ESSENTIAL FUNCTION OF GHRELIN IN CHRONIC STARVATION

by

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To My Parents

ESSENTIAL FUNCTION OF GHRELIN IN CHRONIC STARVATION

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DISSERTATION

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ABSTRACT

Ghrelin, an octanoylated peptide hormone secreted from the stomach, stimulates the release of growth hormone (GH) from the pituitary. Ghrelin *O*-acyltransferase (GOAT) is the enzyme required for the attachment of octanoate to serine-3 of ghrelin, a step essential for making active ghrelin. In this study, we eliminated the *Goat* gene from mice to produce *Goat* $^{-/-}$ mice that lack octanoylated ghrelin. These mice were indistinguishable in weight from their wild-type (WT) littermates in when fed either a normal or a high fat diet. On 60% calorie restriction, WT and *Goat* $^{-/-}$ mice lost 30% of their body weight and 75% of their body fat within the first 4 days. While fasting blood glucose levels declined at the same rate initially in WT and *Goat* $^{-/-}$ mice, levels in the WT mice stabilized at 58–76 mg/dL after 4 days of 60% calorie restriction. In contrast, fasting blood glucose levels in the calorie restricted *Goat* $^{-/-}$ mice continued declining to 12–36 mg/dL by day 7, at which point the mice were moribund. Levels of ghrelin and GH rose progressively in WT mice during the calorie restriction. GH levels in *Goat* $^{-/-}$ mice, which have no ghrelin, rose to a much lesser degree, a phenotype also seen in calorie restricted *Preproghrelin* $^{-/-}$ mice that lack both ghrelin and des-acyl ghrelin. Restoring ghrelin or GH via an osmotic minipump to calorie restricted *Goat* $^{-/-}$ mice rescued their hypoglycemia. Thus, ghrelin is essential for survival during severe calorie restriction by elevating GH levels to preserve blood glucose and maintain life.

The decreased elevation of GH in calorie restricted *Goat* $^{-/-}$ mice was associated with decreased plasma levels of two gluconeogenic substrates: pyruvate and lactate. Injections of exogenous pyruvate, lactate, and alanine to calorie restricted *Goat* $^{-/-}$ mice prevented the development of hypoglycemia. Injections of exogenous octanoate to calorie restricted *Goat* $^{-/-}$ mice, which spares the need to oxidize glucose and gluconeogenic substrate in the

tricarboxylic (TCA) cycle to provide energy for gluconeogenesis, also prevented the hypoglycemia. Therefore, the preservation of blood glucose during calorie restriction by the ghrelin-mediated rise in GH involves the maintenance of adequate plasma levels of gluconeogenic substrates.

The dramatic rise in plasma ghrelin during chronic severe calorie deprivation is essential to maintain life. However, the mechanism for this increase is not understood. From tissue culture cells derived from mice bearing ghrelinomas induced by a tissue-specific SV40 T-antigen transgene, we found that ghrelin secreting cells express high levels of mRNA encoding the β_1-adrenergic receptor. Ghrelin secretion from these cells was stimulated by the addition of norepinephrine or epinephrine, an effect blocked by atenolol, a selective β_1-adrenergic antagonist. Treating WT mice with atenolol or reserpine, a drug that depletes adrenergic neurotransmitters from sympathetic neurons, blocked the fasting-induced increase in plasma ghrelin. Thus, ghrelin secretion during fasting is induced by adrenergic agents released by sympathetic neurons which act directly on β_1 receptors on the ghrelin-secreting cells of the stomach.

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LIST OF ABBREVIATIONS

AGRP: agouti-related protein

ARC: arcuate nucleus

GH: growth hormone

GHRH-R: growth hormone releasing hormone receptor

GHS: growth hormone secretagogues

GHS-R: growth hormone secretagogue receptor

GLP-1: glucagon like peptide-1

GOAT: Ghrelin *O*-acyltransferase

HHAT: Hedgehog Acyltransferase

NMR: nuclear magnetic resonance

NPY: neuropeptide Y

PC 1/3: Prohormone convertase 1/3

PEPCK: phosphoenolpyruvate kinase

PG-1: pancreatic ghrelinoma cell line 1

PK: pyruvate kinase

PMSF: p-hydroxymercuribenzoic acid

RYGB: Roux-en-Y gastric bypass

SG-1: stomach ghrelinoma cell line 1

TCA: tricarboxylic

WT: wild-type

CHAPTER I: INTRODUCTION

The Discovery of Ghrelin

Ghrelin was first identified by Kojima et al. (1) in 1999 from stomach extracts as the endogenous ligand for the growth hormone secretagogue receptor (GHS-R). In the early 1980s, it was found that certain synthetic peptides could stimulate the release of growth hormone (GH) from pituitary extracts (2, 3). These synthetic peptides, later termed growth hormone secretagogues (GHS), do not bind to the growth hormone releasing hormone receptor (GHRH-R) but instead bind to GHS-R (4). Although GHRH-R and GHS-R are both G protein-coupled receptors that stimulate the release of GH by the pituitary, their mechanisms of action are different: GHRH-R acts via G_s to raise intracellular cAMP levels while GHS-R acts via G_q to raise intracellular Ca^{2+} levels. The purification of ghrelin, and the evidence of its conservation from mammals to fish, showed that an alternate, highly conserved, pathway exists to control the release of growth hormone.

The Structure and Post-Translational Processing of Ghrelin

Ghrelin is a 28-amino acid peptide that is modified on its serine-3 residue by *O*-linked acylation with the eight carbon fatty acid octanoate (1). This modification is unique to ghrelin; no other protein is known to be acylated by octanoate. Ghrelin is highly conserved from mammals to fish. Mouse ghrelin is identical with the rat version and differs with human ghrelin by only two amino acids (5). Among mammals, birds, amphibians, and fish, six of the first seven N-terminal residues are identical and the *O*-linked acylation to octanoate is conserved. Reports have shown that only the first four or five residues of ghrelin are needed to efficiently bind

GHS-R (6). Coupled with the high degree of conservation of GHS-R among vertebrates (7), this suggests that N-terminal segment of ghrelin acts as the primary site for GHS-R binding.

Ghrelin is produced in a manner typical of peptide hormones. Preproghrelin, a peptide of 117 amino acids, is translated and subsequently cleaved of its signal sequence to give proghrelin, a peptide of 94 amino acids. Prohormone convertase 1/3 (PC 1/3) removes the C-terminal portion of proghrelin to release the 28-amino acid ghrelin (8). Ghrelin is uniquely modified by *O*-linked acylation to octanoate at serine-3 (1). This modification is conserved among mammals, birds, and fish (Kojima and Kangawa, 2005) and is even found in bullfrog ghrelin, where the octanoylated serine is replaced by an octanoylated threonine (9). Studies have shown that octanoylation of ghrelin is required for its ability to stimulate GH release (1) as well as its other metabolic functions (discussed below).

In 2008, our lab (5) and others (10) identified the enzyme that transfers octanoyl-CoA to serine-3 of ghrelin. This enzyme, which we called ghrelin *O*-acyltransferase (GOAT), is a membrane-bound member of a family of 16 hydrophobic membrane-bound acyltransferases (MBOATs) that includes Porcupine, the enzyme that attaches long-chain fatty acids to Wnt proteins and Hedgehog Acyltransferase (HHAT), the enzyme that palmitoylates Sonic Hedgehog.

The Orexigenic Effect of Ghrelin

Besides its ability to stimulate GH release, ghrelin has been shown to be a potent orexigenic hormone (11). Infusion of ghrelin leads to a marked increase in food intake in rodents (12, 13) and humans (14). Furthermore, plasma ghrelin levels are strongly correlated with food intake, rising immediately prior to meals and decreasing sharply after meal initiation (15, 16).

The Action of Ghrelin in the Hypothalamus

In addition to the pituitary, GHS-R mRNA is also highly expressed in the arcuate nucleus (ARC) of the hypothalamus, the area where ghrelin is proposed to exert its effects on appetite and body weight (17, 18). Binding of ghrelin to its receptor in the ARC increases expression of two orexigenic molecules, neuropeptide Y (NPY) and agouti-related protein (AGRP). Binding of ghrelin to GHS-R in the ARC also counteracts the action of leptin, an anorexic hormone that decreases NPY/AGRP levels (12). Indeed, simultaneous administration of ghrelin and leptin abolishes the anorexigenic effects of leptin (12).

Other Effects of Ghrelin

Though still debated, the majority of studies in rodents have shown that glucose-stimulated insulin secretion and insulin sensitivity are suppressed by ghrelin (19-21). Another study provided evidence for ghrelin's role in the regulation of white adipocytes by increasing triglyceride uptake and lipogenesis and inhibiting lipid oxidation (22). Finally, studies of ghrelin-receptor deficient mice suggest that ghrelin could also play a role in modifying behavior. In particular, ghrelin might have antidepressant properties (23) and be necessary for certain food reward behaviors (24).

Metabolic Effects of Ghrelin Deficiency

In contrast to the dramatic effects of excess ghrelin, mice genetically engineered to lack ghrelin (25, 26) or its receptor (27, 28) grow normally without signs of GH deficiency. With high fat diet feeding, these mice may or may not show a slight resistance to obesity and a slight improvement in glucose tolerance and insulin secretion. Thus, ghrelin is not essential for either growth or for appetite in mice, although the possibility exists for interspecies variations in these

areas. It remains an open question, especially in light of its highly conserved nature, as to what the essential role of ghrelin is.

The Autonomic Control of Glucose Metabolism and Energy Homeostasis

Energy homeostasis is tightly regulated by the autonomic nervous system through the innervation of key metabolic organs as well as the release of epinephrine and norepinephrine from the adrenal medulla. The liver and pancreas, in particular, are richly innervated by both splanchnic sympathetic and vagal parasympathetic nerves (29-31). In the pancreas, increased sympathetic outflow leads to the release of glucagon by α cells while increased parasympathetic outflow leads to insulin release by β cells (30). In the liver, the sympathetic nervous system acts directly to increase glycogenolysis, gluconeogenesis, and glucose export while decreasing glycogen synthesis (29, 30, 32). The parasympathetic nervous system acts in the liver to enhance glucose utilization (29).

Aside from the effects of direct autonomic innervation, liver glucose metabolism is also influenced by the release of glucagon and insulin from the pancreas and epinephrine and norepinephrine from the adrenal medulla (29). Both epinephrine and glucagon increase hepatic glycogen breakdown and glucose production (30, 33, 34) while norepinephrine acts primarily to increase gluconeogenesis only at the high concentrations found at sympathetic nerve terminals (35).

Control of glucose metabolism by the autonomic system also occurs in skeletal muscle and adipose tissue via effects from direct sympathetic innervation and via effects secondary to hormones released from the pancreas and adrenal medulla (36-38). It is well known that a primary action of insulin is to stimulate glucose uptake in skeletal muscle. There is also evidence that a non-insulin dependent glucose uptake pathway exists in skeletal muscle that is mediated by

the release of norepinephrine from sympathetic nerves directly to muscle cells (36). In contrast to norepinephrine, epinephrine acts in skeletal muscle to inhibit insulin-stimulated glucose uptake (38). In white and brown adipose tissue, sympathetic activation via norepinephrine action on β-adrenergic receptors leads to increased glucose uptake, increased lipolysis, and increased thermogenesis (38).

Sympathetic and parasympathetic outflow in relation to metabolism is thought to originate in the ventromedial (VMH) and lateral (LH) nuclei of the hypothalamus, respectively (30). The activity of phosphoenolpyruvate kinase (PEPCK), a key gluconeogenesis enzyme, is increased by electrical stimulation of the VMH and decreased by stimulation of the LH (30). In contrast, the activity of pyruvate kinase (PK), a key glycolytic enzyme, is decreased with VMH stimulation and unchanged with LH stimulation (30).

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CHAPTER II: Ghrelin *O*-acyltransferase (GOAT) is essential for growth hormone-mediated survival of calorie-restricted mice

Introduction

The abilities of ghrelin to stimulate the release of growth hormone (GH) from pituitary cells (1) and to enhance food intake in rodents and humans (2, 3) are well established and define its pharmacologic effects. However, despite the highly conserved nature of ghrelin, the effects of ghrelin deficiency have been much harder to elucidate. Knockout-mice lacking either ghrelin (4, 5) or its receptor (6, 7) grow normally without the signs of GH deficiency when fed a normal chow diet. On high-fat diet, these mice may or may not show a slight resistance to the development of obesity and may or may not have a slight improvement in insulin secretion and glucose tolerance. One study (6) did show that, after 40 days of 50% calorie restriction, ghrelin and ghrelin-receptor knockout mice, having lost the same weight as wild-type (WT) mice, had slight reductions in fasting blood glucose on some, but not all, days.

The stimulation of GH release by ghrelin is entirely dependent on the attachment of octanoate to serine-3 of ghrelin. Conservation of this modification, which is unique to ghrelin, stretches back as far back as fish (8). Recently, our laboratory (9) and others (10) have identified the enzyme responsible for the transfer of octanoate from octanoyl-CoA to serine-3 of ghrelin to form the acyl ester. This enzyme was named ghrelin *O*-acyltransferase or GOAT.

To explain the evolutionary conservation of the octanoate modification of ghrelin and to define the metabolic consequences of a loss of ghrelin octanoylation, we generated mice in which the coding region of the *Goat* gene was deleted via homologous recombination. In the one previous study of *Goat* $^{-/-}$ mice, the only positive finding was a minor reduction in body weight when fed a diet rich in octanoate (11). In our studies, we defined the role of GOAT in the

opposite direction – under conditions of caloric restriction. When given 40% of their normal daily caloric intake (60% calorie restriction), *Goat*$^{-/-}$ mice were increasingly unable to maintain their blood glucose levels and became moribund by the seventh day of calorie restriction. In contrast, wild-type mice were able to maintain their blood glucose levels throughout. Our studies thus reveal that an essential function of ghrelin, and perhaps the explanation for its strong conservation, is to maintain viability in periods of famine.

Results

Generation of *Goat*$^{-/-}$ mice

Goat$^{-/-}$ mice were generated as described in *Materials and Methods*. Briefly, ES cells were obtained from Regeneron Pharmaceuticals in which the coding region of *Goat* was replaced by a cassette containing a *lac*Z reporter gene and a neomycin resistance gene (*neo*) (Fig. 2-1*A*).

The ES cells were injected into C57BL/6J blastocysts which were then implanted into pseudopregnant surrogate mothers. Chimeric offspring were obtained and N2 *Goat*$^{+/-}$ mice were generated by backcrossing the chimeras to C57BL/6J mice. N2F2 *Goat*$^{-/-}$ mice and their wild-type (WT) littermates were then created by crossing N2 *Goat*$^{+/-}$ mice to each other. All experiments were performed with N2F2 *Goat*$^{-/-}$ mice and their WT littermates, which contain a mixture of C57BL/6J and 129SvEv genes. Genomic PCR analysis of DNA from WT, *Goat*$^{+/-}$, and *Goat*$^{-/-}$ mice showed the predicted bands (Fig. 2-1*B*) and quantitative real-time PCR analysis of mRNA from the gastric mucosa of *Goat*$^{-/-}$ mice showed the elimination of the GOAT transcript, whereas the mRNA encoding preproghrelin was unaffected (Fig. 2-1*C*).

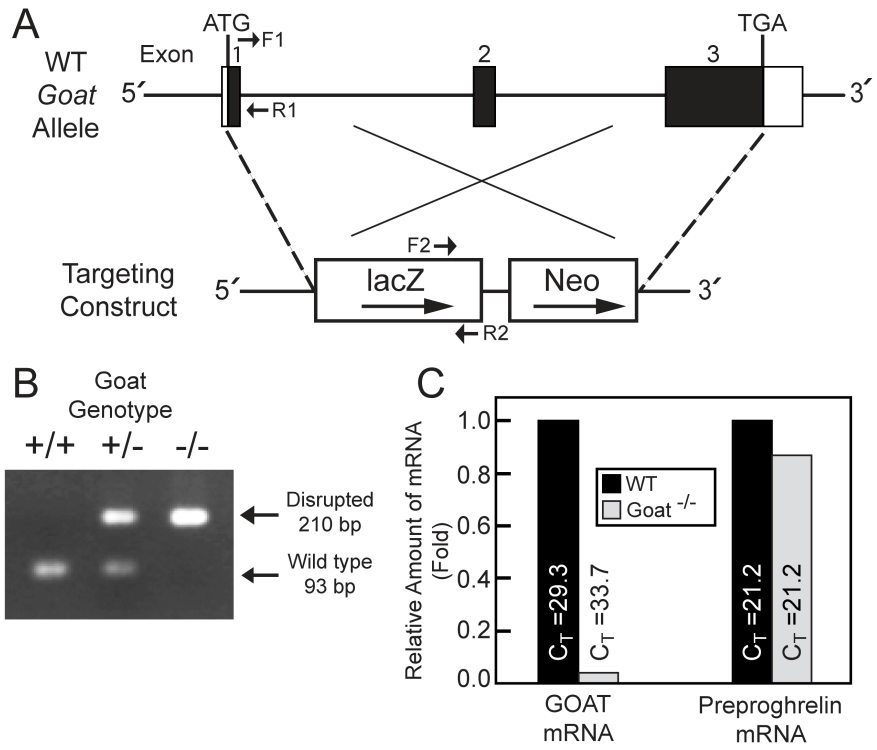

Fig. 2-1. Generation of *Goat*$^{-/-}$ mice. (*A*) The null *Goat* allele was obtained by replacing the entire GOAT coding region with a *lacZ* reporter gene and a *neo* selectable marker. (*B*) Representative PCR analysis of tail DNA from WT, *Goat*$^{+/-}$, and *Goat*$^{-/-}$ mice using primers F1, R1, F2, and R2 as described in *Materials and Methods*. Primers F1 and R1 (depicted in *A*) amplifies a 93-bp fragment from the WT *Goat* gene whereas primers F2 and R2 amplifies a 210-bp fragment from the *lacZ* gene of the null allele. (*C*) Quantitative real-time PCR analysis of total RNA from the stomach mucosa of WT and *Goat*$^{-/-}$ mice. Threshold cycle (C_T) numbers are shown inside the bars.

Chow and High Fat Feeding of *Goat*$^{-/-}$ mice

Goat$^{-/-}$ mice appeared normal through adulthood and produced normal numbers of offspring. After 14-weeks of chow (18% calories from fat) or high-fat feeding (45% calories from fat), *Goat*$^{-/-}$ mice were indistinguishable from their WT littermates in terms of body weight (Fig. 2-2*A*) or fat and lean mass (Fig. 2-2*B* and *C*). Fat mass increased by the same amount in *Goat*$^{-/-}$ mice and their WT littermates after 14-weeks of high-fat diet feeding. There was no difference in daily food intake between WT and *Goat*$^{-/-}$ littermates when fed *ad libitum*.

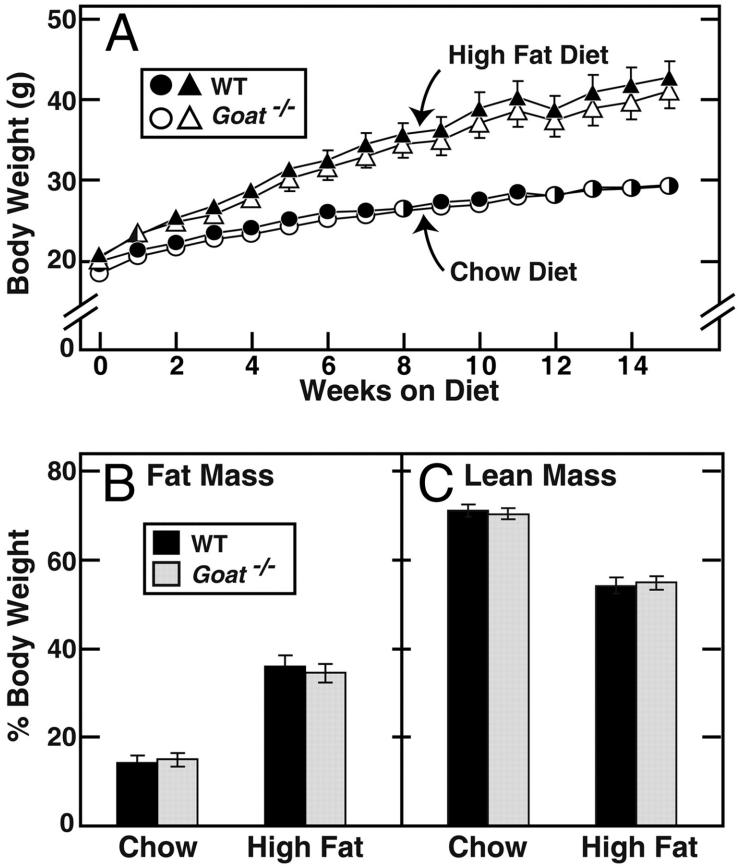

Figure 2-2. Body composition after chow or high fat feeding of WT and *Goat*$^{-/-}$ mice. 4-wk-old male WT and *Goat*$^{-/-}$ littermates were fed *ad libitum* either chow or high fat diet for the indicated time. (*A*) Mice were weighed weekly. (*B* and *C*) The total fat (*B*) and lean (*C*) body mass percentages were determined after 14 weeks of feeding using NMR. Each value represents the mean ± SEM of data from 6 mice.

Calorie Restriction of *Goat*$^{-/-}$ mice

Previous studies have shown that ghrelin levels increase in the fasted state and drop immediately after feeding (12-14). This suggests that ghrelin might play a role during the calorie restricted state. We tested this hypothesis by subjecting *Goat*$^{-/-}$ mice and their WT littermates to 60% calorie restriction in which individually housed 8-wk-old male mice were fed 40% of their measured daily *ad libitum* food intake. One pre-measured pellet was given at 6 p.m. each day, 3 h before the beginning of the dark cycle. Three days after the start of calorie restriction, both WT and *Goat*$^{-/-}$ mice ate the food within 1 h after feeding, a pattern that continued throughout the

experiment. A separate group of *Goat*$^{-/-}$ mice and their WT littermates were fed *ad libitum* chow diet as a control.

Both WT and *Goat*$^{-/-}$ mice lost approximately 28% of their body weight during the first 4 days of calorie restriction, after which their body weights stabilized (Fig. 2-3*A*). Similarly, both groups lost 75% of their fat mass as determined by NMR (Fig. 2-3*B*). At 5:30 p.m. each day, 30 minutes before feeding, fasting blood glucose was measured (Fig. 2-3*C*). Both WT and *Goat*$^{-/-}$ mice showed a similar initial decline in fasting blood glucose during the first two days of calorie restriction. Thereafter, fasting blood glucose in the WT mice remained relatively constant at 54–65 mg/dL through 7 days of calorie restriction. *Goat*$^{-/-}$ mice, on the other hand, could not maintain their fasting blood glucose levels, with levels continuing to decline until they reached 12 mg/dL on day 7. At this point, *Goat*$^{-/-}$ mice were lethargic and moribund whereas their WT littermates appeared healthy and active.

Goat$^{-/-}$ mice and their WT littermates were euthanized at 5:30 p.m. after 7 days of calorie restriction to obtain plasma for hormone measurements. There was an 18-fold increase in plasma ghrelin in calorie-restricted WT mice as compared to *ad libitum* fed WT mice (Fig. 2-3*D*). Ghrelin was undetectable in *Goat*$^{-/-}$ mice on either diet as expected. The level of des-acyl ghrelin in *ad libitum* fed *Goat*$^{-/-}$ mice was 3-fold higher than their *ad libitum* fed WT littermates (Fig. 2-3*E*). Des-acyl ghrelin levels rose markedly in both groups during calorie restriction such that was no longer any difference between the groups after 7 days. Plasma GH levels were similarly low in *Goat*$^{-/-}$ and WT mice under *ad libitum* fed conditions (1.4 ± 0.4 and 1.3 ± 0.6 ng/mL) (Fig. 2-3*F*). After 7 days of calorie restriction, plasma GH levels rose in both groups, but WT mice showed levels 2-fold higher than their *Goat*$^{-/-}$ littermates.

