MOLECULAR
METABOLISM

Loss of FOXO transcription factors in the liver mitigates stress-induced hyperglycemia

Anna E. Garcia Whitlock [1,2], Jamarie Sostre-Colón [2], Matthew Gavin [2], Niels D. Martin [3], Joseph A. Baur [2,4],
Carrie A. Sims [5], Paul M. Titchenell [2,4,*]

ABSTRACT

Objective: Stress-induced hyperglycemia is associated with poor outcomes in nearly all critical illnesses. This acute elevation in glucose after injury or illness is associated with increased morbidity and mortality, including multiple organ failure. Stress-induced hyperglycemia is often attributed to insulin resistance as controlling glucose levels via exogenous insulin improves outcomes, but the mechanisms are unclear. Forkhead box O (FOXO) transcription factors are direct targets of insulin signaling in the liver that regulate glucose homeostasis via direct and indirect pathways. Loss of hepatic FOXO transcription factors reduces hyperglycemia in chronic insulin resistance; however, the role of FOXOs in stress-induced hyperglycemia is unknown.

Methods: We subjected mice lacking FOXO transcription factors in the liver to a model of injury known to cause stress-induced hyperglycemia. Glucose, insulin, glycerol, fatty acids, cytokines, and adipokines were assessed before and after injury. Liver and adipose tissue were analyzed for changes in glycogen, FOXO target gene expression, and insulin signaling.

Results: Stress-induced hyperglycemia was associated with reduced hepatic insulin signaling and increased hepatic FOXO target gene expression while loss of FOXO1, 3, and 4 in the liver attenuated hyperglycemia and prevented hyperinsulinemia. Mechanistically, the loss of FOXO transcription factors mitigated the stress-induced hyperglycemia response by directly altering gene expression and glycogenolysis in the liver and indirectly suppressing lipolysis in adipose tissue. Reductions were associated with decreased IL-6, TNF-α, and follistatin and increased FGF21, suggesting that cytokines and FOXO-regulated hepatokines contribute to the stress-induced hyperglycemia response.

Conclusions: This study implicates FOXO transcription factors as a predominant driver of stress-induced hyperglycemia through means that include cross-talk between the liver and adipose, highlighting a novel mechanism underlying acute hyperglycemia and insulin resistance in stress.

1. INTRODUCTION

Elevated blood glucose levels are associated with poor outcomes in nearly all critically ill conditions, including trauma, sepsis, stroke, myocardial infarction, brain injury, and even novel coronavirus disease 2019 [1–7]. This phenomenon, known as stress-induced hyperglycemia, is a common metabolic derangement characterized by elevated blood glucose levels in the setting of injury or illness [8,9]. Stress-induced hyperglycemia is associated with increased morbidity and mortality in the intensive care unit (ICU), including multiple organ failure, prolonged time on a ventilator, kidney failure, and increased risk of infection [4,10–14]. These complications lead to decreased

survival, especially in trauma, sepsis, and lung injury in which stress-induced hyperglycemia is associated with as high as a two-fold increase in mortality [7,12,15,16]. Patients need not be obese or diabetic to suffer the ill effects of stress-induced hyperglycemia as non-diabetic patients with uncontrolled hyperglycemia in the ICU exhibit worse survival than those diagnosed with diabetes prior to admission [1,17]. These complications can be mitigated by controlling excursions in blood glucose with exogenous insulin, indicating the phenomenon is more than a marker of disease severity [11,18]. Insulin therapy is associated with as much as a 35% reduction in morbidity and mortality; however, controversy remains, as overtreatment with insulin is associated with hypoglycemic events and mortality [19–21]. Thus,

there is a critical need to understand the molecular drivers underlying stress-induced hyperglycemia and its role in adverse outcomes.

Critical illness is associated with multiple metabolic derangements such as increases in glucagon, catecholamines, and cortisol, but many studies on stress-induced hyperglycemia in humans focus on changes in insulin resistance [22—28]. Normally, insulin increases with blood glucose to suppress hepatic glucose production and promote peripheral glucose uptake [29]. Compared to healthy controls, injured and septic patients frequently exhibit a profound increase in hepatic glucose production that can be refractory to exogenous insulin [25—28]. Further, critically ill patients often exhibit increased circulating levels of insulin-like growth factor-binding protein-1 (IGFBP-1), a liver-derived protein that prolongs insulin-like growth factor activity and is normally suppressed by insulin [30]. This increase in IGFBP-1 in humans is associated with hepatic insulin resistance and mortality in critical illness, suggesting a key role of defective hepatic insulin action in the pathophysiology of stress-induced hyperglycemia [31].

Because the timing and type of injury are difficult to control in human patients, animal models of critical illness are a valuable resource for studying stress-induced hyperglycemia [8]. Approaches such as cecal ligation and puncture (CLP) or lipopolysaccharide (LPS)-induced endotoxemia simulate sepsis with variable changes in glucose and insulin [32]. Alternatively, fixed-pressure trauma and hemorrhage is a standard model for studying injury and non-infectious critical illness that consistently results in stress-induced hyperglycemia [33]. Joseph Messina and his team frequently use this approach to recapitulate stress-induced hyperglycemia including producing the aforementioned profound increase in both glucose and insulin [34,35]. This group found that insulin-stimulated phosphorylation of the glucoregulatory kinase, AKT, decreases following injury in the liver, adipose, and muscle [35]. This kinase is central to regulating metabolism and growth, particularly in the liver. Increased insulin-mediated phosphorylation of AKT leads to the phosphorylation and subsequent inhibition of forkhead box protein O (FOXO) transcription factors due to exclusion from the nucleus [36]. This family of transcription factors helps regulate the gluconeogenic gene program in the liver across metabolic states [37]. Stress-induced hyperglycemia is associated with increased gene expression of hepatic glucose-6-phosphatase (G6PC), a gluconeogenic gene regulated in part by FOXO [38]. FOXO also upregulates IGFBP-1, the aforementioned circulating factor associated with hepatic insulin resistance and mortality in critical illness, suggesting a potential role of the transcription factor family in stress-induced hyperglycemia [34,38].

In addition to direct action in the liver, FOXO is also known to regulate glucose homeostasis through cell non-autonomous mechanisms [29]. Loss of FOXO1 in the liver, the predominate hepatic FOXO isoform, improves glucose tolerance and insulin sensitivity in diet-induced obesity and genetic models of insulin resistance, including mouse models lacking the insulin receptor (IR), insulin receptor substrate 1 and 2 (IRS-1 and -2), or AKT1/2 in the liver, demonstrating that this canonical signaling pathway is not required to suppress hepatic glucose production under all conditions *in vivo* [39—42]. Further, research by our lab and other groups suggests that the regulation of peripheral insulin sensitivity by FOXO plays an important role in indirectly regulating hepatic glucose production [40,41,43]. This relationship between peripheral lipolysis and hepatic glucose production also appears to be important in stress-induced hyperglycemia. Increased lipolysis, specifically increased circulating glycerol levels, is associated with increased glucose levels in models of trauma and hemorrhage [44]. However, little is known regarding the role of FOXO in stress-induced hyperglycemia, much less whether the cell non-

autonomous regulation of peripheral lipolysis by the hepatic transcription factor is important.

Our previous research on chronic insulin resistance combined with evidence of increased IGFBP-1 expression in acute hyperglycemia suggests that persistent FOXO activity may be a significant driver of stress-induced hyperglycemia and associated mortality in critical illness. In the current study, we performed loss-of-function experiments to discover that loss of FOXO transcription factors in the liver was sufficient to reduce hyperglycemia and prevent hyperinsulinemia in a mouse model of stress-induced hyperglycemia. These improvements in glucose and insulin were associated with decreased circulating glycerol and improved insulin sensitivity in adipose tissue. This supports the idea that peripheral lipolysis is a driver of hyperglycemia in both acute and chronic insulin-resistant states and that FOXO transcription factors in the liver are a key regulator of stress-induced hyperglycemia through both cell autonomous and non-autonomous pathways.
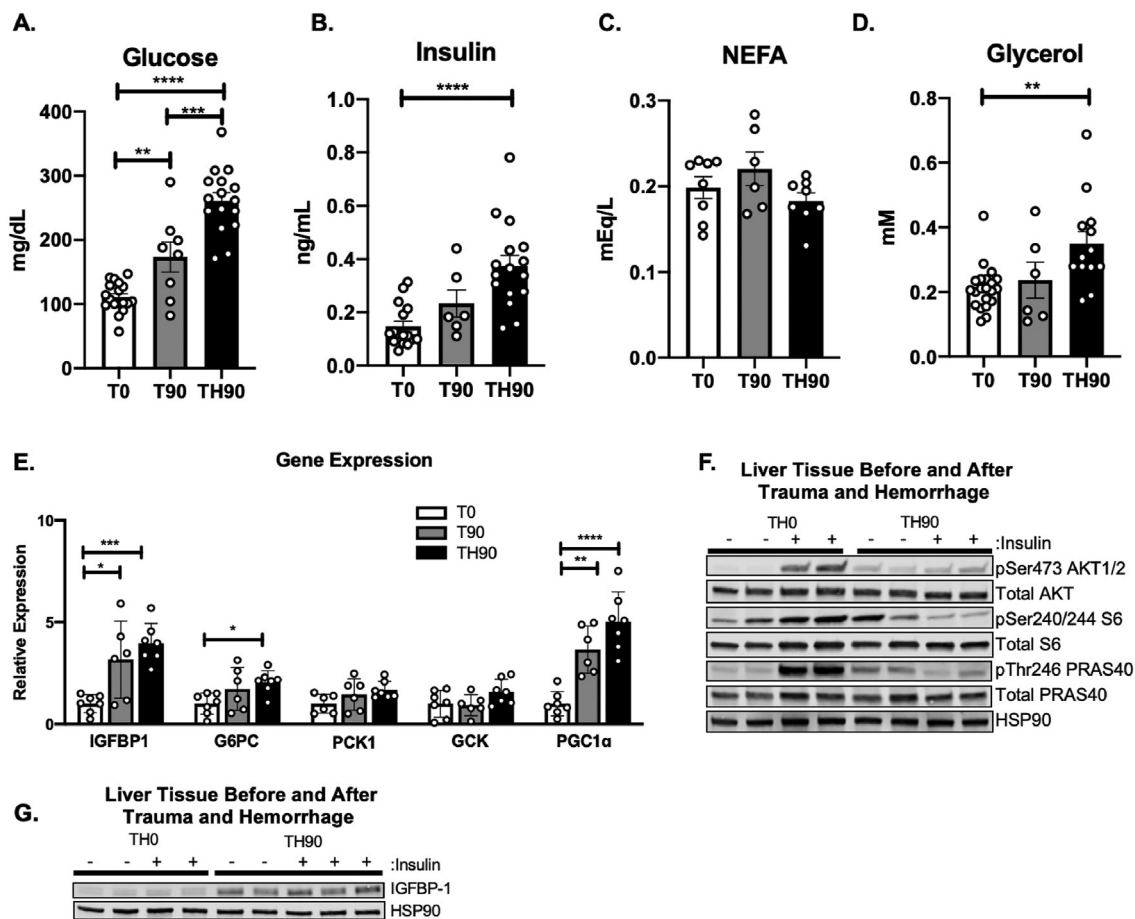
2. RESULTS

