

# Amino Acid–Induced Activation of mTORC1 in Rat Liver Is Attenuated by Short-Term Consumption of a High-Fat Diet<sup>1–3</sup>

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## Abstract

**Background:** The chronic activation of the mechanistic (mammalian) target of rapamycin in complex 1 (mTORC1) in response to excess nutrients contributes to obesity-associated pathologies.

**Objective:** To understand the initial events that ultimately lead to obesity-associated pathologies, the present study assessed mTORC1 responses in the liver after a relatively short exposure to a high-fat diet (HFD).

**Methods:** Male, obesity-prone rats were meal-trained to consume either a control (CON; 10% of energy from fat) diet or an HFD (60% of energy from fat) for 2 wk. Livers were collected and analyzed for mTORC1 signaling [assessed by changes in phosphorylation of 70-kDa ribosomal protein S6 kinase 1 (p70S6K1) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1)] and potential regulatory mechanisms, including changes in the association of Ras-related GTP binding (Rag) A and RagC with mechanistic target of rapamycin (mTOR) and expression of Sestrin1, Sestrin2, and Sestrin3.

**Results:** Feeding-induced activation of mTORC1 was blunted in the livers of rats fed the HFD compared with those fed the CON diet (p70S6K1 phosphorylation, 19% of CON; 4E-BP1 phosphorylation, 61% of CON). The attenuated response was not due to a change in a kinase also referred to as protein kinase B (Akt) signaling but rather to resistance to amino acid–induced activation of mTORC1, as evidenced by a reduction in the interaction of RagA (69% of CON) and RagC (66% of CON) with mTOR and enhanced expression of the mTORC1 repressors Sestrin2 (132% of CON) and Sestrin3 (143% of CON). The consumption of an HFD led to impaired amino acid–induced activation of mTORC1 as assessed in livers perfused in situ with medium containing various concentrations of amino acids.

**Conclusions:** These results in rats support a model in which the initial response of the liver to an HFD is an attenuation of, rather than the expected activation of, mTORC1. The initial response likely represents a counterregulatory mechanism to handle the onset of excess nutrients and is caused by enhanced expression of Sestrin2 and Sestrin3, which, in turn, leads to impaired Rag signaling, resulting in resistance to amino acid–induced activation of mTORC1. *J Nutr* 2015;145:2496–502.

**Keywords:** obesity, liver, high-fat diet, mTORC1, signaling

## Introduction

The consumption of excess nutrients and a sedentary lifestyle are major contributors to the obesity epidemic that affects one-third of

adults in the United States (1). In response to excess nutrients, the energy sensor of the cell, i.e., the mechanistic (mammalian) target of rapamycin in complex 1 (mTORC1)<sup>4</sup>, is chronically activated in multiple tissues, which contributes to obesity-associated pathologies such as type 2 diabetes, cardiovascular disease, and nonalcoholic fatty liver disease. Indeed, previous studies using animal models of diet-induced obesity have shown that mTORC1 in the liver is chronically activated (e.g., 2–4). Activation of mTORC1 has been attributed in part to an elevated concentration of plasma BCAAs. Indeed, in obese humans, plasma BCAA concentrations are elevated, and longitudinal studies have shown this to be predictive for an increased risk of developing insulin resistance and type 2 diabetes (5, 6). Moreover, although the insulin signaling pathway is blunted in the livers of rats fed a high-fat diet (HFD), mTORC1 signaling is enhanced rather than repressed (2), suggesting that in obese animals the kinase is regulated through mechanisms independent of the insulin signaling pathway.

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<sup>3</sup> Supplemental Table 1 and Supplemental Figures 1 and 2 and are available from the “Online Supporting Material” link in the online posting of the article and from the same link in the online table of contents at <http://jn.nutrition.org>.

<sup>4</sup> Abbreviations used: Akt, a kinase also referred to as protein kinase B; CON, control diet; dTORC1, *Drosophila* target of rapamycin complex 1; GAP, GTPase activator protein; HFD, high-fat diet; LAT1, L-type amino acid transporter 1; mTOR, mechanistic (mammalian) target of rapamycin; mTORC1, mechanistic (mammalian) target of rapamycin in complex 1; OP-CD, obesity-prone cesarean-derived; p70S6K1, 70-kDa ribosomal protein S6 kinase 1; Rag, Ras-related GTP binding; Raptor, regulatory associated protein of mechanistic (mammalian) target of rapamycin; Rheb, Ras homolog enriched in brain; *Rps8*, ribosomal protein S8; TBC1D7, Tre2-Bub2-Cdc16 domain family member 7; TSC, tuberous sclerosis complex; 4E-BP1, eukaryotic initiation factor 4E binding protein 1.

