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BRAIN GLYCOGEN SUPERCOMPENSATION IN THE MOUSE AFTER RECOVERY FROM INSULIN-INDUCED HYPOGLYCEMIA

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Abstract

Brain glycogen is proposed to function in both physiological and pathological conditions. Pharmacological elevation of this glucose polymer in brain is hypothesized to protect neurons against hypoglycemia-induced cell death. Elevation of brain glycogen levels due to prior hypoglycemia is postulated to contribute to the development of hypoglycemia-associated autonomic failure (HAAF) in insulin-treated diabetic patients. This latter mode of elevating glycogen levels is termed “supercompensation”. We tested whether brain glycogen supercompensation occurs in healthy, conscious mice after recovery from insulin-induced acute or recurrent hypoglycemia. Blood glucose levels were lowered to less than 2.2 mmol/L for 90 min by administration of insulin. Brain glucose levels decreased at least 80% and brain glycogen levels decreased approximately 50% after episodes of either acute or recurrent hypoglycemia. Following these hypoglycemic episodes, mice were allowed access to food for 6 or 27 hrs. After 6 hrs, blood and brain glucose levels were restored while brain glycogen levels were elevated 25% in mice that were previously subjected to either acute or recurrent hypoglycemia as compared with saline-treated controls. Following a 27 hr recovery period, the concentration of brain glycogen had returned to baseline levels in mice previously subjected to either acute or recurrent hypoglycemia. We conclude that brain glycogen supercompensation occurs in healthy mice but its functional significance remains to be established.

Keywords

hypoglycemia unawareness; hypoglycemia-associated autonomic failure; insulin

INTRODUCTION

Glucose levels in the blood are highly regulated to prevent deleterious effects associated with both hyperglycemia and hypoglycemia. Excess glucose is stored in tissues in the form of glycogen where it can be accessed rapidly in times of need (Roach 2002). Glycogen is found in all regions of adult brain, where it is located predominantly in the astrocytes (Brown 2004). The primary fuel source of brain is glucose, but glucose-6-P produced in astrocytes via glycogenolysis does not appear to be metabolized to glucose, likely due to very low glucose-6-phosphatase activity in the brain and cultured astrocytes (Dienel et al. 1988; Gotoh et al. 2000). Instead, glucose-6-P in cultured astrocytes is metabolized via glycolysis to generate glucose equivalents as lactate (Dringen et al. 1993). The glycogen present in the brain has been suggested to have the capacity to sustain glycolysis from 10

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min (Clark and Sokoloff 1999) to over 100 min (Gruetter 2003). How astrocytic glycogen is metabolized *in vivo* and how it aids surrounding neurons is more controversial (Brown 2004; Dienel and Cruz 2006). One hypothesis is that lactate produced in astrocytes is transported via monocarboxylate transporters to neurons where it is converted to pyruvate, which enters the citric acid cycle to generate ATP (Brown 2004; Cloix and Hevor 2009; Gruetter 2003).

Several physiological and pathological roles have been suggested for brain glycogen (Brown 2004; Brown and Ransom 2007; Hertz et al. 2007). Physiological roles include involvement in the sleep-wake cycle, the consolidation of memory, and neural stimulation. Pathological roles include involvement in seizure and utilization during ischemia and hypoglycemia. In hypoglycemia, brain glycogen is implicated in two processes, neuron function/survival and the development of hypoglycemia-associated autonomic failure (HAAF). In mouse optic nerve preparations, the level of astrocytic glycogen directly correlated with neuron function during aglycemia and hypoglycemia (reviewed in (Brown and Ransom 2007). Treatment of rats with a glucose-dependent inhibitor of glycogen phosphorylase caused an increase in brain glycogen during euglycemia that correlated with increased neuronal survival upon exposure to hypoglycemia, presumably due to utilization of elevated brain glycogen stores (Suh et al. 2007).

