Ghrelin Infusion in Humans Induces Acute Insulin Resistance and Lipolysis Independent of Growth Hormone Signaling

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OBJECTIVE—Ghrelin is a gut-derived peptide and an endogenous ligand for the growth hormone (GH) secretagogue receptor. Exogenous ghrelin stimulates the release of GH (potently) and adrenocorticotropic hormone (ACTH) (moderately). Ghrelin is also orexigenic, but its impact on substrate metabolism is controversial. We aimed to study direct effects of ghrelin on substrate metabolism and insulin sensitivity in human subjects.

RESEARCH DESIGN AND METHODS—Six healthy men underwent ghrelin (5 pmol · kg^{-1} · min^{-1}) and saline infusions in a double-blind, cross-over study to study GH signaling proteins in muscle. To circumvent effects of endogenous GH and ACTH, we performed a similar study in eight hypopituitary adults but replaced with GH and hydrocortisone. The methods included a hyperinsulinemic-euglycemic clamp, muscle biopsies, microdialysis, and indirect calorimetry.

RESULTS—In healthy subjects, ghrelin-induced GH secretion translated into acute GH receptor signaling in muscle. In the absence of GH and cortisol secretion, ghrelin acutely decreased peripheral, but not hepatic, insulin sensitivity together with stimulation of lipolysis. These effects occurred without detectable suppression of AMP-activated protein kinase phosphorylation (an alleged second messenger for ghrelin) in skeletal muscle.

CONCLUSIONS—Ghrelin infusion acutely induces lipolysis and insulin resistance independently of GH and cortisol. We hypothesize that the metabolic effects of ghrelin provide a means to partition glucose to glucose-dependent tissues during conditions of energy shortage. *Diabetes* **57:3205–3210, 2008**

Ghrelin, an endogenous ligand for the growth hormone (GH) secretagogue receptor (GHS-R), stimulates GH and adrenocorticotropic hormone (ACTH) secretion (1) in addition to having orexigenic and gastrokinetic effects (2,3). The observation that GHS-R is located in peripheral tissues suggests that ghrelin may exert direct effects (4). The effects of ghrelin on substrate in humans are uncertain, but insulin resistance and stimulation of lipolysis have been reported (5–7). However, it remains difficult to segregate direct effects from effects related to GH and cortisol, and we have recently demonstrated that somatostatin infusion fails to sufficiently suppress ghrelin-induced GH and cortisol secretion (8). Hormonally replaced hypopituitary patients constitute a means for studying putative GH- and cortisol-independent effects of ghrelin in human subjects in vivo.

We aimed to study potential direct effects of ghrelin on substrate metabolism and insulin sensitivity in the postabsorptive state. In one experiment in healthy adults, we assessed whether ghrelin-induced GH release translated into GH signaling in skeletal muscle, in the event of which the importance of abrogating indirect effects of ghrelin is obvious. Second, we studied the effects of ghrelin exposure on whole-body and regional substrate metabolism in the basal and insulin-stimulated state in hypopituitary patients on stable replacement with GH and hydrocortisone.

RESEARCH DESIGN AND METHODS

The studies were conducted in accordance with the Helsinki Declaration and following the approval by the local ethics committee, the Danish Medicines Agency, and the Good Clinical Practice (GCP) unit of Aarhus University Hospital. Both protocols were registered (Clinicaltrials.gov identification study 1: NCT00116025 and study 2: NCT00139945).

Preparation of synthetic ghrelin. Synthetic human acylated ghrelin (NeoMPS, Strasbourg, France) was dissolved in isotonic saline and sterilized by double passage through a 0.8/0.2-μm pore-size filter (Super Acrodisc; Gelman Sciences, Ann Arbor, MI).

Study 1: subjects and study protocol. Six healthy men (aged 23 ± 1 years, BMI 23.5 ± 0.4 kg/m^2) were examined as previously described (6). They received a constant infusion of saline or ghrelin (5 pmol · kg^{-1} · min^{-1}) starting at 0 min. At 90 min, a muscle biopsy was obtained from the lateral vastus muscle with a Bergström biopsy needle (Fig. 1).

