

Reduced autophagy in livers of fasted, fat-depleted, ghrelin-deficient mice: Reversal by growth hormone

Yuanyuan Zhang, Fei Fang, Joseph L. Goldstein¹, Michael S. Brown¹, and Tong-Jin Zhao

Department of Molecular Genetics, University of Texas Southwestern Medical Center, Dallas, TX 75390

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Plasma growth hormone (GH) and hepatic autophagy each have been reported to protect against hypoglycemia in the fasted state, but previous data have not linked the two. Here we demonstrate a connection using a mouse model of fasting in a fat-depleted state. Mice were subjected to 1 wk of 60% calorie restriction, causing them to lose nearly all body fat. They were then fasted for 23 h. During fasting, WT mice developed massive increases in plasma GH and a concomitant increase in hepatic autophagy, allowing them to maintain viable levels of blood glucose. In contrast, lethal hypoglycemia occurred in mice deficient in the GH secretagogue ghrelin as a result of knockout of the gene encoding ghrelin *O*-acyltransferase (GOAT), which catalyzes a required acylation of the peptide. Fasting fat-depleted *Goat*^{−/−} mice showed a blunted increase in GH and a marked decrease in hepatic autophagy. Restoration of GH by infusion during the week of calorie restriction maintained autophagy in the *Goat*^{−/−} mice and prevented lethal hypoglycemia. Acute injections of GH after 7 d of calorie restriction also restored hepatic autophagy, but failed to increase blood glucose, perhaps owing to ATP deficiency in the liver. These data indicate that GH stimulation of autophagy is necessary over the long term, but not sufficient over the short term to maintain blood glucose levels in fasted, fat-depleted mice.

ghrelin *O*-acyltransferase | *Goat*^{−/−} mice | calorie restriction | hypoglycemia

When animals undergo a complete fast, adaptive mechanisms maintain the supply of energy to vital organs. The classic metabolic response to an acute fast was delineated in humans more than 50 y ago, largely through the work of George Cahill and colleagues (1, 2). The initial response to fasting is glycogenolysis, which releases glucose from liver and muscle. At the same time, the liver begins to synthesize and release glucose *de novo*, initially using lactate, which is returned to the liver from muscle through the Cori cycle. Shortly thereafter, lipolysis is activated in adipose tissue, releasing fatty acids for combustion in muscle and for the supply of energy to fuel gluconeogenesis in the liver. Hepatic fatty acid oxidation also produces the ketone bodies acetoacetate and beta-hydroxybutyrate, which can partially replace glucose for energy in brain and muscle. This entire process is orchestrated by hormones, primarily through a drop in insulin and increases in glucagon, which activates gluconeogenesis in liver (2), and growth hormone (GH), which stimulates lipolysis in adipose tissue (3).

In recent years, another source of gluconeogenic substrates in liver has been identified, namely autophagy (4, 5). Autophagy is the process by which intracellular double-membrane vesicles called autophagosomes ingest cytosolic proteins and organelles (6). Autophagosomes fuse with lysosomes, creating single-membrane bounded autolysosomes that expose the ingested contents to hydrolases that break down the ingested macromolecules. Fasting induces autophagy in liver (4). Some of the autophagic end products are combusted to produce energy, and some are used as substrates for gluconeogenesis. When autophagy is prevented through germline deletion of a required protein, animals develop profound hypoglycemia on fasting (4, 7, 8).

Previous studies of fasting have been conducted in humans or animals that maintain adequate adipose tissue, allowing a continuous supply of fatty acids to liver and muscle. We know little

about the maintenance of blood glucose when adipose triglycerides are exhausted and fatty acids are not available. Such a fat-depleted condition occurs in humans subjected to prolonged calorie deprivation through famine, anorexia, or other causes of cachexia (9, 10).

Recently, our laboratory created an experimental model of calorie restriction in fat-depleted C57BL/6 mice (11–13). In this protocol, mice are fed daily with 40% of the chow diet that the mouse would normally consume. We call this 60% calorie restriction. The mice are fed each day at 6:00 PM. Within 1 d, they become ravenously hungry, and consume all of their food by 7:00 PM. They are then fasted for the next 23 h until they are fed once again. This cycle is repeated each day for 7–9 d. We measure blood glucose each day at 5:30 PM, after the 23-h fast and immediately before the next feeding.

Over the first 4 d of this regimen, WT mice lose ~30% of their body weight, which then stabilizes. By day 4, their body fat, as measured by NMR, constitutes <2% of body weight (11, 13). By day 5 or 6, fasting blood glucose values at 5:30 PM fall to 40–60 mg/dL, and are maintained at that level through day 8. Despite this moderate hypoglycemia, WT mice are alert and active. They have very low concentrations of free fatty acids in plasma (≤0.07 mM) and ketone bodies (≤0.12 mM). The hormonal response includes a marked decrease in insulin, increase in glucagon, and increases in ghrelin and GH, with the latter two rising progressively through 8 d (11, 13).