Recurrent hypoglycemia frequently leads to HAAF, putting patients at a 6-fold higher risk for developing severe hypoglycemia (Geddes et al. 2008) that can cause neuronal cell death in humans (Auer 2004) and rats (Puente et al. 2010; Suh et al. 2007; Tkacs et al. 2005). The mechanism for HAAF is not established but one hypothesis implicates brain glycogen stores. In anesthetized rats, hypoglycemia significantly reduced brain glycogen levels (Choi et al. 2003). As euglycemia was restored, brain glycogen increased to levels higher than those measured before hypoglycemia was induced, i.e. supercompensation (Choi et al. 2003). Choi et al hypothesized that these elevated brain glycogen levels would be available for metabolism during a subsequent bout of hypoglycemia making the brain less dependent on glucose, effectively lowering the glucose threshold at which the brain mounts a counterregulatory response. In contrast, another study using rats was unable to observe brain glycogen supercompensation after either acute or recurrent hypoglycemia (Herzog et al. 2008).

Thus, the role of brain glycogen in both cell survival and the development of HAAF is not completely understood. Given the potential of brain glycogen to function in the above processes, the lack of studies examining brain glycogen supercompensation in the whole mouse, and the utility of genetically modified mouse models, we examined the impact of both acute and recurrent hypoglycemia on brain metabolism in this popular model organism.

MATERIALS AND METHODS

Experimental animals

Three month-old male wild-type mice (68.75% C57B1/6J- 18.75% 129 × SVJ- 12.5% CBA) were used for these studies because they were readily available controls for our genetically modified mouse colony. Animals were maintained in temperature and humidity controlled conditions with a 12-hr light 12-hr dark cycle and were allowed food and water *ad libitum*, except where noted below. All procedures were approved by the Ball State University Animal Care and Use Committee.

Study design

To test the hypothesis that brain glycogen supercompensation occurs in mice after recovery from hypoglycemia, two studies were implemented. The first study was designed to determine if brain glycogen supercompensation occurs after an acute bout of hypoglycemia.

The second study was designed to determine if brain glycogen supercompensation occurs after recurrent, or multiple episodes, of hypoglycemia.

Acute hypoglycemia

Mice were fasted overnight for 17 hrs. Blood glucose levels were measured prior to injection of saline or insulin as well as 30, 60, 90, 120, and 150 min post-injection. Blood glucose levels were measured (HemoCue 201 Analyzer) in blood from the tail vein obtained following the removal of a small section of tail. Mice received intraperitoneal injections of either insulin (Humulin 70/30, 0.39 U/ml, 2U/kg body weight, 5 μ l/g body weight) or 0.9% saline (5 μ l/g body weight). Mice with blood glucose levels higher than 2.2 mmol/L at 30 min and/or 60 min after the initial insulin injection received an additional dose of insulin (0.5U/kg) following the blood glucose measurement at 30 and/or 60 min. Following the blood glucose measurement at 2.5 hrs post-injection, mice were either sacrificed or re-fed and sacrificed 6 or 27 hrs later. Mice were sacrificed by decapitating heads directly into liquid nitrogen. To remove frozen brain tissue, heads were placed on a metal plate cooled with liquid nitrogen. Frozen brain tissue was chipped out of the skull and transferred to tubes in liquid nitrogen. Frozen brain tissue was powdered in a tissue pulverizer cooled in liquid nitrogen. The powdering of whole brain tissue results in a homogenous mixture of the various regions of the brain. Tissue was stored at -80°C until analysis of brain glucose and glycogen levels.

Recurrent hypoglycemia

Injections and blood glucose readings were as described above. Mice were injected and blood glucose readings were taken on the first, fifth, and ninth day of the study. All mice were fasted overnight before each of these days of the study. After the final blood glucose reading on each day of injection, mice were given access to food and water. After the blood glucose measurement at 2.5 hrs post-injection on the ninth day, mice were either sacrificed immediately or re-fed and sacrificed 6 or 27 hrs later. Brain tissue was processed, as described above, for the determination of brain glycogen and brain glucose levels.

Brain Glucose Determination

Brain glucose was measured based on the method described by Passonneau and Lowry (Passonneau and Lowry 1993). Briefly, frozen powdered brain tissue (75–85 mg) was weighed and then incubated on ice with 250 μ l of ice cold 0.6 M HClO_4 and homogenized with a Tissue-Tearor (BioSpec Products) at maximum speed for 20 sec. Samples were then incubated on ice for 15 min and 5 μ l of 50 mM EDTA was added. The samples were centrifuged at $5,000 \times g$ at 4°C for 5 min. The supernatant was neutralized by adding 2M potassium carbonate and centrifuged at $5,000 \times g$ at 4°C for 5 min. The glucose concentration in this supernatant was determined with the method of Bergmeyer (Bergmeyer et al. 1974) as previously described by Suzuki et al (Suzuki et al. 2001).

