The Effect of Glucagon on Protein Catabolism During Insulin Deficiency: Exchange of Amino Acids Across Skeletal Muscle and the Splanchnic Bed

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METABOLISM

Transient insulin deprivation with concurrent hyperglucagonemia is a catabolic state that can occur in type 1 diabetes. To evaluate glucagon's catabolic effect in the setting of its glucogenic effect, we measured the regional exchanges of amino acid metabolites (amino-metabolites) across muscle and splanchnic beds in 16 healthy humans during either somatostatin followed by glucagon or saline infusion alone. Despite a twofold or greater increase in the regional exchange of amino-metabolites by glucagon, whole-body kinetics and concentrations of amino acids (AA) remained stable. Glucagon increased the splanchnic uptake of not only gluconeogenic but also essential (EAA) AA while increasing their release from the muscle bed. Regional tracer-based kinetics and 3-methylhistidine release indicate that EAA release from muscle is likely caused by reduced protein synthesis rather than increased protein degradation. Furthermore, many metabolites known to affect insulin action and metabolism were altered by hyperglucagonemia including increase in branched-chain AA and keto acids of leucine and isoleucine in arterial plasma. Further, an increase in arterial concentrations of α-aminoadipic acid arising from increased conversion from lysine in the splanchnic bed was noted. These results demonstrate that hyperglucagonemia during hypoinsulinemia increases net muscle protein catabolism and substantially increases the exchange of amino metabolites across splanchnic and muscle beds.

Insulin deprivation in people with type 1 diabetes mellitus (T1DM) results in hyperglycemia and hyperglucagonemia. The relative importance of insulin deficiency versus hyperglucagonemia in hepatic release of glucose causing hyperglycemia is not fully understood. Less is known about glucagon's role in the development of a catabolic state following insulin deficiency. Catabolic state associated with weight loss and necrolytic migratory erythema is known to occur in glucagonoma, which is a rare islet cell tumor secreting excessive glucagon (1,2). Persistent insulin deficiency in the preinsulin era was shown to be associated with substantial skeletal muscle (muscle) wasting (3,4). Transient insulin deficiency, whether resulting from deliberate omission of insulin to lose weight (5,6) or from frequent dosing errors (7–9), is also a catabolic state. However, the role of glucagon versus insulin deficiency per se in regional protein catabolism remains to be determined.

While it is well established that insulin deficiency alters concentrations and kinetics of amino acids (AA) and protein turnover in T1DM (10–13), the role of hyperglucagonemia in this setting is not fully understood. Findings of regional studies applying tracer-based kinetics and a somatostatin clamp-based approach (14,15) have shown that the main effect of insulin is the inhibition of muscle protein degradation and that AA replacement is critical for enhancing protein synthesis (16,17). AA released from muscle protein degradation during insulin deficiency are taken up by the splanchnic bed where they are used for gluconeogenesis, facilitated by glucagon and protein synthesis (13–15,18,19). However, glucagon has also been shown to modulate insulin's effect on AA metabolism in the setting of insulin deficiency (20) and inhibit

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AA-induced protein synthesis at the whole-body level (21). Whether the changes during insulin deficiency that are reported to occur are due to insulin deficiency per se or to glucagon excess remains undetermined. Also uncertain is whether these glucagon-induced changes occur in the splanchnic tissues or in other tissues such as skeletal muscle.

In the current study, we sought to clearly define the impact of hyperglucagonemia in the setting of insulin deficiency on plasma concentrations of amino acid metabolites (amino-metabolites) and determine whether arterial amino-metabolites and the protein kinetics reflect the magnitude of muscle and splanchnic bed exchanges of amino metabolites. We also examined how insulin deprivation and concurrent hyperglucagonemia affect protein dynamics using stable isotope tracer techniques across the muscle (leg) and splanchnic beds. Studies were performed in healthy men and women with use of somatostatin to inhibit insulin and glucagon and selective replacement of glucagon to study insulin deficiency and concurrent hyperglucagonemia. We also measured the regional exchange of specific AA across the muscle and splanchnic tissue beds such as lysine, glutamine, and aspartate and their metabolites α-aminoadipic acid (αAA), glutamate, and asparagine, respectively.

RESEARCH DESIGN AND METHODS

Experimental Design

All participants were admitted to the Clinical Research and Trials Unit the evening before the study day. They each received a standard meal (~10 kcal/kg consisting of 15% protein, 30% fat, and 55% carbohydrate), prepared by our metabolic kitchen, at 1800 h and then remained fasting except for water until completion of the study.

At 0600 h on the study day, a primed continuous infusion (1 mg/kg/h) of L-(ring-$^2H_5$)-phenylalanine (99 atom % excess) in normal saline was initiated via peripheral intravenous catheter. Between 0800 and 0900 h, the femoral artery, femoral vein, and hepatic vein were cannulated under fluoroscopic guidance by a vascular interventional radiologist as previously described (14,15). Vascular access and plasma flow measurements as described previously (14,15,22,23) is given in Supplementary Methods A.

Each participant was randomly assigned to the control ($N = 7$; 3 male and 4 female) or intervention ($N = 9$; 4 male and 5 female) group. Participants in the intervention group received an intravenous infusion of somatostatin (0.093 μg/kg fat-free mass/min) through a peripheral vein starting 60 min after the initial blood sample collection from the intravascular catheters followed by a continuous infusion of glucagon (0.003 μg/kg fat-free mass/min) starting 180 min after the initial blood sample collection. The control group received a slow infusion of only normal saline through a peripheral vein. Blood samples were taken from the femoral artery, femoral vein, and hepatic vein three times each after 120 and 240 min to determine the effects of somatostatin alone and somatostatin plus glucagon, respectively. Infusions were then stopped, intravenous catheters were removed, and direct pressure was applied to the puncture sites for 30 min. A pressure bandage was then applied to the puncture sites, and each participant remained in bed with the affected extremity straight for 5 h. For additional information on experimental design see Fig. 1.

Participants

A total of 16 healthy participants (7 in control and 9 in hormone intervention group) enrolled in our study (Supplementary Table 1). We lost hepatic vein access in three participants (one control, two intervention), yielding a smaller sample size for splanchnic analysis (six control, seven intervention). The age, BMI, and body fat percentage were similar in each group, both of which had nearly equal numbers of men and women. Each participant had a normal physical examination and baseline laboratory tests prior to participation, including fasting glucose, electrolytes, complete blood count, and tests of liver, kidney, and thyroid function. None of the participants smoked, ingested alcohol in excess, or used medications known to affect metabolism. The study protocol was reviewed and approved by the institutional review board at Mayo Clinic, and informed consent was obtained for all the study participants.

Sample Analysis: Calculations

Information on analysis of samples can be found in Supplementary Methods B. Whole-body phenylalanine flux and regional dynamics of phenylalanine across muscle and splanchnic beds were calculated with equations previously described (13,22). Muscle and splanchnic exchanges of glucose, AA, and their metabolites were calculated by subtraction of artery concentrations from venous concentrations and multiplication of the difference by the respective muscle and splanchnic plasma flow. Therefore, a positive exchange rate represents net release of substrate from the specified tissue, while a negative exchange rate represents net uptake.