2.1. Stress-induced hyperglycemia is associated with decreased insulin signaling and increased FOXO target gene expression

The fixed-pressure trauma and hemorrhage (TH) protocol is an established mouse model of stress-induced hyperglycemia that includes a laparotomy and cannulation of the bilateral femoral arteries followed by controlled blood loss to a mean arterial pressure of 35—40 mmHg for 90 min [33]. As shown in Figure 1, blood and tissues were collected immediately after surgery (T0 or TH0), 90 min after the completion of surgery (T90), or after the addition of 90 min of hemorrhage-induced hypotension following surgery (TH90). As expected, trauma and hemorrhage reliably induced stress-induced hyperglycemia at the TH90 time point, including a greater than two-fold increase in glucose levels and nearly two-fold increase in insulin levels (Figure 1A—B). While TH90 was not associated with changes in fatty acids, it was associated with a $\sim$75% increase in glycerol, similar to recent reports in the literature (Figure 1C—D) [44,45]. Conversely, T90 or surgery alone was associated with modestly elevated blood glucose levels, but no significant increase in insulin, fatty acids, or glycerol (Figure 1A—D). These data are consistent with published reports that TH90 induces a more profound insulin-resistant phenotype than T90 alone [35].

To evaluate whether stress-induced hyperglycemia was associated with increased FOXO target gene expression, liver lysates were evaluated at T0, T90, and TH90 for changes in transcriptional targets of FOXO (Figure 1E). Livers taken from animals at TH90 exhibited increased expression of IGFBP1 and G6PC (p < 0.05), with a trend toward increased phosphoenolpyruvate carboxykinase 1 (PCK1, p = 0.1), all factors known to be directly induced by FOXO [46]. Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), another gene target and coactivator of FOXO, also significantly increased. Interestingly, IGFBP1 and PGC1α also increased at T90, although to a lesser degree and with increased variability. These effects correlated with earlier findings that trauma alone only modestly increased glucose levels and did not significantly increase insulin or glycerol.

While it is certainly likely that FOXO activity also contributed to elevated glucose levels at T90, we focused our attention on the TH90 condition, given the observation that TH90 elicits robust changes in hyperglycemia, hyperinsulinemia, and glycerol. Thus, we assessed the role of hepatic insulin signaling in mediating the insulin resistance seen in TH90. To this end, the laparotomy was reopened at TH0 or TH90 to

Figure 1: Stress-induced hyperglycemia is associated with decreased insulin signaling and increased FOXO target gene expression. (A–D) Paired analysis of blood glucose, insulin, non-esterified fatty acid (NEFA), and glycerol levels in the mice at baseline (T0) compared to 90 min after surgery alone (T90) or after 90 min of hemorrhage-induced hypotension (TH90), n = 6−20 mice/group. (E) Gene expression analysis in liver lysates from the mice sacrificed at T0 vs T90 vs TH90, n = 6−7 mice/group. (F) Representative Western blotting of AKT, S6, and PRAS40 phosphorylation relative to the total protein in the liver from liver lysates from the mice sacrificed immediately after surgery (TH0) or at TH90. At sacrifice, the animals were injected with either insulin (0.2 U/kg) or vehicle via the portal vein and tissues collected 5 min later. (G) Representative Western blotting of IGFBP-1 in the liver from liver lysates from the mice sacrificed after surgery (TH0) or at TH90 following injection with either insulin (0.2 U/kg) or vehicle via the portal vein and tissues collected 5 min later. Significance was determined in A-E by one-way ANOVA. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ as indicated. Data represented as mean ± SEM.

facilitate identification of the portal vein and subsequent injection of 0.2 U/kg of insulin or vehicle alone. Five minutes later, the animals were sacrificed and tissues were collected and snap frozen for later analysis. Following treatment with exogenous insulin, insulin-mediated phosphorylation of AKT (pAKT) and PRAS40 (pPRAS40) increased significantly at TH0, but not S6 (Figure 1F and Supplemental Figure 1A-C). This lack of phosphorylation of S6 (pS6) could have been because the animals were fasted for only 5−6 h, so pS6 levels in the basal state were still quite high, limiting the induction seen in response to insulin at TH0. When we examined the livers at TH90, we found that the insulin-mediated increase in pAKT was significantly blunted (Figure 1F and Supplemental Figure 1A). This was accompanied by a decrease in the phosphorylation of the downstream signaling target, PRAS40, and a trend toward reduced insulin-mediated phosphorylation of S6 compared to TH0 (p = 0.09, Supplemental Figure 1B and C). Notably, the lack of increased phosphorylation of S6 at TH90 with vehicle or insulin suggested that the suppression in AKT phosphorylation was likely not related to inhibition by mTORC1. The levels of IGFBP-1 protein, a bona fide transcriptional target of FOXO, were dramatically elevated in the liver after injury (Figure 1G and Supplemental

Figure 1D) [34]. Curiously, there was some insulin-mediated induction of IGFBP-1 at TH90, but it was unclear whether this was biologically significant as it was not maintained in later experiments (Supplemental Figure 3D). A striking aspect was that IGFBP-1 was not reduced by insulin but rather increased at TH90. Together, this constellation of signaling defects supports a decrease in insulin-mediated suppression of FOXO at TH90.

2.2. Loss FOXO1 in the liver (L-FOXO1KO) reduces hyperinsulinemia, but not hyperglycemia, in stress-induced hyperglycemia

Our lab and others previously published that loss of FOXO1 activity in the liver is sufficient to prevent hyperglycemia in diet-induced obesity and genetic mouse models of insulin resistance such as those lacking IR, IRS-1/2, or AKT1/2 in the liver [39−41]. To determine whether increased FOXO1 contributes to stress-induced hyperglycemia, the Cre-loxP inducible knockout system was used to create mice lacking FOXO1 in adult liver (L-FOXO1KO). An adeno-associated virus (serotype 8, AAV8) and thyroxine-binding globulin (TBG) promoter provided liver specificity [40]. Knockout was confirmed by decreased FOXO1

gene expression in the livers of L-FOXO1KO mice (Figure 2A). The L-FOXO1KO mice were subjected to the TH protocol using age- and sex-matched littermates injected with AAV-TBG-GFP as controls. As expected, glucose levels increased significantly after trauma and hemorrhage in the controls, increasing 133% (Figure 2B). The L-FOXO1KO mice still exhibited a robust increase in glucose levels in response to injury, with a delta change that was not significantly different from the control mice (Figure 2C). However, insulin levels at TH90 were much lower in the L-FOXO1KO mice compared to the controls (0.608 ± 0.089 vs 0.277 ± 0.041, Figure 2D). The control animals increased 350% from TH0 to TH90 while the L-FOXO1KO mice increased only 98%. This was reflected in the delta change where insulin increased 0.472 ng/mL in the controls but only 0.147 ng/mL in the L-FOXO1KO mice (Figure 2E). Despite little difference in glucose levels, the lower levels of insulin at TH90 suggested that the L-FOXO1KO mice were more insulin sensitive after injury.

To evaluate the implications of acute hepatic FOXO1 transcription factor loss on glycogenolysis, glycogen levels were assessed in the mice sacrificed at TH0 and TH90 (Figure 2F). As expected, glycogen levels significantly decreased between TH0 and TH90 in the controls [44]. In the L-FOXO1KO animals, this significant decrease was lost, although a trend toward decreased glycogen between TH0 and TH90 was noted. There was no difference between the controls or knockouts at TH0 and TH90, although there was a trend toward increased glycogen levels at TH90 in the L-FOXO1KO mice compared to the controls (Figure 2F). Although the findings were limited by variability among the animals in the timing of last feeding immediately prior to a short-term fast, they suggested a trend toward decreased glycogenolysis in the absence of FOXO1.

