DISSERTATION

EFFECTS OF THE POSTPRANDIAL ENVIRONMENT ON THE UNFOLDED PROTEIN RESPONSE

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY KYLE PFAFFENBACH ENTITLED EFFECTS OF THE POSTPRANDIAL ENVIRONMENT ON THE UNFOLDED PROTEIN RESPONSE BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate work

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ABSTRACT OF DISSERTATION

EFFECTS OF THE POSTPRANDIAL ENVIRONMENT ON THE UNFOLDED PROTEIN RESPONSE

BACKGROUND AND SPECIFIC AIMS: Newly synthesized proteins must undergo post-translational modifications such as folding and glycosylation to become fully finctional. The lumen of the endoplasmic reticulum (ER) is a major site of protein folding and processing. The ER can respond to an increase in unfolded proteins (termed "ER stress") by activating the Unfolded Protein Response (UPR), a quality control mechanism which maintains ER homeostasis. Recent studies have demonstrated that the UPR is also involved in the regulation of a diverse array of cellular processes including glucose homeostasis, lipogenesis, and protein synthesis. The liver plays a central role in nutrient metabolism and maintaining glucose homeostasis. Further, in the postprandial state, both lipogenesis and protein synthesis are stimulated in the liver. However, the role of the UPR in the postprandial regulation of these processes has not been studied. Therefore, the first aim of the present study was to examine and characterize the regulation of the UPR in the postprandial state in the liver. One of the main rate limiting steps in protein synthesis is regulatated by the mammalian target of rapamycin complex-1 (mTORC1). Thus, the second aim of the current study was to examine the role of mTORC1 in the postprandial regulation of the UPR. **METHODS:** Rats in Study 1 were fed a single starch or high sucrose meal and sacrificed either 1 or 7 hours post-feeding period. Plasma glucose and insulin were measured and hepatic tissue was examined for

markers of UPR activation. Rats in Study 2 were injected with rapamycin, an inhibitor of mTORC1, prior to meal feeding. Rats were sacrificed 1 or 7 hours post-feeding period and blood and liver tissue were collected for analysis. To examine the role of insulin and glucose in the postprandial activation of the UPR H4IIE liver cells were exposed to varying amounts of glucose and insulin in the presence or absence of rapamycin. **RESULTS:** Feeding activated select components of the UPR, including spliced X-box binding protein-1 (XBP1) and increased GRP78 and GRP94 mRNA expression. Rapamycin inhibited the postprandial increase of these components. The phosphorylation of eif2-α protein was not increased postprandially in the liver. Data from H4IIE cells demonstrate that insulin in the presence of glucose can activate UPR components in an mTORC1 dependent manner. **CONCLUSION:** The current study demonstrates that select components of the UPR are activated in the liver in the postprandial state. This activation appears to be insulin and mTORC1 dependent.

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CHAPTER 1

INTRODUCTION AND SPECIFIC AIMS

The liver plays a central role in nutrient metabolism. In the postabsorptive state the liver is a net glucose producer responsible, along with the kidney, for the maintenance of blood glucose homeostasis. In response to meal ingestion, the liver becomes a net consumer of nutrients and is characterized by increased glycogen, lipid, and protein synthesis.

One of the rate limiting steps in the synthesis of new proteins is regulated by the protein kinase, mammalian target of rapamycin complex-1 (mTORC1). mTORC1 can be activated by insulin and, at least some nutrients, and functions to activate components of protein translation initiation. Membrane bound and secretory proteins are translated by ribosomes associated with the endoplasmic reticulum (ER). The ER is a membranous organelle physically attached to the cell membrane and the nucleus. The lumen of the ER is characterized by a unique oxidative environment and a high concentration of protein chaperones, both of which function to facilitate protein folding.

The lumen of the ER is highly sensitive to perturbations of the cellular environment, such as hypoxia and nutrient deprivation/excess. These perturbations can reduce the ability of the ER lumen to fold or degrade proteins relative to the entering protein client load and result in the accumulation of unfolded or malfolded proteins and protein aggregates, a condition known as "ER stress". The ER responds to this stress by activating the unfolded protein response (UPR). The UPR was originally discovered and characterized in yeast and cell culture models under conditions in which ER stress was induced using pharmacologic agents. Thus, the UPR has, until recently, been considered

a signaling pathway designed to attenuate protein translation and upregulate the capacity of the ER to fold and degrade proteins. More recent studies using genetic models have demonstrated that the UPR plays a vital role in cellular development and nutrient metabolism. These studies have linked components of the activated UPR to a diverse array of cellular functions including cell differentiation, phospholipid biosynthesis and ER membrane expansion, lipogenesis, glucose homeostasis, and insulin action [1-4]. In addition, activation of the UPR has now been observed in cancer cells, in the liver and adipose tissue of humans with non-alcoholic fatty liver disease, obesity and/or type 2 diabetes, and in brain regions of individuals with Alzheimer's disease [5-8]. Thus, while the general structure and function of the UPR has been comprehensively examined, very little is presently known regarding the physiologic factors that promote ER stress and UPR activation, and the role of the UPR in metabolic diseases. Therefore, the current investigation was designed to examine and characterize the UPR in the liver in response to meal ingestion.

Specific Aim 1: To examine and characterize the UPR in response to a single high starch or high sucrose meal in the liver of rats.

Specific Aim 2: To examine the role of the mTORC1 pathway in postprandial regulation of the UPR in the liver of rats.

CHAPTER 2

LITERATURE REVIEW

Introduction.

The postprandial state is marked by significant changes in circulating nutrients and hormones. Carbohydrates and proteins are digested in the gastrointestinal tract, and are absorbed into the portal venous circulation as simple sugars and amino acids. Pancreatic glucagon secretion is suppressed and insulin secretion is stimulated in response to elevated nutrient concentrations. Generally, an elevated glucagon-to-insulin ratio stimulates glycogen breakdown and maintains hepatic glucose output in the fasted state. Conversely, increased insulin concentrations facilitate blood glucose disposal and stimulate protein synthesis in tissues throughout the body. Because portal vein blood flow crosses the liver prior to entering the systemic circulation, the liver is anatomically positioned to act as a nutrient and hormone buffer, and is a vital component in postprandial nutrient disposal. Among other functions, it is involved in the oxidation and storage of glucose, processing of dietary lipids, metabolism of amino acids, and the synthesis of secretory proteins. The focus of the current discussion is on the postprandial regulation of hepatic carbohydrate and amino acid metabolism, with emphasis on key metabolic pathways involved in nutrient oxidation and protein synthesis.

Hepatic glucose metabolism.

The liver is unique in that during the fasted state it is a net glucose producer, but throughout the postprandial period it is a net glucose consumer. The transition from net glucose production to net consumption is rapid and regulated mainly by the rise in portal vein glucose and insulin, and fall in glucagon. Studies in humans, dogs, and rats have

demonstrated that first pass liver uptake accounts for approximately 30% of the disposal of an ingested glucose load [9]. Importantly, this quantity of glucose is sufficient to meet the energy requirements and replenish the glycogen stores of the liver [10].

Overall, net glucose disposal by the liver is dependent on the relationship between the absolute rates of hepatic glucose output and uptake. Several studies in humans and dogs have demonstrated that glucose per se regulates hepatic glucose output. A study done in humans by Sacca et al [11] isolated the effect of glucose on the rate of hepatic glucose output by clamping plasma insulin and glucagon at basal levels and infusing glucose over 2 hours. Plasma glucose levels rose from 4.2mmol/L to 5.5mmol/L, and net hepatic glucose output was decreased by 80% within 20 min. In agreement with this observation, Petersen et al [12] demonstrated that endogenous glucose production was decreased by 66% during a hyperglycemic-euinsulinemic clamp in human subjects. This study also utilized ^{13}C NMR spectroscopy to demonstrate that 92% of the decrease in glucose output was attributed to suppression of glycogenolysis. Together, these studies demonstrated that elevated blood glucose in the presence of basal peripheral insulin and glucagon suppressed hepatic glucose output in humans. However, a weakness in both studies was that neither controlled for portal vein hormone levels. The study by Sacca et al infused basal glucagon into a peripheral vein, hence the amount of glucagon reaching the hepatocyte was likely lower than the endogenous delivery of this hormone under basal conditions. In the study by Petersen et al, glucagon was not replaced and as a result subjects were hypoglucagonemic. Therefore, it is likely that the suppression of hepatic glucose output, as determined in these studies, was not exclusively caused by glucose.

Shulman et al [13] observed a rapid 60% reduction in endogenous glucose production when plasma glucose was raised to 12 mmol/L and basal insulin and glucagon were replaced intraportally in dogs. In the control group, where euglycemia was maintained, net hepatic glucose output did not change. Endogenous glucose production was reduced by 40-50% in dogs when plasma glucose was raised from 5.8 mmol/L to 7.5 mmol/L in the presence of basal intraportal insulin and glucagon [14]. Further, liver glucose output ceased when plasma glucose rose above 10.5 mmol/L [14]. The same investigators later demonstrated that the majority of glucose induced suppression of hepatic glucose output in dogs was attributed to decreased glycogenolysis. Taken together, these studies confirmed that hyperglycemia can decrease hepatic glucose production by ~40-60%, and this effect was primarily due to decreased glycogenolysis.

Along with its ability to decrease glucose output, glucose also contributes to the transition of the liver to a glucose consuming organ in the postprandial state. This latter effect is dependent on both a rise in glucose and the route of glucose entry into the circulation. De Fronzo et al [15] compared glucose uptake across the splanchnic bed (stomach, intestines, and liver) in human subjects in response to either a hyperglycemic-euinsulinemic clamp alone, or a hyperglycemic-euinsulinemic clamp and 100g of orally ingested glucose. Glucose uptake by the splanchnic bed accounted for only 14±4% of total glucose taken up by all tissues in the body during the hyperglycemic-euinsulinemic clamp alone. In contrast, when 100g of glucose was orally ingested in combination with the clamp, the splanchnic bed accounted for 60% of total glucose metabolized. This study demonstrated that orally derived glucose can promote splanchnic glucose uptake, but it did not elucidate a mechanism by which it did so. Subsequent studies demonstrated that

the GI tract was not the source of the signal, rather the liver appears to respond to a signal generated by the presence of a negative arterial-portal glucose gradient (portal vein glucose concentration in excess of arterial glucose concentration), called the portal signal [16]. Pagliassotti et al infused varying amounts of glucose into the portal vein of dogs to create different negative portal-arterial gradients and demonstrated that a small, approximately -10 mg/dl arterial-portal glucose gradient, activated the portal signal and significantly increased net hepatic glucose uptake. A follow up study by Pagliassotti et al [17] demonstrated that the route by which the portal signal increased hepatic glucose uptake was through increased glycogenesis. Therefore, in summary, a rise in peripheral glucose can decrease glycogenolysis and thereby decrease hepatic glucose output, whereas a selective rise in portal vein glucose relative to arterial glucose levels leads to increased hepatic glucose uptake and glycogenesis. Therefore, both the amount and site of entry of glucose can regulate hepatic glucose metabolism.

Insulin action and the liver.

Insulin action on the liver occurs via direct and indirect mechanisms. The direct action is initiated when insulin binds to the insulin receptor on the hepatocyte and activates the insulin signaling cascade. Among other functions, activation of the insulin signaling cascade results in suppression of glucose production and can, under postprandial conditions, facilitate hepatic glucose uptake and glycogenesis. The indirect effect of insulin can generally be described as insulin action on peripheral tissues that, in turn, leads to suppression of hepatic glucose production [18]. The principle example of indirect action is the ability of insulin to suppress adipose tissue lipolysis and decrease

plasma free fatty acids [19]. Lower free fatty acid delivery to the liver can reduce hepatic glucose output via diversion of glucose-6-phosphate to glycolysis.

The relative contribution of the direct vs. indirect actions of insulin on suppression of hepatic glucose output remains controversial. Some investigators maintain that the indirect action of insulin accounts for the majority of hepatic glucose output suppression. A study by Ader et al [19] examined overnight fasted dogs in which endogenous insulin and glucagon were suppressed by somatostatin, and insulin was infused directly into the portal vein or a peripheral vein. To isolate the effect of insulin, glucagon and glucose were replaced at basal levels and peripheral insulin was incrementally raised ~10 fold over the 4.5 hour study period. Portal vein insulin was not controlled and steady state portal insulin ranged from 10 to 345 microunits/ml in the portal infused group, and 12 to 125 microunits/ml in the peripheral infused group. Hepatic glucose production was suppressed to the same degree whether insulin was infused intraportally or into the periphery. Because peripheral insulin was clamped, but portal vein insulin varied, the authors concluded that the indirect effect of peripheral insulin was the primary route by which insulin controlled hepatic glucose output. This conclusion was challenged in a study by Sindelar et al [14] where investigators developed a protocol in which they selectively raised insulin in either the periphery or the portal vein in overnight fasted dogs. In agreement with Ader et al, the rise in peripheral insulin decreased net hepatic glucose output. The decreased hepatic glucose output was mainly accounted for by suppression of lipolysis. In contrast, a selective rise in portal insulin caused an immediate and sustained decrease in net hepatic glucose output. Further, this occurred in the absence of any change in plasma free fatty acid or peripheral insulin

concentrations. The authors calculated that the direct effect of insulin accounted for 85% of the overall reduction in hepatic glucose output. There was no observed change in the rate of gluconeogenesis, which indicated that decreased glycogenolysis was the likely mechanism by which insulin directly regulated hepatic glucose output. This study demonstrated that insulin has both direct and indirect actions on hepatic glucose output, and concluded that the direct action was the primary regulator of hepatic glucose output. In agreement with this conclusion, mice with a liver specific knockout of the insulin receptor failed to suppress hepatic glucose output in response to a hyperinsulinemic-euglycemic clamp, whereas control mice suppressed basal hepatic glucose output by 55% [20]. Collectively, these studies illustrate the important role insulin has in regulating hepatic glucose output via both direct and indirect actions.

Hepatic fructose metabolism.

Fructose is a simple sugar widely used as a sweetener in processed foods and beverages. The per capita consumption of fructose in the US has increased from 0.5 lbs/yr in 1970 to 58.3 lbs/yr in 2006 [21, 22]. The majority of postprandial fructose is metabolized by the liver [23]. As mentioned previously, net postprandial glucose uptake by the liver is approximately 30% of an oral glucose load, and this amount is sufficient to meet the energy needs and replenish glycogen stores of the liver. In contrast to glucose, the liver accounts for the disposal of 50% to 70% of an oral fructose load [23]. One explanation for the excessive uptake of fructose is that the liver has a particularly high concentration of fructokinase compared to glucokinase [23]. Fructokinase and glucokinase are ATP-dependent enzymes responsible for the phosphorylation of fructose and glucose, respectively. Another explanation for the extensive metabolism of fructose

by the liver is that phosphorylated fructose bypasses a key, rate-limiting step of glycolysis, that is the step regulated by phosphofructokinase. The combination of enhanced capacity to phosphorylate and glycolyze fructose implies that the ingestion of diets, meals or drinks containing high concentrations of sucrose (fructose + glucose) or high-fructose corn syrup may lead to a unique postprandial intrahepatic environment. Male rats given ad libitum access to a sucrose-enriched diet (68% of energy from sucrose) for 3 hours were characterized by a decreased concentration of inorganic phosphate in the liver and increased concentrations of lactate, pentose phosphate pathway intermediates, and diacylglycerol compared to rats provided a 68% starch diet for 3 hours [24]. These changes to the hepatic nutrient environment in response to the high sucrose meal are indicative of an excessive substrate load, that is, nutrient delivery in excess of energy and nutrient storage requirements [25]. This excess provokes adaptations in the liver [25]. Rats fed the high sucrose diet for 1 week had elevated plasma and liver triglycerides and increased hepatic saturated fat compared to rats fed a starch diet for 1 week. Further, rats fed sucrose were characterized by an impaired ability to suppress hepatic glucose output in response to a hyperinsulinemic-euglycemic clamp compared to starch fed rats. Thus, one week on a 68% sucrose diet lead to increased saturated fat accumulation in the liver and hepatic insulin resistance in rats [26]. Fructose can also induce hepatic insulin resistance in humans [27]. In a study by Dirlewanger et al a euglycemic, pancreatic clamp was performed in human subjects in the presence or absence of fructose infusion. The fructose infusion group required an insulin infusion rate 133% greater than the non-fructose group to maintain euglycemia. To examine the effect of the increased insulin required in the fructose group, investigators performed a control

experiment using a hyperglycemic-hyperinsulinemic clamp in which the plasma insulin was matched to that of the fructose experiment. Endogenous glucose production was 2.7 fold greater in the fructose group compared with the hyperglycemic-hyperinsulinemic group, demonstrating that an acute fructose infusion can impair the ability of insulin to suppress hepatic glucose output.

One of the primary mechanisms by which fructose induces hepatic insulin resistance is by the activation of c-jun NH_2-terminal Kinase (JNK). JNK is a member of the mitogen-activated protein kinase family that is generally activated in response to stress, inflammation, hypoxia, and is involved in the immune response and apoptosis [28]. Pagliassotti et al [29] demonstrated that insulin resistance and JNK activity were increased in rats fed a high sucrose diet for 1 week. Further, these rats had increased serine phosphorylation of Insulin Receptor Substrate 1 (IRS1), which causes impaired insulin signaling. When hepatocytes were isolated and exposed to either vehicle or a JNK inhibitor, the JNK inhibitor significantly blunted the increased in serine phosphorylation and partially restored insulin signaling in hepatocytes from sucrose-fed animals. This experiment demonstrated that fructose induced hepatic insulin resistance was, in part, due to activation of JNK and serine phosphorylation of IRS1 [29]. In summary, fructose is taken up and metabolized by the liver at an excessive rate compared to glucose. The excess metabolism provokes a metabolic stress on the hepatocyte which appears to involve activation of JNK and subsequent insulin resistance.

Hepatic protein metabolism

The liver plays an integral role in the postprandial metabolism of ingested protein. Ingested protein is digested to individual amino acids in the gastrointestinal tract and

absorbed through the small intestine. A portion of ingested amino acids are utilized for protein synthesis and oxidation by the gut, while the remainder are released into the portal circulation and pass through the liver prior to entering the peripheral circulation. The postprandial hepatic utilization of exogenous amino acids is disproportionally high compared to other tissues. It has been estimated in humans that ~30% of meal protein is utilized by splanchnic tissue [30]. This is in agreement with animal studies where hepatic amino acid utilization was measured [31]. The rate of protein turnover in the liver is high and protein synthesis is the primary result of amino acid utilization by the liver[32-35]. The main regulators of hepatic amino acid uptake and protein synthesis are glucose, insulin, and amino acids.

The liver preferentially takes up essential amino acids compared to branch chain amino acids. Essential amino acids accounted for 78% of retained amino acids in the liver of pigs administered 440g of maltose and 110g of amino acid duodenally over 1 hour. In contrast, branch chain amino acids accounted for only 43% of retained amino acids in livers of the same pigs [31]. The rate of first pass hepatic amino acid uptake also varies widely among individual amino acids. Collectively, stable isotope studies in humans have demonstrated abundant first pass hepatic extraction of glutamate, glutamine, alanine, arginine, leucine, and phenylalanine [36].

The composition of a meal can also influence postprandial hepatic amino acid extraction and utilization. Amino acid catabolism by the liver was decreased in pigs administered a mixed meal of protein and glucose compared to pigs given protein alone [37]. Fouillet et al [30] examined first pass splanchnic nitrogen uptake in humans by feeding them meals containing labeled nitrogen consisting of either protein or protein

combined with sucrose. Insulin levels rose significantly in response to the combined meal vs. the protein meal. First pass splanchnic nitrogen uptake accounted for 46% of the protein meal compared to 56% of the combined meal. Compared to the mixed meal, ingestion of the protein meal led to rapid and excessive incorporation of labeled nitrogen into the peripheral free amino acid pool, which demonstrated less hepatic amino acid uptake and utilization. In agreement with these results, dogs fed 5g/kg raw beef had a rapid increase in amino acid in both the portal vein and arterial circulation, indicating a low net amino acid uptake for both the intestine and liver. Conversely, when beef was ingested in the presence of 1.75 g/kg glucose there was significantly less portal vein and arterial amino acid, and the rate at which amino acid was increased was significantly slower than that of the beef group. Further, hepatic amino acid uptake correlated with a rise in insulin [38]. Taken together, these studies demonstrate that the postprandial splanchnic uptake and utilization of amino acids is greater in the presence of carbohydrate. Further, slowed intestinal absorption and increased insulin are two mechanisms by which carbohydrate can increase hepatic amino acid uptake.

The two main fates of dietary amino acids taken up by the liver postprandially are protein synthesis and oxidation. Many secretory and plasma proteins are exclusively synthesized in the liver. A minimally invasive and useful technique used to indirectly examine postprandial hepatic protein metabolism is to examine the incorporation of labeled exogenous amino acids into secretory proteins released into the peripheral circulation. In humans, Cayol et al [39] used the incorporation of labeled leucine into very low density lipoprotein (VLDL) apolipoprotein (apo) B100 to compare postprandial protein synthesis between subjects that ingested protein or were fasted. Subjects fed the

protein meal had higher leucine oxidation as well as higher leucine deposition. Leucine incorporation was 60% greater in meal fed subjects vs. control. Further, despite the observed leucine oxidation, splanchnic leucine balance was positive in the protein fed group, whereas splanchnic balance in the control group was negative. Similar techniques were used in studies by Stoll et al [32, 34] in which pigs were given mixed meals containing 600 mg/kg protein with labeled phenylalanine. The liver took up 18% of phenylalanine that reached the portal vein, and it was estimated that 64% of phenylalanine taken up was incorporated into hepatic proteins. Serum albumin is another protein used to examine hepatic protein synthesis. Serum albumin is the most abundant plasma protein constituting ~60% of plasma proteins, and is synthesized exclusively in the liver [40]. A study by De Feo et al [35] used leucine incorporation into serum albumin to measure postprandial hepatic protein synthesis in humans. Baseline albumin synthesis was established with a fasted control group and compared to subjects administered a glucose and amino acid mixture nasogastrically over 6 hours. Results demonstrated that whole body protein synthesis increased 23% and albumin synthesis increased 90% in the meal infused group compared to baseline. Further, the increase in albumin synthesis accounted for nearly 30% of the whole body increase in protein synthesis. Importantly, the rate at which albumin synthesis increased was significantly correlated with increased plasma insulin during the infusion.

Overall, exogenous amino acids are readily taken up by the liver. Postprandial hepatic amino acid metabolism is regulated by the presence of amino acids, glucose, and insulin. Each of these plays an important role in regulating amino acid uptake and

metabolism. Further, the majority of postprandial hepatic amino acids taken up are used for protein synthesis.

Protein synthesis and translation.

Protein synthesis is a broad term that involves post-transcriptional processes, translation, and post-translational modification, which together, culminate in the production of functioning proteins. Briefly, transcription describes the production of messenger RNA (mRNA) in the nucleus. Translation occurs after the mRNA exits the nucleus and associates with the ribosome. The ribosome translates the mRNA code and facilitates the formation of a polypeptide from individual amino acids. Finally, post-translational modification describes the process by which the newly synthesized polypeptide chain is folded and processed to produce a fully functional protein. The net production of protein via protein synthesis requires both available mRNA and mRNA translation, and is therefore is regulated to some extent at both the transcriptional and translational level. Further, protein synthesis is an energy dependent process and requires the cellular capacity to carry out post-translational modifications. The focus of this section will be on protein translation and its regulation in the postprandial state.

The ribosome is the principal molecular machine involved in the translation of proteins. It consists of two different cytosolic proteins, the 40S subunit and the 60S subunit. Each subunit has a core made of ribosomal RNA where various ribosomal proteins assemble. The subunits remain separate until translation initiation, when they come together to form the 80S translational unit. The 80S ribosome simultaneously conveys mRNA through its core, decodes genetic information from mRNA, and assembles the primary amino acid sequence of a new protein. After the

primary sequence is assembled, the newly formed protein is released and the ribosomal subunits separate, completing the cycle [41]. The efficiency of translation can be increased by the formation of polysomes. A polysome is composed of multiple ribosomes, which simultanesously bind to and translate one mRNA [42].

The initiation of protein translation involves several eukaryotic initiation factors (eIF). These eIF's guide interactions between the ribosomal subunits, mRNA, and transfer RNA (tRNA), and facilitate translation initiation. The process begins with the activation of protein kinase p70S6 (p70S6k), which subsequently phosphorylates and activates ribosomal protein S6 (RPS6) and eIF-4B. RPS6 then combines with eIF2-GTP, initiator tRNA bound with methionine, and the 40S subunit of the ribosome to form the 43S pre-initiation complex. The 43S pre-initiation complex then combines with the eIF4F complex. The eIF4F complex is formed when eIF-4E combines with eIF-4G, eIF4B, and the capped end of the mRNA. The eIF4F complex guides the mRNA to the 43S pre-initiation complex where eIF4F and the 43S pre-initation complex form the 48S initiation complex. Translation initiation is completed with the addition of the 60S ribosomal unit and the loss of eIF4F and eIF3, which completes the assembly of the 80S translational unit [43-45]. The formation of the initiation complexes is a highly regulated process. The mammalian target of rapamycin complex 1 (mTORC1) is a principle regulator of translation initiation. mTORC1 is composed of the proteins mammalian target of rapamycin (mTOR), mlST8, and PRAS40. It lies upstream of p70S6 kinase and eIF-4E, and regulates the activation of both [45]. p70S6 kinase appears to be directly phosphorylated and activated by mTORC1, and in turn activates RPS6 and eIF-4B. In contrast, mTORC1 indirectly activates eIF-4E via 4E-BP1 phosphorylation. Under basal

conditions, eIF-4E is held in the inactive state through the constitutive binding of the 4E-BP1 protein. Once mTORC1 is activated it acts to hyperphosphorylate 4E-BP1, which causes its release from eiF-4E [46]. This allows eIF-4E to bind with eIF-4G and capped mRNA, which facilitates the formation of the 43S pre-initiation complex.

Insulin is a potent activator of mTORC1. In isolated hepatocytes, mTOR activity increased 3 fold in response to a 3 hour exposure to insulin [47]. In agreement with this, H4IIE liver cells expressing a regulatable version of Akt, a protein involved in insulin signaling, demonstrated increased phosphorylation of p70S6 kinase and RPS6 when Akt was active. Conversely, the phosphorylation of p70S6 kinase and RPS6 was completely inhibited when mTORC1 activation was blocked [48]. Interestingly, insulin per se does not appear to have the same effects in vivo. Yoshizawa et al [49] demonstrated that rats fed a meal consisting of 20% protein had increased hepatic p70S6 kinase activation, RPS6 phosphorylation, eIF-4F associated with eIF-4G, and decreased association of 4EBP1 with eIF-4F. Additionally, global hepatic protein synthesis was increased. In contrast, rats fed a meal containing no protein, showed no activation of these protein synthetic components or increased hepatic protein synthesis, despite the meal eliciting a similar insulin response [49]. Taken together, these studies suggest that insulin is capable of activating mTORC1 and components of translation initiation in the liver, but the presence of amino acids is necessary to carry out protein synthesis in vivo.

The insulin signaling cascade is one mechanism by which insulin dependent mTORC1 activation takes place. Initiation of the cascade occurs when insulin binds to and activates the insulin receptor tyrosine kinase. An insulin receptor substrate (IRS-1, IRS-2, IRS-3) attaches to the insulin receptor and is activated through tyrosine

phosphorylation. IRS proteins phosphorylate and activate phosphoinositide-3 kinase, which converts phosphoinositide-2 phosphate 2 (PIP2) into its activated form, phosphoinositide-3 phosphate 3 (PIP3), at the cell membrane. PIP3 then phosphorylates and activates the serine/threonine kinase Akt which, once activated, phosphorylates a protein complex known as Tumor Suppressor Complex (TSC). In the absence of insulin stimulation, TSC inhibits the guanine nucleotide exchange factor Rheb by keeping it in its guanine diphosphate (GDP) bound form. Akt phosphorylation of TSC relieves the inhibition on Rheb, allowing it to take on its active guanine triphosphate (GTP) bound state [45, 47]. The mTORC1 is a direct target of and is activated by Rheb-GTP. In this manner, insulin can activate mTORC1 activation and can initiate the formation of downstream translation initiation complexes.

Certain amino acids can also activate hepatic mTORC1, and similar to insulin, do not necessarily increase overall rates of global protein synthesis. A 3 hour exposure to a complete amino acid mixture increased mTORC1 activation by 5.6 fold in isolated rat hepatocytes [47]. Fasted rats administered 135mg/100g body weight of either leucine, isoleucine, or valine diluted in water and administered through a feeding tube showed increased phosphorylation of hepatic 4EBP1. Further, leucine administration was associated with increased phosphorylation of hepatic p70S6 kinase and RPS6. Despite the activation of these translational proteins, the fractional synthetic rate of protein synthesis in the liver of rats exclusively administered a branch chain amino acid did not increase above fasted controls [50]. In agreement with this, a study in rats by Reiter et al [44] showed that leucine administration through a feeding tube activated hepatic 4EBP1, p70s6k, and RPS6, but did not increase global rates of protein synthesis. Together, these

studies suggest that amino acids alone do not increase protein synthesis, despite exhibiting the ability to activate protein translation. Currently, the mechanism by which amino acids activate mTORC1 is not well understood. However, the activation appears to function independently of Akt and the insulin signaling cascade [51].

The solitary administration of branch chain amino acids or an increase in insulin in the absence of protein fails to stimulate hepatic protein synthesis. However, when the liver is exposed to both protein and insulin in the context of a mixed meal, there is activation of both mTORC1 and global protein synthesis. Rats given access to a standard chow meal and sacrificed at 15, 60, or 180 minutes following the introduction of the meal had significantly greater hepatic protein synthesis at 60 and 180 minutes compared to fasted controls. In agreement with this, polysome formation was significantly greater and there were fewer free 60S and 40S ribsomal subunits in the liver of rats killed at 60 minutes compared to controls. Further, the phosphorylation of p70S6 kinase and 4EBP1 were maximally stimulated at 15 minutes and this activation was sustained throughout 180 minutes. The association of EIF-4E and EIF-4G was increased at 15 and 60 minutes, but not at 180 minutes [52]. Taken together, these results demonstrate that in response to a mixed meal, hepatic mTORC1 activation is rapid, sustained, and paralleled by an increase in polysome formation and global rates of protein synthesis. However, this study did not examine whether the contributions of insulin and amino acids were equally important in stimulating postprandial hepatic protein synthesis.