Statistics

Data are presented as means ± SEM. ΔExchange rates of glucose, AA, and their metabolites as well as the arterial concentrations of AA and their metabolites from baseline to 120 min (reflecting somatostatin's effects) and from 120 to 240 min (reflecting glucagon's effects) were compared in each group across one time point with another with the paired *t* test. Phenylalanine dynamics were compared across time points with ANOVA. Given the small sample size in each group, the effect of sex was not factored into these statistical analyses.

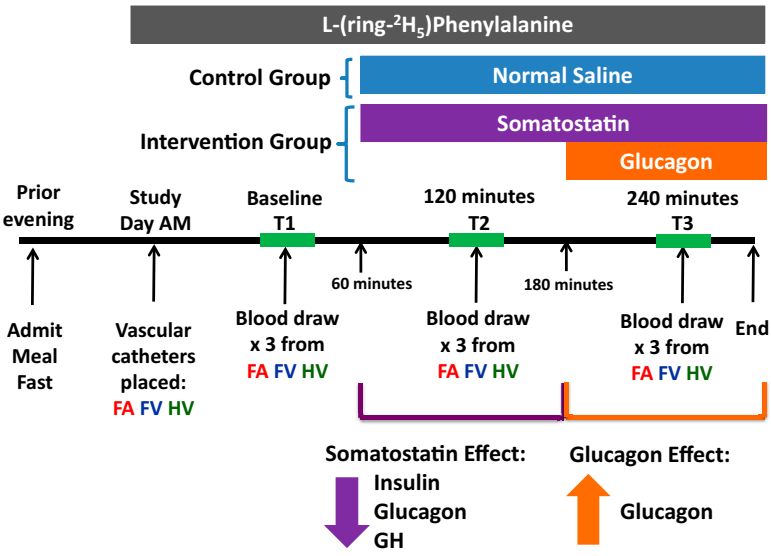

Figure 1—Study schema. FA, femoral artery; FV, femoral vein; HV, hepatic vein.

Data and Resource Availability

Data sets and resources are available on request.

RESULTS

Somatostatin Reduces Plasma Concentrations of Insulin, Glucagon, and Growth Hormone Without Altering Arterial Glucose Concentrations or Net Regional Glucose Balances

Participants received an intravenous continuous infusion of either somatostatin or saline for 240 min following baseline measurements (Fig. 1). Glucagon was infused during the last 120 min of the somatostatin infusion to create a low insulin/high glucagon state. Mean plasma flow, measured with indicator dye dilution (Supplementary Fig. 1), showed no change at all three time points in the group that received saline alone. In contrast, in the group that received the somatostatin infusion followed by the addition of glucagon, there was a decrease in splanchnic plasma flow on infusion of somatostatin that was reversed by glucagon infusion. The somatostatin infusion alone (120 min) led to a significant decrease in arterial plasma C-peptide, insulin, glucagon, and growth hormone (GH) without increasing glucose concentrations or glucose exchange (micromoles per minute) across the splanchnic and muscle beds (Fig. 2*A*).

Glucagon Increased Arterial Glucose Concentrations Through Greater Release of Glucose From Splanchnic Bed

The addition of glucagon (240 min) and subsequent increase in plasma concentrations of glucagon resulted in increased glucose concentrations without any breakthrough increase in insulin secretion, as indicated by suppressed C-peptide concentrations. Insulin and GH were suppressed during the entire period of somatostatin infusion, showing that during the last 120 min only glucagon concentrations increased in comparison with somatostatin infusion alone. Adding glucagon increased the appearance of glucose from the splanchnic bed (~165 mg/min) with a reciprocal increase in uptake of glucose by the muscle bed (~28 mg/min, one leg) (Fig. 2*B*). It is possible that there was increased glucose output from the kidney (28) that was not measured. During the same period, participants receiving the saline infusion did not experience changes in arterial concentrations of any of the hormones or glucose or glucose exchanges across the muscle and splanchnic beds. No changes were observed in glucose exchange across the skeletal muscle and splanchnic beds during somatostatin treatment without glucagon. In the intervention group, there was a slight increase in insulin levels between 120 min and 240 min, but C-peptide level continued to decrease, indicating that the slight increase in insulin is likely related to reduced clearance of insulin.

More Pronounced Regional Arteriovenous Exchanges of AA Occurred Than Changes in Arterial Concentrations of AA

Addition of glucagon caused a substantial increase in the exchange of total AA, essential AA (EAA), and glucogenic AA (GAA) across muscle and splanchnic beds, but the changes in concentrations of these AA in the arterial blood were smaller. Among the EEA, branched-chain AA (BCAA) were pronouncedly increased on addition of glucagon (Table 1). However, there was heterogeneity in the response of their respective keto acids; there was no change in the concentrations of the α-ketovalerate metabolite of valine in response to somatostatin alone or in the presence of glucagon. In contrast, α-keto-β-methylvalerate

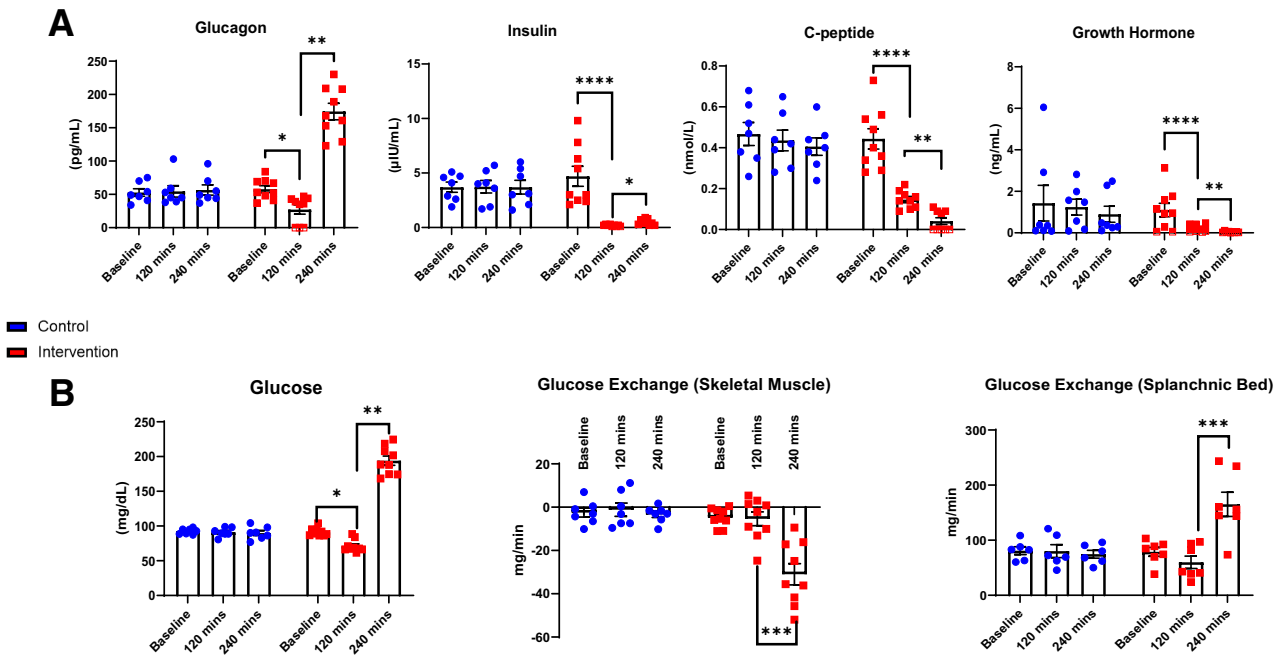

Figure 2—*A*: Mean arterial hormone concentrations in control and intervention participants. *B*: Mean arterial glucose concentration and exchange across leg and splanchnic bed at baseline, 120 min (60 min after initiation of somatostatin infusion in intervention group), and 240 min (60 min after initiation of glucagon infusion in intervention group). In the exchange graphs, positive values demonstrate release into circulation and negative values demonstrate uptake into the circulation. Error bars reflect SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