Study 2: subjects and study protocol. Eight hypopituitary men (aged 53 ± 4 years, BMI 31.6 ± 1.0 kg/m^2) on stable replacement therapy with GH and hydrocortisone (for >3 months) participated. None of the patients had diabetes (A1C 5.7 ± 0.1% [range 4.9–6.0]) or any concomitant chronic disease. Each patient was studied on two occasions with 5-h infusions of saline or ghrelin (5 pmol · kg^{-1} · min^{-1}) in a randomized double-blind, cross-over design. Both study days commenced at 0800 h after an overnight fast (>9 h), with the subjects remaining fasting.

One intravenous cannula was inserted in the antecubital region for infusion, and one intravenous cannula was inserted in a heated dorsal hand vein for sampling of arterialized blood. At t = 0 min, saline or a primed-continuous ghrelin infusion (5 pmol · kg^{-1} · min^{-1}) was commenced. The bolus dose was estimated from the elimination rate constant of ghrelin (k_{01}) (6) and infused over a 20-min interval to avoid an overshoot of steady-state levels. Muscle biopsies were obtained at 120 min, as described above. A hyperinsulinemic-euglycemic clamp (insulin 0.6 mU · kg^{-1} · min^{-1}; Actrapid, Novo Nordisk, Denmark) was performed from 120 to 300 min. Plasma glucose was clamped at 5.0 mmol/l by adjusting the rate of infusion of 20% glucose according to plasma glucose measurements every 10 min. Insulin sensitivity was calculated from the glucose infusion rate (GIR) during the clamp. The period from 0 to 120 min is referred to as the basal period and the period from 120 to 300 min as the clamp period. Blood samples were obtained as indicated in Fig. 2.

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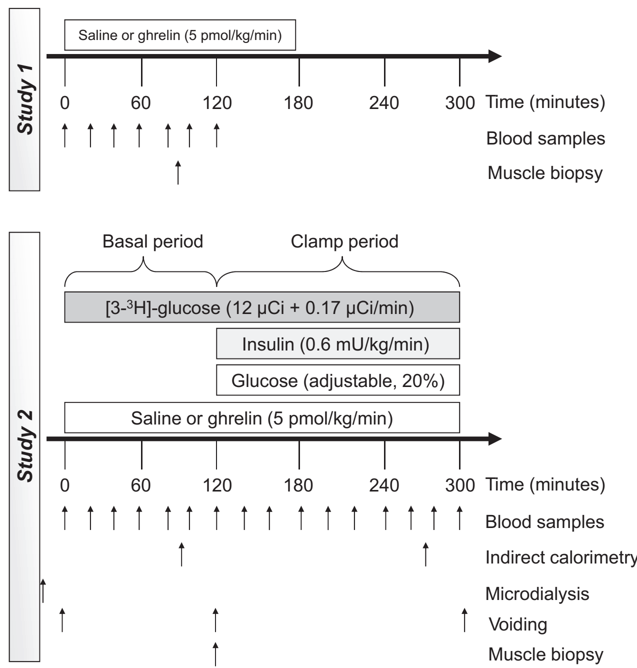

FIG. 1. Study protocol. Please refer to RESEARCH DESIGN AND METHODS for further details.

Tracers. A primed-continuous infusion of [3-^{3}H]-glucose (bolus 12 μCi, 0.17 μCi/min; NEN Life Science Products, Boston, MA) was initiated at $t = 0$ min and continued throughout. Glucose rate of appearance (R_a) was calculated at 10-min intervals from 90 to 120 min and 270 to 300 min using Steele's non–steady-state equations (9). During the clamp, endogenous glucose production was calculated by subtracting the GIR from R_a. Oxidative rates of glucose (G_{OX}) and lipids were calculated from indirect calorimetry (Deltatrac; Datex Instruments, Helsinki, Finland) after correction for protein oxidation, which was estimated from the urinary excretion of urea. Nonoxidative glucose disposal was calculated as whole-body glucose disposal (R_d) minus the rate of G_{OX} (10).