A study by O'Connor et al [53] examined the relative contributions of insulin and protein to the stimulation of hepatic protein synthesis and translation initiation using pancreatic and amino acid clamps in neonatal pigs. Somatostatin inhibited endogenous

insulin production and insulin was replaced peripherally at one of four levels; less than fasting, fasting, intermediate, or fed. Amino acids were administered at fasted or fed levels, for each insulin condition, and protein synthesis was measured using labeled phenylalanine. The fractional synthetic rate of hepatic protein synthesis did not increase when insulin was elevated in the presence of fasted amino acids. This is in agreement with the previously discussed study by Yoshizawa et al [49] where rats fed a meal lacking protein did not increase hepatic protein synthesis. In contrast, there was a significant increase in the fractional synthetic rate of hepatic protein synthesis when amino acids were raised from the fasted to fed level, under all four insulin conditions. Additionally, the phosphorylation of 4EBP1 and p70S6 kinase was positively correlated with fed amino acid levels. A positive correlation was also observed between the rate of hepatic protein synthesis and phosphorylation of 4EBP1 and p70S6 kinase. The results from this study suggest that the postprandial rise in hepatic protein synthesis has greater sensitivity to the rise in amino acids rather than the rise in insulin. However, maximal stimulation of hepatic protein synthesis requires postprandial levels of amino acids and insulin [53].

Another requirement for the optimal activation of postprandial hepatic protein synthesis is the presence of particular amino acids. Using labeled phenylalanine, Anthony et al [54] demonstrated that rats had lower hepatic protein synthesis and translation initiation when fed a meal lacking tryptophan, leucine, or branch chain amino acids (BCAA) compared to rats fed a meal deficient in glycine, or a meal including all amino acids. Protein synthesis was decreased 40-50% in livers of rats fed the meal lacking tryptophan, leucine, and BCAA meals. Further, rats fed these diets had significantly

lower phosphorylation of 4EBP1 and p70S6 kinase [54]. This is in agreement with another study by Anthony et al [50], which demonstrated increased phosphorylation of p70S6 kinase and RPS6 in response to BCAA administration. Tryptophan, leucine, and BCAAs are essential amino acids, whereas glycine is not. Thus, along with insulin, it appears that these essential amino acids are required for the maximal stimulation of postprandial hepatic protein synthesis.

The above studies demonstrate that insulin and amino acids can activate translation initiation through mTORC1, and together activate protein synthesis. However, these studies do not address whether increased postprandial hepatic protein synthesis is activated in an mTORC1 dependent manner. To address the relationship between protein synthesis and mTORC1, investigators utilize the drug rapamycin. Rapamycin is a potent inhibitor of mTORC1 activation [55]. It occurs naturally as a bacterial product, is used clinically as an immunosuppressent, and has been widely used to study mTORC1 [56]. Rapamycin functions by binding to the cytosolic protein FK binding protein 12 (FKB-12). Once bound, the rapamycin/FKB-12 complex directly binds to and inhibits the activation of MTORC1. Preliminary evidence that protein synthesis was mTORC1 dependent came from an in vitro study where rapamycin inhibited the insulin dependent increase in cellular size by 70% in H4IIE liver cells. The phosphorylation of p70S6K and RPS6 was completely blocked in cells administered rapamycin [48]. In agreement with in vitro data, the acute postprandial increase in hepatic protein synthesis was also dependent on MTORC1 activation. Hepatic protein content was significantly increased in rats refed after a 48 hour fast compared to rats administered rapamycin prior to refeeding [57]. In neonatal pigs, the meal-induced increase in hepatic protein synthesis was completely

prevented by a pre-injection of rapamycin. The presence of rapamycin also reduced phosphorylation of p70S6 kinase and 4EBP1 in fed pigs. These results demonstrated that mTORC1 activation is a rate limiting step in the activation of postprandial hepatic protein synthesis. Further, they demonstrated that a functioning mTORC pathway is required for optimal growth in cells and animals.

In summary, the activation of postprandial hepatic protein synthesis is a highly regulated process dependent on the presence of particular amino acids and insulin. Postprandial protein synthesis is regulated in an mTORC1 dependent fashion, and the stimulation of mTORC1 leads to translation initiation. The process of protein translation occurs at the ribosome and functions to produce the primary amino acid sequence of the newly synthesized protein.

Endoplasmic reticulum structure and function.

Once the primary sequence of a nascent protein has been assembled and released from the ribosome, it has to undergo several posttranslational modifications to become fully functional. The main posttranslational modifications are the formation of disulfide bonds, conformational folding, and protein glycosylation. These are carried out by a group of proteins collectively known as chaperone proteins. Approximately one third of all nascent proteins are membrane bound proteins or destined for the secretory pathway [58]. These proteins are translocated into the lumen of the endoplasmic reticulum (ER) where they are folded, processed, and packaged. The following section will discuss the structure and function of the ER, and the regulation of protein folding in the ER.

The ER is a large, membranous organelle that is in physical contact with almost all other organelles in the cell including the nucleus, mitochondria, golgi, and plasma

membrane [59, 60]. Despite the lumen of the ER being a single compartment there are many subdomains. The rough endoplasmic reticulun (RER) is associated with ribosomes. The nuclear envelope surrounds the nucleus and communicates with the nuclear membrane. The plasma membrane of the ER contains membrane proteins that are involved in synthesis, elongation, and desaturation of lipids and fatty acids [61]. The ER is the starting point of the secretory pathway, and is also involved in the synthesis of phospholipids and steroids, calcium storage, and regulation of calcium concentrations in the cytosol.

Proteins destined for the secretory pathway, or a plasma membrane protein, have a signal sequence on the N terminus that is exposed as the protein begins to emerge from the ribosome. The signal sequence is recognized and bound to a signal recognition particle (SRP), such as SRP54. Signal recognition particles bind to translating ribosomes whether the protein has signal sequence or not, however their affinity for ribosomes with emerging signal sequences is greater [62]. Once the SRP binds to the ribosome, and recognizes and reads the signal sequence, the complex is transported to the ER membrane where it binds to the SRP receptor (SR)[63]. The SRP/SR complex positions the ribosome over a translocon and the protein being translated enters the lumen of the ER as it is being translated [62, 63].

After the translated protein is released from the ribosome and has entered the lumen of the ER it undergoes postranslational folding and glycosylation, processes that are required to produce a functional protein, prior to being released from the ER lumen and transported to its final destination (e.g. plasma membrane, secretory pathway, golgi). The lumen of the ER has a high concentration of chaperone proteins which promote

protein folding and, via binding to hydrophobic regions, prevent the formation of potentially harmful protein aggregates. Various chaperone proteins work in concert and form complexes which effectively process proteins and ensure the proper folding and glycosylation [58].

The most abundant chaperone in the ER is GRP78 (glucose regulated protein 78 also known as immunoglobulin binding protein or BiP). GRP78 action is dependent on ATPase activity, and its main function as a chaperone is to bind to exposed hydrophobic residues on nascent proteins and prevent them from forming protein aggregates. GRP78 also serves to retain misfolded proteins in the lumen of the ER. The amount of GRP78 in the ER is tightly regulated and reflects the folding status of the ER. For example, chinese hamster ovary (CHO) cells containing a vector expressing a dysfunctional GRP78 responded by increasing the transcription of GRP78 [64]. Importantly, an increase in GRP78 mRNA alone does not necessarily lead to increased GRP78 protein expression. Gülow et al [65] studied the regulation of GRP78 expression in human HeLa cells. They used a system whereby they could conditionally overexpress the mouse form of GRP78 mRNA which could be translated into a functional GRP78 protein in the cell. When GRP78 transcription was turned on, the amount of mouse mRNA was 8 to 10 times greater than the human GRP78 mRNA. Despite this excessive increase in GRP78 mRNA, the amount of GRP78 protein expression was not different than control cells. To study the regulation of GRP78 in response to an increase in unfolded proteins, investigators have utilized drugs such as tunicamycin and thapsigargin. These compounds cause "ER stress", an environment characterized by an accumulation of unfolded or misfolded proteins in the ER lumen. Tunicamycin inhibits the glycosylation of nascent proteins and

causes a rapid increase in the amount of unfolded and misfolded proteins in the ER [66]. Thapsigargin, an inhibitor of the ER-associated calcium ATPase, results in the loss of luminal calcium stores and a reduced capacity for protein chaperones to fold proteins 183. When cells overexpressing mouse GRP78 mRNA were exposed to tunicamycin there was increased mouse GRP78 protein prior to increased human GRP78 mRNA transcription, indicating distinct regulation of translational vs. transcriptional control of GRP78 [65].

GRP78 can also be regulated at the transcriptional level in response to an impaired ability to respond to the accumulation of unfolded proteins. A study in CHO cells overexpressing either functional GRP78 protein, GRP78 protein with impaired ATPase activity, or wild type cells. The study showed that cells overexpressing GRP78 responded to tunicamycin by increasing GRP78 by 1.8 fold whereasGRP78 was increased by 19 fold in wild type cells. This suggests that cells have the ability to sense the ratio of GRP78 to unfolded proteins and can respond by increasing transcription of chaperones according to need. These investigators also looked at how overexpression of GRP78 would affect protein synthesis. They showed that cells overexpressing GRP78 and exposed to tunicamycin had protein synthetic rates that were 57% of control cells not exposed to tunicamycin. Conversely, protein synthesis was reduced by only 17% in wild type cells exposed to tunicamycin, compared to non-treated controls [64]. Thus, the ratio of GRP78 to the unfolded protein load appears to also influence the overall protein synthetic rate.

GRP78 plays an essential role in development. Homozygous GRP78 knockout mice die at ~ 3.5 days of embryonic development [67]. In contrast, heterozygous

GRP78+/- mice, in which there was a 40% reduction in GRP78 protein compared to wild types, displayed no signs of ER stress under basal conditions. Further, the chaperone function of the ER appeared to be maintained by a compensatory increase in two other chaperones, glucose regulated protein 94 (GRP94) and protein disulfide isomerase (PDI). This observation demonstrates that luminal chaperone capacity is not only regulated but that protein chaperones can compensate for each other.

Several studies have shown that nutritional status can affect hepatic GRP78 expression. Dhabi et al examined the effect of chronic calorie restriction on GRP78 protein expression in the liver in mice aged for 28 mos [68]. Mice received either 95 kcal per week or 52 kcal per week (calorie restriction). Calorie restriction caused a 65% decrease in GRP78 protein expression in the liver of mice. In a follow up study by the same group the investigators looked at how rapidly GRP78 hepatic mRNA was induced in response to feeding and compared calorie restricted (55% of normal fed)(4.1 g/day versus 2.3 grams /day) mice vs. normal fed mice over 24 mos. After depriving both groups of food for 24 hours, calorie restricted mice exhibited lower hepatic GRP78 mRNA expression. Mice were then meal fed for 2 hours and sacrificed 1.5, 5, 12, and 24 hours post feeding. Interestingly, both groups were characterized by a significant increase in hepatic GRP78 mRNA 1.5 hours after feeding which decreased over the 24 hour period. Investigators also completely deprived mice of food for 4 days and examined GRP78 protein. Food deprived mice were characterized by a 30% decrease in hepatic GRP78 protein expression. Further, upon refeeding 5.5 grams/day standard chow for 4 days, hepatic GRP78 protein returned to control levels. In total, these data suggest that GRP78 content in the liver can be regulated by nutrient exposure. The final study Dhahbi

et al undertook was to inhibit protein translation with puromycin, a drug that inhibits protein translation by polysome dissociation. Puromycin was injected 30 min before feeding and 30 min after feeding and mice were sacrificed 1.5 hours after feeding. Puromycin inhibited protein synthesis by 95% but did not inhibit hepatic induction of GRP78 postprandially. The authors concluded that the induction and regulation of GRP78 mRNA expression was not dependent on protein synthesis or an increase in ER protein traffic. Taken together these studies illustrate the vital importance of the chaperone GRP78 and illustrate that GRP78 can be modulated by the presence of folded or unfolded proteins in the ER and by nutritional status. Whether nutritional status affects other components of the ER protein chaperone machinery and whether the effects of nutritional status operate via affects on protein folding per se is presently unknown.

Glucose regulated protein 94 (GRP94) is another abundant chaperone protein found in the ER that facilitates proper protein folding. It was originally thought that GRP78 and GRP94 worked in a similar fashion and could both bind to unfolded proteins simultaneously. However, Melnick et al [69]provided evidence that GRP78 and GRP94 work in a sequential manner in cells. Whereas GRP78 efficiently hydrolyzes ATP and binds to hydrophobic regions of proteins over a period of a few minutes, GRP94 does not utilize ATP efficiently and binds to proteins with a half time dissociation constant of 50 minutes [69]. Further, very little GRP94 was detected on proteins saturated with GRP78 and vice a versa. Thus, it would appear that GRP78 and 94 are regulated in a distinct manner and promote protein folding via different mechanisms [69]. It has been postulated that GRP78 binds to hydrophobic regions of proteins to prevent protein aggregation with other unfolded protein while they are in the process of being folded, whereas GRP94

binds to folded proteins that are undergoing final processing for entry into the secretory pathway. Similar to GRP78, GRP94 is essential for development. Homozygous GRP94 knockout mice die at day 7 of embryonic development due to an inhibition of skeletal muscle differentiation. Embryonic stem cells from GRP94 knockout mice were capable of differentiating into several cell types except skeletal muscle, due to a deficiency in insulin like growth factor II [70]. GRP94 also appears to respond to nutritional status. Food-deprived rats were characterized by a reduction in GRP94 mRNA that was restored upon re-feeding [68].

Along with GRP78 and GRP94 there are other chaperones that carry out essential protein folding functions. A major contributing factor to the folding of proteins is the creation of disulfide bonds. Disulfide bonds form between native thiols in a polypeptide and help fold a protein into its native conformation. The two principle chaperones involved in disulfide bond formation are protein disulfide isomerase (PDI) and endoplasmic reticulum oxidase-1 (ERO1). PDI and ERO1 are found in both yeast and higher eukaryotes, and act in concert to facilitate the transfer of reducing equivalents from sulfur containing groups within a protein so that disulfide bonds can form. The cycle of disulfide bond formation begins with PDI being reduced and an unfolded protein becoming oxidized, allowing for disulfide bond formation [71, 72]. PDI also facilitates the reconfiguration of non-native disulfide bonds to their native conformation through oxidation [73]. Reduced PDI is then oxidized by ERO1 so that it can interact with proteins again. Dithiothrietol (DTT) is a potent reducing agent that is used to disrupt the oxidative environment of the ER and causes the accumulation of unfolded proteins by inhibiting disulfide bond formation. Yeast with a genetic mutation in ERO1 were

characterized by constitutively reduced PDI and increased sensitivity to DTT [71]. Conversely, HeLa cells overexpressing ERO1 were resistant to the effects of DTT [74]. ERO1 is expressed in the ER membrane and is associated with flavoprotein FAD which can transfer reducing equivalents to oxygen, the final electron acceptor in the chain of disulfide bond formation [75].

Two more prominent ER chaperone proteins are the calcium requiring lectins, calnexin and calreticulin. These chaperones are involved in processing carbohydrate residues which are attached to nitrogen (N) residues on nascent proteins. N-linked glycans serve a wide array of functions including the stabilization of proteins against denaturation, enhance solubility, orient proteins to membranes, add structural rigidity, regulate protein turnover, and mediate pathogen interaction [76]. When a newly synthesized protein enters the ER it is attached with pre-synthesized oligosaccharides on N residues. Calnexin and calreticulin interact with these oligosacharides and expose the unfolded protein to endoplasmic reticulum protein 57 (ERp57), which is a thiolreductase and acts to form disulfide bonds. Calnexin and calreticulin are released from the protein when the glucose residue that they are associated with is cleaved. If the protein is folded into its proper conformation upon release of the lectin, it is transported to the golgi for final processing and release [76]. Calreticulin and ERp57 mRNA expression were reduced in mice that had been food deprived for 48 hours. Calreticulin mRNA was slightly increased and ERp57 mRNA expression was induced 2 fold, 1.5 hours after the reintroduction of food to food-deprived mice. These results demonstrate that both calreticulin and ERp57 are modulated by nutrient status [68].

Taken together, these studies demonstrate the necessary function of chaperones in post-translational modification that are required for proper cellular functioning, and in some instances, development. Further, protein folding requires energy, oxygen, and calcium. Chaperone content in the liver appears to be modulated by nutritional status. Since protein synthesis and translation are activated in the liver in the postprandial state and newly formed proteins require post-translational processing it is postulated that chaperone protein content is upregulated postprandially to deal with the increased client load presented to the ER.

Despite the presence of chaperones, some proteins fail to be folded and processed properly, which can lead to the formation of non-functioning protein aggregates. If proteins cannot be properly folded or misfolded proteins then these proteins are marked for degradation and can be translocated back to cytosol were they are ubiquitinated and degraded by the proteasome. The integrated processes of protein folding and targeted protein degradation are collectively known as quality control [77]. The ER is not a proteolytic organelle and misfolded proteins must be retrotranslocated out of the ER for degradation [78]. Proteins such as ER degradation enhancing alpha-mannosidase-like protein (EDEM) recognize and bind to folding incompetent proteins [79]. These proteins are retrotranslocated out of the ER lumen to the cytosol, where they are ubiquinated and degraded by the proteasome. This process is important to maintaining the quality of proteins entering the secretory pathway and is an additional mechanism that helps to prevent the accumulation of unfolded proteins.

The unfolded protein response.

There are circumstances where the balance between unfolded proteins entering the lumen exceeds the folding and degradative capacity of the ER. As mentioned previously, the accumulation of mal-folded or unfolded proteins in the lumen of the ER is termed "ER stress". Conditions such as hypoxia, nutrient deprivation, and exposure to chemical agents such as thapsigargin or tunicamycin can lead to this accumulation. The ER is equipped with a signaling pathway which acts to reestablish the balance between folding capacity/degradation and unfolded proteins termed the Unfolded Protein Response (UPR) (28). Originally, the UPR was thought of as a pathway singularly involved in responding to accumulation of unfolded proteins in the ER. However, more recent research has shown that the UPR is involved in a wide array of cellular functions, and is implicated in a number of diseases [1-8]. The following section will discuss the UPR.

The UPR is a highly conserved pathway that was originally described in yeast. The proximal sensor of ER stress in yeast is the transmembrane protein inositol requiring enzyme-1 (Ire1). In response to ER stress, Ire1 is activated through dimerization and trans-autophosphorylation [80]. Ire1 contains a unique endoribonuclease in its cytosolic domain that carries out the unconventional splicing of Hac1 mRNA [81]. Hac1 mRNA is found constitutively in the cytosol, where its native nucleotide sequence is inefficiently recognized and translated. However, in response to ER stress, the activated endoribonuclease on Ire1 splices out a specific intron causing a frame-shift in the Hac1 nucleotide sequence. The new nucleotide sequence is recognized and efficiently translated into the transcription factor Hac1p [81]. Hac1p subsequently enters the nucleus and induces genes which aid in folding and degradation of proteins.

The UPR is also ubiquitously expressed in mammalian cells. The mammalian UPR is more developed than that of yeast, and consists of three ER membrane bound proximal sensors. These are the mammalian homologue of yeast IRE1, protein kinase R like ER protein kinase (PERK), and activating transcription factor 6 (ATF6). In response to ER stress, these proteins act in a coordinated fashion to initially decrease general protein translation, and subsequently increase transcription of genes that encode proteins to aid in both protein folding and degradation [82].

Mammalian IRE1 is expressed in two isoforms, alpha and beta. IRE1-β is found in the epithelial cells of the gut and intestines, whereas IRE1-α is expressed in all other tissues [83]. Similar to yeast, mammalian IRE1 is activated through dimerization and transautophosphorylation [84, 85]. Mammalian IRE1 also contains an endoribonuclease that is activated in response to ER stress. The exclusive target of the endoribonuclease is the mRNA for X-box binding protein-1 (XBP1). Similar to Hac1 mRNA, unspliced XBP1 is inefficiently translated. ER stress activated IRE1 splices out a 26 nucleotide intron from XBP1 mRNA. The removal of the intron causes a translational frame shift which results in a spliced form of XBP1 (XBP1s) that is not only efficiently translated but also a highly potent transcription factor [86, 87]. Overall, the main function of the IRE1/XBP1 branch of the UPR in response to ER stress is to increase the capacity for degradation via upregulation of the ER quality control apparatus, such as EDEM [88].

It is presently thought that IRE1 activation is inhibited by the constitutive binding of GRP78 to its luminal domain. Upon the accumulation of unfolded proteins in the lumen of the ER, GRP78 dissociates from IRE1 to bind with unfolded proteins. This dissociation releases the inhibition on IRE1 and allows for the dimerization,

transautophosphorylation, and activation of IRE1. Bertolotti et al demonstrated that GRP78 co-immunoprecipated with IRE1 in unstressed AR42J cells [89]. This association was significantly reduced when cells were treated with either thapsigargin or DTT. Further, chinese hamster ovary cells (CHO) where GRP78 was overexpressed showed increased GRP78 associated with IRE1 compared to control cells. Additionally, cells overexpressing GRP78 were characterized by a delayed and significantly reduced phosphorylation of IRE1 in response to thapsigargin treatment compared to control [89]. These data support the notion that the association of GRP78 with IRE1 is likely an important determinant of IRE1 activation.

Along with its ability to respond to ER stress and activate components of the UPR, the IRE1/XBP1 pathway also appears to be involved in a wide array of basic cellular functions. Both IRE1 and XBP1 are essential for development. IRE1 knockout mice die of unknown causes between 9.5 and 11.5 days of gestation [90]. Mice expressing a homozygous knockout of XBP1 die at embryonic day 12.5 or 13.5. The embryos of XBP1 knockout mice were characterized by anemia and hypoplastic livers. These results suggest that XBP1 is required for proper liver development. To further examine the role of XBP1 in liver development investigators performed partial hepatectomies on adult mice. The normal response to partial hepatectomy includes rapid cell division and replacement of the lost liver mass within 10 days. Within 30 minutes post surgery there was a significant increase in XBP1 mRNA expression, XBP1 splicing and protein synthesis, and the magnitude of the increase in protein synthesis was correlated with XBP1 splicing [91]. These data suggest that the IRE1/XBP1 pathway may be activated under conditions characterized by increased growth and/or protein

synthetic rates. Indeed, a recent study demonstrated that cells deficient in TSC, a negative inhibitor of mTOR and therefore characterized by constitutive activation of mTOR, were characterized by increased XBP1 splicing. Additionally, XBP1 splicing was inhibited upon the administration of rapamycin [92]. Thus, these studies provide the first evidence that the IRE1/XBP1 pathway of the UPR may play an important role in cell growth and division and may be regulated by the mTOR pathway.

The IRE1/XBP1 pathway appears to be involved in metabolism and regulation of certain nutrient biosynthetic pathways. Lipson et al demonstrated IRE1 activation in pancreatic beta cells in response to exposure to glucose. Further, insulin secreting INS-1 cells were examined to look at IRE1 activation and insulin biosynthesis. INS-1 cells exposed to 10, 20, or 25 mM glucose were characterized by increased insulin secretion and phosphorylation of IRE1. IRE1 was then inhibited in INS-1 cells through the use of small interfering RNA to knockdown IRE1 function or by the expression of an IRE1 mutant that could not be phosphorylated. Under both conditions, insulin secretion was decreased in response to glucose exposure when compared to control INS-1 cells [93]. This study demonstrated that IRE1 is involved in the ability of pancreatic beta cells to sense glucose and respond by processing and releasing insulin. XBP1 appears to also regulate lipid biosynthetic pathways. Lee et al [3] developed a model where they could disrupt liver XBP1 of adult mice. The expression of a non-functional XBP1, denoted as XBP1Δ, in the liver did not on its own cause ER stress. Mice with XBP1Δ had lower plasma triglycerides (TG), cholesterol, and free fatty acids (FFA). This decrease in plasma lipids was not due to an increase in lipid retention by the liver, as livers of XBP1Δ and control mice had the same lipid content. Further, livers from XBP1Δ mice were

characterized by a downregulation of several genes important in lipid synthesis including stearoyl coenzyme A (CoA) desaturase 1, diacylglycerol acyltransferase 2, and acetyl CoA carboxylase [3]. Taken together, these studies illustrate functions of the IRE1/XBP1 pathway that transcend its traditional role in ER stress. In addition, these studies demonstrate that components of the UPR can respond to signals from nutrients and play important roles in development and nutrient biosynthetic pathways.

In addition to mammalian homologues of yeast IRE1, the mammalian UPR includes two other ER bound proximal sensors. These are the PKR like ER protein kinase (PERK) and activating transcription factor 6 (ATF6). Overall, the activation of PERK leads to a generalized decrease in global protein translation which decreases the entry of newly synthesized proteins and allows for selective translation of specific mRNAs that encode proteins necessary for ER homeostasis. . Similar to IRE1, the activation of PERK is regulated by the constitutive binding of GRP78. Upon the accumulation of unfolded proteins, GRP78 dissociates from PERK to bind to nascent proteins. This dissociation allows for the dimerization, trans-autophosphorylation, and activation of PERK [94]. Activated PERK rapidly phosphorylates the alpha subunit of eiF2 [95]. Phosphorylation of eif2-α inhibits eiF2B from converting GDP bound eiF2 to its active GTP bound form. This inhibits the formation of the 43S preinitiation complex and significantly reduces the rate of protein translation [96]. Reduced protein translation decreases the number of nascent proteins entering the lumen of the ER that require folding, and in this way PERK activation reduces the protein load on the ER luminal folding apparatus.

The phosphorylation of eif2-α is not exclusively controlled by PERK. There are three additional eif2-α kinases that respond to various conditions to decrease protein

synthesis via this mechanism. Protein kinase R (PKR) is activated in response to viral infections. The genetic material produced by some viruses is double stranded mRNA. PKR has a double stranded (ds) mRNA binding motif and is activated in response to dsRNA binding. PKR phosphorylates eif2-α to slow protein translation and promotes apoptosis in cells with viral infection [97]. Heme regulated protein (HRI) is another eif2-α kinase that is activated in red blood cells in response to a decrease in heme [98]. Finally, GCN2 is an eif2-α kinase that is activated in response to decreased amino acid availability. When amino acid levels fall, there is an increase in uncharged tRNA. Uncharged tRNA can bind to and activate GCN2 [99, 100]. The activation of GCN2 is not a proximal sensor of unfolded proteins in the lumen of the ER, but by decreasing the level of translation in response to amino acid availability, GCN2 aids in decreasing the entry of unfolded proteins into the ER lumen. The redundant actions of these kinases on eif2-α are illustrated in genetic knockout mice. Mice with homozygous knockout of PERK were viable out to six weeks and characterized by hyperglycemia [4], where as mice with a mutation on the eif2-α phosphorylation site died within 18 hours after birth due to hypoglycemia [101]. Thus, the ability to phosphorylate eif2-α is vital to survival, but in the absence of PERK other kinases can likely assume this role.

Along with its role in rapidly phosphorylating eif2-α to decrease protein translation, the induction of a host of UPR related genes are controlled by PERK in response to ER stress. Using genomic analysis Harding et al examined PERK KO cells and demonstrated reduced expression of 28 out of 88 UPR target genes in response to tunicamycin treatment [102]. One of the main transcription factors that respond to PERK activation is activating transcription factor 4 (ATF4). There is a constitutive level of

ATF4 mRNA present in the cytosol, and under non-stressed conditions ATF4 mRNA is inefficiently translated due to an inhibitory upstream open reading frame (uORF). Conversely, under conditions when eif2-α is phosphorylated, ribosomal scanning is less efficient due to the decreased formation of the 43s preinitation complex. This decreased scanning efficiency allows the ribosome to scan past the uORF and initiate translation at the ATF4 start codon [103]. Mouse embryonic stem cells exposed to thapsigargin had increased eif2-α phosphorylation and increased ATF4 protein compared to controls. Cells in which PERK and GCN2 were genetically ablated showed an abundance of ATF4 mRNA but significantly lower levels of ATF4 protein compared to controls [103].

One of the principle genes induced by ATF4 in response to ER stress is the growth arrest and DNA damage 34 (GADD34) gene [104]. The protein encoded by the GADD34 gene binds to and activates protein phosphatase 1 (PP1), which facilitates the dephosphorylation of eif2-α, resulting in restored protein translation [105]. This cyclical system ensures that attenuation of protein translation in response to ER stress is transient, thus ensuring that genes encoding proteins required to increase the capacity to fold and degrade proteins can be translated [82]. W4 embryonic stem cells expressing mutant GADD34 were characterized by decreased protein synthesis and increased GRP78 and GRP94 mRNA in response to thapsigargin. However, the mutant cells failed to increase GRP78 and GRP94 protein levels [105]. Ma et al [104] demonstrated a decrease in protein translation in response to thapsigargin in MEFs. Protein rates recovered back to pre-thapsigargin levels within two hours after removal of thapsigargin. However, protein synthesis rates did not recover in GADD34 KO MEFs [104]. Taken together, these studies illustrate the importance of GADD34 in the restoration of normal protein

translation following ER stress-mediated attenuation of translation. Interestingly, GADD34 induction appears to be unique to the UPR, as it is not induced in response to amino acid deprivation [104]. Under conditions of prolonged ER stress, in which the UPR is unable to restore ER homeostasis, the UPR can initiate apoptosis. Thus, activation of the UPR not only initiates responses designed to restore ER homeostasis but also upregulates pro-apoptotic gene expression. It is presently unclear how the cell integrates and responds to these opposing responses. One of the primary UPR-related pro-apoptotic proteins is CCAAT-homologous protein (CHOP). Increased CHOP expression, mediated in part by ATF4, appears to contribute to the induction of apoptosis in response to ER stress [106]. Akita mice have a mutation on the Ins2 gene which encodes for preproinsulin, and develop hyperglycemia and type 2 diabetes at 4 to 8 weeks of age. Oyadomari et al demonstrated that Akita mice were characterized by increased pancreatic CHOP expression and pancreatic beta cell apoptosis. Conversely, CHOP deficient, Akita mice were characterized by decreased beta cell apoptosis [98]. This study and others carried out in cells indicate that CHOP potentiates apoptosis during prolonged ER stress [97, 106]. However, it is presently unclear how CHOP induces apoptosis.