and α-ketoisocaproate, metabolites of isoleucine and leucine, respectively, changed significantly on addition of glucagon (Fig. 3).

Other AA

In comparison with saline, there were significant and specific changes in the concentration of many individual AA (Figs. 3–5) following somatostatin and on addition of glucagon indicating specificity of hormonal effects on individual AA (Table 1). Among aromatic AA (AAA), only tyrosine arterial concentration showed a continuous increase during somatostatin infusion compared with baseline, but surprisingly no changes in the net release or uptake of tyrosine from the skeletal muscle bed or the splanchnic bed, respectively, were observed. A likely explanation is that in the postabsorptive state a key organ involved in tyrosine release is the kidney (24), measurements for which were not included in the current study. In contrast, among sulfur-containing AA, methionine concentration increased with somatostatin and increased further with glucagon—unlike cystine and taurine. However, histidine was not affected by either hormone.

Lysine arterial concentrations decreased in response to glucagon (Fig. 4). Of note, there was increased release of lysine from the skeletal muscle bed and increased uptake by the splanchnic bed during the glucagon infusion with a net higher uptake by the splanchnic bed (23 μmol/min) than muscle (estimated 17 μmol/min release by total body muscle mass if leg muscle represents one-third of total muscle mass). The arterial concentration of lysine's metabolic product, αAA, increased in response to glucagon due to greater release from the splanchnic bed without any change in muscle uptake (Fig. 4). Finally, proline concentrations also increased with somatostatin but tended to decrease with glucagon infusion.

Other key GAA, such as glutamine, alanine, glycine, asparagine, and serine, increased with somatostatin but decreased with the addition of glucagon. More importantly, these AA exhibited greater release from the skeletal muscle bed with increased uptake in the splanchnic bed (Fig. 5). Of interest, aspartate, which is the precursor of asparagine, showed a reciprocal relationship in their exchanges across the muscle and splanchnic beds (Fig. 5). This ammonia-dependent reaction along with the conversion of glutamine to glutamate and vice versa is of great clinical interest, as the ammonia accumulation observed during liver failure concurrent to hyperglucagonemia (25) is possibly derived from the impaired conversions of these metabolites leading to excess ammonia formation (26).

Muscle Protein Synthesis and Degradation

We used [ring-$^{13}C_6$]phenylalanine as a tracer to measure phenylalanine kinetics. An EAA with no fate other than incorporation into protein in muscle, the disappearance rate of phenylalanine represents protein synthesis in muscle and its appearance rate represents protein degradation

Table 1—Arterial concentrations of AA and their metabolites at time points baseline, 120 min, and 240 min and their differences between time points

		Baseline		120 min		240 min		Δ120 min–baseline			Δ240–120 min			Δ240 min–baseline		
AA and metabolites	Groupings	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	*P*	Mean	SEM	*P*	Mean	SEM	*P*
BCAA																
Valine	Control	188.9	13.3	190.4	14.0	188.6	13.5	1.4	2.1	**0.005**	−1.7	4.0	**0.0002**	−0.3	4.3	**0.0002**
	Intervention	191.5	14.9	209.9	13.3	235.6	13.9	18.4	4.3		25.7	2.5		44.1	4.2	
α-Ketoisovalerate	Control	10.8	0.58	11.7	0.35	11.1	0.24	0.9	0.4	0.662	−0.6	0.3	0.364	0.3	0.5	0.777
	Intervention	12.1	0.70	12.9	0.94	12.3	0.85	0.8	0.4		−0.5	0.9		0.2	0.6	
Isoleucine	Control	45.6	2.96	49.8	3.52	50.9	3.35	4.23	1.5	**0.008**	1.02	3.01	**0.0007**	5.26	3.5	**0.0002**
	Intervention	47.3	3.39	60.7	3.00	76.6	3.52	13.3	1.7		15.9	1.1		29.3	1.8	
α-Keto-β-methylvalerate	Control	15.1	1.09	16.3	1.16	16.1	0.92	−1.1	1.1	0.076	2.0	0.7	**0.028**	0.9	1,1	**0.0021**
	Intervention	17.0	1.13	17.5	1.66	20.7	1.7	−3.9	0.5		−0.8	0.8		−4.6	0.9	
Leucine	Control	90.3	3.9	97.1	5.9	96.9	5.7	6.74	2.9	**0.0021**	−2.24	6.8	**0.0311**	6.58	4.9	**0.0002**
	Intervention	98.3	7.6	127.3	6.3	157.9	8.1	29.0	3.5		31.9	9.8		59.6	3.9	
α-Ketoisocaproate	Control	21.9	1.6	23.9	1.2	22.4	1.1	2.0	1.0	0.211	−1.5	1.2	**0.015**	0.5	1.7	0.114
	Intervention	26.0	2.0	25.6	2.3	30.4	2.7	−0.5	1.7		4.8	1.8		4.3	1.0	
Total BCAA	Control	324.9	18.4	337.3	20.1	336.3	19.3	12.4	5.3	**0.0021**	−0.89	11.8	**0.0002**	11.5	12.4	**0.0002**
	Intervention	337.1	25.8	397.9	22.3	470.2	25.2	60.8	9.3		72.2	5.9		133.0	9.65	
AAA																
Phenylalanine	Control	51.8	3.2	53.6	3.7	51.9	2.7	1.75	0.90	**0.012**	−1.68	1.16	0.174	0.07	0.83	0.174
	Intervention	49.2	2.3	55.8	2.4	51.9	2.4	6.62	1.05		−3.83	1.25		2.79	1.33	
Tryptophan	Control	36.84	2.24	35.17	1.98	32.33	1.58	−1.67	1.12	**0.001**	16.70	2.66	0.055	−4.51	1.68	0.607
	Intervention	36.03	2.08	26.40	2.41	30.19	2.06	−9.63	1.32		25.59	2.74		−5.83	1.73	
Tyrosine	Control	46.28	3.93	46.19	3.47	43.96	2.88	−0.09	1.30	**0.012**	−2.23	1.57	**0.0002**	−2.32	2.45	**0.0002**
	Intervention	42.56	3.97	47.24	4.04	56.65	4.65	4.68	0.91		9.41	1.14		14.09	1.05	
Sulfur-containing AA																
Methionine	Control	17.08	0.80	17.38	0.96	16.15	0.65	0.30	0.36	**0.003**	−1.23	0.61	**0.012**	−0.94	0.37	**0.0002**
	Intervention	17.35	1.15	19.81	1.38	20.97	1.50	2.46	0.55		1.16	0.37		3.62	0.55	
Cystine	Control	39.47	1.92	37.07	2.98	35.87	2.49	−2.4	2.26	0.607	−1.20	1.31	0.737	−3.6	2.12	0.252
	Intervention	32.69	1.61	32.93	1.90	32.57	1.75	0.24	0.99		−0.36	1.22		−0.12	1.01	
Taurine	Control	36.51	2.48	38.37	3.29	38.49	3.55	1.86	3.88	0.758	0.12	3.40	0.681	1.99	2.18	0.408
	Intervention	41.61	2.48	41.29	3.09	39.92	2.03	−0.32	2.27		−1.37	2.62		−1.69	2.16	
Other EAA																
Histidine	Control	88.47	10.50	91.68	12.49	90.31	9.24	3.20	5.90	0.470	−1.37	4.78	0.536	1.83	3.06	0.536
	Intervention	97.11	8.42	110.69	9.86	103.16	10.48	13.58	4.07		−7.53	4.37		6.05	6.22	
Lysine	Control	142.11	9.77	144.43	12.56	136.22	11.15	2.32	3.18	**0.003**	−8.21	4.98	0.999	−5.89	5.09	**0.031**
	Intervention	152.21	10.26	174.54	12.53	166.31	13.50	22.33	4.01		−8.23	3.15		14.10	5.15	
α-Aminoadipic acid	Control	0.49	0.05	0.47	0.06	0.50	0.06	−0.02	0.01	**0.0008**	0.03	0.03	**0.006**	0.01	0.04	**0.0006**
	Intervention	0.53	0.07	0.64	0.08	0.83	0.09	0.11	0.02		0.19	0.03		0.30	0.04	
Proline	Control	127.63	12.44	124.00	11.42	116.02	11.96	−3.63	2.34	**0.0033**	−7.98	3.39	0.408	−11.61	2.54	**0.0007**
	Intervention	127.37	14.72	134.44	14.9	130.36	13.64	7.07	1.92		−4.08	1.81		2.99	1.85	
Threonine	Control	104.77	8.69	102.63	9.81	95.71	9.33	−2.14	2.01	**0.0052**	−6.92	2.38	0.209	−9.06	2.31	**0.0079**
	Intervention	107.94	9.80	117.75	10.98	111.43	11.35	9.81	1.84		−6.32	2.23		3.49	2.49	