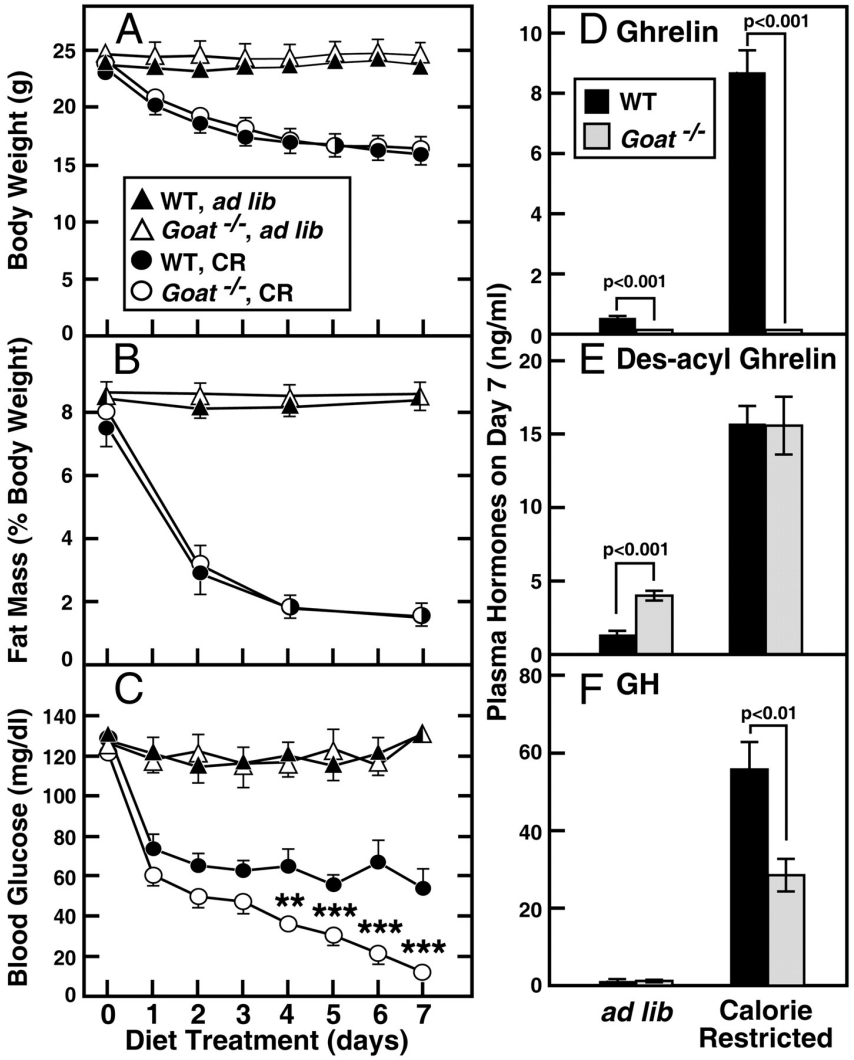

Figure 2-3. Response of WT and *Goat*$^{-/-}$ mice to *ad libitum* chow feeding or 60% calorie restriction. 8-wk-old male WT and *Goat*$^{-/-}$ littermates were fed *ad libitum* chow diet or subjected to 60% calorie restriction as described in *Materials and Methods*. Daily measurements of body weight (*A*) were made at 5:30 p.m. Total fat mass (*B*) was measured by NMR every 2 or 3 days at 5 p.m. Blood glucose (*C*) was measured using a Bayer Contour Glucometer at 5:30 p.m. (30 minutes before feeding). (*D-F*) On the seventh day of calorie restriction, the mice were euthanized at 5:30 p.m. (before feeding) to collect plasma for the measurement of plasma levels of ghrelin (*D*), des-acyl ghrelin (*E*), and GH (*F*). Each value represents the mean ± SEM of data from 6 mice. Asterisks (*) denote the level of statistical significance (Student's *t* test) between WT and *Goat*$^{-/-}$ mice under calorie-restricted conditions. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Metabolic Changes to WT and Goat $^{-/-}$ mice after Calorie Restriction

Various metabolic parameters were measured at 5:30 p.m. on day 7 of calorie restriction in the *ad libitum* fed and calorie-restricted WT and *Goat* $^{-/-}$ mice described in Fig. 2-3. The data are summarized in Table 2-1. Changes in body weight, fat mass, blood glucose levels, and plasma ghrelin and GH levels were described previously. Despite the elevation in GH after calorie restriction, plasma IGF1 levels were reduced by >90% in both WT and *Goat* $^{-/-}$ mice. Although GH is known to elevate plasma free fatty acid levels (16), they were in fact extremely low in the calorie-restricted WT and *Goat* $^{-/-}$ mice, with no difference between the two groups. This may be explained by the observation that both groups showed an extreme depletion of fat mass, which limits the source of fatty acids. This is also the likely explanation for the low levels of plasma β-hydroxybutyrate, a product of free-fatty acid oxidation, in both groups.

Plasma insulin was nearly undetectable in both groups after calorie restriction whereas plasma glucagon levels rose by approximately 2-fold in both groups. Plasma corticosterone and cortisol were not significantly different between WT and *Goat* $^{-/-}$ littermates after calorie restriction, but were higher than levels during *ad libitum* feeding. It should be noted that the higher levels observed are those normally associated with stress and some of this elevation may be caused by stress induced by animal handling.

The transcriptional response of the liver during calorie restriction was analyzed by using quantitative real-time PCR to measure the levels of various mRNAs from pooled liver RNA samples. The data are summarized in Table 2-2 and are consistent with a low insulin/high glucagon state.

Table 2-1. Metabolic Response of WT and *Goat*$^{-/-}$ mice after 60% calorie restriction.

	Ad libitum Fed		Calorie-restricted	
Parameter	WT	*Goat*$^{-/-}$	WT	*Goat*$^{-/-}$
Body weight, g	23.7 ± 0.6	24.7 ± 1.0	15.9 ± 0.6	16.4 ± 0.5
Fat mass, % body weight	8.4 ± 0.3	8.6 ± 0.2	1.5 ± 0.2	1.5 ± 0.1
Lean mass, % body weight	74.2 ± 0.7	73.1 ± 0.7	78.1 ± 0.1	78.6 ± 0.2
Blood glucose, mg/dL	131 ± 4	133 ± 0.7	54 ± 10	12 ± 1***
Ghrelin, ng/mL	0.5 ± 0.1	<0.004***	8.7 ± 0.7	<0.004***
Des-acyl ghrelin, ng/mL	1.3 ± 0.1	4.0 ± 0.3***	15.7 ± 1.2	15.6 ± 2.0
Growth hormone, ng/dL	1.3 ± 0.6	1.4 ± 0.4	66 ± 8	34 ± 5***
IGF1, ng/mL	32 ± 3	37 ± 3	3.1 ± 1.5	1.1 ± 0.7
Free fatty acids, mM	0.44 ± 0.06	0.46 ± 0.05	0.07 ± 0.02	0.04 ± 0.02
β-hydroxybutyrate, mM	0.25 ± 0.02	0.27 ± 0.04	0.12 ± 0.01	0.10 ± 0.01
Insulin, ng/mL	0.38 ± 0.04	0.66 ± 0.09*	0.006 ± 0.005	0.016 ± 0.016
Glucagon, pg/mL	46 ± 7	59 ± 4	83 ± 16	106 ± 19
Corticosterone, ng/mL	158 ± 62	189 ± 45	225 ± 28	172 ± 23
Cortisol, pg/mL	531 ± 117	556 ± 128	844 ± 71	620 ± 73

8-wk-old male WT and *Goat*$^{-/-}$ littermates were fed *ad libitum* a chow diet or subjected to 60% calorie restriction for 7 days (same animals used for Fig. 2.). Each value represents the mean ± SEM of data from 6 mice. Measurements of blood glucose and plasma parameters were performed as described in *Materials and Methods*. Asterisks (*) denote the level of statistical significance (Student's *t* test) between WT and *Goat*$^{-/-}$ mice under the same *ad libitum* fed or calorie-restricted condition. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

SREBP-1c mRNA was reduced by 90% in both calorie restricted WT and *Goat*$^{-/-}$ groups with corresponding decreases in levels of mRNAs encoding lipogenic proteins, which are SREBP-1c targets. mRNA encoding two gluconeogenic genes, PEPCK and glucose-6-phosphatase, were elevated, which is consistent with the low insulin/glucagon ratio. IGFBP-1 mRNA was markedly elevated in the calorie-restricted livers, again consistent with the low insulin/high glucagon state. Despite the elevated plasma GH levels, mRNA encoding IGF1 was similarly reduced in the livers of both groups after calorie restriction.

Table 2-2. Comparison of mRNA amounts in livers of calorie restricted WT and *Goat*$^{-/-}$ mice.

	Ad libitum Fed		Calorie-restricted	
mRNA	WT	*Goat*$^{-/-}$	WT	*Goat*$^{-/-}$
SREBP pathway				
SREBP-1c	1.0	0.85	0.08	0.08
SREBP-2	1.0	1.07	0.48	0.38
Lipogenesis				
ATP citrate lyase	1.0	1.32	0.13	0.13
ACC-1	1.0	1.10	0.50	0.51
FAS	1.0	1.33	0.14	0.14
SCD-1	1.0	1.25	0.40	0.52
Gluconeogenesis				
PEPCK	1.0	1.08	4.46	4.78
G6Pase	1.0	1.05	2.84	3.82
Others				
IGF1	1.0	1.12	0.41	0.38
IGFBP-1	1.0	0.89	36.7	74.5
IGFBP-3	1.0	1.65	0.76	0.66

8-wk-old male WT and *Goat*$^{-/-}$ littermates were fed *ad libitum* a chow diet or subjected to 60% calorie restriction for 7 days and euthanized at 5:30 p.m. Pooled RNA from 4 to 6 mouse livers per group were quantified by real-time PCR as described in *Materials and Methods*. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was used as the invariant control and each value represents the mRNA concentration relative to *ad libitum* fed WT mice, which is arbitrarily defined as 1.0. Primers for real-time PCR are described in *Materials and Methods*. SREBP, sterol regulatory element-binding protein; ACC-1, acetyl-CoA carboxylase-1; FAS, fatty acid synthase; SCD-1, stearoyl-CoA desturase-1; PEPCK, phosphoenolpyruvate carboxylase; G6Pase, glucose-6-phosphatase.

Fasting Blood Glucose and Plasma Ghrelin Levels in *Goat*$^{-/-}$ Mice during Calorie Restriction

To better understand the metabolic changes during calorie restriction, 8-week-old male WT and *Goat*$^{-/-}$ littermates were subjected to 60% calorie restriction for 8 days. At 5:30 p.m. on the indicated days, 30 minutes before feeding, blood was collected from the tail vein of each mouse and assayed for blood glucose and plasma GH levels. As seen in previous experiments, blood glucose levels dropped in both WT and *Goat*$^{-/-}$ groups during the first 4 days of calorie restriction. Afterwards, blood glucose levels in WT mice stabilized but continued to decline in

the *Goat*$^{-/-}$ mice (Fig. 2-4*A*). Plasma ghrelin levels rose linearly in the WT mice (Fig. 2-4*B*) but, as expected, were undetectable in *Goat*$^{-/-}$ mice. Both groups of mice showed increase in plasma des-acyl ghrelin (Fig. 2-4*C*). Plasma GH levels rose moderately in both groups up through day 4 (Fig. 2-4*D*). Subsequently, and synchronously with the stabilization of their blood glucose levels, plasma GH levels in the WT mice started rising much more rapidly than their *Goat*$^{-/-}$ littermates. By day 8, fasting plasma levels of GH in the WT mice were three times higher than those in the *Goat*$^{-/-}$ mice.

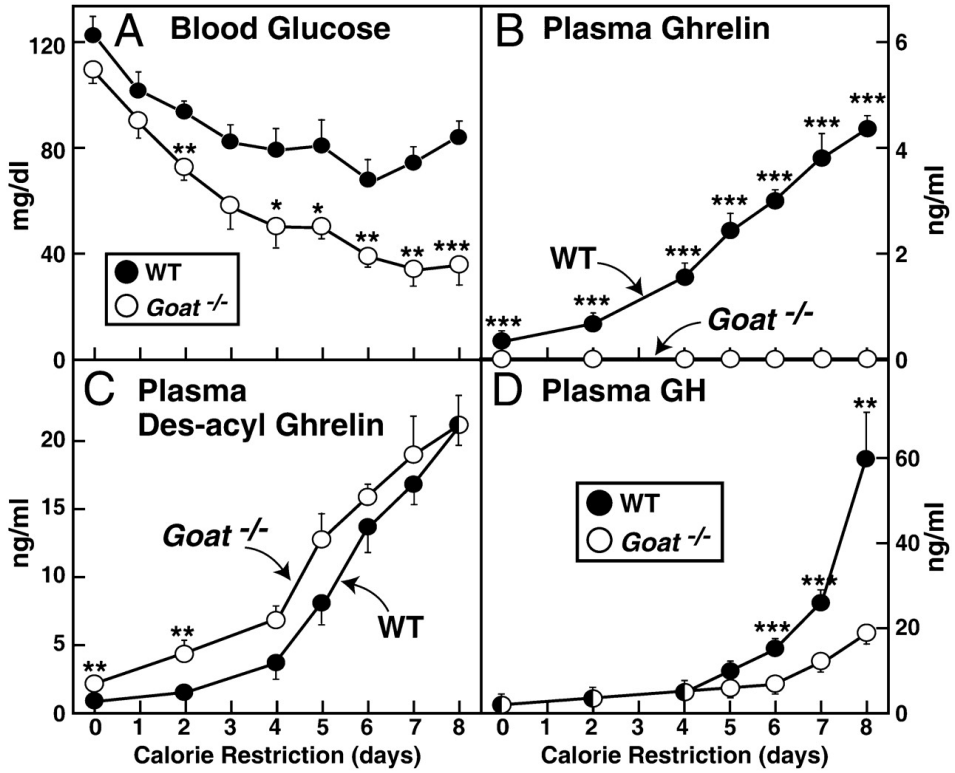

Figure 2-4. Different responses in blood glucose and GH levels in WT and *Goat*$^{-/-}$ mice during 60% calorie restriction. 8-wk-old male WT and *Goat*$^{-/-}$ littermates were subjected to 60% calorie restriction as described in *Materials and Methods*. At 5:30 p.m. on the indicated days, the concentrations of blood glucose (*A*), plasma ghrelin (*B*), plasma des-acyl ghrelin (*C*), and plasma GH (*D*) were measured. Each value represents the mean ± SEM of data from 5 mice. Asterisks (*) denote the level of statistical significance (Student's *t* test) between WT and *Goat*$^{-/-}$ mice. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Prevention of Hypoglycemia in *Goat*$^{-/-}$ Mice during Calorie Restriction by Infusion of Ghrelin

To examine the role of ghrelin in stabilizing blood glucose levels in WT vs. *Goat*$^{-/-}$ mice during calorie restriction, an osmotic pump delivering ghrelin or saline was implanted in 8-week-old male WT and *Goat*$^{-/-}$ littermates 3 days before they were subjected to 60% calorie restriction. In WT mice, infusion of ghrelin had no effect on blood glucose versus the infusion of saline (Fig. 2-5*A*). In *Goat*$^{-/-}$ mice, infusion of saline resulted in the expected fasting hypoglycemia. However, infusion of ghrelin restored blood glucose levels to those seen in WT mice (Fig. 2-5*B*). After 7 days of calorie restriction, plasma hormone levels were measured at 5:30 p.m. Both WT and *Goat*$^{-/-}$ mice showed similar elevations of plasma ghrelin and des-acyl ghrelin (Fig. 2-5*C* and *D*). More importantly, infusion of ghrelin to *Goat*$^{-/-}$ mice normalized their plasma GH level (Fig. 2-5*E*). The restoration of GH did not, however, result in a rise in plasma IGF1 (WT, 2.2 ± 0.8 ng/mL; *Goat*$^{-/-}$, 2.6 ± 0.9 ng/mL; n=6 mice per group). Plasma levels of free fatty acids (WT, 0.22 ± 0.04 mM; *Goat*$^{-/-}$, 0.23 ± 0.09 mM) and β-hydroxybutyrate (WT, 0.34 ± 0.10 mM; *Goat*$^{-/-}$, 0.35 ± 0.13 mM) remained low.

Prevention of Hypoglycemia in *Goat*$^{-/-}$ Mice during Calorie Restriction by Infusion of GH

The ability of ghrelin infusion to restore blood glucose levels in *Goat*$^{-/-}$ mice during calorie restriction suggests that, due to the absence of ghrelin, *Goat*$^{-/-}$ mice develop hypoglycemia because they fail to maximally stimulate the release of plasma GH, a hormone known to counteract hypoglycemia (15). To examine the role of GH in stabilizing blood glucose levels in WT vs. *Goat*$^{-/-}$ mice during calorie restriction, an osmotic pump delivering GH or its vehicle was implanted in 8-week-old male WT and *Goat*$^{-/-}$ littermates 3 days before they were subjected to 60% calorie restriction. In WT mice, infusion of GH had no effect on their blood glucose as

compared to infusion of vehicle (Fig. 2-6*A*). In *Goat*$^{-/-}$ mice, infusion of vehicle resulted in the expected fasting hypoglycemia. However, infusion of GH restored blood glucose levels to those seen in WT mice (Fig. 2-6*B*). After 8 days of calorie restriction, plasma hormone levels were measured at 5:30 p.m. Infusion of GH did not significantly affect plasma ghrelin (Fig. 2-6*C*) or plasma des-acyl ghrelin (Fig. 2-6*D*) levels. Importantly, infusion of GH resulted in similar elevations of plasma GH in WT and *Goat*$^{-/-}$ mice (Fig. 2-6*E*). As with ghrelin infusion, the infusion of GH did not result in a rise in plasma IGF1 (WT, 2.2 ± 1.0 ng/mL; *Goat*$^{-/-}$, 2.4 ± 1.0 ng/mL; *n*=5 mice per group).

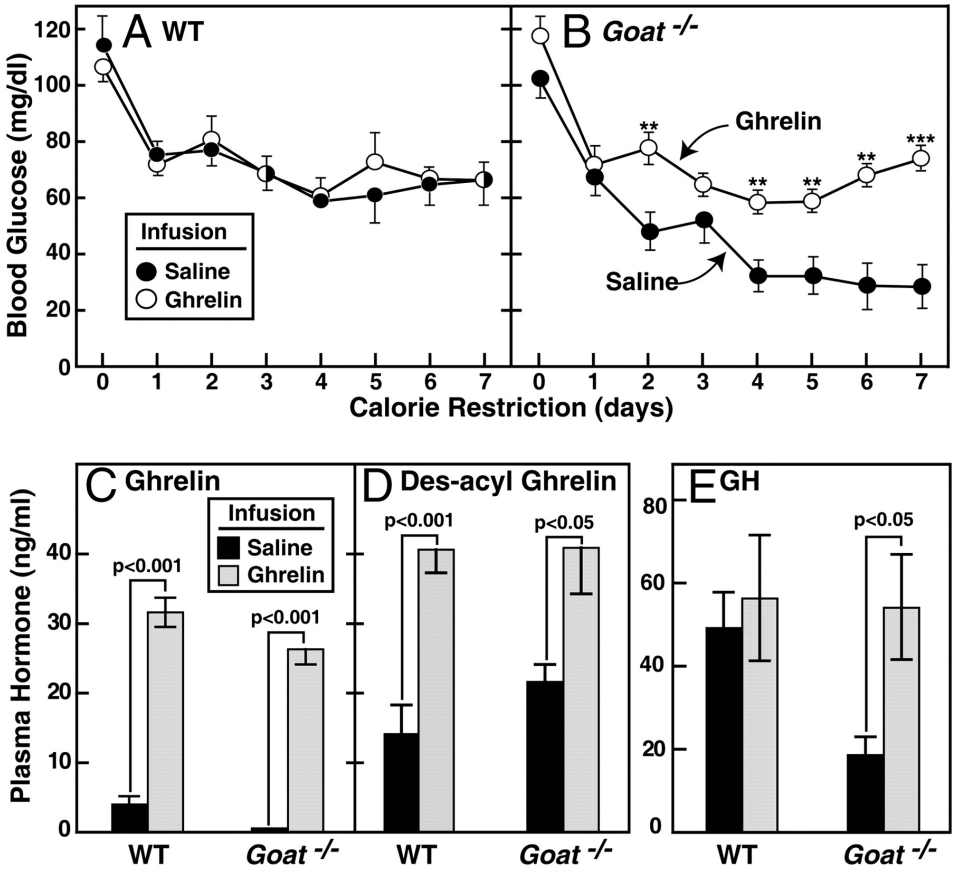

Figure 2-5. Response to 60% calorie restriction by WT and *Goat*$^{-/-}$ mice undergoing continuous subcutaneous infusion of saline or ghrelin. Three days prior to the initiation of 60% calorie restriction, 8-wk-old male WT and *Goat*$^{-/-}$ littermates were implanted subcutaneously with Alzet osmotic pumps delivering saline (●) or saline containing 5 mg/ml ghrelin (○) as described in *Materials and Methods*. During calorie restriction, blood glucose (*A* and *B*) was measured daily at 5:30 p.m. (30 minutes before

feeding). (*C* – *E*) On the seventh day of calorie restriction, mice were euthanized at 5:30 p.m. (30 minutes before feeding) to collect plasma for the measurement of plasma ghrelin (*C*), plasma des-acyl ghrelin (*D*), and GH (*E*). Each value represents the mean ± SEM of data from 6 mice. Asterisks (*) denote the level of statistical significance (Student's *t* test) between WT and *Goat*$^{-/-}$ mice infused with saline or ghrelin. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

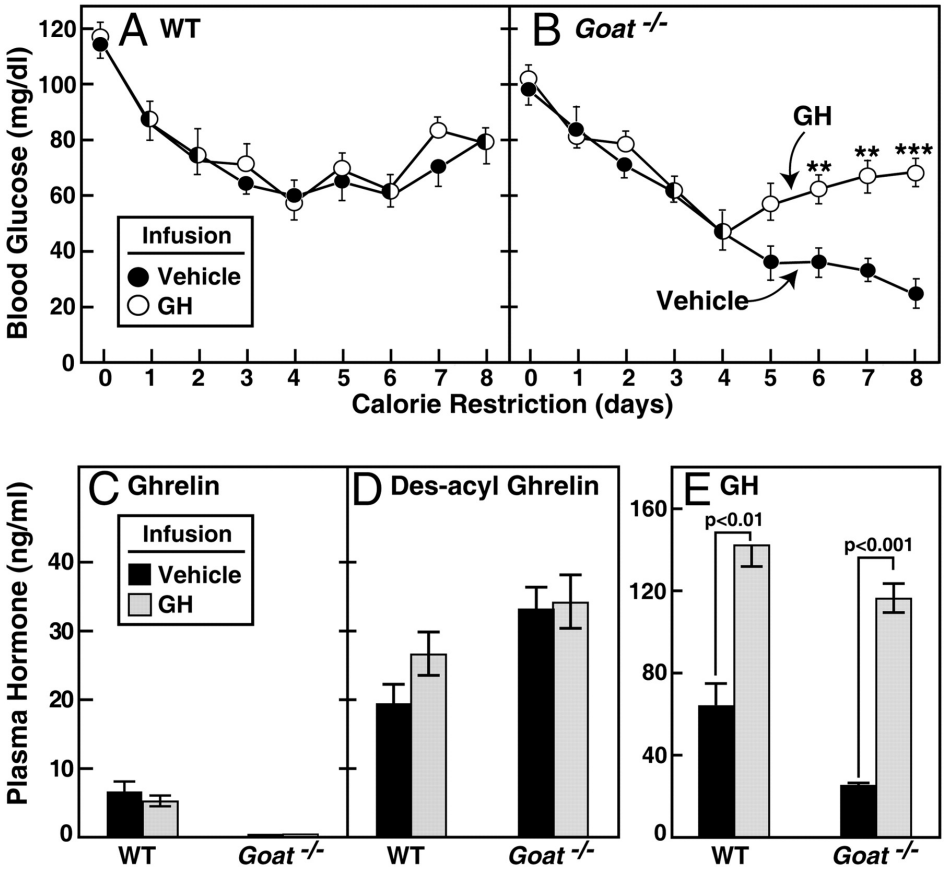

Figure 2-6. Response to 60% calorie restriction by WT and *Goat*$^{-/-}$ mice undergoing continuous subcutaneous infusion of vehicle or GH. Three days prior to the initiation of 60% calorie restriction, 8-wk-old male WT and *Goat*$^{-/-}$ littermates were implanted subcutaneously with Alzet osmotic pumps delivering vehicle (●) or vehicle containing 2.5 mg/ml GH (○) as described in *Materials and Methods*. During calorie restriction, blood glucose (*A* and *B*) was measured daily at 5:30 p.m. (30 minutes before feeding). (*C* – *E*) On the seventh day of calorie restriction, mice were euthanized at 5:30 p.m. (30 minutes before feeding) to collect plasma for the measurement of plasma ghrelin (*C*), plasma des-acyl ghrelin (*D*), and GH (*E*). Each value represents the mean ± SEM of data from 5 mice. Asterisks (*) denote the level of statistical significance (Student's *t* test) between WT and *Goat*$^{-/-}$ mice infused with vehicle or GH. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Oral Glucose Tolerance of *Goat*$^{-/-}$ Mice after Fasting or 60% Calorie Restriction

Compared to their WT littermates, chow-fed *Goat*$^{-/-}$ mice had modestly enhanced oral glucose tolerance (Fig. 2-7*A*), which was associated with a slight but significant increase in insulin release at the 30-min time point (Fig. 2-7*C*). Oral glucose tolerance was worse in both WT and *Goat*$^{-/-}$ groups after 8 weeks of high fat feeding but *Goat*$^{-/-}$ mice still showed an improvement in oral glucose tolerance compared to their WT littermates (Fig. 2-7*B*). There was a marked elevation in insulin levels in the *Goat*$^{-/-}$ mice at the 30-min time point (2-fold) (Fig. 2-7*D*). These results are consistent with previous studies in mice lacking ghrelin (16).