The activation of PERK and its downstream components in response to ER stress have been well characterized, but few studies have examined whether PERK plays a role in normal cellular functions. However, studies using genetic manipulation have begun to characterize the physiologic importance of PERK, and these indicate that an intact PERK pathway is required for the maintenance of glucose homeostasis. Harding et al developed PERK KO mice in which there was significantly less PERK expression in the pancreas, lungs, and thymus compared to wild type mice. There was no difference in

PERK expression in the liver between KO mice and controls. Characterization of PERK KO mice indicated pancreatic endocrine and exocrine dysfunction. The pancreatic beta cells of KO mice were incapable of producing insulin and mice exhibited hyperglycemia due to an inability to facilitate systemic glucose uptake and suppress hepatic glucose production [4]. It may have been expected that the ablation of pancreatic PERK would have resulted in hyperinsulinemia due to an inability to phosphorylate eif2-α and suppress protein synthesis, but this was not the case. Rampant insulin production may have occurred early on, but prolonged ER stress due to an inability to control protein translation in the highly secretory cells of the pancreas could not be maintained and lead to apoptosis, significantly less beta cell mass, and the development of type 1 diabetes [4]. The phenotype of PERK KO mice is similar to that of humans with the genetic disease known as Wolcott-Rallison syndrome. Wolcott-Rallison syndrome is caused by a genetic mutation in the EIF2AK3 gene, which is responsible for encoding PERK [107]. Thus, it would appear the PERK is an important component of pancreatic beta cell function.

In support of the observation that an intact PERK pathway is required for glucose homeostasis, Scheuner et al demonstrated that a homozygous mutation of the phosphorylation site (Ser51) on eif2-α, which inhibited eif2-α phosphorylation, caused mice to die of hypoglycemia 18 hours after birth. Ser51 mutated mice had impaired pancreatic beta cell differentiation during development, indicating a vital role for eif2-α in beta cell differentiation [101]. These mice differed from the PERK knockout mice of the Harding et al study which showed normal beta cell development, but had beta cell dysfunction due to apoptosis after birth [4]. Ser51 mice also failed to express phosphenolpyruvate carboxykinase (PEPCK) and liver glycogen synthase. PEPCK is an

important protein in gluconeogenesis, and glycogen synthase is the enzyme responsible for glycogen synthesis. Expression and functioning of both proteins represent crucial steps in maintaining the ability of the liver to produce glucose, which was absent in Ser51 mice [101]. This study reinforced the observations of Harding et al, in that it further illustrated the importance of PERK and eif2-α in the maintenance of glucose homeostasis. Taken together, these studies present the possibility that the PERK pathway can sense and respond to changes in glucose and insulin, such as those observed in the postprandial state. However, to date, PERK regulation in the postprandial state has not been studied.

The final proximal sensor in the mammalian UPR is ATF6. ATF6 is a membrane bound protein located in the ER and has two forms, ATF6-α and ATF6-β. ATF6-α is the predominant form expressed. Under basal conditions, ATF6 is constitutively expressed as a 90 kDa protein. Similar to IRE1 and PERK, the luminal domain of ATF6 is bound by GRP78 under basal conditions. In response to ER stress, GRP78 dissociates from the luminal domain and ATF6 is released from the ER membrane [108]. ATF6 travels to the golgi where it is cleaved and processed into a 50 kDa transcription factor that enters the nucleus and activates transcription of UPR target genes [109]. ATF6 90 is cleaved and processed in the golgi by site 1 and site 2 proteases (S1P, S2P) to produce the active transcription factor. A study in Hela cells where S1P was mutated and cells were stained showed that ATF6 did not appear in the nucleus in response to DTT but did translocate to the golgi [110]. Further, M19 mutant CHO cells, which do not express S2P, showed no nuclear ATF6 in response to tunicamycin [81].

Activated ATF6 enters the nucleus, binds to the ER stress response element (ERSE) motif and activates genes mainly encoding for ER chaperones [111]. Yamamoto et al [112] developed an ATF6-α knock out mouse and examined gene expression in MEFs from wild type and ATF6-α deficient mice in response to tunicamycin. Using a luciferase reporter to examine activation of the ERSE they observed that reporter activity was induced in wild type cells, but not in ATF6-α knock out cells. Additionally, tunicamycin induced GRP78, GRP94, and PDI mRNA in wild type but not ATF6-α knock out cells [112]. While ATF6 appears to be mainly involved in the regulation of chaperone genes, there is some evidence that it can also induce XBP1 mRNA expression. Yoshida et al demonstrated that the overexpression of ATF6 in Hela cells lead to induction of the XBP1 promoter, where as overexpression of an ATF6 mutant failed to do so [113]. It is thought that ATF6 increases XBP1 mRNA expression which can then be spliced by IRE1 and translated during ER stress [113].

There is little evidence describing a physiologic role of ATF6 in cellular functioning. However, mice with a double knock out of both ATF6-α and ATF6-β were not viable, indicating a necessary function in development [112]. Interestingly, M19 cells, which cannot process ATF6, have constitutive activation of markers of ER stress. In cells unstressed by chemicals, IRE and PERK were phosphorylated and XBP1 splicing was present. This indicates that in the basal state processing of ATF6 may serve to maintain ER homeostasis. This balance is disrupted and ER stress ensues when ATF6 is absent [81]. However, these studies were done in cells, and the role and regulation of ATF6 in vivo have not been examined.

Summary.

The ability to sense and respond to the accumulation of misfolded or unfolded proteins is a central component of the cellular defense against environmental insult. The importance of proper protein folding to cell survival may best be appreciated by the observation that, for every compartment in which proteins fold there is a quality control apparatus and a system for sensing protein misfolding. The accumulation of unfolded or misfolded proteins in the ER lumen results in activation of the UPR. It has been postulated that ER stress can result from a number of insults including pharmacologic agents that perturb protein folding, genetic mutations, and viral infection. Recent studies have suggested that the UPR may have an expanded role in cellular function. These studies have linked components of the activated UPR to a diverse array of cellular functions including cell differentiation, phospholipid biosynthesis and ER membrane expansion, lipogenesis, glucose homeostasis, and insulin action [1-4]. Activation of the UPR has also been observed in cancer cells, in the liver and adipose tissue of humans with non-alcoholic fatty liver disease, obesity and/or type 2 diabetes, and in brain regions of individuals with Alzheimer's Disease [5-8]. Thus, while the general structure and function of the UPR has been comprehensively examined, very little is presently known regarding the physiologic factors that promote ER stress and UPR activation, and the role of the UPR in metabolic diseases. Given the UPR's putative involvement in lipogenesis and glucose homeostasis we hypothesized that it may be a component of the postprandial response of the liver in vivo. In particular, we wanted to examine whether the UPR was activated in response to specific nutrient combinations in the liver and whether this regulation could be linked to the regulation of nutrient synthetic pathways. Therefore,

the purpose of the current study was to characterize postprandial regulation of the UPR, and to determine whether postprandial activation of the UPR was regulated by mTORC1.

CHAPTER 3

METHODS

Animals. Male Wistar Crl(WI)BR rats (Charles River Laboratories, Wilmington, MA) weighing ~150 g upon arrival were individually housed in a temperature and humidity controlled environment. Animals were maintained on a reverse 12 hour light:dark cycle and were given free access to water. Animals were provided a purified high starch diet, consisting of 68% cornstarch, 20% casein, and 12% corn oil (Research Diets Inc., New Brunswick, NJ) for 3 hours per day, starting 1 hour after the dark cycle, for 11 days. Food intake and body weight were monitored over this 11 d period. All procedures involving rats were reviewed and approved by the Colorado State University Institutional Animal Care and Use Committee.

Experimental procedures. Study 1 was designed to examine the hepatic UPR in response to a high starch or high sucrose meal. On the day of the study, rats were randomly assigned to one of five groups (n=6/group): Fasted (FAST), in which rats were fasted for 24 hours prior to sacrifice, starch-fed rats (STA), in which rats were provided ad libitum access to the high starch diet for 3 hours and were sacrificed either 1 hour or 7 hours following the 3 hour feeding period, sucrose-fed rats (SUC), in which rats were provided ad libitum access to a high sucrose diet (68% sucrose, 20% casein, 12% corn oil, Research Diets, Inc., New Brunswick, NJ) for 3 hours and were sacrificed either 1 hour or 7 hours following the 3 hour feeding period. Study 2 was designed to examine the role of mTOR in postprandial activation of the UPR. On the day of study 2, all of the rats were provided ad libitum access to the high starch diet for 3 hours and were either sacrificed 1 hour or 7 hours following the 3 hour feeding period. Rapamycin (RAP; 1

mg/kg; n=4) or carrier (VEH; DMSO n=5) was injected (IP) into rats one hour prior to the start of the feeding period.

Rats were anesthetized with sodium pentobarbital (IP; ~70 mg/kg). Once deeply anesthetized (absence of response to toe pinch and eye reflex) rats were placed on a heating pad, the abdominal cavity was exposed and portal vein and inferior vena cava blood samples were obtained. Portions of the liver, kidney, and gastrocnemius muscle were removed and processed for subsequent analyses. Epididymal and retroperitoneal fat pads, as well as the stomach were removed and weighed.

Cell culture. H4IIE liver cells (American Type Culture Collection, Manassas, VI), a rat hepatoma cell line, were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum, penicillin, and streptomycin sulfate (REF). Control medium, referred to as low glucose medium or LG, contained 8 mM glucose. Each experiment was performed in triplicate and 5 to 8 independent experiments were performed.

Processing and analysis of blood samples. Blood samples were immediately centrifuged at 1000 x g for 2 minutes. Plasma was collected and frozen at -80°C for later analysis of glucose (Beckman glucose analyzer, Fullerton, CA), fructose (Sigma-Aldrich, St. Louis, MO), and insulin (kit # 1395418, Linco, St Charles, MO).

Tissue preparation and isolation of polyribosome fractions. Sucrose solutions consisting of 50%, 38.2%, 26.6%, and 15% sucrose were prepared and 2.5 ml of each of these solutions was placed into an RNase free tube and quick frozen at -80°C. . A continuous sucrose gradient was poured after tubes were thawed at 4°C the evening before each analysis.

Frozen liver tissue (200-300 mg) was placed in 1 ml of 0.1 mg/ml cyclohexamide for 2 minutes at room temperature and then homogenized in a glass douncer. Tissue was then placed in 1 ml of low salt buffer (LSB) (20mM Tris pH7.5, 10mM NaCl and 3mM $MgCl_2$) containing 1mM dithiothreitol and 50microunits/375ml recombinant RNase inhibitor. The tissue was then treated with 225 microliters of lysis buffer (0.2M sucrose and 1.2% Triton x-100) and centrifuged at 13,000 x g for 5 minutes to pellet nuclei and mitochondria. The supernatant was transferred to a new tube on ice. The supernatant was then layered over the sucrose gradient and centrifuged at 200,000 x g in a Beckman SW41Ti rotor for 2.5 hours at 4°C. After centrifugation, the top layer was removed and discarded. The remaining fractions were separated into 18, 0.5 ml aliquots that were placed into 1.5 ml tubes. Each fraction was analyzed by UV spectrometry at a wavelength of 254 nm to identify 40S and 60S ribosomal peaks. Fractions were then pooled 8 groups (group 1 = first three fractions, group 2 = fractions 4-6, etc), with the first group representing the smallest polyribosome and the last group representing the largest polyribosome. Following denaturation (Ambion, Austin, TX) RNA was isolated from each fraction using the TōTally RNA™ kit (Ambion, Austin TX).

Tissue preparation and RNA analysis. Fresh liver was immediately placed into RNALater solution and frozen at -80°C. Total RNA was isolated using TRIzol reagent (Life Techonologies) as per the Manufacturer's instructions. Purified RNA was DNase-treated (Ambion, Austin, TX) and reverse transcription (RT) was performed using Superscript II RNaseH- and random hexamers (Life Technologies, Carlsbad, CA). Transcribed cDNA was subjected to duplex polymerase chain reaction (PCR) amplification using a primer specific for X-Box Binding Protein-1 (XBP-1), forward

sequence 5'-TTGTCTCAGTGAAGGAAGAACC, reverse sequence 5'-TAGGCAGGAAGATGGCTTTGG. The XBP-1 PCR product was separated on 4% gel and visualized with ethidium bromide staining. A digital image of the gel was taken and the presence of unspliced and spliced XBP-1 was detected using an UVP Bioimaging System (UVP, Upland, CA).

Real time PCR was performed in 96-well plates using transcribed cDNA and IQ-SYBR green master mix (Bio-Rad, Hercules, CA). Primer sets were designed by Beacon designer program version 3.1. GRP78, forward sequence 5'-GAGGCGTATTTGGGAAAGAAGG, reverse sequence 5'-GCTGCTGTAGGCTCATTGATG. CHOP, forward sequence 5'-CGCTCTCCAGATTCCAGTCAG, reverse sequence 5'-GTTCTCCTGCTCCTTCTCCTTC. GADD34, forward sequence 5'-CACAGGGCAGGGAAGTATCAAC, reverse sequence 5'-CGTCATCCTCGGTGTCCTCTC. FASn, forward sequence 5'-TGCTGCCGTGTCCTTCTACTAC, reverse sequence 5'-CACCCAAGTCCTCGCCGTAG. SREBP1c, forward sequence 5'-TGGTGGGCACTGAAGCAAAG, reverse sequence 5'-CACTTCGTAGGGTCAGGTTCTC. SRP54, forward sequence 5'-GTCTGCGATTGATCTTGAGGAG, reverse sequence 5'-TGCTTTCCCTTAGTCGGTGTC. SRPR, forward sequence 5'-TACAGTGCCTGGTAGACAAGTG, reverse sequence 5'-CAGACAGCCTTCACCTTATTGC. ATF4, forward sequence 5'-GAATGGATGACCTGGAAACC, reverse sequence 5'-

GGCTCCTTATTAGTCTCTTGG. DAD, forward sequence 5'-GAACAAGGCGGATTTCCAAGG, reverse sequence 5'-GTCTCCAACTCCACGGTAAGG. GK, forward sequence 5'-AGGCACGAAGACCTAGACAAG, reverse sequence 5'-CCACCACATCCATCTCAAAGTC. GRP78, forward sequence 5'-GAGGCGTATTTGGGAAAGAAGG, reverse sequence 5'-GCTGCTGTAGGCTCATTGATG. GRP94, forward sequence 5'-CTGCGTCCTGCTGACCTTC, reverse sequence 5'-CATCGTCTGTCCGTGAGCC. Mgat2, forward sequence 5'-CTACACCACCATTCGGAGTTTC, reverse sequence 5'-GCATCTCGGGTCAAGGCTAG. Ketohexokinase (FK), forward sequence 5'-GCTGTTCGGCTATGGAGAGG, reverse sequence 5'-CCAGGCACAGATGAGCGTAG. PCR efficiency was between 90% and 105% for primer and probe sets and was linear over five orders of magnitude. Specificity of products generated for each set of primers was examined for each fragment using a melting curve analysis and gel electrophoresis. Reactions were run in duplicate and data calculated as the change in cycle threshold for the target gene relative to the change in target threshold for B2-microglobulin according to the procedures described by Muller et al [114].

Tissue preparation and western blotting. Fresh liver was immediately homogenized on ice in a buffer containing 150mmol/L NaCL, 1% Triton X100, 10mmol/L Tris-HCL, 5 mmol/L EDTA, 1 mmol/L sodium vanadate, 2 mmol/L dithiothreitol, 1 mmol/L phenylmethylsulfonylflouride, 50 mmol B-glycerophosphate, 3mmol/L benzamidine, 10 micromol/L lleupeptin, 5 micromol/L pepstatin and 10 mg/L

aprotinin. Samples were rotated for 30 min. at 4°C and centrifuged at 15,000 x g for 30 min. Total protein was measured using a modified Lowry protocol. Equivalent amounts of protein (50 micrograms) were subjected to SDS-PAGE and transferred to Hybond-P membranes (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were blocked and incubated with antibodies against total and phosphorylated (Ser51) eif2-alpha (Cell Signaling, Danvers, MA), XBP-1 (M-186) (Santa Cruz, Santa Cruz, CA), total and phosphorylated IRE-1 alpha (Abcam, Cambridge, MA), and ribosomal protein S6 (Cell Signaling, Danvers, MA). Proteins were detected using horseradish peroxidase-conjugated secondary antibodies and a chemiluminescence reagent (Santa Cruz, Santa Cruz, CA.). Detection and analysis of density was assessed with a UVP Bioimaging system (Upland, CA, USA).

Data analysis and statistics.

Statistical comparisons were calculated using one-way ANOVA. *Post hoc* comparisons were made using Least Significant Difference (LSD) test. The level of significance was $P < 0.05$. Data are reported as means ± SEM.

CHAPTER 4

RESULTS

Study 1

Food intake and body weight. Study 1 was undertaken to examine whether meal ingestion activated the UPR in the liver, and, in particular, whether the composition of carbohydrate in the meal influenced activation of the UPR. Rats were trained to consume food over the same three hour period each day for 10 to 12 days. Average food intake on the first day was only 1.7±2.1 grams but increased to 14.0±2.1 grams by day 12 (Figure 1). Average body weight was 149.1±3 grams on the first day and increased to 197.3±2.7 grams by day 12.

Study day food consumption and plasma parameters. On the day of study, fed rats were sacrificed either 1 or 7 hours after the termination of the 3 hour feeding period. Starch fed rats consumed significantly more food on the study day than sucrose fed rats in both the 1 hour (14.6±2.0 vs 11.7±2.3 grams, respectively) and 7 hour (13.2±2.3 vs 10.7±1.6 grams, respectively) groups (Figure 2). Additionally, the starch fed 1 hour group (STA1) consumed significantly more food than the sucrose fed 7 hour group (SUC7) (Figure 2).

Plasma samples from the portal vein and vena cava were analyzed for glucose and insulin. At 1 hour, portal vein glucose levels of fed rats were significantly greater than fasted rats (Figure 3a). Portal vein glucose levels were significantly greater than vena cava glucose in the STA1 and STA7 groups, indicating that glucose was still being absorbed in these groups. Glucose levels measured in the vena cava were not significantly different when fed groups were compared to fasted rats, indicating that at

both 1 and 7 hours systemic plasma glucose had returned to or not changed from basal, fasted levels (Figure 3a).

Portal vein insulin levels were significantly increased in STA1 and SUC1 compared to all other groups (Figure 3b). Vena cava insulin levels were significantly increased in STA1 compared to all other groups except SUC1 (Figure 3b). Additionally, vena cava insulin levels were significantly higher in SUC1 vs SUC7 (Figure3b). As would be expected, portal vein insulin levels were significantly increased relative to vena cava insulin levels in STA1 and SUC1 (Figure 3b).

We examined fatty acid synthase (FAS) and sterol regulatory element binding protein 1c (SREBP1c) mRNA as markers of a feeding response in the liver. Both of these genes are involved in lipid synthesis and are induced in the liver in the postprandial state (168, 169). FAS mRNA was increased 35-65 fold in fed vs fasted rats (Figure 4a). However, due to high variability within each group, some of these changes did not reach statistical significance (Figure 4a). SREBP1c mRNA was increased 7-28-fold in fed vs fasted rats (Figure 4b). Taken together, these data provide evidence that the meal ingestion protocol provoked a "feeding response" in the liver.

We next examined glucokinase (GK) and fructokinase (FK) mRNA expression, both of which are regulated by the postprandial environment (170, 171). Glucokinase mRNA was increased 12-30 fold in STA rats and 2-12 fold in SUC rats compared to FAST (Figure 4c). Only minimal changes in fructokinase mRNA were observed between fed and fasted rats (Figure 4d).

Overall, these data indicate that rats were sacrificed in the postprandial state. There was an increase in portal vein glucose and insulin observed in the fed groups vs.

fasted group. Furthermore, there was a robust induction of two lipogenic genes which have been demonstrated to be increased in the postprandial state. There were several significant differences among postprandial markers between the one and seven hour time points, demonstrating the transient nature of the postprandial state.

XBP1 splicing. The unfolded protein response has three proximal sensors, IRE1, PERK and ATF6. Upon activation, these sensors regulate the translational and transcriptional machinery, ultimately resulting in attenuation of global protein synthesis and upregulation of the folding and degradative capacity of the ER [82]. One of these components, IRE1, possesses an endoribonuclease which is only activated following IRE1 activation. XBP1 mRNA is the only known substrate for IRE1 ribonuclease activity and, therefore, activation of IRE1 results in the splicing of XBP1 mRNA (Figure 5a). Thus, the presence of spliced XBP1 is a reliable indicator of activation of the IRE1 branch of the UPR [92].

We examined the presence of spliced XBP1 in the fasted and postprandial states. Only unspliced XBP1 mRNA was detected in the FAST group (Figure 5b). All STA1 rats and 5 out of the 6 SUC1 rats were characterized by varying degrees of spliced XBP1 (Figure 5b). At the 7 hour time point, small amounts of spliced XBP1 were detected in 2-3 STA rats and 1-2 SUC rats (Figure 5b). However, the spliced band at 7 hours appeared lighter than the spliced band at 1 hour, indicating that activation of IRE1 may have been reduced at the later time point. Importantly, XBP1 splicing was not significantly different between STA and SUC, suggesting that the type of carbohydrate and therefore the quantity of sugar removed by the liver may not be a determinant of IRE1 activation.

Response of UPR gene markers. Activation of the UPR leads to the induction of several genes which aid in the folding and degradation of proteins. While they are not direct measures of UPR activation (i.e. they are downstream of the proximal sensors of ER stress), these genes are always increased in response to pharmacologic induction of ER stress and typically reflect the magnitude or severity of that stress [82, 115]. In addition, these gene markers provide insight into which branches of the proximal UPR (i.e. IRE1, ATF6, PERK) may be activated. For example, induction of CHOP and GADD34 mRNA are primarily regulated by the PERK arm of the UPR, whereas upregulation of GRP78 mRNA is primarily governed by ATF6 and IRE1 [87, 99, 104, 106]. We examined several of these UPR gene markers to gain insight into the regulation of the UPR in the postprandial state.

Pharmacologic-induction of ER stress, using tunicamycin or thapsigargin, produces severe ER stress and simultaneous activation of all three branches of the UPR. Under such conditions, activation of the PERK arm leads to attenuation of general translation [96]. Paradoxically, under these conditions, the translation of ATF4 is increased [103]. Two primary targets of ATF4 are the pro-apoptotic genes CHOP and GADD34, the latter encodes for a subunit of protein phosphatase 1. Therefore, we examined the expression of ATF4, CHOP and GADD34 mRNA in the liver from fasted and fed rats. Changes in ATF4 mRNA were extremely modest and ATF4 mRNA was only significantly increased in STA7 compared to FAST (Figure 6a). CHOP mRNA was not increased in any of the refed groups, and in fact was significantly reduced in STA1, SUC1, and STA7 compared to FAST (Figure 6b). Similar results were observed for GADD34 mRNA (Figure 6c). Therefore, in contrast to the postprandial activation of

XBP1 splicing and therefore IRE1, these data suggest that the postprandial environment may not activate PERK in the liver

We also examined a subset of chaperone genes which are typically upregulated in response to ER stress. Regulation of chaperone gene expression is generally thought to be the main role of the ATF6 branch, but several studies have shown crosstalk between the arms of the UPR and subsequent regulation of chaperone expression [87, 113]. Two of the most abundant chaperone proteins in the lumen of the ER are GRP78 and GRP94 [58]. Therefore, we next examined the expression of GRP78 and GRP94 mRNA. In the present study, GRP78 mRNA was increased ~ 2.4 fold in STA1 and 3.1 fold in SUC1 when compared to FAST, the latter reaching statistical significance (Figure 6d). The response of GRP94 mRNA was similar to that of GRP78. GRP94 mRNA was increased ~ 2.9 fold in STA1 and 3.2 fold in SUC1 when compared to FAST, the latter again reaching statistical significance (Figure 6e).

Both ATF6 and XBP1 protein can bind to and upregulate XBP1 mRNA [87]. Therefore, we also examined XBP1 mRNA using Real Time PCR. Surprisingly, XBP1 mRNA was reduced in all four fed groups compared with FAST (Figure 6f).

These data suggest that meal feeding elicits an environment that results in XBP1 splicing, suggesting that the postprandial environment activates the proximal sensor IRE1. However, other gene markers typically associated with ER stress and activation of the UPR were not significantly increased, and in some cases these markers were reduced in fed rats. Therefore, these data suggest that the postprandial environment may regulate/activate only selective components of the UPR, namely IRE1, and may do so in a manner that is independent of the classic definition of ER stress. A recent study

examined gene targets of the spliced form of XBP1 by selectively overexpressing XBP1s [116]. The study identified a broad array of genes that were upregulated in the presence of spliced XBP1 but in the absence of general ER stress. We examined four of these gene targets to determine whether they were upregulated in response to feeding. The first two genes examined were mannoside acetylglucosaminyltransferase-2 (Mgat2) and defender against cell death-1 (Dad1). Both of these genes are involved in the N-linked glycosylation of nascent proteins [116]. Mgat mRNA was significantly induced in the STA1, SUC1, and SUC7 groups compared to FAST (Figure 6g), whereas Dad1 mRNA was only induced in the STA7 group (Figure 6h). We also examined the gene for signal recognition particle-54 (SRP54) and signal recognition particle receptor (SRPR). Proteins encoded from these genes facilitate the translocation of newly synthesized, nascent proteins from the cytosol to the lumen of the ER [62, 63]. There was modest but statistically significant rise in SRP54 mRNA expression in the STA1 and SUC1 groups (Figure 6i), and SRPR mRNA was induced ~2 fold in the STA1, STA7, and SUC7 groups compared to FAST (Figure 6j). These data are consistent with the notion that feeding induces XBP1 splicing and regulation of XBP1s gene targets.

Phosphorylation of eIF2α. Phosphorylation of the alpha subunit of eIF2 is a direct downstream action of activated PERK, as well as the ER-associated kinases, GCN2, HRE, and PKR [95, 97-99]. PERK-mediated phosphorylation of eIF2α serves to attenuate general protein synthesis in order to reduce the protein load that requires post-translational modification in the ER lumen. Phosphorylation of eIF2-α was significantly decreased in STA1 compared to both FAST and STA7 (Figure 7a, 7b). Phosphorylation of eIF2α was not significantly different among FAST, SUC1 and SUC7 (Figure 7c, 7d).

These data suggest that the postprandial environment does not induce phosphorylation of eIF2α, and therefore does not appear to activate PERK, or other ER-associated kinases that can phosphorylate eIF2α.

Association of mRNA with polysomes. Polysome formation occurs when multiple ribosomes simultaneously bind to and translate a single mRNA, thereby enhancing translational efficiency. To further examine regulation of UPR gene markers in the postprandial state, we next determined the association of feeding-induced (e.g. FAS) and UPR target genes with polysomes. We first identified fractions that contained free 40S and 60S ribosomal subunits, that is, nonpolysomal fractions. The remaining 16 fractions were pooled in sequential groups of 2 (i.e. fraction 1 = fractions 1 and 2; fraction 2 = fractions 3-4, etc). Thus, in our analysis Fraction 1 represents the smallest polysomal fraction and fraction 8 the largest polysome. We measured target mRNA's using real time RT-PCR and have reported the data based on the genes cycle threshold (CT) relative to a housekeeping gene, which reflects the number of cycles required to produce a significant increase in gene amplification. CT is inversely related to the amount of starting mRNA, that is, the lower the CT the higher the amount of starting mRNA. Thus, throughout Figure 8, a lower bar is representative of greater mRNA associated with a given polysome.

For this analysis, we focused on starch fed and fasted animals, primarily due to the similarity between starch- and sucrose-fed animals with respect to most of the measurements already performed and the large number of samples required to perform the polysome analyses. There was significantly more FAS mRNA associated with all polysome fractions in fed vs fasted rats (Figure 8a). When considered in total, GRP78,

GRP94 and XBP1 mRNA associated with polysomes was increased in fed vs fasted rats, although this was not statistically significant within any single polysome fraction (Figure 8b-8d). The association of ATF4 and CHOP mRNA to polysomes was not different among groups, and was, in fact, decreased in the higher compared to lower polysome fractions (Figure 8e, 8f). Since, ATF4 translation is increased in response to eif2-α phosphorylation, these polysome data are in agreement with our previous data which demonstrated that feeding did not increase phosphorylation of eif2-α (Figure 7).

Summary. The data from Study 1 provide the first evidence characterizing the hepatic UPR by the postprandial environment. Specifically, our data demonstrate that the ingestion of either a high starch or high sucrose diet induced XBP1 splicing, increased expression of GRP78 and GRP94 mRNA, and increased the association of GRP78, GRP94 and XBP1 mRNA with polysomes. In contrast to chemically-induced ER stress and UPR activation, we did not observe increased phosphorylation of eIF2α. In fact, phosphorylation of eIF2α was reduced in one group of fed rats, STA1, compared to fasted rats. Together, these data suggest that select components of the UPR, that is the IRE1/XBP1 branch, appear to be activated in the postprandial state.

Figure 1

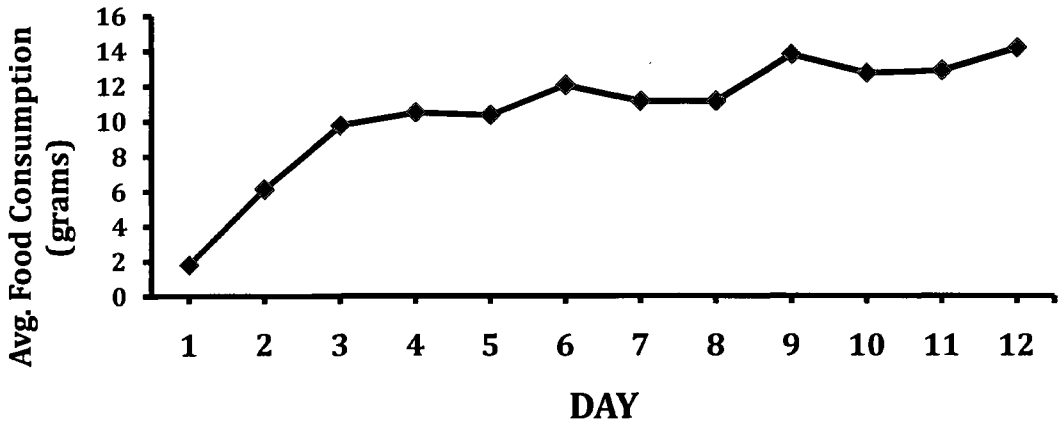

Figure 1. Daily food consumption. Average daily food intake of rats (n=30) over the meal training period.