Continued on p. 1641

Table 1—Continued

AA and metabolites	Groupings	Baseline		120 min		240 min		Δ120 min–baseline			Δ240–120 min			Δ240 min–baseline		
		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	*P*	Mean	SEM	*P*	Mean	SEM	*P*
Nonessential AA																
Glycine	Control	217.08	19.51	214.95	17.78	212.36	20.04	−2.13	3.02	**0.012**	−2.59	6.00	**0.005**	−4.72	5.53	0.351
	Intervention	225.37	21.45	242.27	20.18	210.07	15.87	16.90	3.99		−32.20	5.28		−15.30	6.71	
Sarcosine	Control	4.54	0.49	4.12	0.44	4.12	0.53	−0.42	0.37	0.607	0.00	0.42	0.837	−0.42	0.19	0.408
	Intervention	3.77	0.63	3.65	0.63	3.50	0.58	−0.12	0.19		−0.14	0.14		−0.26	0.10	
Alanine	Control	181.14	15.59	170.94	13.83	156.35	9.07	−10.21	6.94	**0.0012**	−14.58	7.27	0.918	−24.79	9.08	**0.0021**
	Intervention	193.19	22.16	213.32	21.93	200.59	22.19	20.14	3.95		−12.73	4.55		7.41	4.02	
Asparagine	Control	40.83	1.58	40.17	2.08	38.47	2.00	−0.66	1.27	**0.0052**	−1.70	1.11	**0.012**	−2.36	0.68	00.456
	Intervention	41.42	2.19	47.15	2.18	40.26	2.62	5.73	0.97		−6.90	1.29		−1.16	1.05	
Arginine	Control	69.11	5.76	71.42	6.88	67.79	6.74	2.31	1.92	**0.031**	−3.62	2.44	0.114	−1.32	2.18	**0.012**
	Intervention	68.98	6.29	79.00	7.72	80.00	8.11	10.01	2.06		1.00	1.94		11.01	2.79	
Citrulline	Control	25.58	2.28	24.77	2.52	23.72	2.17	−0.81	0.46	0.091	−1.05	0.54	0.219	−1.86	0.41	**0.016**
	Intervention	22.27	1.31	23.02	1.45	22.87	1.44	0.75	0.62		−0.15	0.44		0.60	0.68	
Ornithine	Control	36.74	3.22	36.01	3.14	34.15	3.39	−0.74	1.05	**0.042**	−1.86	1.20	0.252	−2.59	0.98	**0.042**
	Intervention	32.75	3.84	35.11	4.01	33.20	3.92	2.36	0.41		−1.91	0.55		0.45	0.71	
Glutamine	Control	548.81	26.61	549.71	23.05	538.74	22.91	0.90	12.52	**0.0021**	−10.97	14.37	0.114	−10.07	20.23	0.091
	Intervention	529.35	17.25	599.91	26.59	567.61	26.35	70.56	10.24		−32.30	11.22		38.26	12.49	
Glutamate	Control	40.76	4.11	38.85	3.43	38.77	4.22	−15.99	5.26	0.536	−0.08	1.82	0.999	−2.00	1.30	**0.0021**
	Intervention	40.90	1.86	33.06	2.53	31.36	2.65	−17.88	2.35		−1.70	0.92		−9.55	1.24	
Aspartate	Control	1.79	0.14	1.75	0.14	1.61	0.17	−0.04	0.09	0.470	−0.14	0.10	**0.015**	−0.19	0.06	**0.005**
	Intervention	1.96	0.13	1.80	0.17	1.23	0.10	−0.15	0.07		−0.57	0.12		−0.73	0.11	
Serine	Control	100.09	9.84	100.70	11.39	95.62	10.51	0.61	2.92	**0.016**	−5.08	2.27	0.299	−4.47	2.21	0.055
	Intervention	100.10	5.41	110.61	4.86	102.44	4.13	10.51	2.37		−8.17	1.98		2.35	3.28	
α-Amino-N-butyric acid	Control	21.6	2.1	22.3	2.0	23.0	1.9	0.76	0.54	**0.004**	0.70	0.50	**0.004**	1.46	0.54	**0.004**
	Intervention	22.6	2.6	26.0	3.0	27.8	2.9	3.32	0.70		1.82	0.45		5.14	0.74	
β-Aminoisobutyric acid	Control	1.3	0.2	1.4	0.2	1.5	0.2	0.13	0.07	**0.012**	0.10	0.07	**0.004**	0.23	0.12	**0.004**
	Intervention	1.3	0.1	1.5	0.1	1.7	0.1	0.18	0.04		0.19	0.03		0.37	0.05	

Bold indicates that the difference is statistically significant at $P < 0.05$.