Liver lysates from L-FOXO1KO livers were then assessed for changes in gene expression after 90 min of trauma and hemorrhage and compared to GFP-injected controls also subjected to the trauma and hemorrhage protocol. The L-FOXO1KO mice exhibited a significant decrease in IGFBP1 and GCK compared to the controls, suggesting a decrease in FOXO activity (Figure 2G). A significant decrease in IGFBP-1 was also seen at the protein level as demonstrated by Western blotting (Figure 2I and Supplemental Figure 2A). The expression levels of two gluconeogenic genes, G6PC and PCK1, were no different between the control and L-FOXO1KO mice, suggesting changes in gluconeogenic gene expression were not required for the reduction in hyperinsulinemia, similar to what we previously reported [40,43]. We then evaluated the ratio of G6PC to GCK, as published work suggests that a reduction in the relative amounts of G6PC to GCK is associated with decreased hepatic glucose production [37]. This ratio decreased at TH90 in the L-FOXO1KO mice compared to the controls (Figure 2H), suggesting that the ratio of GCK to G6PC could play a role in the regulation of glucose homeostasis in stress-induced hyperglycemia.
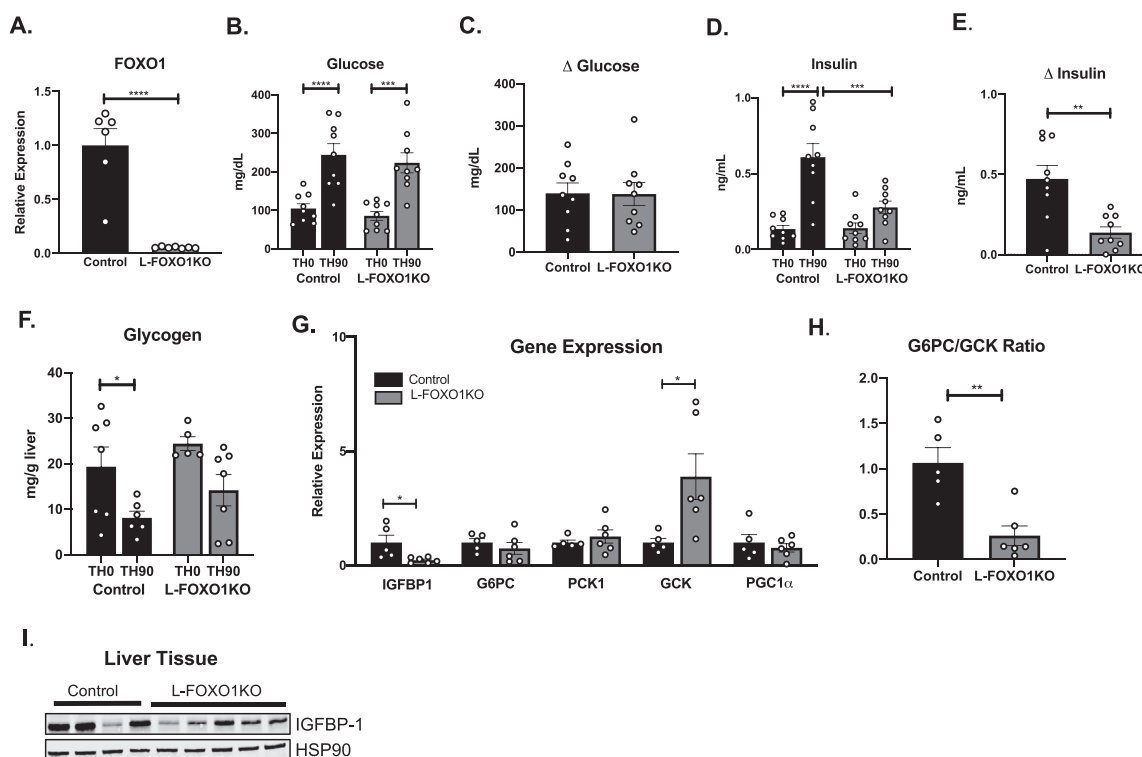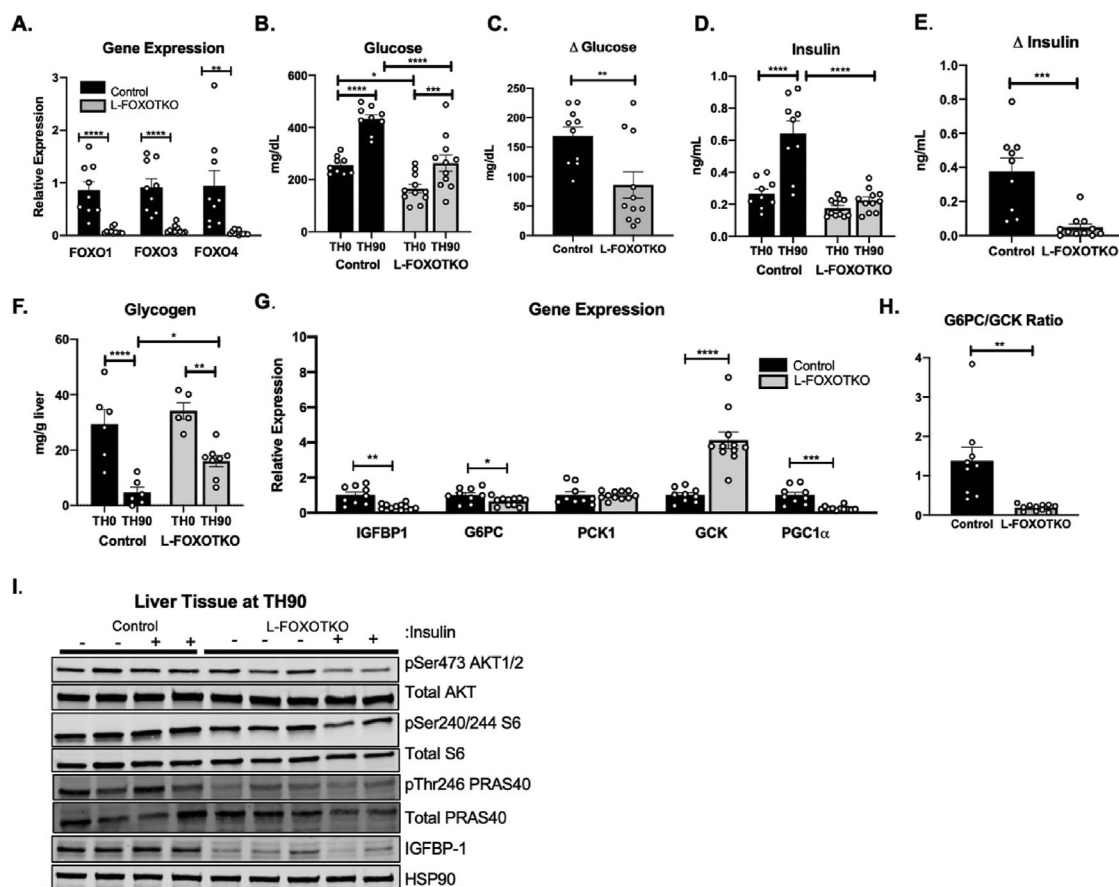

Figure 2: Loss FOXO1 in the liver (L-FOXO1KO) reduces hyperinsulinemia, but not hyperglycemia, in stress-induced hyperglycemia. (A) FOXO1 gene expression levels after 90 min of trauma and hemorrhage (TH90) in the controls vs mice lacking FOXO1 in the liver (L-FOXO1KO), n = 6–7 mice per group. (B) Glucose levels following trauma and hemorrhage at baseline (TH0) and TH90 in the L-FOXO1KO mice compared to the controls. (C) Difference in glucose levels at TH90 relative to TH0 in the L-FOXO1KO mice compared to the controls. (D) Insulin levels at TH0 and TH90 in the L-FOXO1KO mice compared to the controls. (E) Difference in insulin levels at TH90 relative to TH0 in the L-FOXO1KO mice compared to the controls, all n = 9 mice/group. (F) Glycogen levels following trauma and hemorrhage at TH0 and TH90 in the L-FOXO1KO mice compared to the controls, n = 4–7 per group. (G) Gene expression analysis in the liver at TH90 in the L-FOXO1KO mice compared to the controls, n = 5–6 mice/group. (H) Ratio of G6PC to GCK gene expression in livers of the L-FOXO1KO mice compared to the controls, n = 6. (I) Representative Western blotting of IGFBP-1 expression in the liver at TH0 vs TH90 without exogenous insulin. Statistical significance in A, C, E, G, and H was determined by unpaired Student's t tests, two-way repeated-measures ANOVA was used in B and D, and two-way ANOVA was used in F. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ as indicated. Data represented as mean ± SEM.

2.3. Loss of transcription factors, FOXO1, 3, and 4, in the liver (L-FOXOTKO) reduces hyperglycemia and hyperinsulinemia in stress-induced hyperglycemia

Although loss of FOXO1 alone is sufficient to reduce hyperglycemia in chronic insulin-resistant states, other FOXO isoforms significantly contribute to hepatic glucose metabolism. Specifically, Haeusler et al. found that combined loss of all three FOXO transcription factors in the liver, FOXO1, FOXO3, and FOXO4, had an additive effect on reducing hepatic glucose production in mice, notably improved glucose tolerance and insulin sensitivity [37,47]. Given that our L-FOXO1KO mice exhibited reductions in insulin levels, but not glucose levels, we sought to determine if loss of FOXO1, 3, and 4 in the liver results in additive protection from stress-induced hyperglycemia. To test this, the AAV Cre-loxP-inducible knockout system was used to create mice lacking all three FOXO transcription factors in the liver (L-FOXOTKO) and the mice were subjected to the TH protocol as described. Age-matched littermates injected with GFP served as controls. Gene expression confirmed loss of FOXO1, 3, and 4 mRNA in the liver from the L-FOXOTKO mice (Figure 3A).

Consistent with the literature, the L-FOXOTKO mice had lower fasting glucose levels (Figure 3B). When subjected to the TH protocol, the L-FOXOTKO mice still exhibited a modest increase in glucose levels between TH0 and TH90 (170.6 vs 263.6 mg/dL); however, glucose levels at TH90 were significantly lower than in the controls (263.6 vs 432.3 mg/dL). This was reflected in the mean difference in glucose levels between TH0 and TH90, as the change in glucose was nearly twice as large in the controls compared to the L-FOXOTKO mice (Figure 3C). Further, the L-FOXOTKO mice also exhibited lower insulin levels at TH90 (0.642 ± 0.080 vs 0.265 ± 0.047, Figure 3D). While the controls exhibited a 152.3% increase in insulin levels, the L-FOXOTKO mice only increased 26.6%. This was also reflected in the delta change, with the L-FOXOTKO mice exhibiting a limited increase in insulin at TH90 (Figure 3E).