Microdialysis. Microdialysis was performed and analyzed as described previously (8). Catheters (CMA 60, molecular cutoff of 20 kDa, membrane length 30 mm; CMA, Stockholm, Sweden) were placed in the lateral vastus muscle, in the subcutaneous adipose tissue, and in the femoral subcutaneous adipose tissue. The abdominal and femoral adipose tissue blood flow was estimated by Xe washout (11).

Blood samples and measurements. Plasma glucose was analyzed in duplicate using the glucose oxidase method (Beckman Instruments, Palo Alto, CA). Serum ghrelin (total levels) was measured in duplicate by an in-house assay (12). Serum GH, cortisol, and insulin were analyzed with a double monoclonal immunofluorometric assay (Delfia; Perkin Elmer, Wallac Oy, Turku, Finland). Serum free fatty acids (FFAs) were determined using a commercial kit (Wako Chemicals, Neuss, Germany). Plasma catecholamines were measured by liquid chromatography (13). Plasma glucagon was measured by radioimmunoassay (14). Glucose, glycerol, lactate, and urea in the microdialysis dialysate were measured in duplicate by an automated spectrophotometric kinetic enzymatic analyzer (CMA 600; CMA).

Western blotting and phosphatidylinositol 3-kinase assay. Muscle biopsies were homogenized as previously described (15). Aliquots of protein were resolved by SDS-PAGE, and proteins were electroblotted onto nitrocellulose membranes. Immunoblotting was performed using primary antibodies as follows: p signal transducers and activators of transcription (STAT5)a and -b, STAT5, pSTAT3, STAT3, p extracellular signal–regulated kinase (ERK)1 and -2, ERK-1 and -2, p AMP-activated protein kinase (AMPK)α, AMPKα-pan, p acetyal-CoA carboxylase (ACC), pAkt, pAkt substrate, and Akt substrate 160 (AS160). Membranes were incubated with horseradish peroxidase–coupled secondary antibodies, visualized by BioWest enhanced chemiluminescence (UVP LabWorks, Upland, CA) and quantified by the UVP BioImaging System. Densitometric measurements were adjusted to an internal control. Phosphatidylinositol 3-kinase (PI3K) activity was assessed, as previously described (15).

Statistics. Results are expressed as means ± SE. Systemic levels of hormones, metabolites, and GIR were analyzed by two-way ANOVA. The interaction between time and treatment (time × treatment) was considered the term of interest. The Bonferroni correction was used to account for multiple comparisons when appropriate. Pairwise comparisons were carried out by Student's two-tailed paired t test when appropriate. P values <0.05 were considered significant. Statistical analysis was performed using SPSS version 14.0 for Windows.

RESULTS

Study 1. Ghrelin infusion stimulated endogenous GH secretion, which peaked at $t = 60$ min (1.1 ± 0.9 μg/l [saline] vs. 33.3 ± 8.0 μg/l [ghrelin]; $P = 0.008$). A significant elevation in serum FFA levels was recorded (0.4 ± 0.04 μg/l [saline] vs. 1.0 ± 0.1 μg/l [ghrelin]; $P = 0.003$). The levels of serum cortisol (268 ± 24 nmol/l [saline] vs. 400 ± 57 nmol/l [ghrelin]; $P = 0.06$) and plasma glucose (5.2 ± 0.1 mmol/l [saline] vs. 5.5 ± 0.1 mmol/l [ghrelin]; $P = 0.16$) were similar. Western blots performed on skeletal muscle biopsies revealed distinct STAT5 phosphorylation in all six subjects 30 min after the endogenous GH burst (Fig. 3).

Study 2. Pituitary surgery had been performed in all cases, and GH deficiency was documented by GH stimulation tests (insulin tolerance test [$n = 7$] or arginine test [$n = 1$]; means ± SE peak GH 0.3 ± 0.1 μg/l).