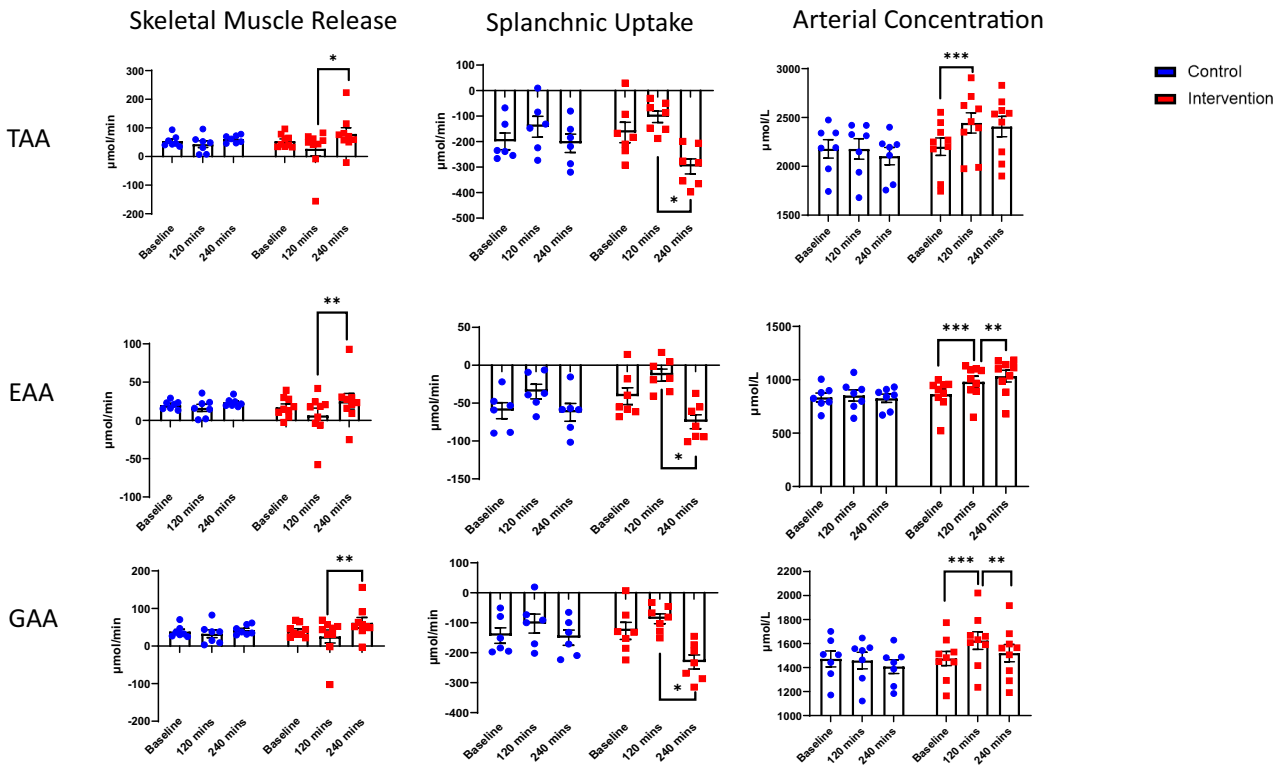

Figure 3—Mean total AA (TAA), EAA, and GAA exchange across leg and splanchnic bed as well as arterial concentrations at baseline, 120, and 240 min. In the exchange graphs, positive values demonstrate release into circulation and negative values demonstrate uptake into circulation. Error bars reflect SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

(13,27,28). Phenylalanine kinetics across the splanchnic bed is more complex, as its disappearance rate includes not only protein synthesis but also conversion to tyrosine and degradation. Here we have mainly used the phenylalanine tracer approach to determine the dynamics across muscle bed and to measure its appearance rate from the splanchnic bed. The results from these dynamic studies demonstrated that phenylalanine flux at the whole-body level remained stable during hormone infusions and appearance from muscle protein was unchanged by either infusion of somatostatin alone or on addition of glucagon (Fig. 5). The above result is supported by measurement of the 3-methylhistidine concentrations in femoral venous samples and its release from muscle protein showing no increase with either somatostatin or glucagon. 3- methylhistidine is an accepted measure of myofibrillar protein degradation (29), although there are concerns about small contributions from the gut when urinary 3-methylhistidine excretion is used as a measure of whole-body muscle protein degradation. Here, we used the femoral vein concentration of 3-methylhistidine, which represents its appearance or release from the muscle bed. Thus, both isotope-based measurement and 3-methylhistidine–based measurement support that glucagon has no effect on muscle protein degradation. In contrast, addition of glucagon decreased phenylalanine incorporation into muscle protein, based on its disappearance rate. A net release of phenylalanine from the muscle bed without increased muscle protein degradation supports the mechanistic explanation that addition of glucagon to somatostatin resulted in decreased muscle protein synthesis. There was a nonsignificant trend for splanchnic phenylalanine release from protein degradation following addition of glucagon, but net phenylalanine uptake was increased, suggesting that phenylalanine may be converted to tyrosine, degraded, or incorporated into protein (13). Whole-body phenylalanine flux representing protein degradation also remained stable throughout the study with no significant differences in the flux changes across time points between the intervention and control groups (Fig. 6).

DISCUSSION

Findings of the current study provide new insight into the regional metabolic effects of hyperglucagonemia during hypoinsulinemia across the muscle and splanchnic tissue beds, which are the predominant organs involved in fuel metabolism. We observed that the predominant effect of glucagon during insulin deficiency was substantially increase in the exchange (by a mean of twofold to fourfold) of AA across the muscle and splanchnic tissue beds, which is not reflected in the arterial concentrations of the respective