We then evaluated glycogen levels in the livers of the L-FOXOTKO animals at TH90 (Figure 3F). As expected, there was a dramatic reduction in glycogen levels in the controls between TH0 and TH90; however, unlike in the L-FOXO1 experiments, the reduction between TH0 and TH90 was also significant in the triple knockouts. When the

Figure 3: Loss of transcription factors FOXO1, 3, and 4, in the liver (L-FOXOTKO) reduces hyperglycemia and hyperinsulinemia in stress-induced hyperglycemia. (A) FOXO1, 3, and 4 gene expression in the liver after 90 min of trauma and hemorrhage (TH90), n = 9−11 mice per group (B) Glucose levels at baseline (TH0) and TH90 in the L-FOXOTKO mice compared to the controls. (C) Difference in glucose levels at TH90 relative to TH0 in the L-FOXOTKO mice compared to the controls. (D) Insulin levels at TH0 and TH90 in the L-FOXOTKO mice compared to the controls. (E) Difference in insulin levels at TH90 relative to TH0 in the L-FOXOTKO mice compared to the controls, all n = 9−11 mice/group. (F) Glycogen levels following trauma and hemorrhage at TH0 and TH90 in the L-FOXOTKO mice compared to the controls, n = 5−8 per group. (G) Gene expression in the liver at TH90 in the L-FOXOTKO mice compared to the controls, n = 9−11 mice/group. (H) Ratio of G6PC to GCK expression at TH90 in the L-FOXOTKO mice compared to the controls, n = 9−11 mice/group. (I) Representative Western blotting of IGFBP-1 relative to HSP90 and phosphorylation AKT, S6, and PRAS40 phosphorylation relative to the total protein in the liver from liver lysates from the control and L-FOXOTKO mice sacrificed at TH90 following injection with either insulin (0.2 U/kg) or vehicle via the portal vein and tissues collected 5 min later. Statistical significance in A, C, E, G, and H was determined by unpaired Student's t tests, two-way repeated-measures ANOVA was used in B and D, and two-way ANOVA was used in F. ****p < 0.0001, ***p < 0.001, **p < 0.01, and *p < 0.05 as indicated. Data represented as mean ± SEM.

controls and L-FOXOTKO animals were further compared, there was no difference in glycogen levels at TH0 between the groups; however, there was more glycogen in the livers of the L-FOXOTKO animals at TH90 compared to the controls. This suggested that delayed glycogenolysis in the triple knockout may have played a role in the reduced glucose levels after trauma and hemorrhage compared to the controls [37].

To determine whether there were transcriptional changes associated with the protective effect of hepatic FOXO1, 3, and 4 deletion in regard to stress-induced hyperglycemia, liver lysates were assessed at TH90 for differences in gene expression between the controls and L-FOXOTKO mice (Figure 3G). As expected, IGFBP1 gene expression was reduced, confirming a functional FOXO knockout. We observed a significant reduction in the glucoregulatory gene G6PC and the FOXO coactivator PGC1α. Interestingly, we found a large increase in GCK in the L-FOXOTKO mice compared to the controls that was higher than in the single knockout (Figure 3G). As described, the ratio of G6PC to GCK is associated with decreased hepatic glucose production [37]. Here we observed a large decrease in the ratio of G6PC/GCK at TH90 in the L-FOXOTKO mice compared to the controls, suggesting a role of the ratio in regulation of hepatic glucose homeostasis, including the potential reduction in glycogenolysis observed in the triple knockout livers (Figure 3H).

Evidence of reduced FOXO activity was further confirmed by immunoblotting analysis, which found a significant reduction in IGFBP-1 protein expression at TH90 in the L-FOXOTKO mice compared to the controls (Figure 3I and Supplemental Figure 3D). Despite the implied improvements in insulin sensitivity, phosphorylation of AKT did not increase in the L-FOXOTKO mice compared to the controls (Supplemental Figure 3A). Although variability limited conclusions on S6 activity, insulin-mediated phosphorylation of PRAS40 was significantly reduced at TH90 in the L-FOXOTKO mice (Supplemental Figure 3B and C). This was consistent with findings in chronic insulin-resistant models lacking FOXO1 in the liver where there are improvements in insulin sensitivity, including decreased hepatic glucose production, despite a lack of increased AKT signaling in the liver [39—43]. Collectively, our findings between the controls and L-FOXOTKO mice suggested that FOXO-related reductions in stress-induced hyperglycemia were not necessarily due to increased insulin signaling via AKT in the liver alone.

2.4. Reduction in stress-induced hyperglycemia with loss of hepatic FOXO1, 3, and 4 is associated with inhibition of peripheral lipolysis

Both type II diabetes and stress-induced hyperglycemia involve an association between insulin resistance and increased lipolysis [48—52]. Raje et al. recently demonstrated that increased lipolysis is a primary driver of hyperglycemia in mice subjected to the TH protocol and that direct inhibition of peripheral lipolysis reduces hyperglycemia [44]. Further, research from our lab and others found that hepatic FOXO plays a significant role in regulating peripheral lipolysis in adipose tissue and that loss of FOXO1 in the liver can indirectly inhibit hepatic glucose production by reducing peripheral lipolysis and improving insulin signaling via AKT in adipocytes [40,41,43]. With this in mind, we hypothesized that liver FOXO transcription factors help regulate peripheral lipolysis in this model. Peripheral lipolysis was assessed in the control, L-FOXO1KO, and L-FOXOTKO mice before and after the TH protocol by measuring circulating levels of fatty acids, ketones, and glycerol. Similar to our baseline studies shown in Figure 1 and published research from others, there was no difference in non-esterified fatty acids with increased stress-induced hyperglycemia in

any group in the trauma and hemorrhage model (Figure 4A) [45]. To this end, circulating ketones were assessed to address potential changes in fatty acid oxidation, but no differences were observed (Figure 4B). Because adipocytes lack glycerol kinase, circulating levels of glycerol can be a more reliable marker of peripheral lipolysis. Glycerol levels increased at TH90 in both the control and L-FOXO1KO mice compared to TH0 (Figure 4C). Interestingly, when we assessed glycerol in the L-FOXOTKO mice, there was no longer an increase at TH90, suggesting decreased lipolysis in the triple knockouts.

Mechanistically, insulin inhibits lipolysis in adipose tissue through increased phosphorylation of AKT, a process that appears to be defective in stress-induced hyperglycemia and type II diabetes [51,52]. Studies in mouse models of chronic insulin resistance found that this defect in AKT phosphorylation in the adipose tissue is restored upon deletion of FOXO in the liver [40,41]. To determine whether the reduction in circulating glycerol in the L-FOXOTKO mice could be a reflection of decreased lipolysis secondary to increased insulin action in the adipose tissue, insulin signaling was evaluated in the adipose tissue across the groups following trauma and hemorrhage. Here, AKT phosphorylation increased at TH90 in the L-FOXOTKO mice compared to the controls or L-FOXO1KO mice (Figure 4D and Supplemental Figure 4A) [40,41]. This was particularly notable given circulating insulin levels were much lower in the L-FOXOTKO mice compared to the controls (Figure 3D). When changes in peripheral insulin signaling were assessed in the skeletal muscle, there was no evidence of increased AKT phosphorylation in the L-FOXO1KO or L-FOXOTKO animals compared to the controls (Figure 4E and Supplemental Figure 4B). Collectively, this suggests that FOXO in the liver cell non-autonomously regulates peripheral insulin sensitivity in adipose tissue in acute stress-induced hyperglycemia and that changes in adipose insulin signaling may translate into a reduction in adipose tissue lipolysis, circulating glycerol, and subsequent hepatic glucose production.

The cross-talk between the liver and adipose tissue, particularly in the setting of FOXO depletion, is an area of intense investigation in chronic insulin-resistant states, including the role of IL-6- and FOXO-regulated hepatokines such as follistatin and FGF21 [40,41,43,53]. Cytokines such as IL-6 and TNF-α and adipokines such as resistin and leptin have been implicated in the pathophysiology of both chronic and stress-induced hyperglycemia [44,54—56]. We sought to determine whether any of these factors could play a role in mediating the communication between the liver and adipose tissue in the L-FOXOTKO animals. To this end, paired plasma was assayed at TH0 and TH90 via Luminex for changes in circulating levels of resistin, leptin, IL-6, and TNF-α. Similar to previous reports, resistin increased with trauma; however, it was unlikely to be a driver in our model as resistin levels also increased similarly in the L-FOXOTKO animals (Figure 4F) [44,56]. Interestingly, circulating levels of IL-6 and TNF-α increased between TH0 and TH90 in the controls, but both cytokines were significantly lower at TH90 in the knockouts compared to the controls at TH90 (Figure 4H—I). Leptin also increased in the controls at TH90 but not in the L-FOXOTKO mice, although there was not a significant difference in TH90 for the knockouts compared to the controls (Figure 4G).