Hormones and metabolites. Hormones and metabolites are shown in Fig. 2. Serum ghrelin concentrations were similar at baseline on the 2 study days (0.51 ± 0.06 μg/l [saline] vs. 0.49 ± 0.06 μg/l [ghrelin]; $P = 0.38$) and correlated inversely with BMI (saline $r = -0.83$, $P = 0.01$; ghrelin $r = -0.73$, $P = 0.04$). Plasma levels of norepinephrine and epinephrine were comparable on both study days, and plasma levels of glucagon were also similar (10.9 ± 0.9 pmol/l [saline] vs. 9.3 ± 0.5 pmol/l [ghrelin], $P = 0.10$ at $t = 120$ min; and 8.6 ± 0.5 pmol/l [saline] vs. 7.1 ± 0.8 pmol/l [ghrelin], $P = 0.14$ at $t = 300$ min).

Resting energy expenditure and glucose and lipid metabolism. Data on resting energy expenditure and respiratory quotient (RQ) in study 2 are given in Table 1. Energy expenditure, RQ, or lipid oxidation were not significantly affected by ghrelin in the basal or in the clamp period. The increase in RQ ($RQ_{clamp} - RQ_{basal}$) during the clamp, however, was larger in the saline study (0.07 ± 0.01 vs. 0.03 ± 0.01 [ghrelin], $P = 0.03$).

FFAs. Ghrelin infusion induced an 80% increase in FFAs to 0.62 ± 0.03 mmol/l at $t = 120$ ($P < 0.05$), followed by a return to placebo levels during the clamp period (Fig. 2*C*).

Glucose. Ghrelin induced a rapid increase in plasma glucose levels with a peak value of 6.1 ± 0.2 mmol/l at $t = 120$ min ($P = 0.009$) (Fig. 2*E*). During the clamp, glucose levels gradually decreased toward postabsorptive levels on the ghrelin day, resulting in comparable glucose levels during the final 30 min of the clamp. The GIR was significantly decreased during ghrelin administration (Fig. 4*A*) ($P < 0.01$), and the corresponding M value was reduced by ~60% ($P < 0.001$). Ghrelin did not significantly impact glucose metabolism in the basal state (Table 1 and Fig. 4*C*) but reduced the rates of oxidative, nonoxidative, and total glucose disposal during the clamp period.

Regional substrate metabolism (microdialysis). Interstitial muscle glucose levels fluctuated in parallel with those in the circulation (Fig. 2*F*). By contrast, interstitial glucose in fat remained stable also during ghrelin infusion. Ghrelin did not significantly influence the levels of interstitial glycerol, lactate, or urea in either tissue (data not shown).

GH, insulin, and AMPK signaling. Densitometric quantitative bar graphs and representative Western blots from skeletal muscle biopsies are provided in Fig. 3. Adminis-

