Suppression of the mTORC1/STAT3/Notch1 pathway by activated AMPK prevents hepatic insulin resistance induced by excess amino acids

HONGLIANG LI, JIYEON LEE, CHAOYONG HE, MING-HUI ZOU, and ZHONGLIN XIE

Section of Molecular Medicine, Department of Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma

Submitted 12 April 2013; accepted in final form 25 November 2013

Li H, Lee J, He C, Zou M, Xie Z. Suppression of the mTORC1/STAT3/Notch1 pathway by activated AMPK prevents hepatic insulin resistance induced by excess amino acids. Am J Physiol Endocrinol Metab 306: E197–E209, 2014. First published December 3, 2013; doi:10.1152/ajpendo.00202.2013.—Nutrient overload is associated with the development of obesity, insulin resistance, and type 2 diabetes. However, the underlying mechanisms for developing insulin resistance in the presence of excess nutrients are incompletely understood. We investigated whether activation of AMP-activated protein kinase (AMPK) prevents the hepatic insulin resistance that is induced by the consumption of a high-protein diet (HPD) and the presence of excess amino acids. Exposure of HepG2 cells to excess amino acids reduced AMPK phosphorylation, upregulated Notch1 expression, and impaired the insulin-stimulated phosphorylation of Akt Ser473 and insulin receptor substrate-1 (IRS-1) Tyr612. Inhibition of Notch1 prevented amino acid-induced insulin resistance, which was accompanied by reduced expression of Rbp-Jk, hairy and enhancer of split-1, and forkhead box O1. Mechanistically, mTORC1 signaling was activated by excess amino acids, which then positively regulated Notch1 expression through the activation of the signal transducer and activator of transcription 3 (STAT3). Activation of AMPK by metformin inhibited mTORC1–STAT3 signaling, thereby preventing excess amino acid–impaired insulin signaling. Finally, HPD feeding suppressed AMPK activity, activated mTORC1/STAT3/Notch1 signaling, and induced insulin resistance. Chronic administration of either metformin or rapamycin inhibited the HPD-activated mTORC1/STAT3/Notch1 signaling pathway and prevented hepatic insulin resistance. We conclude that the upregulation of Notch1 expression by hyperactive mTORC1 signaling is an essential event in the development of hepatic insulin resistance in the presence of excess amino acids. Activation of AMPK prevents amino acid–induced insulin resistance through the suppression of the mTORC1/STAT3/Notch1 signaling pathway.

signal transducer and activator of transcription 3; amino acids; insulin resistance; mammalian target of rapamycin complex 1; AMP-activated protein kinase; Notch

CHRONIC NUTRIENT OVERLOAD is associated with high incidence of chronic metabolic diseases, including obesity, insulin resistance, and type 2 diabetes. In Western cultures, dietary fat has long been considered a driver of insulin resistance (28–30). Prolonged exposure to high concentrations of saturated fatty acids leads to oxidative stress and endoplasmic reticulum stress, which may impair insulin signaling and contribute to the development of type 2 diabetes (8). During the past 50 years, meat consumption has increased by one-third in industrialized countries (13). An increase in the intake of protein is associated with the development of type 2 diabetes (35, 45). Thus, nutrient overload leading to insulin resistance and type 2 diabetes involves not only a higher intake of fat but also a higher intake of protein. High plasma levels of amino acids have been found in obese and insulin-resistant individuals (1, 9, 10). Moreover, supplementation of a high-fat diet (HFD) with branched-chain amino acids caused insulin resistance in animals (40). The identification of the mechanistic link between the presence of an excess of amino acids and insulin resistance might help to define novel nutritional and pharmacological approaches for the treatment of diabetes, obesity, and insulin resistance.