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The role of mTORC1 in the development of the deleterious effects of excess nutrient consumption in the liver has been shown in a study in which the selective repression of the kinase was accomplished by hepatocyte-specific ablation of the canonical mTORC1 component regulatory associated protein of mechanistic (mammalian) target of rapamycin (Raptor) (7). In that study, the deletion of Raptor led not only to a dramatic attenuation of high-fat/high-cholesterol diet-induced hepatic lipid accumulation and induction of genes involved in FA and cholesterol biosynthesis but also to almost complete resolution of diet-induced hyperlipidemia and hypercholesterolemia. Moreover, intermittent administration of the mTORC1-specific inhibitor rapamycin to mice fed an HFD was shown to reduce diet-induced hyperinsulinemia and the development of fatty liver (8, 9). Notably, insulin resistance and hepatic steatosis are blunted in mice with a liver-specific deletion of the mTORC1 substrate 70-kDa ribosomal protein S6 kinase 1 (p70S6K1) (10), suggesting that mTORC1-mediated activation of p70S6K1 is an important component in the development of the deleterious effects of an HFD in the liver.

Amino acids, and in particular the BCAAs, activate mTORC1 through a pathway involving a GTPase heteroduplex consisting of either Ras-related GTP binding (Rag) A or RagB in association with either RagC or RagD (11, 12). Binding of mTORC1 to a Rag heterodimer results in its recruitment to the late endosomal/lysosomal membrane where it interacts with and is activated by the Ras homolog enriched in brain (Rheb) GTPase (13–15). Rag function is controlled in part by Sestrins 1, 2, and 3 (16–18). In the absence of amino acids, the Sestrins act to promote the accumulation of RagA and RagB in the inactive, GDP-bound form, thereby repressing mTORC1 signaling. In contrast to amino acids, hormones, such as insulin, activate mTORC1 through a kinase also referred to as protein kinase B (Akt)-mediated phosphorylation of tuberous sclerosis complex (TSC) 2, which functions in a complex with TSC1 and Tre2-Bub2-Cdc16 domain family member 7 (TBC1D7) (19). TSC2 is a GTPase activator protein (GAP) for Rheb, which activates mTORC1 when it is associated with GTP but not GDP (20). Phosphorylation of TSC2 by Akt results in the inhibition of its GAP activity toward Rheb, leading to increased Rheb GTP loading and consequently to activation of mTORC1.

Recent studies (21, 22) have shown that, in contrast to mice given ad libitum access to an HFD, mice fed the same diet for  $\leq 9$  h/d gain significantly less weight and remain insulin sensitive, even though total daily energy consumption is similar in the 2 feeding paradigms. Moreover, time-restricted feeding is associated with reduced hepatic steatosis compared with ad libitum consumption. Similar results were reported in mice restricted to 4 h of daily access to an HFD (23). However, even though hepatic steatosis is improved, the livers of meal-trained (e.g., time-restricted feeding) mice fed the HFD still accumulate more lipid than do livers of meal-trained controls. Moreover, as was observed in studies in which animals consumed ad libitum (2–4), mTORC1 signaling is elevated in the livers of meal-trained mice fed an HFD (21). In previous studies involving both ad libitum and meal-trained consumption paradigms, mice were exposed to the HFD for  $\geq 4$  wk, time periods in which the HFD-induced metabolic and phenotypic changes in the liver have already occurred. Therefore, the overall objective of the present study was to assess the effect of consumption of an HFD for a shorter period of time on activation of mTORC1 in the livers of meal-trained rats. Surprisingly, we found that feeding-induced activation of mTORC1 was dramatically attenuated in the livers of rats after only 2 wk of consuming an HFD. Moreover, the data

demonstrate an impairment at this early time in amino acid-induced activation of mTORC1 rather than a reduction in insulin signaling to the kinase.

## Methods

**Animals.** Male, obesity-prone cesarean-derived (OP-CD) Sprague-Dawley rats ( $\sim 6$  wk of age; Charles River) were randomly divided into 2 groups. One group was fed a control diet containing 10% of energy from fat (CON diet; Research Diets D12450J; Supplemental Table 1) and the other was fed an HFD containing 60% of energy from fat (Research Diets D12492; Supplemental Table 1) for 2 wk. For both groups, the light:dark cycle was reversed (lights off at 0700 and on at 1900).

Rats consumed ad libitum for the first 2 d to allow them to adapt to the new diet and light/dark timing; beginning on the third day, access to food was restricted to 3 h/d (0800–1100). On the day of the study, rats were fed their respective diet (i.e., rats that had been eating the CON diet were given the CON diet, and rats that had been eating the HFD were given the HFD) for 60 min, after which they were deeply anesthetized by isoflurane inhalation (EZ-Anesthesia). Livers were rapidly removed, a portion of which were rapidly frozen between aluminum blocks that were precooled in liquid nitrogen, and the remainder was homogenized for Western blot analysis. Three independent studies were performed following the above protocol. For studies involving liver perfusion, rats were not fed on the morning of the study (and thus were food-deprived for 21 h). Livers were perfused in situ for 30 min as described previously (24). The perfusate contained amino acids at the same concentrations found in the arterial plasma of food-deprived rats ( $1\times$ ) or at 4 times those concentrations ( $4\times$ ) (25). The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the Penn State College of Medicine.