Figure 2

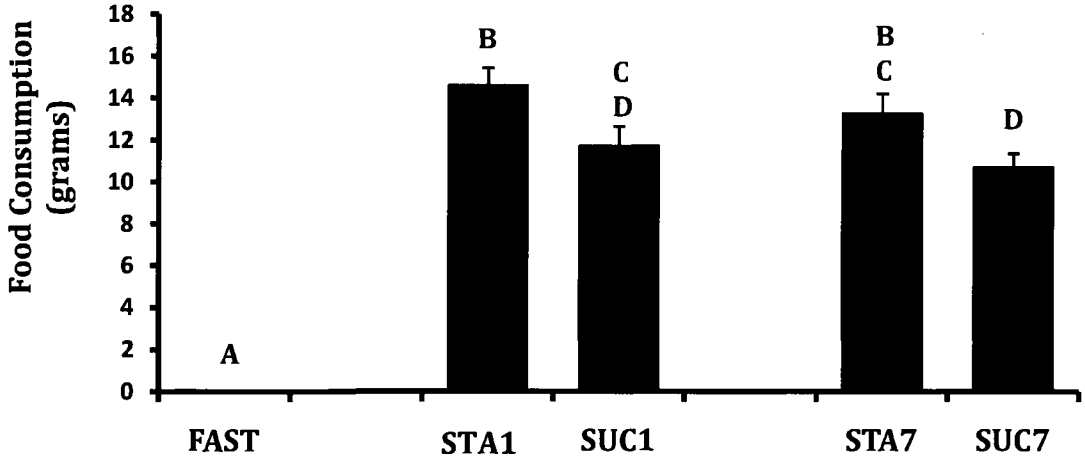

Figure 2. Study day food consumption. Food intake during the 3 hour meal feeding period for fasted (FAST), starch fed (STA1, STA7), or sucrose fed (SUC1, SUC7) rats sacrificed 1 or 7 hours post feeding period. Data are reported as the mean±SE. n=6 rats per group. Bars without a common letter differ, $p < 0.05$

Figure 3a

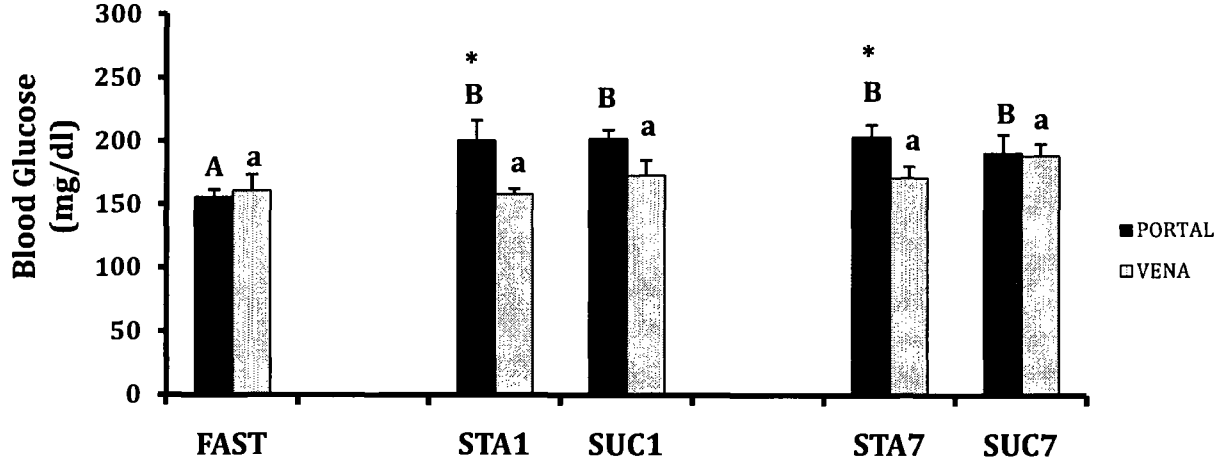

Figure 3b

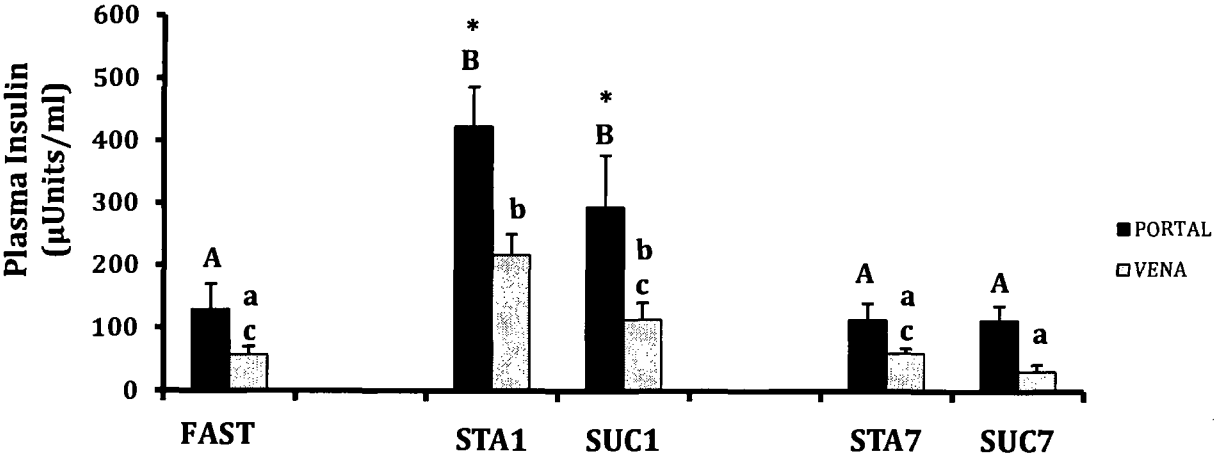

Figure 3. Plasma parameters. Plasma glucose (a) and insulin (b) concentrations taken from the portal vein (black bars) and the descending vena cava (grey bars) of rats from each group. Data are reported as the mean±SE. n=6 rats per group. Capital letters denote comparison of portal vein concentrations between groups. Lower case letters denote comparison of descending vena cava concentrations between groups. * denote differences between portal vein and descending vena cava within a group. Bars without a common letter differ, $p < 0.05$.

Figure 4a

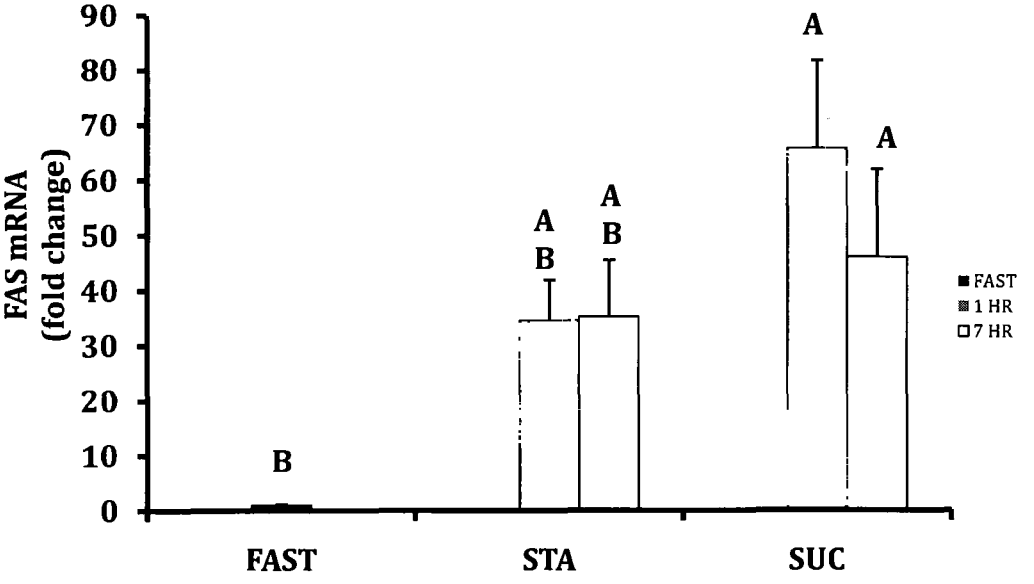

Figure 4b

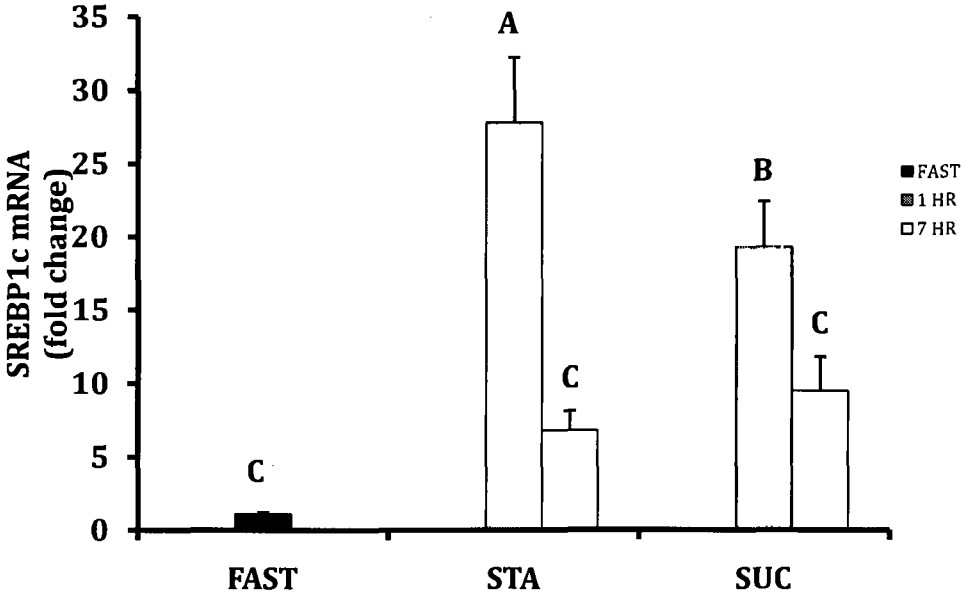

Figure 4c

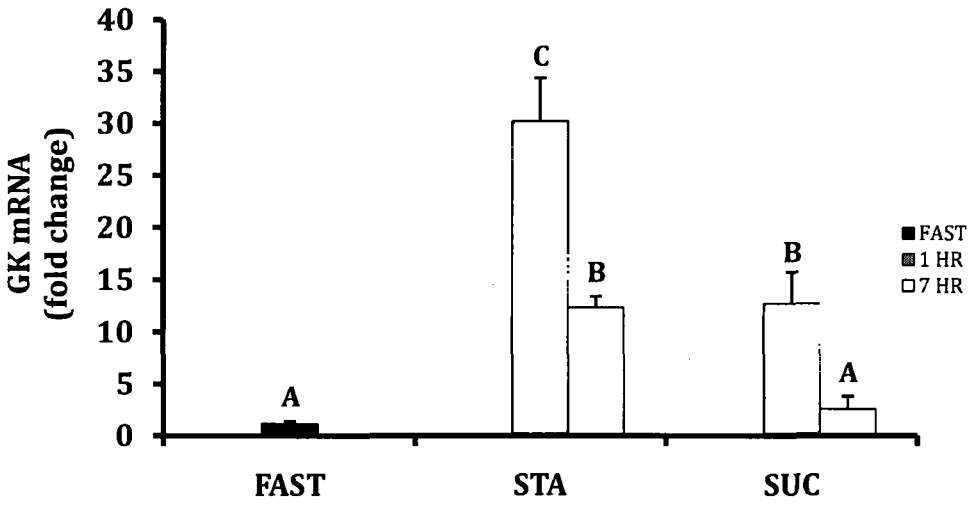

Figure 4d

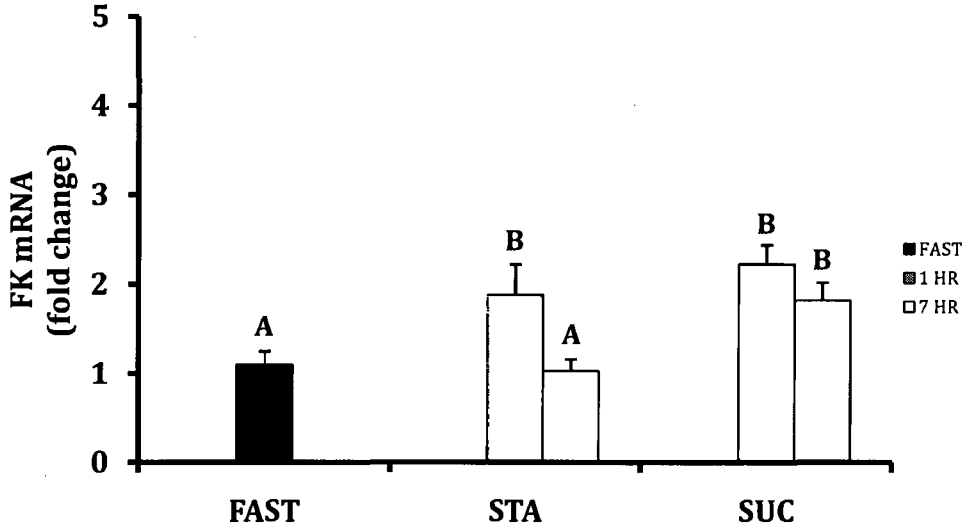

Figure 4. Postprandial gene markers. Expression of FAS (a), SREBP1c (b), GK (c), and FK (d), mRNA in the liver of fasted (FAST), starch fed (STA) and sucrose fed (SUC) rats after a 24 hour fast (black bar), 1 hour post (grey bars) or 7 hours post (white bars) meal feeding period. Data are reported as the mean±SE. n=4-6 rats per group. Bars without a common letter differ, $p < 0.05$.

Figure 5a

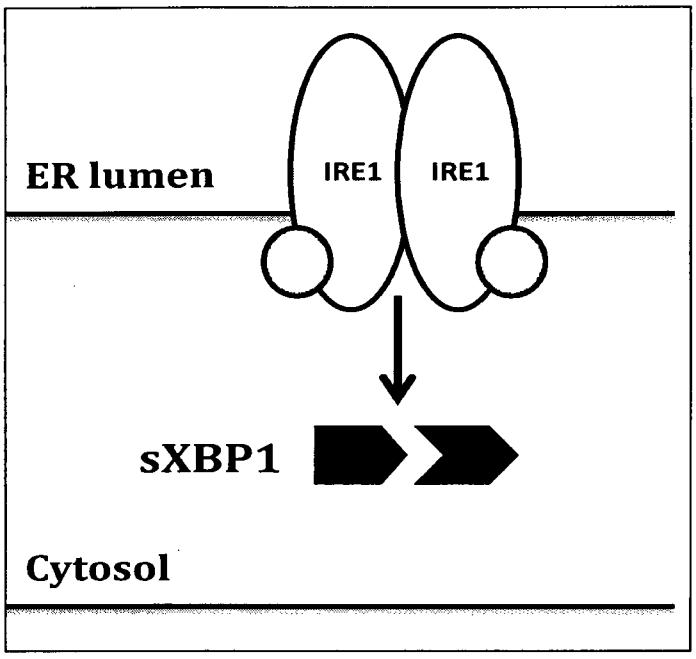

Figure 5b

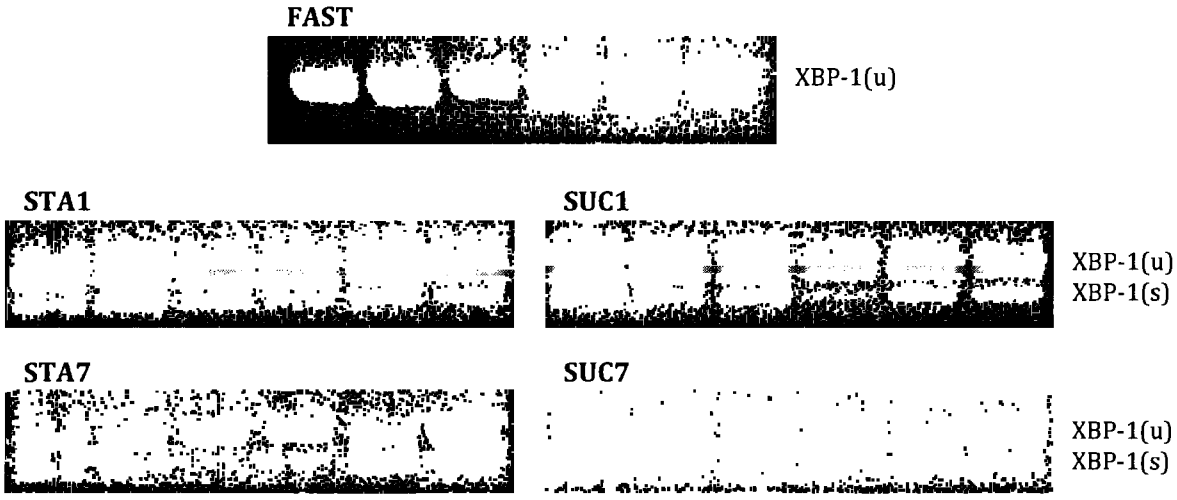

Figure 5. XBP1 splicing. Schematic of IRE1 dependent splicing of XBP1 mRNA (a). The presence of unspliced (u) and spliced (s) XBP1 mRNA in hepatic tissue of rats either fasted (FAST), starch fed (STA1, STA7), or sucrose fed (SUC1, SUC7) and sacrificed at 1 or 7 hours post feeding period (b). n=6 rats per group.

Figure 6a

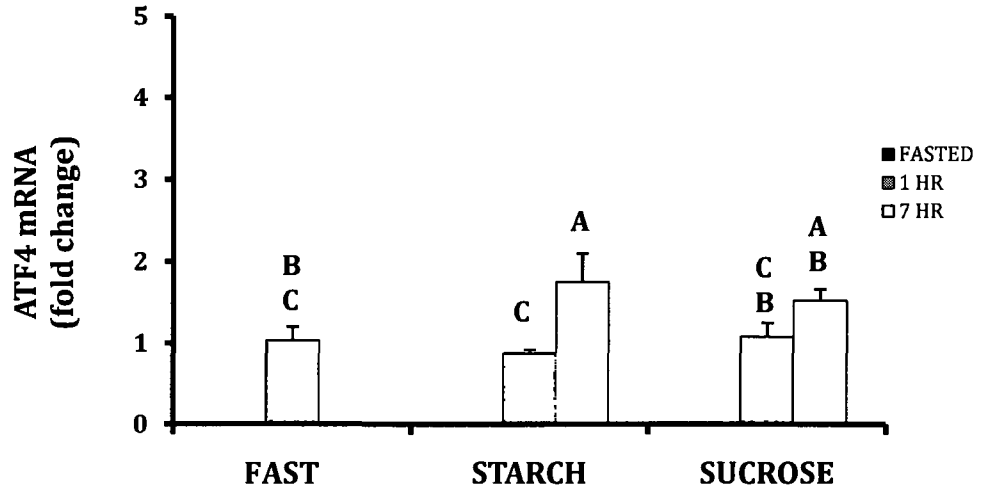

Figure 6b

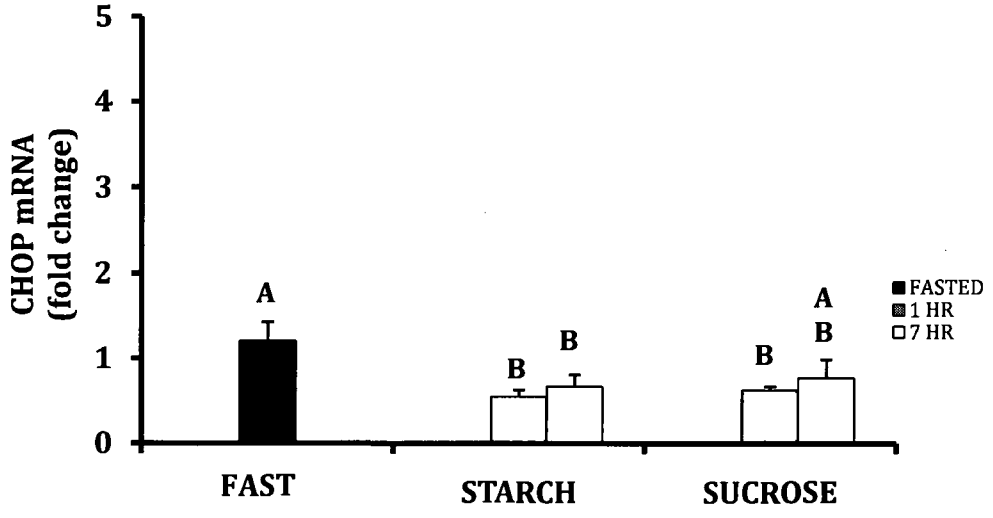

Figure 6c

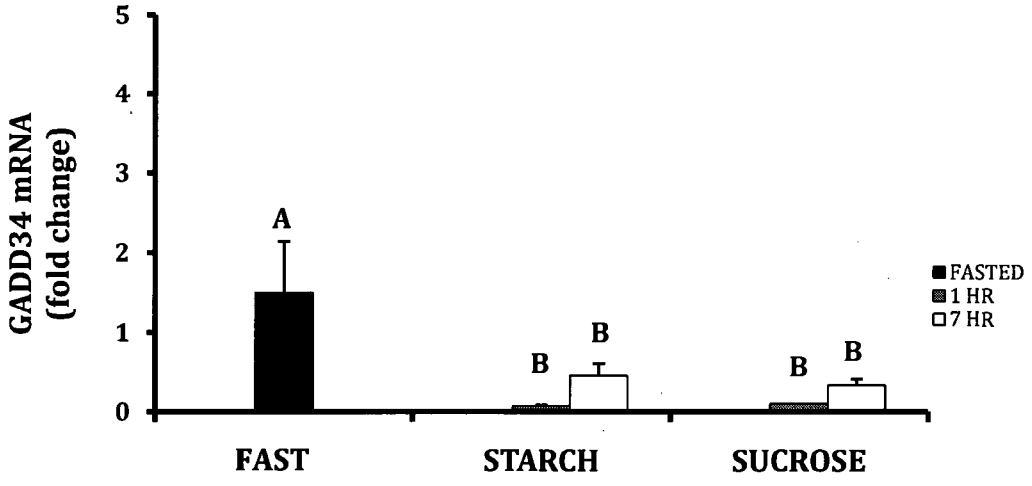

Figure 6d

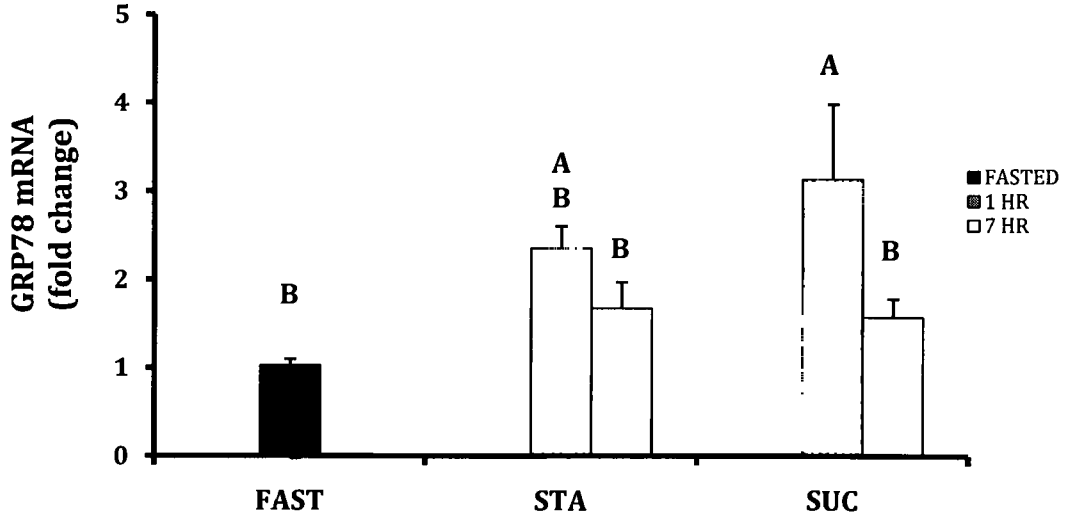

Figure 6e

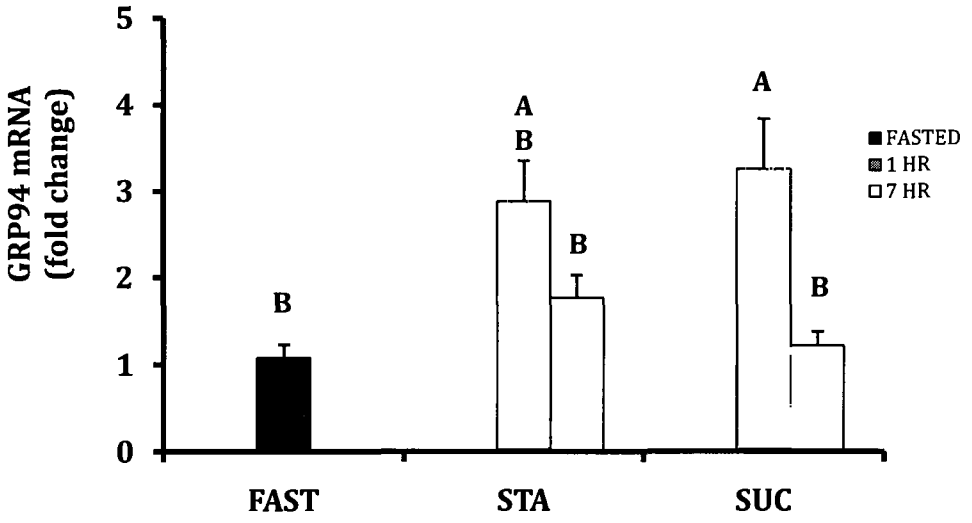

Figure 6f

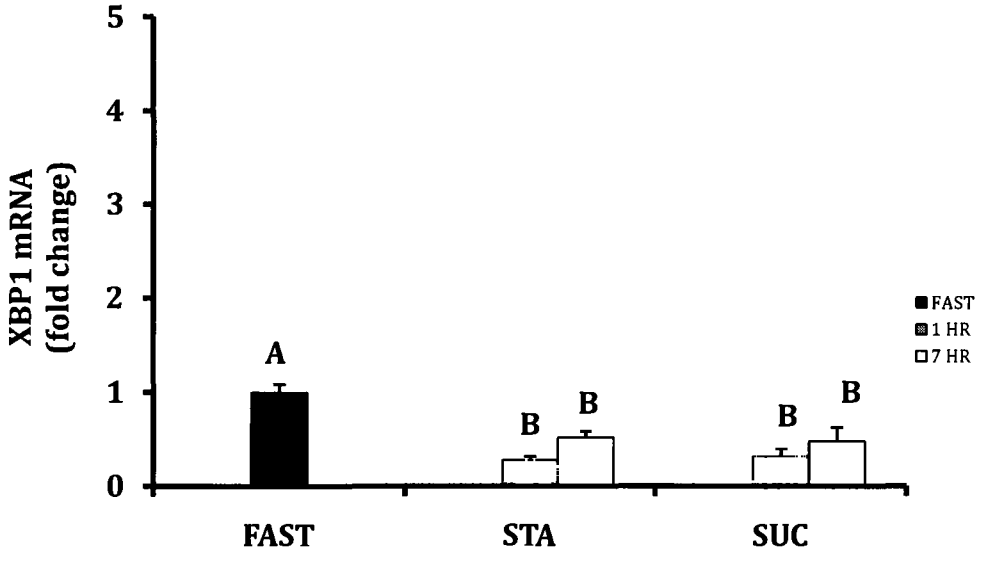

Figure 6g

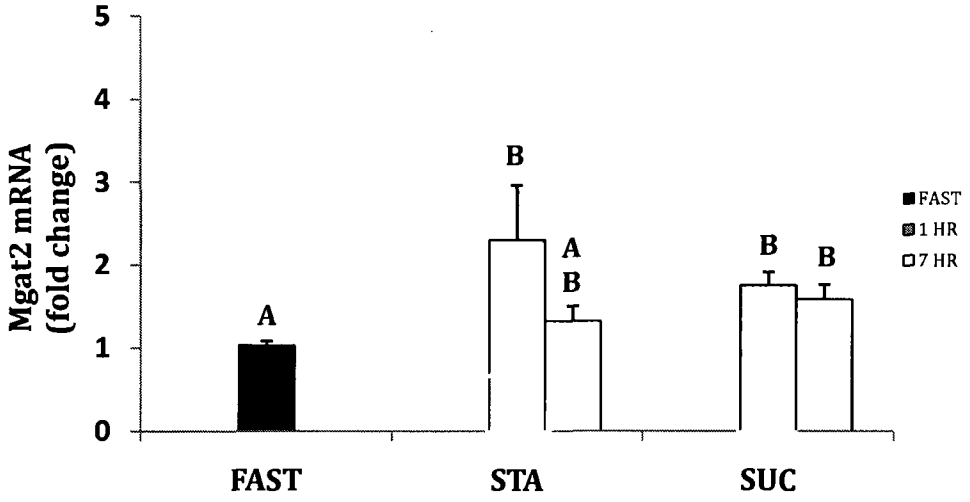

Figure 6h

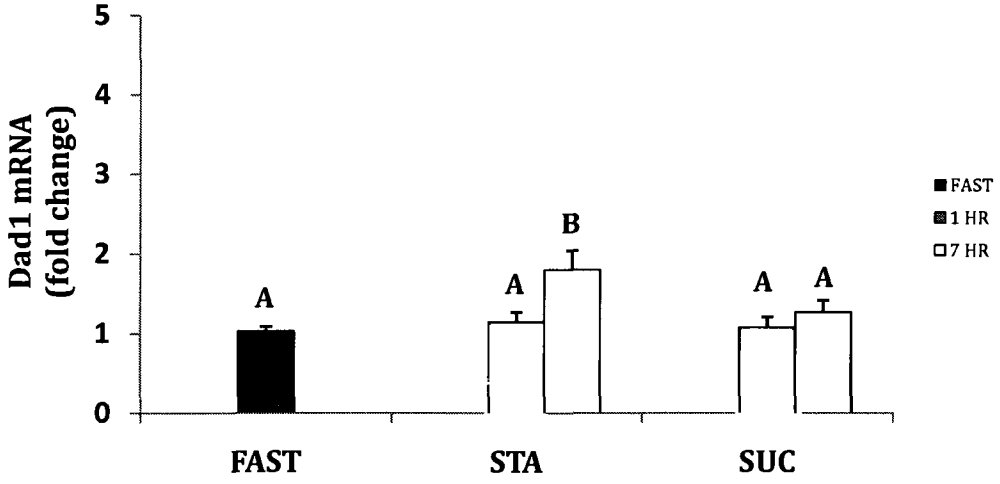

Figure 6i

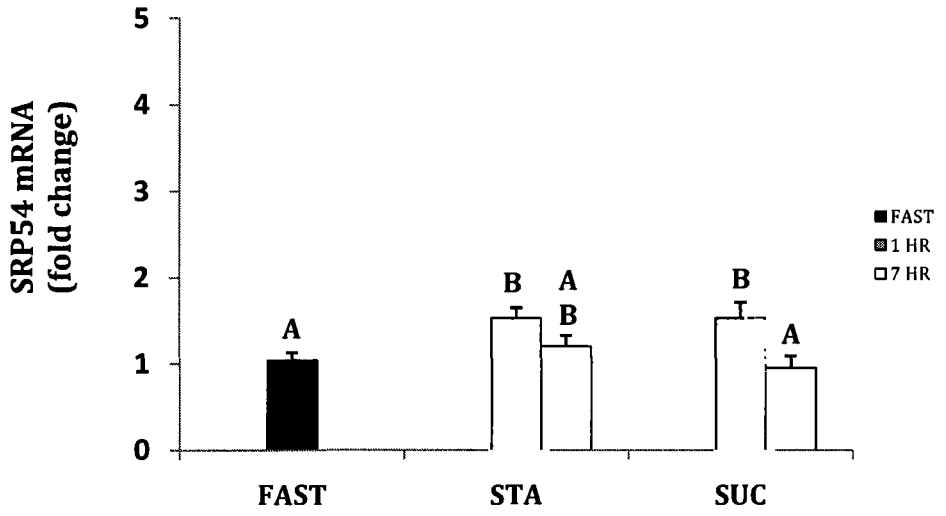

Figure 6j

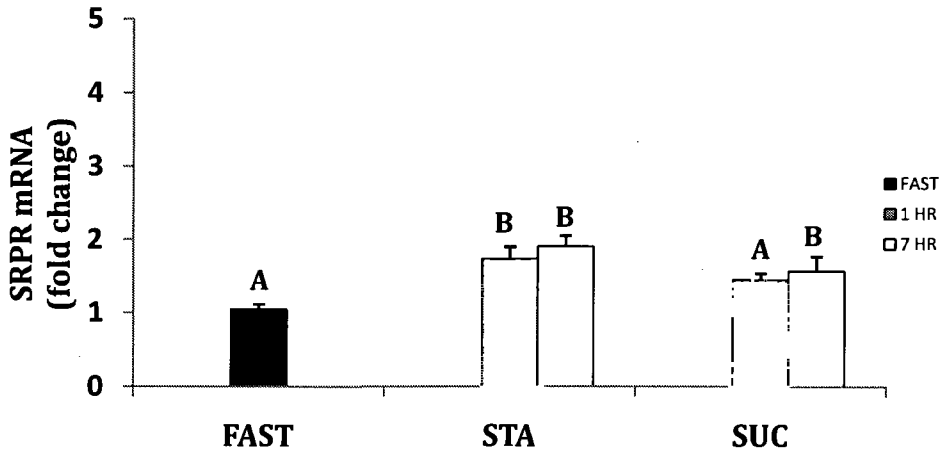

Figure 6. UPR gene markers. Expression of ATF4 (a), CHOP (b), GADD34 (c), GRP78 (d), GRP94 (e), XBP1 (f), Mgat2 (g), Dad1 (h), SRP54 (i), and SRPR (j) mRNA in the liver of fasted (FAST), starch fed (STA) and sucrose fed (SUC) rats after a 24 hour fast (black bar), 1 hour post (grey bars) or 7 hours post (white bars) meal feeding period. Data are reported as the mean±SE. n=4-6 rats per group. Bars without a common letter differ, $p < 0.05$.