We then evaluated livers at TH0 and TH90 for changes in gene expression of follistatin and FGF2;1 FOXO regulated factors thought to influence insulin sensitivity [41,53,57]. Total follistatin levels increased between TH0 and TH90, but the gene expression of hepatokine was completely reduced at both TH0 and TH90 in the L-FOXOTKO animals (Figure 4J). We also assayed the two alternative isoforms of follistatin, the membrane-bound form Fst288, and the longer circulating form
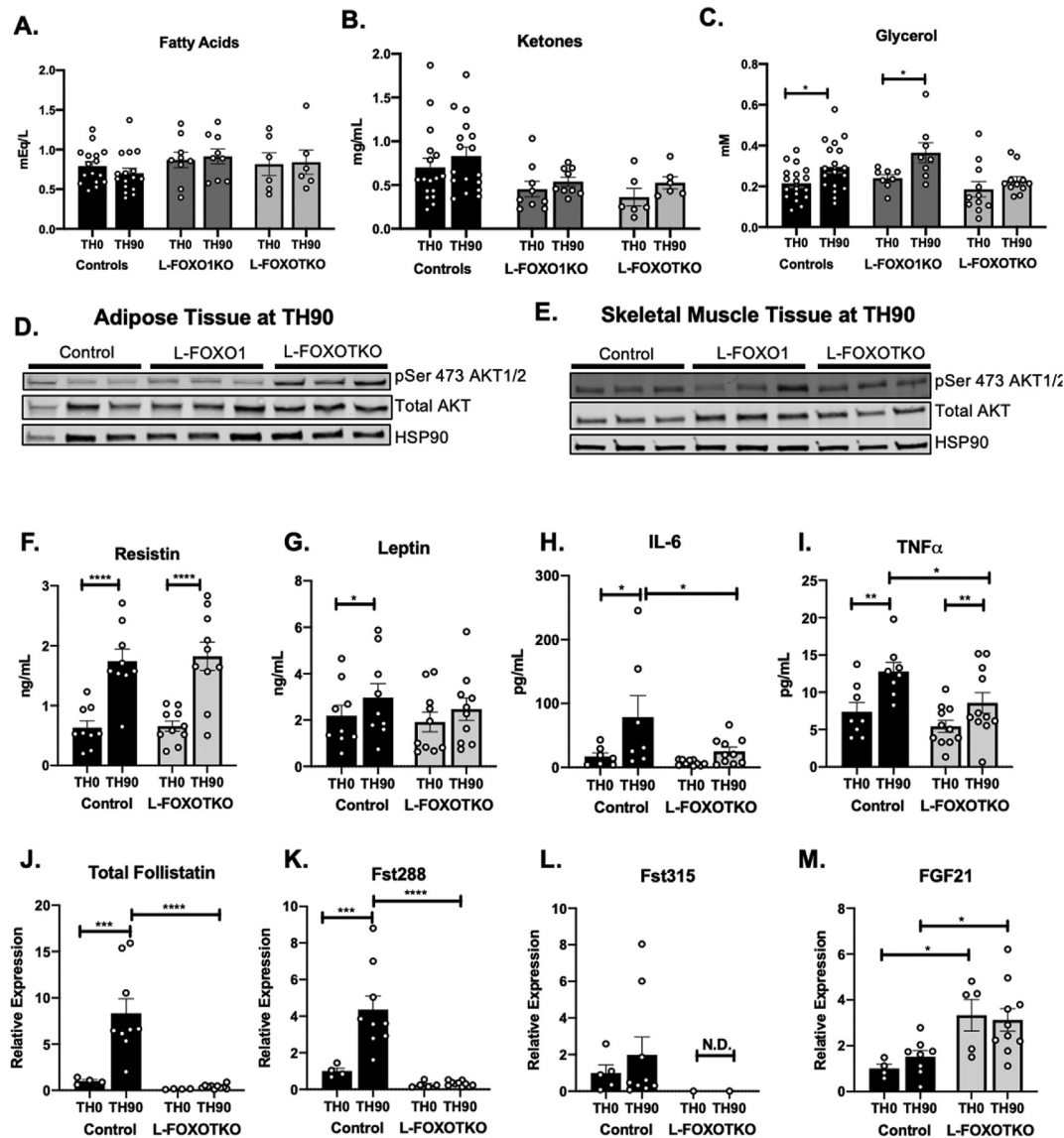
Figure 4: Reduction in stress-induced hyperglycemia with loss of hepatic FOXO1, 3, and 4 is associated with inhibition of peripheral lipolysis. (A–C) Fatty acid, ketone, and glycerol levels following trauma and hemorrhage at TH0 and TH90 in the control, L-FOXO1, and L-FOXOTKO, n = 6–16. (D) Representative Western blotting of AKT phosphorylation at TH90 in the adipose tissue. (E) Representative Western blotting of AKT phosphorylation at TH90 in the quadriceps of the skeletal muscle. (F–I) Circulating levels of resistin, leptin, IL-6, and TNF-α at TH0 and TH90 in the plasma of the controls and L-FOXOTKO mice as assayed by Luminex, n = 7–11 each. (J–M) Gene expression of total follistatin, Fst288, Fst315, and FGF21 in the liver at TH0 and TH90 in the controls and L-FOXOTKO mice, n = 4–10. Statistical significance in A-C and F–I was determined via two-way repeated-measures ANOVA. Statistical significance in J-M was determined by two-way ANOVA. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ as indicated. Data represented as mean ± SEM.

Fst315. This yielded similar results, including expression that was below the limit of detection for Fst315 in the L-FOXOTKO mice (Figure K–L) [41]. Conversely, we found that FGF21 was elevated in the L-FOXOTKO animals compared to the controls at TH0 and TH90, supporting its regulation by FOXO. However, FGF21 did not vary between TH0 and TH90, making it unclear whether hepatokine contributes to insulin sensitivity in acute stress-induced hyperglycemia.

3. DISCUSSION

Stress-induced hyperglycemia was first observed in 1878 and today this phenomena is associated with poor outcomes in nearly all critical illness disciplines [58]. Hepatic insulin resistance, specifically the

inability of insulin to suppress hepatic glucose production, is thought to be the central defect in stress-induced hyperglycemia. Studies evaluating glucose turnover in critically ill patients demonstrate increased hepatic glucose production that is difficult to suppress even with supraphysiological doses of insulin [8,27]. Studies in mouse models indicate that this may be related to defects in hepatic insulin signaling, but the exact mechanisms are unclear [34,35,44]. This study implicated persistent activity of FOXO transcription factors in the liver as direct and indirect promoters of altered glucose homeostasis in stress-induced hyperglycemia. Specifically, we found that FOXO activity was upregulated in stress-induced hyperglycemia, and loss of FOXO1, 3, and 4 in the liver attenuated hyperglycemia and prevented hyper-insulinemia, suggesting an improvement in insulin sensitivity.

Exploration of insulin signaling in the liver and adipose tissues revealed that the mechanisms by which FOXO mitigated stress-induced hyperglycemia were multi-modal, namely that FOXO promoted hyperglycemia through direct and indirect pathways in the liver and adipose tissue, respectively.

FOXO transcription factors in the liver are key regulators of both hepatic glucose homeostasis and systemic insulin sensitivity. Normally, hepatic FOXO is inhibited at least in part via insulin's action in the liver. Canonically, this is thought to occur through increased phosphorylation of AKT, leading to increased phosphorylation of the transcription factor, subsequently excluding it from the nucleus. Previous studies have suggested that insulin signaling is defective at TH90, including decreased phosphorylation of IR, IRS-1, and AKT and increased IGFBP1 mRNA by Northern blotting, the latter suggesting decreased insulin-mediated signaling downstream of AKT [34,35]. Interestingly, we still observed some increase in AKT phosphorylation in a subset of our controls at TH90, although more variable than what was seen at TH0 (Supplemental Figures 1A and 3A). This could have been due to differences in the insulin dosing, as our study used a ∼100- to 200-fold lower dose of insulin than previously published studies, a dose that could invoke a relatively submaximal response and subsequently underappreciate differences in AKT phosphorylation at TH90 [34,35,44]. Regardless, downstream insulin-mediated signaling in the liver consistently decreased at TH90, including decreased phosphorylation of PRAS40 and increased IGFBP-1. This collectively supports the idea that insulin-mediated signaling is defective in the liver after trauma and hemorrhage. Our previous research demonstrated that AKT is not required for suppression of hepatic glucose production in the liver in response to insulin when FOXO1 is also deleted, highlighting that AKT is not required for insulin-mediated glucose homeostasis under all conditions [40].

FOXO activity in the liver is associated with an increased gluconeogenic gene profile, and such changes are often cited as playing a central role in FOXO's regulation of hepatic glucose production [36]. Specifically, FOXO inhibits GCK and promotes gluconeogenic genes such as G6PC and PCK1. However, reductions in hepatic glucose production with loss of FOXO activity are thought to be due to not only changes in individual genes, but a decrease in the ratio of G6PC to GCK gene expression, a model proposed by Haeusler et al. [37]. Similarly, the use glucokinase overexpression to mimic the loss of FOXO's inhibitory effect on gene expression is sufficient to improve hyperglycemia and insulin resistance in mice lacking AKT in the liver [40]. Our evaluation of gene expression in the liver was consistent with this notion and was associated with decreased glucose levels, although we were unable to directly evaluate endogenous glucose production. Our study did however find that loss of FOXO transcription factors was associated with a delayed depletion in glycogen levels following TH90 relative to the controls. Further research will explore the impact of FOXO depletion and subsequent gene changes on gluconeogenesis in stress, including the relative rates of glycogenolysis vs *de novo* gluconeogenesis and how alterations in these processes translate to increased hepatic glucose production under duress.

While direct mechanisms are paramount to insulin sensitivity, research from our lab and others found that insulin can suppress hepatic glucose production through means independent of hepatic gluconeogenic gene expression. In particular, loss of FOXO is known to sensitize peripheral adipose tissue to insulin, inhibit peripheral lipolysis, and indirectly reduce hepatic glucose production [40,41,43]. Hyperinsulinemic-euglycemic clamps in mice lacking both AKT isoforms and FOXO1 in the liver found that free fatty acid levels correlate with hepatic glucose production and that preventing the insulin-mediated suppression of free fatty acids prevents the reduction in glucose levels independent of changes in gluconeogenic gene expression [40]. This was corroborated by Tao et al. who also found that persistent hepatic glucose production in a comparable knockout of proximal insulin signaling was reversed with concomitant deletion of hepatic FOXO1 and correlated with improved adipose tissue insulin sensitivity [41]. The authors attributed this finding to the FOXO-mediated regulation of hepatokines, namely follistatin. Interestingly, we also found that follistatin increased at TH90 but was nearly abrogated in the L-FOXOTKO mice at both TH0 and TH90. Collectively, this research supports a dual role of hepatic FOXO transcription factors in the regulation of hepatic glucose production via both direct processes in the liver and indirect processes in the adipose tissue and that associated changes in liver-derived follistatin could play a significant role.

Increased peripheral lipolysis has long been associated with injury or illness, but only recently has the importance of this relationship in the severity of stress-induced hyperglycemia become better understood [48–51]. Recent research by Raje et al. convincingly demonstrated that decreased lipolysis via genetic or pharmacological inhibition of adipose triglyceride lipase (ATGL) correlated with dramatic reductions in glucose levels and increased hepatic AKT phosphorylation in the same model of stress-induced hyperglycemia used herein [44]. The authors specifically documented a role of glycerol in these processes by demonstrating that mice lacking aquaporin 7, the main glycerol-release channel in adipocytes, were protected from stress-induced hyperglycemia. Further, adding back glycerol abolished the previously observed protection from stress-induced hyperglycemia in mice pretreated with atglistatin, an ATGL inhibitor. Interestingly, changes in fatty acids were not consistent, including significant variability among the controls. This variability in fatty acid release despite increased lipolysis was similar to what has been described in the literature [44,59]. In particular, re-esterification of fatty acids in the adipocytes is sensitive to alterations in perfusion and tissue acidification, both of which are side effects of hemorrhage-induced hypotension and could be of consequence in this model.