Brain Glycogen Determination

Brain glycogen content was measured using a modification of the protocol by Hutchins and Rogers (Hutchins and Rogers 1970). Frozen brain tissue (40–50 mg) was weighed and then solubilized by adding 200 μ l of preheated (100°C) 30% potassium hydroxide and heated at 100°C for 30 min. After heating, the samples were cooled on ice and 65 μ l of cold 2% sodium sulfate and 540 μ l of cold 100% ethanol were added to precipitate glycogen. After incubation at -20°C for at least 20 min, tubes were heated at 100°C for 2 min and then centrifuged at $17,500 \times g$ at 4°C for 20 min. The pellet was re-suspended in 100 μ l of high purity water (Millipore) and 1 mL of 4:1 (v/v) methanol: chloroform was added to aid in lipid solubilization and glycogen precipitation. The samples were heated for 5 min at 80°C ,

cooled on ice and centrifuged at $17,500 \times g$ at 4°C for 15 min. The pellets were again resuspended and centrifuged as above. The pellets were re-suspended a third time with 100 μl of high purity water (Millipore) and 200 μl of 100% ethanol was added. The samples were incubated at -20°C for at least 20 min followed by heating for 2 min at 100°C . The tubes were centrifuged at $17,500 \times g$ at 4°C for 20 min and the pellet was dried in a Speed-Vac. The pellet was re-suspended in 0.3 mg/mL amyloglucosidase dissolved in 0.2 M sodium acetate, pH 4.8. Samples were incubated overnight at 40°C to facilitate amyloglucosidase digestion of glycogen to release glucose. The glucose concentration was determined by the method of Bergmeyer (Bergmeyer et al. 1974) as previously described by Suzuki et al (Suzuki et al. 2001).

Statistical Analyses

Prism (GraphPad Software, Inc) was used to analyze data. All data are presented as mean values and standard deviation. Statistical tests used are noted in figure legends.

RESULTS

Acute Hypoglycemia

Blood glucose—Administration of insulin effectively induced hypoglycemia with a fall in blood glucose levels to ~ 2 mmol/L by 60 min post-injection. Blood glucose levels were maintained at or below this level over the remaining 90 min (Fig. 1A). Following a recovery period of 6 hrs, during which time food was available, blood glucose levels were elevated similarly in mice previously treated with saline (11.3 mmol/L (SD 0.8, $n=7$)) or insulin (12.0 mmol/L (SD 1.1, $n=8$)). Blood glucose values 27 hrs after re-introduction of food were 10.1 mmol/L (SD 1.2, $n=5$) in formerly saline-treated mice and 12.7 mmol/L (SD 2.3, $n=5$) in formerly hypoglycemic mice. The blood glucose level in fed mice was 11.6 mmol/L (SD 1.3, $n=6$). There were no statistically significant differences when comparing any of these blood glucose measurements.

Brain glucose—Brain glucose was measured in the insulin and saline-treated mice at the end of a single bout of hypoglycemia as well as 6 and 27 hrs after recovery from acute hypoglycemia. Hypoglycemia elicited a dramatic reduction ($p<0.001$) in brain glucose levels to near zero in the hypoglycemic (I-0) mice 2.5 hrs after injection (Fig. 1B). Brain glucose levels were similar between the formerly hypoglycemic as compared to saline-treated mice both 6 and 27 hrs after recovery from the treatment (Fig. 1B). There were no significant differences in brain glucose levels in the fed mice compared to the recovered saline or insulin-treated mice (Fig. 1B). Saline treated control blood glucose values (S-0) were lower than fed values and those measured 6 and 27 hrs after re-feeding. This is consistent with studies by Poitry-Yamate (Poitry-Yamate et al. 2009) showing a good correlation between blood and brain glucose levels in the rat, an effect also reported between glycogen in cultured astrocytes and the glucose concentration of the media (Cummins et al. 1983a). The ratio of brain to blood glucose was not different when comparing S-0 (0.079 SD 0.016) with S-6 (0.095 SD 0.019) or S-27 (0.092 SD 0.010).