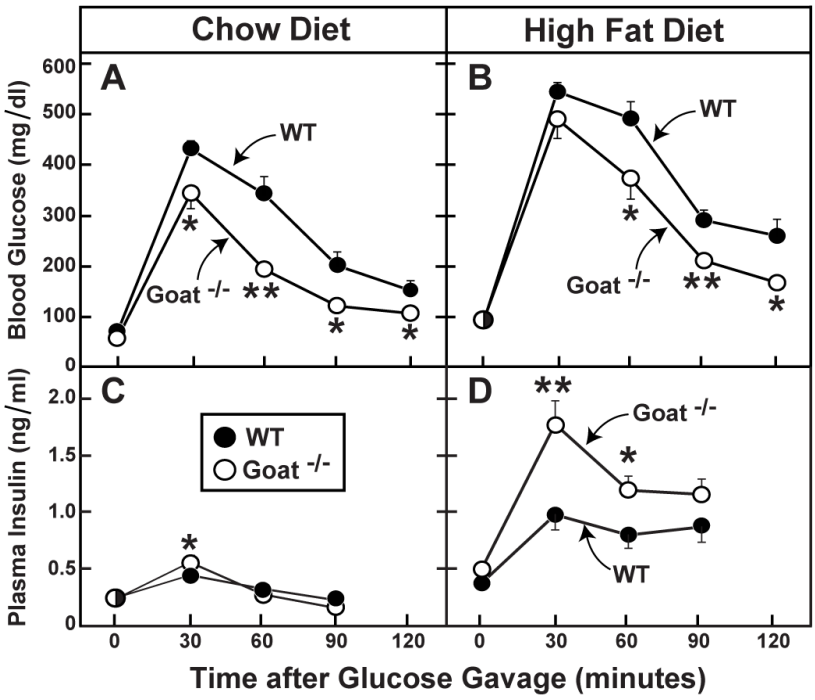

Fig. 2-7. Oral glucose tolerance of WT and *Goat*$^{-/-}$ mice fed a chow or high fat diet. 4-wk-old male WT and *Goat*$^{-/-}$ littermates were fed *ad libitum* either chow (*A* and *C*) or high fat diet (*B* and *D*) for 8 weeks. Mice were fasted 16 h prior to the start of the experiment (5 p.m. to 9 a.m.) and then orally gavaged with 25% D-Glucose (2.5 mg/g body weight) as described in *Materials and Methods*. Blood samples were taken from the tail vein at the indicated times for blood glucose and plasma insulin measurement. Each value represents the mean ± SEM of data from 6 to 8 mice. Asterisks (*) denote the level of statistical significance (Student's *t* test) between WT and *Goat*$^{-/-}$ mice. *, $P < 0.05$; **, $P < 0.01$.

After being subjected to 60% calorie restriction for 5 days, *Goat*$^{-/-}$ mice continued to show improved oral glucose tolerance compared to their WT littermates (Fig. 2-8) with blood glucose levels significantly lower at 15 and 30 min after glucose gavage.

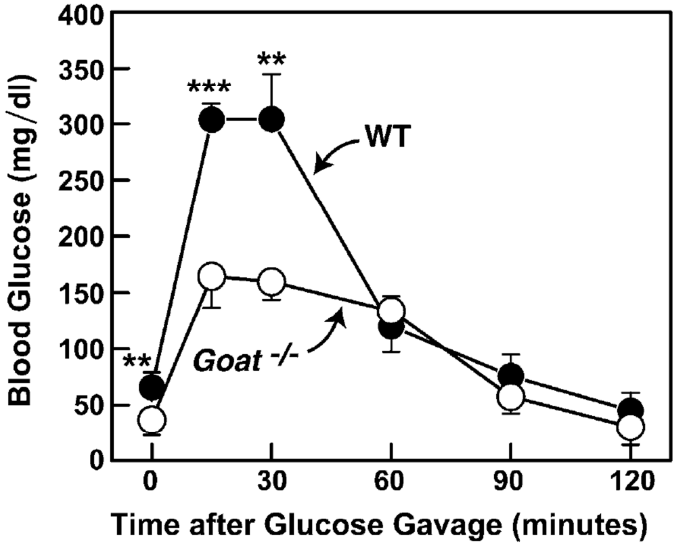

Fig. 2-8. Oral glucose tolerance of WT and *Goat*$^{-/-}$ mice subjected to 5 days of 60% calorie restriction. 8-wk-old male WT and *Goat*$^{-/-}$ littermates were placed under 60% calorie restriction as described in *Materials and Methods*. At 5 p.m. on the fifth day of calorie restriction, WT and *Goat*$^{-/-}$ mice were orally gavaged with 25% D-Glucose (2.5 mg/g body weight) as described in *Materials and Methods*. Blood samples were taken from the tail vein at the indicated times for blood glucose and plasma insulin measurement. Each value represents the mean ± SEM of data from 5 mice. Asterisks (*) denote the level of statistical significance (Student's *t* test) between WT and *Goat*$^{-/-}$ mice. **, $P < 0.01$; ***, $P < 0.001$.

Discussion

The data show that an essential function of ghrelin is to elevate plasma GH levels during periods of severe caloric restriction to ensure sufficient blood glucose to permit survival. After 4 days of 60% calorie restriction, during which WT and *Goat*$^{-/-}$ mice lost similar amounts of body weight (30%) and body fat (75%) (Fig. 2-2), WT mice continued to maintain their blood glucose at levels (58–76 mg/dL) sufficient for viability while the *Goat*$^{-/-}$ mice exhibited increasingly severe fasting hypoglycemia with blood glucose levels reaching 12–36 mg/dL. After 7 days of calorie restriction, *Goat*$^{-/-}$ mice became moribund and had to be euthanized.

Plasma ghrelin levels during calorie restriction progressively rose in WT mice, reaching levels 12- to 18-fold higher than levels seen in *ad libitum* fed mice after 7 days (Fig. 2-3 and 2-4). *Goat*$^{-/-}$ mice had no detectable ghrelin as expected. Both WT and *Goat*$^{-/-}$ mice showed large, increases in plasma des-acyl ghrelin (Figs. 2-3 and 2-4). Interestingly, ghrelin deficiency had no effect on food intake in *Goat*$^{-/-}$ mice as they ate all the given food within 1 h of delivery, thus showing that ghrelin is not essential for hunger during periods of starvation.

It is important to note that the hypoglycemia seen in *Goat*$^{-/-}$ mice during calorie restriction was only observed at 5:30 p.m., 30 minutes before feeding and approximately 22.5 h after the last feeding. Thus, the blood glucose levels reflect both long term calorie restriction and short term fasting. When blood glucose was measured at 9 a.m. on day 7 of calorie restriction, WT and *Goat*$^{-/-}$ mice had similar levels (76 ± 6.2 and 65 ± 6.5 mg/dL, respectively, n=10 mice per group). At 9 a.m., liver glycogen levels were similar in both groups (17 ± 0.3 and 18 ± 1.0 µg/mg tissue, respectively, n=5 mice per group). At 5:30 p.m., liver glycogen levels fell, but were still similar in both groups (3.6 ± 0.7 and 2.0 ± 0.6 µg/mg tissue, respectively, n=23 mice per group). In *Goat*$^{-/-}$ mice fed *ad libitum* and fasted for 24 h, hypoglycemia did not develop. Thus, these

data reveal that the hypoglycemia seen in *Goat*$^{-/-}$ mice develops as a result of both: (*i*) severe prolonged calorie restriction leading to a depletion of nearly all body fat stores and (*ii*) acute food deprivation leading to a loss of liver glycogen.

The hypoglycemia seen in *Goat*$^{-/-}$ mice during calorie restriction was not caused by excess insulin or a deficiency in glucagon. At 5:30 p.m. on day 7 of calorie restriction (Table 2-1), insulin was barely detectable and glucagon in both WT and *Goat*$^{-/-}$ mice were at the same elevated level. Liver responses to the low insulin/high glucagon levels were as expected, at least transcriptionally, resulting in the upregulation of mRNAs for gluconeogenic enzymes and downregulation of mRNAs for lipogenic enzymes (Table 2-2).

An oral glucose tolerance test performed after 5 days of calorie restriction showed a dramatically improved glucose tolerance in the *Goat*$^{-/-}$ mice vs. their WT littermates (Fig. 2-8). Glucose levels in the WT mice reached a maximum of 300 mg/dL whereas levels in *Goat*$^{-/-}$ mice attained only 150 mg/dL ($p < 0.001$). These data suggest that the *Goat*$^{-/-}$ mice have an increased glucose disposal rate and their livers cannot compensate by increased gluconeogenesis. Whether the failure to increase blood glucose levels is due to downregulation of enzymes in the gluconeogenic pathway or the depletion of gluconeogenic substrates is a question that requires further study.

The hypoglycemia seen in *Goat*$^{-/-}$ mice during calorie restriction is associated with a relative, but not absolute, deficiency in plasma GH levels (Figs. 2-4, Table 2-1). On day 5 of calorie restriction, GH levels begin to rise in both WT and *Goat*$^{-/-}$ mice (Fig. 2-4*D*). However, GH rises much more dramatically in WT mice and is coincident with the stabilization of their blood glucose. These data indicate that while other signals can raise GH levels in calorie restricted mice, ghrelin is essential for the maximal stimulation of GH release in order to prevent

hypoglycemia and death. When ghrelin is infused back to the *Goat*$^{-/-}$ mice, GH levels are restored during calorie restriction and blood glucose is normalized (Fig. 2-5). More importantly, when GH itself is directly infused back to the *Goat*$^{-/-}$ mice, the restoration of GH resulted in the rescue of the hypoglycemia (Fig. 2-6). It should be noted that plasma GH levels are not reduced in *Goat*$^{-/-}$ mice when fed a chow diet (Table 2-1). Therefore, ghrelin appears to be required for GH secretion only when demanded by conditions of severe calorie restriction. This conclusion supports the finding that *Goat*$^{-/-}$ mice show normal growth and previous findings in which *Preproghrelin*$^{-/-}$ mice also exhibit normal growth (4-6).

IGF1 levels remained low in the calorie restricted animals despite significant rises in plasma GH levels (Table 2-1) and remained low despite infusion of GH to either WT or *Goat*$^{-/-}$ mice. Thus, the ability of GH to maintain blood glucose levels during calorie restriction is independent of IGF1 action, a finding that makes sense since IGF1 typically acts to lower, not raise, glucose.

Normally, the ability of GH to maintain blood sugar is thought to be from increased lipolysis in adipose tissue to release fatty acids that spare glucose utilization in muscle (15). This mechanism is unlikely to occur in the calorie restricted mice because, after 4 days of calorie restriction, the mice have very little adipose tissue from which to release fatty acids. Plasma free fatty acid and β-hydroxybutyrate levels were similar and low in both calorie restricted WT and *Goat*$^{-/-}$ mice (Table 1), and were not elevated by infusion of ghrelin (mean levels, 0.23 ± 0.08 mM) or GH (mean levels, 0.20 ± 0.06 mM).

GOAT is essential for survival in times of famine in mice but appears to not be essential for energy storage in times of plenty. When fed a high fat diet, *Goat*$^{-/-}$ mice were not different from WT littermates in terms of either body weight or fat mass (Fig. 1). These findings are generally consistent with previous findings in mice lacking ghrelin or ghrelin receptor (4-7).. Thus, ghrelin

is a hormone that promotes survival in times of famine by maintaining adequate levels of blood glucose.

A previous study by Sun et al. (6) in which ghrelin and ghrelin receptor knockout mice were subjected to 50% calorie restriction showed slight, but inconsistent hypoglycemia. In contrast, the hypoglycemia seen in *Goat*$^{-/-}$ mice after 60% calorie restriction was much more profound. This could be because the mice used by Sun et al. retained some body fat, as we have found that hypoglycemia during calorie restriction does not occur until body fat has declined below 2% of body mass. In spite of the differences, the conclusion reached by Sun et al. is similar to ours, namely that a major action of ghrelin is to maintain blood sugar levels in the undernourished state. Cummings et al. have also advanced a similar hypothesis (17).

There is an interesting parallel between the calorie restricted mice and humans with calorie restriction due to anorexia nervosa. Anorexic humans, like calorie restricted mice, have elevated ghrelin and GH levels (18) as well as reduced IGF1 levels. It is possible that, like the mice, the maintenance of blood glucose in anorexic humans is due to the maximal stimulation of GH by elevated levels of ghrelin.

Materials and Methods

Mice. Approval for all animal experiments was obtained from the Institutional Animal Care and Research Advisory Committee at the University of Texas Southwestern Medical Center at Dallas. Mice were placed on a 12-h light/12-h dark cycle with the dark cycle beginning at 9:00 or 10:00 p.m. Standard colony cages were used and the chow diet used was Teklad Mouse/Rat Diet 7002 from Harlan Teklad Global Diets (3.0 kcal/g metabolizable energy, 18% of calories from fat, 49% from carbohydrates, and 33% from protein).

High Fat Diet. The high fat diet used was obtained from Research Diets (Cat. No. D12451) and consisted of 24% (w/w) fat, 41% (w/w) carbohydrates, and 24% (w/w) protein. 45% of the total metabolizable energy was from fat.

Metabolic Parameters. After anesthetization with isoflurane, mice were bled from the retro-orbital sinus and blood collected in EDTA-coated microfuge tubes containing p-hydroxymercuribenzoic acid (PMSF; final concentration, 1 mM). The plasma was separated by centrifugation and stored immediately at –80°C. Samples used for the measurement of ghrelin and IGF1 were pretreated with HCl (final concentration , 0.1 M) or MIX diluent (Assaypro), respectively, before freezing. Ghrelin and des-acyl ghrelin were measured using specific EIA kits from Cayman Chemical (Cat. No. A05117 and A05118). Plasma concentrations of the following compounds were measured using commercial kits from the indicated vendor in parentheses: growth hormone (Cayman Chemical), corticosterone (Cayman Chemical), cortisol (Cayman Chemical), free fatty acids (Wako Chemicals), β-hydroxybutyrate (Pointe Scientific), IGF1 (Assaypro), glucagon (Millipore), and glycogen (Biovision). The measurement of plasma glucose, cholesterol, and triglycerides was performed by the Mouse Metabolic Phenotyping Core at University of Texas Southwestern using the Vitros 250 Chemistry Analyzer (Ortho Clinical Diagnostics).

Calorie Restriction and Infusion of Hormones. 8-wk-old male WT and *Goat*$^{-/-}$ littermates were separated and individually housed one week before the start of calorie restriction. The mice were given chow diet *ad libitum* and daily food intake was measured. The mice were then randomly placed into two groups: a control group that continued to receive food *ad libitum* and a 60% calorie restricted group. Mice in the 60% calorie restricted group were fed such that at 6

p.m., each mouse received a food pellet equal to 40% by weight of its average daily food intake measured in the previous week.

During calorie restriction, daily measurements of body weight and blood glucose were performed 30 minutes before feeding at 5:30 p.m.; total body fat was measured by NMR (Bruker Minispec mq7.5) every 2 to 3 days at 5 p.m.

On the seventh or eighth day of calorie restriction, mice were euthanized with isoflurane at 5:30 p.m. to collect blood and tissues for analysis.

The infusion of recombinant ghrelin or GH into calorie-restricted mice was performed as follows. Three-days before calorie restriction, mice were anesthetized and implanted with an Alzet osmotic pump (Cat. No. 1002; DURECT) subcutaneously in the interscapular region. For ghrelin infusion, the pump contained either saline or saline with 5 mg/ml rat recombinant ghrelin (GenScript; Cat. No. RP10781) and delivered at a rate of 0.25 µl/h (30 µg/24 h). For GH infusion, the pump contained either vehicle (70 mM sodium bicarbonate, pH 9.5, 137 mM NaCl, and 100 mg/mL rat albumin) or vehicle with 2.5 mg/mL recombinant rat GH (National Hormone and Peptide Program at the National Institute of Diabetes and Digestive and Kidney Diseases through A.F. Parlow) and delivered at a rate of 0.25 µl/h (15 µg/24 h).

Quantitative Real-Time PCR. Total RNA was extracted from various tissues from 4 to 6 mice per group and real-time PCR measurements were made on pooled RNA samples using the primers described (9, 19, 20) except for the following: IGF1 (NM_010512): 5'-CCACACTGACATGCCCAAGA-3' and 5'-TCCTTTGCAGCTTCGTTTTCT-3'; IGFBP-3 (NM_008343): 5'-GAGTGTGGAAAGCCAGGTTGTC-3' and 5'-GCATGGAGTGGATGGAACTTG-3'. PCR reactions were performed in triplicate and the mean range of variation for all values was 0.40±0.03%. Cyclophilin or GAPDH mRNA was used as

the invariant control to calculate the relative amount of all mRNAs using the comparative threshold cycle (C_T) method.

Generation of *Goat*$^{-/-}$ Mice. Mouse ES cells containing a disrupted *Goat* allele were generated using VelociGene technology by Regeneron Pharmaceuticals. The entire GOAT coding region was replaced on a bacterial artificial chromosome (BAC)-based targeting vector by a gene encoding *lac*Z and a neomycin-selectable marker. Hybrid C57BL6/129SvEv F1 (VGF1) ES cells were electroporated with this vector and correctly targeted ES clones were identified using a quantitative PCR assay as described.

Genotyping was performed using PCR with the following primers: 5'-TGCATCTTGGACACCTTTTCC-3' (F1), 5'-GCGCGTTCCACCCTATTACTG-3' (R1), 5'-GGTAAACTGGCTCGGATTAGGG-3' (F2), and 5'-TTGACTGTAGCGGCTGATGTTG-3' (R2). F1 and R1 specifically amplifies a 93-bp fragment from the wild-type *Goat* gene. F2 and R2 specifically amplifies a 210-bp fragment from the *lac*Z gene.

Three independent, correctly targeted, ES clones from Regeneron were injected into C57BL/6J blastocysts by the Transgenic Core Facility at University of Texas Southwestern. Chimeric males with >80% *agouti* coat color were obtained from all three ES clones. After the establishment of germ-line transmission, N2 *Goat*$^{+/-}$ mice were generated by backcrossing to C57BL/6J. These mice were then crossed with each other to generate N2F2 *Goat*$^{-/-}$ mice along with their wild-type littermates. All experiments were carried out in these N2F2 mice which contain genes from both the C57BL/6J and 129SvEv parental strains.

Oral Glucose Tolerance Test. After fasting for 16 h or calorie-restriction for 5 days, mice were orally gavaged with 25% D-glucose in water (2.5 mg/g body weight) with animal feeding

needles (Popper and Sons; Cat. No. 9921-20 × 1/1/2). The tail vein was bled to obtain blood samples for the measurement of blood glucose (Bayer Contour Glucometer) and plasma insulin (Crystal Chemicals; Ultra Sensitive Insulin ELISA kit).

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Chapter III: Fasting-induced Hypoglycemia in the Setting of Calorie Restriction and Ghrelin Deficiency Due to Shortage of Gluconeogenic Substrates

Introduction

Ghrelin, a 28-amino acid peptide hormone secreted by the stomach, is uniquely modified by *O*-acylation of octanoate to serine-3. Octanoylation of ghrelin is essential for the ability of ghrelin to stimulate the release of GH by the pituitary (1, 2). Recently, our lab (3) and others (4), identified ghrelin *O*-acyltransferase (GOAT) as the enzyme responsible for the transfer of octanoate from octanoyl-CoA to serine-3 of ghrelin. Mice deficient for GOAT ($Goat^{-/-}$) have no detectable ghrelin (2) but have normal or increased levels of des-acyl ghrelin. When food intake is reduced to 40% of normal levels (60% calorie restriction), both WT and $Goat^{-/-}$ mice show similar drops in body weight (30%), fat mass (60%), and blood glucose (to 60 mg/dL) over the first 3 days. Afterwards, blood glucose levels in WT mice stabilized at 58–76 mg/dL and the mice behaved normally over the next 4 d. In contrast, blood glucose levels in the $Goat^{-/-}$ mice failed to stabilize and progressively dropped until they reached 12–36 mg/dL after 7 d. At this point, $Goat^{-/-}$ mice became moribund and had to be euthanized. Stabilization of glucose levels in WT mice was associated with a progressive rise in plasma ghrelin levels and a consequent rise in plasma growth hormone. $Goat^{-/-}$ mice, which lack detectable ghrelin, exhibited significantly lower increases in growth hormone during calorie restriction. When ghrelin or growth hormone was infused back to calorie-restricted $Goat^{-/-}$ mice, hypoglycemia did not develop (2).

Growth hormone is secreted by the pituitary primarily as a 22 kDa polypeptide of 191 amino acids (5, 6). Two hormonal pathways stimulate the secretion of GH: the classic growth hormone releasing hormone (GHRH) pathway, and the more recently discovered ghrelin pathway in which ghrelin from the stomach directly binds to growth hormone secretagogue receptors (GHS-

R) in the pituitary to stimulate GH release (1). The effects of growth hormone vary depending on the nutritional state during which it acts. Given sufficient nutrients and elevated portal insulin levels, GH stimulates IGF-1 production in liver (7) and acts through IGF-1 to exert its protein anabolic effects (8). During times of food shortage, the IGF-1 independent effects of GH become more prominent. These are primarily the stimulation of lipolysis (9, 10) and the prevention of muscle protein breakdown (10-13).

Hypoglycemia is commonly found in GH deficient patients (14, 15) and is rescued with GH replacement therapy (16-20). The mechanisms by which GH acts to restore blood glucose are controversial and include: (*i*) increasing the rate of lipolysis to correct either hypoketonemia (20) or gluconeogenic substrate deficiency (primarily alanine and glutamine) (19), (*ii*) restoration of a deficiency in the rate of glucose production (16), and (*iii*) resetting the homeostatic balance between insulin vs. glucose and free fatty acids to a higher level of blood glucose (17).

In an attempt to explain the protective role of ghrelin and GH against hypoglycemia during calorie restriction, we sought to define the changes in plasma parameters of various hormones and gluconeogenic substrates during the terminal stages of calorie restriction when *Goat* $^{-/-}$ mice exhibited severe hypoglycemia. We found that *Goat* $^{-/-}$ mice started to develop hypoglycemia at 2 p.m. on day 8 of calorie restriction, 20 h after the last meal, which worsened until feeding. Coincident with the drop in blood glucose levels, plasma levels of two gluconeogenic substrates, pyruvate and lactate, also dropped in calorie restricted *Goat* $^{-/-}$ mice. This drop was correlated in time and magnitude to a rise in the levels of ghrelin and GH in calorie restricted WT mice. These findings were confirmed in *Preproghrelin* $^{-/-}$ mice. Injections of exogenous gluconeogenic substrate to *Goat* $^{-/-}$ and *Preproghrelin* $^{-/-}$ mice were protective against the development of hypoglycemia, as were injections of exogenous octanoate. These data suggest that the function of

the ghrelin-mediated rise in GH during calorie restriction is to preserve gluconeogenic substrate to maintain an adequate rate of gluconeogenesis.

Results

Calorie Restriction of *Preproghrelin*$^{-/-}$ Mice

Previous studies of mice genetically modified to lack Ghrelin *O*-acyltransferase (GOAT) demonstrated an essential role of ghrelin in the preservation of blood glucose levels during conditions of severe calorie restriction (2). Because *Goat*$^{-/-}$ mice lack only the octanolyated form of ghrelin and, in fact, have higher levels of circulating des-acyl ghrelin than their WT littermates, it is possible that des-acyl ghrelin may also play a role in the development of fasting hypoglycemia during calorie restriction.

To test this hypothesis, we obtained genetically engineered *Preproghrelin*$^{-/-}$ mice which lack both the octanoylated and des-acylated forms of ghrelin and subjected them, along with their WT littermates, to the same 60% calorie restriction that we used previously for the *Goat*$^{-/-}$ mice (2). Briefly, individually housed 8-wk-old male *Preproghrelin*$^{-/-}$ mice and their WT littermates were fed 40% of their measured daily *ad libitum* food intake. One pre-measured pellet was given at 6 p.m. each day (3 h before the beginning of the dark cycle). Both WT and *Preproghrelin*$^{-/-}$ mice ate the food within 1 h after feeding, a pattern that continued throughout the experiment. A separate group of *Preproghrelin*$^{-/-}$ mice and their WT littermates were fed a chow diet *ad libitum* as a control.

Both WT and *Preproghrelin*$^{-/-}$ mice lost approximately 30% of their body weight during the first 4 days of calorie restriction, after which their body weights stabilized (Fig. 3-1*A*). Fasting blood glucose was measured at 5:30 p.m. each day, 30 minutes before feeding (Fig. 3-1*B*). During the first two days of calorie restriction, both WT and *Preproghrelin*$^{-/-}$ mice showed a

similar initial decline in fasting blood glucose. In the following days, fasting blood glucose in the WT mice stabilized at a range of 55–70 mg/dL through 9 days of calorie restriction. *Preproghrelin* $^{-/-}$ mice, on the other hand, failed to stabilize their fasting blood glucose levels, which continued to decline until they reached 30 mg/dL on day 9. The *Preproghrelin* $^{-/-}$ mice were lethargic and nearly moribund at this point whereas their WT littermates appeared healthy and active.

Preproghrelin $^{-/-}$ mice and their WT littermates were euthanized at 5:30 p.m. after 9 days of calorie restriction to obtain plasma for hormone measurements. Plasma ghrelin (Fig. 3-1*C*) and des-acyl ghrelin (Fig. 3-1*D*) increased in WT mice under calorie-restriction compared to *ad libitum* feeding (ghrelin, 0.6 ± 0.1 to 2.7 ± 0.9 ng/dL; des-acyl ghrelin, 0.7 ± 0.2 to 6.1 ± 1.3 ng/dL). As expected, neither ghrelin nor des-acyl ghrelin were detectable in *Preproghrelin* $^{-/-}$ mice on either diet. Plasma GH levels were similarly low in WT and *Preproghrelin* $^{-/-}$ mice under *ad libitum* fed conditions (5.2 ± 1.4 and 4.1 ± 1.7 ng/mL, respectively) (Fig. 3-1*E*). After 9 days of calorie restriction, plasma GH levels rose in WT and *Preproghrelin* $^{-/-}$ mice, but were 2.4-fold higher in the WT group (43.5 ± 4.6 ng/dL vs. 17.8 ± 2.7 ng/dL). These data are consistent with our previous data in calorie restricted *Goat* $^{-/-}$ mice (2), and suggest that the rise in plasma GH levels during calorie restriction is mediated by ghrelin and not des-acyl ghrelin.