The potential importance of glycerol in influencing hepatic glucose production is not limited to stress-induced hyperglycemia. Perry et al. not only observed that inhibition of peripheral lipolysis via atglistatin decreased hepatic glucose production in mice lacking the insulin receptor, but explicitly found a strong correlation between rates of glycerol turnover and hepatic glucose production during hyperinsulinemic-euglycemic clamps [43]. They also found that insulin-mediated suppression of hepatic glucose uptake was abolished by co-infusion of acetate and glycerol in both controls and mice lacking AKT1, AKT2, and FOXO in the liver, suggesting a role of glycerol in promoting hyperglycemia. Wondisford et al. tackled this question in short and prolonged fasted mice using infusions of $^{13}C_3$ glycerol, $^{13}C_3$ lactate, and $^{13}C_3$ glucose. The authors found that glycerol became a major carbon source for gluconeogenesis with increased fasting, a stressed metabolic setting that may be related to stress-induced hyperglycemia [60]. Although our findings echo this potential role of glycerol in promoting hepatic glucose production, additional research is needed to better understand the relative contribution of this substrate to *de novo* gluconeogenesis compared to circulating fatty acids, lactate, or other factors in acute and chronic hyperglycemia.

Our data expand on the complex relationship between glucose levels and peripheral lipolysis by demonstrating the role of the liver in regulating adipose insulin sensitivity in stress-induced hyperglycemia. Specifically, we found that FOXO activity was upregulated in stress-induced hyperglycemia and that loss of FOXO transcription factors in

the liver reduced hyperglycemia in part due to enhanced adipose tissue insulin sensitivity, inducing the same cross-talk seen between the liver and adipose described in chronic insulin resistance [40,41,43]. The next frontier is to determine which factors mediate the cross-talk between the liver and periphery in acute insulin resistance and whether these factors can be harnessed for pharmacological treatment of stress-induced hyperglycemia. In addition to glycerol and fatty acids, research into chronic insulin resistance found a role of other substrates in promoting hyperglycemia [29,43]. For example, Perry et al. found that hyperglycemia was associated with increased adipose tissue-derived hepatic acetyl CoA and that IL-6-mediated lipolysis plays a central role in the relationship [43]. Conversely, Tao et al. found that loss of IL-6 was not associated with an improved metabolic phenotype, instead favoring a role of the FOXO-regulated hepatokine follistatin [41]. Since then, IL-6 has reemerged in acute insulin resistance after a recent study proposed a role of brown adipocyte-derived IL-6 in promoting hepatic gluconeogenesis in acute stress [54]. Our research was consistent with both of these studies, as both follistatin and IL-6 increased between TH0 and TH90 and were reduced at TH90 in the L-FOXOTKO mice, suggesting a role of either of these factors in the cross-talk between the liver and adipose.

In addition to follistatin and IL-6, other circulating factors have also been implicated in promoting hepatic glucose production in stress-induced hyperglycemia. Most notably, previous research demonstrated a relationship between increased levels of TNF-α and hyperglycemia in the trauma and hemorrhage model including that infusion of a neutralizing TNF-α antibody reversed many of the signaling defects associated with hemorrhagic shock [55]. Our research also potentially implicates TNF-α, as we found that the L-FOXOTKO mice exhibited reduced cytokine levels at TH90. In addition, increased levels of adipokines are implicated in the detrimental metabolic effects of stress-induced hyperglycemia, in particular resistin [44,56]. We found that resistin similarly increased at TH90 in both the controls and L-FOXOTKO mice, suggesting that at least in our paradigm, resistin levels, or lack thereof, were not a major contributor to the observed reduced stress-induced hyperglycemia response seen in the triple knockout. Interestingly, leptin was significantly elevated at TH90 in the controls, but not in the L-FOXOTKO mice, suggesting a possible role of the adipokine in the FOXO paradigm described herein.

FGF21 is a FOXO-regulated hepatokine that is associated with improved systemic insulin sensitivity [53,57]. As expected, we also found that FGF21 levels increased in the L-FOXOTKO mice in general at both TH0 and TH90, without a particular trend in relation to the trauma intervention itself, suggesting that hepatokines may have an effect on insulin sensitivity that is independent of stress insults per se. Although the responsible culprit is rightfully debated, defining how hepatic transcription factors such as FOXO regulate the cell non-autonomous control of hepatic glucose metabolism through circulating factors could open novel avenues for treating acute and chronic insulin-resistant states. Our data suggest that perhaps multiple FOXO-regulated factors are necessary to promote stress-induced hyperglycemia. Additional loss-of-function experiments are needed to define which factors are truly required and sufficient.

A limitation of this study was its inability to definitively confirm improvements in insulin sensitivity, specifically changes in hepatic glucose production or peripheral glucose uptake. Although the decreases in glucose and insulin levels were impressive, adding a hyperinsulinemic-euglycemic clamp would directly assess changes in insulin sensitivity in a tissue-specific manner. Our findings do not rule out the possibility of increased skeletal muscle glucose uptake in our model, and a clamp would help answer this question by directly quantifying glucose uptake in muscle. Alternatively, we find it less likely that adipose tissue glucose uptake per se is playing a role as much as the regulation of lipolysis, although this would be better investigated with labeling studies. Developing more advanced infusion protocols in the trauma model will enable us to answer these questions on peripheral glucose uptake and lipolysis, including explicitly quantifying changes in glycerol and fatty acid turnover.

We are similarly limited in our assessment of the contribution of other metabolic perturbations to stress-induced hyperglycemia. Injury and illness are associated with multiple hormone derangements, including increases in glucagon, cortisol, and epinephrine, the latter of which is of particular importance to the regulation of peripheral adipose tissue lipolysis [58]. Others suggest a role of tissue inflammation in defective insulin signaling, which was not explored in this study outside of measuring circulating cytokines. In particular, TNF-α blockade and reduced reactive oxidative species are associated with improved hepatic insulin sensitivity [35,55]. Evidence suggests that reactive oxidative species may impact or reflect changes in FOXO activity, presenting another angle for investigation as to how FOXO regulates stress-induced hyperglycemia [61]. Stress-induced hyperglycemia also occurs in sepsis and other forms of critical illness. FOXO gene target expression has been implicated in the proteolysis and insulin resistance seen in skeletal muscle in response to lipopolysaccharide (LPS)-induced endotoxemia [62]. Future research will confirm whether FOXO-dependent mechanisms apply to stress-induced hyperglycemia broadly or just injury and hemorrhage.

4. CONCLUSIONS

We found that persistent upregulation of hepatic FOXO activity is central to stress-induced hyperglycemia through both direct and indirect mechanisms. Loss of multiple FOXO transcription factors in the liver leads to a reduction in the hyperglycemia and hyperinsulinemia normally seen in stress-induced hyperglycemia. These preclinical animal studies support the notion that inhibition of FOXO or excess adipocyte lipolysis may be an effective strategy to combat stress-induced hyperglycemia. More research is needed to translate these findings into a human clinical setting.

5. MATERIALS AND METHODS

5.1. Animals

The institutional animal care and use committees of the University of Pennsylvania approved all of the animal studies, which adhered to the National Institutes of Health guidelines for the care and use of laboratory animals. Male mice (*Mus musculus*) were used in all of the experiments. The mice were maintained on a 12-hour light/dark cycle. The L-FOXO1 and L-FOXOTKO mice were previously described [63,64]. Adult *Foxo1*^fl/fl or *Foxo1*^fl/fl, *Foxo3*^fl/fl, and *Foxo4*^fl/Y mice were injected at 8−10 weeks of age with an adeno-associated virus containing a liver-specific promoter, thyroxine-binding globulin (TBG), driving either GFP or Cre recombinase to generate control or knockout mice, respectively. The control mice were GFP-injected littermates floxed for indicated genotypes while the knockout mice were injected with Cre recombinase to generate L-FOXO1KO or L-FOXOTKO. Experiments were performed 2−3 weeks after virus injection.

5.2. Trauma and hemorrhage protocol

We used an established mouse model of trauma and hemorrhage (TH) to induce stress-induced hyperglycemia [8,33]. The mice were

fasted 6 h prior to surgery with access to water ad libitum. The mice were anesthetized with 3% isoflurane and 97% air via the SomnoSuite Low-Flow Anesthesia System (Kent Scientific, Torrington, CT, USA), with levels reduced to 1—1.5% isoflurane by the end of surgery and maintained there for the duration of the protocol. A 2-cm ventral midline laparotomy was performed to mimic soft tissue trauma and subsequently closed in layers with 5-0 silk sutures. The wounds were bathed in 1% lidocaine to minimize pain. Incisions were made in the left and right groins to identify the bilateral femoral arteries. Polyethylene catheters were placed in each artery. One catheter was connected to a blood pressure transducer as part of the PowerLab data acquisition device (ADInstruments, Colorado Springs, CO, USA), allowing for real-time invasive blood pressure monitoring. Enough blood was removed via the other arterial catheter to maintain a mean arterial pressure of 35—40 mmHg for 90 min (TH90). A subset of animals underwent surgery and was sacrificed immediately after (T0 or TH0), while another subset were sacrificed after 90 min of anesthesia but without induction of hemorrhage (T90). Blood was collected before sacrifice in the T0 mice and T90 mice, whereas blood was collected both immediately after surgery and 90 min later (TH0 and TH90) in the hemorrhaged mice to perform paired analyses.