Survival of these fasted, fat-depleted WT mice requires ghrelin, which acts by stimulating secretion of GH (11). Ghrelin, a 28-aa peptide produced primarily by enteroendocrine cells in the stomach and duodenum, acts in the arcuate nucleus of the hypothalamus and in somatotrophs in the pituitary to release GH (14–16). Ghrelin activity requires ghrelin *O*-acyltransferase (GOAT), which attaches octanoate, an eight-carbon fatty acid, to serine-3 of ghrelin before secretion (17, 18). Mice with germline

Significance

Famine kills millions of people each year. Survival requires the maintenance of blood glucose. Famine depletes body fat, thereby removing a source of energy for hepatic glucose production. Here we used a mouse model of fat depletion to show that growth hormone (GH) maintains blood sugar by stimulating hepatic autophagy, the process by which the liver digests its organelles to provide energy and substrates for producing glucose. When fat-depleted mice are fasted, their stomachs secrete ghrelin, a GH secretagogue. The resultant elevation in GH stimulates hepatic autophagy. In mice lacking ghrelin, GH fails to rise appropriately, hepatic autophagy is reduced, and mice die from hypoglycemia. These studies demonstrate a ghrelin-GH-autophagy axis that is required for survival in famine.

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The authors declare no conflict of interest.

¹To whom correspondence may be addressed. Email: joe.goldstein@utsouthwestern.edu or mike.brown@utsouthwestern.edu.

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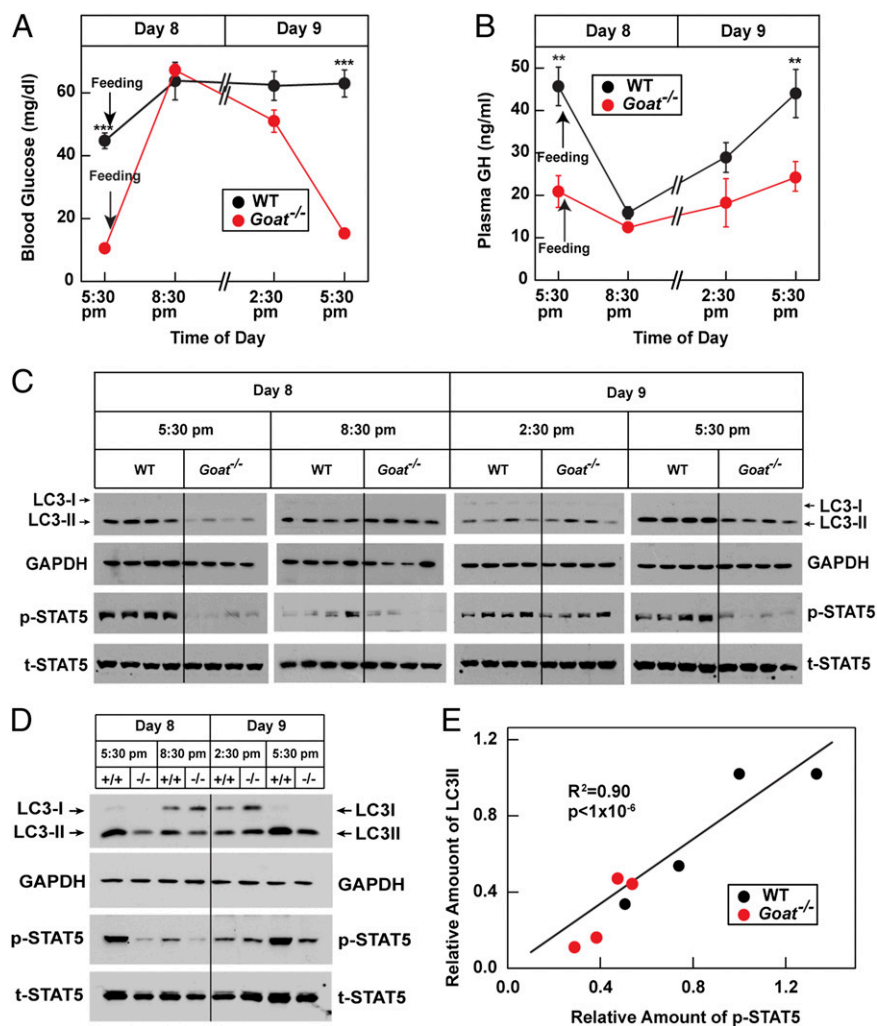


Fig. 1. Levels of blood glucose (A), plasma GH (B), and hepatic LC3-II (C–E) before and after feeding in calorie-restricted WT and *Goat*^{−/−} mice. Sixteen male WT and 16 *Goat*^{−/−} littermates (8 wk old) were subjected to 60% calorie restriction for 8–9 d as described in *Materials and Methods*. Four mice from each group were killed at the indicated time. (A and B) At 1 h before sacrifice, mice were injected i.p. with leupeptin (15 mg/kg). Immediately before sacrifice, blood was obtained from the retro-orbital sinus for measurement of blood glucose and plasma GH. Each value in A and B represents mean \pm SEM of data from four mice. Asterisks denote the level of statistical significance (Student *t* test) between WT and *Goat*^{−/−} mice: ***P* < 0.01; ****P* < 0.001. (C–E) Liver supernatants were processed as described in *Materials and Methods*, after which individual (C) or pooled (D) supernatants were subjected to immunoblotting with the indicated antibody. For immunoblotting of LC3 and GAPDH, 20 μ g of liver supernatant was loaded in each lane; for p-STAT5 and t-STAT, 60 μ g was loaded. Each pooled sample in D was derived from equal amounts of the four individual samples in C. (E) Relationship between the amounts of LC3II and p-STAT5. The relative amounts of LC3II (normalized to GAPDH) and p-STAT5 (normalized to total STAT5) were quantified by scanning the autoradiograms in D. The data were analyzed with ImageJ.