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase and functions in an intracellular signaling pathway that senses the availability of amino acids. mTOR can exist as two distinct protein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (15, 22, 24, 44). mTORC1 regulates protein synthesis, cell growth, and insulin sensitivity in a rapamycin-sensitive manner (11, 17). Activation of mTORC1 and its downstream molecule S6 kinase 1 (S6K1) phosphorylates insulin receptor substrate-1 (IRS-1), leading to the degradation of IRS-1 and impaired phosphoinositide 3-kinase (PI3K) signaling (16). In contrast, mTORC2 phosphorylates and activates Akt, which is a key kinase involved in cell growth and apoptosis. The activation of Akt by mTORC2 is not inhibited by rapamycin (22, 44). Recently, the signal transducer and activator of transcription 3 (STAT3) was identified as the downstream signal of mTOR (3, 49) that mediates amino acid–inhibited insulin signaling (26). STAT3 also has a close interaction with Notch receptors in various physiological and pathological conditions, including proliferation, differentiation, and apoptosis (12). Upon ligand-dependent activation, Notch is cleaved and releases the Notch intracellular domain, which participates in a transcriptional complex in the nucleus to regulate Notch-dependent gene expression (38). Inhibition of Notch has been reported to ameliorate insulin resistance in a diet-induced insulin-resistant animal model (41). However, whether Notch signaling is involved in amino acid–induced hepatic insulin resistance remains to be determined.

AMP-activated protein kinase (AMPK) functions as an intracellular energy sensor that maintains energy homeostasis (5) in addition to regulating protein synthesis (46), apoptosis (4), and autophagy (18, 52, 53, 58). Activation of AMPK leads to the phosphorylation of a number of target molecules, resulting in the downregulation of anabolic pathways (to conserve ATP) and the upregulation of catabolic pathways (to generate more ATP) (5). AMPK regulates the insulin-signaling pathway at the level of the tuberous sclerosis complex 2 (TSC2). Activation of AMPK reverses mTOR-mediated inhibition on PI3K/Akt sig-
nating and suppresses IRS-1 phosphorylation (48). Thus, activation of AMPK has a beneficial effect on glucose homeostasis and peripheral insulin sensitivity (39, 57). We investigated whether AMPK prevents hepatic insulin resistance induced by elevated amino acid concentrations and examined the mechanisms by which AMPK suppresses amino acid-induced hepatic insulin resistance. We found that activation of AMPK improved insulin sensitivity by suppressing the mTOR/STAT3/Notch1 signaling pathway.

MATERIALS AND METHODS

Materials. Human hepatocarcinoma HepG2 cells were obtained from Cascade Biologies (Portland, OR). Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Mediatech (Herndon, VA). Fetal bovine serum (FBS) was from Invitrogen (Carlsbad, CA). Rapamycin and AG490 were obtained from Calbiochem (San Diego, CA). Human recombinant insulin was purchased from Sigma-Aldrich (St. Louis, MO). Antibodies against phosphorylated IRS-1 (Ser307) and hairy and enhancer of split-1 (Hes1) were purchased from Santa Cruz Biotechnology (Dallas, TX). The antibody against Rbp-Jκ was obtained from Abcam (Cambridge, MA). The following antibodies were from Cell Signaling Technology (Beverly, MA): phosphorylated Akt (Ser473), Akt, phosphorylated mTOR (Ser2448), mTOR, Notch1, phosphorylated AMPK (Thr172), AMPKα, phosphorylated acetyl-CoA carboxylase (ACC; Ser79), STAT3, phosphorylated STAT3 (Ser277), phosphorylated STAT3 ( Tyr235/246), phosphorylated 4E-binding protein 1 (4EBP1; Thr37/46), phosphorylated IRS-1 (Tyrosine), phosphorylated S6K1 (Thr389), phosphorylated glycogen synthase kinase-3β (GSK-3β; Ser9), GSK-3β, and forkhead box protein 1 (FoxO1). All secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA).