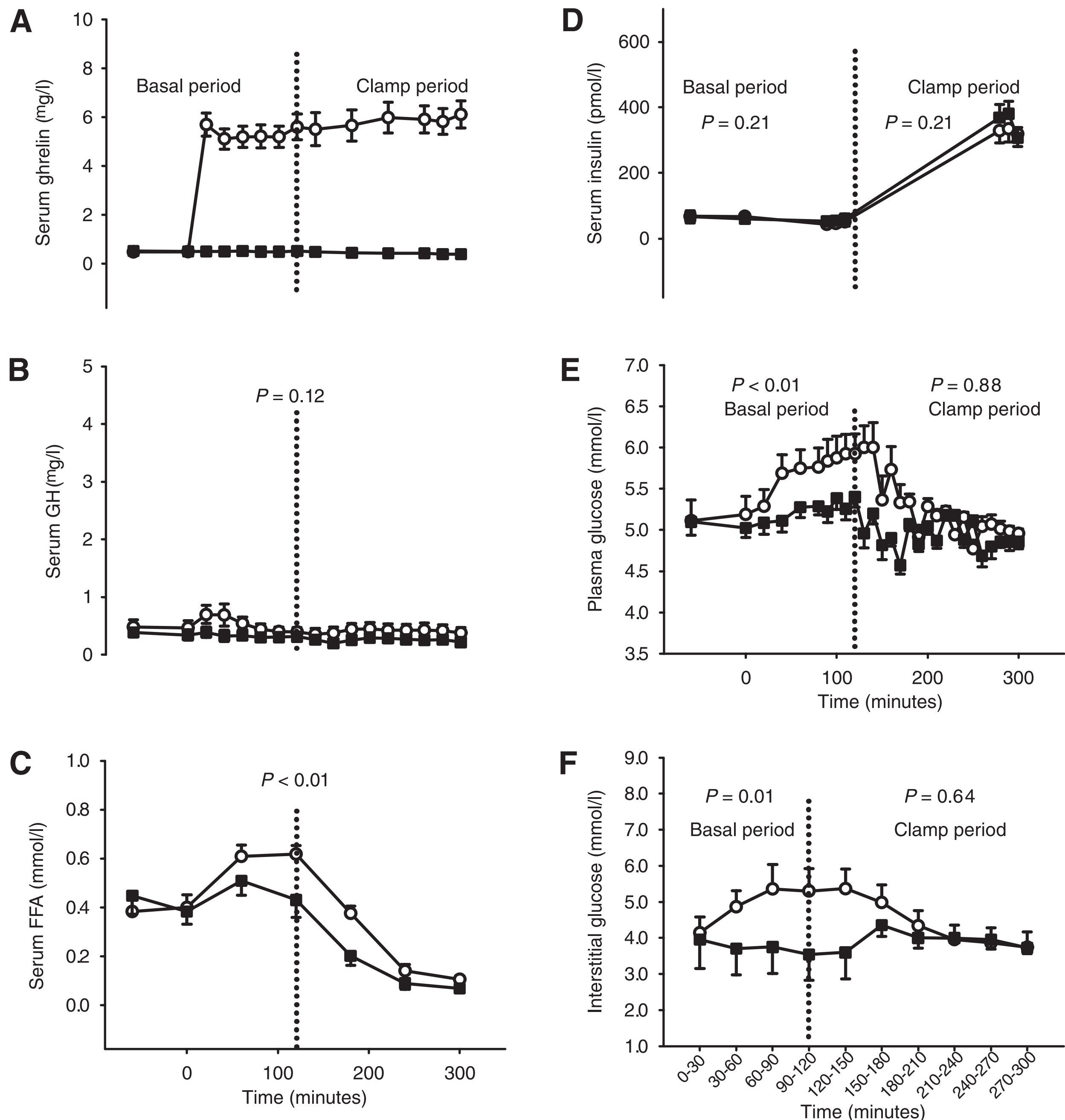

FIG. 2. Hormones and metabolites during saline and ghrelin administration in study 2. *A*: Serum levels of ghrelin increased in response to ghrelin infusion to a plateau of 5.33 ± 0.45 μg/l in the basal state and a higher plateau of 5.86 ± 0.50 μg/l during the clamp period (mean ghrelin levels basal period vs. clamp period *P* = 0.001). *B*: Serum levels of GH. *C*: Serum levels of FFA. *D*: Serum levels of insulin. Serum insulin was similar during both basal and clamp conditions. *E*: Plasma glucose levels. *F*: Interstitial skeletal muscle glucose levels. Printed *P* values refer to two-way ANOVA significance levels. ■, saline infusion; □, ghrelin infusion. All data are presented as means ± SE.

tration of ghrelin translated into STAT5 phosphorylation in the healthy subjects but not in the hypopituitary patients. Ghrelin exerted no effects on total protein levels, and no effects were recorded with regard to AMPK, ACC, STAT3, ERK-1 or -2, Akt, or AS160 phosphorylation in study 2. Insulin receptor substrate–associated PI3K activity was also not modified by ghrelin infusion (data not shown).

DISCUSSION

We document for the first time that ghrelin induces peripheral insulin resistance and stimulates lipolysis in the absence of GH and cortisol release. This investigation is also the first to document that ghrelin-induced endogenous GH release translates into Janus kinase/STAT signaling in skeletal muscle.