Figure 7a

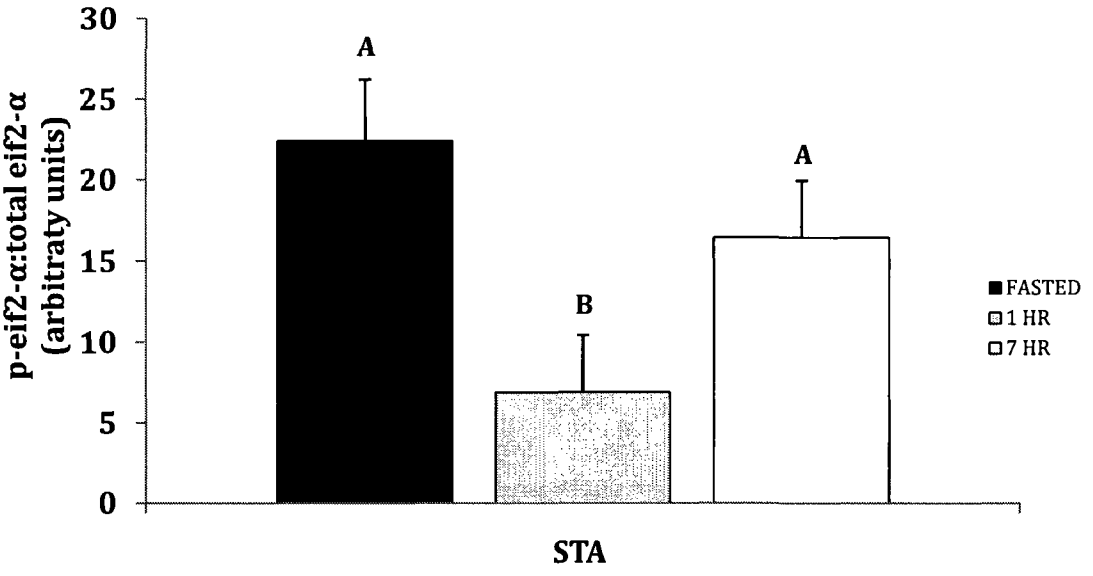

Figure 7b

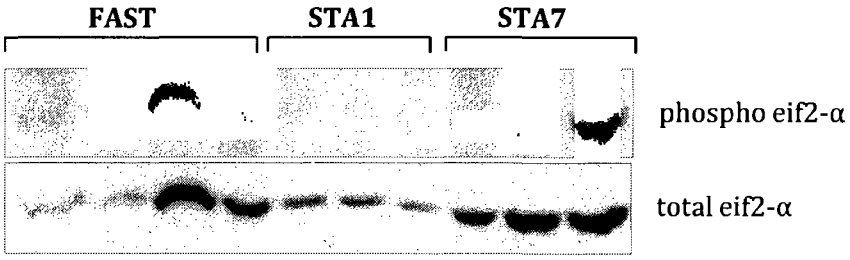

Figure 7c

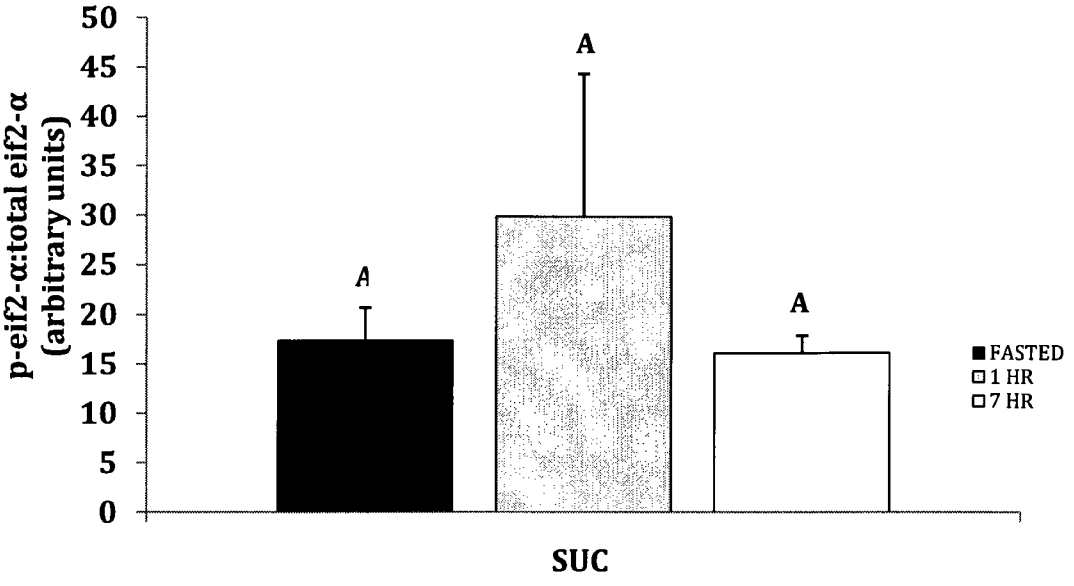

Figure 7d

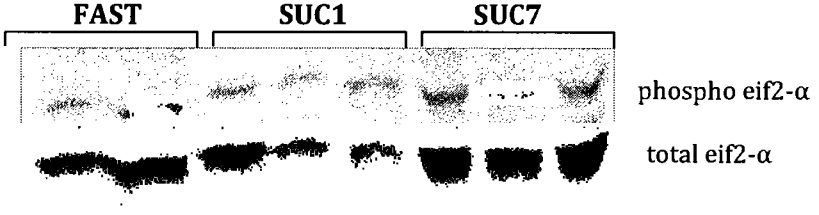

Figure 7. Phosorylation of eif-2α. Ratio of phosphorylated hepatic eif2-α protein to total eif2-α in starch (a) and sucrose (c) fed rats 1 hour post (grey bars) or 7 hours post (white bars) meal feeding period compared to fasted rats (black bars). Representative blots for starch (b) and sucrose (d) fed rats. Data are reported as the mean±SE. n=4-6 rats per group. Bars without a common letter differ, $p < 0.05$.

Figure 8a

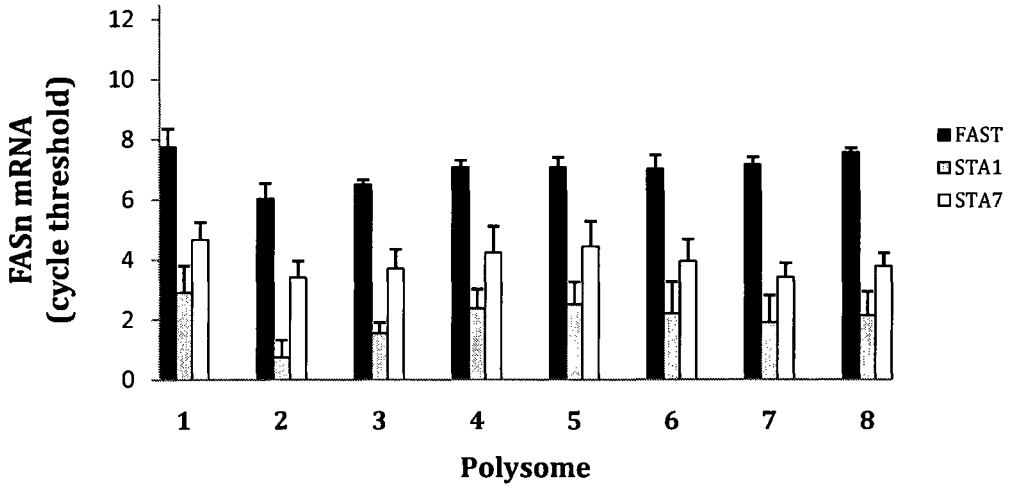

Figure 8b

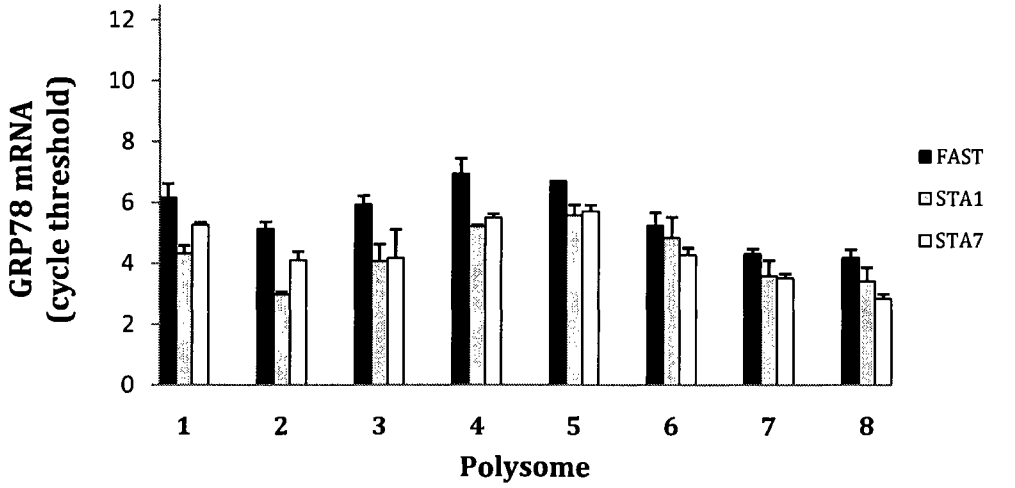

Figure 8c

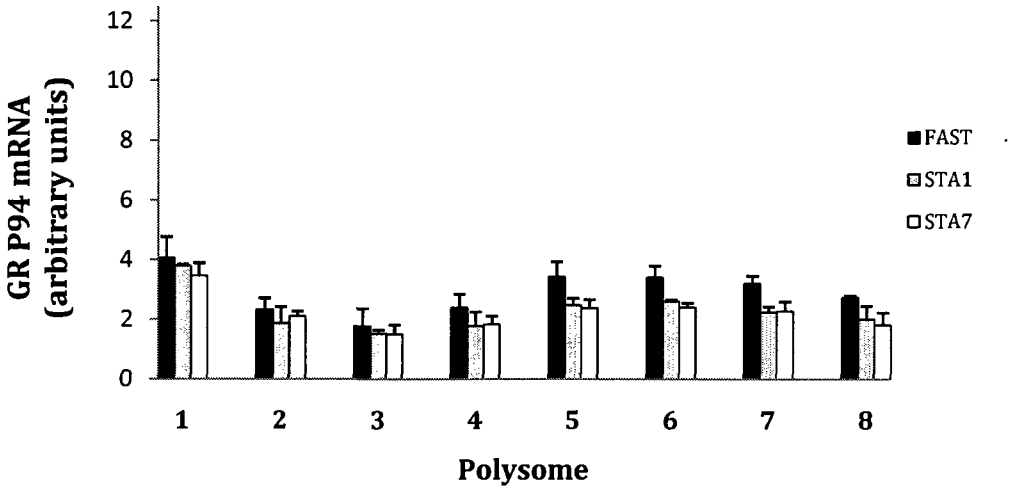

Figure 8d

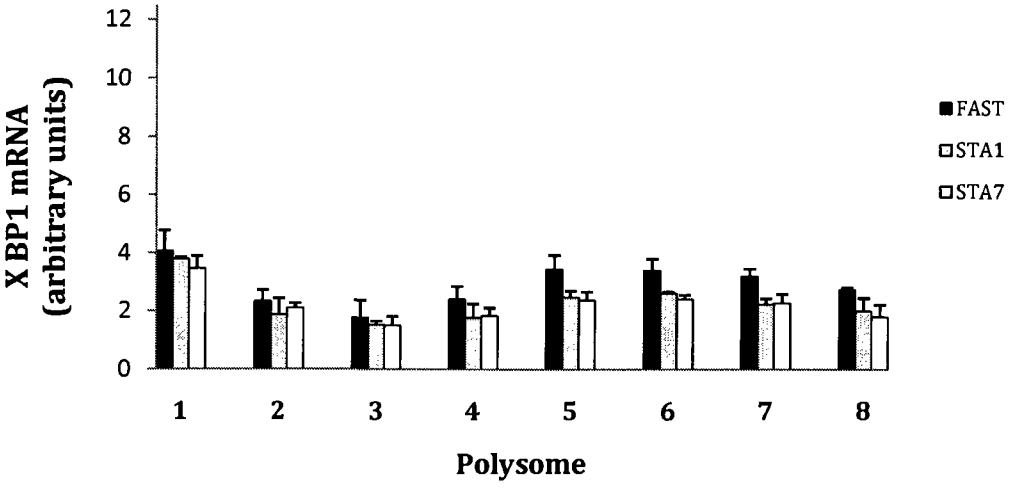

Figure 8e

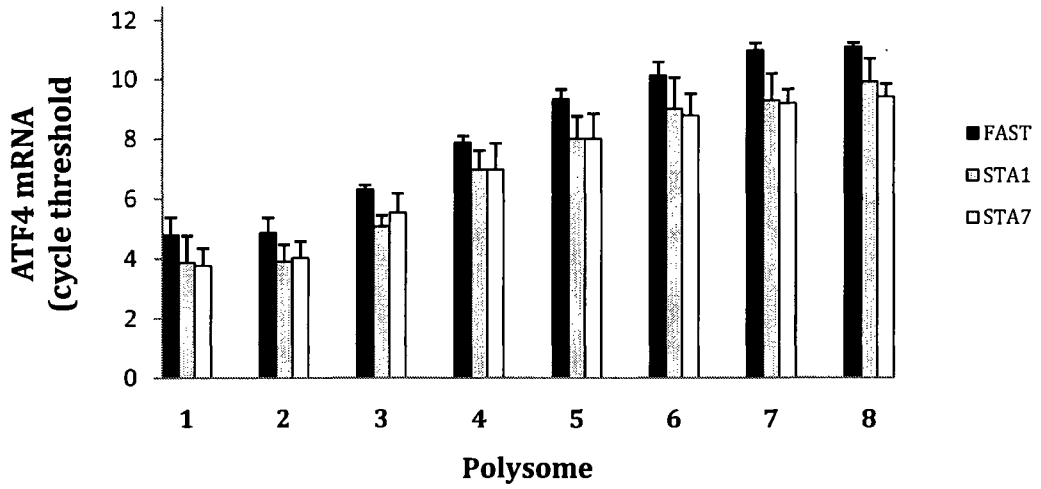

Figure 8f

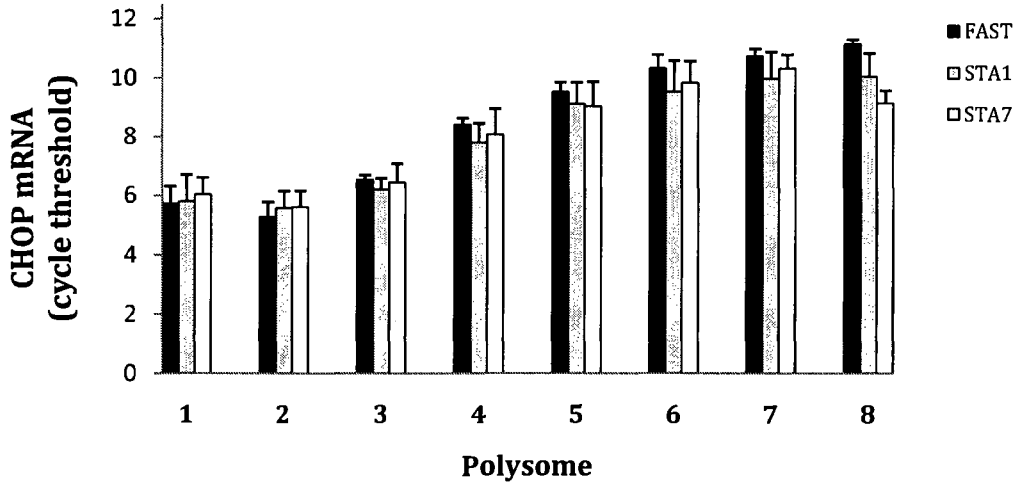

Figure 8. mRNA association with polysomes. Expression of FAS (a), GRP78 (b), GRP94 (c), XBP1 (d), ATF4 (e), and CHOP (f) mRNA associated with polysome fractions in the liver of rats after a 24 hour fast (black bars), and 1 hour (grey bars) or 7 hours (white bars) after consumption of a starch meal. Polysome 1 represents the smallest fraction and polysome 8 the largest. Bars represent the number of cycles required to reach a significant increase in gene amplification. Data are reported as the mean±SE. n=3 rats per group.

Study 2

Mammalian target of rapamycin, mTOR, is a major sensor of nutrient and energy availability and regulates a variety of cellular processes, including growth, proliferation and metabolism [48, 57]. Loss of the tuberous sclerosis complex genes, TSC1 and 2, leads to constitutive activation of mTOR [92]. A recent study from Ozcan et al [92] demonstrated that the loss of TSC1 and 2 triggers the activation of the UPR, and via this mechanism regulates insulin signaling and apoptosis. In addition, the activity of SREBP1c, a transcription factor involved in lipogenesis, and insulin-stimulated lipogenesis appears to be regulated by mTORC1 [117]. Therefore, we sought to determine whether mTOR was activated in the liver in fed compared to fasted rats from Study 1.

Ribosomal protein-s6 (RPS6) is a target of mTORC1 and, when phosphorylated, acts to facilitate the initiation of protein synthesis. A number of studies have demonstrated the activation of hepatic RPS6 in the postprandial state [50, 54]. RPS6 phosphorylation was increased in STA1 compared to both FAST and STA7 (Figure 9a, 9b). Due to high variability in both the STA1 group and small sample size for the FAST group, the difference, however did not reach statistical significance. However, the p-RPS6 to actin ratio of STA1 was 74.1±31.3, whereas the fasted group was 8.4±6.2. Phosphorylation of RPS6 was significantly increased in SUC1 compared to FAST or SUC7 (Figure 9c, 9d). Based on these data, we next sought to determine whether mTOR regulated the postprandial UPR.

Food intake and body weight. The purpose of Study 2 was to address whether the postprandial activation of components of the UPR was regulated by the mTORC1 pathway. We employed the same feeding model in Study 2 as was described for Study 1, but because there were no drastic differences between STA and SUC groups in Study 1, we used only a high starch meal for Study 2. Additionally, one hour prior to the onset of the study day feeding period, half of the rats in both the 1 hour group and the seven hour group were injected with rapamycin, a potent mTORC1 inhibitor [55]. Figure 10 shows average daily food intake during the meal training period. Rats rapidly adapted to the 3 hour feeding period and consumed an average of 15.7±8.7 grams per day by day 15-16. Average body weight was 142.9±1.9 grams on the first day and 201.9±3.4 grams by day 16.

Study day food consumption and plasma parameters. There were no significant differences in food consumption between rats administered rapamycin or vehicle and sacrificed 1 hour post feeding period (RAP1 and VEH1), or rats administered rapamycin or vehicle and sacrificed 7 hours post feeding period (RAP7and VEH7). However, RAP7 and VEH7 rats did consume significantly less food during the three hour feeding period compared to the VEH1 group (12.5±1.6 and 13.2±2.1, respectively vs 16.7±0.9 grams) (Figure 11).

Portal vein glucose levels were significantly increased in response to feeding in RAP1 and VEH1, and returned to fasting levels by 7 hours (Figure 12a). Portal vein glucose levels were significantly increased relative to vena cava glucose levels in RAP1 and VEH1 groups, demonstrating that glucose absorption was present (Figure 12a). Although portal vein insulin levels were not significantly increased (Figure 12b), their

elevation relative to fasting combined with portal vein glucose data strongly suggests that fed rats were in a postprandial state.

Activation of the mTORC1 pathway. The phosphorylation of RPS6 is an established indicator of mTORC1 activation. Hepatic RPS6 phosphorylation was significantly increased at 1 hour and the presence of rapamycin prevented this increase (Figure 13a-13c).

Lipogenic gene expression. In Study 1 we observed an increase in both FAS and SREBP1c mRNA in the liver of rats that were sacrificed 1 hour following meal feeding. In Study 2, FAS mRNA was significantly increased in VEH at 1 and 7 hours, whereas SREBP1c was increased in VEH at 1 hour (Figure 14a, 14b). In RAP, the increase in FAS mRNA was reduced by ~50% (Figure 14a). Conversely, rapamycin did not significantly reduce SREBP1c mRNA (Figure 14b).

XBP1 splicing. Spliced XBP1 was present in 3 of the 5 VEH1 rats and in 1 rat from the RAP1 group (Figure 15). There were 2 rats in the VEH7 group and no rats in the RAP7 group that displayed spliced XBP1.

The presence of spliced XBP1 in the VEH1 group of Study 2 was somewhat less consistent than the presence of spliced XBP1 in the STA1 group of Study 1. There were a few differences between Study 1 and Study 2 that may explain this discrepancy. First, the post feeding sacrifice of rats was significantly delayed in Study 2 compared to Study 1. This difference was due to the longer duration required to adequately anesthetize rats in study 2 (Figure 16a). Another possible contributing factor to the difference between the frequency of spliced XBP1 in Study 1 compared to Study 2 may involve the variability of individual insulin levels. The three VEH1 rats that displayed spliced XBP1 in study 2

represented the three highest individual portal vein insulin levels (Figure 16b). Additionally, the one rat with spliced XBP1 in the RAP1 group had the second highest insulin level of that group (Figure 16b).

Gene markers of the UPR. We next examined the UPR gene markers GRP78, GRP94, XBP1, CHOP, and ATF4 in fed rats in the absence and presence of rapamycin. GRP78 mRNA was increased 6 fold, GRP94 mRNA was increased 2.5 fold, and XBP1 mRNA was increased 6.5 fold in the VEH1 group vs. the FAST group (Figure 17a-17c). In contrast, feeding had no effect on these genes in the presence of RAP (Figure 17a-17c). There were no significant differences in GRP78, GRP94 or XBP1 mRNA among VEH7, RAP7, and FAST groups (Figure 17a-17c). There were small but significant increases in CHOP (2 fold) and ATF4 (1.7 fold) mRNA's in VEH1 compared to the FAST group (Figure 17d, 17e). Feeding had no effect on CHOP and ATF4 mRNA in the presence of RAP (Figure 17d, 17e).

Association of mRNA with polysomes. Polysomal fractions were isolated at the 1 hour time point to examine the effects of rapamycin on translational efficiency of lipogenic and UPR gene markers. Similar to study 1, feeding resulted in an increase in the association of FAS, GRP78 and GRP94 with polysomes (Figure 18a-18c). Rapamycin decreased the amount of FAS, GRP78, and GRP94 mRNA associated with polysomes (Figure 18a-18c). However, rapamycin did not prevent the feeding-mediated increase in the association of these mRNAs with polysomes. Feeding did not increase the association of ATF4 or CHOP mRNA with polysomes, and rapamycin did not significantly affect the pattern of mRNA association with polysomes (Figure 18d, 18e).

Chaperone protein expression. Based on the observation that there were increases in both GRP78 mRNA expression and GRP78 mRNA association with polysome fractions, we next examined GRP78 protein expression. GRP78 protein expression was significantly increased in VEH7 compared to FAST. In contrast, GRP78 protein was not increased in RAP7 (Figure 19a-19c).

Summary. The purpose of Study 2 was to examine the relationship between the postprandial activation of liopogenic and UPR gene markers and mTORC1 activation. Spliced XBP1, the expression of GRP78, GRP94 and XBP1 mRNA, and GRP78 protein expression were increased in an mTORC1 dependent manner in the postprandial state. Although, we observed small but significant increases in ATF4 and CHOP mRNA expression, polysome data suggest that these two genes may not be efficiently translated in the postprandial setting. The feeding induced increase in FAS mRNA appears to be partially dependent on mTORC1 activation. Taken together, these data demonstrate that the postprandial activation of UPR components and FAS gene expression is regulated in an mTORC1 dependent manner.

Figure 9a

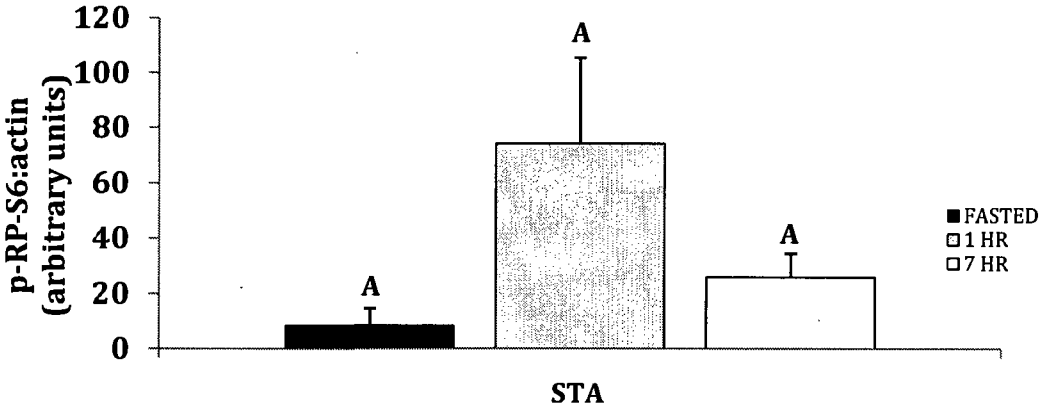

Figure 9b

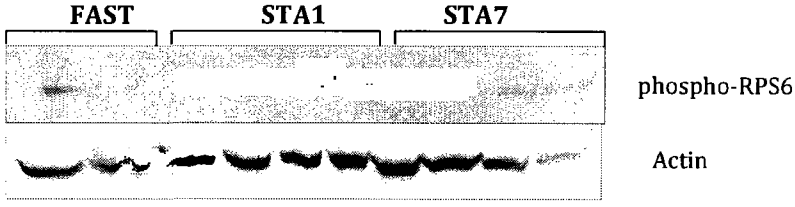

Figure 9c

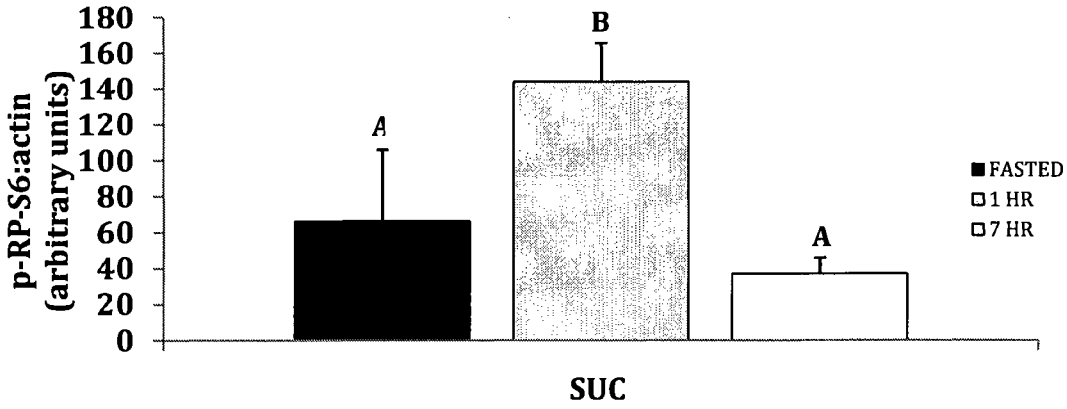

Figure 9d

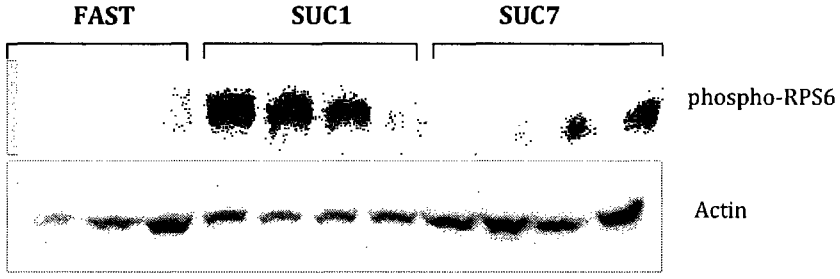

Figure 9. Phosorylation of RPS6. Expression of phosphorylated RPS6 protein in the liver of starch (a) and sucrose (c) fed rats 1 hour post (grey bars) or 7 hours post (white bars) meal feeding period compared to fasted rats (black bars). Representative blots for starch (b) and sucrose (d) fed rats. Data are reported as the mean±SE. n=3-6 rats per group. Bars without a common letter differ, $p < 0.05$.