Experimental animals and treatments. The animal protocol was reviewed and approved by the University of Oklahoma Institutional Animal Care and Use Committee. At 12 wk of age, male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were fed a normal diet (ND; protein 23.5%; Picolab Rodent Diet 205053; LabDiet, Brentwood, MO) or a high-protein diet (HPD; protein 42.6%, fat 13.2%; TD90018; Harlan Laboratories, Houston, TX). The antibody against Rbp-Jκ was obtained from Abcam (Cambridge, MA). The following antibodies were from Cell Signaling Technology (Beverly, MA): phosphorylated Akt (Ser473), Akt, phosphorylated mTOR (Ser2448), mTOR, Notch1, phosphorylated AMPK (Thr172), AMPKα, phosphorylated acetyl-CoA carboxylase (ACC; Ser79), STAT3, phosphorylated STAT3 (Ser277), phosphorylated STAT3 ( Tyr235/246), phosphorylated 4E-binding protein 1 (4EBP1; Thr37/46), phosphorylated IRS-1 (Tyrosine), phosphorylated S6K1 (Thr389), phosphorylated glycogen synthase kinase-3β (GSK-3β; Ser9), GSK-3β, and forkhead box protein 1 (FoxO1). All secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA).

Cell culture and treatment. HepG2 cells were maintained in DMEM with 1 g/l glucose containing 10% FBS at 37°C with 5% CO2. All DMEM lacking amino acids and containing different combinations of amino acids were made according to the formulations provided by Sigma (St. Louis, MO). Mouse primary hepatocytes were isolated from 10 wk-old mice and cultured as described previously (27).

Adenoviral infection. HepG2 cells were infected with the adenovirus encoding constitutively active AMPK adenoval vector (CA-AMPK) or dominant-negative AMPK adenoval vector (DN-AMPK) at a multiplicity of infection of 50 in serum-free medium overnight, using adenovirus encoding green fluorescent protein (GFP) as a control. The cells were then washed and incubated in fresh serum-free medium for an additional 18–24 h before they were used in any experiment. Under these conditions, infection efficiencies were typically >75%, as determined by measuring GFP expression (50, 51).

siRNA gene silencing of mTOR, STAT3, and Notch1. Scrambled siRNA, and mTOR-, STAT3-, and Notch1-specific siRNAs were obtained from Applied Biosystems (Foster City, CA). Transfection was performed according to the manufacturer’s instructions. The efficiencies of siRNA-silenced genes were evaluated by Western blotting of the targeted proteins with specific antibodies (19).

Western blotting. Proteins were extracted from cultured cells and mouse livers as described previously (19, 33, 54). The lysates were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and incubated with the appropriate specific antibody. Bands were visualized by using enhanced chemiluminescence, and the membranes were stripped and probed with total protein and/or β-actin to verify equal loading of proteins. The optical density of each band was quantified by AlphaEase (Alpha Innotech) and expressed as an arbitrary unit (18, 19, 53, 55).

Glucagon synthase assay. HepG2 cells seeded in six-well plates were serum starved overnight and then amino acid starved for 1 h. After the indicated treatments, the cells were incubated in DMEM containing d-[1-3H]glucose (0.6 μCi/well; GE Healthcare, Little Chalfont, UK) at 37°C for 3 h. The supernatants were discarded, and the cells were washed twice with ice-cold PBS. Afterward, 500 μl of KOH (30%) was added to the cells at room temperature for 30 min. The precipitation solution was then mixed with 1 mg of glycogen and heated for 30 min at 95°C. Samples were washed with ice-cold ethanol twice. The glycogen pellets were resuspended in distilled water and analyzed with a β-counter, as described previously (14).

Glucose uptake assay. Glucose uptake was determined as described previously (20). TSC2−/− MEFs were plated in a 12-well plate (5 × 104/well) and incubated overnight in DMEM containing 10% serum. The cells were washed twice in PBS, incubated in serum-free medium for 2 h, and then incubated in 1 ml of PBS containing 200 nM insulin for 30 min. After being washed with PBS, the cells were incubated in 1 ml of PBS containing 0.1 mM 2-deoxyglucose and 1 μCi/ml 2-deoxy-d-[1-3H]glucose for 5 min, washed three times in ice-cold PBS, and solubilized in 0.4 ml of 1% SDS. The [3H]glucose uptake was detected in 4 ml of scintillant by using a Beckman LS6500 scintillation counter. Nonspecific deoxyglucose uptake was measured in the presence of 20 mM cytochalasin B and was subtracted from the total uptake to calculate the specific glucose uptake.