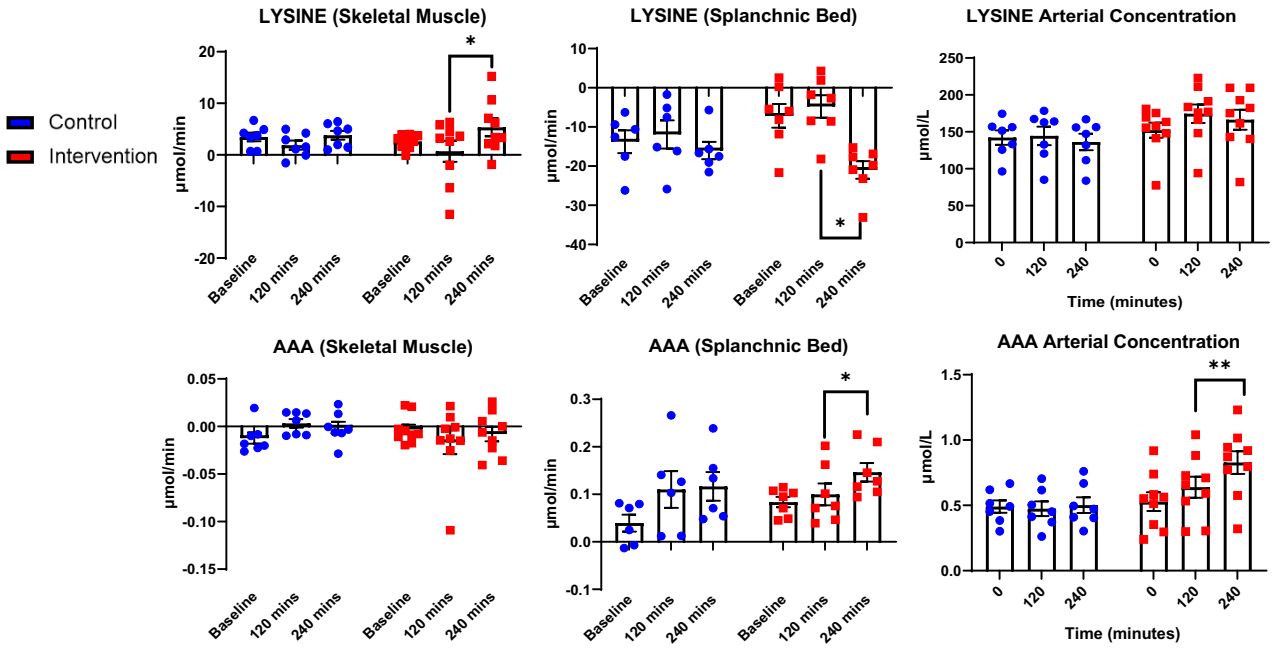

Figure 4—Lysine and αAA (AAA) exchange across leg and splanchnic bed at baseline, 120 min (60 min after initiation of somatostatin infusion in intervention group), and 240 min (60 min after initiation of glucagon infusion in intervention group). In the exchange graphs, positive values demonstrate release into circulation and negative values demonstrate uptake into the circulation. Error bars reflect SEM. $*P < 0.05$, $**P < 0.01$.

AA because the increased release in one organ bed occurred concurrent to increased uptake in the other tissue bed. At the whole-body level, tracer-based flux measurements also failed to reflect the accelerated exchange of amino-metabolites between muscle and splanchnic beds. However, regional kinetic studies demonstrated that hyperglucagonemia in the setting of low insulin concentrations inhibits muscle protein synthesis with no effect on muscle protein degradation. We also observed substantial differences among individual AA and their metabolites in their response to hyperglucagonemia during insulin deficiency. Some of these AA and their metabolites, such as αAA and α- and β-aminoisobutyric-acid, showed responses to glucagon that have a potential impact on the pathophysiology of diabetes and cardiovascular risk (30–32).

The reciprocal roles of insulin and glucagon are critical to the maintenance of glucose homeostasis in humans (33). These roles are disrupted in insulin-resistant and insulin-deficient states (34), and glucagon was thought to contribute to hyperglycemia in diabetes (35). However, it has been argued that insulin deficiency alone is the primary cause of hyperglycemia in diabetes (36). The findings of the current study clearly demonstrate the critical role of glucagon in increasing arterial glucose concentrations, since during the period of somatostatin infusion causing insulin deficiency, glucose arterial concentrations and output from the splanchnic bed did not change, whereas addition of glucagon dramatically increased arterial glucose concentration and splanchnic glucose output. Furthermore, the results of the current study support the notion that glucagon during hypoinsulinemia enhanced the release of the GAA from muscle that are taken up by the splanchnic bed, presumably to be used for gluconeogenesis by the liver (37). It has been proposed that AA and glucagon are linked in a feedback cycle involving liver and pancreatic α-cells (2).

The results of the current study demonstrate that glucagon during insulin deficiency not only increases the release of GAA from the muscle bed but also, similarly, increases the release of EAA. Although the nonessential AA such as GAA can be synthesized in muscle, EAA can be released in the fasted state from the muscle bed only if muscle protein degradation exceeds its synthesis. Based on the stable isotope tracer of phenylalanine complemented by measurement of the arteriovenous concentrations of 3-methylhistidine, we demonstrate in the current study that glucagon during insulin deficiency does not increase muscle or splanchnic protein degradation but, rather, decreases skeletal muscle protein synthesis. This leads to a net increase in muscle protein degradation and the release of EAA from the muscle bed. These findings are somewhat comparable with those of a recent study in T1DM and ketosis-prone individuals during ketoacidosis with high glucagon suggesting a lack of increase in muscle protein degradation (based on 3-methylhistidine) and decrease in muscle protein synthesis potentially explaining catabolic state in diabetic ketoacidosis (38). However, 3-methyhistidine released by the skeletal muscle from

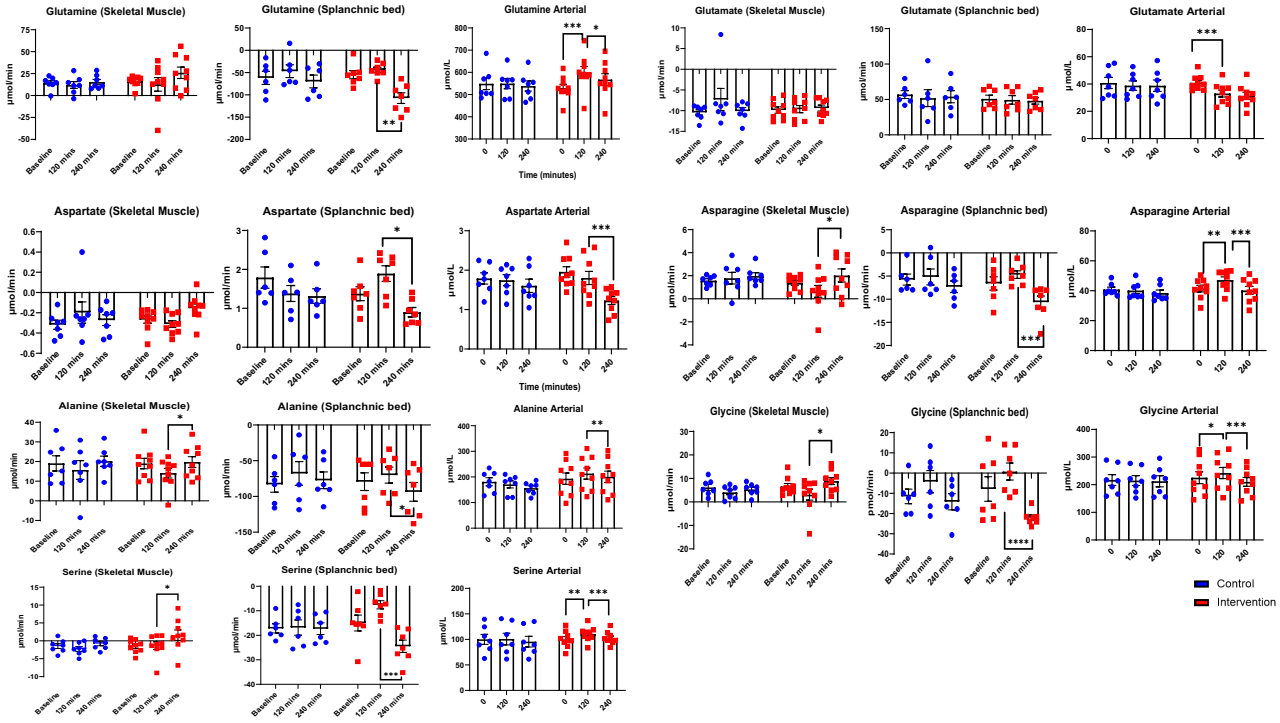

Figure 5—Glutamine, glutamate, aspartate, asparagine, alanine, glycine, and serine exchange across leg and splanchnic bed at baseline, 120 min (60 min after initiation of somatostatin infusion in intervention group), and 240 min (60 min after initiation of glucagon infusion in intervention group). Error bars reflect SEM. *$P < 0.05$, **$P < 0.01$. Mins, minutes.