5.3. Tissue collection

At the respective time point, the laparotomy was reopened to inject 0.2 U/kg of insulin in vehicle (3% BSA in normal saline) or vehicle alone into the portal vein. Tissues were then collected 5 min later including the liver, epididymal fat, and quadriceps muscle. The tissues were then quickly snap frozen in liquid nitrogen and stored at −80 °C until experimental use.

5.4. Study design

Because it is not possible to noninvasively sample femoral artery blood or inject the portal vein, we were unable to have a completely trauma-free group. For this reason, the mice labeled T0 or TH0 in Figure 1 are mice that underwent anesthesia and catheter placement but were sacrificed immediately after placement of femoral artery catheters and collection of baseline arterial blood. The mice labeled TH90 are mice that went on to complete the 90 min of trauma- and hemorrhage-induced hypotension and sacrificed after 90 min. The mice labeled T90 underwent surgery and 90 min of anesthesia but no hemorrhage. In Figures 2—4, arterial blood was collected from the same animal immediately after surgery (TH0) and after 90 min of hemorrhage-induced hypotension (TH90), allowing for paired analysis. Tissues were collected at TH90 as well as in a subset of animals at TH0 to provide baseline levels for tissue studies such as glycogen, gene expression, and Western blotting.

5.5. Metabolite and hormone assays

Blood was sampled from femoral artery catheters. Glucose was directly measured via glucometers at the respective time points. Blood was collected in commercially available heparinized tubes and centrifuged for 15 min at 4 °C to isolate plasma. Plasma was stored at −80 °C until later analysis. Plasma was assayed for insulin via ELISA (Crystal Chem, IL, USA). Plasma was assayed for fatty acids, ketones, and glycerol using colorimetric assays from FUJIFILM Medical Systems (Lexington, MA, USA), Stanbio Laboratory (Boerne, TX, USA), and Sigma—Aldrich (St. Louis, MO, USA), respectively. Plasma was assayed for TNF-α, leptin, IL-6, and resistin via the Luminex mouse adipokine panel (Millipore, Billerica, MA, USA) run on a Luminex MAGPIX machine and analyzed via xPONENT software.

5.6. Quantification of relevant gene expression in the liver

Total RNA was isolated from frozen livers using the RNeasy Plus Kit (Qiagen) according to the manufacturer's directions. Complementary DNA was synthesized using Moloney murine leukemia virus reverse transcriptase. Relative expression of genes of interest was quantified by real-time qPCR performed with SYBR Green dye-based assays and the Applied Biosystems QuantStudio instrument (Applied Biosystems). The analysis was performed via the ΔΔCT method. A list of mouse primers used is shown in Supplemental Table 1.

5.7. Tissue protein extraction for Western blotting

Protein lysates were prepared from frozen livers and muscle in a modified cell lysis or RIPA buffer, respectively, with phosphatase and protease inhibitors as previously described [42]. Protein was isolated from adipose in the RIPA buffer using a similar approach with the added step of acetone precipitation to remove excess lipids. The following antibodies from Cell Signaling Technology were used for immunoblotting: pAKT Ser473 (#9271), Akt (#2964), PRAS40 (#2610), pS6 (#2215), S6 (#2217), and HSP90 (#4874). IGFBP-1 (sc-6000) was from Santa Cruz. pPRAS40 (07—888) was from Millipore. Blots were developed with the LI-COR Odyssey Imaging System including quantification via the associated Image Studio Software (LI-COR Biosciences, Lincoln, NE, USA). Quantifications are expressed as changes relative to controls.

5.8. Liver glycogen determination

Glycogen was extracted from ∼100 mg of liver in 6% perchloric acid by digesting the samples in KOH followed by digestion with amyloglucosidase (Sigma—Aldrich, St. Louis, MO). Resulting free glycosyl units were assayed spectrophotometrically using a hexokinase-based glucose assay kit (Sigma—Aldrich, St. Louis, MO, USA) and compared to the glucose levels in the samples prior to digestion.

5.9. Statistical power and data analysis

Data between two groups were compared using paired or unpaired Student's t tests depending on the experimental design. One-way ANOVA was used for three groups. Two-way ANOVA was used when comparing the knockouts to controls at two time points, except in the plasma studies, in which two-way repeated-measures ANOVA was used given the paired-samples approach. Grubbs' test was used to identify outliers. All the statistics were completed using statistical programming within Prism 9 macOS by GraphPad Software, Inc. (San Diego, CA, USA). The statistical study design was confirmed via consultation with the Biostatistical Analysis Center at the University of Pennsylvania. Data are available for review upon request.

CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2021.101246.

REFERENCES

[1] Wang, S., Ma, P., Zhang, S., Song, S., Wang, Z., Ma, Y., et al., 2020. Fasting blood glucose at admission is an independent predictor for 28-day mortality in patients with COVID-19 without previous diagnosis of diabetes: a multi-centre retrospective study. Diabetologia 63(10):2102—2111.

[2] Zhu, B., Pan, Y., Jing, J., Meng, X., Zhao, X., Liu, L., et al., 2019. Stress hyperglycemia and outcome of non-diabetic patients after acute Ischemic stroke. Frontiers in Neurology 10:1003.

[3] Bosarge, P.L., Shoultz, T.H., Griffin, R.L., Kerby, J.D., 2015. Stress-induced hyperglycemia is associated with higher mortality in severe traumatic brain injury. Journal of Trauma and Acute Care Surgery 79(2):289—294.

[4] Sperry, J.L., Frankel, H.L., Vanek, S.L., Nathens, A.B., Moore, E.E., Maier, R.V., et al., 2007. Early hyperglycemia predicts multiple organ failure and mortality but not infection. The Journal of Trauma 63(3):487—493 discussion 493-484.

[5] Ali, N.A., O'Brien Jr., J.M., Dungan, K., Phillips, G., Marsh, C.B., Lemeshow, S., et al., 2008. Glucose variability and mortality in patients with sepsis. Critical Care Medicine 36(8):2316—2321.

[6] Lee, T.F., Burt, M.G., Heilbronn, L.K., Mangoni, A.A., Wong, V.W., McLean, M., et al., 2017. Relative hyperglycemia is associated with complications following an acute myocardial infarction: a post-hoc analysis of HI-5 data. Cardiovascular Diabetology 16(1):157.

[7] Carrasco-Sánchez, F.J., López-Carmona, M.D., Martínez-Marcos, F.J., Pérez-Belmonte, L.M., Hidalgo-Jiménez, A., Buonaiuto, V., et al., 2021. Admission hyperglycaemia as a predictor of mortality in patients hospitalized with COVID-19 regardless of diabetes status: data from the Spanish SEMI-COVID-19 Registry. Annals of Medicine 53(1):103—116.

[8] Li, L., Messina, J.L., 2009. Acute insulin resistance following injury. Trends in Endocrinology and Metabolism 20(9):429—435.

[9] Lesperance, R.N., Guillamondegui, O.D., 2016. Glycemic control and insulin resistance. In: Martin, N.D., Kaplan, L.J. (Eds.), Principles of adult surgical critical care. Cham: Springer International Publishing. p. 343—50.

[10] Umpierrez, G.E., Isaacs, S.D., Bazargan, N., You, X., Thaler, L.M., Kitabchi, A.E., 2002. Hyperglycemia: an independent marker of in-hospital mortality in patients with undiagnosed diabetes. The Journal of Clinical Endocrinology & Metabolism 87(3):978—982.

[11] Jacobi, J., Bircher, N., Krinsley, J., Agus, M., Braithwaite, S.S., Deutschman, C., et al., 2012. Guidelines for the use of an insulin infusion for the management of hyperglycemia in critically ill patients. Critical Care Medicine 40(12):3251—3276.

[12] Bochicchio, G.V., Joshi, M., Bochicchio, K.M., Pyle, A., Johnson, S.B., Meyer, W., et al., 2007. Early hyperglycemic control is important in critically injured trauma patients. The Journal of Trauma 63(6):1353—1358 discussion 1358-1359.

[13] Falciglia, M., Freyberg, R.W., Almenoff, P.L., D'Alessio, D.A., Render, M.L., 2009. Hyperglycemia-related mortality in critically ill patients varies with admission diagnosis. Critical Care Medicine 37(12):3001—3009.

[14] Krinsley, J.S., 2003. Association between hyperglycemia and increased hospital mortality in a heterogeneous population of critically ill patients. Mayo Clinic Proceedings 78(12):1471—1478.

[15] Sung, J., Bochicchio, G.V., Joshi, M., Bochicchio, K., Tracy, K., Scalea, T.M., 2005. Admission hyperglycemia is predictive of outcome in critically ill trauma patients. The Journal of Trauma 59(1):80—83.

[16] van Vught, L.A., Wiewel, M.A., Klein Klouwenberg, P.M., Hoogendijk, A.J., Scicluna, B.P., Ong, D.S., et al., 2016. Admission hyperglycemia in critically ill sepsis patients: association with outcome and host response. Critical Care Medicine 44(7):1338—1346.

[17] Kerby, J.D., Griffin, R.L., MacLennan, P., Rue 3rd, L.W., 2012. Stress-induced hyperglycemia, not diabetic hyperglycemia, is associated with higher mortality in trauma. Annals of Surgery 256(3):446—452.

[18] Van den Berghe, G., 2004. How does blood glucose control with insulin save lives in intensive care? Journal of Clinical Investigation 114(9):1187—1195.

[19] van den Berghe, G., Wouters, P., Weekers, F., Verwaest, C., Bruyninckx, F., Schetz, M., et al., 2001. Intensive insulin therapy in critically ill patients. New England Journal of Medicine 345(19):1359—1367.

[20] Krinsley, J.S., 2004. Effect of an intensive glucose management protocol on the mortality of critically ill adult patients. Mayo Clinic Proceedings 79(8):992—1000.

[21] Investigators, N.-S.S., Finfer, S., Chittock, D.R., Su, S.Y., Blair, D., Foster, D., et al., 2009. Intensive versus conventional glucose control in critically ill patients. New England Journal of Medicine 360(13):1283—1297.

[22] Thorell, A., Efendic, S., Gutniak, M., Haggmark, T., Ljungqvist, O., 1994. Insulin resistance after abdominal surgery. British Journal of Surgery 81(1):59—63.