**Blood glucose measurements.** Blood was collected from non-anesthetized rats on the day before killing (i.e., after 13 d being fed the HFD or CON diet). The tail was cleaned with an alcohol wipe, air-dried, and a drop of blood was collected by puncturing a tail vein with a sterile 27-gauge needle. Blood glucose concentrations were measured by using a OneTouch Ultra2 meter (Lifescan).

**Western blot analysis.** Livers were processed as described previously (26). Briefly, a portion of liver was homogenized in 7 volumes of ice-cold buffer containing 20 mM HEPES (pH 7.4), 100 mM KCl, 0.2 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol, 50 mM sodium fluoride, 50 mM  $\beta$ -glycerophosphate, and 10  $\mu$ L/mL protease inhibitor mixture (P8343; Sigma-Aldrich). The homogenate was centrifuged at  $1000 \times g$  for 3 min at 4°C, and a sample of the supernatant was subjected to Western blot analysis as described previously (24). Antibodies against total p70S6K1 (A300–510A), eukaryotic initiation factor 4E binding protein 1 (4E-BP1; custom antibody L090700), and TSC2 (A300–463A) were purchased from Bethyl Laboratories; anti-GAPDH (32233) was purchased from Santa Cruz Biotechnology; and anti-Sestrin1 (NBP196045), anti-Sestrin2 (10795–1-AP), and anti-Sestrin3 (AP12471C) antibodies were purchased from Novus Biologicals, ProteinTech Group, and Abgent, respectively. All other antibodies were purchased from Cell Signaling Technology: phospho-p70S6K1 T389 (9205), phospho-4EBP1 S65 (9451), mechanistic (mammalian) target of rapamycin (mTOR; 2972), phospho-Akt S473 (4060), total Akt (9272), phospho-TSC2 S939 (3615), RagA (4357), RagC (4935), Raptor (4978), and L-type amino acid transporter 1 (LAT1; 5347).

**Quantification of the proportion of individual mRNAs in polysomes.** Liver homogenates were prepared and analyzed by sucrose density gradient analysis as described previously (27). Representative profiles from livers of rats consuming a CON or an HFD are shown in Supplemental Figure 1A, B, respectively. RNA was isolated from fractions corresponding to the nonpolysomal and polysomal portions of the gradient by using Trizol (Invitrogen) as described previously (28). Briefly, the polysome fraction (5 mL) was diluted with an equal volume

of water and then 10 mL Trizol and 2 mL chloroform were added. Trizol (5 mL) and 1 mL chloroform were added to the nonpolysome fraction (5 mL). RNA was then isolated according to the protocol provided by the manufacturer and stored in 25  $\mu$ L Ambion Storage Solution at  $-80^{\circ}\text{C}$  until analyzed. Before use, the RNA concentration of each sample was assessed by using a Nanodrop 1000 spectrophotometer with the use of the Nucleic Acid function of the software provided by the manufacturer. RNA (1  $\mu$ g) was reversed-transcribed by using a kit (High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor; catalog no. 4374966) according to the manufacturer's instructions. Real-time PCR was performed by using a kit (QuantiTect SYBR Green PCR Kit; Life Technologies catalog no. 204143) from Qiagen and 384-well PCR plates that were purchased from Denville Scientific (catalog no. C18384-BC). Before use, cDNA was diluted 1:16 in water and 1  $\mu$ L of the diluted sample was analyzed in a 10- $\mu$ L final reaction volume with the use of a QuantStudio 12K Flex Real-Time PCR System (Life Technologies). The cycling parameters were as follows:  $50^{\circ}\text{C}$  for 2 min and  $95^{\circ}\text{C}$  for 10 min, followed by 45 cycles of  $95^{\circ}\text{C}$  for 15 s,  $55^{\circ}\text{C}$  for 30 s, and  $72^{\circ}\text{C}$  for 30 s. A melting curve was performed at the end of each run. The primers used for  $\beta$ -actin were purchased from Qiagen (Rn\_Actb\_1\_SG QuantiTect Primer Assay, QT00193473); the primers used for ribosomal protein S8 (*Rps8*) were purchased from Integrated DNA Technologies and were as follows—forward: 5'-CAACACTAAGATTGGCCCTCG-3', and reverse: 5'-GCAGTTCTTCACCAGGTCT-3'. As shown in Supplemental Figure 2A, B, the amplification of both  $\beta$ -actin and *Rps8* was linear over a minimum of a 1000-fold range of concentrations with an efficiency of  $\sim 100\%$ . The cycle threshold value for each sample was compared to the standard curve generated for the respective mRNA. Moreover, melting curve analysis showed that the primers for both mRNAs yielded a single product, with no evidence of primer dimer formation revealed by using a no-template control (Supplemental Figure 2C, D).