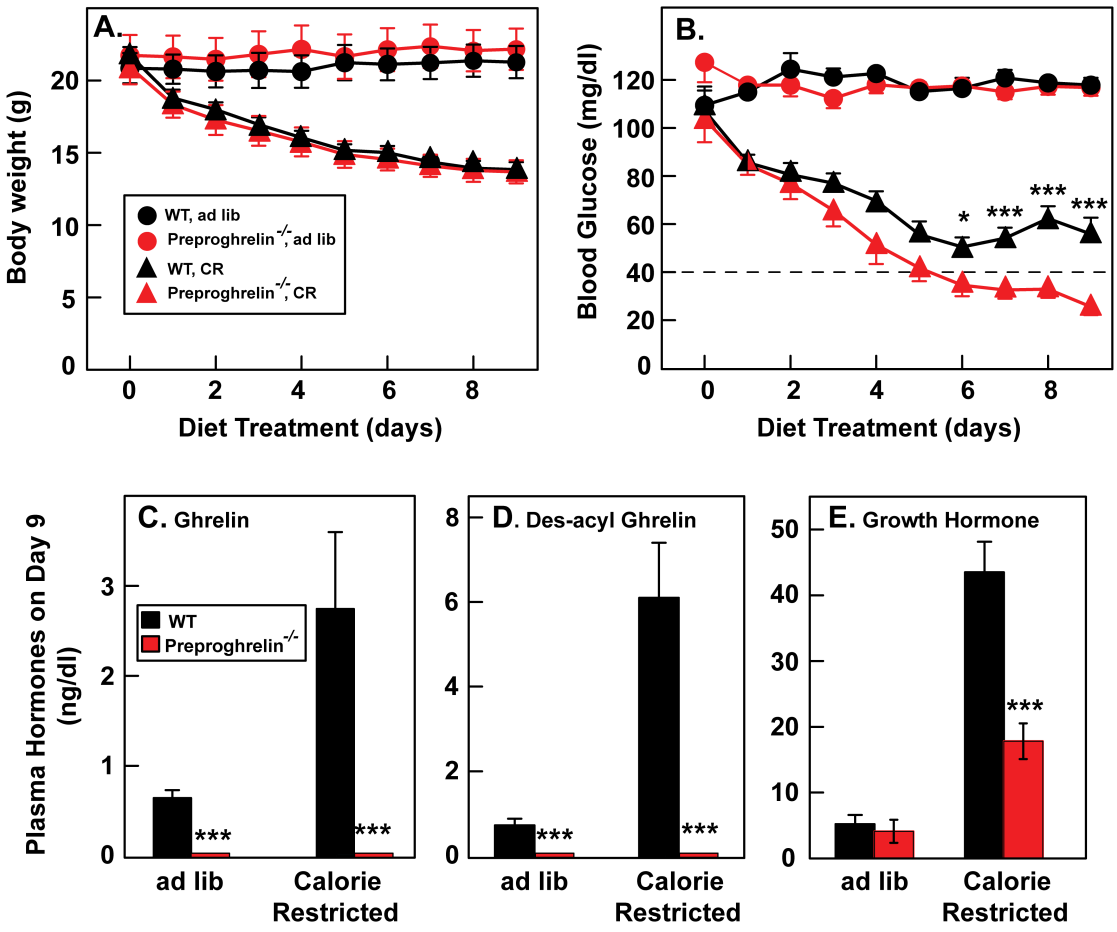

Figure 3-1. Comparison of WT and *Preproghrelin*$^{-/-}$ mice fed the chow diet *ad libitum* or subjected to 60% calorie restriction. Male littermates (8-wk-old) were housed in individual cages and fed *ad libitum* with the chow diet or subjected to 60% calorie restriction as described in *Materials and Methods*. Body weight (*A*) and blood glucose were measured daily at 5:30 p.m. (30 min before feeding). (*C–E*) the mice were euthanized at 5:30 p.m. (before feeding) on the ninth day of calorie restriction, and plasma levels of ghrelin (*C*), des-acyl ghrelin (*D*) and GH (*E*) were determined. Each value represents mean ± SEM of data from six mice. Asterisks (*) denote level of statistical significance (Student's *t* test) between WT and *Preproghrelin*$^{-/-}$ mice. *, $P < 0.05$; ***, $P < 0.001$.

Changes in Blood Glucose, Plasma Ghrelin, and Plasma Growth Hormone Levels during Day 8 of Calorie Restriction

Hypoglycemia in *Goat*$^{-/-}$ mice requires both chronic calorie restriction and acute food deprivation (2). In our previous study, we found hypoglycemia in *Goat*$^{-/-}$ mice only at 5:30 p.m., 22 hours after feeding. At 9 a.m. on day 7 of calorie restriction, *Goat*$^{-/-}$ mice were not hypoglycemic and showed similar blood glucose levels as WT littermates (65 ± 6.5 and 76 ± 6.2 mg/dL, respectively). To better understand the time course of the development of hypoglycemia in the setting of calorie restriction, we subjected *Goat*$^{-/-}$ mice and their WT littermates to 7 days of 60% calorie restriction such that the *Goat*$^{-/-}$ mice developed severe hypoglycemia at 5:30 p.m. on day 7. Both WT and *Goat*$^{-/-}$ mice were fed at 6 p.m. on day 7 and ate their food within 1 h. The next day, blood glucose levels were measured at two to three hour intervals starting at 9 a.m. and concluding at 8 p.m., 2 h after feeding. At each time point, blood was also collected from the tail vein for plasma hormone measurement.

In agreement with previous data, *Goat*$^{-/-}$ mice were not hypoglycemic at 9 a.m. and had similar blood glucose levels compared to their WT littermates (63.3 ± 10.7 and 66.8 ± 3.8 mg/dL, respectively) (Fig. 3-2*A*). Blood glucose was maintained in *Goat*$^{-/-}$ mice until 12 p.m., when levels started declining, reaching 30 mg/dL by 6 p.m. In contrast, WT mice maintained their blood glucose levels throughout the day at approximately 63–77 mg/dL. After mice were fed at 6 p.m., blood glucose levels in both WT and *Goat*$^{-/-}$ mice rose within 2 h to similar levels (108.4 ± 8.6 and 107.5 ± 13.2 mg/dL, respectively).

Coinciding with the onset of hypoglycemia in of *Goat*$^{-/-}$ mice, plasma ghrelin levels in WT mice rose after 12 p.m. and reached a peak value of 4.55 ± 0.31 ng/ml at 6 p.m. (Fig. 3-2*B*). GH levels in WT mice followed the trend of ghrelin, rising after 12 p.m. and peaking at 52.6 ± 2.9 ng/ml 6 p.m. (Fig. 3-2*D*). After feeding, levels of both hormones dropped within 2 h to pre-rise

levels. As expected, *Goat*$^{-/-}$ mice had no detectable plasma ghrelin (Fig. 3-2*B*). Importantly, GH levels in *Goat*$^{-/-}$ mice, although elevated at 6 p.m., did not exhibit the same dramatic rise seen in WT mice (Fig. 3-2*D*). Whereas levels of GH in WT mice rose to 52.6 ± 2.9 ng/ml at 6 p.m., levels of GH in *Goat*$^{-/-}$ mice only reached 18.3 ± 2.6 ng/ml. Plasma des-acyl ghrelin levels were similar in both WT and *Goat*$^{-/-}$ mice and rose throughout the day until feeding (Fig. 3-2*C*).

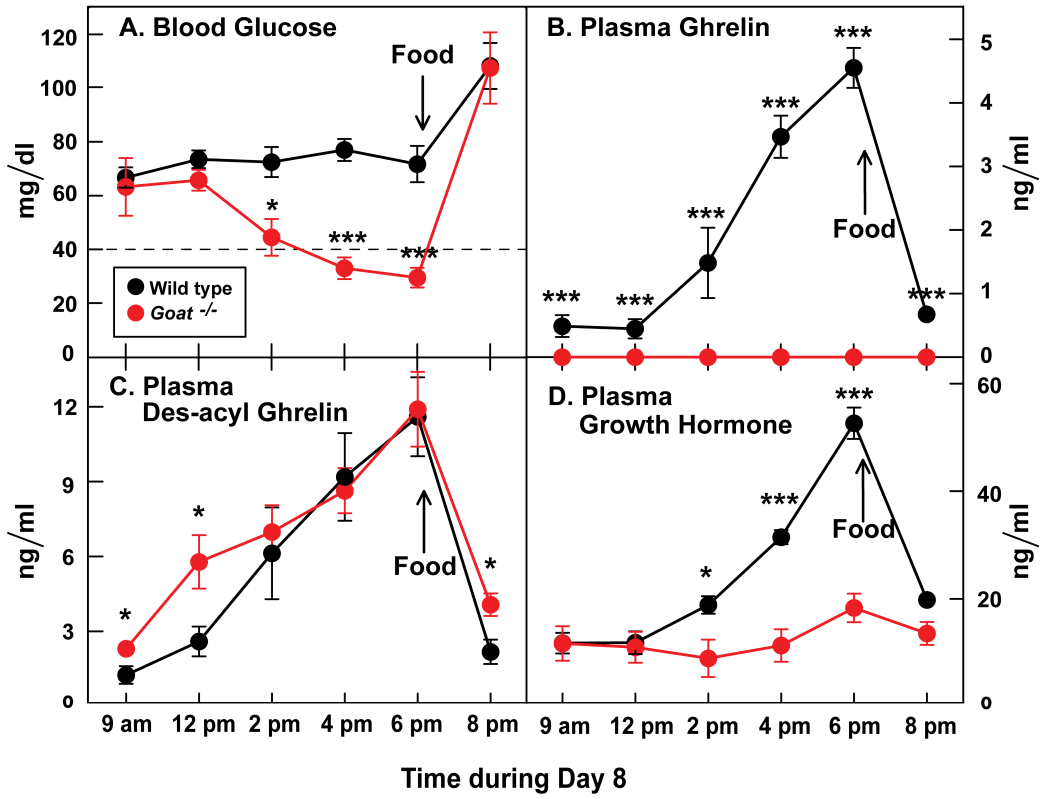

Figure 3-2. Differential changes in blood glucose and GH levels in WT and *Goat*$^{-/-}$ mice during day 8 of calorie restriction. Male WT and *Goat*$^{-/-}$ littermates (8-wk-old) were subjected to a 60% calorie restriction as described in *Materials and Methods*. Concentrations of blood glucose (*A*), plasma ghrelin (*B*), plasma des-acyl ghrelin (*C*), and plasma GH (*D*) were measured on day 8 of calorie restriction at the indicated times. Food was given at 6 p.m. Each value represents mean ± SEM of data from four mice. Asterisks (*) denote level of statistical significance (Student's *t* test) between WT and *Goat*$^{-/-}$ mice. **, $P < 0.01$; ***, $P < 0.001$.

The experiment was repeated in calorie restricted *Preproghrelin* $^{-/-}$ mice with similar results. After 6 days of calorie restriction, hypoglycemia developed in *Preproghrelin* $^{-/-}$ mice on day 7 after 2 p.m. and worsened until feeding at 6 p.m. (Fig. 3-3*A*). Plasma ghrelin, des-acyl ghrelin, and growth hormone rose in WT mice in a pattern coincident in time and magnitude to the development of hypoglycemia in *Preproghrelin* $^{-/-}$ mice (Fig. 3-3*B* and *D*). Neither forms of ghrelin were detectable in *Preproghrelin* $^{-/-}$ mice as expected. Significantly, *Preproghrelin* $^{-/-}$ mice, like *Goat* $^{-/-}$ mice, failed to exhibit the dramatic afternoon rise in plasma GH levels seen in WT mice (Fig. 3-3*D*).

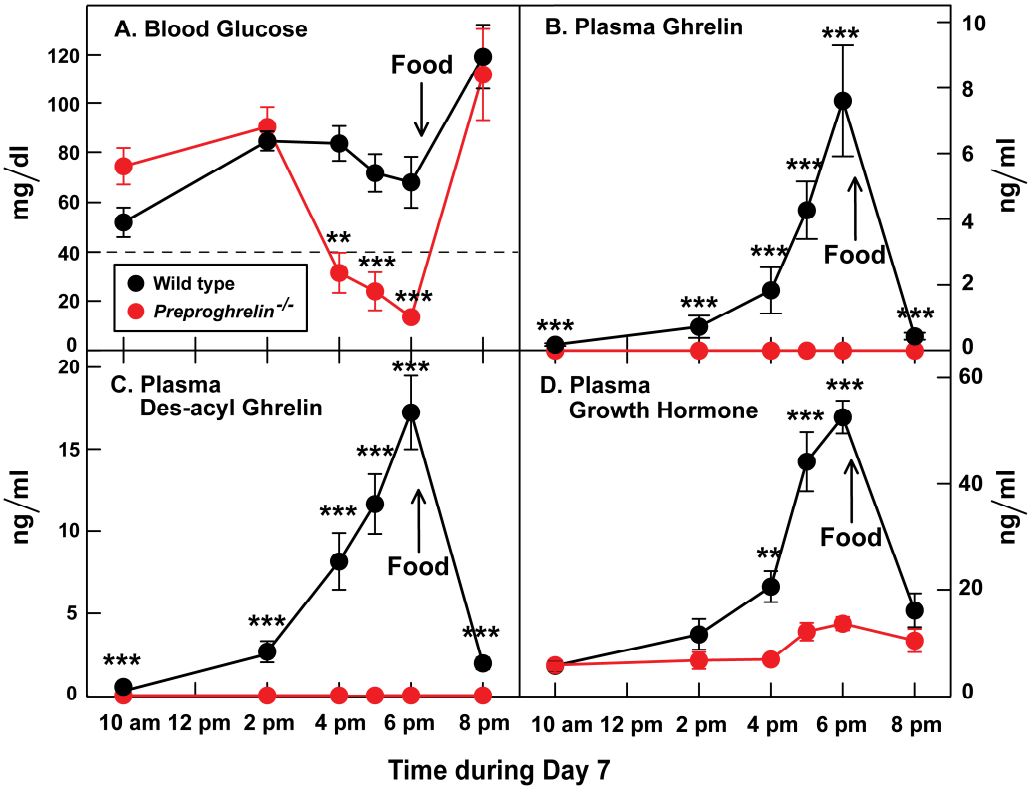

Figure 3-3. Differential changes in blood glucose and GH levels in WT and *Preproghrelin*$^{-/-}$ mice during day 8 of calorie restriction. Male WT and *Prephroghrelin*$^{-/-}$ littermates (8-wk-old) were subjected to a 60% calorie restriction as described in *Materials and Methods*. Concentrations of blood glucose (*A*), plasma ghrelin (*B*), plasma des-acyl ghrelin (*C*), and plasma GH (*D*) were measured on day 8 of calorie restriction at the indicated times. Food was given at 6 p.m. Each value represents mean ± SEM of data from four mice. Asterisks (*) denote level of statistical significance (Student's *t* test) between WT and *Preproghrelin*$^{-/-}$ mice. **, $P < 0.01$; ***, $P < 0.001$.

Changes in Gluconeogenic Substrates and Hormones during Day 8 of Calorie Restriction

Maintenance of blood glucose during fasting requires gluconeogenesis, which is dependent on both the availability of substrate and the hormonal milieu (21). A deficiency in the rate of gluconeogenesis could explain the fasting hypoglycemia that develops in calorie restricted *Goat*$^{-/-}$ mice. To examine this, we subjected *Goat*$^{-/-}$ mice and their WT littermates to 7 days of 60% calorie restriction such that the *Goat*$^{-/-}$ mice developed severe hypoglycemia at 5:30 p.m. on day 7. Both WT and *Goat*$^{-/-}$ mice were fed at 6 p.m. on day 7 and ate their food within 1 h. The next day, blood glucose levels were measured at two hour intervals starting at 10 a.m. and concluding at 8 p.m., 2 h after feeding. At each time point, blood was also collected from the tail vein for measurement of two key gluconeogenic substrates, lactate and pyruvate, and two hormones, insulin and glucagon.

In agreement with previous experiments, *Goat*$^{-/-}$ mice developed hypoglycemia at 2 p.m. that worsened until feeding at 6 p.m. (Fig. 3-4*A*). Strikingly, this decline in blood glucose levels corresponded to similar drops in levels of plasma lactate (2.1 ± 0.2 to 0.8 ± 0.1 mM) and pyruvate (1.2 ± 0.3 to 0.2 ± 0.01 mM), compared to values at 10 a.m. (Fig. 3-4*B* and *C*). In contrast, WT mice maintained their blood glucose, plasma lactate, and plasma pyruvate levels until feeding. When measured at 2 p.m., 4 p.m., and 6 p.m., levels of blood glucose, plasma lactate, and plasma pyruvate were significantly in *Goat*$^{-/-}$ mice vs. the WT group. After feeding, levels of blood glucose, plasma lactate, and plasma pyruvate rose to similar levels in both WT and *Goat*$^{-/-}$ mice.

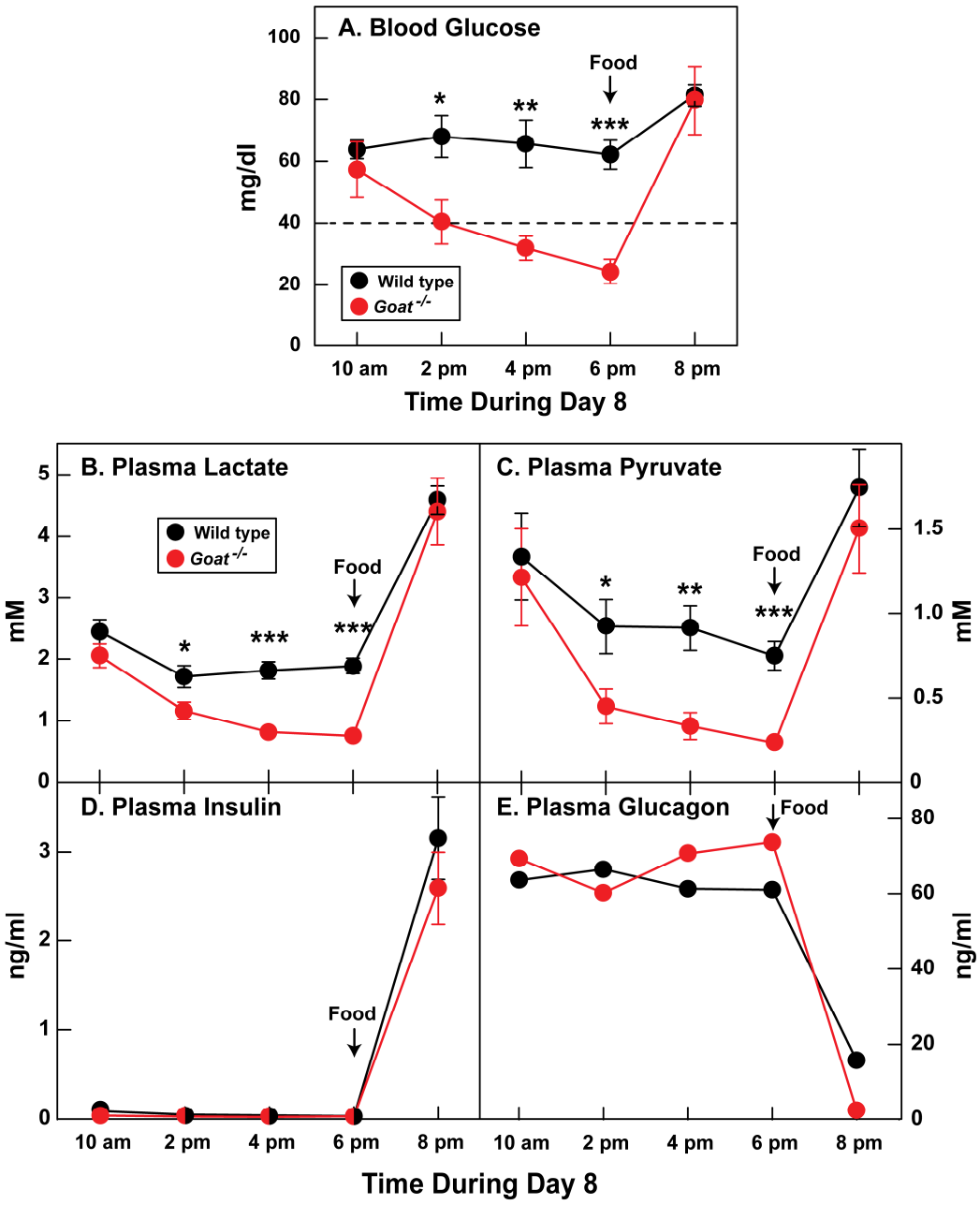

Figure 3-4. Changes in Plasma Parameters in WT and *Goat*$^{-/-}$ Mice after 7 Days of Calorie Restriction. Male WT and *Goat*$^{-/-}$ littermates (8-week-old) were subjected to 60% calorie restriction for 7 days as described in *Materials and Methods*, having received their last food at 6 pm on day 7. On day 8, blood was obtained at the indicated times for measurement of glucose (*A*), lactate (*B*), pyruvate (*C*), insulin (*D*) and glucagon (*E*). Food was given on day 8 at 6 pm (arrow). Each value represents mean ± SEM of data from 6 mice except for glucagon, which was measured using pooled samples from 6 mice. Asterisks (*) denote level of statistical significance (Student's *t* test) between WT and *Goat*$^{-/-}$ mice. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Studies in perfused rat liver and isolated rat hepatocytes have shown that the extracellular lactate/pyruvate (L/P) ratio, a reflection of the intracellular NADH/NAD^+ ratio, affects the rate of gluconeogenesis (22, 23). For a given level of pyruvate, gluconeogenesis is maximally stimulated at an L/P ratio of 10. WT and *Goat*$^{-/-}$ mice had similar L/P ratios at 10 a.m. (1.9 and 1.8, respectively). However, the L/P ratio rose to a much greater extent in *Goat*$^{-/-}$ mice by 6 p.m. (4.0 vs. 2.7 in WT mice). At 8 p.m., 2 h after feeding, the L/P ratios in both groups of mice were similar (WT, 2.7; *Goat*$^{-/-}$, 2.9).

The levels of insulin and glucagon, two hormones that directly regulate blood glucose levels were similar between WT and *Goat*$^{-/-}$ mice and were consistent with a catabolic state (Fig. 3-4*D* and *E*). Insulin levels were nearly undetectable in both WT and *Goat*$^{-/-}$ mice from 10 a.m. until feeding at 6 p.m., after which levels rose dramatically. Plasma glucagon was correspondingly elevated from 10 a.m. in both WT and *Goat*$^{-/-}$ mice until food was given at 6 p.m., after which levels dropped. There was no significant difference in glucagon levels between WT and *Goat*$^{-/-}$ mice.

Prevention of Hypoglycemia in *Goat*$^{-/-}$ Mice during Calorie Restriction by Injection of Gluconeogenic Substrates

To examine the role of deficiencies in gluconeogenic substrates as a cause for the fasting hypoglycemia found in *Goat*$^{-/-}$ mice during calorie restriction, hourly injections of either lactate, pyruvate, or alanine were given to 8-week-old male WT and *Goat*$^{-/-}$ littermates on day 7, 8, or 9 of 60% calorie restriction. Compared to saline injection, injections of lactate or pyruvate to WT mice raised blood glucose levels (Fig. 3-5*A* and *C*) while injections of alanine had no significant effect (Fig. 3-5*E*). When given injections of saline, *Goat*$^{-/-}$ mice developed hypoglycemia as expected, with blood glucose levels dropping to around 20 mg/dL at 6 p.m. (Fig. 3-5*B*, *D*, and

F). Importantly, the development of hypoglycemia was blocked by injections of either lactate, pyruvate, or alanine, all of which preserved blood glucose until 6 p.m. (Fig. 3-5*B*, *D*, and *F*).