deletions of *ghrelin* or *Goat* are unable to maintain viable levels of blood glucose when subjected to 60% calorie restriction as described above (11, 13). The calorie-restricted, ghrelin-deficient mice lose body weight and body fat as rapidly as WT mice, and become just as hungry, consuming all of their food within 60 min. The ghrelin-deficient mice are unable to stabilize their blood glucose. By day 7–8, their fasting blood glucose falls below 20 mg/dL. It rises promptly after each meal, only to fall progressively during the next 23-h fast. Profound hypoglycemia can be prevented by chronic infusion of either ghrelin or GH, begun before the onset of calorie restriction and maintained throughout (11, 13).

Tracer experiments demonstrate that hypoglycemia in ghrelin-deficient, fat-depleted mice is caused by reduced production of glucose rather than by enhanced clearance (13). Plasma lactate, the major substrate for gluconeogenesis, falls in parallel with blood glucose (13). On day 7 of calorie restriction, blood glucose can be restored to WT levels by injections of pyruvate, lactate, or alanine, which provide energy and substrates for gluconeogenesis. Blood glucose is also restored by octanoate, a fatty acid that cannot be converted to glucose but can be oxidized to provide energy (13).

Inasmuch as both hepatic autophagy and plasma GH are required to maintain blood glucose during fasting, the present study was designed to test the hypothesis that GH stimulates hepatic autophagy in starved, fat-depleted mice. The results demonstrate that hepatic autophagy is diminished in ghrelin-deficient mice and can be restored acutely by GH infusion. Acute restoration of

hepatic autophagy does not prevent hypoglycemia, however. These results raise the possibility that GH-stimulated hepatic autophagy is necessary, but not sufficient, to maintain blood glucose in fasted, fat-depleted mice.

Results

The experiment shown in Fig. 1 was performed to assess the relationships among fat depletion, starvation, plasma GH level, and indicators of autophagy in livers of WT and *Goat*^{−/−} mice. Sixteen WT and 16 *Goat*^{−/−} mice were depleted of fat by being placed on a diet containing 40% of the calories that they normally consumed. We previously showed that body fat in both groups falls to <2% of body weight by day 5 on this diet (11, 13). Mice were fed each day at 6:00 PM. As a result of the calorie restriction, the WT and *Goat*^{−/−} mice were hungry, and they consumed all of their food within 1 h. By 5:30 PM each day, the mice had been fasted for 23 h. At the time points indicated in Fig. 1A, groups of four WT mice and four *Goat*^{−/−} mice were killed for measurement of blood and liver parameters. As shown in Fig. 1A, at the 5:30 PM time point on day 8, blood glucose in WT mice averaged 45 mg/dL, compared with only 11 mg/dL in the *Goat*^{−/−} mice. By 8:30 PM, 2.5 h after feeding, blood glucose in both groups had risen to 65 mg/dL. At 2:30 PM on the next afternoon (day 9), blood glucose in the *Goat*^{−/−} mice was maintained at 51 mg/dL. Over the next 3 h, it plummeted to 15 mg/dL. In contrast, the WT mice were able to maintain their blood glucose levels throughout the day in the range of 50–68 mg/dL.

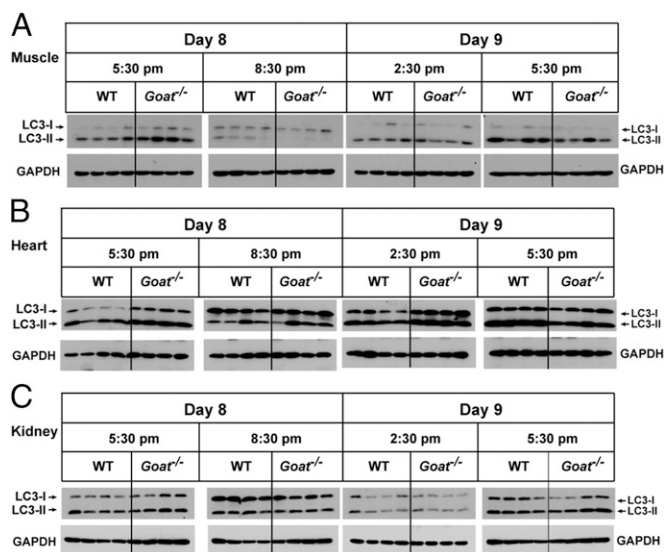


Fig. 2. LC3 levels in muscle (A), heart (B), and kidney (C) before and after feeding in calorie-restricted WT and *Goat*^{-/-} mice. The mice used in this experiment are the same as those used in the experiment shown in Fig. 1. Tissues were processed as described in *Materials and Methods*, after which 20 μ g of each supernatant was subjected to immunoblot analysis with the indicated antibody.

Plasma GH levels are shown in Fig. 1B. As we reported previously (11, 13), at the 5:30 PM time point on day 8, plasma GH was markedly elevated in WT mice and much lower in *Goat*^{-/-} mice. After feeding, GH levels fell sharply in WT mice, reaching the same level as in *Goat*^{-/-} mice. On the next day, GH levels

rose progressively in WT mice, reaching a peak at 5:30 PM. The rise in the *Goat*^{-/-} mice was much less pronounced.