Statistical analysis. Data are presented as means ± SE. The differences between experimental groups were determined by using Student’s t-tests (2 groups) or one- or two-way ANOVAs followed by Bonferroni’s post hoc test for multiple comparisons (≥3 groups). P values <0.05 were considered statistically significant.

RESULTS

Activated AMPK prevents hepatic insulin resistance induced by elevated amino acid concentrations. Since AMPK activation improves insulin sensitivity (57), we examined whether excess amino acids affect AMPK activity in HepG2 cells. Increasing amino acids from one- to fourfold of normal concentrations significantly inhibited the phosphorylation of AMPK (Thr172) and its downstream molecule ACC (Ser79), indicating that excess amino acids suppressed AMPK activity (Fig. 1A). In the presence of normal concentrations of amino acids, metformin, a well-known AMPK activator, increased AMPK phosphorylation in a dose-dependent manner (Fig. 1B).

Two micromolars of metformin also activated AMPK in the
absence of amino acids (Fig. 1C). Therefore, we used metformin as an AMPK stimulator to determine the role of AMPK in the prevention of amino acid-induced insulin resistance. In the presence of excess amino acids, insulin stimulation reduced AMPK phosphorylation, and the suppression of AMPK activity was attenuated by metformin treatment (Fig. 1D).

Although metformin had no effect on phosphorylation of IRS-1 (Tyr612) and Akt (Ser473) in the absence of amino acids (Fig. 1E), it abolished the inhibitory effect of amino acids on insulin-stimulated phosphorylation of IRS-1 and Akt (Fig. 1F). In the presence of insulin, metformin increased phosphorylation of AMPK and ACC (Ser\textsuperscript{792}) and prevented amino acid-inhibited phosphorylation of IRS-1, Akt, and GSK-3\textbeta{} (Ser\textsuperscript{9}) (Fig. 1, G–I). To determine the metabolic endpoints of insulin action in the liver, we measured glycogen synthesis in HepG2 cells, which has been reported to mirror the activity of insulin signaling (23). Consistently, excess amino acids diminished insulin-stimulated glycogen synthesis, and the administration of metformin restored the effect of insulin on glycogen synthesis (Fig. 1J). The effect of AMPK on amino acid-induced insulin resistance was further assessed using genetic means. Excess amino acids suppressed AMPK phosphorylation, inhibited insulin-stimulated phosphorylation of IRS-1 and Akt, and reduced glycogen synthesis in the cells infected with GFP adenovirus. These effects were prevented by transfection of CA-AMPK adenovirus (Fig. 1, K and L).

Activation of AMPK suppresses excess amino acid-induced insulin resistance through upregulation of Notch1. The result of the inhibition of Notch increasing insulin sensitivity in diet-induced hepatic insulin-resistant animals (41) prompted us to determine the role of Notch signaling in amino acid-induced insulin resistance. We found that elevated amino acid concentrations increased Notch1 expression (Fig. 2A). To determine the role of Notch1 in the modulation of insulin signaling, we treated HepG2 cells with LY-374973, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyI ester (DAPT), a γ-secretase inhibitor that blocks Notch signaling (41). Although DAPT alone had no effect on the phosphorylation of IRS-1 and Akt (Fig. 2B), it eliminated the inhibitory effects of amino acids on insulin-stimulated phosphorylation of IRS-1 and Akt (Fig. 2C). In the presence of excess amino acids, the expression of glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) was increased, and the upregulation of G6Pase and PEPCK mRNA was eradicated by inhibition of Notch1 (Fig. 2D). Consistently, insulin-stimulated glycogen synthesis was inhibited by the presence of excess amino acids; the inhibition was attenuated by administration of DAPT (Fig. 2E).