Figure 10

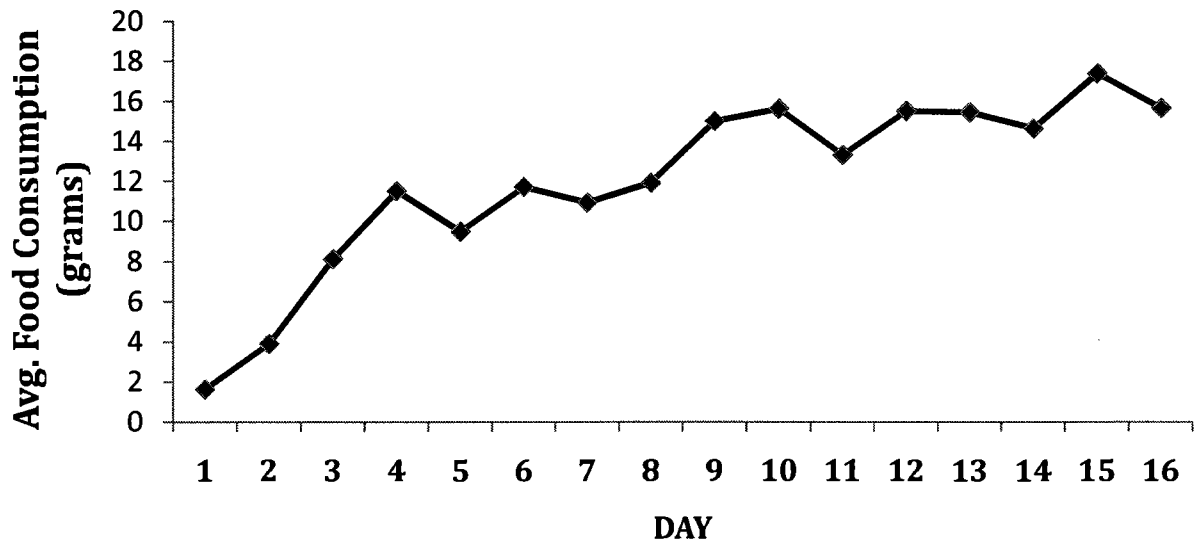

Figure 10. Daily food consumption. Average daily food intake of rats (n=21) over the meal training period.

Figure 11

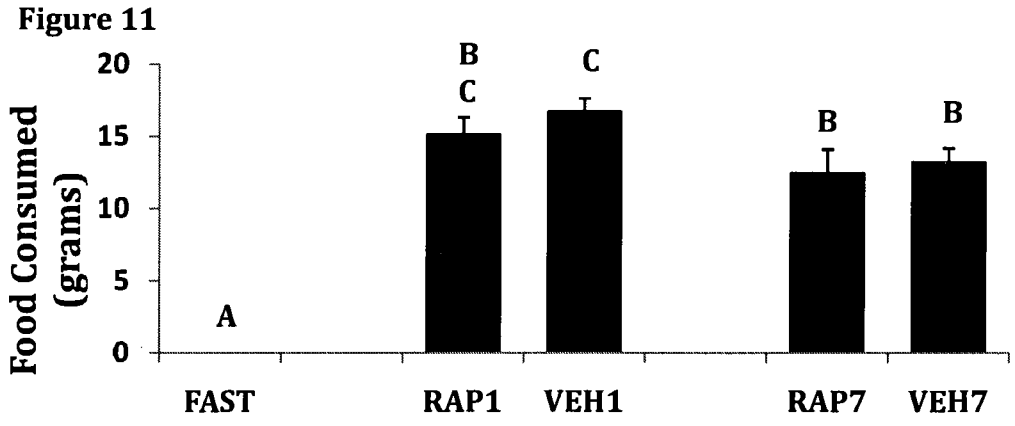

Figure 11. Study day food consumption. Starch meal intake during the 3 hour feeding period in fasted (FAST) or fed rats administered rapamycin (RAP1, RAP7) or carrier (VEH1, VEH7) and sacrificed 1 or 7 hours post feeding period. Data are reported as the mean±SE. n=4-6 rats per group. Bars without a common letter differ, $p < 0.05$

Figure 12a

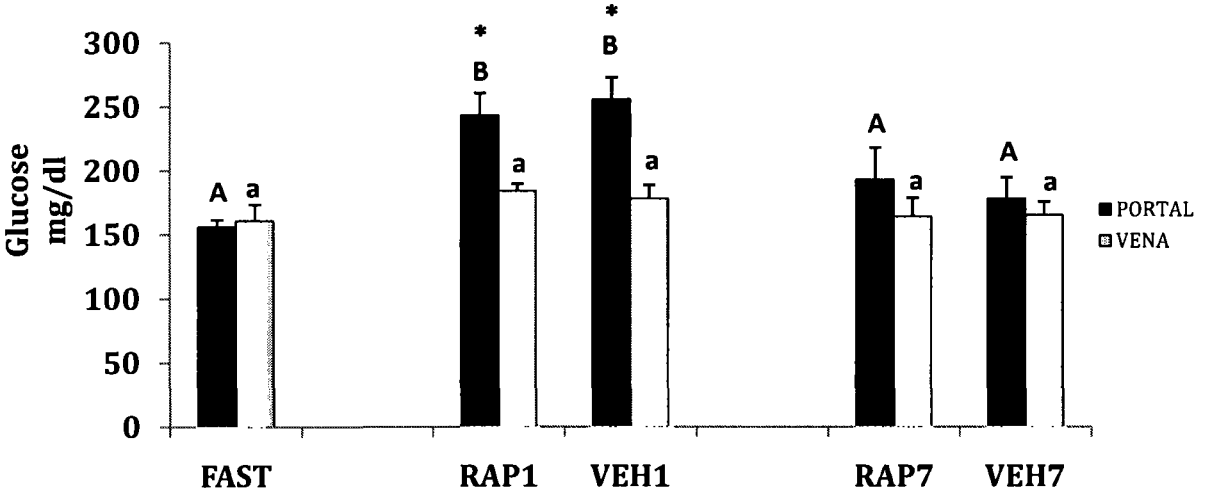

Figure 12b

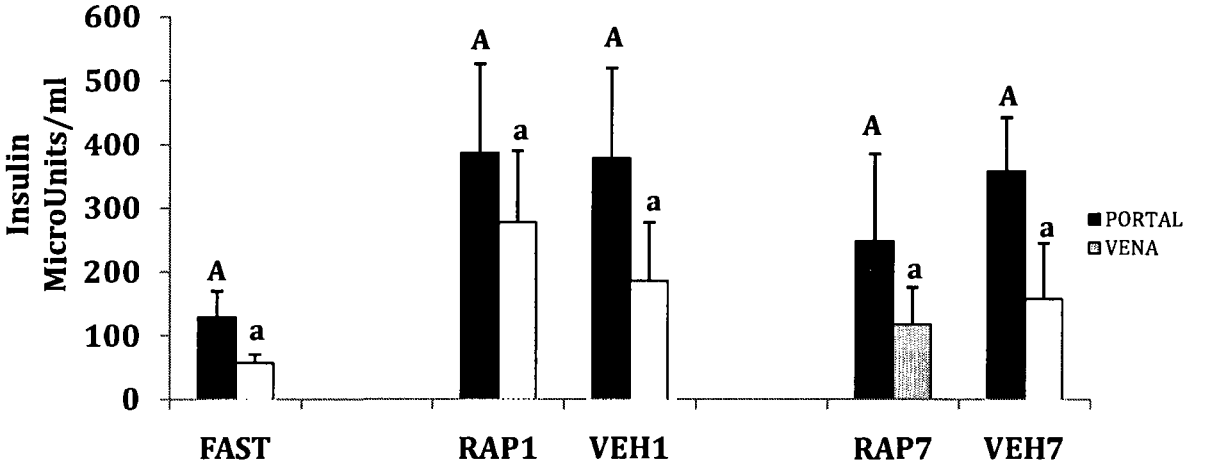

Figure 12. Plasma parameters. Plasma glucose (a) and insulin (b) concentrations taken from the portal vein (black bars) and the descending vena cava (grey bars) of rats from each group. Data are reported as the mean±SE. n=4-6 rats per group. Capital letters denote comparison of portal vein concentrations between groups. Lower case letters denote comparison of descending vena cava concentrations between groups. * denote significant differences between portal vein and descending vena cava within a group. Bars without a common letter differ, $p < 0.05$.

Figure 13a

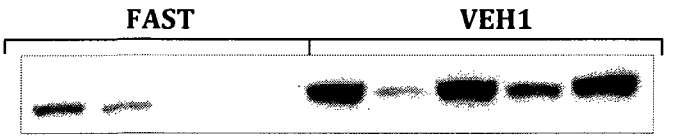

Figure 13b

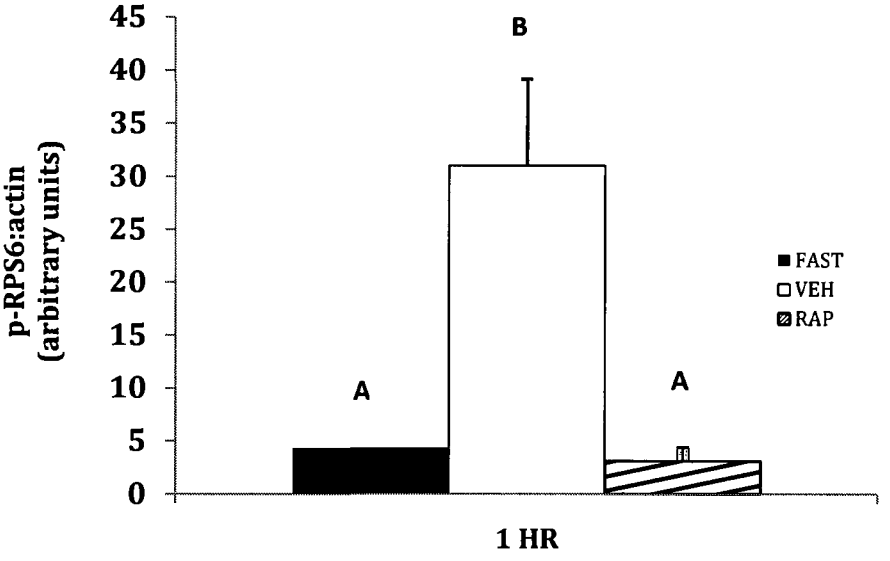

Figure 13c

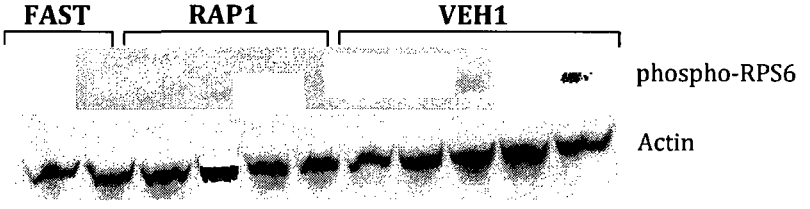

Figure 13. Effect of rapamycin on the postprandial phosorylation of RPS6. Representative blot for phosphorylated RPS6 protein expression in fasted (FAST) and starch fed vehicle injected rats (VEH1) sacrificed 1 hour post feeding period (a). Ratio of phosphorylated hepatic RPS6 protein to actin protein expression in the liver of fasted (black bar), carrier injected (white bar), and rapamycin injected (striped bar) rats 1 hour post meal feeding period (b). Representative blot (c). Data are reported as the mean±SE. n=4-6 rats per group. Bars without a common letter differ, $p < 0.05$.

Figure 14a

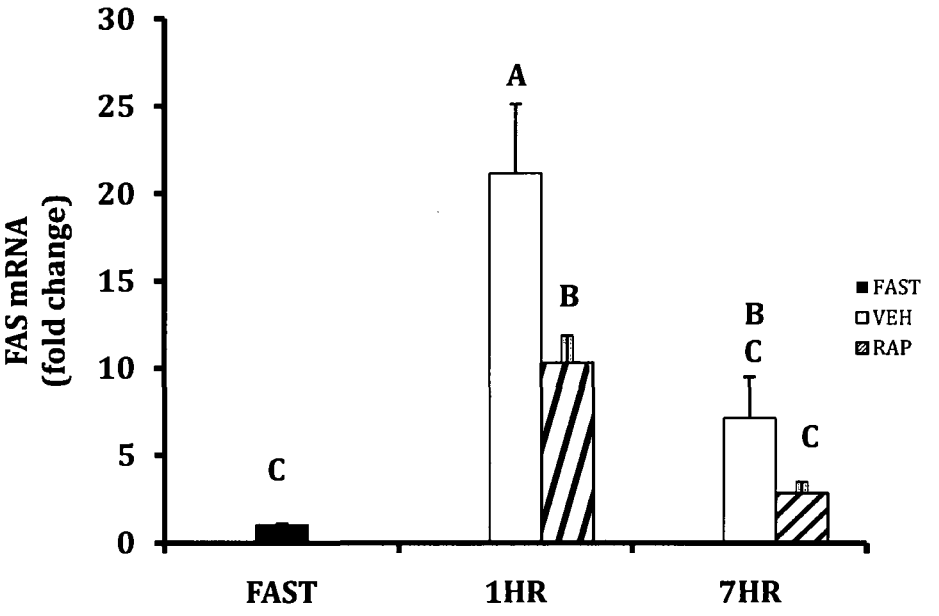

Figure 14b

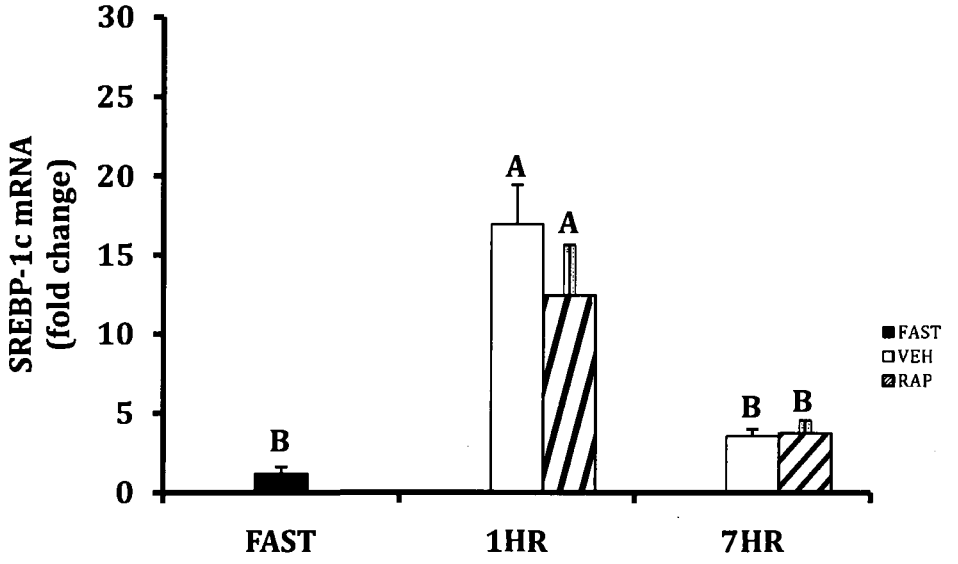

Figure 14. Effect of rapamycin on postprandial gene markers. Expression of FAS (a) and SREBP1c (b) mRNA in the liver of fasted (black bar), vehicle injected (white bars), and rapamycin injected (striped bars) rats sacrificed after a 24 hour fast, 1 hour post, or 7 hours post meal feeding period. Data are reported as the mean±SE. n=4-6 rats per group. Bars without a common letter differ, $p < 0.05$.

Figure 15

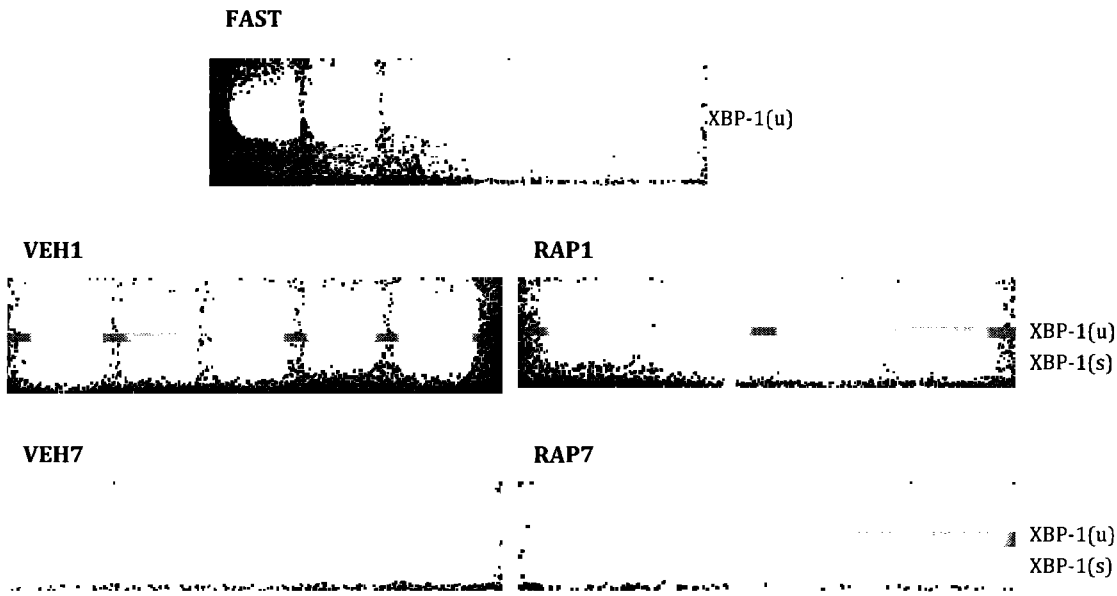

Figure 15. Effect of rapamycin on XBP1 splicing. The presence of unspliced (u) and spliced (s) XBP1 mRNA in hepatic tissue of rats either fasted (FAST), or injected with rapamycin (RAP1, RAP7) or vehicle (VEH1, VEH7) and sacrificed 1 or 7 hours post feeding period. n=4-6 rats per group.

Figure 16a

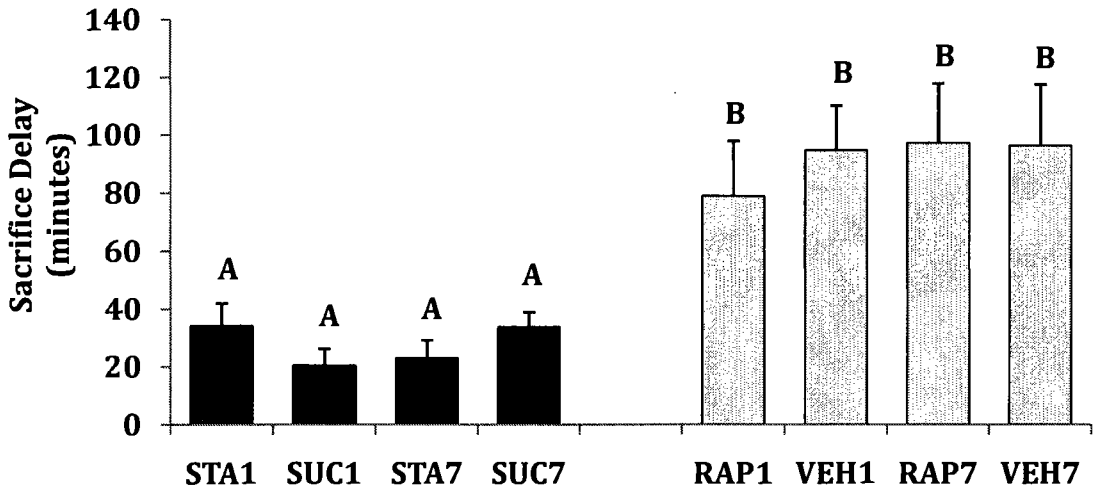

Figure 16b

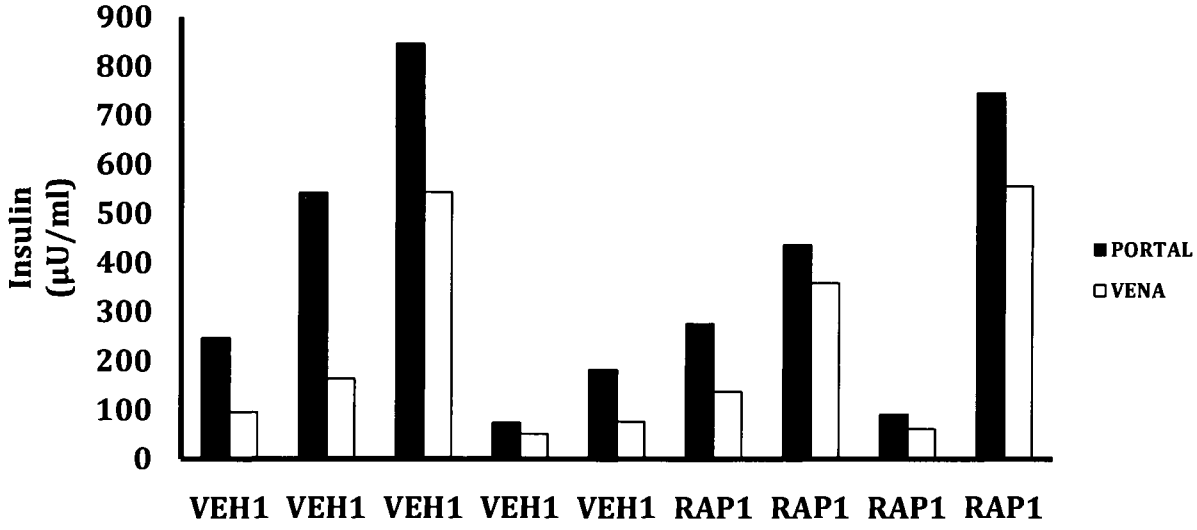

Figure 16. Comparison of sacrifice time points between Study1 and Study 2 and individual insulin levels from Study2. Delay of post feeding period sacrifice for fed rats from Study 1 (black bars) and Study 2 (grey bars) (a). Individual plasma insulin concentrations in the portal vein (black bars) and descending vena cava (white bars) in vehicle injected (VEH1) or rapamycin injected (RAP1) rats sacrificed 1 hour post meal feeding. n=4-6 rats per group. Bars without a common letter differ, $p < 0.05$.

