon levels, producing a correlation coefficient (R^2) of 0.81 (Fig. 2).

The percentage of leucine flux oxidized was significantly higher during the intervention period of protocol V (when

TABLE 5
Leucine kinetics values for phase 2 experiments

	Protocol IV		Protocol V	
	Baseline, insulin deprivation (0–120 min)	Intervention, insulin deprivation + SRIH (120–360 min)	Baseline, insulin deprivation (0–120 min)	Intervention, insulin deprivation + SRIH + high level of glucagon replacement (120–360 min)
RMR (calories/24 h)	2,100 ± 85	1,950 ± 60*‡	2,050 ± 90	2,250 ± 110†
Respiratory quotient	0.76 ± 0.05	0.74 ± 0.03	0.75 ± 0.04	0.76 ± 0.05
O_2 consumption (ml/min)	265 ± 14	251 ± 11*‡	262 ± 13	284 ± 16†
Leucine C flux (Q_c)	150 ± 9	130 ± 6*	154 ± 7	140 ± 5
Leucine oxidation (E)	40 ± 2.5	32 ± 2.7*	36 ± 2.0	48 ± 3.0†
Leucine → protein	110 ± 8.2	98 ± 6	119 ± 5.3	93 ± 6
Leucine N flux	290 ± 22	370 ± 28	305 ± 14	350 ± 22
Leucine → KIC (X_O)	290 ± 22	370 ± 28	305 ± 14	350 ± 22
KIC → leucine (X_N)	250 ± 22	340 ± 27	270 ± 13	315 ± 21
% Flux oxidized (C/Q_c)	27 ± 1.7	24 ± 2.0	23 ± 0.6	35 ± 1.9†

Data are means ± SE. *A value significantly lower than during the baseline period of protocol IV; †a value significantly higher than that obtained during the baseline period of protocol V; ‡a value significantly lower than seen during either the baseline period of protocol IV and the intervention period of protocol V.

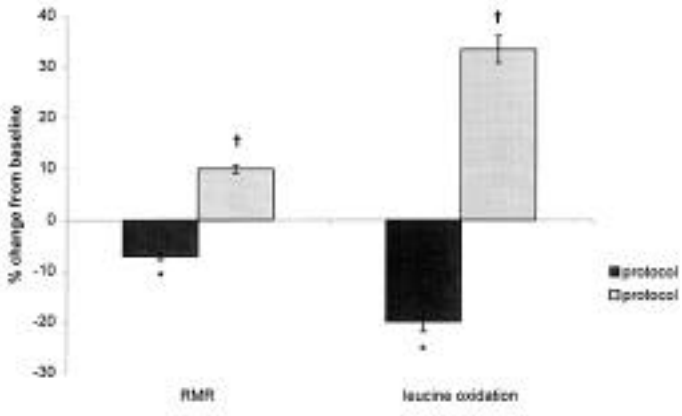

FIG. 1. The effects of suppression of endogenous glucagon production during insulin deprivation (■, protocol IV) and of glucagon replacement during insulin deprivation with concomitant SRIH infusion (□, protocol V) on leucine oxidation and the RMR are shown: *a value significantly lower than seen during the baseline period of protocol IV (insulin deprivation alone); †a value significantly higher than either the baseline period of protocol V (insulin deprivation alone) or the intervention period of protocol IV (insulin deprivation with SRIH infusion).

glucagon levels were high) than that of protocol IV ($P < 0.02$). The same was true for the percentage of KIC that was oxidized ($P < 0.014$).

While the nonoxidative portion of leucine flux, a putative index of protein synthesis, was similar during the intervention periods of protocols IV and V, the percentage decline in nonoxidative flux when compared with baseline periods of these protocols was 11% for protocol IV versus 22% in protocol V. Although this difference in percentage change from baseline between the protocols did not reach statistical significance ($P < 0.09$), there was a trend for hyperglucagonemia to inhibit nonoxidative flux. Whole-body leucine nitrogen flux (Q_N) and transamination rates of leucine to KIC were similar in protocols IV and V.