**Immunoprecipitation of mTOR.** Frozen liver samples were powdered under liquid nitrogen and the powder was used for mTOR immunoprecipitation as described previously (26). Results for RagA and RagC present in the mTOR immunoprecipitate were expressed relative to the amount of mTOR in the immunoprecipitate.

**Statistical analysis.** Statistical analysis was performed by using Prism 6.0f for Macintosh (GraphPad Software). Comparison of data from 2 conditions was performed by unpaired *t* test. All other data were analyzed by 2-factor ANOVA with Holm-Sidak correction for multiple comparisons. In the analysis of blood glucose concentrations, the 2 factors were diet (CON compared with HFD) and feeding condition (food-deprived compared with refed). In the analysis of data from liver perfusion studies, the 2 factors were diet (CON compared with HFD) and perfusate amino acid concentration. For body weight analysis, repeated-measures ANOVA was performed. Results were qualitatively indistinguishable between the 3 indepen-

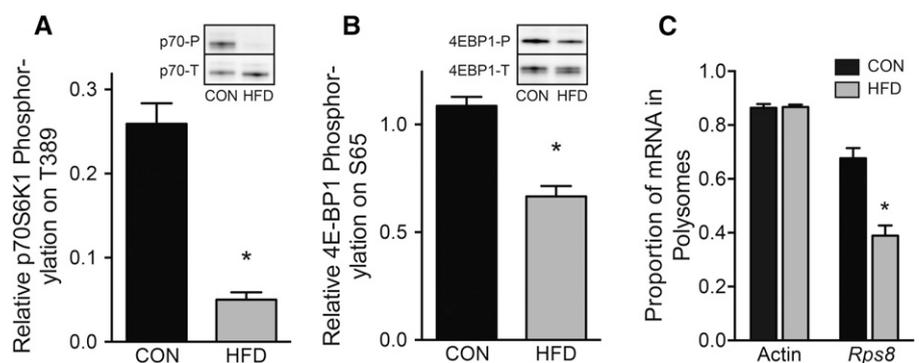
dent studies (i.e., mTORC1 signaling was reduced in HFD compared with CON rats in each study), so data from all 3 studies were pooled for analysis. In all cases,  $P < 0.05$  was considered significant.

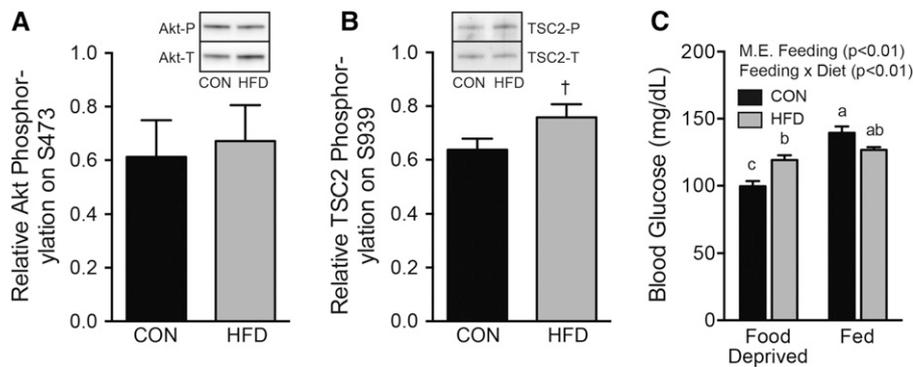
## Results

Initial body weight was the same in both groups of rats ( $159 \pm 14$  g for each group;  $n = 17$ ). However, over the 2-wk feeding period, rats fed the HFD gained 20 g more body weight than did those fed the CON diet, such that the final body weights were  $271 \pm 13$  g and  $250 \pm 13$  g, respectively ( $P < 0.01$ ). Average food consumption on the first 2 d after the start of the feeding protocol ( $13.0 \pm 0.02$  g/d) was significantly less ( $P < 0.05$ ) than the amount consumed during the last 7 d ( $16.06 \pm 0.2$  g/d). However, the amount consumed on days 3 and 4 ( $15.2 \pm 0.4$ ) was not significantly different ( $P = 0.1$ ) compared with the amount consumed during the last 7 d of the experiment. Because the caloric density of the 2 diets is different, the mean daily calorie intake of the rats fed the HFD ( $83.3 \pm 2.1$  kcal/d) was higher than for rats fed the CON diet ( $57.5 \pm 1.0$  kcal/d) ( $P < 0.01$ ). However, on the day of the study, there was no significant difference in energy intake between rats fed the HFD ( $37 \pm 4$  kcal) compared with those fed the CON diet ( $30 \pm 3$  kcal) ( $P = 0.16$ ). Rats were fed during the dark period because previous studies (e.g., 29) showed that mice fed only during the light period gained more weight than mice fed during the dark period, even though both groups of mice consumed the same number of calories.