Figure 17a

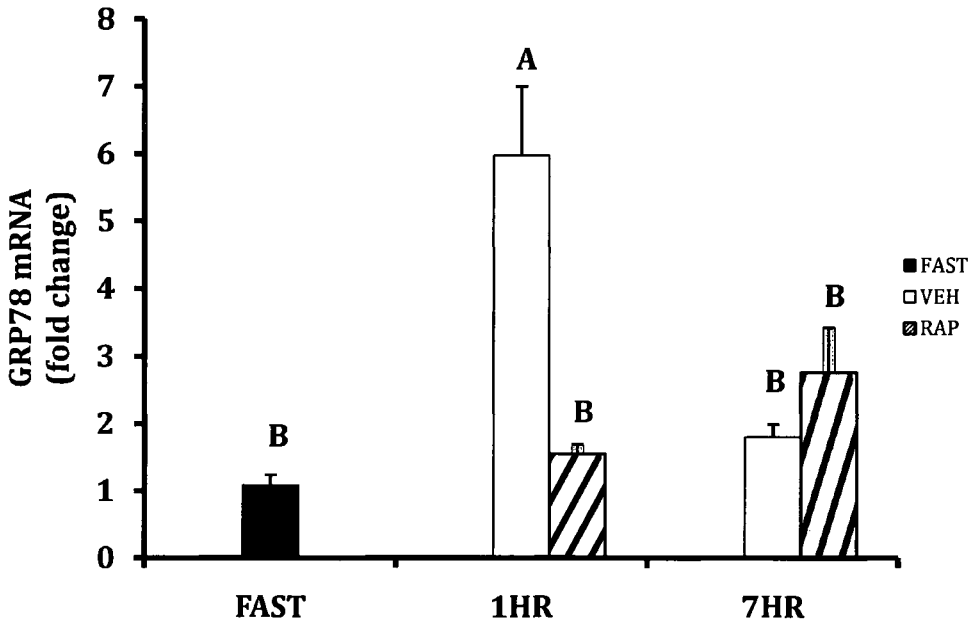

Figure 17b

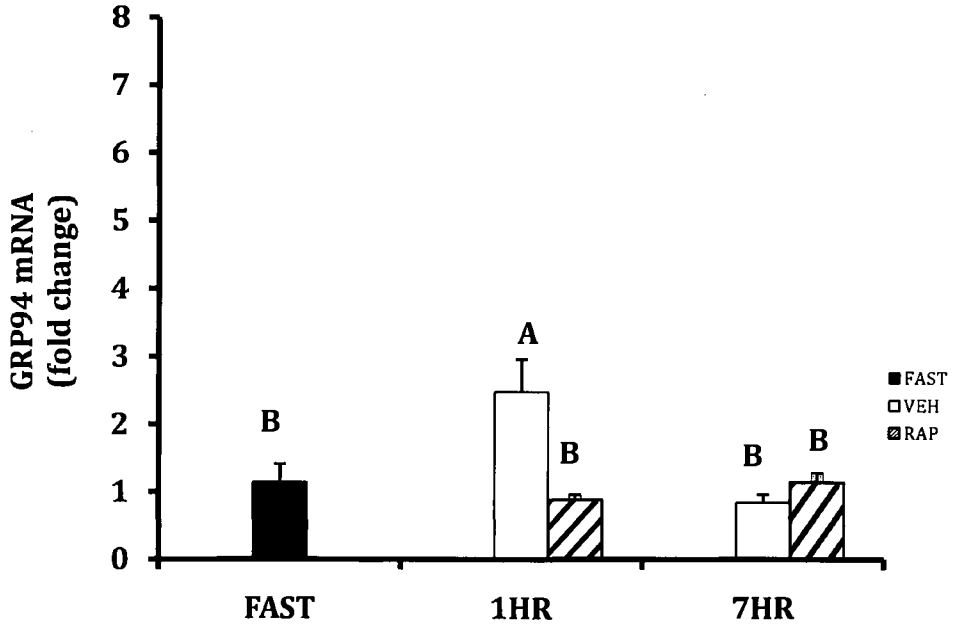

Figure 17c

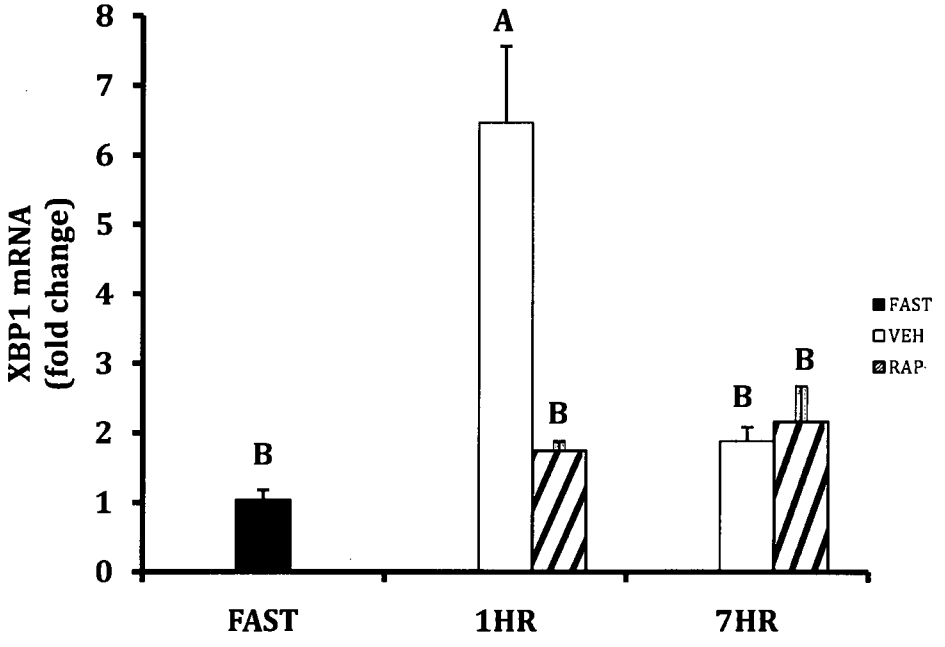

Figure 17d

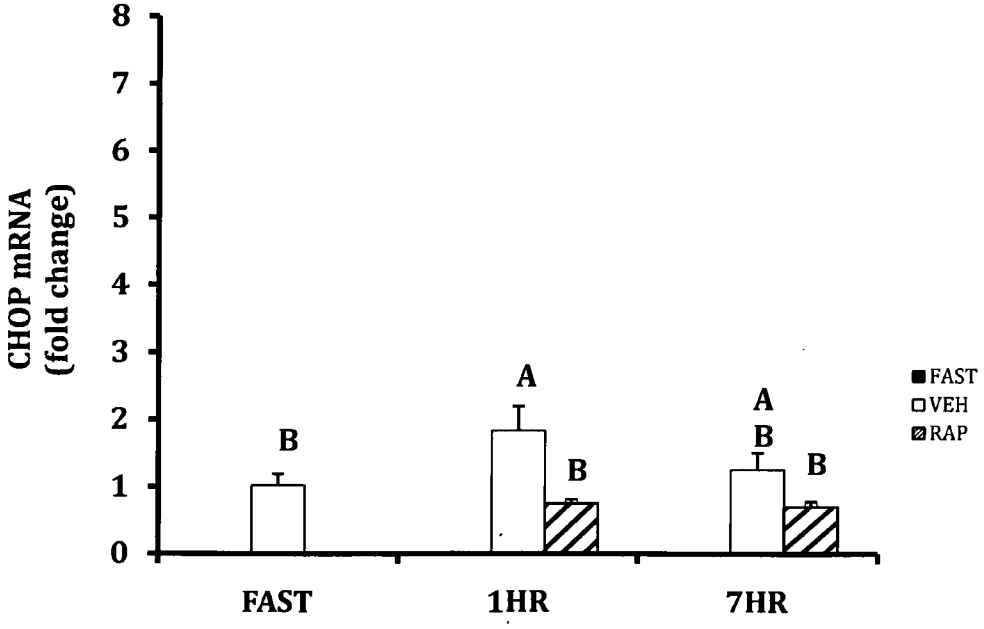

Figure 17e

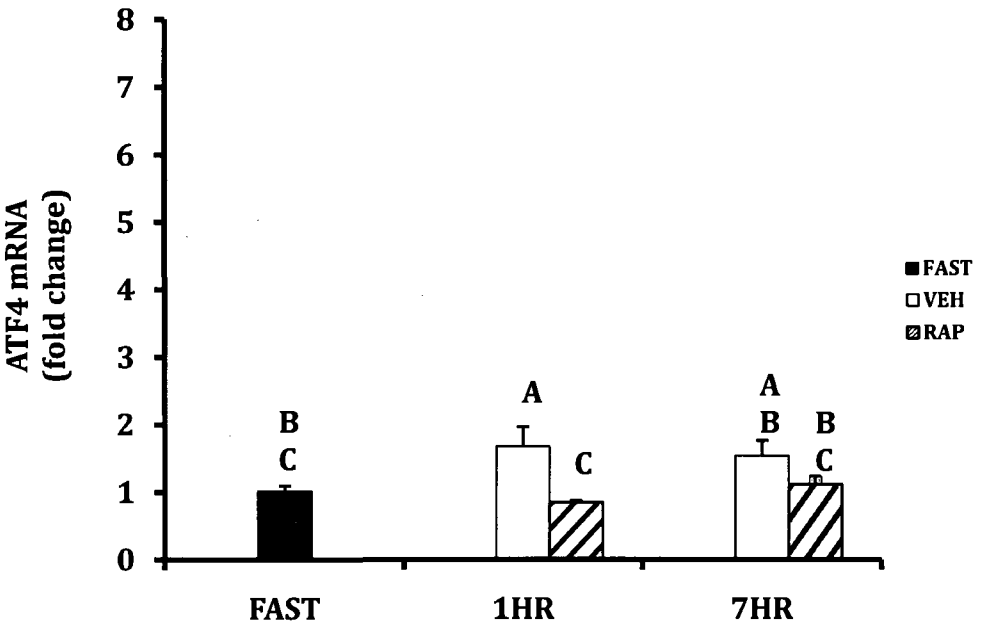

Figure 17. Effect of rapamycin on UPR gene markers. Expression of GRP78 (a), GRP94 (b), XBP1 (c), CHOP (d), and ATF4 (e) mRNA in the liver of fasted (black bar), vehicle injected (white bars), and rapamycin injected (striped bars) rats sacrificed after a 24 hour fast, 1 hour post, or 7 hours post meal feeding period. Data are reported as the mean±SE. n=4-6 rats per group. Bars without a common letter differ, $p < 0.05$.

Figure 18a

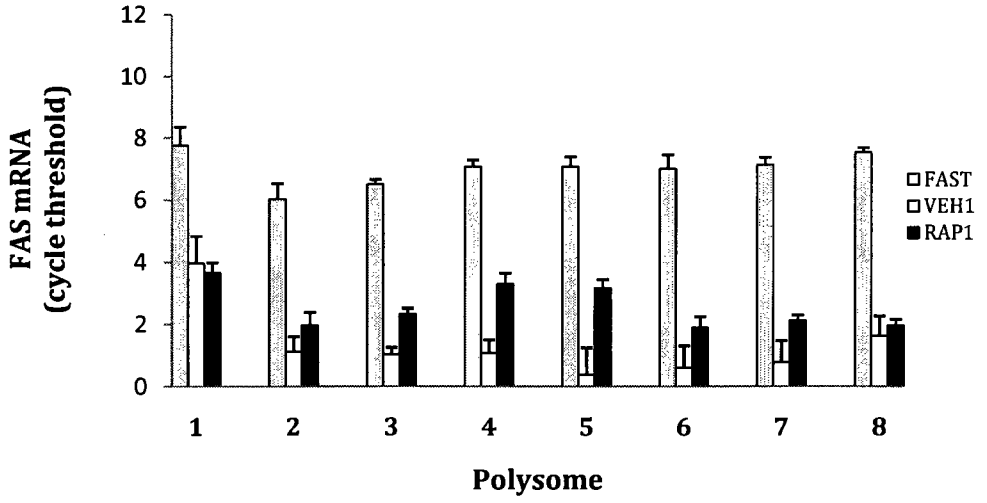

Figure 18b

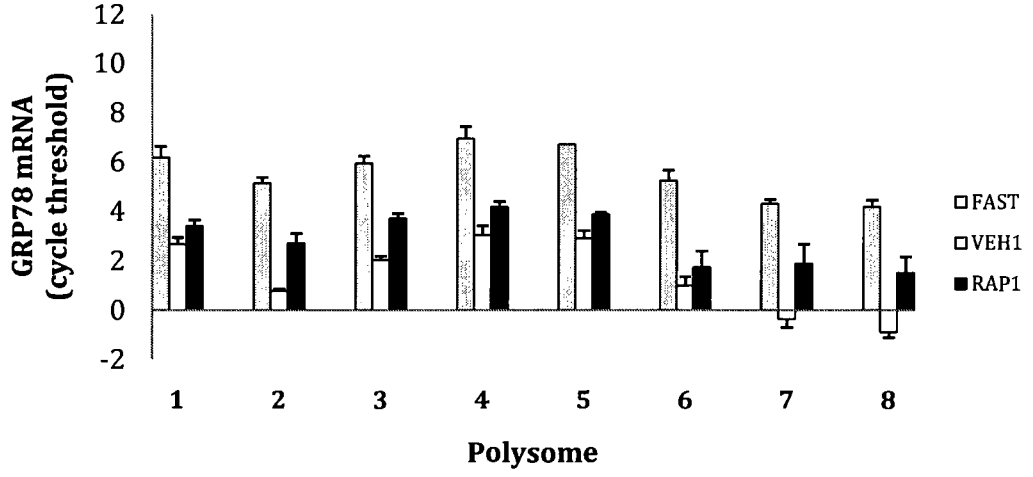

Figure 18c

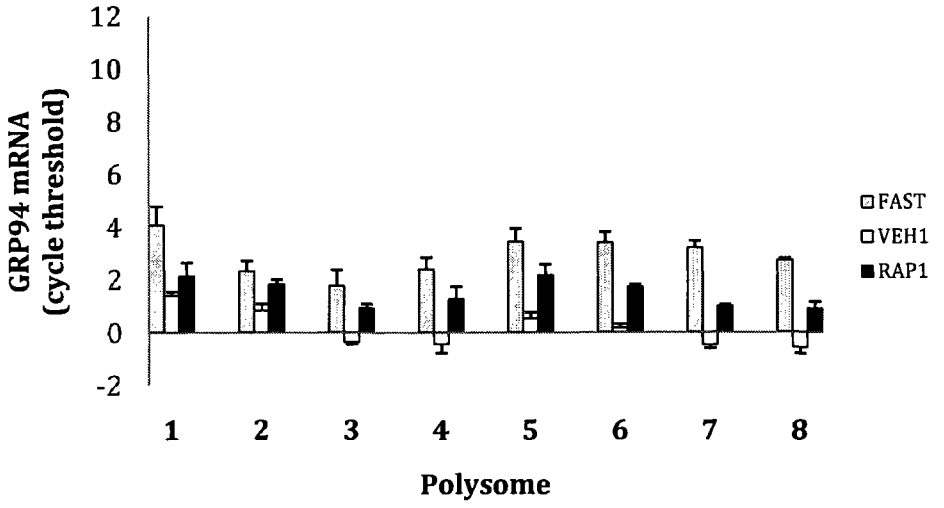

Figure 18d

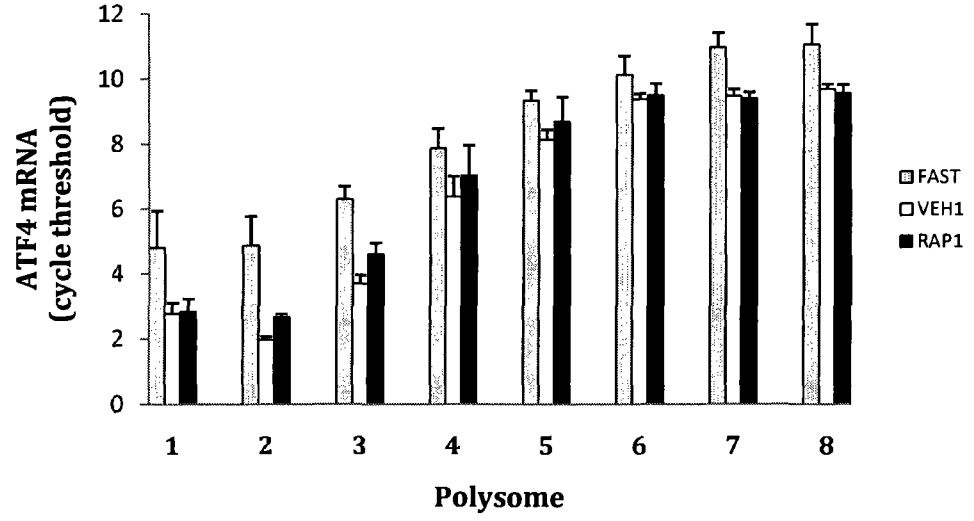

Figure 18e

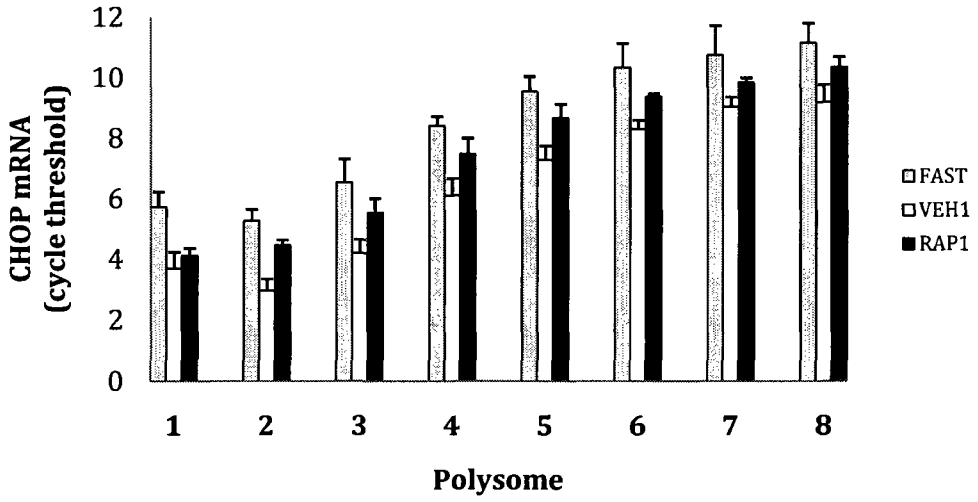

Figure 18. Effect of rapamycin on mRNA association with polysomes. Expression of FAS (a), GRP78 (b), GRP94 (c), ATF4 (d), and CHOP (e) mRNA associated with polysome fractions in the liver of rats after a 24 hour fast (grey bars) and vehicle injected (white bars) or rapamycin injected (black bars) rats sacrificed 1 hour post feeding period. Polysome 1 represents the smallest fraction and polyssome 8 is the largest. Bars represent the number of cycles required to reach a significant increase in gene amplification. Data are reported as the mean±SE. n=3 rats per group.

Figure 19a

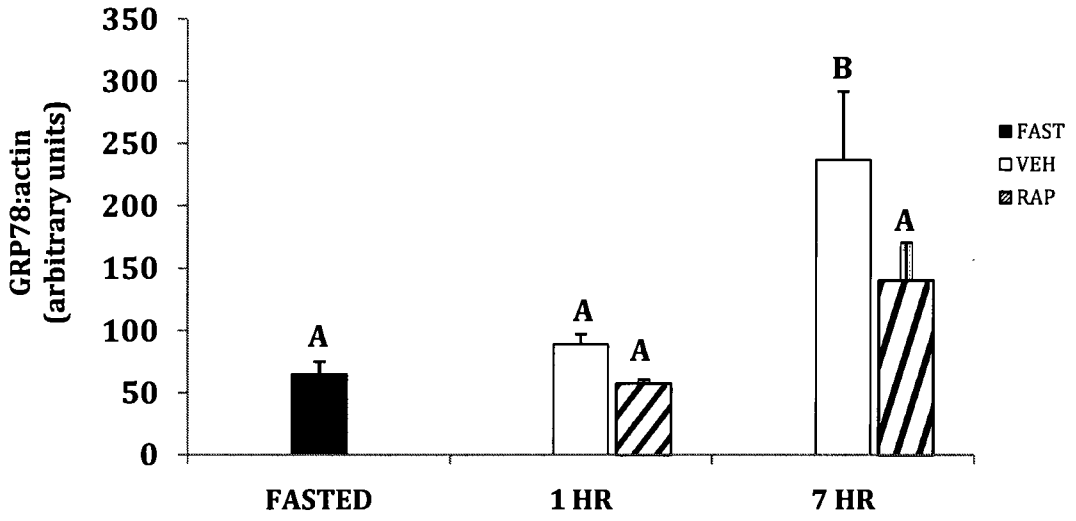

Figure 19b

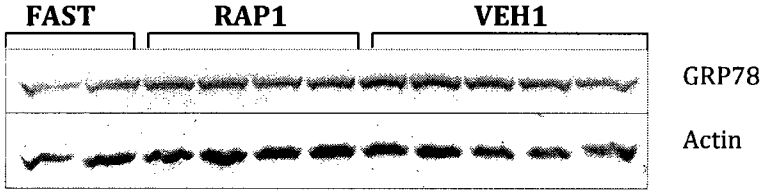

Figure 19c

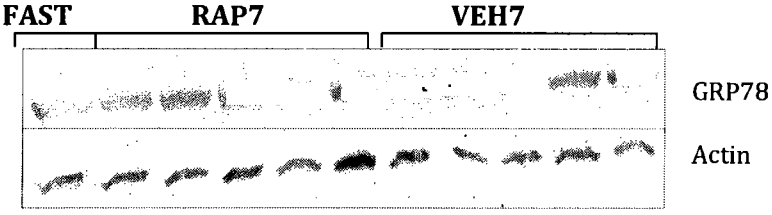

Figure 19. Effect of rapamycin on hepatic GRP78 protein expression. Expression of GRP78 protein in the liver of fasted (black bar), vehicle injected (white bars), or rapamycin injected (striped bars) rats sacrificed 1 or 7 hours post feeding period (a). Representative blots (b,c). Data are reported as the mean±SE. n=3-6 rats per group. Bars without a common letter differ, $p < 0.05$.

Study 3

The postprandial rise in glucose and insulin has been shown to stimulate protein synthesis in the liver, in part, via mTORC1 activation [57]. Data from Study 1 and Study 2 demonstrate that components of the UPR were activated postprandially and that this activation was dependent onmTORC1. The aim of study 3 was to examine the independent and combined roles of glucose and insulin in the activation of UPR components, and whether any activation was dependent on mTORC1. For these studies, we utilized H4IIE liver cells in order to carefully control the delivery of glucose and/or insulin.

XBP1 splicing. Feeding induced XBP1 splicing in both Study 1 and Study 2. To examine whether glucose and/or insulin mediates this effect, H4IIE liver cells were exposed to 0, 1, or 10 nM insulin in combination with 8 mM or 15 mM glucose for 6 or 16 hours. There was no evidence of XBP1 splicing in cells exposed to either 8 or 15 mM glucose in the absence of insulin (Figure 20a-20c). However, XBP1 splicing was detected when insulin was present (Figure 20a-20c). Rapamycin prevented insulin-mediated induction of XBP1 splicing (Figure 20a-20c).

UPR gene markers. We next examined CHOP, GADD34, GRP78, and GRP94 mRNA. Glucose alone did not increase any of the UPR gene markers tested (Figure 21a-21h). In contrast, the presence of insulin significantly increased CHOP, GADD34, and GRP78 mRNA at 6 and 16 hours (Figure 21a-21f). Rapamycin prevented the increase in these genes in response to insulin. Overall, these data suggest that insulin may regulate the UPR in an mTORC1 dependent fashion in liver cells.

Figure 20a

Insulin (nM)	0	1	10	0	1	10
Glucose (mM)	8	8	8	8	8	8
Rapamycin	-	-	-	+	+	+

6 hr

Insulin (nM)	0	1	10	0	1	10
Glucose (mM)	15	15	15	15	15	15
Rapamycin	-	-	-	+	+	+

6 hr

Insulin (nM)	0	1	10	0	1	10
Glucose (mM)	8	8	8	8	8	8
Rapamycin	-	-	-	+	+	+

16 hr

Insulin (nM)	0	1	10	0	1	10
Glucose (mM)	15	15	15	15	15	15
Rapamycin	-	-	-	+	+	+

16 hr

Figure 20b

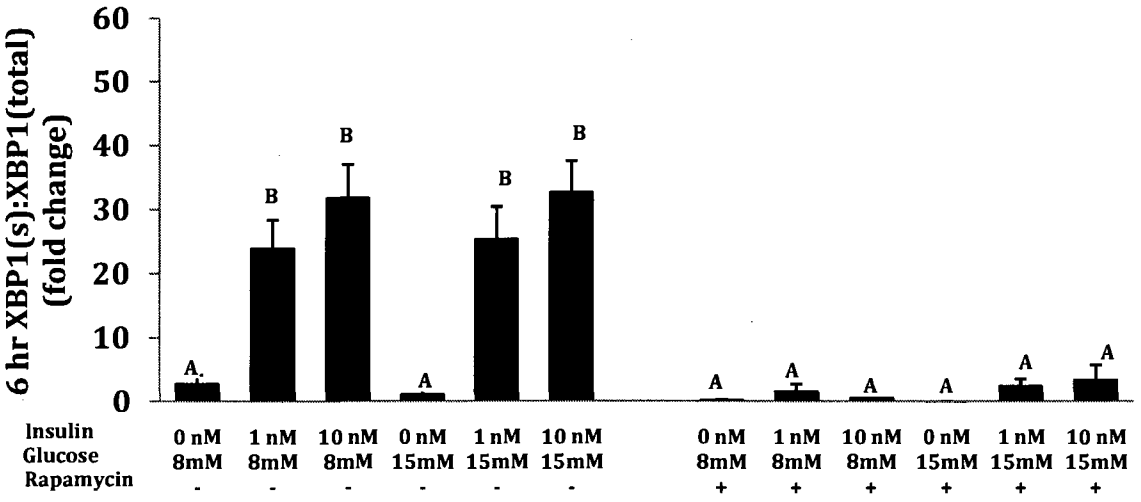

Figure 20c

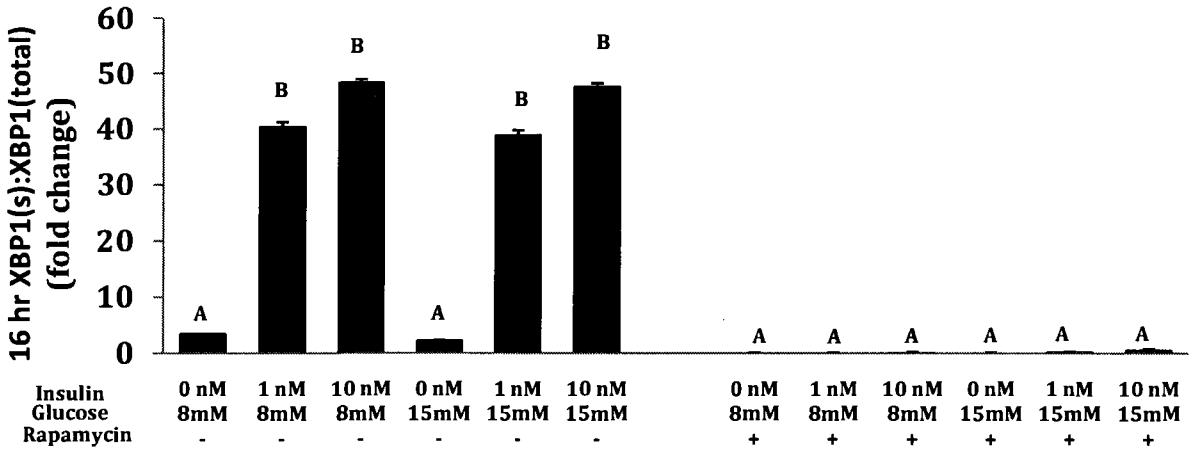

Figure 20. Effect of glucose, insulin, and rapamycin on XBP1 splicing. Presence of unspliced (u) and spliced (s) XBP1 in H4IIE cells that were exposed to varying insulin and glucose concentrations in the presence or absence of rapamycin, for either 6 or 16 hours (a). Quantification of spliced XBP1 at 6 (b) and 16 (c) hours. Data are reported as mean±SE. n=5-8 experiments. Bars without a common letter differ, $p < 0.05$.