As an index of autophagy in liver, we used immunoblotting to estimate the relative amount of microtubule-associated protein 1A/1B light chain 3 (LC3). This protein exists in two forms: LC3-I, a soluble cytosolic form, and LC3-II, a form coupled covalently to phosphatidylethanolamine and inserted into autophagosomal membranes (19). To prevent lysosomal degradation of LC3-II, we injected all mice with leupeptin, a lysosomal protease inhibitor, at 1 h before sacrifice (4). Under these conditions, the amount of LC3-II reflects the overall rate of autophagy (19). Immunoblotting data for each of the 32 individual mice are shown in Fig. 1C. In addition, equal aliquots from the individual extracts in each of the eight groups were pooled and immunoblotted (Fig. 1D). In WT mice, the LC3-II level was highest at 5:30 PM on both days, and it fell by 8:30 PM (Fig. 1C and D). Compared with WT mice, *Goat*^{-/-} mice had a reduced LC3-II level at 5:30 PM, and the level increased at 8:30 PM after feeding (Fig. 1C and D).

Two closely related genes, *LC3A* and *LC3B*, encode LC3-I and LC3-II (20). To determine whether the difference in LC3-II in the liver of WT and *Goat*^{-/-} mice was the result of transcriptional changes in *LC3A* and/or *LC3B*, we checked their mRNA expression levels and found no difference at various time points on days 8 and 9 of calorie restriction (Fig. S1). These data suggest that the decreased LC3-II level in *Goat*^{-/-} mice is a reflection of a deficiency of autophagy rather than of reduced gene expression. We also checked LC3-II levels in skeletal muscle, heart, and kidney from the same experiment described in Fig. 1. In muscle and kidney, the LC3-II level was highest at 5:30 PM, but there was no difference in level between WT and *Goat*^{-/-} mice (Fig. 2). In the heart, LC3-II level did not change during the 24-h period, and it was similar in WT and *Goat*^{-/-} mice.

GH signals by inducing phosphorylation of the transcription factor STAT-5 (21). Immunoblotting revealed elevated phospho-STAT-5

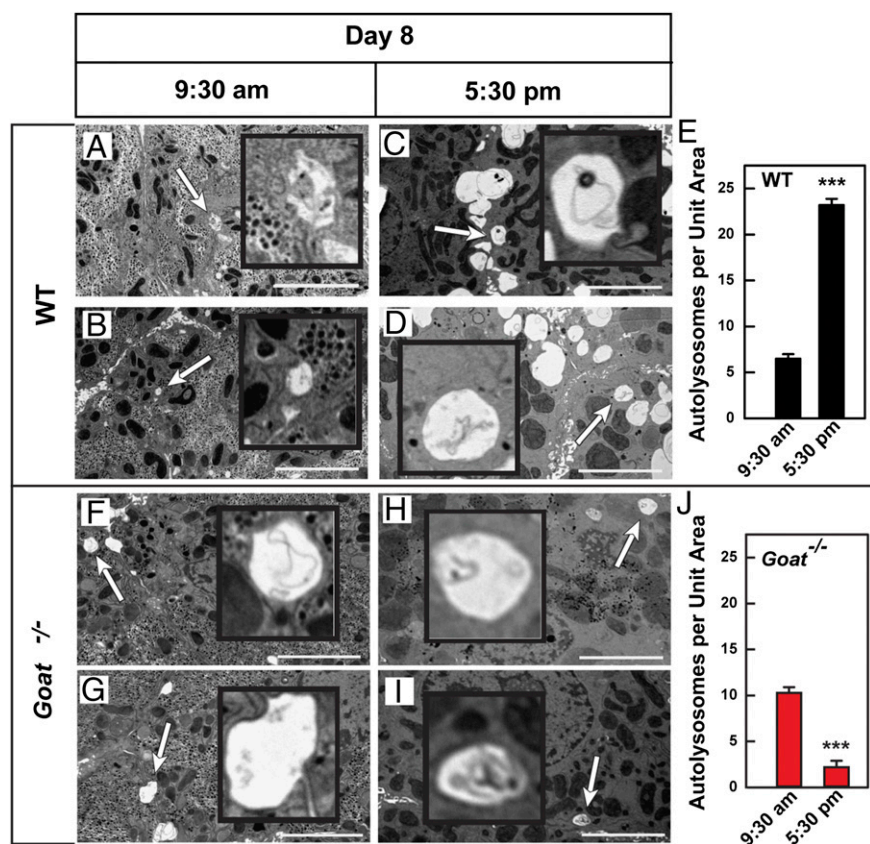


Fig. 3. Electron micrographs of liver from calorie-restricted WT (A–D) and *Goat*^{-/-} (E–F) mice at 9:30 AM and 5:30 PM on day 8. WT and *Goat*^{-/-} littermates (8 wk old) were subjected to 60% calorie restriction for 8 d. Mice were perfused at 9:30 AM or 5:30 PM on day 8 of calorie restriction, after which the liver was fixed and processed as described in *Materials and Methods*. Pictures from 20 different unit areas (36 \times 26 μ m per unit area) from two WT and two *Goat*^{-/-} livers at each time point were taken at 5,000 amplitude; representative pictures are shown. White arrows denote typical autolysosomes. (Scale bar: 5 μ m; magnification, 5,000 \times .) (Insets) Enlarged images of each arrow-denoted autolysosome (magnified 5 \times). (E and F) The number of autolysosomes per unit area was determined by three independent examiners as described in *Materials and Methods*. Each value represents mean \pm SEM of data from 20 images. Asterisks denote level of statistical significance (Student *t* test) between autolysosome numbers at 9:30 AM and 5:30 PM of WT (E) and *Goat*^{-/-} (F) mice: ****P* < 0.001.