The activation of mTORC1 was assessed 60 min after refeeding by examining the phosphorylation state of 2 direct targets of the kinase, p70S6K1 on Thr389 (Figure 1A) and 4E-BP1 on Ser65 (Figure 1B). Feeding-induced phosphorylation of both proteins was significantly blunted in rats fed an HFD compared with those fed a CON diet. To assess the functional consequences of attenuated activation of mTORC1, translation of an mRNA known to be regulated by the signaling pathway (i.e., *Rps8*) (30) was examined. As shown in Figure 1C, the proportion of *Rps8* mRNA in polysomes was  $\sim 50\%$  lower in the livers of rats fed the HFD compared with those fed the CON diet, whereas the reference mRNA,  $\beta$ -actin, was present almost entirely in the polysome fraction regardless of diet. This finding shows that the efficiency of *Rps8* mRNA translation was impaired in response to the consumption of an HFD, confirming a functional consequence to the attenuated activation of mTORC1.

**FIGURE 1** Feeding-induced activation of mTORC1 and translation of the *Rps8* mRNA are blunted in the livers of rats fed an HFD compared with a CON diet. Representative blots showing phosphorylation of p70S6K1 on Thr389 (A) and 4E-BP1 on Ser65 (B). (C) The proportion of  $\beta$ -actin and *Rps8* mRNAs in polysomes. Values are means  $\pm$  SEMs;  $n = 10$  (polysome analysis) or  $n = 15$ –16 (p70S6K1 and 4E-BP1 phosphorylation). \*Different from CON,  $P < 0.01$ . CON, control diet; HFD, high-fat diet; mTORC1, mechanistic (mammalian) target of rapamycin in complex 1; -P, phosphorylated; p70S6K1, 70-kDa ribosomal protein S6 kinase 1; *Rps8*, ribosomal protein S8; S65, Ser65; -T, total; T389, Thr389; 4E-BP1, eukaryotic initiation factor 4E binding protein 1.



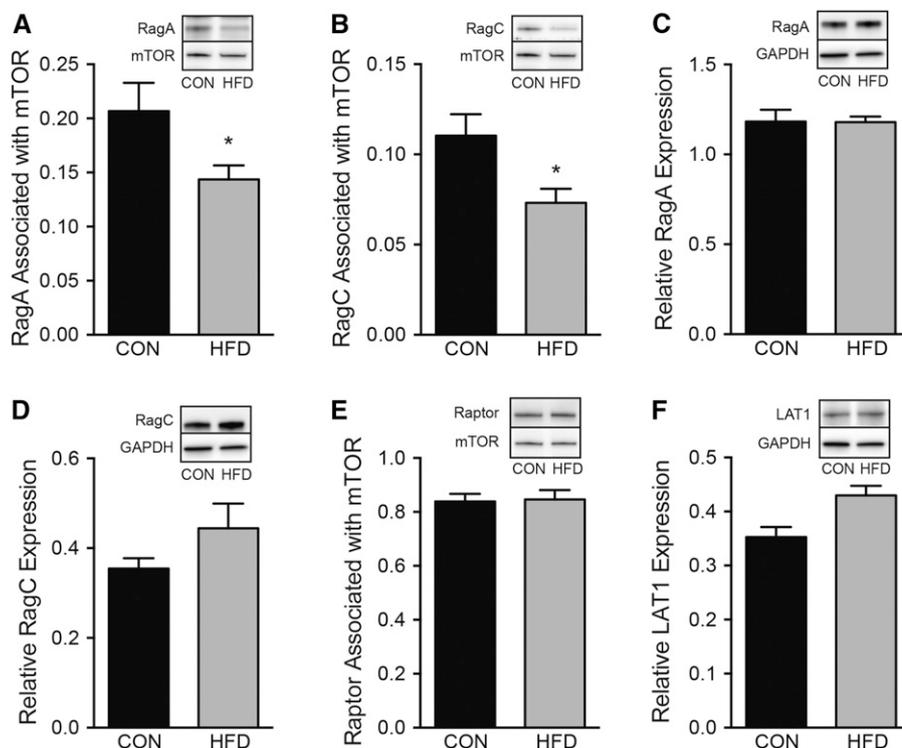


**FIGURE 2** Insulin signaling through the Akt/TSC2 pathway is maintained in the livers of rats fed an HFD compared with a CON diet. Representative blots showing phosphorylation of Akt on Ser473 (A) and TSC2 on Ser939 (B), assessed by Western blot analysis. (C) Blood glucose concentrations were assessed before or after 60 min of refeeding. Values are means  $\pm$  SEMs;  $n = 9-10$  (A, B) or 17-18 (C). Labeled means without a common letter differ,  $P < 0.05$ . <sup>†</sup>Different from CON,  $P = 0.07$ . Akt, a kinase also referred to as protein kinase B; CON, control; HFD, high-fat diet; M.E., main effect; -P, phosphorylated; S473, Ser473; S939, Ser939; -T, total; TSC2, tuberous sclerosis complex 2.