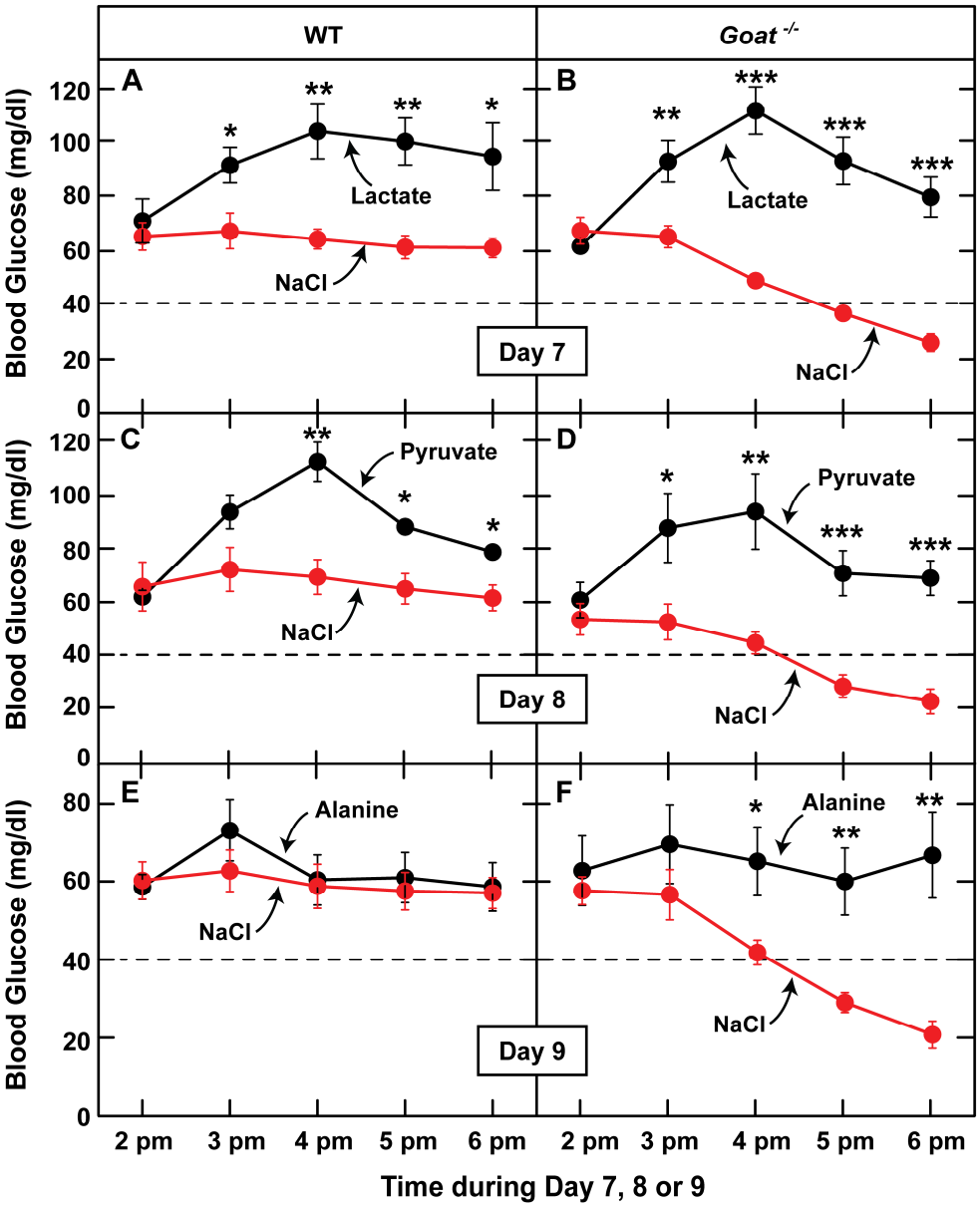

Figure 3-5. Comparison of calorie-restricted WT and *Goat*$^{-/-}$ mice after multiple injections of saline, lactate, pyruvate, or alanine. Male WT and *Goat*$^{-/-}$ littermates (8-wk-old) were subjected to a 60% calorie restriction as described in *Materials and Methods*. From 2 p.m. on day 7 of calorie restriction, WT (*A*) or *Goat*$^{-/-}$ (*B*) mice were given four hourly intraperitoneal injections of either sodium lactate (18 μmol/g body weight, 20% solution in water, pH 7.2) or sodium chloride (18 *μ*mol/g body weight, 10.5% solution in water, pH 7.2). Blood glucose was measured immediately before each injection and at 6 p.m.,

one hour after the last injection. From 2 p.m. on day 8 of calorie restriction, WT (*C*) or *Goat*$^{-/-}$ (*D*) mice were given four hourly intraperitoneal injections of either sodium pyruvate (18 μmol/g body weight, 20% solution in water, pH 7.2) or sodium chloride (18 μmol/g body weight, 10.5% solution in water, pH 7.2). Blood glucose was measured immediately before each injection and at 6 p.m., one hour after the last injection. From 2 p.m. on day 9 of calorie restriction, WT (*E*) or *Goat*$^{-/-}$ (*F*) mice were given four hourly intraperitoneal injections of either alanine (18 μmol/g body weight, 12.5% solution in water) or sodium chloride (18 *μ*mol/g body weight, 7% solution in water). Blood glucose was measured immediately before each injection and at 6 p.m., one hour after the last injection. Each value represents mean ± SEM of data from five mice. Asterisks (*) denote level of statistical significance (Student's *t* test) between WT and *Goat*$^{-/-}$ mice. *, $P < 0.05$; **, $P < 0.01$.

Prevention of Hypoglycemia in *Goat*$^{-/-}$ Mice during Calorie Restriction by Injection of Octanoate

Oxidation of free fatty acids (FFAs) is known to increase hepatic gluconeogenesis both *in vitro* and *in vivo* (24-27) via several mechanisms. These include: (*i*) generation of acety-CoA, which allosterically activates pyruvate carboxylase , (*ii*) generation of NADH, which is used to form glyceraldehyde 3-phosphate from 1,3-bisphosphoglycerate, and (*iii*) generation of ATP, which is used as an energy source for gluconeogenesis. To assess the role of the rate of gluconeogenesis in *Goat*$^{-/-}$ mice as a cause for the development of hypoglycemia during calorie restriction, two injections of octanoate were given to 8-week-old male WT and *Goat*$^{-/-}$ littermates at 2 p.m. and 4 p.m. on day 7 of 60% calorie restriction. In WT mice, injections of octanoate had no effect on their blood glucose as compared to injections of saline (Fig. 3-6*A*). In *Goat*$^{-/-}$ mice, injections of saline resulted in the expected fasting hypoglycemia. This effect was prevented by injections of octanoate, which restored blood glucose levels to those seen in WT mice (Fig. 3-6*B*).

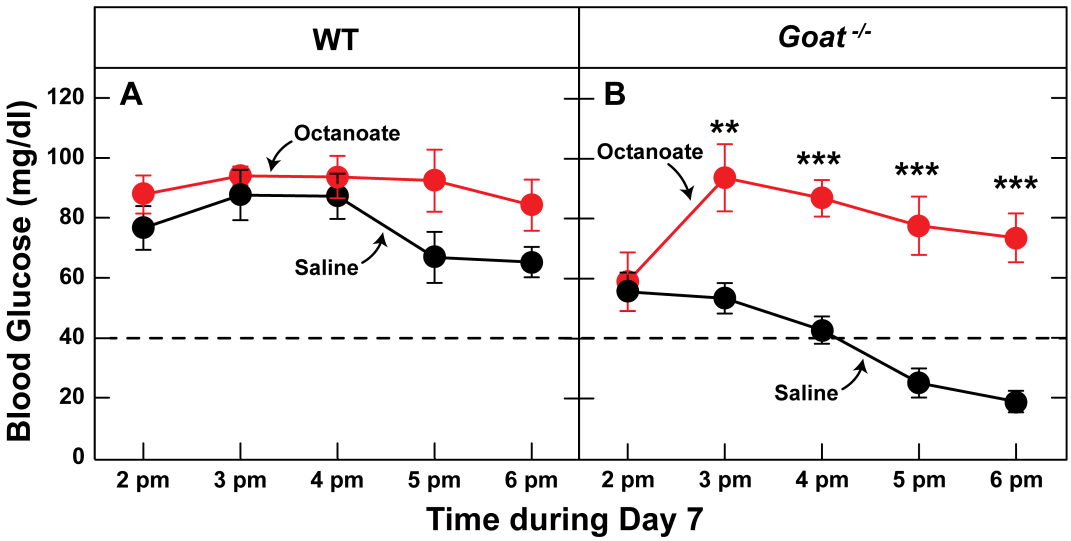

Figure 3-6. Response of Calorie-Restricted WT and *Goat*$^{-/-}$ Mice to Injections of Octanoate. Male WT and *Goat*$^{-/-}$ littermates (8-week-old) were subjected to 60% calorie restriction for 7 days as described in *Materials and Methods*. On day 7, WT (A) and *Goat*$^{-/-}$ (B) mice were injected intraperitoneally with either saline or octanoate. Each mouse received 2 injections (2 pm and 4 pm) of either saline or octanoate (3 μmol/g body weight per injection) in a volume of 140-170 μl (sodium octanoate was dissolved in saline to a final concentration of 0.3 M and adjusted to pH 7.6). Blood glucose was measured at 2 pm, 3 pm, 4 pm, 5 pm and 6 pm before any injections. Each value represents mean ± SEM of data from 5 mice. Asterisks (*) denote level of statistical significance (Student's *t* test) between WT and *Goat*$^{-/-}$ mice. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Discussion

The data show that protection against hypoglycemia in the setting of 60% calorie restriction requires an increase in levels of octanoylated ghrelin (Fig. 3-2*B*, 3-3*B*) and is associated with a coincident rise in levels of plasma GH (Fig. 3-2*D*, 3-3*D*) and the preservation of plasma lactate and pyruvate (Fig. 3-4*B* and *C*). The presence or absence of des-acyl ghrelin has no effect on blood glucose levels during calorie restriction. In experiments comparing levels of blood glucose at 5:30 p.m. during each day of 60% calorie restriction (Fig. 3-1*B* vs. Fig. 3-3*A*) or intra-day changes in blood glucose and GH during day 7 of calorie restriction (Fig. 3-2*A* vs. Fig. 3-3*A*), *Goat*$^{-/-}$ mice, which have high levels of plasma des-acyl ghrelin, behaved similarly to

Preproghrelin$^{-/-}$ mice, which have no detectable levels of plasma des-acyl ghrelin. This confirms the idea that ghrelin, not des-acyl ghrelin, is responsible for the elevation of GH and the protection against hypoglycemia during calorie restriction.

Time course studies of the changes in blood glucose, plasma hormones, and plasma substrates during the 8th day of calorie restriction (Fig. 3-2, 3-4) reveal a correlation in the timing and magnitude between the drop in blood glucose, plasma pyruvate, and plasma lactate in *Goat*$^{-/-}$ mice and the rise of plasma ghrelin and GH in WT mice. The ability of WT mice to preserve glucose and the gluconeogenic substrates pyruvate and lactate is correlated with a rises in plasma ghrelin (0.49 ± 0.07 to 4.55 ± 0.31 ng/ml, Fig. 3-2*B*) and plasma GH (11.8 ± 1.9 to 52.6 ± 2.9 ng/ml, Fig. 3-2*D*). *Goat*$^{-/-}$ mice, devoid of detectable plasma ghrelin, fail to maximally increase plasma GH (18.3 ± 2.6 vs. 52.9 ± 2.9 ng/ml in WT mice at 6 p.m., Fig. 3-2*D*). Correspondingly, levels of blood glucose, plasma pyruvate, and plasma lactate in *Goat*$^{-/-}$ mice fell to less than half that of WT mice by 6 p.m. (Fig. 3-4*A*-*C*). When exogenous pyruvate or lactate was injected into *Goat*$^{-/-}$ mice from 2 p.m. to 6 p.m., hypoglycemia was prevented (Fig. 3-5*B* and *D*). Similarly, injections of alanine, which can be converted to pyruvate in the liver by alanine transaminase, also rescued the hypoglycemia (Fig. 3-5*F*). This suggests that the ghrelin-mediated rise in GH acts to maintain blood glucose levels by preserving plasma levels of gluconeogenic substrates.

No differences were seen in the levels of insulin and glucagon in WT and *Goat*$^{-/-}$ mice during the course of the 8th day of calorie restriction (Fig. 3-4*D* and *E*). This is in agreement with previous measurements at 5:30 p.m. on day 7 of calorie restriction (2). Thus, differences in insulin and glucagon levels cannot explain the hypoglycemia found in calorie restricted *Goat*$^{-/-}$ mice.

Gluconeogenesis consumes ATP and NADH to generate glucose. In the setting of severe calorie restriction in which adipose tissue is depleted, the anaplerotic reactions necessary to regenerate ATP and NADH require the oxidation of glucose or the irreversible entry of pyruvate, lactate, or amino acids into the tricarboxylic acid (TCA) cycle. If excessive amounts of glucose or gluconeogenic substrates are consumed, then a feed-forward cycle can develop in which the need to maintain blood glucose levels drives the increased oxidation of glucose and gluconeogenic substrates, thus lowering levels of both at an increasingly faster rate. Evidence for both an increased drive towards gluconeogenesis and an increased rate of oxidation of glucose and gluconeogenic substrates are found in calorie restricted *Goat*$^{-/-}$ mice. Initially similar at 10 a.m. on day 8 of calorie restriction, levels of blood glucose, plasma lactate, and plasma pyruvate drop at a faster rate in *Goat*$^{-/-}$ mice than in WT mice (Fig. 3-4*A*–*C*). This is evidence of a faster rate of oxidation of these substances by *Goat*$^{-/-}$ mice. Furthermore, the lactate/pyruvate (L/P) ratio in *Goat*$^{-/-}$ mice during this time rises faster than the ratio in WT mice. Previous studies have shown that the L/P ratio directly affects the rate of gluconeogenesis (22, 23) and that increasing L/P ratios up to 10, with the concentration of pyruvate fixed, are associated with increasing rates of gluconeogenesis. Thus, the faster rise in the L/P ratio in *Goat*$^{-/-}$ mice (1.8 at 10 a.m. to 4.0 at 6 p.m.) vs. WT mice (1.9 at 10 a.m. to 2.7 at 6 p.m.) indicates an increased stimulus towards gluconeogenesis in *Goat*$^{-/-}$ mice.

To further test whether calorie restricted *Goat*$^{-/-}$ mice are trapped in a futile feed-forward cycle that wastes glucose and gluconeogenic substrates, we injected octanoate to these mice during the day 8 of calorie restriction. Octanoate and other free fatty acids can regenerate ATP and NADH through β-oxidation to spare the consumption of glucose and gluconeogenic substrates in the TCA cycle. Octanoate also been shown to increase the rate of gluconeogenesis

from lactate, pyruvate, and alanine in perfused rat liver (28). Tellingly, injections of octanoate rescued the hypoglycemia (Fig. 3-6). Furthermore, the energy provided by the octanoate injections was approximately 30% of that provided by the injections of gluconeogenic substrates (see *Materials and Methods* for calculations). These results suggest that: (*i*) *Goat*$^{-/-}$ mice excessively consume glucose and gluconeogenic substrates which in turn drives a feed-forward cycle that further accelerates the consumption, and (*ii*) introduction of exogenous gluconeogenic substrate or exogenous octanoate interrupts the feed-forward cycle and prevents the development of hypoglycemia.

The sharp ghrelin-mediated rise in GH seen in WT mice preserves plasma levels of gluconeogenic substrates, thus preventing the development of a feed-forward cycle in which hypoglycemia can develop. This is the mechanism by which the protective role of the ghrelin-induced rise in GH during calorie restriction is mediated. Further studies are required to delineate the pathways through which the substrate-preserving actions of GH act.

Materials and Methods

Materials. We obtained sodium lactate, sodium pyruvate, alanine, and sodium octanoate from Sigma. The glucometer was obtained from Bayer. Other chemicals were obtained from Sigma unless otherwise specified.

Mice. *Goat*$^{-/-}$ mice were generated as previously described (2). *Preproghrelin*$^{-/-}$ mice were generously provided by Mark Sleeman (Regeneron Pharmaceuticals, Tarrytown, NY). All mice were housed in colony cages with 12-h light/12-h dark cycles. The dark cycle began at 9:00 or 10:00 p.m. The chow diet consisted of Teklad Mouse/Rat Diet 7002 from Harlan Teklad Global Diets. This diet contains 3.0 kcal/g of metabolizable energy, of which 18% of calories are from

fat, 49% from carbohydrates, and 33% from protein. All animal experiments were performed with the approval of the Institutional Animal Care and Research Advisory Committee at University of Texas Southwestern Medical Center at Dallas.

Metabolic Parameters. Blood was drawn from tail vein or retro-orbital sinus and collected on ice in EDTA-coated tubes containing *p*-hydroxymercuribenzoic acid (final concentration, 1 mM) and/or Aprotinin (250 KIU/mL for glucagon measurement). Plasma was separated immediately and stored without treatment at −80 °C except for samples used for measurement of ghrelin, lactate, and pyruvate. For measurement of ghrelin, plasma samples were treated with HCl (final concentration, 0.1 M) before freezing. For measurement of lactate and pyruvate, plasma samples were deproteinized using 70% acetone and then lyophilized. Octanoylated ghrelin and des-acyl ghrelin were measured by immunoassay kits that distinguished both forms of the peptide (Cat. No. A05117 and A05118; Cayman Chemical). Lactate and pyruvate were measured with commercial assay kits from Biovision. Plasma levels of the following hormones were measured with commercial kits from the indicated vendor: growth hormone (Cayman Chemical), *insulin (Crystal Chem.),* and glucagon (Millipore).

Calorie Restriction. Calorie restriction was carried out as previously described (2). One week before initiation of calorie restriction, 8-wk-old male littermates of WT and *Goat* $^{-/-}$, or WT and *Preproghrelin* $^{-/-}$, were placed in individual cages and fed the chow diet ad libitum. During this week of acclimation, food intake was monitored to determine the average amount of food consumed daily by each mouse. Thereafter, the mice were subjected to 60% calorie restriction such that each mouse was fed at 6 p.m. every day with an amount of food equal to 40% of the daily amount consumed by the same mouse during the week of acclimation.

Energy Provided by Substrate and Octanoate Injections. The oxidation of acetyl-CoA, $FADH_2$, and NADH generate 10, 1.5, and 2.5 ATPs, respectively (29). β-oxidation of octanoate requires 3 oxidation cycles, each generating 1 acetyl-CoA, 1 $FADH_2$ and 1 NADH. Accounting for the final acetyl-CoA released after the last oxidation cycle and the 2 ATPs consumed to activate octanoate, the net production of ATP from 1 molecule of octanoate is:

$$3 \times (10 + 1.5 + 2.5) + 10 - 2 = 50 \text{ ATP}$$

Decarboxylation of pyruvate to acetyl-CoA generates 1 NADH. Coupled with the 10 ATPs generated from the oxidation of acetyl-CoA, the oxidation of pyruvate generates approximately 12.5 ATPs. Alanine is interchangeable with pyruvate and also generates 12.5 ATPs. The dehydrogenation of lactate to pyruvate produces an extra NADH so the oxidation of lactate generates 15 ATPs.

Four injections of gluconeogenic substrates were given at 18 μmol/g body weight while two injections of octanoate were given at 3 μmol/g body weight. Therefore 12 times as much gluconeogenic substrate was given as octanoate. From the above calculations, a molecule of octanoate generates 4 times the ATP of a molecule of pyruvate and alanine, and 3.3 times the ATP of a molecule of lactate. Thus, the total energy provided by the octanoate injections is 28 – 30% of that provide by the gluconeogenic substrate injections.

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Chapter IV: Ghrelin secretion stimulated by β_1-adrenergic receptors in cultured ghrelinoma cells and in fasted mice

Ghrelin is an octanoylated peptide hormone secreted by highly specialized cells in the gastric epithelium (1). Regulation of ghrelin secretion is tightly regulated and occurs in response to circadian rhythms, eating, stress, and other factors. Plasma ghrelin levels peak before meals and drop immediately after food ingestion (2-4). After release from the stomach, ghrelin binds to its receptors in the cells of the pituitary to stimulate growth hormone secretion. Other sites in the central nervous system, including those involved in body weight determination, also contain ghrelin receptors (1, 5). When ghrelin is given pharmacologically in excess to rodents and humans, it leads to significantly increased food intake. Thus, ghrelin is thought to play an important role in regulating appetite (1, 6).

Despite the appetite-stimulating effect of excess ghrelin, ghrelin deficiency has little to no effect on food intake. Mice lacking ghrelin or ghrelin receptor from birth gain nearly the same amount of weight as normal mice except when placed on high-calorie diets early in life (7-10). Ghrelin, however, has been found to be required for certain food reward behaviors that occur in the setting of chronic calorie restriction (11).

Aside from regulating food intake, the presence of ghrelin has been shown to be essential in mice to prevent hypoglycemia and death under conditions of severe calorie restriction. Genetically engineered mice that lack the gene encoding ghrelin *O*-acyltransferase (GOAT), the enzyme that attaches octanoate to ghrelin, an essential step to producing the biologically active hormone, have normal to excess levels of des-acyl ghrelin but no detectable plasma ghrelin (12). When food intake is reduced to 40% of their normal caloric intake (60% calorie restriction), both WT and *Goat*$^{-/-}$ mice show similar drops in body weight (30%), fat mass (60%), and blood

glucose (to 60 mg/dL) over the first 3 days. Afterwards, blood glucose levels in WT mice stabilized at 58–76 mg/dL and the mice behaved normally over the next 4 d. In contrast, blood glucose levels in the *Goat* $^{-/-}$ mice failed to stabilize and progressively dropped until they reached 12–36 mg/dL after 7 d. At this point, the mice became moribund and had to be euthanized. Stabilization of glucose levels in WT mice was associated with a progressive rise in plasma ghrelin levels and a consequent rise in plasma growth hormone. *Goat* $^{-/-}$ mice, which lack detectable ghrelin, exhibited significantly lower increases in growth hormone during calorie restriction. When ghrelin or growth hormone was infused back to calorie-restricted *Goat* $^{-/-}$ mice, hypoglycemia did not develop and the mice were able to survive the calorie restriction like their WT littermates (12).

If ghrelin is protective in preventing hypoglycemia and death during periods of famine, the question arises as to how ghrelin secretion is regulated. Previous studies have implicated adrenergic stimulation, whether by artificial stimulation of sympathetic nerves (13) or by infusion of adrenergic hormones locally into the stomach lining (14). However, the precise mechanism by which these events lead to ghrelin secretion is unknown.

Studies of ghrelin secretion in isolated cells have been complicated by the rarity of ghrelin secreting cells (< 1%) in the gastric epithelium (1, 15) and the lack of immortalized cell lines which can produce and secrete ghrelin. To solve this problem, we produced transgenic mice expressing SV40 T-antigen under the control of the *preproghrelin* promoter. These mice developed ghrelin-secreting gastric and pancreatic tumors from which we established permanent tissue culture cell lines that secrete ghrelin constitutively.

Ghrelin secretion in these cells are stimulated by the adrenergic agents norepinephrine and epinephrine and inhibited by atenolol, a selective antagonist of the β_1 class of adrenergic

receptors (16). Quantitative real-time PCR analysis of these cells show a several hundred-fold upregulation of mRNA encoding the β_1-adrenergic receptor as compared with the level in mRNA prepared from total stomach. These results were then tested *in vivo* role by administration to mice of reserpine, a drug that depletes presynaptic sympathetic neurons of catecholamines, or atenolol, a drug that selectively antagonizes β_1-adrenergic receptors. In contrast to vehicle treated mice, mice depleted of catecholamines or whose β_1-adrenergic receptors were antagonized failed to increase plasma ghrelin levels after fasting. Thus, we conclude that fasting-induced ghrelin secretion is mediated via the sympathetic nervous system acting through the β_1-adrenergic receptor.

Results

Generation of Transgenic Mice Containing Gastric and Pancreatic Ghrelinomas.

Transgenic mice expressing SV40 large T-antigen in ghrelin cells (hereafter referred to as TgGhrelin-SV40-T) were generated by first engineering a BAC-based transgene construct containing an insertion of the SV40 T-antigen coding region immediately downstream of the start codon of *preproghrelin* with 60 kb of flanking upstream and 104 kb of flanking downstream genomic DNA. This modified BAC allowed both the transcription and translation of SV40 T-antigen to be controlled by *preproghrelin* regulatory elements and was used to generate four independent lines of TgGhrelin-SV40-T mice. In all transgenic mice, SV40 T-antigen expression was highest in the stomach at 13-14 wk of age, reaching levels more than 500-fold higher than those in the intestine and pancreas. The studies in this report were carried out in line H7 and identical findings were obtained in studies of line H2.

Plasma ghrelin and des-acyl ghrelin levels rose progressively with age in TgGhrelin-SV40-T mice (Fig. 4-1*B* and *C*). At 20 wk of age in *ad libitum* fed animals during the light cycle,

TgGhrelin-SV40-T mice had 25-fold higher plasma ghrelin levels and 37-fold higher plasma des-acyl ghrelin levels than their WT littermates (Fig 4-1*D*). In spite of the large increase in plasma ghrelin levels, TgGhrelin-SV40-T mice were indistinguishable from WT animals in terms of body weight up to 20 wk of age (Fig. 4-1*E*) and there were no differences in food intake between the two groups of mice studied in metabolic cages at 16 wk of age.

Visible gastric tumors containing thickened gastric epithelium developed in all 22 of the autopsied TgGhrelin-SV40-T mice by 24 wk of age (Fig. 4-1*F* and *G*). Of the 22 mice, 11 also had tumors in the pancreas, 10 in the kidney, and 7 in the liver. All 7 of the liver tumors were associated with a stomach tumor and either a pancreatic or kidney tumor. Morphologically, the liver tumors were multifocal and suggest a metastatic origin.

Derivation and Establishment of Ghrelinoma Cell Lines.

Pancreatic and stomach ghrelinomas were used to establish ghrelinoma cell lines. In order to minimize contamination by fibroblasts and other cell types, tumor cells were not treated with trypsin, instead being mechanically dispersed after the removal of the tumor capsule. Tumor cells were incubated at a high density (3×10^6 cells per 35-mm well) and did not attach to the plastic. On a weekly basis, tumor cells were centrifuged and resuspended in fresh medium. Medium des-acyl ghrelin levels were measured weekly and after 3 months of culture, levels of des-acyl ghrelin in the medium rose to >20 ng/mL. The ghrelinoma cell lines from the pancreas and stomach are designated as PG-1 and SG-1, respectively.