Figure 21a

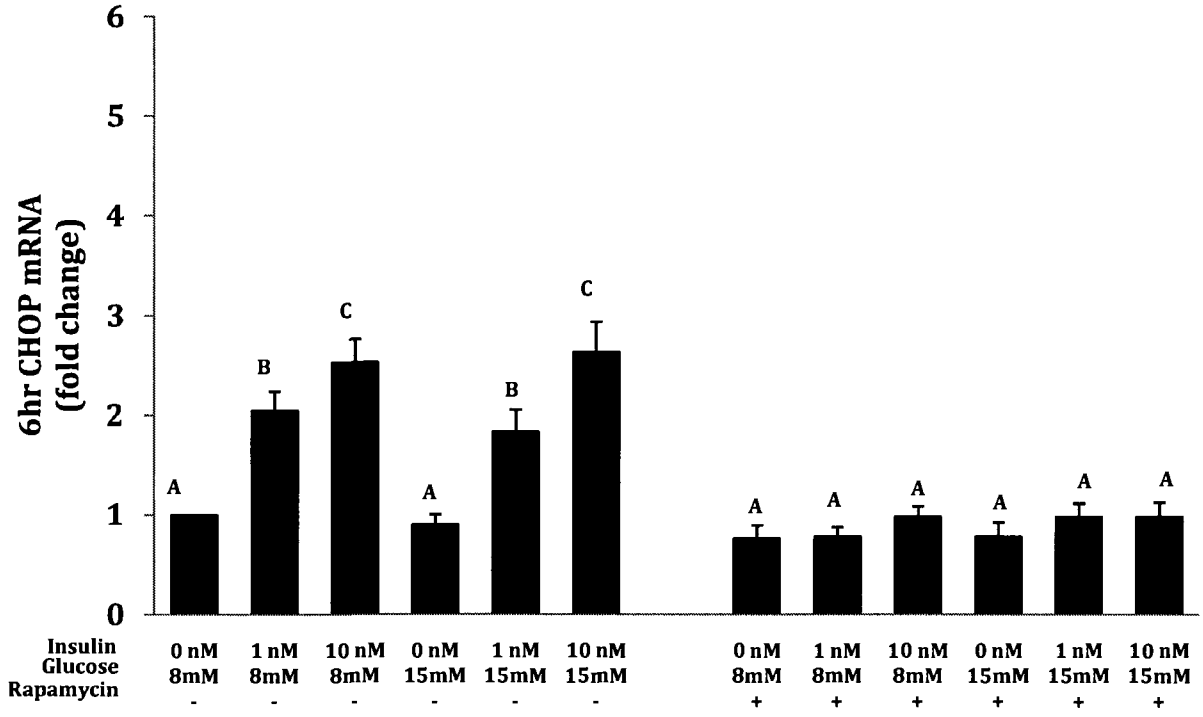

Figure 21b

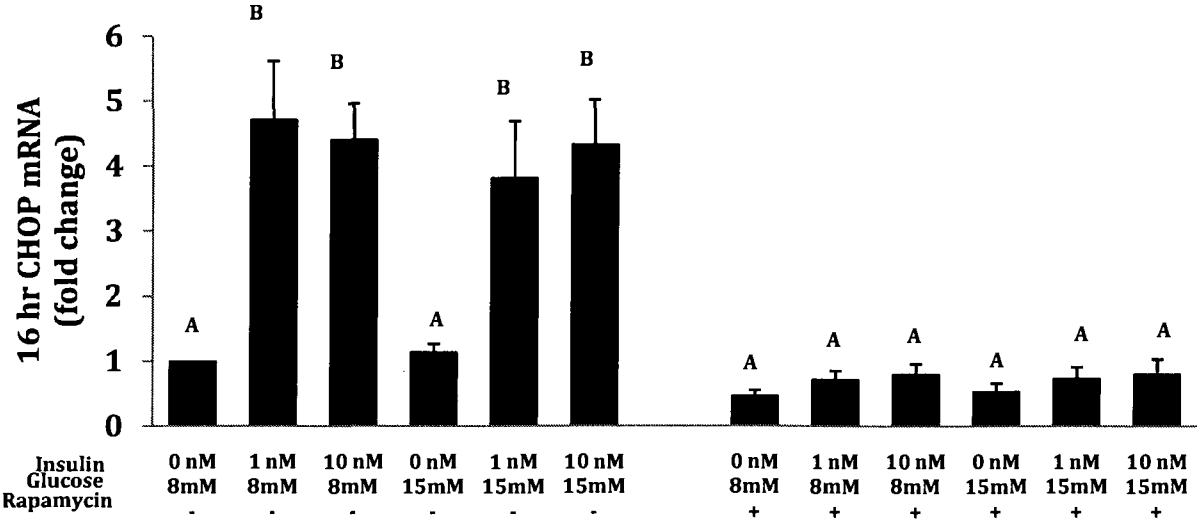

Figure 21c

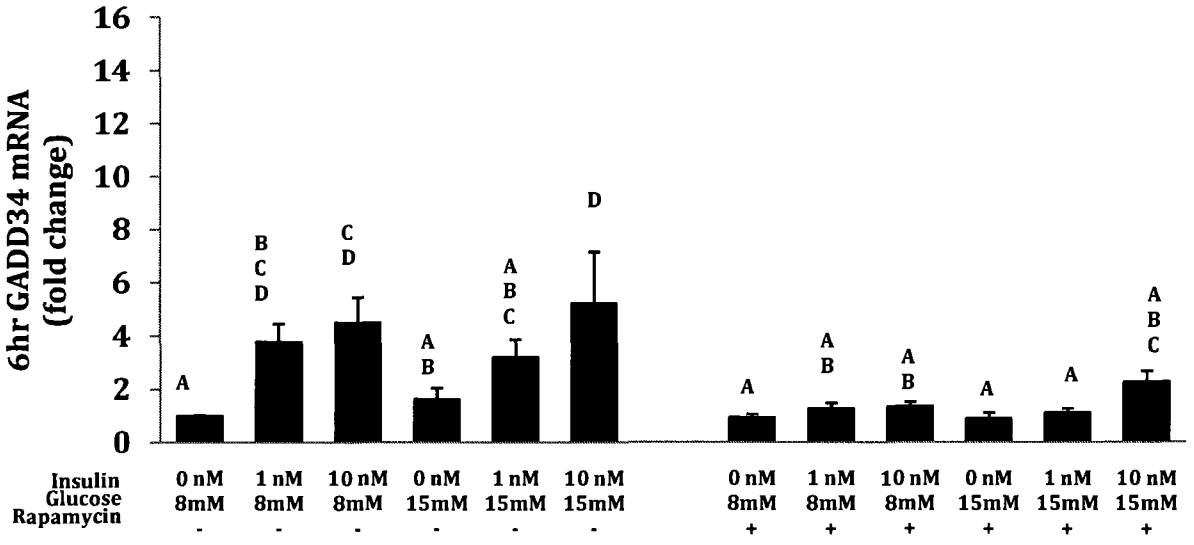

Figure 21d

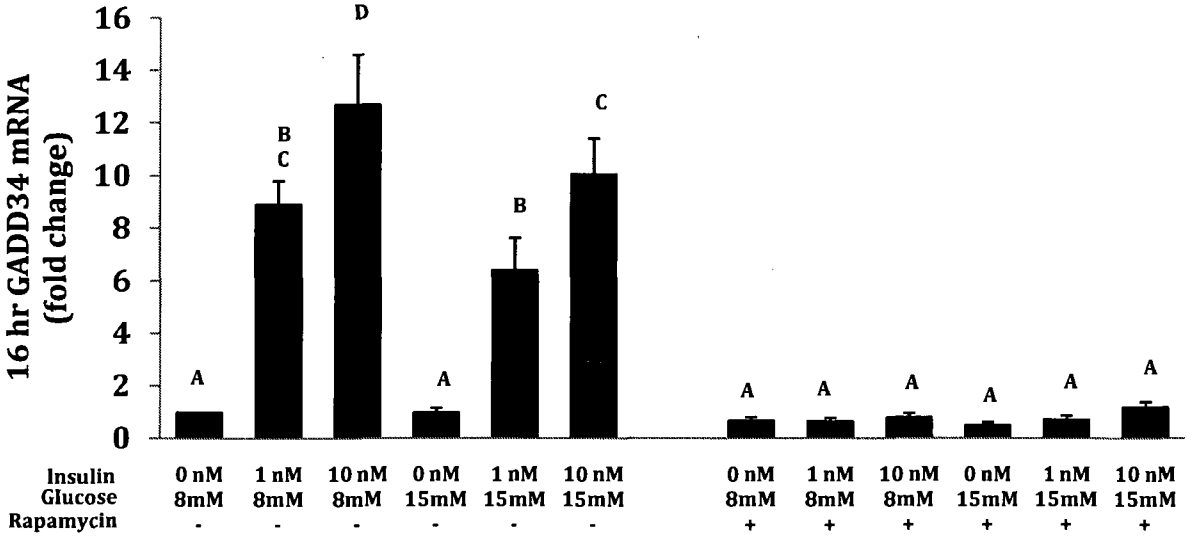

Figure 21e

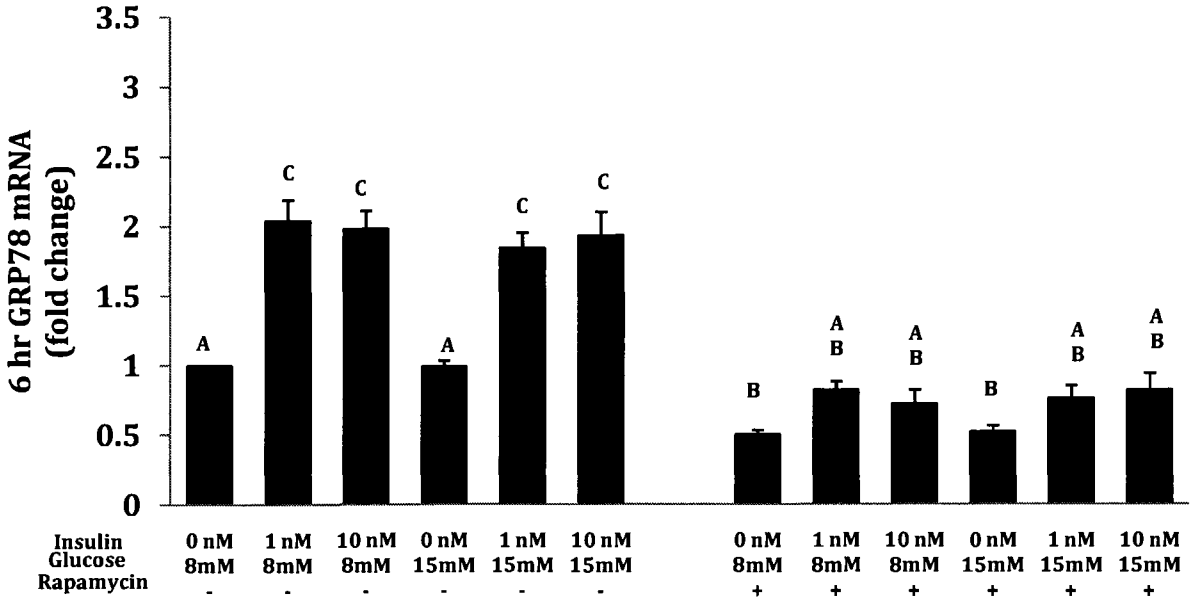

Figure 21f

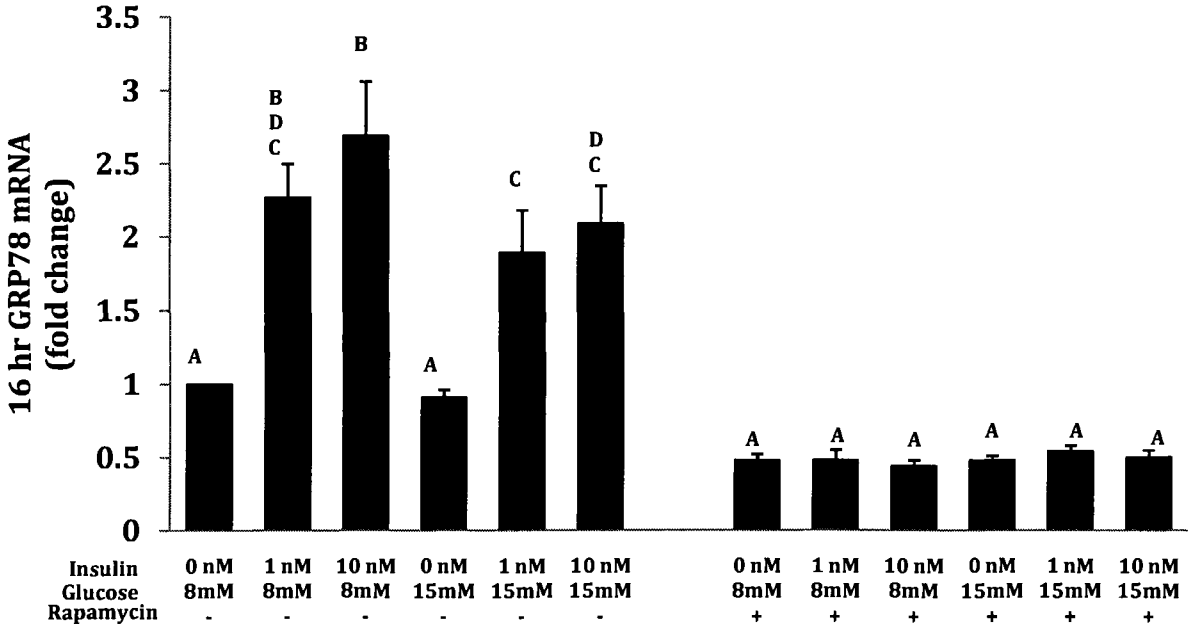

Figure 21g

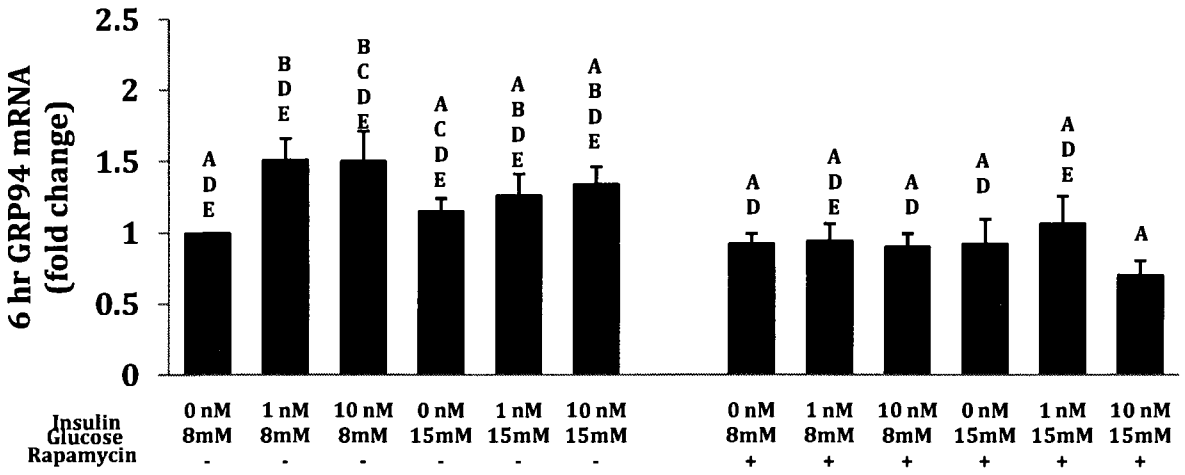

Figure 21h

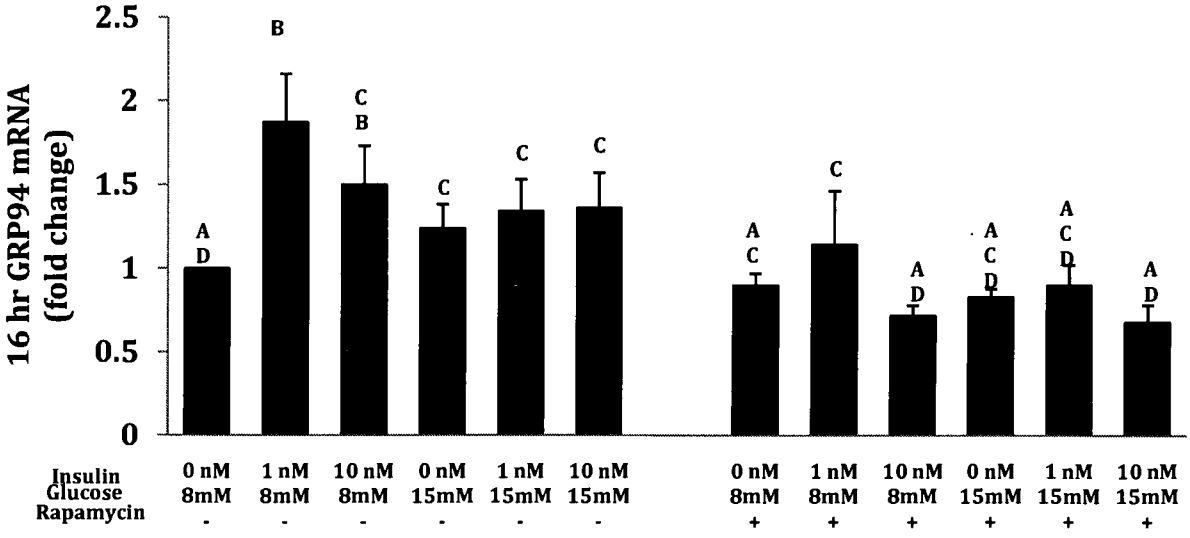

Figure 21. Effect of glucose, insulin, and rapamycin on UPR gene markers. Expression of CHOP (a,b), GADD34 (c,d), GRP78 (e,f), and GRP94 (g,h) mRNA expression in H4IIE cells that were exposed to varying insulin and glucose concentrations in the presence or absence of rapamycin, for wither 6 or 16 hours.Data are reported as mean±SE.
n=5-8 experiments. Bars without a common letter differ, $p < 0.05$.

CHAPTER 5

DISCUSSION

Introduction.

The accumulation of unfolded proteins in the lumen of the ER induces a coordinated adaptive program called the unfolded protein response (UPR). The UPR serves to alleviate the ER stress provoked by accumulation of unfolded proteins by upregulating protein folding and degradation pathways in the ER and inhibition global protein synthesis. Although groundbreaking research over the past few years has led to a comprehensive description of the basic pathways involved in UPR activation, signal transduction and transcriptional activation in mammalian systems, much less is known about the physiologic regulation of the UPR and the potential for the UPR to influence cellular function beyond ER luminal homeostasis. Recent studies have suggested that the UPR participates in a diverse array of cellular functions including differentiation, ER and mitochondrial biogenesis, insulin action, glucose metabolism, and lipogenesis ([1-4]. Other studies have demonstrated that both nutrient deprivation or excess can lead to activation of the UPR in a number of cell types [93, 118]. Indeed, Kaufman and colleagues have hypothesized that the UPR represents a "primordial signaling pathway for communication between the cytoplasm and nucleus that evolved to deal with the most significant stress encountered by single-cell organisms – nutrient limitation" [119]. Given the putative role of the UPR in the regulation of insulin action, glucose homeostasis and lipogenesis and the central role of the liver in postprandial nutrient disposal the present studies were undertaken to examine postprandial regulation of the UPR in the liver.

Results from the present studies demonstrate that meal feeding activates selected components of the UPR in the liver of rats. Activation of the UPR, in particular the IRE1/XBP1 branch, appears to be independent of the carbohydrate composition of the diet. Using H4IIE liver cells, we have demonstrated that insulin, but not glucose, can activate the UPR. In addition, data demonstrate that rapamycin reduced or prevented meal-induced and insulin-induced activation of several gene, and some protein markers of the UPR. In total, results from the present series of studies demonstrate that the postprandial environment activates the UPR in a manner that may be distinct from chemical induction of ER stress and UPR activation.

XBP1 splicing and mTORC1.

One of the early events of the UPR in response to ER stress is the unconventional splicing of a 26 nucleotide intron from XBP1 mRNA by IRE1. IRE1 splicing of XBP1 leads to a sequence frameshift that allows for the recognition and efficient translation of XBP1 mRNA. In the context of ER stress, XBP1 upregulates ER degradation-enhancing α-mannosidase-like protein (EDEM) and therefore the capacity for protein degradation [88]. In some studies, XBP1 also appears to regulate the expression of protein chaperones, such as GRP78 [87]. Overexpression of the spliced version of XBP1 in 3T3 fibroblasts resulted in increased phospholipid synthesis and ER membrane biogenesis (150). In addition, overexpression of the spliced version of XBP1 in primary mouse hepatocytes increased lipogenesis and the binding of XBP1 to promoter regions on lipogenic genes [3]. Importantly, these data demonstrate that not only does XBP1 play a role in metabolic pathways that are activated in the postprandial state but that this regulation can occur independently of ER stress. In the current study, we observed XBP1

splicing in the liver of rats 1 hour following the completion of a 3 hour meal feeding period in which either a high starch or high sucrose diet was provided. Since XBP1 is the only known substrate of IRE1 nuclease activity, these data suggest that meal feeding activates the IRE1/XBP1 branch of the UPR in the liver. To examine putative postprandial signals that mediate XBP1 splicing we exposed H4IIE liver cells to insulin, glucose or their combination. Insulin, but not glucose, induced XBP1 splicing. Therefore, we hypothesize that meal-induced pancreatic insulin secretion and the resultant high portal vein insulin concentration may mediate postprandial regulation of XBP1 splicing in the liver.

Hepatic protein synthesis is increased under postprandial conditions [35]. Several studies have described an association between protein synthesis and XBP1 splicing. Shaffer et al [120] demonstrated that the overexpression of spliced XBP1 in B cells lead to increased 80S ribosomal formation, a 50% increase in protein synthesis, and increased cell size. Reimold et al [91] demonstrated that the embryos of XBP1 knockout mice were characterized by underdeveloped livers, and implicated insufficient protein synthesis as a cause for the impairments in liver development. To further examine the link between protein synthesis and XBP1 splicing, these investigators performed partial hepatectomies on adult mice and monitored liver regeneration. Within 30 minutes post surgery the liver of hepatectomized mice exhibited XBP1 splicing, and this coincided with an increase in acute phase proteins and growth factors [91]. A direct link between a protein synthetic pathway and increased XBP1 splicing was demonstrated by Ozcan et al [92] in mouse embryonic fibroblasts that contained a genetic knockout of TSC1. TSC1 is an inhibitor of mTORC1 activation, and deletion of TSC1 leads to constitutive activation of mTORC1

activity and protein translation. TSC1 knockout cells were characterized by PERK phosphorylation, increased UPR gene markers, and XBP1 splicing. Activation of UPR markers were inhibited when TSC1 knockout cells were exposed to rapamycin for 24 hours. To ensure that UPR activation was a result of ER stress and not due to mTORC1 activity, TSC1 knockout cells were exposed to cyclohexamide, which inhibits the translocation of the 48S initiation complex to the ribosome, for 24 hours. Exposure to cyclohexamide produced the same effect as rapamycin [92]. Thus, it was concluded that under conditions of constitutive activation of protein synthesis, uncontrolled protein translation leads to ER stress through an imbalance between the protein load generated and the ability of the cell to fold and process that load. In the current study, the frequency of XBP1 splicing in meal fed rats was decreased when rapamycin was provided to rats prior to initiation of meal feeding. In addition, rapamycin prevented insulin-mediated XBP1 splicing in H4IIE liver cells. Taken together, these data suggest that the postprandial regulation of XBP1 splicing in the liver is an mTORC1 dependent process. It should be noted that whether the acute regulation of mTOR by the postprandial environment and insulin involves direct actions of mTOR activity or is related to acceleration of protein synthesis in excess of protein folding/degradation cannot be determined at this time.

Given that XBP-1 splicing was rapid and apparently transient (based on reduced XBP1 splicing at 7 vs. 1 hour), occurred in response to a daily, physiologic event (feeding), and occurred in the absence of an increase in eIF2-α phosphorylation or ATF4 mRNA association with polysomes, it seems unlikely that meal feeding-induced XBP1 splicing was due to ER stress caused by the accumulation of unfolded proteins. Indeed,

indirect evidence from the present study suggests that protein synthesis/translation were increased in response to meal feeding. This conclusion is supported by the observation that phosphorylation of RPS6 and the association of several genes to polysomes was increased in fed compared to fasted rats. We propose three potential scenarios to explain the activation of the IRE1/XBP1 branch of the UPR in the absence of activation of the PERK/eIF2α branch. One possibility is that selected components of the UPR can be activated in response to an increased protein load delivered to the ER lumen and a small, non-stressful accumulation of misfolded or unfolded proteins. Current evidence which demonstrates that all components of the UPR, that is PERK, IRE1 and ATF6, are typically activated in response to mild, moderate or severe ER stress do not support this scenario [121]. Alternatively, selected components of the UPR may be activated in response to an increase in the protein load per se, that is independent of any accumulation of unfolded proteins. One might envision that such a response may represent a pre-emptive program but would still require differential sensitivity and responsiveness among the three proximal UPR sensors. A third scenario can be envisaged in which insulin activates mTORC1, which in turn directly activates IRE1 mediated XBP1 splicing. Such communication would allow the cell to upregulate folding and quality control machinery in preparation for an oncoming protein load, and would also allow the IRE1 branch of the UPR to contribute to the regulation of lipogenesis in the postprandial state. We propose mTORC mediated XBP1 splicing occurs not as a response to the accumulation of unfolded proteins, but in preparation for, the postprandial increase in hepatic protein synthesis and the need to couple this synthesis to the biosynthesis of membrane and cellular lipids.

Chaperone gene expression.

Chaperone proteins facilitate the proper folding and processing of nascent proteins and prevent the formation of harmful protein aggregates [58]. Two of the most common and widely studied ER chaperones are GRP78 and GRP94. These glucose regulated proteins are ER-localized members of the heat shock protein family that were first identified based on their responsiveness to glucose deprivation [122]. In the present study, we observed an increase in hepatic GRP78 and GRP94 mRNA expression at the early postprandial time point, which was 1 hour following the 3 hour meal feeding period. In Study 1, GRP78 mRNA was increased 2 to 3 fold and GRP94 mRNA was increased ~3 fold in starch and sucrose-fed rats compared to fasted controls. In Study 2, GRP78 mRNA was increased ~6 fold and GRP94 mRNA was increased 2.5 fold in the VEH1 group compared to fasted controls. Others have reported similar levels of hepatic GRP78 mRNA induction in response to feeding. Dhahbi et al [68] trained mice to feed over a two hour period and examined the induction of hepatic GRP78 and GRP94 0.5, 1.5, 10, and 24 hours following the 2 hour feeding period. They reported a 2 to 3 fold increase in hepatic GRP78 mRNA and a slightly less than 2 fold increase in GRP94 mRNA. They also demonstrated that hepatic GRP78 mRNA expression decreased in response to food deprivation, and upon the reintroduction of food, GRP78 mRNA returned to levels present prior to food deprivation. Taken together, the results from the current study and the previous results from Dhahbi et al demonstrate that the genes encoding the chaperone proteins GRP78 and 94 are responsive to the nutritional status of the organism. In addition, insulin but not glucose increased the expression of GRP78 mRNA in H4IIE liver cells. Insulin also induced upregulation of GRP78 in murine

peritoneal macrophages [123]. Therefore, we hypothesize that postprandial-induced hyperinsulinemia is an important regulator of GRP gene expression. This regulation may involve signals that transit through the ER or may occur independently of the ER, for example via transcription factors that are directly activated or de-repressed by insulin (e.g. Foxo1).

Similar to XBP1 splicing, both the meal-induced increase in hepatic GRP78 mRNA in vivo and the insulin-induced increase observed in H4IIE liver cells were reduced or prevented by rapamcyin. Interestingly, Dhahbi et al [68], in the same feeding study discussed above, hypothesized that increased protein translation and ER protein trafficking were the signals that induced the postprandial increase in hepatic GRP78 mRNA expression. To address this hypothesis, they administered an injection of puromycin to mice both prior to and after the completion of the 2 hour feeding period. Puromycin is an antibiotic that acts to prematurely terminate ribosomal translation. Puromycin caused a 95% decrease in overall protein synthesis, but failed to inhibit the postprandial induction of hepatic GRP78 mRNA expression. Thus, the authors concluded that the feeding-induced increase in hepatic GRP78 mRNA was not a response to increased protein synthesis or an accumulation of proteins in the ER lumen. Results from the current study show that the postprandial induction of hepatic GRP78 mRNA was inhibited by rapamycin. When considered in conjunction with the data from Dhahbi et al, it would appear that the postprandial and insulin regulation of GRP78 occurs upstream of protein translation and, therefore accumulation of unfolded proteins in the ER lumen. In fact, these data are consistent with the notion described above, in which insulin activates mTORC1, which in turn directly activates not only IRE1 mediated XBP1 splicing but

also increased expression of GRP78. Both XBP1 and ATF6 can regulate the expression of GRP78 [80, 87]. It is tempting to speculate that mTORC1 dependent activation of ATF6 and/or XBP1 splicing leads to increased hepatic GRP78 mRNA expression in the postprandial state.

Lipogenic gene expression.

In the postprandial state, not only is hepatic lipogenesis increased but the hepatic lipogenic gene expression program is upregulated, in part due to the regulation of sterol regulatory element binding protein (SREBP) activity [124]. In the current study, FAS mRNA was increased by meal feeding and this induction of FAS mRNA was partially suppressed in rats injected with rapamycin. As noted above, rapamycin also inhibited meal-induced XBP1 splicing. Several reports have recently demonstrated a direct link between the expression of the spliced form of XBP1 and cellular biosynthetic pathways. Sriburi et al [2] demonstrated that overexpression of the spliced form of XBP1 increased membrane phospholipids and stimulated ER expansion in 3T3 fibroblast cells. The differentiation of B cells into immunoglobulin-secreting plasma cells is controlled by two transcription factors, Blimp-1 and XBP1 [120]. Ectopic expression of XBP1 in B cells induced a wide spectrum of secretory pathway genes and physically expanded the ER. In addition, XBP1 increased cell size, lysosome content, mitochondrial mass and function, ribosome number and total protein synthetic capacity [120]. Selective deletion of XBP1 in the liver of adult mice resulted in lower plasma triglycerides, cholesterol, and free fatty acids, as well as reduced hepatic lipid content compared to wild type mice [3]. Further, livers from XBP1 liver deficient mice were characterized by decreased expression of several genes involved in lipid synthesis. Finally, lipid content and lipogenic gene

expression were increased when the spliced form of XBP1 was overexpressed in primary hepatocytes. These data demonstrate that the spliced form of XBP1 plays an important role in an array of cellular functions. The diverse set of cellular functions affected by XBP1 also strongly suggests that generation of the spliced, active form of XBP1 may occur in the context of normal physiologic perturbations, for example by nutritional status. Although, we did not in the current study directly demonstrate that the postprandial-mediated increase in FAS was the result of spliced XBP1, we did demonstrate that they were both reduced in the presence of rapamycin. Therefore both events appear to depend, at least in part, on mTORC1 activation.

Alternative XBP1 target genes.

A recent study examined gene targets of the spliced form of XBP1 by selectively overexpressing XBP1s [116]. The study identified a broad array of genes that were upregulated in the presence of spliced XBP1 but in the absence of general ER stress. We examined four of these genes in order to acquire more correlative evidence for functional, downstream consequences of postprandial-mediated XBP1 splicing. Mgat2 and Dad1 mRNA, genes that encode proteins involved in N-linked glycosylation, and SRP54 and SRPR mRNA, genes that encode proteins involved in the translocation of newly synthesized, nascent proteins from the cytosol to the ER lumen, were generally increased by meal feeding. Although we recognize that the changes in the expression of these mRNA's were small, their statistically significant increase does lend support to the notion that XBP1 splicing in the postprandial state may have functional consequences to a global gene network that includes FAS, as well as Mgat2, Dad1, SRP54 and SRPR.

Postprandial regulation of the PERK pathway.

The ability to phosphorylate eIF2α, and therefore transiently reduce protein translation, is critical to survival. Mice with a homozygous mutation at the eIF2α phosphorylation site (serine 51 to an alanine) died within 18 hours of birth due to hypoglycemia associated with defective gluconeogenesis in the liver [101]. Phosphorylation of eIF2α is governed by four kinases, PERK, PKR, GCN2 and HRE, with each kinase being regulated by distinct cellular signals [95, 97, 98, 100]. PERK-mediated phosphorylation of eIF2α is a fundamental response to ER stress induced by pharmacologic agents, such as tunicamycin and thapsigargin [95]. In the present study, eIf2α phosphorylation was not increased in the liver in response to meal feeding. In addition, meal feeding did not induce an increase in the association of ATF4 mRNA with polysomes, which would be expected if global protein translation were attenuated. Since phosphorylation of eIf2α results in the selective translation of ATF4 mRNA, as well as other mRNA's that contain upstream open reading frames [103, 125], these data suggest that ER stress mediated translational control was not initiated by the postprandial environment. In fact, phosphorylation of eIf2α was decreased in the liver of starch-fed rats that were sacrificed 1 hour after the feeding period. This reduction may allow for greater utilization of available nutrients for protein synthesis in the postprandial state. Thus, the postprandial UPR may maximize the capacity to synthesize and process proteins by decreasing the inhibition on translation while simultaneously increasing folding capacity via the IRE1/XBP1 pathway.

mRNA association with polysomes.

In the present study we examined mRNA association with polysomes in order to obtain information related to the targeting of genes for translation. This analysis was

undertaken, in part, because of the relatively short time frame of the study and therefore the limited duration of time available for specific proteins to increase, as well as the limited availability of antibodies for UPR proteins. We compared the association of mRNAs with polysomes between fasted and fed animals, as well as between vehicle- and rapamycin-treated fed animals. In general, genes that were upregulated in the postprandial state, such as FAS, GRP78, and XBP1 were increased in polysomal fractions when compared to fasted conditions. Such data imply that these genes were therefore also targeted for translation in the postprandial state. In contrast, genes such as CHOP and ATF4 were not significantly different when fed rats were compared to fasted rats. Rapamycin reduced but did not prevent the increased association of FAS, GRP78 and XBP1 with polysomes in the fed state. These data suggest that increased targeting of mRNA's in the fed state was not solely determined by mTOR activation.

Summary.

The results from the present study demonstrate that the postprandial environment activates the IRE1/XBP1 branch of the UPR and upregulates genes that encode for glucose regulated proteins in the liver. Insulin, but not glucose, activates the IRE1/XBP1 branch of the UPR and downstream UPR target genes in H4IIE liver cells. Rapamycin prevents both postprandial-mediated regulation and insulin-mediated regulation of the IRE1/XBP1 branch of the UPR, downstream UPR target genes, and/or GRP78 protein expression. In addition, rapamycin also reduced the postprandial-mediated increase in FAS mRNA in vivo. In contrast, to the postprandial-mediated regulation of the IRE1/XBP1 branch of the UPR, phosphorylation of eIF2α was not increased in the liver of fed rats. These data therefore suggest that the postprandial environment, potentially

mediated via increased insulin secretion, regulates selective branches of the UPR and may do so prior to or in the absence of ER stress. We propose that in the postprandial state, the hepatic UPR functions to facilitate the increase in protein synthesis and lipogenesis.

Caveats, limitations and future directions.

In the current study we utilized a meal feeding paradigm in which rats were trained to consume food over a three hour period each day. The study design allowed for the measure of the UPR in the postprandial state. However, there are several limitations which should be considered with such a model. First, we were unable to control when rats consumed the bulk of their meal during the three hour period. These differences may explain some of the individual variability in glucose, insulin, and other outcome variables. One possible way to control the timing of food consumption would be to tube feed or directly infuse a given amount of nutrients into the gut. Another limitation with the meal feeding paradigm is that there may be confounding consequences due to limiting food availability to three hours per day. In the present study, rats ate ~12-15 grams of food over a 3 hour period. This mass represents ~60-75% of the food consumption of rats fed this same diet but in which ad libitum access was provided over the entire dark/light cycle. Thus, the relatively large amount of food eaten over the 3 hour period may influence the magnitude of response by the liver. We have suggested that the postprandial environment may only activate select components of the UPR (e.g. IRE1/XBP1 pathway). However, in the present study we have only examined the UPR at two postprandial time points. It is possible that activation of other components of the UPR, for example PERK/eIF2α, may have occurred over a time course not captured by our

measurement time points. Future studies should evaluate a broader array of postprandial time points and additional tissues/organs.

The difference in time of sacrifice between Study 1 and Study 2 was another limitation of the current study. The pattern of gene expression differed between the two studies, in particular with respect to the frequency of XBP1 splicing and the expression of genes such as XBP1 and CHOP. However, we do not know whether these differences were due to a difference in sacrifice time or some other factor.

Finally, due to a paucity of reliable and commercially available antibodies we were unable to provide evidence for direct activation of any of the proximal UPR sensors, IRE1, PERK or ATF6. . It would be of interest to determine whether the postprandial environment causes release of GRP78 from these proximal sensors and whether phosphorylation of IRE1 and PERK occur under feeding conditions. It would also be interesting to examine whether the postprandial environment increases the amount of ATF6 in the nucleus. We continue to test antibodies and should valid antibodies become available, future studies will evaluate these possibilities.

To examine the effects of insulin and/or glucose on UPR activation we employed H4IIE liver cells, a rat hepatoma cell line. We have used this cell line in other studies to examine the role of lipids on the UPR and results have been consistent among both H4IIE liver cells, primary hepatocytes and whole livers. However, caution is always warranted when examining the effects of growth factors in hepatoma cells, due to the critical role these factors play in cellular proliferation. Thus, future studies should also examine the role of insulin and glucose on the UPR and the dependency of mTOR in this regulation using primary hepatocytes or glucose clamp studies in vivo.

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