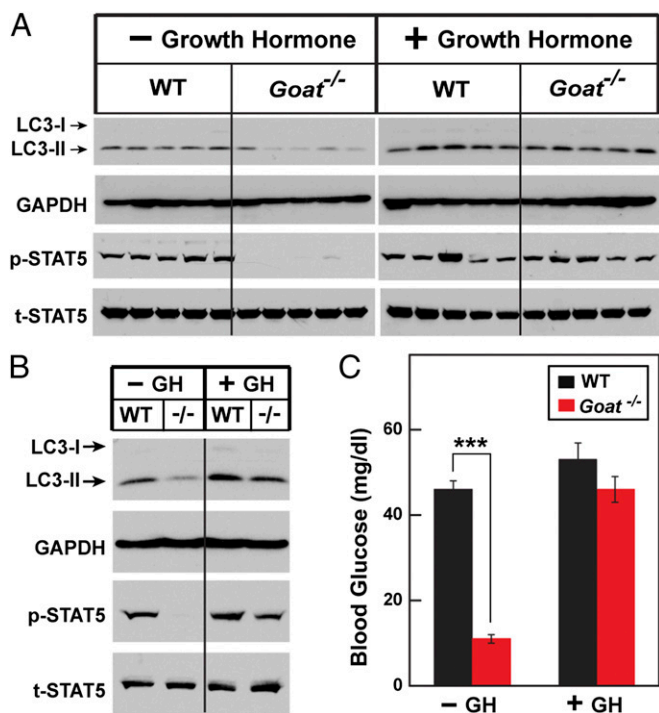


Fig. 4. Preservation of autophagy in calorie-restricted *Goat*^{-/-} mice by infusion of GH. Alzet osmotic minipumps containing either vehicle or GH (15 μ g/24 h) were implanted at 3 d before calorie restriction as described in *Materials and Methods*. Mice were then subjected to 60% calorie restriction for 8 d. Mice were dissected at 5:30 PM on day 8, at 1 h after leupeptin was injected i.p. (A and B) Immunoblot analysis with the indicated antibody was carried out on liver supernatants from individual mice (A) or pooled supernatants (B) from the five individual mice as described in Fig. 1. (C) Blood glucose levels were measured at 5:30 PM on day 8. Each value represents mean \pm SEM of data from five mice. Asterisks denote level of statistical significance (Student *t* test) between WT and *Goat*^{-/-} mice infused with vehicle or GH: ****P* < 0.001.

(p-STAT5) levels in WT livers at 5:30 PM on both days (Fig. 1 C and D), which correlates with the elevated plasma GH levels at those times (Fig. 1B). In contrast, p-STAT5 was relatively low in *Goat*^{-/-} livers at all time points. Fig. 1E plots the relative amount of LC3-II vs. the relative amount of p-STAT5 in livers from the eight groups of mice as determined by scans of the immunoblots. The plot reveals a strong correlation between LC3-II level and p-STAT5 level ($R^2 = 0.90$). Inasmuch as p-STAT5 is produced by GH, the data suggest that GH may be responsible for the increase in autophagy in fat-depleted, WT mice subjected to a 23-h fast.

To confirm that the reduction in LC3-II reflects reduced autophagy in livers of calorie-restricted, fat-depleted *Goat*^{-/-} mice, we used electron microscopy (EM) to examine livers from WT and *Goat*^{-/-} mice that had been starved for 16 h or 23 h on

day 8 of calorie restriction. At 9:30 AM, after 15 h of starvation, livers from WT and *Goat*^{-/-} mice had very few autolysosomes (Fig. 3 A, B, F, and G). By 5:30 PM, after 23 h of starvation, the number of autolysosomes in WT livers had increased markedly (Fig. 3 C and D) compared with those in *Goat*^{-/-} livers (Fig. 3 H and I). Autolysosomes (denoted by white arrows) were defined by their characteristic single membranous vacuole-containing amorphous and multilamellar structures (shown in *Insets*, magnified fivefold). To quantify the data in Fig. 3, we asked three colleagues to count the number of autolysosomes in random EM images. The colleagues were blinded as to the source of the specimens. In the images from WT mice, the observers found that the mean number of autolysosomes per image had increased fourfold, from 6 at 9:30 AM to 23 at 5:30 PM (Fig. 3E). In striking contrast, the mean autolysosome count in the *Goat*^{-/-} livers decreased by 80%, from 10 autolysosomes per image at 9:30 AM to 2 at 5:30 PM (Fig. 3J).