[23] Black, P.R., Brooks, D.C., Bessey, P.Q., Wolfe, R.R., Wilmore, D.W., 1982. Mechanisms of insulin resistance following injury. Annals of Surgery 196(4):420—435.

[24] Shaw, J.H., Wolfe, R.R., 1989. An integrated analysis of glucose, fat, and protein metabolism in severely traumatized patients. Studies in the basal state and the response to total parenteral nutrition. Annals of Surgery 209(1):63—72.

[25] Dahn, M.S., Mitchell, R.A., Lange, M.P., Smith, S., Jacobs, L.A., 1995. Hepatic metabolic response to injury and sepsis. Surgery 117(5):520—530.

[26] Thorell, A., Rooyackers, O., Myrenfors, P., Soop, M., Nygren, J., Ljungqvist, O.H., 2004. Intensive insulin treatment in critically ill trauma patients normalizes glucose by reducing endogenous glucose production. The Journal of Clinical Endocrinology & Metabolism 89(11):5382—5386.

[27] Nelson, K.M., Long, C.L., Bailey, R., Smith, R.J., Laws, H.L., Blakemore, W.S., 1992. Regulation of glucose kinetics in trauma patients by insulin and glucagon. Metabolism 41(1):68—75.

[28] Long, C.L., Kinney, J.M., Geiger, J.W., 1976. Nonsuppressability of gluconeogenesis by glucose in septic patients. Metabolism 25(2):193—201.

[29] Titchenell, P.M., Lazar, M.A., Birnbaum, M.J., 2017. Unraveling the regulation of hepatic metabolism by insulin. Trends in Endocrinology and Metabolism 28(7):497—505.

[30] Baxter, R.C., 2001. Changes in the IGF-IGFBP axis in critical illness. Best Practice & Research Clinical Endocrinology & Metabolism 15(4):421—434.

[31] Mesotten, D., Delhanty, P.J., Vanderhoydonc, F., Hardman, K.V., Weekers, F., Baxter, R.C., et al., 2002. Regulation of insulin-like growth factor binding protein-1 during protracted critical illness. The Journal of Clinical Endocrinology & Metabolism 87(12):5516—5523.

[32] Buras, J.A., Holzmann, B., Sitkovsky, M., 2005. Animal models of sepsis: setting the stage. Nature Reviews Drug Discovery 4(10):854—865.

[33] Ayala, A., Wang, P., Chaudry, I., 2001. Shock models: hemorrhage. In: Souba, W.W.D. (Ed.), Surgical research. San Diego, CA: Academic PRess. p. 325—7.

[34] Ma, Y., Wang, P., Kuebler, J.F., Chaudry, I.H., Messina, J.L., 2003. Hemorrhage induces the rapid development of hepatic insulin resistance. American Journal of Physiology - Gastrointestinal and Liver Physiology 284(1):G107—G115.

[35] Zhai, L., Ballinger, S.W., Messina, J.L., 2011. Role of reactive oxygen species in injury-induced insulin resistance. Molecular Endocrinology 25(3):492—502.

[36] Golson, M.L., Kaestner, K.H., 2016. Fox transcription factors: from development to disease. Development 143(24):4558—4570.

[37] Haeusler, R.A., Hartil, K., Vaitheesvaran, B., Arrieta-Cruz, I., Knight, C.M., Cook, J.R., et al., 2014. Integrated control of hepatic lipogenesis versus glucose production requires FoxO transcription factors. Nature Communications 5:5190.

[38] Maitra, S.R., Pan, W., Lange, A.J., el-Maghrabi, M.R., Abumrad, N.N., Pilkis, S.J., 1994. Glucose-6-phosphatase gene expression and activity are modulated in hemorrhagic shock: evidence for a new heat-sensitive activator. Biochemical and Biophysical Research Communications 204(2):716—724.

[39] I, O.S., Zhang, W., Wasserman, D.H., Liew, C.W., Liu, J., Paik, J., et al., 2015. FoxO1 integrates direct and indirect effects of insulin on hepatic glucose production and glucose utilization. Nature Communications 6:7079.

[40] Titchenell, P.M., Quinn, W.J., Lu, M., Chu, Q., Lu, W., Li, C., et al., 2016. Direct hepatocyte insulin signaling is required for lipogenesis but is dispensable for the suppression of glucose production. Cell Metabolism 23(6):1154—1166.

[41] Tao, R., Wang, C., Stohr, O., Qiu, W., Hu, Y., Miao, J., et al., 2018. Inactivating hepatic follistatin alleviates hyperglycemia. Nature Medicine 24(7):1058—1069.

[42] Titchenell, P.M., Chu, Q., Monks, B.R., Birnbaum, M.J., 2015. Hepatic insulin signalling is dispensable for suppression of glucose output by insulin in vivo. Nature Communications 6:7078.

[43] Perry, R.J., Camporez, J.G., Kursawe, R., Titchenell, P.M., Zhang, D., Perry, C.J., et al., 2015. Hepatic acetyl CoA links adipose tissue inflammation to hepatic insulin resistance and type 2 diabetes. Cell 160(4):745—758.

[44] Raje, V., Ahern, K.W., Martinez, B.A., Howell, N.L., Oenarto, V., Granade, M.E., et al., 2020. Adipocyte lipolysis drives acute stress-induced insulin resistance. Scientific Reports 10(1):18166.

[45] Li, L., Thompson, L.H., Zhao, L., Messina, J.L., 2009. Tissue-specific difference in the molecular mechanisms for the development of acute insulin resistance after injury. Endocrinology 150(1):24—32.

[46] Matsumoto, M., Pocai, A., Rossetti, L., Depinho, R.A., Accili, D., 2007. Impaired regulation of hepatic glucose production in mice lacking the forkhead transcription factor Foxo1 in liver. Cell Metabolism 6(3):208—216.

[47] Haeusler, R.A., Kaestner, K.H., Accili, D., 2010. FoxOs function synergistically to promote glucose production. Journal of Biological Chemistry 285(46): 35245—35248.

[48] Belfrage, E., Hjemdahl, P., Fredholm, B.B., 1979. Metabolic effects of blood flow restriction in adipose tissue. Acta Physiologica Scandinavica 105(2):222—227.

[49] Klein, S., Peters, E.J., Shangraw, R.E., Wolfe, R.R., 1991. Lipolytic response to metabolic stress in critically ill patients. Critical Care Medicine 19(6):776—779.

[50] Ilias, I., Vassiliadi, D.A., Theodorakopoulou, M., Boutati, E., Maratou, E., Mitrou, P., et al., 2014. Adipose tissue lipolysis and circulating lipids in acute and subacute critical illness: effects of shock and treatment. Journal of Critical Care 29(6):1130 e1135—1139.

[51] Williams, V.L., Martin, R.E., Franklin, J.L., Hardy, R.W., Messina, J.L., 2012. Injury-induced insulin resistance in adipose tissue. Biochemical and Biophysical Research Communications 421(3):442—448.

[52] Czech, M.P., 2020. Mechanisms of insulin resistance related to white, beige, and brown adipocytes. Molecular Metabolism 34:27—42.

[53] Kliewer, S.A., Mangelsdorf, D.J., 2019. A dozen years of discovery: insights into the physiology and pharmacology of FGF21. Cell Metabolism 29(2):246—253.

[54] Qing, H., Desrouleaux, R., Israni-Winger, K., Mineur, Y.S., Fogelman, N., Zhang, C., et al., 2020. Origin and function of stress-induced IL-6 in murine models. Cell 182(6):1660.

[55] Xu, J., Kim, H.T., Ma, Y., Zhao, L., Zhai, L., Kokorina, N., et al., 2008. Trauma and hemorrhage-induced acute hepatic insulin resistance: dominant role of tumor necrosis factor-alpha. Endocrinology 149(5):2369—2382.

[56] Hajri, T., Gharib, M., Kaul, S., Karpeh Jr., M.S., 2017. Association between adipokines and critical illness outcomes. Journal of Trauma and Acute Care Surgery 83(3):507—519.

[57] Li, H., Wu, G., Fang, Q., Zhang, M., Hui, X., Sheng, B., et al., 2018. Fibroblast growth factor 21 increases insulin sensitivity through specific expansion of subcutaneous fat. Nature Communications 9(1):272.

[58] Marik, P.E., Bellomo, R., 2013. Stress hyperglycemia: an essential survival response! Critical Care Medicine 41(6):e93—94.

[59] Cree, M.G., Wolfe, R.R., 2008. Postburn trauma insulin resistance and fat metabolism. American Journal of Physiology. Endocrinology and Metabolism 294(1):E1—E9.

[60] Wang, Y., Kwon, H., Su, X., Wondisford, F.E., 2020. Glycerol not lactate is the major net carbon source for gluconeogenesis in mice during both short and prolonged fasting. Molecular Metabolism 31:36—44.

[61] Klotz, L.O., Sanchez-Ramos, C., Prieto-Arroyo, I., Urbanek, P., Steinbrenner, H., Monsalve, M., 2015. Redox regulation of FoxO transcription factors. Redox Biology 6:51—72.

[62] Crossland, H., Constantin-Teodosiu, D., Gardiner, S.M., Constantin, D., Greenhaff, P.L., 2008. A potential role for Akt/FOXO signalling in both protein loss and the impairment of muscle carbohydrate oxidation during sepsis in rodent skeletal muscle. Journal of Physiology 586(22):5589—5600.

[63] Lu, M., Wan, M., Leavens, K.F., Chu, Q., Monks, B.R., Fernandez, S., et al., 2012. Insulin regulates liver metabolism in vivo in the absence of hepatic Akt and Foxo1. Nature Medicine 18(3):388—395.

[64] Lee, S.X., Heine, M., Schlein, C., Ramakrishnan, R., Liu, J., Belnavis, G., et al., 2018. FoxO transcription factors are required for hepatic HDL cholesterol clearance. Journal of Clinical Investigation 128(4):1615—1626.

12   MOLECULAR METABOLISM 51 (2021) 101246   www.molecularmetabolism.com