We further examined the role of Notch1 in the modulation of insulin signaling by using a gene-silencing approach. Notch1 siRNA, but not control siRNA, significantly reduced Notch1 protein levels (data not shown). Excess amino acids activated Notch1 signaling by increasing the expression of Hes1, RbpJ, and FoxO1 in the cells transfected with control siRNA. The activation of Notch1 signaling was attenuated by transfection of Notch1 siRNA (Fig. 2F). Similar results were also observed in the presence of insulin (Fig. 2G). Similarly to the effect of DAPT, transfection of Notch1 siRNA eliminated amino acid-enhanced G6Pase and PEPCK mRNA (Fig. 2H). Furthermore, in the presence of insulin, excess amino acids inhibited insulin-stimulated phosphorylation of IRS-1 and Akt (Fig. 2, I and J) and synthesis of glycogen in the cells transfected with control siRNA (Fig. 2K). This inhibitory effect was absent in the cells transfected with Notch1 siRNA (Fig. 2, I–K). These data suggest that upregulation of Notch1 mediates excess amino acid-induced insulin resistance.

We next investigated whether AMPK regulates Notch1 expression by measuring Notch1 protein levels after manipulation of AMPK activity. The administration of metformin to activate AMPK dose-dependently reduced the amino acid-enhanced Notch1 protein levels (Fig. 2L). We further determined the role of AMPK in the regulation of Notch1 signaling after transfecting the cells with adenovirus encoding GFP, DN-AMPK, or CA-AMPK. In GFP-transfected cells, the presence of excess amino acids increased the expression of Notch1; this effect was lessened by either metformin treatment or CA-AMPK overexpression. The overexpression of DN-AMPK adenovirus increased Notch1 expression and abolished metformin-reduced expression of Notch1 (Fig. 2M). These data suggest that activation of AMPK prevents amino acid-induced insulin resistance through the downregulation of Notch1.

Activation of mTORC1 signaling by excess amino acids is required for upregulation of Notch1. mTORC1 signaling is essential for amino acid signal transduction (17). We studied the effect of mTORC1 on Notch1 expression in the presence of excess amino acids. In HepG2 cells, amino acids activated the mTORC1 signaling pathway in a concentration-dependent manner, as estimated by the reduction in phosphorylation of Raptor (a major component of the mTORC1 complex) at Ser\textsuperscript{2448} and increases in phosphorylation of mTOR at Ser\textsuperscript{922} and Thr\textsuperscript{389} (Fig. 3A). The activation of mTORC1 signaling was accompanied by upregulation of Notch1 protein levels. Administration of the mTORC1-specific inhibitor rapamycin attenuated excess amino acid-stimulated phosphorylation of S6K1 and 4EBP1 (Fig. 3B) and concomitantly reduced the Notch1 protein levels, suggesting that the mTORC1 pathway regulates Notch1 signaling.

To further clarify the relationship between mTORC1 and Notch1 signaling pathways, we investigated whether hyperactive mTORC1 could increase Notch1 expression in TSC\textsuperscript{2/−/−} MEFs, in which mTORC1 is constitutively active, as evidenced by enhanced phosphorylation of mTOR and S6K1. The activation of mTORC1 by deletion of TSC2 increased Notch1 expression. This effect was prevented by administration of either rapamycin or mTOR siRNA (Fig. 3, C and D), indicating that activation of the mTORC1 signaling pathway is required for the amino acid-enhanced Notch1 expression. To confirm the role of the mTORC1 signaling pathway in amino acid-induced insulin resistance, we detected insulin signaling after inhibition of mTORC1 with rapamycin. As expected, elevated amino acids inhibited the insulin-stimulated phosphorylation of IRS-1 at both Tyr\textsuperscript{612} (Fig. 3E) and Ser\textsuperscript{307} (Fig. 3F) as well as reduced phosphorylation of Akt at Ser\textsuperscript{473} (Fig. 3E). The inhibition was eliminated completely by rapamycin treatment (Fig. 3E). Similarly, the inhibitory effect of excess amino acids on insulin-stimulated glycogen synthesis was attenuated by the administration of rapamycin (Fig. 3G). These data suggest that the amino acid-enhanced Notch1 expression is mTORC1 dependent.