We previously reported that hypoglycemia is prevented in calorie-restricted, fat-depleted *Goat*^{-/-} mice through GH infusion throughout the period of calorie restriction (11, 13). To determine whether this chronic GH treatment prevents the loss of autophagy, we used an Alzet minipump to administer a constant infusion of GH beginning 3 d before the onset of calorie restriction. At 5:30 PM on day 8 of calorie restriction, blood glucose in vehicle-infused *Goat*^{-/-} mice was severely reduced compared with WT mice (11 mg/dL vs. 46 mg/dL) (Fig. 4C). The GH infusion had no effect on blood glucose in WT mice (52 mg/dL), but it prevented profound hypoglycemia in *Goat*^{-/-} mice (46 mg/dL). Mice were killed at 5:30 PM on day 8, and liver extracts were subjected to immunoblotting. In the vehicle-infused *Goat*^{-/-} mice, LC3-II levels were reduced both in individual mice (Fig. 4A) and in pooled extracts from all of the mice in each group (Fig. 4B). LC3-II levels were nearly as high in *Goat*^{-/-} mice that received the GH infusion as in WT mice. p-STAT5 levels were low in vehicle-infused *Goat*^{-/-} mice, and were returned to near-normal levels by GH infusion.

To determine whether acute s.c. injections of GH would have the same effects as chronic infusions, we injected calorie-restricted WT and *Goat*^{-/-} mice with GH on day 7 at 2:30 PM, 3:30 PM, and 4:30 PM and examined liver autophagy using EM. In the absence of GH injection, WT livers (Fig. 5 A and B) had significantly more autolysosomes than *Goat*^{-/-} livers (Fig. 5 F and G), consistent with the results shown in Fig. 2. Acute injections of GH had no effect on the number of autolysosomes in WT livers (Fig. 5 A–E), but markedly increased the number of autolysosomes in *Goat*^{-/-} livers (Fig. 5 F–J). Despite the increase in hepatic autophagy, GH did not restore blood glucose levels in the *Goat*^{-/-} mice (average values, 20 mg/dL in vehicle-injected mice and 16 mg/dL in GH-injected mice; also see Fig. 5K).

To confirm that the acute injections of GH did not desensitize hepatic GH receptors, we treated WT and *Goat*^{-/-} mice exactly as shown in Fig. 5 A–J and then assayed the livers for p-STAT5. On day 7 of calorie restriction at 5:30 PM, *Goat*^{-/-} mice showed a decrease in immunoblotable p-STAT5 that subsequently increased markedly after the three acute injections of GH (Fig. 5L), indicating that hepatic GH receptors were not desensitized by the repeated GH injections.

Table 1. Liver content of ATP, ADP, and AMP in calorie-restricted WT and *Goat*^{-/-} mice injected with GH

Genotype	Liver content, nmol/mg protein					
	ATP		ADP		AMP	
	–GH	+GH	–GH	+GH	–GH	+GH
WT	2.3 \pm 0.2	2.6 \pm 0.1	2.6 \pm 0.3	2.5 \pm 0.3	3.5 \pm 0.7	2.8 \pm 0.6
<i>Goat</i> ^{-/-}	1.6 \pm 0.1**	1.5 \pm 0.1***	3.2 \pm 0.4	3.3 \pm 0.2*	7.9 \pm 1.4**	8.3 \pm 1.3***

These data were obtained from the mice described in Fig. 6 (13 mice in each group). Liver content of ATP and ADP was measured as described in *Materials and Methods*. Liver content of AMP was calculated according to Burgess et al. (22). Each value represents mean \pm SEM of data from 13 mice. Asterisks denote level of statistical significance between vehicle or GH-injected WT vs. *Goat*^{-/-} mice: **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

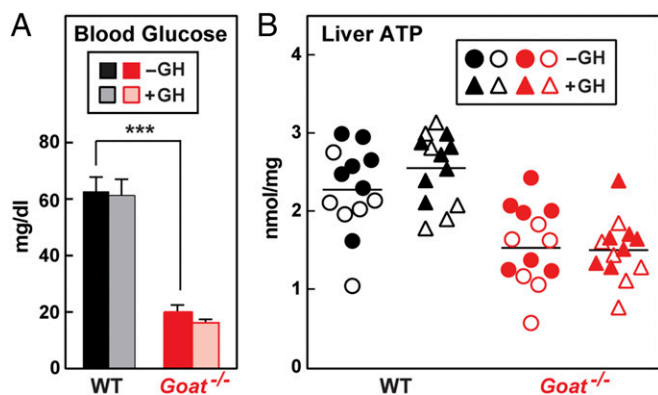


Fig. 6. Failure to restore liver content of ATP in calorie-restricted *Goat*^{-/-} mice by acute injection of GH. Male WT and *Goat*^{-/-} mice littermates (8 wk old) were subjected to 60% calorie restriction for 8 d. On day 8, each mouse received three s.c. injections of vehicle (-GH) or 30 μ g of GH (+GH) at 2:30 PM, 3:30 PM, and 4:30 PM. Mice were killed at 5:30 PM, liver samples were freeze-clamped, and blood glucose level (A) and hepatic content of ATP (B) were measured as described in *Materials and Methods*. In A, individual values from two independent experiments ($n = 13$ mice) were combined (mean \pm SEM; *** $P < 0.001$). In B, each individual value from the two experiments is shown. Results from one experiment are denoted by open symbols; results from the other experiment, by closed symbols.

infusion of GH, started before the onset of calorie restriction, prevents hypoglycemia in the ghrelin-deficient mice, indicating that the major role of ghrelin is to facilitate the secretion of GH (11).