To assess whether insulin signaling to mTORC1 was impaired in the HFD compared with CON rats phosphorylation of Akt and TSC2 was examined. In contrast to earlier studies showing impaired activation of Akt (2), there was no difference in feeding-induced phosphorylation of the kinase in rats fed the HFD compared with those fed the CON diet (Figure 2A). Moreover, there was no significant difference in the feeding-induced phosphorylation of a direct target of Akt, TSC2, on Ser939 (Figure 2B) between rats fed the HFD compared with those fed the CON diet. Indeed, although not significant, there was a trend ( $P = 0.07$ ) for phosphorylation of TSC2 on Ser939 to increase rather than decrease in rats fed the HFD compared with those fed the CON diet. Consistent with the finding that insulin signaling to mTORC1 was not different between the 2 groups of rats, no difference in plasma glucose concentrations between rats fed the HFD compared with those fed the CON diet was observed 60 min after refeeding (Figure 2C).

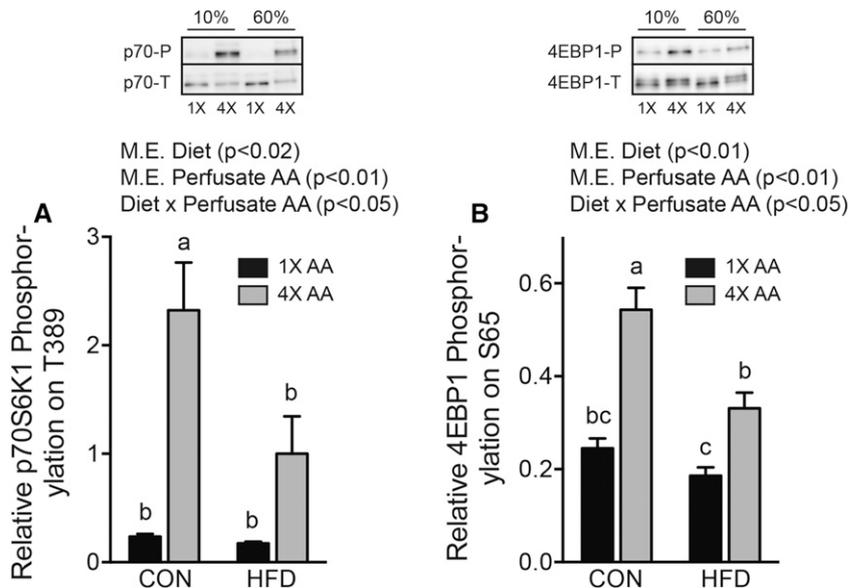
A critical step in the feeding-induced activation of mTORC1 involves the action of amino acids to enhance the interaction of the kinase complex with the Rag GTPases (12). The interaction

of mTORC1 with RagA and RagC was assessed by immunoprecipitation of mTOR followed by Western blot analysis of RagA and RagC in the immunoprecipitate. As shown in Figure 3A, B, the relative amount of RagA and RagC was lower in mTOR immunoprecipitates from the livers of HFD-fed compared with CON rats. The difference was not due to reduced expression of the Rag proteins, because there was no significant difference in the abundance of either RagA or RagC between the livers of rats fed the HFD compared with those fed the CON diet (Figure 3C, D, respectively). The Rag GTPases bind to mTORC1 by interacting with Raptor (12). Thus, a reduction in Rag binding to mTORC1 could be a consequence of reduced Raptor interaction with the kinase complex. However, there was no difference in the amount of Raptor present in mTOR immunoprecipitates from livers of rats fed an HFD compared with those fed a CON diet (Figure 3E), and consequently, the HFD-induced reduction in RagA and RagC binding to mTOR was not due to an attenuated Raptor-mTOR interaction. Similarly, there was no difference in the expression of the high-affinity LAT1 leucine transporter in the livers of HFD-fed



**FIGURE 3** Consumption of an HFD results in impaired feeding-induced interaction of hepatic mTOR with RagA and RagC in rats. The interaction of RagA (A) and RagC (B) with mTORC1 was assessed in mTOR immunoprecipitates. Results are expressed relative to mTOR. Relative expressions of RagA (C) and RagC (D) in liver homogenates are shown. Results are expressed relative to GAPDH expression. (E) The interaction of Raptor with mTOR was assessed in the same mTOR immunoprecipitates depicted in panels A and B. (F) LAT1 expression expressed relative to GAPDH. Representative blots are shown. Values are means  $\pm$  SEMs;  $n = 10$ . <sup>\*</sup>Different from CON,  $P < 0.05$ . CON, control; HFD, high-fat diet; LAT1, L-type amino acid transporter 1; mTOR, mechanistic (mammalian) target of rapamycin; mTORC1, mechanistic (mammalian) target of rapamycin in complex 1; Rag, Ras-related GTP binding; Raptor, regulatory associated protein of mechanistic (mammalian) target of rapamycin.