Activation of mTORC1 enhances Notch1 expression through activation of STAT3. STAT3 has been identified as a downstream molecule of mTORC1 (26) and acts as a mediator between mTORC1 and Notch signaling pathways (37). Therefore, we investigated whether mTORC1 upregulates Notch1
E200

AMPK PREVENTS AMINO ACID-INDUCED INSULIN RESISTANCE

AJP-Endocrinol Metab • doi:10.1152/ajpendo.00202.2013 • www.ajpendo.org

Downloaded from http://ajpendo.physiology.org by 10.220.33.6 on June 25, 2017

by 10.220.33.6 on June 25, 2017 http://ajpendo.physiology.org/ Downloaded from

E200

AMPK PREVENTS AMINO ACID-INDUCED INSULIN RESISTANCE

AJP-Endocrinol Metab • doi:10.1152/ajpendo.00202.2013 • www.ajpendo.org

Downloaded from http://ajpendo.physiology.org by 10.220.33.6 on June 25, 2017

by 10.220.33.6 on June 25, 2017 http://ajpendo.physiology.org/ Downloaded from
through stimulation of STAT3 in the presence of excess amino acids. Excess amino acids significantly increased the phosphorylation of STAT3 at Ser\(^{727}\) (Fig. 4A), which was dose-dependently inhibited by rapamycin (Fig. 4B). In TSC2\(^{-/-}\) MEFs, the activation of mTORC1 increased STAT3 phosphorylation. The effect was prevented by the administration of either rapamycin (Fig. 4C) or mTOR siRNA (Fig. 4D), indicating that mTORC1 acts upstream of STAT3. Consistent with previous findings that activation of mTORC1 by amino acids can lead to insulin resistance through mechanisms involving activation of S6K1 (47) and suppressor of cytokine signaling-3 (SOCS3) induction (25, 26), we found that excess amino acids increased SOCS3 protein levels (Fig. 4E, top). We next determined the role of STAT3 in the regulation of Notch1 by examining Notch1 expression after treating the HepG2 cells with AG490, a STAT3-specific inhibitor. As expected, inhibition of STAT3 by AG490 reduced the amino acid-enhanced Notch1 expression (Fig. 4E, bottom). We further identified Notch1 expression in TSC2\(^{-/-}\) MEFs after treating the cells with either AG490 or STAT3 siRNA. The inactivation of STAT3 by AG490 or siRNA led to a reduction in Notch1 expression but had no effect on S6K1 phosphorylation (Fig. 4, F and G). Moreover, activation of AMPK by metformin could not prevent the upregulation of Notch1 in TSC2\(^{-/-}\) MEFs (Fig. 4H). Taken together, these results suggest that mTORC1 stimulates Notch1 expression through activation of STAT3.

**Activation of AMPK by metformin inhibits mTORC1/STAT3 signaling.** To establish the role of the mTORC1/STAT3/Notch1 pathway in excess amino acid-induced insulin resistance, we examined the alteration of insulin signaling and glucose uptake in TSC2\(^{-/-}\) MEFs after treating the cells with inhibitors of mTOR, STAT3, or Notch1. In MEFs, GLUT1 is the major glucose transporter (32). GLUT1-mediated glucose transport has been reported to be regulated by insulin in human osteosarcoma cells (7). Under basal conditions, insulin stimulated the phosphorylation of IRS-1 (Fig. 5A) and increased glucose uptake (Fig. 5B). Treatment of cells with rapamycin, AG490, or DAPT alone did not affect IRS-1 phosphorylation or glucose uptake, whereas the combination of these agents with insulin induced an additional increase in the phosphorylation of IRS-1 and glucose uptake (Fig. 5, A and B). These results suggest that the mTORC1/STAT3/Notch1 pathway negatively regulates insulin signaling.