Resting metabolic rate

Phase 1 (protocols I–III). Insulin treatment (protocol II) produced an 11% fall in the RMR when compared with

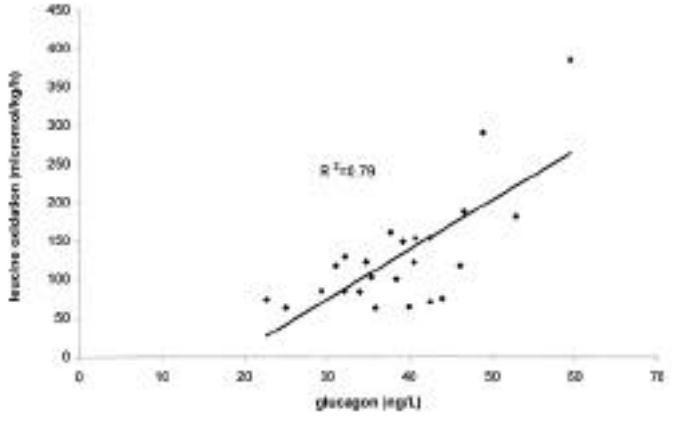

FIG. 2. A regression plot of the rate of leucine oxidation rate with individual plasma glucagon values is shown. The correlation coefficient (R^2) is 0.79.

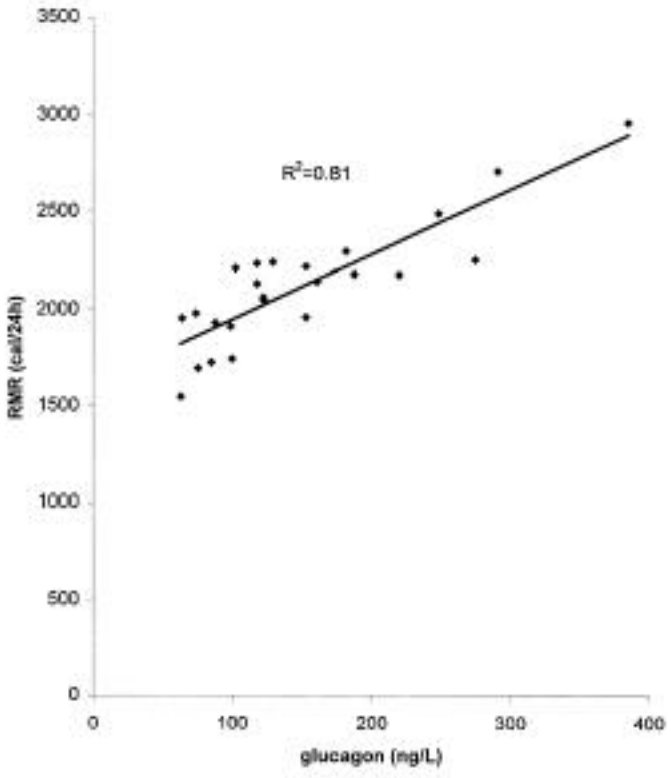

FIG. 3. A regression plot of the RMR with individual plasma glucagon values is shown. The correlation coefficient (R^2) is 0.81.

insulin deprivation (protocol I) ($P < 0.02$). SRIH infusion (suppressing endogenous glucagon production) during insulin deprivation (protocol III) also lowered the RMR (by 5%) when compared with insulin deprivation alone ($P < 0.05$). A similar relationship was observed for VO_2. Respiratory quotients were similar in protocols I–III (Table 4).

Phase 2 (protocols IV and V). The RMR was similar during the baseline periods of protocols IV and V. During the intervention period of protocol IV, in which endogenous glucagon was suppressed through the infusion of SRIH, the RMR fell 7% below baseline levels ($P < 0.019$). During the intervention period of protocol V, during which SRIH was infused with a concomitant infusion of high levels of glucagon, the RMR rose 9% above baseline levels ($P < 0.04$). The RMR during the intervention period of protocol V was 13% higher than during the intervention period of protocol IV ($P < 0.015$). A similar relationship was observed for VO_2. (Fig. 1). By regression analysis it was seen that the RMR in protocols IV and V varied with glucagon levels, producing a correlation coefficient (R^2) of 0.81 (Fig. 3). The respiratory quotient did not vary between protocols IV and V or between the baseline and intervention periods within these protocols (Table 5).

DISCUSSION

The current study demonstrated that hyperglucagonemia contributes to the catabolic state, which is a hallmark of insulin deficiency in type 1 diabetic patients. In addition to promoting hyperglycemia, and thus facilitating the loss of glucose in urine, glucagon was seen to exert a catabolic effect by increasing both the RMR and leucine oxidation.