The link between GH and hepatic autophagy was established by the experiment shown in Fig. 1. After 8 d of calorie restriction in WT mice, GH levels rose during the 23-h period when food was not available, peaking at 5:30 PM just before the animals were fed and declining immediately thereafter. The rise in GH was paralleled by a rise in hepatic p-STAT5, the mediator of GH action (21), and in LC3-II, a marker of autophagy (19). In *Goat*^{-/-} mice deficient in ghrelin, the fasting-induced rise in GH was blunted, and there were corresponding decreases in p-STAT5 and LC3-II levels ($R^2 = 0.9$), supporting the notion that the rise in GH was responsible for the increase in autophagy (Fig. 1E). The changes in LC3-II were mirrored by changes in autophagic vacuoles in liver as assessed by EM (Fig. 3).

Hepatic autophagy was maintained, and hypoglycemia was prevented when the ghrelin-deficient mice were subjected to continuous infusion of GH, started before calorie restriction and continued throughout (Fig. 4). Autophagy was also restored

when three injections of GH were given at hourly intervals to the ghrelin-deficient mice (Fig. 5); however, these injections were unable to raise the blood glucose level (Fig. 6A). A possible explanation for this finding is provided by the observation that hepatic ATP was depleted and AMP accumulated on day 8 in the fasted, ghrelin-deficient mice, neither of which was restored to normal by the GH injections (Table 1).

Considered together, the foregoing data suggest that GH maintains blood glucose levels in these calorie-restricted mice in part by activating hepatic autophagy. However, once the animals have become depleted of fat, the increase in hepatic autophagy is not sufficient to restore blood glucose. In addition to autophagy, a source of ATP is required. Indeed, we have shown previously that blood glucose is restored to WT levels when the fasted, fat-depleted, ghrelin-deficient mice are injected acutely with sources of energy that include lactate, pyruvate, alanine, and octanoate (13).

Although GH and hepatic autophagy are recognized to be important factors in maintaining blood glucose level during fasting, we are unaware of previous data linking these two agents. During an ordinary fast, GH is thought to support blood glucose primarily by activating adipose tissue lipolysis, thereby providing fatty acids that the liver can use as a source of energy to fuel gluconeogenesis (3). Our mice represent a different phenomenon, because the week-long calorie restriction depletes fat stores, making lipolysis an inadequate source of energy. Under these fat-depleted conditions, GH-induced hepatic autophagy may assume a greater role in maintaining blood glucose than it does in animals with adequate fat stores.

A major unanswered question is why chronic GH infusion is able to prevent hypoglycemia, whereas acute injections of GH fail to do so, even though both treatments raise hepatic p-STAT5 and increase hepatic autophagy. It may be that maintenance of blood glucose requires the presence of GH during the time when fat stores are being depleted. Perhaps GH acts to preserve some hidden store of energy during this depletion period. Experiments designed to solve this puzzle are underway.

Materials and Methods

The following previously described methods are summarized in *SI Materials and Methods*: generation of *Goat*^{-/-} mice, protocol for 60% caloric restriction, GH administration, and measurement of metabolic parameters. Detailed information on electron microscopy for quantifying autolysosomes, immunoblotting, and real-time PCR is provided in *SI Materials and Methods*.

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Supporting Information

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SI Materials and Methods

Materials. We obtained Contour blood glucose test strips from Bayer, protease inhibitor mixture tablets from Roche, phosphatase inhibitor mixtures I and II from Calbiochem, and leupeptin from Sigma-Aldrich. All other chemicals were obtained from Sigma-Aldrich unless specified otherwise.

Mice. *Goat*^{-/-} mice were generated as described previously (1). All mice were housed in colony cages under a 12-h light/12-h dark cycle. The dark cycle began at 9 or 10:00 PM. The chow diet (Teklad Mouse/Rat Diet 7002; Harlan Teklad Global Diets) contains 3.0 kcal/g of metabolizable energy, of which 18% of calories are from fat, 49% are from carbohydrates, and 33% are from protein. All animal experiments were performed with approval of the University of Texas Southwestern Medical Center's Institutional Animal Care and Research Advisory Committee.

Calorie Restriction. Calorie restriction was implemented as described previously (1). At 1 wk before the initiation of calorie restriction, 8-wk-old male WT and *Goat*^{-/-} littermates 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 6:00 PM every day with an amount of food equal to 40% of the daily amount consumed by the same mouse during the week of acclimation.

Infusions and Injections. Infusion of GH was carried out as described previously (1). An Alzet osmotic pump (catalog no. 1002; DURECT) was inserted s.c. in the interscapular region of each mouse at 3 d before the initiation of calorie restriction. Each pump was filled with vehicle (70 mM sodium bicarbonate pH 9.5, 137 mM NaCl, and 100 µg/mL rat albumin) or vehicle containing 2.5 mg/mL recombinant rat GH (obtained from the National Hormone and Peptide Program of the National Institute of Diabetes and Digestive and Kidney Disorders through A. F. Parlow). GH was delivered at a rate of 0.25 µL/h (15 µg/24 h). For GH injection, GH (30 µg/mouse) or vehicle was injected s.c. at 2:30 PM, 3:30 PM, and 4:30 PM on day 7 of calorie restriction.