Brain glycogen—Brain glycogen levels were measured in saline and insulin-treated mice sacrificed at the end of hypoglycemia as well as in mice sacrificed 6 and 27 hrs after recovery from treatment. Brain glycogen decreased ($p<0.001$) in the hypoglycemic (I-0) compared to saline-treated (S-0) mice at the end of the treatment period (Fig. 1C). Brain glycogen levels 6 hrs after re-introduction of food were increased in formerly hypoglycemic (I-6) as compared to saline-treated animals before (S-0, $p<0.01$) or after (S-6, $p<0.001$) 6 hrs of food re-introduction. However, after 27 hrs of recovery, mean brain glycogen levels were not significantly different when comparing the formerly hypoglycemic (I-27) mice with

saline-treated (S-27) mice (Fig. 1C). There were no significant differences in brain glycogen levels in the fed mice compared to the saline-treated or hypoglycemic mice with the exception of I-0 and I-6 (Fig. 1C).

Recurrent hypoglycemia

Blood glucose—The administration of insulin on days 1, 5, and 9, reduced blood glucose levels below 3 mmol/L by 30 min post-injection for all three of the hypoglycemic episodes (Fig. 2A). At 60 min, blood glucose levels of the 1st, 2nd, and 3rd hypoglycemic episodes had fallen below 2 mmol/L and remained at or below this level through the remaining 90 min of observation. There were no differences in area under the curve (AUC) when comparing the 3 episodes in saline-treated animals with the exception of an 8% lower AUC for blood glucose in episode 1 as compared to episodes 2 and 3. The blood glucose concentration for the previously saline-treated mice 6 hours after recovery from episode 3 was 11.1 mmol/L (SD 1.1, n=9), and the value for mice that were previously hypoglycemic was 11.6 mmol/L (SD 1.8, n=10). The blood glucose concentration for the saline-treated mice 27 hrs after recovery from episode 3 was 10.3 mmol/L (SD 0.6, n=6), and the corresponding value for mice formerly hypoglycemic was 10.0 mmol/L (SD 1.1, n=7). The blood glucose concentration for fed mice was 11.6 mmol/L (SD 1.3, n=6). There were no statistically significant differences between any of the above blood glucose levels.

Brain glucose—A dramatic decrease ($p<0.001$) in brain glucose was observed in the hypoglycemic (I-0) compared to control (S-0) mice at the end of the third hypoglycemic episode (Fig. 2B). Though there is a trend for the levels to be higher than that observed with our acute hypoglycemic studies, it is not statistically significant ($p>0.05$). After a 6 hr recovery period, brain glucose levels rose to fed levels in both the formerly saline (S-6) and insulin-treated (I-6) mice. Brain glucose levels were not different between the hypoglycemic as compared to saline-treated mice either 6 hrs or 27 hrs after recovery from the 3rd episode (Fig. 2B). Similarly to our observations with acute hypoglycemia, the ratio of brain to blood glucose after recurrent hypoglycemia was not different when comparing S-0 (0.098 SD 0.027) with S-6 (0.104 SD 0.016) or S-27 (.090 SD 0.013).

Brain glycogen—A decrease ($p<0.001$) in brain glycogen was observed in the hypoglycemic (I-0) versus control (S-0) mice at the conclusion of episode 3. Brain glycogen levels were higher ($p<0.01$) between the hypoglycemic (I-6) versus saline-treated (S-6) mice 6 hours after recovery from episode 3 (Fig. 2C). However, after 27 hrs of recovery, brain glycogen levels were not significantly different between the hypoglycemic (I-27) compared to saline-treated (S-27) mice (Fig. 2C).

DISCUSSION

Recent rat and human studies suggest two pathological roles in which brain glycogen stores could be involved, namely, promoting cell survival and contributing to the development of HAAF (Choi et al. 2003; Herzog et al. 2008; Morgenthaler et al. 2006; Oz et al. 2009; Suh et al. 2007). Measuring glycogen in the brain is inherently difficult due to the fact that this glucose polymer is known to undergo rapid post-mortem degradation (Hutchins and Rogers 1970; Lowry et al. 1964). Numerous techniques have been used on rodents to minimize loss of brain metabolites (Ponten et al. 1973a; Ponten et al. 1973b). These techniques include decapitation into liquid nitrogen, whole body immersion into liquid nitrogen, funnel freezing, freeze blowing and head-focused microwave. For our purposes, a caveat for many of these procedures is the requirement of anesthesia. Because anesthesia is known to promote the accumulation of glycogen (Nelson et al. 1968), we chose a technique that does not require anesthesia, i.e. decapitation into liquid nitrogen (-196°C). This method reduces