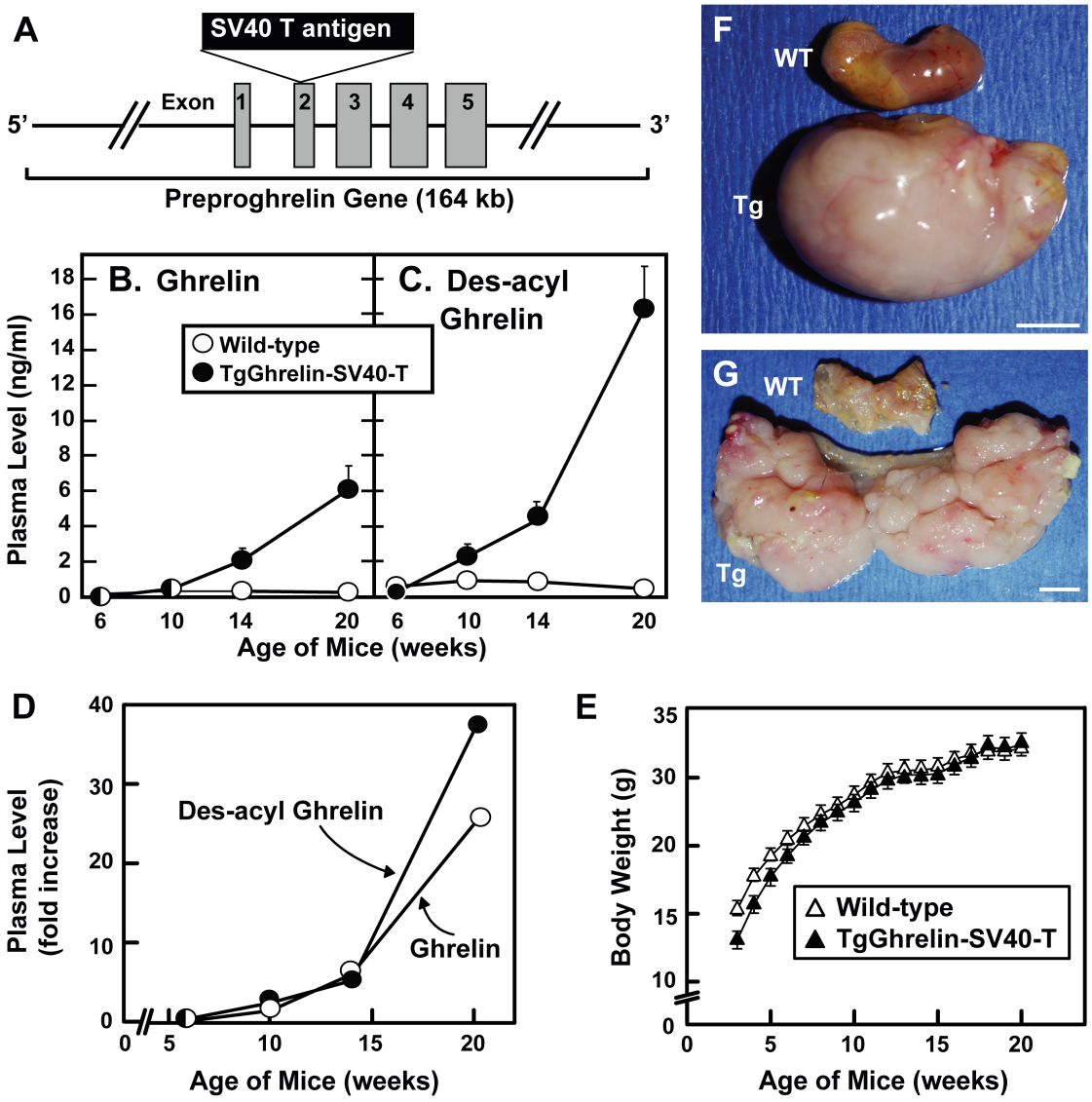

Figure 4-1. Generation of transgenic TgGhrelin-SV40-T mice. (*A*) Schematic of the construct used to generate transgenic mice that express SV40 large T-antigen in ghrelin cells. This construct was made by BAC engineering as described in *Materials and Methods* using a BAC clone (RP23-62H1) that contains the entire coding region of the mouse *preproghrelin* gene plus a total of 164 kb of genomic DNA flanking its start and stop codons. The SV40 T-antigen coding region (17) was inserted immediately downstream of the start codon of *preproghrelin*, thereby allowing both the transcription and translation of SV40 T antigen to be controlled by preproghrelin regulatory elements. Plasma levels of ghrelin (*B*) and des-acyl ghrelin (*C*) from WT and TgGhrelin-SV40-T male littermates at the indicated time after birth. The same cohort of WT mice and TgGhrelin-SV40-T mice was fed *ad libitum* on a chow diet (4% fat) and followed serially from 4–20 wk of age. Plasma levels were measured during the light cycle (between noon and 4:00 PM). Each value represents the mean ± SEM of values from 13 WT mice and 17 TgGhrelin-SV40-T mice. (*D*) Increase in plasma levels of ghrelin (○) and des-acyl ghrelin (●) in TgGhrelin-SV40-T mice as

compared with littermate WT mice. Each value represents the fold increase in TgGhrelin-SV40-T mice (*n* = 17) relative to WT mice (*n* = 13) as calculated from the data in Fig. 1 *B* and *C*. (*E*) Body weight in WT (△) and TgGhrelin-SV40-T (▲) mice measured weekly at 9:00 to 10:00 AM. These mice are the same as those studied in Fig. 1 *B*–*D*. Each value represents the mean ± SEM of values from 13 (△) and 17 (▲) mice. (*F*) Representative photographs of stomachs from 6-mo-old WT and TgGhrelin-SV40-T mice. (*G*) Same stomachs from *D* opened and photographed. (Scale bar: *F* and *G*, 0.5 cm).

Stimulation of Ghrelin Secretion in PG-1 Cells by Octanoate

In order to characterize the ghrelinoma cell lines, PG-1 cells were treated with octanoate and assessed to determine its effects on the synthesis and secretion of ghrelin. In the presence of octanoate, both intracellular (Fig. 4-2*A*) and medium (Fig. 4-2*B*) ghrelin levels in PG-1 cells increased over time, reaching 5-fold higher (from 11 to 54 pg/μg of cell protein) intracellular and 39-fold higher medium (from 6.7 to 264 pg/μg of cell protein) levels after 24 h of incubation. Incubation in the absence of octanoate resulted in only slight increases in intracellular and medium ghrelin over 24 h. Octanoate incubation did not affect levels of des-acyl ghrelin in the cells (Fig. 4-2*C*) or in the medium (Fig. 4-2*D*). The results were confirmed by SDS/PAGE and immunoblotting analysis of cells and medium with antibodies specific for the two forms of ghrelin (18), which showed bands consistent with the known molecular weights of the two peptides.

Lack of Effect of Long-chain Fatty Acids on Ghrelin Secretion by PG-1 Cells

Although octanoate stimulates ghrelin synthesis (Fig. 4-3*A*) and secretion (Fig. 4-3*B*), two 16-carbon fatty acids, palmitate and oleate, failed to have any effect on ghrelin synthesis or secretion. Treatment with any of the fatty acids did not affect des-acyl ghrelin levels in the cells (Fig. 4-3*C*) or medium (Fig. 4-3*D*). In all subsequent experiments, we included 50 μM octanoate in the culture medium to provide substrate for ghrelin synthesis.

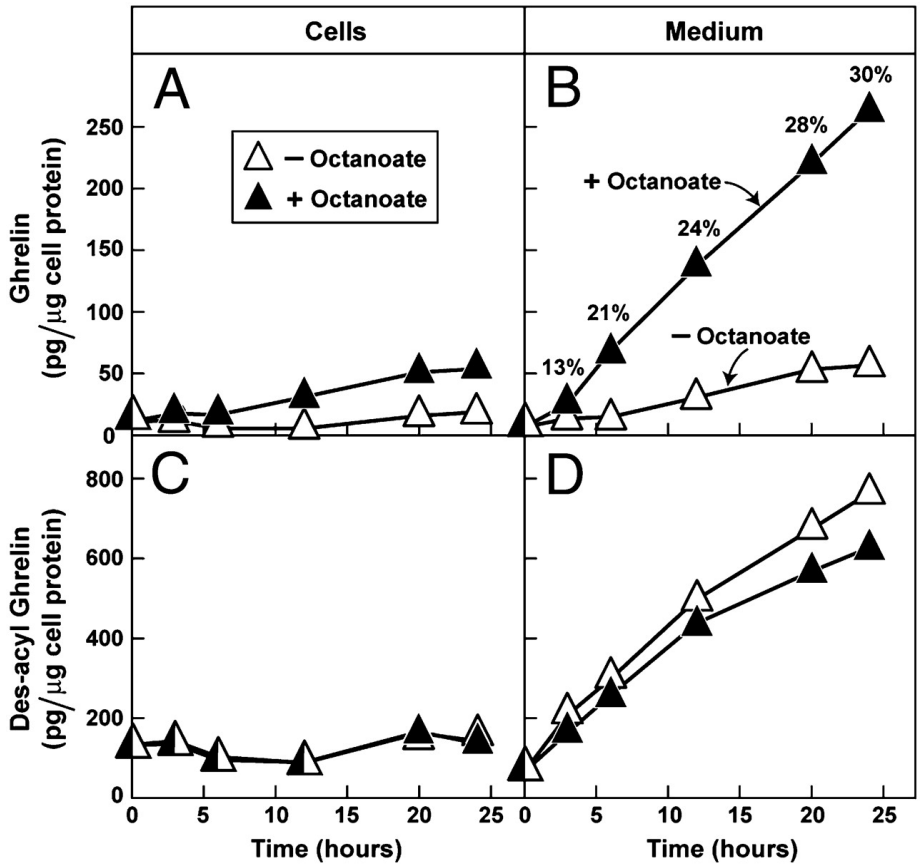

Figure 4-2. Time-dependent stimulation of synthesis and secretion of ghrelin by octanoate in PG-1 cells. On day 0, PG-1 cells were set up in medium A with 10% delipidated FBS as described in *Materials and Methods*. On day 3, cells were centrifuged, resuspended in serum-free medium B, aliquoted into a 24-well plate (5×10^4 cells per well), and treated in the absence (△) or presence (▲) of 50 μM sodium octanoate-albumin. After incubation at 37 °C for the indicated time, the medium and cells from each well were harvested for measurement of ghrelin (*A* and *B*) and des-acyl ghrelin (*C* and *D*) levels. Each value is the average of duplicate incubations. Numbers in *B* denote the percentage of ghrelin relative to the total amount of ghrelin plus des-acyl ghrelin secreted into the medium.

Stimulation of Ghrelin Secretion in PG-1 and SG-1 Cells by β-Adrenergic Agonists

Studies in rats have shown that the stimulation of the sympathetic nervous system can raise plasma ghrelin levels (13, 14). PG-1 cells were treated with two adrenergic agonists, norepinephrine and epinephrine in the presence of 50 μM octanoate. Both compounds stimulated the secretion of ghrelin (Fig. 4-4*B*) and des-acyl ghrelin (Fig. 4-4*D*) in a dose-dependent manner. There was near maximal stimulation of ghrelin secretion (2.5-fold vs. no treatment) at a

concentration of 1 μM for both agents. Norepinephrine was more potent than epinephrine in stimulating ghrelin secretion at lower concentrations, with the half-maximal stimulation occurring at 0.1 vs. 0.6 μM, respectively (Fig. 4-5). Intracellular levels of ghrelin (Fig. 4-4*A*) and des-acyl ghrelin (Fig. 4-4*C*) decreased with adrenergic agonist treatment, which suggests that the adrenergic agents primarily stimulate ghrelin release without immediately increasing synthesis.

Because epinephrine and norepinephrine stimulate the production of cAMP, PG-1 cells were incubated with forskolin, a compound that directly activates adenylyl cyclase (19). Like the two adrenergic agonists, forskolin also stimulated ghrelin secretion in a dose dependent manner (Fig. 3). Neither a $GABA_A$ receptor agonist (muscimol) nor a cholinergic receptor agonist (carbachol), had any effect on intracellular or secreted medium ghrelin levels in PG-1 cells (Fig. 4-4*A* and *B*).

To measure the time course of ghrelin secretion, PG-1 cells were treated with various agonists for 1, 3, or 6 h. Compared to no treatment, epinephrine, norepinephrine, and forskolin all increased the rate of ghrelin and des-acyl ghrelin secretion in a fashion that was linear with time (Fig. 4-4*B* and 4-4*D*). Intracellular levels of ghrelin (Fig. 4-4*A*) and des-acyl ghrelin (Fig. 4-4*C*) initially dropped following treatment with adrenergic agents, but then stabilized, suggesting the initial stimulation of ghrelin and des-acyl ghrelin is followed by increased synthesis. Neither muscimol nor carbachol had any effect on ghrelin or des-acyl ghrelin synthesis or secretion.

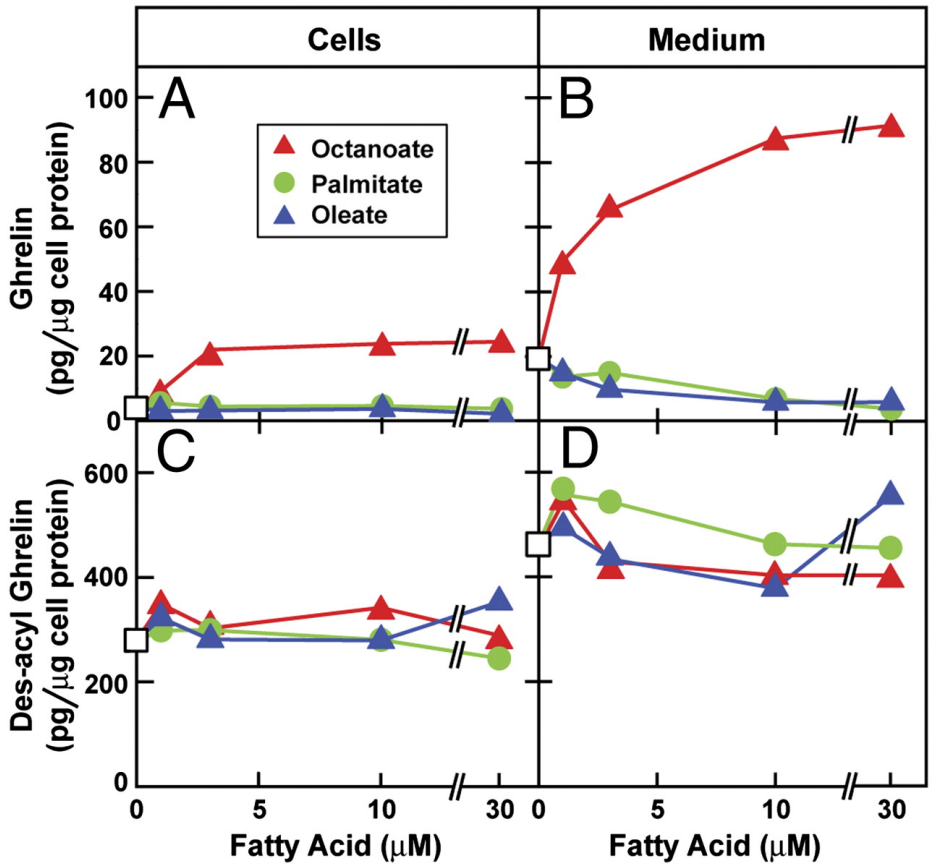

Figure 4-3. Dose-dependent stimulation of synthesis and secretion of ghrelin by octanoate in PG-1 cells. On day 0, PG-1 cells were set up in medium A with 10% delipidated FBS as described in *Materials and Methods*. On day 3, cells were centrifuged, resuspended in serum-free medium B, aliquoted into 24-well plates (5×10^4 cells per well), and treated with varying concentrations of the indicated fatty acid. After incubation at 37°C for 24 h, the medium and cells from each well were harvested for measurement of ghrelin (*A* and *B*) and des-acyl ghrelin (*C* and *D*). Each value is the average of duplicate incubations. □, none.

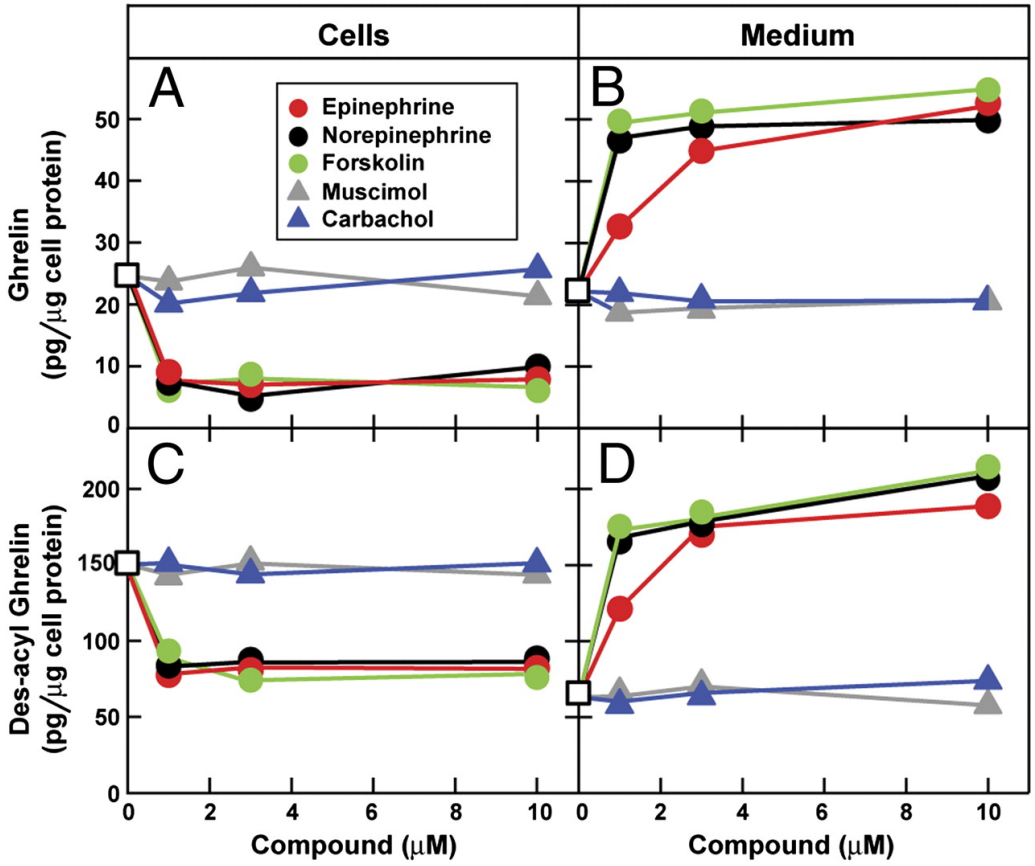

Figure 4-4. Dose-dependent stimulation of ghrelin secretion by adrenergic but not cholinergic or GABA agonists in PG-1 cells. On day 0, PG-1 cells were set up in medium A with 10% FBS. On day 2, octanoate was added to the medium at a final concentration of 50 μM. On day 3, cells were centrifuged, resuspended in serum-free medium B with 50 μM octanoate, aliquoted into 24-well plates (5×10^4 cells per well), and treated with varying concentrations of the indicated compound. After incubation at 37 °C for 6 h, the medium and cells from each well were harvested for measurement of ghrelin (*A* and *B*) and des-acyl ghrelin (*C* and *D*). Each value is the average of duplicate incubations. □, none.

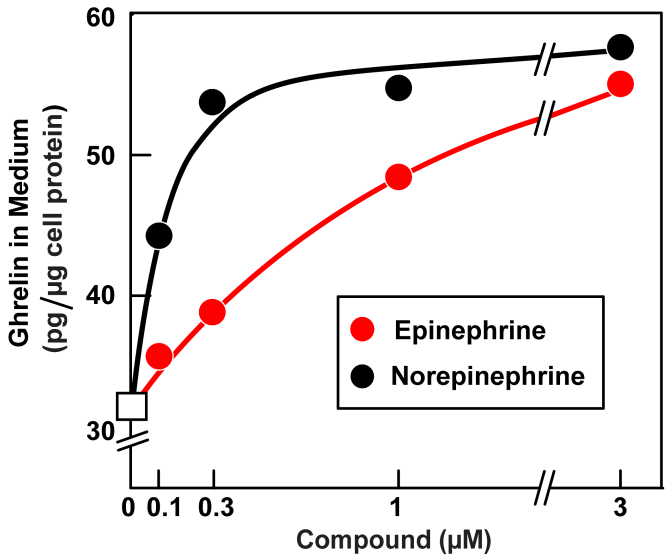

Figure 4-5. Norepinephrine is more potent than epinephrine in stimulating ghrelin secretion. On day 0, PG-1 cells were set up in medium A with 10% FBS. On day 2, sodium octanoate was added to the medium at a final concentration of 50 μM. On day 3, cells were centrifuged, resuspended in serum-free medium B with 50 μM sodium octanoate, aliquoted into 24-well plates (5×10^4 cells per well), and treated with varying concentrations of norepinephrine and epinephrine as indicated. After incubation at 37 °C for 6 h, the medium from each well was harvested for measurement of ghrelin levels. Each value is the average of duplicate values. This experiment was repeated with virtually identical results.

Inhibition of Adrenergic Stimulation of Ghrelin Secretion by β_1- but not β_2-selective Receptor Antagonists

Binding of epinephrine and norepinephrine to β-adrenergic receptors increases intracellular cAMP levels (20). To determine the adrenergic receptor subtypes involved in ghrelin secretion, PG-1 cells were treated with norepinephrine in the presence of different receptor agonists and antagonists. Atenolol, a β_1-selective adrenergic receptor antagonist, blocked the norepinephrine-stimulated secretion of ghrelin and des-acyl ghrelin (Fig. 4-6*B* and *D*) in a dose-dependent manner. Consistent with a primary inhibition of secretion, atenolol increased intracellular levels of ghrelin and des-acyl ghrelin (Fig. 4-6*A* and 4-6*C*). On the other hand, treatment with ICI118,551, a selective β_2-selective adrenergic receptor antagonist, had no effect on the ability of

norepinephrine to stimulate the secretion of ghrelin and des-acyl ghrelin. Neither muscimol nor carbachol treatment had any effect on the stimulation of ghrelin secretion by norepinephrine.

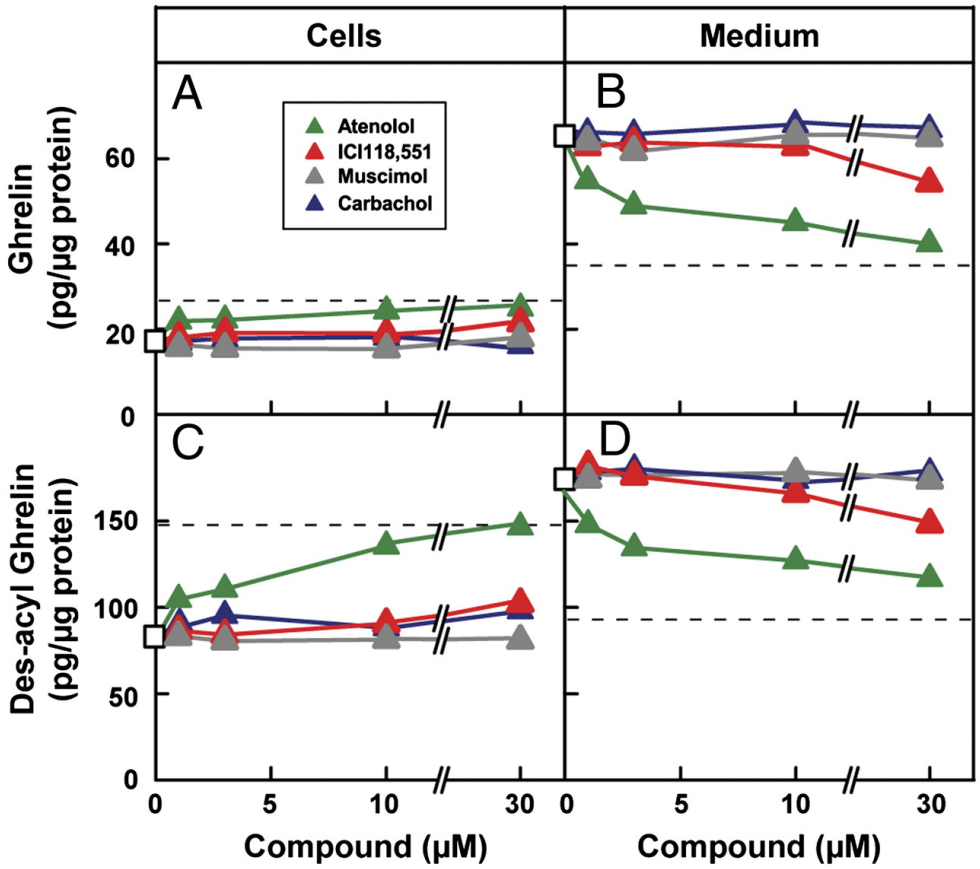

Figure 4-6. Effect of various receptor agonists and antagonists on norepinephrine-mediated stimulation of ghrelin secretion in PG-1 cells. On day 0, PG-1 cells were set up in medium A with 10% FBS. On day 2, octanoate was added to a final concentration of 50 µM. On day 3, cells were centrifuged, resuspended in serum-free medium B with 50 µM octanoate, aliquoted into 24-well plates (5×10^4 cells per well), and treated with 1 µM norepinephrine and different concentrations of the indicated compounds. After incubation at 37 °C for 6 h, the medium and cells from each well were harvested for measurement of ghrelin (*A* and *B*) and des-acyl ghrelin (*C* and *D*) levels. The dashed lines indicate the basal levels of ghrelin or des-acyl ghrelin without any treatment. Each value is the average of duplicate incubations.