We next examined whether AMPK regulates mTORC1/STAT3 signaling pharmacologically or genetically. Metformin treatment restored AMPK phosphorylation in the presence of excess amino acids and inhibited excess amino acid-enhanced phosphorylation of mTOR, S6K1, 4EBP1, and STAT3 (Fig. 5C). In the cells transfected with GFP adenovirus, excess amino acids increased the phosphorylation of mTOR, 4EBP1, S6K1, and STAT3. The activation of mTOR/STAT3 signaling was attenuated by administration of metformin and overexpression of CA-AMPK adenovirus. However, the protective effects of metformin were abrogated by the overexpression of DN-AMPK adenovirus (Fig. 5D). Thus, inhibition of mTORC1/STAT3 signaling is essential for AMPK to prevent excess amino acid-impaired insulin signaling.

We further examined the role of the mTORC1/STAT3/Notch1 pathway in excess amino acid-induced insulin resistance in primary hepatocytes. Similar to the findings in HepG2 cells and MEFs, excess amino acids inhibited AMPK phosphorylation. This was accompanied by increases in phosphorylation of mTOR and STAT3 as well as upregulation of Notch1 and Rbp-Jk. Administration of metformin restored AMPK phosphorylation, inhibited mTOR and STAT3 phosphorylation, and reduced Notch1 and Rbp-Jk expression. Notably, rapamycin treatment inhibited mTOR and STAT3 phosphorylation and reduced Notch1 and Rbp-Jk protein levels but did not restore AMPK phosphorylation (Fig. 5E). Glycogen synthesis assay further confirmed that excess amino acids inhibited insulin-stimulated glycogen synthesis, and administration of either metformin or rapamycin prevented the inhibitory effect of amino acids on glycogen synthesis (Fig. 5F).

**Chronic metformin treatment improves insulin sensitivity in HPD-fed mice through inhibition of mTORC1/STAT3/Notch1 signaling.** To extend our in vitro findings, we investigated whether metformin and rapamycin prevent HPD-induced insulin resistance in vivo. Although there was no difference in daily food intake, body weight, or fasting blood glucose levels between the groups (Fig. 6, A–C), HPD-fed mice had significantly higher expression of G6Pase and PEPCK mRNA than did ND-fed mice. The high levels of mRNA were attenuated by administration of either metformin or rapamycin (Fig. 6D). In HPD-fed mice, administration of metformin led to an increase in AMPK phosphorylation and a reduction in S6K1 and 4EBP1 phosphorylation (Fig. 6E). The suppression of mTORC1 signaling was accompanied by decreases in STAT3 phosphorylation and Notch1 protein levels, indicating that chronic metformin therapy inhibited the mTORC1/STAT3/Notch1 signaling pathway (Fig. 6F). To determine the effect of metformin...
and HPD on liver insulin signaling, we analyzed IRS-1 and Akt phosphorylation after a bolus injection of insulin. Acute administration of insulin did not alter total Akt protein levels in either ND- or HPD-fed mice. However, insulin stimulation significantly enhanced phosphorylation of IRS-1 and Akt in ND-fed mice but failed to enhance the phosphorylation in HPD-fed mice (Fig. 6G). Chronic metformin therapy prevented the HPD-inhibited AMPK phosphorylation (Fig. 6H) and re-