the temperature of the mouse brain cortex to 0° within 6 sec and to -30° within 12 sec (Swaab 1971). Freezing heads with CCl₂F₂ at its melting point (-150° C) caused deeper regions of the mouse brain to reach 0° within 33 sec (Swaab 1971). It is worth noting that the majority of studies examining brain glycogen are in rats where, due to the size of the head and brain, brain freezes more slowly promoting greater loss of metabolites. Another species-dependent difference is that glycogen phosphorylase is approximately 60% active in rat brain after decapitation (Lust et al. 1973), but only 30% active in mouse brain following decapitation (Lust and Passonneau 1976); this would favor preservation of brain glycogen in the mouse. Rapid freezing of decapitated mouse heads in liquid nitrogen was reported to yield a brain glycogen concentration of ~2.5 µmol/g (Hutchins and Rogers 1970; Lowry et al. 1964) which is almost identical to the values we report in this study. Using what many consider to be the gold standard technique of head-focused microwave, Franken et al (Franken et al. 2003) reported a concentration of ~3 µmol/g tissue for brain glycogen in anesthetized C57Bl/6J mice. Using this technique with nominal power of 3.5 kW, the temperature of the mouse brain was increased to 85°C in 0.5 to 0.6 sec, thereby inactivating brain enzymes. To reach the same temperature in rat brain, 10 kW for 1.2 s is required (Kong et al. 2002). Besides the differential preservation of brain metabolite levels by the methods above, it has been reported that subjection of rats to various types of stress or sensory activation impacts brain glycogen level (Cruz and Dienel 2002). These factors make it challenging to compare levels of metabolites in different studies. Our data indicate that the decapitation method is effective at reproducibly preserving and measuring brain glycogen and glucose in the mouse. But we cannot exclude the possibility that this method may underestimate the levels of metabolites in the brain.

Our primary finding is that brain glycogen supercompensation occurs in healthy, conscious mice after recovery from either acute or recurrent hypoglycemia. In addition, we found that recurrent and acute hypoglycemic episodes both elicit the same degree of brain glycogen degradation and supercompensation. This suggests that multiple hypoglycemic episodes are not causing further adaptations that affect the metabolism of glycogen. Our studies are in qualitative agreement with Choi et al (Choi et al. 2003) who reported that brain glycogen labeling increases 7 hrs after a single hypoglycemic episode in rats. However, quantitatively, the increase in brain glycogen in these rats was 4-fold greater than basal levels, whereas we observed an increase of approximately 25%. This more modest increase in brain glycogen levels is similar to a report in humans (Oz et al. 2009) where the rate of brain glycogen synthesis, measured by ¹³C NMR, was higher after moderate hypoglycemia than after euglycemia. Oz et al calculated that 34 hrs after hypoglycemia, this enhanced rate would lead to 10–15% supercompensation. In contrast, Herzog et al (Herzog et al. 2008) concluded that brain glycogen supercompensation does not occur in conscious rats after either 6 or 24 hr recovery from acute or recurrent hypoglycemia. Possible explanations for the differences between these studies include different initial levels of blood glucose experienced by the subjects, the severity of hypoglycemia induced, and the level to which glucose levels rose when hypoglycemia was ended. Further variables include the degree of hypoglycemia-induced glycogen depletion, the use of anesthetics, and different methods for monitoring brain glycogen levels. For instance, ¹³C NMR monitors glycogen labeling rather than glycogen content, therefore glycogen turnover can increase labeling without increasing glycogen accumulation.

Glycogen supercompensation has been reported in a number of different situations including human skeletal muscle following recovery from glycogen-depleting bicycling (Bergstrom and Hultman 1966), rat cardiac muscle following swimming (Poland and Trauner 1973), and rat brain after ischemia (Folbergrova et al. 1996). The phenomenon has also been observed in cultured astrocytes following depletion of glycogen by neurotransmitters (Cummins et al. 1983b; Sorg and Magistretti 1992). If brain glycogen supercompensation has a functional