Lack of Effect of Peptide Hormones on Ghrelin Secretion by PG-1 Cells

PG-1 cells were treated for 6 h with 100 nM of 10 peptide hormones that regulate fuel metabolism or hormone secretion (Fig. 4-7). None affected intracellular or medium levels of ghrelin or des-acyl ghrelin. Norepinephrine was used in the experiment as a positive control.

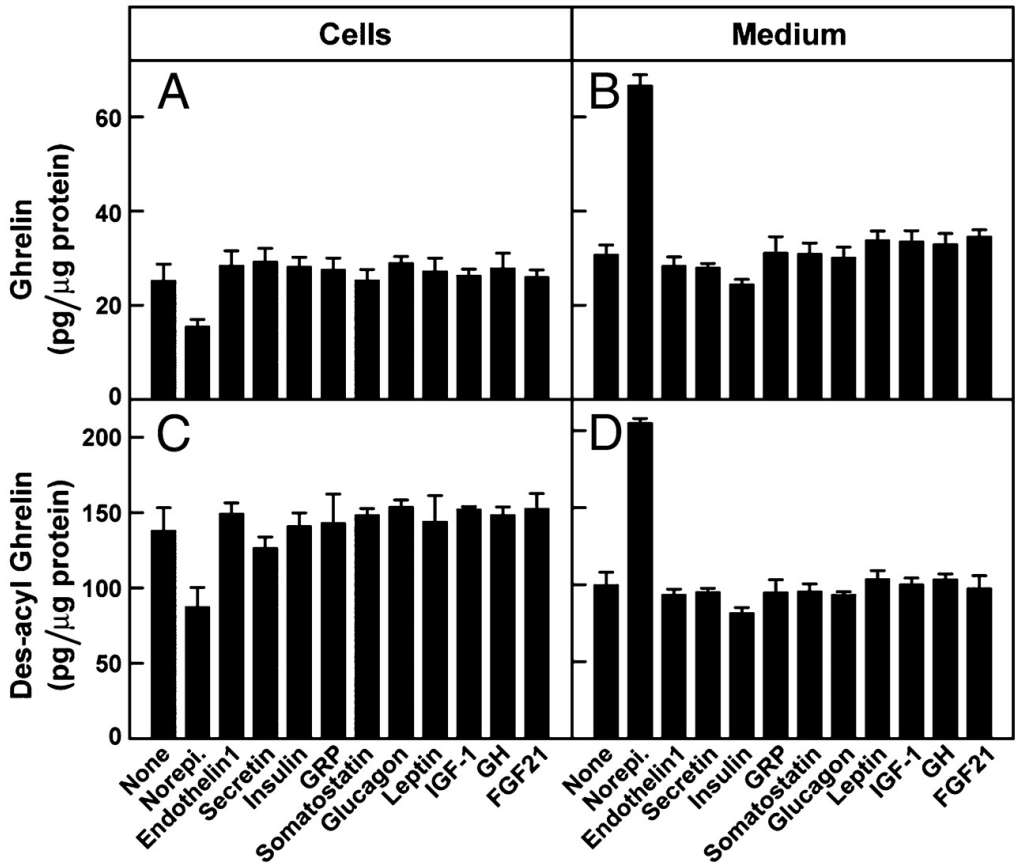

Figure 4-7. Lack of effect of peptide hormones on secretion of ghrelin in PG-1 cells. On day 0, PG-1 cells were set up in medium A with 10% FBS. On day 2, octanoate was added to the medium at a final concentration of 50 μM. On day 3, cells were centrifuged, resuspended in serum-free medium B with 50 μM octanoate, aliquoted into 24-well plates (5×10^4 cells per well), and treated with 100 nM of the indicated peptide hormone. Norepinephrine (Norepi., added at 10 μM) was included as a positive control. After incubation at 37 °C for 6 h, the medium and cells from each well were harvested for measurement of ghrelin (*A* and *B*) and des-acyl ghrelin (*C* and *D*). Each bar represents the mean ± SEM of three incubations. GRP, gastrin-releasing peptide.

SG-1 Cells Behave in a Similar Manner as PG-1 Cells

All of the preceding studies were repeated using stomach SG-1 ghrelinoma cells with similar results. Like PG-1 cells (Figs. 4-3 and 4-4), epinephrine, norepinephrine, and forskolin stimulated the secretion of ghrelin (Fig. 4-8*A*) and des-acyl ghrelin (4-8*B*). Neither muscimol nor carbachol had any effect. Similar to PG-1 cells, none the 10 peptide hormones tested (Fig. 4-7)

had any effect on the secretion of either ghrelin or des-acyl ghrelin (Fig. 4-8*C* and *D*) by SG-1 cells.

Quantitative Real-Time PCR Profiling of mRNA Expression Levels in PG-1 Cells, SG-1 Cells, and Ghrelin Cells Isolated from the Stomach.

Using quantitative real-time PCR, we compared the mRNA expression levels of various hormones, hormone receptors, adrenergic receptors, and neuroendocrine markers in PG-1 and SG-1 ghrelinoma cells using the mRNA expression levels of the same genes in stomachs from WT and TgGhrelin-SV40-T mice as controls (Table 4-1). Because ghrelin-secreting cells comprise a small proportion of stomach cells, mRNAs that are selectively expressed in ghrelin-secreting cells should show a relative increase in the tumor cells when compared with whole stomach.

Relative to stomach from WT mice, PG-1 and SG-1 cells had 5.5- to 7.7-fold increases in mRNA levels of preproghrelin and 13.4- to 13.8-fold increases in levels for GOAT (Table S1A). Similar increases in mRNA levels of preproghrelin (7.1-fold) and GOAT (8.3-fold) were found in stomach ghrelinomas from TgGhrelin-SV40-T mice.

Both PG-1 and SG-1 cells are highly enriched for mRNA expressing the β_1-adrenergic receptor (138- to 416-fold increases vs. stomach from WT mice, respectively). There was low to undetectable levels of mRNA for the β_2 and β_3 subtypes, giving further support to the idea that ghrelin secretion in PG-1 and SG-1 cells is stimulated by norepinephrine acting on the β_1-adrenergic receptor (Figs. 4-3, 4-4 and Fig. 4-8)

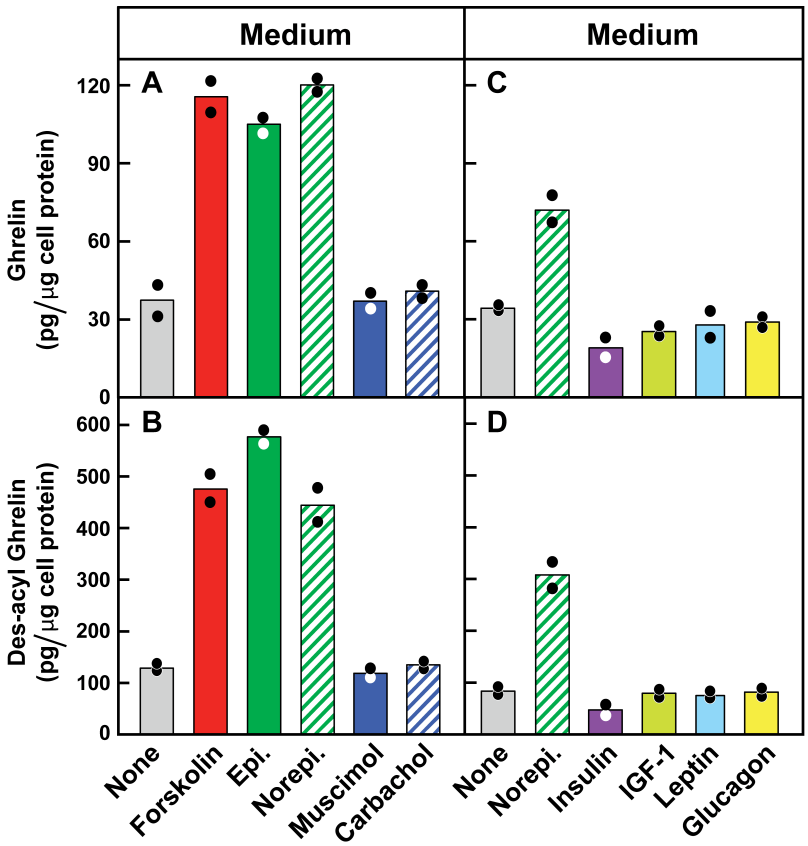

Figure 4-8. Stimulation of ghrelin secretion by adrenergic compounds in SG-1 cells. On day 0, SG-1 cells were set up in medium A with 10% FBS. On day 2, octanoate was added to the medium at a final concentration of 50 μM. On day 3, cells were centrifuged, resuspended in serum-free medium B with 50 μM octanoate, aliquoted into 24-well plates (5×10^4 cells per well), and treated with the indicated compound at a final concentration of either 10 μM [forskolin, epinephrine (Epi.), norepinephrine (Norepi.), muscimol, and carbachol] or 100 nM (insulin, glucagon, IGF-1, and leptin). After incubation at 37 °C for 6 h, the medium and cells from each well were harvested for measurement of ghrelin (*A* and *C*) and des-acyl ghrelin (*B* and *D*). Each bar denotes the average of duplicate incubations, the individual values of which are shown in circles.

PG-1 and SG-1 cells showed high levels of expression of mRNAs characteristic of neuroendocrine cells, including prohormone convertases PC1/3 and PC2; chromogranin A; and secretogranin II, III, and V However, there was no detectable expression of mRNAs for hormones found in the pancreatic islets including insulin, preproglucagon, pancreatic polypeptide, and somatostatin (Table 4-1B).

Using FACS, mucosal cells from the stomach of transgenic mice expressing GFP under the control of the mouse *preproghrelin* promoter (15) were isolated as previously described (21). Quantitative real-time PCR was then used to compare the expression of mRNAs encoding various adrenergic receptors in the ghrelin-enriched (GFP+) and nonenriched (GFP−) gastric mucosal cells (Table 4-2). As expected, ghrelin-enriched cells showed high levels of expression of mRNA for preproghrelin (8,870-fold) and GOAT (90-fold) compared to nonenriched, GFP− cells. Expression of mRNA for the β_1-adrenergic receptor was also highly increased(59-fold).

With the exception of the α_{1B} receptor (37- to 65-fold increase in PG-1 and SG-1 cells), no other adrenergic receptor subtype showed enrichment. The increase in mRNA expression of the α_{1B} receptor did not appear to be functionally significant for ghrelin secretion. Secretion of ghrelin and des-acyl ghrelin was not affected in PG-1 cells treated with an α_1-selective agonist (methoxamine) and was not blunted with a α_1-selective antagonist (prazosin) (16) in the presence of norepinephrine (Table 4-3). Expression of mRNA for the α_{1B} receptor was also undetectable in the FACS-enriched ghrelin cells (Table 4-2).

Table 4-1. Relative amount of mRNAs in PG-1 and SG-1 cells

Experiment A

mRNA	Stomach WT	Stomach Ghrelinoma	PG-1 Cells	SG-1 Cells
Preproghrelin	1.0 (20.7 ± 0.3)*	7.1 ± 0.7	5.5 ± 0.7	7.7 ± 0.9
GOAT	1.0 (28.9 ± 0.6)	8.3 ± 1.7	13.8 ± 2.7	13.4 ± 3.0
Adrenergic Receptors				
α_{1A}	1.0 (34.4 ± 0.2)	2.1 ± 0.6	1.5 ± 0.4	1.3 ± 0.8
α_{1B}	1.0 (32.2 ± 0.3)	1.5 ± 0.1	65 ± 8.1	37 ± 5.3
α_{1D}	1.0 (32.3 ± 0.3)	1.8 ± 0.1	1.2 ± 0.2	0.60 ± 0.20
α_{2A}	1.0 (29.0 ± 0.1)	0.46 ± 0.04	0.52 ± 0.04	0.05 ± 0.01
α_{2B}	1.0 (32.2 ± 0.3)	1.4 ± 0.14	1.2 ± 0.15	1.3 ± 0.15
α_{2C}	1.0 (33.9 ± 0.7)	2.4 ± 0.73	1.3 ± 0.41	0.61 ± 0.29
β_1	1.0 (33.2 ± 0.4)	138 ± 23	416 ± 46	138 ± 16
β_2	1.0 (28.8 ± 0.2)	1.1 ± 0.02	0.52 ± 0.03	0.07 ± 0.01
β_3	1.0 (32.2 ± 0.4)	0.49 ± 0.10	0.10 ± 0.02	0.09 ± 0.03
Neurocrine Markers				
Chromogranin A	1.0 (23.6 ± 0.3)	13 ± 0.8	5.8 ± 0.6	5.4 ± 0.7
Secretogranin II	1.0 (29.5 ± 0.3)	438 ± 26	311 ± 3.0	220 ± 1.0
Secretogranin III	1.0 (29.6 ± 0.1)	73 ± 6.3	94 ± 9.1	77 ± 9.1
Secretogranin V	1.0 (28.0 ± 0.3)	189 ± 7.0	134 ± 9.0	115 ± 2.0
Prohormone Convertase 1/3	1.0 (28.2 ± 0.6)	36 ± 3.5	231 ± 55	188 ± 49
Prohormone Convertase 2	1.0 (28.3 ± 0.1)	80 ± 15	57 ± 12	75 ± 16
Hormone Receptors				
GHSR	Not detected†	Not detected	Not detected	Not detected
Growth Hormone	1.0 (24.7 ± 0.2)	1.3 ± 0.10	0.88 ± 0.04	0.41 ± 0.03
Glucagon	1.0 (33.3 ± 0.5)	1.7 ± 0.73	0.37 ± 0.08	1.1 ± 0.30
Leptin	1.0 (27.5 ± 0.2)	1.7 ± 0.03	0.01 ± 0.002	0.005 ± 0.003
IGF1	1.0 (26.1 ± 0.3)	0.20 ± 0.01	0.04 ± 0.003	Not detected
Insulin	1.0 (25.6 ± 0.2)	0.91 ± 0.03	1.3 ± 0.08	0.69 ± 0.03

Experiment B

mRNA	Stomach WT	PG-1 cells	SG-1 cells	Pancreatic islets WT
Preproghrelin	1.0 (20.9 ± 0.1)	7.2 ± 0.8	3.6 ± 0.3	Not detected†
GOAT	1.0 (31.2 ± 0.2)	26 ± 2.0	36 ± 3.7	Not detected
Neuroendocrine Markers				
Chromogranin A	1.0 (23.8 ± 0.4)	7.0 ± 1.0	14 ± 4.5	17 ± 0.5
PC 1/3	1.0 (30.5 ± 0.3)	751 ± 102	577 ± 37	265 ± 7
PC 2	1.0 (31.9 ± 0.1)	319 ± 44	581 ± 66	2,737 ± 155
Pancreatic hormones				
Insulin	Not detected†	Not detected	Not detected	1.0 (14.4 ± 0.02)
Preproglucagon	Not detected	Not detected	Not detected	1.0 (16.8 ± 0.2)
Pancreatic Polypeptide	Not detected	Not detected	Not detected	1.0 (18.6 ± 0.1)
Somatostatin	Not detected	Not detected	Not detected	1.0 (19.0 ± 0.1)

Total mRNA from the indicated tissues or cells was extracted and quantified by quantitative real-time PCR as described in *Materials and Methods*. 36B4 mRNA (*A*) and cyclophilin mRNA (*B*) were used as invariant controls. Each value represents the amount of mRNA relative to that in WT stomach or WT pancreatic

islets, which is arbitrarily defined as 1.0 and shown as the mean ± SEM of three different experiments. Each determination in each experiment was done in duplicate (*B*) or triplicate (*A*). GHSR, growth hormone secretagogue receptor; PC, prohormone convertase.

*Values in parentheses denote the mean ± SEM of threshold cycle values.

†Not detected, threshold cycle value ≥35.

Table 4-2. Relative amount of mRNAs for adrenergic receptors in FACS-separated pools of ghrelin-enriched gastric mucosal cells.

mRNA	Gastric Cells Expressing Ghrelin	
	Non-enriched	**Enriched**
Preproghrelin	1.0 (26.5 ± 0.4)*	8,870 ± 2346
GOAT	1.0 (31.5 ± 0.9)	90 ± 39
Adrenergic Receptors		
α_{1A}	Not detected†	Not detected
α_{1B}	1.0 (30.4 ± 0.4)	Not detected
α_{1D}	1.0 (29.3 ± 0.3)	Not detected
α_{2A}	1.0 (28.9 ± 0.1)	Not detected
α_{2B}	Not detected	Not detected
α_{2C}	Not detected	Not detected
β_1	1.0 (32.2 ± 0.3)	59 ± 15
β_2	1.0 (29.8 ± 0.4)	0.3 ± 0.1
β_3	Not detected	Not detected

Gastric mucosal cells were isolated from mice containing a transgene encoding GFP driven by the mouse *preproghrelin* promoter as described in *Materials and Methods*. FACS was used to sort the cells into ghrelin-enriched (GFP^+) and unenriched (GFP^-) populations. Total mRNA from each cell population was extracted and quantified by quantitative real-time PCR as described in *SI Materials and Methods*. Cyclophilin mRNA was used as an invariant control to calculate relative amounts of all mRNA using the comparative threshold cycle (C_T) method. Each value represents the amount of mRNA relative to that in GFP^- cells, which is arbitrarily defined as 1.0 and is shown as the mean ± SEM of three different experiments. Each determination was done in duplicate.

*Values in parentheses denote the mean ± SEM of threshold cycle values.

†Not detected, threshold cycle value ≥ 35.

Table 4-3. Effects of adrenergic agonists and antagonists on ghrelin secretion in PG-1 cells

	Medium (pg/μg protein)		
Addition to Medium	Ghrelin	Des-acyl Ghrelin	Total Ghrelin
None	39 ± 1.5	99 ± 7.7	137 ± 8.8
Norepinephrine, 1 μM	81 ± 57 (2.1)*	214 ± 9.8 (2.2)	295 ± 15 (2.1)
+ Prazosin, 10 μM	80 ± 64 (2.1)	199 ± 7.9 (2.0)	279 ± 13 (2.0)
+ Atenolol, 10 μM	53 ± 1.5 (1.4)	115 ± 2.3 (1.2)	168 ± 2.6 (1.2)
Methoxamine	41 ± 1.7 (1.1)	107 ± 3.8 (1.1)	148 ± 4.3 (1.1)

On day 0, PG-1 cells were set up in medium A with 10% FBS. On day 2, octanoate was added to a final concentration of 50 μM. On day 3, cells were centrifuged, resuspended in serum-free medium B with 50 μM octanoate, aliquoted into 24-well plates (5×10^4 cells per well), and treated with the indicated compounds at the indicated final concentration. After incubation at 37 °C for 6 h, the medium from each well was harvested for measurement of ghrelin and des-acyl ghrelin levels. Each value represents the mean ± SEM of four incubations. Prazosin is an α_1-adrenergic antagonist, atenolol is a β_1-adrenergic antagonist, and methoxamine is an α_1-adrenergic agonist.

*Denotes fold increase relative to no addition.

Table 4-4. Real-time PCR primer sources

Mouse Gene	Source of Primer Sequences
Growth hormone receptor	PrimerBank (6857795a1)
Glucagon receptor	PrimerBank (6679965a1)
Leptin receptor	PrimerBank (1139593a1)
Secretogranin II	PrimerBank (6677865a1)
Secretogranin III	PrimerBank (6677867a1)
Secretogranin V	PrimerBank (31982002a1)
Adrenergic receptor α_{1A}	PrimerBank (31542114a1)
Adrenergic receptor α_{1B}	PrimerBank (6680660a1)
Adrenergic receptor α_{1D}	PrimerBank (34328059a1)
Adrenergic receptor α_{2A}	PrimerBank (6680662a1)
Adrenergic receptor α_{2B}	PrimerBank (6752994a1)
Adrenergic receptor α_{2C}	PrimerBank (6680664a1)
Adrenergic receptor β_1	Mouse Genome Informatics (MGI:1204525)
Adrenergic receptor β_2	PrimerBank (34328092a1)
Adrenergic receptor β_3	PrimerBank (298113a1)
Growth hormone secretagogue receptor	5'-TGGAGATCGCGCAGATCAG-3' 5'-CCGGGAACTCTCATCCTTCAG-3'
Insulin receptor	5'-CGAGTGCCCGTCTGGCTATA-3' 5'-GGCAGGGTCCCAGACATG-3'
IGF1 receptor	5'-AGCGCAGCTGATGTGTACGT-3' 5'-GCTCCCGGTTCATGGTGAT-3'
Chromagranin A	5'-GCAGGCTACAAAGCGATCCA-3' 5'-CTCTGTCTTTCCATCTCCATCCA-3'
Prohormone convertase 1/3	5'-GGCACCTGGACATTGAAAATTAC-3' 5'-TTCATGTGCTCTGGTTGAGAAGA-3'
Prohormone convertase 2	5'-CAAGCGGAACCAGCTTCA-3' 5'-ATTCCAGGCCAACCCCA-3'
Insulin	5'-TGAAGTGGAGGACCCACAAGT-3' 5'-AGATGCTGGTGCAGCACTGAT-3'
Preproglucagon	5'-ATTCACCAGCGACTACAGCAA-3' 5'-TCATCAACCACTGCACAAAATC-3
Pancreatic polypeptide	5'-GAAACTCAGCTCCGCAGATACA-3' 5'- TGTTCTCCTCTTCGGCTCTCT-3'
Somatostatin	5'-CCCAGACTCCGTCAGTTTCT-3' 5'-GGGCATCATTCTCTGTCTGG-3'

* Available at http://pga.mgh.harvard.edu/primerbank

In Vivo Role of Sympathetic Nervous System in Fasting-Induced Ghrelin Secretion.

The previous data suggest that increased adrenergic activity directly stimulates ghrelin secretion. We next asked if adrenergic activity *in vivo* is essential for the rise in plasma ghrelin induced by fasting. First, the sympathetic nervous system of mice were depleted of catecholamines by treatment with reserpine. This compound did not affect the plasma ghrelin level in the fed state (Fig. 4-9*A*) but completely blocked the increase in plasma ghrelin (Fig. 4-9*A*) and des-acyl ghrelin (Fig. 4-9*B*) after a 24-h fast. In order to ascertain whether β_1-adrenergic receptors mediate the adrenergic response, as is the case in PG-1 and SG-1 cells, we treated mice with atenolol, a selective β_1-antagonist and as a control, we also treated mice with ICI118,551, a selective β_2-antagonist. Treatment with atenolol completely blocked the increase in ghrelin (Fig. 4-9*C*) and des-acyl ghrelin (Fig. 4-9*D*) induced by 24-h fast. Furthermore, atenolol lowered plasma levels of ghrelin (Fig. 4-9*C*) and des-acyl ghrelin (Fig. 4-9*D*) even in the fed state. ICI118,551 treatment had no effect on ghrelin or des-acyl ghrelin levels.

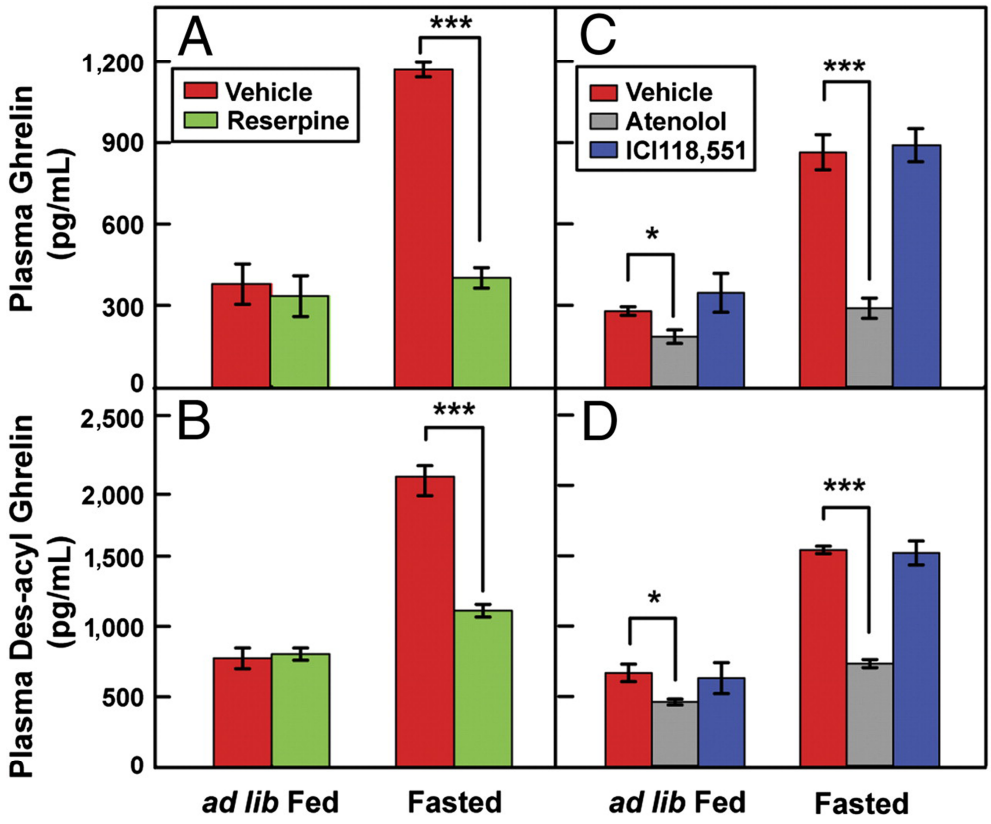

Figure 4-9. Suppression of fasting-induced ghrelin secretion by reserpine (*A* and *B*) and atenolol (*C* and *D*). Male C57BL/6J mice (8 wk of age) were injected intraperitoneally with either reserpine (0.5 mg/kg of body weight) or its vehicle (0.02% acetic acid) once daily (8:30 AM) for 3 d (*A* and *B*). A separate group of mice was injected intraperitoneally with atenolol (10 mg/kg), ICI118,551 (1.0 mg/kg), or their vehicles (2 mM HCl) twice daily (8:30 AM and 8:30 PM) for 3 d (*C* and *D*). [A 10-fold lower dose of ICI118,551 relative to atenolol was used because of ICI118,511's 550-fold higher affinity for β_2 than β_1 receptor (22). On day 2, blood samples were obtained at 10:00 AM from all mice (ad lib Fed), after which the same mice were fasted for 24 h. On day 3, mice were euthanized at 10:00 AM, and blood was collected (Fasted). The blood samples were used for measurement of levels of plasma ghrelin (*A* and *C*) and des-acyl ghrelin (*B* and *D*). Each value represents the mean ± SEM of data from four mice. Asterisks denote the level of statistical significance (Student's *t* test). * $P < 0.05$; *** $P < 0.001$.