Metabolic Parameters. Blood glucose was measured from tail-vein blood using a Bayer blood glucose meter. For measurement of plasma GH, blood was drawn from the retro-orbital sinus and collected in EDTA-coated tubes. Plasma was separated, and GH plasma levels were measured with a commercial kit (Cayman; catalog no. 589601). For liver ATP and ADP measurements, the liver was freeze-clamped immediately after dissection. Approximately 50 mg of tissue was homogenized in 0.5 mL of ice-cold 3.5% perchloric acid and neutralized with 0.25 mL of 2.2 M potassium bicarbonate. The supernatant was used to measure ATP and ADP with commercial kits (Biovision; catalog nos. K354-100 and K355-100).

Electron Microscopy for Quantifying Autolysosomes. For each experiment, two WT and two *Goat*^{-/-} mice were anesthetized with pentobarbital at 9:30 AM or 5:30 PM on day 8 of calorie restriction. Their bodies were perfused with 25 mL of solution containing 4% (vol/vol) paraformaldehyde, 1% (wt/vol) glutaraldehyde, and 250 mM sucrose in 0.1 M cacodylate buffer (pH 7.4) injected into the left ventricle of the heart. Mice were then dissected, and tissues were fixed in a solution containing 0.1 M cacodylate (pH 7.4) and 2.5% glutaraldehyde. The samples were postfixed with 1% (wt/vol) OsO₄, embedded, and sectioned. Specimens were visualized on a JEOL 1200 EX transmission electron microscope. Twenty digital electron micrographs (10 from each mouse) were obtained from random areas at a magnification of 5,000×. An autolysosome was defined as a single membranous vacuole containing amorphous and multilamellar structures. The number of autolysosomes per unit area (36 × 26 µm) were counted by three examiners who were blinded to the experimental parameters. Their results varied by <2%. Each value represents mean ± SEM of data obtained from 20 images of WT or *Goat*^{-/-} mice.

Immunoblotting. For samples used for LC3 blotting, mice were injected i.p. with leupeptin (15 mg/kg) at 1 h before sacrifice (2). Frozen tissue samples (~50 mg) were homogenized on ice in 1 mL of lysis buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, and 1% (vol/vol) Triton X-100] supplemented with protease inhibitor mixture and phosphatase inhibitor mixtures I and II. Homogenates were then incubated on ice for 20 min and then centrifuged at 20,000 × g for 20 min at 4 °C. Supernatants were collected, and protein concentrations were determined using the Pierce BCA Protein Assay Kit. Samples were then denatured in SDS buffer, boiled at 95 °C for 5 min, and loaded onto SDS gels for immunoblotting. The following antibodies were used: LC3 (1/500 dilution; Novus; catalog no. 100-2331), GAPDH (1/10,000; Cell Signaling; catalog no. 5174); t-STAT5 (1/1,000; Cell Signaling; catalog no. 9363); p-STAT5 (1/1,000; Cell Signaling; catalog no. 9351). Blots were exposed to film for 1–10 s. Films were scanned and quantified using ImageJ (rsbweb.nih.gov/ij/).

Quantitative Real-Time PCR. Total RNA was isolated, and real-time PCR measurements were performed on pooled RNA samples from four mice in each group. Primer sequences of LC3A (ID no. 23956148a1) and LC3B (ID no. 13385664a1) were obtained from Primerbank (pga.mgh.harvard.edu/primerbank). All reactions were done in triplicate, with a mean range of variation of 0.40 ± 0.03% for all values. The relative amount of all mRNAs was calculated using the comparative threshold cycle (C_T) method, with 36B4 mRNA as the invariant control.

Reproducibility. All results were confirmed in two or three independent experiments.

1. Zhao T-J, et al. (2010) Ghrelin O-acyltransferase (GOAT) is essential for growth hormone-mediated survival of calorie-restricted mice. *Proc Natl Acad Sci USA* 107(16): 7467–7472.

2. Ezaki J, et al. (2011) Liver autophagy contributes to the maintenance of blood glucose and amino acid levels. *Autophagy* 7(7):727–736.

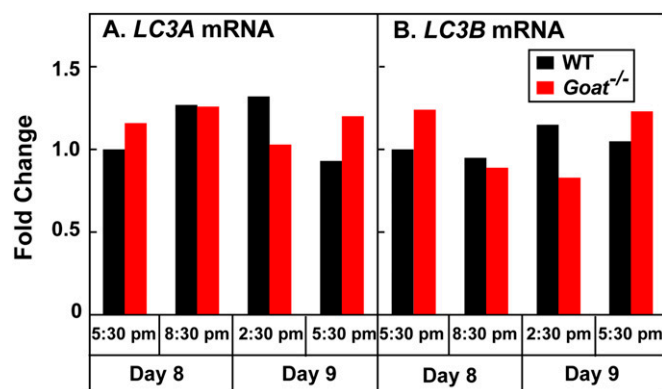


Fig. S1. Quantitative real-time PCR analysis of *LC3A* and *LC3B* mRNAs from calorie-restricted WT and *Goat*^{-/-} mice. The mice used in this experiment are the same as those used in the experiment described in Fig. 1. Liver total RNA was extracted, and quantitative real-time PCR was performed as described in *Materials and Methods*. Each measurement was made on pooled mRNA samples from four mice in each group. Expression of *LC3A* or *LC3B* was normalized to its expression in WT at 5:30 PM on day 8. The C_T values of *LC3A* and *LC3B* in WT mice at 5:30 PM on day 8 were 22.4 and 20.8, respectively. Similar results were obtained in an independent experiment.