In some human studies, ghrelin administration increases plasma levels of glucose and FFAs (6–8,16) and reduces glucose disposal (5,8), indicating insulin resistance. These effects are, however, partly attributable to GH and/or cortisol secretion (5–8). There are both clinical and in vitro data to suggest that ghrelin directly suppresses glucose-induced insulin secretion from the β-cell (17), whereas administration of a ghrelin antagonist does the opposite (18). Moreover, ghrelin knockout mice display enhanced glucose-induced insulin release from isolated

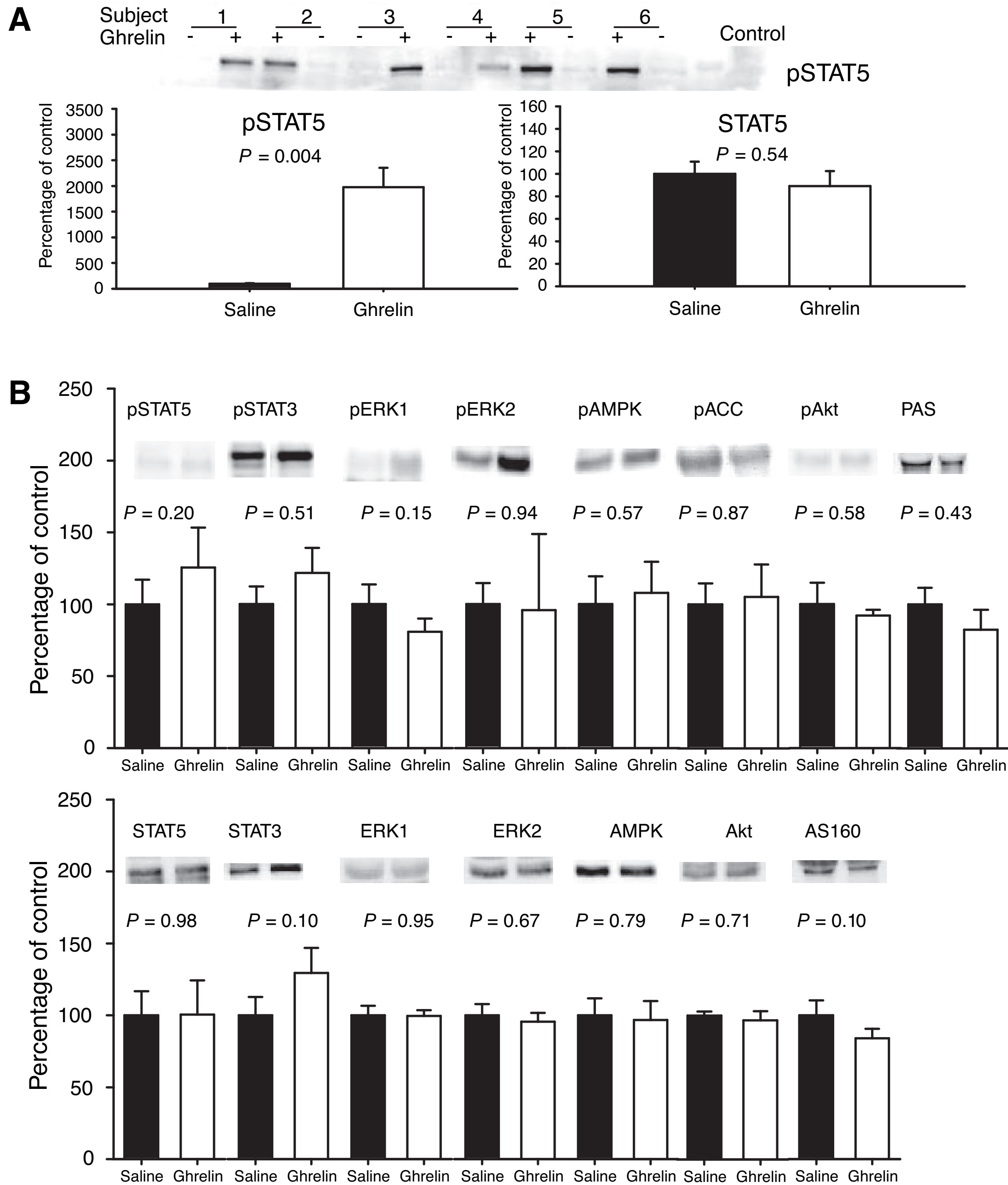

FIG. 3. ***A*: Effects of ghrelin infusion on STAT5 phosphorylation and total STAT5 levels in skeletal muscle in healthy subjects (study 1). Values are means ± SE. *B*: Representative Western blots and quantitative bar-graphs regarding JAK/STAT, MAPK, AMPK, and insulin signaling pathways in skeletal muscle in hypopituitary patients (study 2) during ghrelin and saline infusion. PAS, pAkt substrate.**

TABLE 1
Metabolic parameters during saline and ghrelin infusion in hypopituitary men (study 2)

	Basal period			Clamp period		
	Saline	Ghrelin	*P* value	Saline	Ghrelin	*P* value
RQ (ratio O_2/CO_2)	0.82 ± 0.02	0.83 ± 0.01	0.24	0.89 ± 0.01	0.86 ± 0.01	0.06
Energy expenditure (kcal/24 h)	1902 ± 58	1916 ± 61	0.80	1931 ± 49	1906 ± 66	0.72
Lipid oxidation ($mg \cdot kg^{-1} \cdot min^{-1}$)	0.74 ± 0.08	0.67 ± 0.05	0.37	0.34 ± 0.05	0.48 ± 0.08	0.19
Endogenous glucose production ($mg \cdot kg^{-1} \cdot min^{-1}$)	1.59 ± 0.18	1.63 ± 0.16	0.74	0.58 ± 0.19	0.57 ± 0.15	0.95

Data are means ± SE. Paired analysis of treatments.