Consistent with previous observations (30,31), we found that insulin deprivation in type 1 diabetic patients is associated with an elevation in the RMR when compared with insulin treatment. Lowering glucagon levels during insulin deprivation, by the infusion of somatostatin, decreased the RMR by 7.1% ($P < 0.01$). Conversely, when exogenous glucagon was infused during insulin deprivation with a concomitant somatostatin infusion, a substantial increase (9.8%, $P < 0.04$) in the RMR was seen. While there was a trend for the growth hormone levels to be lower during the SRIH infusion of protocol IV (growth hormone deficiency is associated with lowered RMR [32,33]), the growth hormone levels were identical during the SRIH and glucagon infusion of protocol V, when the RMR was increased. The RMR thus changed independently of growth hormone levels. The rise and fall of the RMR during insulin deficiency in association with isolated changes in glucagon levels indicates that glucagon is largely responsible for the increase in the RMR that occurs during insulin deprivation. The effect of insulin treatment on lowering the RMR thus is not entirely due to a direct effect of insulin but is also due to the insulin-induced fall in glucagon levels. These findings are consistent with the previous demonstration of an increased RMR in nondiabetic subjects, in whom glucagon was infused during SRIH-induced insulinopenia (34). Recently, it has also been demonstrated that high glucagon levels enhance the RMR even in the presence of basal insulin (35). Although the biochemical basis of the hypermetabolic effect of hyperglucagonemia remains to be fully elucidated, glucagon is known to enhance gluconeogenesis, urea production, and fibrinogen synthesis, all of which are energy-consuming reactions (36–38). That leucine oxidation and the RMR were both lowered during insulin deprivation by somatostatin infusion (protocols III and IV) might be attributed to a direct effect of somatostatin, rather than occurring secondary to the inhibition of glucagon secretion by somatostatin. Protocol V directly tested and excluded this possibility. Protocols IV and V both involved the infusion of somatostatin during insulin deprivation. The singular difference between these protocols was that glucagon was replaced exogenously during protocol V. During protocol V, we infused glucagon at a rate that was intended to achieve peripheral levels that would produce hepatic levels similar to those seen during insulin deficiency in patients with type 1 diabetes. Approximately 40% of glucagon is extracted during first-pass metabolism through the liver (39,40). By extrapolation, the mean peripheral glucagon level achieved during the intervention period of protocol V would have produced hepatic levels well within the range reported to occur in type 1 diabetes during insulin deprivation (7–10). To measure hepatic levels of glucagon directly, it would have required cannulation of the portal vein, a procedure that poses an unacceptable risk to study participants.

Further evidence of a catabolic effect of glucagon was demonstrated by the increased rates of leucine oxidation that were seen during hyperglucagonemia. This finding is consistent with the previous observation in nondiabetic subjects that leucine oxidation, urinary nitrogen losses, and urea production are substantially enhanced by high glucagon levels during relative insulin deficiency (11).

Physiological hyperglucagonemia has also previously been shown to increase phenylalanine oxidation (41). In the current study, the rate of leucine oxidation was greatest when glucagon levels were high and insulin was deficient (protocols I and V), suggesting that glucagon's stimulatory effect on leucine oxidation is enhanced by insulin deficiency in patients with type 1 diabetes (Table 4). Inhibition of glucagon secretion during insulin deprivation was associated with a decline in leucine oxidation (protocols III and IV). That the decline in leucine oxidation during protocol IV was due to lower levels of circulating glucagon, rather than a direct effect of SRIH or due to the trend for lower growth hormone levels seen in both protocol IV and V, is supported by the observation that leucine oxidation increased when glucagon was replaced during an SRIH infusion without concomitant changes in the level of circulating growth hormone (protocol V) (Table 5). Insulin replacement, and the subsequent decline in glucagon levels, was associated with the maximum decrease in leucine oxidation (protocol II). This may represent either an additive effect of insulin in inhibiting glucagon's stimulation of leucine oxidation or result from the greater magnitude in the decline of glucagon levels. Insulin replacement was not only associated with a fall in glucagon levels and a diminished rate of leucine oxidation, but also a decrease in the rate of leucine flux. Glucagon-induced enhancement of leucine oxidation was also associated with a trend for decreased protein synthesis. Regression analysis demonstrated that leucine oxidation significantly correlated with circulating glucagon levels and not with changes in the levels of other hormones ($R^2 = 0.79$, $P < 0.001$).