**FIGURE 4** Amino acid–induced activation of mTORC1 is blunted in the livers of HFD-fed compared with CON rats. Rats were fed a CON diet or an HFD for 2 wk, and livers were perfused in situ with medium containing amino acids at the concentrations observed in food-deprived rats (1×) or 4 times those concentrations (4×). Phosphorylation of p70S6K1 on Thr389 (A) and 4E-BP1 on Ser65 (B) was assessed by Western blot analysis. Representative blots are shown. Values are means ± SEMs;  $n = 5$ –6. Labeled means without a common letter differ,  $P < 0.05$ . AA, amino acids; CON, control; HFD, high-fat diet; M.E., main effect; mTORC1, mechanistic (mammalian) target of rapamycin in complex 1; -P, phosphorylated; p70S6K1, 70-kDa ribosomal protein S6 kinase 1; S65, Ser65; -T, total; T389, Thr389; 4E-BP1, eukaryotic initiation factor 4E binding protein 1.



compared with CON rats (Figure 3F), suggesting that the import of leucine into the liver through this transporter is unlikely to account for the reduced mTORC1 signaling in HFD rats.

Phosphorylation of both p70S6K1 (Figure 4A) and 4E-BP1 (Figure 4B) in the livers of CON rats was increased in response to perfusion with amino acids at 4 times the concentration found in plasma of food-deprived rats compared to control. However, in agreement with the finding that the binding of RagA and RagC to mTOR was reduced in the livers of rats fed an HFD compared with a CON diet, amino acid–induced phosphorylation of both proteins was significantly blunted in livers of rats fed the HFD compared with those fed the CON diet (Figure 4).

Amino acid–induced activation of mTORC1, is, in part, regulated by the Sestrins (16, 17). In the present study, the expression of Sestrin1 was the same in the livers of rats fed either a CON diet or an HFD (Figure 5A). In contrast, the expressions of both Sestrin2 (Figure 5B) and Sestrin3 (Figure 5C) were significantly increased in the livers of rats fed the HFD compared with those fed the CON diet.

## Discussion

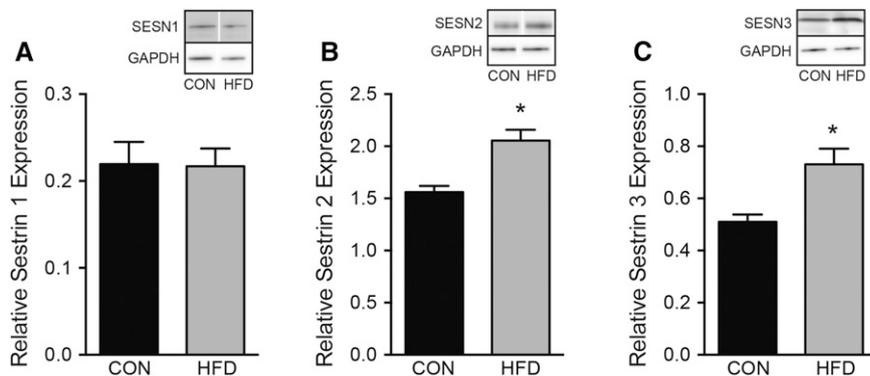
Instead of the expected reduction in the Akt signaling pathway to mTORC1, the results of the present study support a model in which short-term consumption of an HFD induces impairment in the amino acid signaling pathway. Previous studies have shown that in rats fed an HFD for 4 wk, insulin-induced phosphorylation of Akt on both Thr308 and Ser473 is severely

blunted relative to rats fed a CON diet (2). An HFD-induced attenuation of Akt phosphorylation on Ser473 was also shown in mice fed an HFD for 10 wk, in conjunction with development of insulin resistance as assessed by impaired glucose tolerance test values and elevated fasting serum insulin concentrations (31). Surprisingly, in contrast to attenuated activation of Akt, mTORC1 signaling is dramatically elevated in HFD-fed rats in the fasted condition, as assessed by phosphorylation of both p70S6K1 on Thr389 and mTOR on Ser2448 (2). These findings suggest that mTORC1 signaling under the conditions used in that study was elevated despite the development of insulin resistance.

A novel finding in the present study is that an early response of the liver to an HFD is the development of resistance of mTORC1 to activation by amino acids, an effect that may initially serve to counteract the deleterious effect of excess nutrient consumption on hepatic lipid metabolism. The continued consumption of an HFD eventually results in failure of this compensatory mechanism, leading to constitutive mTORC1 activation and dysregulation of lipogenesis. Therefore, knowledge gained about this counterregulatory mechanism would be highly useful in developing approaches aimed at combating the development of fatty liver disease.