degradation of myofibrillar proteins (29) by itself does not rule out degradation of nonmyofibrillar proteins including those involved in fuel metabolism and plasma proteins. In fact, insulin deprivation for longer periods (6–8 h) with concurrent hyperglucagonemia has been shown to accelerate muscle protein degradation especially of proteins involved in functions of mitochondria, proteostasis, nDNA support, glucose metabolism, and contractile/cytoskeletal and cell adhesion (13,39) via oxidative damage (39–41). Increased oxidative stress is related to

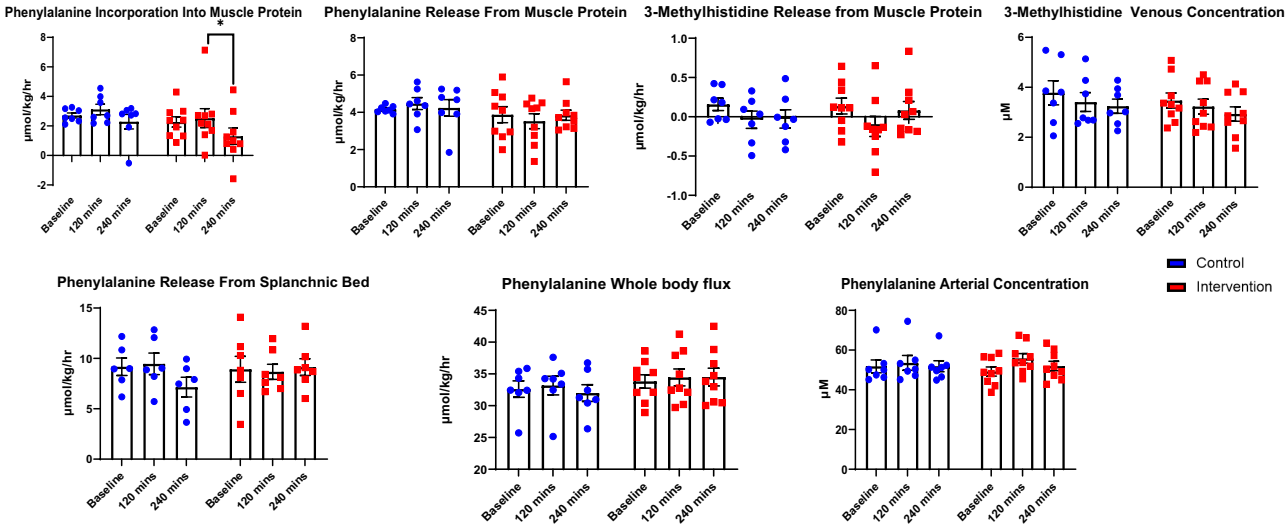

Figure 6—Regional phenylalanine dynamics and venous histidine concentrations. Phenylalanine incorporation into leg protein was significantly decreased in the intervention group at 240 min during low insulin/high glucagon state. Error bars reflect SEM. *$P < 0.05$. In the exchange graphs, positive values demonstrate release into circulation and negative values demonstrate uptake into the circulation.

hyperglucagonemia that increases oxidative metabolism, hyperglycemia, and AA oxidation (42–44). Oxidative stress increases intracellular protein oxidative modifications and activates both ubiquitin protease and autophagy pathways that accelerate protein degradation (39). Insulin deprivation and resulting oxidative stress increase degradation of muscle proteins in diabetic mice (41). Moreover, human studies have demonstrated that the main in vivo effect of insulin is inhibition of whole-body (45,46) and muscle (14,15) protein degradation without increasing muscle protein synthesis and this lack of muscle protein synthesis is explained based on lowering plasma EAA concentrations (15–17) and thus preventing the activation of mTOR anabolic signaling. However, mTOR activation requires the replacement of AA to enhance muscle protein synthesis (15,39,47,48). Although it has been shown that high glucagon concentrations inhibit liver protein synthesis in a dog model (49), it is not entirely clear how the relative hyperglucagonemia in the setting of hypoinsulinemia inhibits muscle protein synthesis, especially since there are no glucagon receptors in muscle. A potential explanation is that this inhibition of muscle protein synthesis arises from the absence of a full complement of AA required for peptide synthesis, secondary to increased uptake and consumption of AA by the splanchnic bed.

Hyperglucagonemia has been strongly associated with various protein catabolic conditions that lead to muscle wasting such as trauma, burns, sepsis, cirrhosis, glucagonoma, the postoperative state, and poorly controlled T1DM (34,50,51). Of interest, the catabolic state in glucagonoma, especially the necrolytic lesions in the skin, suggests the catabolic effect of glucagon on skin also potentially results from excessive AA consumption by the liver during hyperglucagonemia (2) and glucagon has been shown to inhibit AA-induced enhancement of whole-body protein synthesis by reducing the AA availability (21). Whether any other factors facilitating release of AA from muscle for synthesizing essential liver-derived plasma proteins and gluconeogenesis are active in this situation remains to be determined. Translational regulation depends on multiple factors including acylation of AA to tRNA, ribosomal proteins, and availability of transcriptomes. Thus, further tissue-based studies are needed to carefully look at these multiple components of protein synthetic machinery including mTOR signaling in the context of hyperglucagonemia and hypoinsulinemia.

It has been well established that insulin deficiency increases plasma concentrations of many EAA, especially of BCAA (13). Of note, in the current study, the addition of glucagon during the insulin-deficient state caused a clear increase in many EAA, especially of all three of the BCAA. Increase in peripheral blood concentrations of BCAA has been reported on the basis of a substantially increased transamination rate that occurs during insulin deficiency (13) and in insulin-resistant type 2 diabetes (52), both of which are associated with variable degrees of hyperglucagonemia, although the specific role of glucagon has not been studied. It has also been shown that BCAA such as leucine, isoleucine, and valine, as well as the AAA such as phenylalanine and tyrosine, are present at higher concentrations in insulin-resistant states years before disease onset of diabetes (53,54). One reason for this observation may be that skeletal muscle uptake of BCAA is less persistent in humans with diabetes after protein ingestion compared with humans without diabetes (55). However, in our study where participants were fasting throughout the study, there was still a continuous increase in the arterial concentrations of all BCAA in the intervention group, as they experienced a low insulin/high glucagon state similar to that in T1DM compared with the control group without diabetes. Potentially, increased transamination of BCAA contributes to increased synthesis of both GAA such as alanine and glutamine (56,57) with contribution of the amino group from BCAA transamination. The transamination rate measurements require double-labeled (^{15}N and 1-^{13}C) leucine infusion (13), which was not done in the current study, but elevated keto acids of leucine and isoleucine is consistent with increased transamination. The kidney also may play a role AA metabolism (24,58), but our study did not measure the contribution of the kidneys in the exchange of AA or glucose.