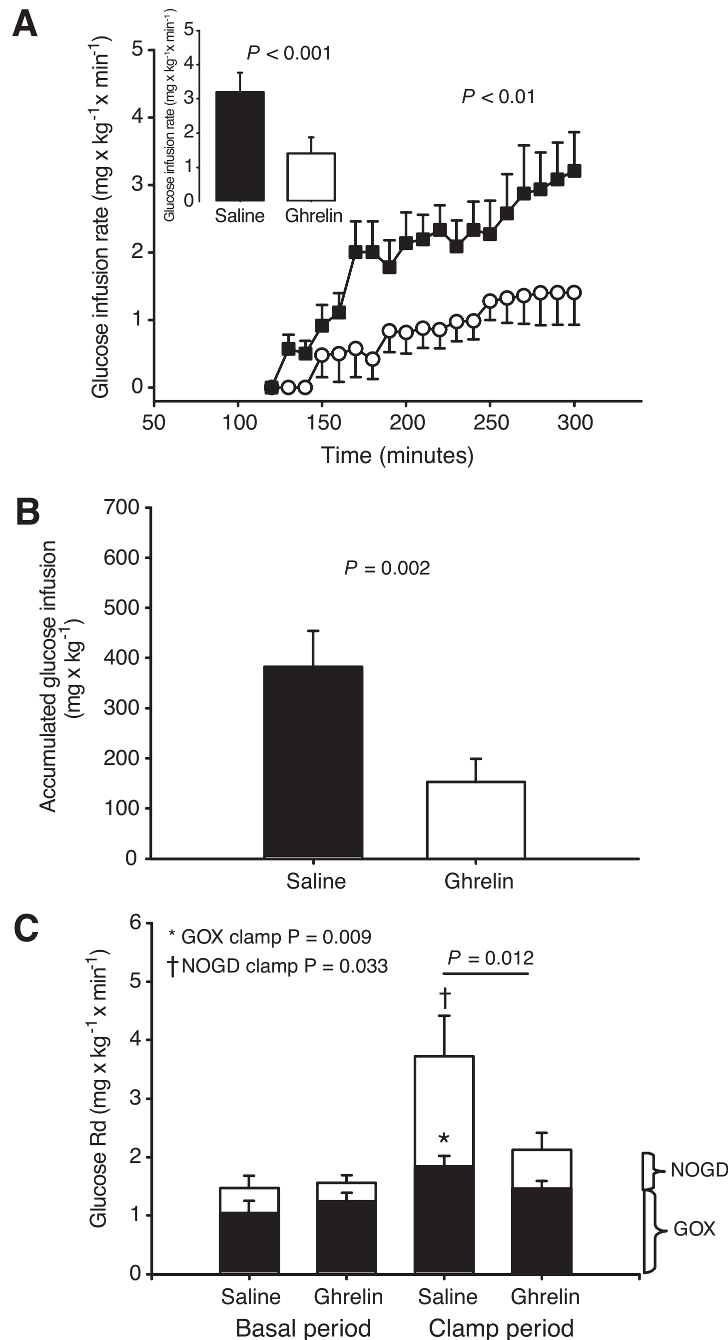

FIG. 4. ***A*: Glucose metabolism during saline and ghrelin administration in study 2. Hyperinsulinemic clamp. Glucose infusion rates and *M* value during saline and ghrelin administration. ●, saline infusion; □, ghrelin infusion. *B*: Accumulative glucose infusion dosage during saline and ghrelin administration. The accumulative glucose dose was significantly decreased during the clamp period in the ghrelin study (P = 0.002). *C*: Glucose utilization during the terminal 30 min of basal and clamp periods in saline and ghrelin studies. Ghrelin did not significantly impact glucose metabolism in the basal period. During the clamp ghrelin infusion reduced the rates of oxidative, nonoxidative, and total glucose disposal (P = 0.009, P = 0.03, and P = 0.012, respectively). All data are presented as means ± SE.**

islets (18,19) and exhibit increased peripheral insulin sensitivity (19). Ghrelin/GHS-R double knockout mice show lower glucose levels after a glucose tolerance test and a more rapid drop in plasma glucose levels after an insulin tolerance test (20).

Our study in hypopituitary men demonstrates that ghrelin in humans directly suppresses insulin-stimulated glucose disposal and stimulates FFA release. The observations at the whole-body level were corroborated by the demonstration of increased concentrations of glucose in skeletal muscle interstitial tissue. Previously, it has been demonstrated that in humans glucose disposal rates during hyperinsulinemic clamp conditions are predominantly determined by the rate of glucose uptake into skeletal muscle (21). By contrast, we observed no significant effect of ghrelin on hepatic insulin sensitivity. AMPK appears to be a key messenger in ghrelin signaling