The impairment in the amino acid signaling pathway observed in the present study is likely due to HFD-induced expression of Sestrin2 and Sestrin3. Sestrins are universally expressed in metazoans, and a number of studies have shown

**FIGURE 5** Sestrin2 and Sestrin3, but not Sestrin1, expression is higher in the livers of HFD-fed compared with CON rats. Sestrin1 (A), Sestrin2 (B), and Sestrin3 (C) are expressed relative to GAPDH. Representative blots are shown. The samples shown in each panel were analyzed on the same blot, but in panels A and B they were not contiguous lanes; a white line separates noncontiguous lanes. Values are means ± SEMs;  $n = 11$ –12. \*Different from CON,  $P < 0.01$ . CON, control diet; HFD, high-fat diet; SESN, Sestrin.



that they play a role in the regulation of fat metabolism. For example, the deletion of the single Sestrin gene in *Drosophila* results in fat accumulation that occurs in a *Drosophila* target of rapamycin complex 1 (dTORC1)–dependent manner (32). Similarly, the deletion of Sestrin2 in mice exacerbates both HFD-induced hepatosteatosis (33, 34) and activation of mTORC1 (33). Moreover, the combined deletion of Sestrin2 and Sestrin3 leads to the development of insulin resistance and constitutive activation of mTORC1 in the liver, even in animals fed a low-fat diet (33).

Recent studies (16, 18) suggest that the Sestrins modulate mTORC1 signaling by altering the GTP-loading status of RagA and RagB. Specifically, the Sestrins are proposed to enhance the GTPase activity of RagA and RagB, leading to reduced interaction of mTORC1 with the Rag heterodimer and consequently to the repression of mTORC1 signaling. The finding in the present study that short-term consumption of an HFD is associated with both increased Sestrin2 and Sestrin3 expression and reduced interaction of RagA and RagC with mTOR is consistent with such a model. A caveat to this idea is the finding in a previous study (33) that mTORC1 signaling is elevated in the livers of mice fed an HFD for 4 mo concomitant with increased Sestrin 2 expression. However, in mice lacking Sestrin2, the HFD-induced activation of mTORC1 is even greater than in wild-type mice, which is consistent with the idea that the induction of Sestrin2 expression is a compensatory mechanism that initially is sufficient to restrain mTORC1 activation, although after prolonged periods of nutrient excess its effect is overcome through an as yet undefined mechanism. In this regard, on the basis of previous studies showing no difference in Sestrin3 expression in the livers of mice fed an HFD for 3 mo compared with CON mice (33), it is tempting to speculate that the combined upregulation of Sestrin2 and Sestrin3 expression is necessary to maintain suppression of mTORC1 activity under conditions of nutrient excess. In support of this idea, liver-specific ectopic expression of Sestrin3 is associated with elevated mTORC1 signaling in food-deprived mice (35). However, that study also showed that ectopic hepatic expression of Sestrin3 protected mice from HFD-induced insulin resistance, an effect opposite to what would be expected on the basis of the studies in mice lacking Sestrin2. Possible explanations for the discrepancy between the study by Tao et al. (35) and others include different lengths of time that animals were on fed an HFD and different genetic backgrounds.

A caveat to the conclusion that a shorter exposure to excess nutrients causes an attenuated activation of mTORC1 compared with a longer one is that the strain of rat used in the present study (OP-CD) is different than in previous studies, and the effect of longer-term consumption of an HFD in OP-CD rats is unknown. A second caveat is that, in contrast to most previous studies in which animals were freely fed, the rats in the present study were meal-trained and were only allowed access to food for 3 h each day. We chose this experimental paradigm because previous studies have shown that in mice fed a regular feed pellet diet, most of the daily food intake occurs during the dark period (36). In contrast, mice fed an HFD exhibit a rapid (within 1 wk) shift in the pattern of food intake and consume a significantly greater proportion of food during the light period, with no overall difference in amount of food consumed. Notably, the change in the timing of food consumption occurs without a corresponding alteration in the percentage of total activity occurring in the light period. The increase in food consumption taking place during the light period may contribute to the development of obesity and insulin resistance because mice fed

an HFD only during the light period gain significantly more weight than mice fed only during the dark period (29). Moreover, an extended feeding window in combination with a reduced feed-deprived period has been reported to contribute to the development of insulin resistance and hepatic steatosis in HFD-fed mice, in association with dysregulated mTORC1 signaling (22). However, the finding that mTORC1 signaling is elevated in the livers of meal-trained fed mice compared with mice that consumed ad libitum after being fed an HFD for 12 wk (22) suggests that the inhibition of mTORC1 signaling observed in the present study is not maintained in animals subjected to long-term meal training regimens.

In summary, the results of the present study are consistent with a model in which increased Sestrin2 and Sestrin3 expression in the livers of rats fed an HFD acts to repress amino acid-induced activation of mTORC1 by attenuating the interaction of RagA and RagC with the complex. Elevated Sestrin2 expression is maintained during the continued consumption of excess nutrients, but in the absence of increased Sestrin3 expression is insufficient to completely restrain mTORC1, leading to constitutive activation of the kinase complex. The goal of future studies will be to delineate the mechanism or mechanisms through which Sestrin2 and Sestrin3 expression is regulated by consumption of excess nutrients.

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