Of interest, during somatostatin and glucagon infusion, the plasma concentrations of the keto acids derived from leucine and isoleucine increased, except for ketoisovalerate derived from valine. Keto acids of BCAA are mostly produced in the skeletal muscle and are oxidized in liver, and their concentrations represent the net changes in production and catabolism (oxidation). One potential explanation for the lack of increase of ketoisovalerate with glucagon is valine contribution to gluconeogenesis that is enhanced by glucagon. Valine does this by forming both α-ketovalerate and 3-hydroxybutyrate in the muscle, and 3-hydroxybutyrate subsequently serves as a precursor for gluconeogenesis. In contrast, Jahoor et al. (38) showed increased branched-chain α-keto acids, especially ketoisocaproic acid and α-keto-β-methylvalerate, during diabetic ketoacidosis, suggestive of an impairment in the BCAA–to–branched-chain α-keto acid pathway, which cannot be verified in the current study.

Of interest, hyperglucagonemia in the setting of insulin deficiency increased arterial αAA, which is a metabolic product of lysine. Epidemiological data suggest that αAA is associated with type 2 diabetes, and elevated blood concentrations may be found up to 12 years before diabetes diagnosis (31) and in insulin-resistant states such as in polycystic ovarian syndrome (59). Furthermore, the insulin-sensitizing medications pioglitazone and metformin decrease plasma concentrations of αAA and lysine (32). Though biologic effects of αAA are not fully known, preclinical studies in mice and human islet cell lines demonstrate that αAA induced insulin release (31). The current study demonstrates that hyperglucagonemia in the setting of insulin deficiency increases arterial concentrations of αAA by increasing splanchnic uptake of lysine and

splanchnic release of its metabolite, αAA. Thus, it appears that glucagon-induced elevation in αAA concentration represents a compensatory mechanism, which could potentially increase insulin secretion in the setting of insulin resistance to maintain glucose homeostasis.

Glucagon has effects on the metabolism of glutamine, glutamate, aspartate, and asparagine, which constitute an intertwined amide AA metabolic network. Glutamine and asparagine form glutamate and aspartate intracellularly via the enzyme glutaminase and asparagine synthase. Asparagine concurrently uses ammonia generated from the conversion of glutamine to glutamate for its synthesis. At baseline, there was a net release of glutamine and asparagine from the skeletal muscle bed with a concurrent net uptake by the splanchnic tissue bed. This contrasts with a net release of glutamate and aspartate from the splanchnic bed tissue and concomitant uptake by the skeletal muscle bed, suggesting that the splanchnic tissue bed and skeletal muscle are intimately involved in the regional metabolism of these four AA in human physiology. Furthermore, hyperglucagonemia in the setting of low insulin greatly increases uptake of glutamine and asparagine by splanchnic tissue while decreasing release of aspartate by splanchnic tissue. As a result, in this study we observed increased glutamine concentrations and decreased glutamate concentrations during somatostatin and glucagon infusion, as also previously noted by others (42). Finally, we also observed that tryptophan levels decreased during somatostatin infusion but tended to increase on addition of glucagon, and this was similarly observed in other reports (38).

There are a several limitations inherent to in vivo human studies that limit the interpretation of the results of our study. First, the duration of the low insulin ± high glucagon state in the intervention group was limited to $<$2 hours each. Thus, it is possible that we were unable to observe the time-related physiological effects of these hormonal states in the participants in the intervention group. In a prior study (20) with similar low insulin and glucagon states, we did not observe any changes in EAA concentrations or flux in the first 2–4 h. Furthermore, Matsuda et al. (60) demonstrated that most changes in glucose metabolism in humans is observed in the first 2 h of glucagon infusions. In addition, we were constrained by the unavoidable complexity and length of the study period involving use of multiple vascular catheters and potential risk of platelets adhesion to the catheters and potential clots if the study duration was prolonged. Nevertheless, in our previous studies we already demonstrated that in T1DM patients (13,39,40) and mice with diabetes on insulin treatment, longer duration of insulin deprivation with concurrent hyperglucagonemia increased oxidative stress and oxidative damage to proteins and thus increased their degradation. Thus, for the current study, we focused specifically on the effect of transient and shorter duration of hypoinsulinemia and hyperglucagonemia. Secondly, use of somatostatin to inhibit endogenous hormone secretion also lowers GH concentrations. However, the acute GH effect is mainly on serum fatty acid levels and GH deficiency has not been shown to produce changes in AA concentrations in adults (61,62). Even though, following a prolonged fast of 40 h, increased GH levels may affect protein retention and degradation that seem dependent on free fatty acid release (63), no acute effect of GH has been observed on AA flux or concentrations. Nevertheless, in this study, it is important to note that GH levels remained low during both phases of the study protocol and the main hormone change from 120 to 240 min was in glucagon levels. GH is known to enhance AA uptake in tissues and reduce their release (64) but not have any acute impact on AA, as demonstrated in this study where GH deficiency was evident both during the somatostatin infusion alone and during addition of glucagon. Finally, the small sample size and high interindividual variability in metabolite measurements created some borderline nonsignificant differences in the measurements of metabolites and hormones between the two groups at the baseline level and furthermore potentially limited our ability to detect relatively small and subtle differences. Despite these shortcomings, we report several significant and important differences related to the glucagon effect on AA exchanges and flux between two important regions of AA metabolism that are interesting and require further mechanistic tissue-based studies for understanding of their clinical significance.

In summary, we demonstrate that hyperglucagonemia to a degree commonly seen during transient insulin deficiency results in substantial upregulation of AA exchanges between muscle and splanchnic beds. Hyperglucagonemia during insulin deficiency also results in increased net release of EAA from the muscle bed, likely due to reduced protein synthesis contributing to muscle protein catabolism. The current study also provides experimental data offering insights on glucagon effect in the regulation of tissue specific release and uptake of certain AA and release of their metabolites into the circulation that may contribute to the pathogenesis of diabetes and its complications.

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Author Contributions. H.J., W.I.G., and S.M. were involved directly in the accrual of participants on the study and performed study-related procedures, analyzed data, and drafted the manuscript. I.R.L. and K.A.K. was involved in performing study-related procedures and critically reviewing the manuscript. S.D. was involved in performing the analysis of data and critically reviewing the manuscript. J.C.A. was the interventional radiologist involved in the placement of all the arterial and venous catheters in participants enrolled in this study and critically reviewing the manuscript. A.V. was involved in critically reviewing the manuscript. K.S.N. designed the study, supervised data collection, and helped analyze and interpret data and draft the manuscript. K.S.N. is the guarantor of this work and, as such, had full access to all the

data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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