significance in neuronal cell survival and/or HAAF, one might expect brain glycogen levels to be elevated at a time where cell survival is promoted and when HAAF is present. However, the duration of glycogen supercompensation is not well characterized. Muscle glycogen supercompensation persists for 5 days in humans but disappears by 7 days post-exercise (Arnall et al. 2007). Supercompensated glycogen in mouse muscle and liver was observed at both 5 and 24 hrs post-exercise (Ryder et al. 1999). In the brain, glycogen supercompensation was reported at 34 hrs following the end of hypoglycemia in humans (Oz et al. 2009), 7 hrs following hypoglycemia in rats (Choi et al. 2003), and in our hands, 6 hrs following hypoglycemia in the mouse. Whether brain glycogen supercompensation contributed to enhanced neuronal cell survival after recurrent hypoglycemia in a recent study (Puente et al. 2010) is not known because brain glycogen levels were not reported. Hypoglycemia unawareness, a component of HAAF, reportedly persists for 1–2 wks following a hypoglycemic bout in humans (Cryer 2002). However, it remains to be established whether brain glycogen supercompensation remains for 1–2 wks in humans or rats. Impaired counterregulatory response, another component of HAAF, has been reported in mice subjected to two episodes of hypoglycemia separated by 24 hrs (Jacobson et al. 2006). Our observation that mice do not have elevated brain glycogen stores at a comparable time suggests that brain glycogen supercompensation is not a critical factor in this impaired response in the mouse.

In conclusion, our studies indicate that brain glycogen supercompensation does occur in the mouse following hypoglycemia. Based on the length of time that these elevated glycogen stores are maintained, it appears any functional significance would be limited to events occurring within a few hrs after a hypoglycemic episode.

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Figure 1. Effects of acute hypoglycemia on metabolite levels

(A) Blood glucose levels were monitored in wild-type mice injected with saline (open squares; $n=17$) or insulin (closed squares; $n=18$). Area under the curve (AUC) calculations are 1108 (SD 116), saline treated and 362 (SD 54), insulin treated, $n=18-20$, $p<0.0001$. Statistical significance was determined using Students t-test. Brain glucose (B) and glycogen (C) levels in saline (S) or insulin (I)-treated mice 150 min after injection (S-0 and I-0), 6 hrs (S-6 and I-6) and 27 hrs (S-27 and I-27) after re-feeding, and in fed mice (Fed); $n=5-9$ for all groups. Data are presented as mean and SD. ^a $p<0.001$ compared to S-0, S-6, S-27, I-6, I-27, and fed, ^b $p<0.05$ compared to S-0, ^c $p<0.001$ compared to S-0, S-6, S-27, I-6, I-27, and fed, ^d $p<0.001$ compared to S-6, S-27, I-27, and fed and $p<0.01$ compared to S-0. Statistical significance was determined using one-way ANOVA followed by a Newman-Keuls multiple comparison post-test.

Figure 2. Effects of recurrent hypoglycemia on metabolite levels

(A) Blood glucose levels were monitored in mice injected with saline (n=19–21, open symbols) or insulin (n=24, closed symbols) on three separate occasions; episode 1 (squares) episode 2 (circles), and episode 3 (triangles). AUC calculations for saline-treated animals are 1119 (SD 79), episode 1; 1210 (SD 149), episode 2; 1198 (SD 138), episode 3; and for insulin-treated mice 365 (SD 53), episode 1; 352 (SD 50), episode 2; 342 (SD 46), episode 3; n=19–24. $p < 0.01$ comparing episode 1 with episodes 2 and 3 for saline-treated mice. AUC for the three insulin-treated episodes are indistinguishable. $p < 0.001$ when comparing all episodes of saline-treated AUC with episodes of insulin-treated AUC. Brain glucose (B) and glycogen (C) levels in saline (S)- or insulin (I)- treated mice 150 min after injection (S-0 and I-0), 6 hrs (S-6 and I-6) and 27 hrs (S-27 and I-27) after re-feeding, and in fed mice (Fed); n=6–10 for each group. Data are presented as means and standard deviation. ^a $p < 0.001$ compared to S-0, S-6, S-27, I-6, I-27, and fed, ^b $p < 0.05$ compared to S-0, ^c $p < 0.05$ compared to I-6, ^d $p < 0.001$ compared to S-0, S-6, S-27, I-6, I-27, and fed, ^e $p < 0.01$ compared to S-6, I-27, S-27, and fed and $p < 0.05$ compared to S-0. Statistical significance was determined using one-way ANOVA followed by a Newman-Keuls multiple comparison post-test.