Discussion

We undertook the current studies to further understand the mechanisms by which plasma ghrelin levels change in response to circadian rhythms and fasting. Because ghrelin-secreting cells make up <1% of the gastric mucosa and due to a lack of an existing cell culture model for ghrelin secretion, we followed the precedent set by Hanahan and colleagues (17, 23-25) to produce ghrelinoma-bearing mice. From the ghrelinomas, we derived lines of immortalized tissue culture cells that secrete ghrelin.

Both the pancreatic (PG-1) and stomach (SG-1) ghrelinoma cells show features of neuroendocrine cells, including an upregulation of mRNAs encoding components of the secretory pathway such as chromogranins, secretogranins, and prohormone convertases. We found little difference between PG-1 and SG-1 cells (Table 4-1*A*); both cell lines are enriched in the mRNAs encoding preproghrelin and GOAT but not for a variety of peptide hormone receptors. There was no enrichment for mRNAs encoding insulin, preproglucagon, somatostatin, or other pancreatic islet hormones (Table 4-1*B*). Most notably, PG-1 and SG-1 cells were highly enriched by several hundred-fold of mRNA encoding the β_1-adrenergic receptor, as were gastric ghrelin secreting cells isolated by FACS from transgenic *preproghrelin-GFP* mice (Table 4-2).

Secretion of ghrelin by the cultured ghrelinoma cells require an exogenous source of octanoate (Fig. 4-2). Substitution of octanoate with longer chain fatty acids such as oleate and palmitate failed to stimulate ghrelin secretion (Fig. 4-3), indicating that the ghrelinoma cells cannot shorten long-chain fatty acids. These data are in agreement with previous whole-animal studies which showed that ghrelin secretion is dependent on an exogenous source of octanoate (26).

Most importantly, we found that ghrelin secretion is specifically stimulated by the direct action of the adrenergic hormones norepinephrine and epinephrine on the ghrelin-producing cells

(Fig. 4-3 to 4-6). In previous studies, it was found that direct stimulation of sympathetic nerves (13, 27) or local infusion adrenergic hormones onto the stomach lining (14) but not intravenous infusion of epinephrine (13) can stimulate ghrelin secretion. This is consistent with our results and suggests that local release of adrenergic agents onto ghrelin-secreting cells is required.

Several lines of evidence point to the role of the β_1-adrenergic receptor as the mediator of the stimulatory effect of norepinephrine and epinephrine on ghrelin secretion. β-adrenergic receptor activation triggers an increase intracellular cAMP levels. When ghrelinoma cells are treated forskolin, a compound that raises cAMP levels by directly activating adenylyl cyclase (19, 20), ghrelin secretion is stimulated in an identical manner to norepinephrine and epinephrine (Fig. 4-3 and 4-4). Atenolol, a selective β_1 adrenergic receptor antagonist, but not ICI 118,551, a selective β_2 adrenergic receptor antagonist, blocks the stimulatory effects of norepinephrine in a dose-dependent manner. Both ghrelinoma cell lines as well as ghrelin expressing cells isolated directly from stomach show a marked enrichment for mRNA encoding the β_1-adrenergic receptor (Table 4-1). We also observed that norepinephrine was 6-fold more potent on a molar basis than epinephrine in stimulating ghrelin secretion (Fig. 4-5), a finding that is consistent with the differential affinities of the β_1 receptor for these agents (28).

The role of β_1-adrenergic receptors was confirmed *in vivo* by the administration of atenolol to mice (Fig. 4-9*C* and *D*) which prevented the normal increase in plasma ghrelin following a 24-h fast. Depletion of neuronal but not adrenal catecholamines by treatment of mice with reserpine (16) also prevented the normal increase in plasma ghrelin following a 24-h fast (Fig. 4-9*A* and *B*).

The reserpine data suggests that fasting acts through the sympathetic nervous system to release catecholamines in proximity to the ghrelin-secreting cells. Direct involvement of the

sympathetic nervous system is further supported by the fact that norepinephrine rather than epinephrine is the primary sympathetic neurotransmitter (16).

In summary, the data strongly supports the notion that the elevation in plasma ghrelin after fasting is mediated by locally released norepinephrine acting directly on the β_1-adrenergic receptors of ghrelin-secreting cells. Involvement of the sympathetic nervous system in elevating ghrelin levels is likely in several models of acute or chronic stress (reviewed in (29)). Because atenolol is commonly used in humans to antagonize β-adrenergic effects, it should be possible to determine whether this drug can block rises in plasma ghrelin caused by fasting or other settings of stress. If this is the case, atenolol might be very useful in studies of the physiologic function of ghrelin in humans.

Materials and Methods

Establishment of Ghrelinoma Cell Lines from Pancreas and Stomach. Tumor-bearing pancreas and stomach were dissected from anesthetized (i.p. chloral hydrate 500 mg/kg) 6-month-old TgGhrelin-SV40-T mice. Organ capsules were removed, washed with PBS, and placed in 10 mL of

medium A [DMEM/F-12 medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin sulfate, and 1% (vol/vol) insulin-transferrin-selenium-X (Invitrogen)] containing 10% (v/v) PBS. After mincing into 1- to 2-mm^2 pieces, the tissues were mechanically dispersed by pipetting up and down 20–30 times.

To establish the pancreatic ghrelinoma cell line, dispersed cells were first passed through a 100-μm filter and then centrifuged at 500 × g for 5 min at room temperature. Approximately 9 × 10^6 cells from the cell pellet were resuspended in 9 mL of medium A with 10% (vol/vol) FBS. Aliquots of the suspension were seeded into a six-well Costar plate (Cat. No. 3516; Corning) at a

density of ~3×10^6 cells per 35-mm well and cultured in a 37°C incubator with 5% (v/v) CO_2 and 5% (v/v) O_2. Every week, the medium from each well, along with unattached cells, was collected and centrifuged at 1,000 × g for 5 min. The cell pellet was resuspended in 3 mL of fresh medium A with 10% FBS and reseeded onto new plates. Meanwhile, the centrifugation medium was assayed to determine the concentration of des-acyl ghrelin. Weekly reseeding and des-acyl ghrelin measurements were made for 3 months, after which the level of des-acyl ghrelin in the medium reached a concentration of ≥20 ng/mL. At this point, the cells were split at a ratio of 1:1.5, passaged every 3 or 4 d, and frozen in multiple aliquots in liquid nitrogen. We designated the resulting pancreatic ghrelinoma cell line, which was derived from TgGhrelin-SV40-T mouse 8175, PG-1.

Using essentially the same procedure as described above, we also generated a stomach ghrelinoma cell line, which we named SG-1 from TgGhrelin-SV40-T mouse 1672.

Cell Culture. Cells were maintained in medium A with 10% FBS and grown in a 37 °C incubator with 5% CO_2 and 5% O_2. Experiments were performed by first plating cells onto 100-mm dishes at a density of 5×10^4 cells/mL (Day 0). After 2 days, sodium octanoate-albumin was added to the medium to a final concentration of 50 μM (Day 2). The next day, cells were centrifuged, resuspended in serum-free medium B (DMEM low-glucose containing 100 U/mL penicillin and 100 μg/mL streptomycin sulfate), aliquoted into Costar 24-well plates (1 mL per 18-mm well), and treated with different compounds as described in the figure legends (Day 3). Following incubation at 37 °C for periods indicated by each experiment, the medium and cells from each well were harvested for measurement of ghrelin and des-acyl ghrelin levels.

Materials. Reagents and kits were purchased from the following companies: ghrelin enzyme immunoassay kits from Cayman Chemical Co.(unacylated: Cat. No. A05117; acylated: Cat. No. A05118); DMEM/F-12 50:50 medium from Mediatech, Inc. (Cat. No. 10-090-CV); insulin-transferrin-selenium-X (ITS-X) from Invitrogen; fatty acids, BSA (fatty acid-free), forskolin, epinephrine, norepinephrine, muscimol, carbachol, S(−)-atenolol, ICI118,551, reserpine, glucagon, insulin, secretin, somatostatin, and gastrin-releasing peptide from Sigma; mouse recombinant IGF-1, FGF-21, and leptin from ProSpecTany Technogene, Ltd.; endothelin-1 from Peptides International; and growth hormone from the National Hormone and Peptide Program of National Institute of Diabetes and Digestive and Kidney Diseases.

FBS was delipidated by solvent extraction with l-butanol and isopropyl ether as described (1). BSA-conjugated fatty acids were prepared as described in (1) by dissolving palmitate (5 mM final concentration), octanoate (10 mM final concetration) or oleate (10 mM final concentration) in 0.15 M sodium chloride containing 10% (w/v) BSA. Stock solutions of the following compounds were made as follows: reserpine, 0.5 mg/mL in 0.1% (v/v) acetic acid; forskolin, 10 mM in DMSO; epinephrine and norepinephrine, 5 mM in 0.01 M HCl; atenolol, 5 mM (for cell culture) or 10 mg/mL (for *in vivo* experiments) in 0.01 M HCl; all other compounds, 5 mM in PBS or water.

Generation of Transgenic Ghrelin-SV40 T-Antigen Mice. Mice expressing SV40 large T-antigen in ghrelin cells, hereafter referred to as TgGhrelin-SV40-T mice, were generated by first engineering a BAC clone (RP23-62H1) containing the coding region of mouse *preproghrelin* plus 60 kb of flanking upstream and 104 kb of flanking downstream genomic DNA to insert the SV40 early region [which contains the T-antigen coding sequence and corresponds to the XbaI/BamHI fragment of the previously reported RIP1-tag plasmid (17); kindly provided by

Douglas Hanahan, University of California, San Francisco, CA] immediately downstream of the start codon of *preproghrelin* (15). As a result of the insertion (which also removes the first 29 bp of *preproghrelin*), transcription and translation of SV40 T-antigen are placed under the control of *preproghrelin* regulatory elements.

The SV40-*preproghrelin* construct was microinjected into pronuclei of fertilized one-cell stage C57BL/6J embryos by the Transgenic Core Facility at University of Texas Southwestern Medical Center at Dallas. Several lines of transgenic mice expressing SV40 T-antigen in ghrelin cells of the stomach and pancreas were generated and backcrossed so that all the TgGhrelin-SV40-T mice used in this study were performed on a pure C57BL/6J genetic background.

Mice were housed on a 12-h light/12-h dark cycle in standard cages and fed a chow diet containing 4% (w/w) fat (Teklad Mouse/Rat Diet 7001; Harlan Teklad Premier Laboratory Diets).

All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center at Dallas.

Isolation of Enriched Pools of Gastric Ghrelin Cells. FACS was used to isolate gastric mucosal cells from mice containing a transgene encoding GFP driven by the mouse *preproghrelin* promoter (15) as previously described (21).

Ghrelin Measurements in Plasma and in Cultured Cells. Measurement of plasma ghrelin and des-acyl ghrelin levels were previously described (12). Ghrelin and des-acyl ghrelin measurements in cultured cells were performed by centrifuging cell suspensions from each well at 1,000 × g for 5 min at 4 °C. The supernatant (medium) was treated with HCl to a final concentration of 0.1 M and stored at −20 °C until the assay. Meanwhile, the cell pellets were

resuspended in 1 mL of boiling water, boiled for 10 min, and then treated with acetic acid and HCl to final concentrations of 1 and 0.02 M, respectively. Following homogenization by passage through a 22-gauge needle 10 times, the cell lysates were spun at 20,000 × g for 10 min at 4 °C to remove cell debris. The cleared supernatants were then lyophilized for 6–12 h, resuspended in 200 μL of 0.1 M HCl, and assayed immediately, along with the medium from the same wells, for ghrelin and des-acyl ghrelin. Enzyme Immunoassay Kits (Cayman Chemical Co.) were used to determine the medium and intracellular levels of ghrelin and des-acyl ghrelin. In each experiment, two wells of untreated cells were lysed in 0.1 M NaOH and assayed for cell protein concentration using a BCA kit (Pierce). Ghrelin levels are expressed as picograms per microgram of cell protein.

Quantitative Real-Time PCR. Total RNA was extracted from tissues and cells, and real-time PCR measurements were carried out as described (18, 30). Sequences of the primer used are listed in Table 4-4. mRNAs for 36B4 and cyclophilin were used as invariant controls to calculate the relative amounts of all mRNAs using the comparative threshold cycle (C_T) method.

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CHAPTER V: Conclusion

Our studies show that an essential role of ghrelin is to preserve glucose during times of famine. When subjected to 60% calorie restriction, *Goat* $^{-/-}$ and *Preproghrelin* $^{-/-}$ mice develop severe fasting hypoglycemia and become moribund. This vital, protective role of ghrelin helps to explain its strong evolutionary conservation from fish to mammals.

The metabolic effects of ghrelin deficiency are discussed in Chapters II and III. *Goat* $^{-/-}$ and *Preproghrelin* $^{-/-}$ mice develop severe fasting hypoglycemia after 7 days of 60% calorie restriction and become lethargic and moribund. Calorically restricted WT mice are able to maintain their blood glucose, an ability which is associated with daily and intra-day rises in levels of plasma ghrelin and plasma GH. *Goat* $^{-/-}$ and *Preproghrelin* $^{-/-}$ mice, in contrast, fail to raise GH on a daily or intra-day basis to levels seen in WT mice. This inability to raise GH levels leads to the development of increasingly severe hypoglycemia as the caloric restriction progresses. Restoration of GH levels by infusion of ghrelin or GH rescues the hypoglycemia in *Goat* $^{-/-}$ mice.

The intra-day rise in growth hormone in WT mice corresponds in time and magnitude to falls in the levels of blood glucose, plasma pyruvate, and plasma lactate in *Goat* $^{-/-}$ mice. This suggests that increasingly higher levels of GH are needed to counteract the hypoglycemia experienced during fasting under calorie restricted conditions by preserving levels of gluconeogenic substrates. When the deficiencies in gluconeogenic substrates in *Goat* $^{-/-}$ mice are corrected by injection of exogenous substrate (pyruvate, lactate, or alanine), development of hypoglycemia is prevented.

Hypoglycemia in *Goat* $^{-/-}$ mice can also be prevented by injection of exogenous octanoate. Fatty acids are known to increase the rate of gluconeogenesis through mechanisms that include

the regeneration of the ATP and NADH consumed in the production of glucose. Notably, there is a difference between the efficacy of free fatty acids and gluconeogenic substrate to correct hypoglycemia. On a caloric basis versus octanoate, at least 3 times gluconeogenic substrate is required to prevent hypoglycemia. This phenomenon supports the idea that, in the absence of a maximal rise in GH, gluconeogenic substrate clearance rates are substantially higher in *Goat*$^{-/-}$ mice which, in the setting of severe calorie restriction, leads to a feed-forward cycle that lowers levels of both blood glucose and plasma gluconeogenic substrates.

Chapter IV describes a second series of studies that examine the mechanism by which ghrelin release is controlled. Two ghrelin-secreting cell lines were established from mice engineered to develop ghrelin secreting tumors. Using this system, we found that the control of ghrelin secretion is mediated by β_1-adrenergic receptors on surface of ghrelin secreting cells. Adrenergic agonists such as norepinephrine and epinephrine bind to β_1 receptors to stimulate ghrelin release. The addition of atenolol, a selective β_1-adrenergic antagonist, blocks stimulation of ghrelin secretion by norepinephrine. We confirmed the results in *vivo* by showing that both atenolol and reserpine, a drug that blocks catecholamine release from pre-synaptic sympathetic neurons, inhibit the fasting-induced rise in plasma ghrelin in mice.

The sum of our data describes a novel and highly conserved system by which glucose homeostasis is maintained in times of famine (Fig. 5-1). During calorie restriction, low blood glucose levels lead glucose sensing neurons to stimulate the sympathetic nervous system to release norepinephrine onto β_1-adrenergic receptors of ghrelin secreting cells. This results in the release of ghrelin which then acts to stimulate the release of GH. The increased levels of plasma GH is essential to protect against the development of hypoglycemia and act, at least in part, to preserve plasma levels of the gluconeogenic substrates lactate and pyruvate.

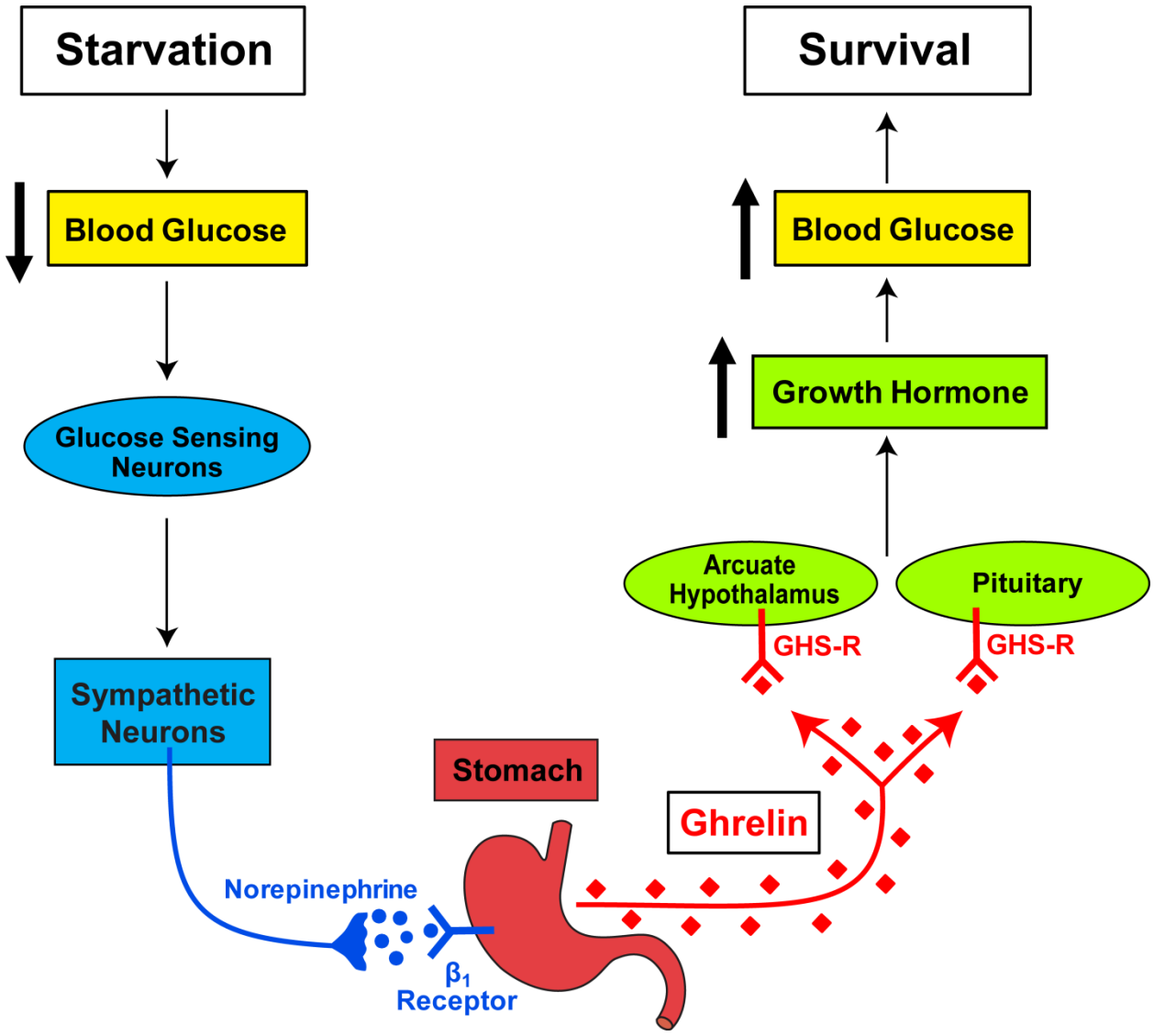

Figure 5-1. Model for the protective role of ghrelin and GH during calorie restriction. During starvation, glucose sensing neurons react to low blood glucose levels by stimulating sympathetic neurons to release norepinephrine onto β_1-adrenergic receptors of ghrelin secreting cells in the stomach. Rising levels of ghrelin stimulate the release of GH which acts to preserve blood glucose and ensure survival.

The still unanswered question is the mechanism by which GH is protective against hypoglycemia. Current research on the relationship between GH and glucose are conflicting. One study showed that an acute injection of GH during exercise exaggerates post-exercise increases in plasma lactate (1) while other studies have suggested that GH increases the rate of gluconeogenesis (2-7). GH is also known to inhibit glucose uptake acutely, increase lipid oxidation, and suppress glucose oxidation (8-14). Thus, there is evidence that GH can preserve blood glucose in 3 ways: (*i*) by increasing release of gluconeogenic substrates, (*ii*) by increasing the rate of gluconeogenesis, and (*iii*) by decreasing the rate of glucose oxidation, directly or indirectly through increased lipolysis. Additional studies are required to determine which of these modalities of GH action are present in calorie restricted mice.

In the setting of severe calorie restriction in which adipose tissue is depleted, the deficit in calories between the food given and the energy required for viability can only be made up by the release of amino acids through protein catabolism (5). The urinary excretion of the amino acid 3-methylhistidine, a component of actin and myosin, has been used as an index for the rate of muscle protein breakdown (15). Assaying the urinary excretion of 3-methylhistidine in calorie restricted mice could help answer the question of whether the stimulation of GH release during calorie restriction increases the release of gluconeogenic substrates.

Various methods exist to assay the rates of gluconeogenesis and glucose oxidation i*n vivo*. These include a glucose turnover study in which [^{13}C]glucose is infused to calorie restricted mice and mass spectrometry or nuclear magnetic resonance (NMR) is used to examine the quantities of ^{13}C-labeled metabolites that are produced. This method can also be adapted to measure glucose production and tissue glucose uptake. In addition to *in vivo* experiments, the rates of gluconeogenesis and glucose oxidation can be determined on a cellular level by isolating primary

hepatocytes or mitochondria from calorie restricted mice and measuring their respective production of [^{14}C]glucose from [^{14}C]pyruvate or [^{14}C]CO_2 from [^{14}C]pyruvate. Performing these experiments would help define the roles that gluconeogenesis and glucose oxidation play in the development of hypoglycemia in calorie restricted *Goat*$^{-/-}$ mice and reveal their relationship to changes in the plasma levels of GH.

Elucidating the relationship between ghrelin, GH, and glucose homeostasis has important clinical implications for the treatment of diabetes. In the last 20 years, bariatric surgery has gained popularity as an effective tool for the management of diabetes, with nearly 200,000 people each year undergoing the procedure (16). Two general forms of bariatric surgery are commonly performed: (*i*) gastric restrictive procedures such as gastric banding in which the size of the stomach is physically restricted, and (*ii*) gastrointestinal bypass procedures such as the Roux-en-Y gastric bypass (RYGB) in which the proximal stomach is connected to the jejunum so that ingested food bypasses the absorptive cells of the proximal small intestine. Both forms of bariatric surgery result in sustained 10-year weight losses of 14 – 25%, improvements in diabetic morbidities, and a significant improvement in overall mortality up to 16 years (hazard ratio, 0.76 vs. control) (17). However, whereas weight loss is thought to be the dominant means by which diabetes is resolved in patients with gastric banding (18), patients with a RYGB often become euglycemic within days after surgery, before any significant weight loss occurs (19, 20). Hormonal changes are thought to mediate these early metabolic improvements; satiety signals such as peptide YY and glucagon like peptide-1 (GLP-1) increase in patients with a RYBG (21, 22). Interestingly, patients with a RYBG also show a dramatic 72–77% post-surgery reduction in plasma ghrelin levels and a complete loss of the normal prandial and circadian oscillations associated with ghrelin secretion (23).

Our data show that *Goat*$^{-/-}$ mice have better oral glucose tolerance and higher insulin secretion vs. their WT littermates, especially in the setting of high-fat diet induced obesity (Fig. 2-7). Thus, there could be a link between the ablation of ghrelin secretion in patients with gastric bypass and the dramatic resolution of their diabetic symptoms. Although it has been suggested that the anti-diabetic effects of ghrelin loss in patients with gastric bypass are mediated via decreased appetite stimulation (23), our data show no loss of appetite in *Goat*$^{-/-}$ mice but instead directly links the maintenance of blood glucose levels during caloric restriction with the ghrelin-mediated secretion of GH.

Diabetes is characterized by the maintenance of pathologically high levels of blood glucose. A better understanding of the pathway by which GH maintains blood glucose levels during caloric restriction could lead to novel treatments for this disease. It would be an ironic twist of fate if a hormone vital for survival during caloric restriction could lead to the treatment of a disease caused by caloric excess.

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