in several tissues (22–24), and AMPK is also a recognized cellular energy sensor (25). In our study, we did not record any significant effect of ghrelin on the activation of either AMPK or ACC in skeletal muscle. The biopsies, however, were only obtained in the basal period, which limits assessment of aberrations in insulin signaling pathways. The

molecular mechanisms by which ghrelin causes insulin resistance in humans thus remain to be further ascertained and should be studied in both basal and insulin-stimulated conditions.

Serum FFA levels increased in response to ghrelin, which did not translate into increased lipid oxidation. It should be noted that hypopituitary adults are moderately obese, which is likely to influence any effect of ghrelin on FFA turnover. More importantly, infusion of ghrelin does not imitate the secretory pattern of endogenous ghrelin. Furthermore, this study was powered to detect effects of ghrelin on insulin sensitivity (8), and lack of significance with regard to lipid metabolism may be due to a β error.

In conclusion, we demonstrate for the first time in humans that ghrelin directly induces lipolysis and resistance to insulin-stimulated glucose disposal. We also demonstrate that ghrelin-induced GH release translates into GH signaling in skeletal muscle, emphasizing the significance of accounting for GH when evaluating any effects of ghrelin. The physiological significance of the direct metabolic effects of endogenous ghrelin remains unclear, but we propose that ghrelin in concert with GH partition substrate metabolism during conditions of energy shortage in such a way as to restrict glucose utilization to insulin-independent tissues such as the brain.

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