To gain mechanistic insight into changes in leucine oxidation that occurred during protocols IV and V, leucine transamination rates were measured. Leucine oxidation rates were higher during the intervention period of protocol V (SRIH and glucagon infusion during insulin deprivation) than those seen during the intervention period of protocol IV (SRIH infusion without glucagon replacement during insulin deprivation). This increase in leucine oxidation between the protocols IV and V remained after normalization of leucine oxidation for the rate of leucine deamination to KIC, suggesting that hyperglucagonemia increases leucine oxidation through enhancing the rate of decarboxylation of KIC. This mechanism is made more likely by the finding of a lack of any changes in the rate of leucine transamination (KIC to leucine and leucine to KIC) despite substantial isolated changes in glucagon levels during protocols IV and V. The major site of decarboxylation of KIC is the liver (42), an organ rich in glucagon receptors (43). A previous study demonstrated that leucine oxidation and transamination substantially increases in type 1 diabetic patients during insulin deprivation (3). Since elevated circulating glucagon levels were observed in these type 1 diabetic patients and because glucagon has been reported to activate branched-chain amino acid dehydrogenase in the liver (44), it was proposed that the increased leucine transamination during insulin deprivation is related to high glucagon levels (3,45). The current study, however, demonstrated that increased leucine transamination is related to insulin deficiency, and not hyperglucagonemia. This finding is not surprising since most of the increase in leucine transamination during insulin deprivation in type 1 diabetic patients occurs in skeletal muscle (3), which has no demonstrable glucagon receptors in humans (46). In rodents, glucagon receptors have been demonstrated at low levels in skeletal muscle and other tissues, including the small intestine (47).

From the current study, it appears that insulin deficiency is crucial for enhanced protein breakdown and, thereby, the provision of amino groups from branched-chain amino acids for the production of glucogenic precursors such as glutamine and alanine. Glucagon is known to stimulate gluconeogenesis and glycogenolysis in the liver (48,49), potentiating the hyperglycemia seen during insulin deprivation in type 1 diabetic patients. Hyperglucagonemia contributes to the increased glucose levels, as evident from lower glucose levels in protocols III and IV (when glucagon levels were decreased by somatostatin infusion). In contrast, insulin deficiency and hyperglucagonemia (protocols I and V) caused the highest circulating glucose levels. The degree of hyperglucagonemia seen in association with insulin deficiency during these experiments is similar to the range reported by other authors in patients with poorly controlled type 1 diabetes and is higher than the degree of hyperglucagonemia experienced by patients with type 2 diabetes (50,51). The relative concentrations of glucagon and insulin have been reported to be major determinants of glucagon's action on the liver (49). In the case of glucose metabolism, there is considerable evidence to indicate that it is the insulin:glucagon ratio, rather than the absolute amount of glucagon, that determines glucose production (18,49,52). The effects of hyperglucagonemia on the RMR and protein metabolism in the setting of a normal insulin:glucagon ratio cannot be extrapolated from the results of our current study, as hyperglucagonemia always occurred during insulin deprivation (i.e., the insulin:glucagon ratio was always low). It is likely that the catabolic effects of hyperglucagonemia are potentiated by concomitant insulin deficiency through the provision of amino acids from protein breakdown as substrates for oxidation and gluconeogenesis. Changes in glucagon levels do not alter either the baseline (natural) abundance of $^{13}CO_2$ (11) or CO_2 trapping, based on our own unpublished studies of ^{14}C-labeled bicarbonate trapping during different rates of glucagon infusion.

Together, the results of this study are further proof of glucagon's role in the pathogenesis of the catabolic state in patients with poorly controlled type 1 diabetes and may imply a catabolic role for hyperglucagonemia in other clinical conditions. The metabolic abnormalities of poorly controlled type 1 diabetes result not only from insulin deficiency but also from hyperglucagonemia. This study provides further support to the hypothesis that diabetes is a bihormonal disease (49).

ACKNOWLEDGMENTS

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Author Queries (please see Q in margin and underlined text)

Q1:Correct that SRIH stands for somatostatin?
Q2: Correct that you mean micrograms per hour for mcg/h?
Q3: Micrograms correct?
Q4: Can you provide manufacturer and location of GC/MS?
Q5: Please provide manufacturer and location of Vacutainer.
Q6: Correct that GGON stands for high level of glucogon replacement?
Q7: Do you wish to add HIS and MET to this paragraph?
Q8: OK to combine ‘isotopes’ and ‘leucine kinetics’ here as a level 2 head?
Q9: This sentence okay as edited?
Q10: Please add title for Table 3. Please spell out HIS, MET, and PHE.
Q11: In Tables 4 and 5 are data in row two, “Respiratory quotient,” correct as edited?
In Table 4, correct that leucine → protein?
Q12: Please check this sentence carefully and make sure your meaning is correct?

Refs 31, 32: Please provide initials for Hurter and Cerchio.
Ref 43: Do you mean beta-cells?