Fig. 3. Elevated AA activate Notch1 by stimulating mammalian target of rapamycin (mTOR) signaling. A: HepG2 cells were treated with the indicated concentrations of AA for 1 h. Expression of P-Raptor (Ser970), P-mTOR-Ser2448, phosphorylated S6 kinase 1 (P-S6K1)-Thr389, phosphorylated 4E-binding protein 1 (P-4EBP1)-Thr473, and β-actin was analyzed by WB (\(P < 0.05\) vs. control; \(n = 4\)). B: Cells were pretreated with rapamycin (Rap; 100 nM) for 1 h and then stimulated with the indicated concentrations of AA. Expression of P-S6K1, P-4EBP1, Notch1, and β-actin was determined by WB (\(P < 0.05\) vs. control, \(\dagger P < 0.05\) vs. 1× AA, and \(\ddagger P < 0.05\) vs. 2× AA; \(n = 4\)). C: Wild-type (WT) and tuberous sclerosis complex 2 (TSC2) knockout (KO) MEFs were treated with Rap (100 nM) for 24 h. Cell lysates were subjected to WB to determine the expression of P-mTOR, P-S6K1, Notch1, and β-actin (\(P < 0.05\) vs. WT control and † \(P < 0.05\) vs. 1× AA, and ‡ \(P < 0.05\) vs. 2× AA; \(n = 4\)). D: TSC2−/− MEFs were transfected with C-siRNA or mTOR siRNA (mTOR-si) for 48 h. Expression of P-S6K1, P-4EBP1, Notch1, and β-actin was analyzed by WB (\(P < 0.05\) vs. control; \(\dagger P < 0.05\) vs. 2× AA, and \(\ddagger P < 0.05\) vs. 2× AA/insulin; \(n = 4\)). E: phosphorylation of IRS-1 at Ser636 was assayed by WB (\(P < 0.05\); \(n = 3\)). F: glycogen synthesis was assayed as described in MATERIALS AND METHODS (\(P < 0.05\) vs. control, \(\dagger P < 0.05\) vs. 2× AA, and \(\ddagger P < 0.05\) vs. 2× AA/insulin; \(n = 4\)).
markedly increased the insulin-stimulated phosphorylation of IRS-1 and Akt (Fig. 6H).

**Rapamycin prevents insulin resistance in HPD-fed mice through the inhibition of STAT3/Notch1 signaling.** To determine the role of mTORC1 signaling in amino acid-induced insulin resistance in vivo, we treated the HPD-fed mice with rapamycin. STAT3 phosphorylation and Notch1 protein levels were significantly reduced in rapamycin-treated and HPD-fed mice (Fig. 7A), but no change in AMPK phosphorylation occurred in these mice (data not shown). In addition, acute administration of insulin did not alter the phosphorylation of IRS-1 or Akt in HPD-fed mice but significantly enhanced the phosphorylation of IRS-1 and Akt in rapamycin-treated and HPD-fed mice (Fig. 7B).

**DISCUSSION**

Compared with the established roles of dietary fat in the development of insulin resistance and type 2 diabetes, the roles of proteins and amino acids in glucose homeostasis and insulin resistance are still poorly characterized. The present study indicates that, like HFD, HPD has detrimental effects on glucose homeostasis by promoting insulin resistance and increasing gluconeogenesis. Amino acid signaling is integrated by AMPK and mTORC1, two important signaling pathways in sensing nutrient and energy status, which coordinately regulate Notch1 and insulin-signaling pathways in the presence of excess amino acids. Our data indicate that the upregulation of Notch1 protein levels was involved in amino acid-induced insulin resistance. In the presence of excess amino acids, inhibition of AMPK triggered mTORC1/STAT3 signaling, leading to the upregulation of the Notch1 protein levels and impairment of insulin signaling. Activation of AMPK suppressed the mTORC1-stimulated STAT3 phosphorylation, reduced the Notch1 expression, and consequently prevented insulin resistance. Furthermore, administration of either metformin or rapamycin reduced Notch1 expression and prevented insulin resistance in HPD-fed mice. These findings establish a novel and attractive model that excess amino acids induce hepatic insulin resistance through regulation of the AMPK/mTORC1/STAT3/Notch1 axis (Fig. 7C).
Since AMPK is an important enzyme in maintaining cellular energy homeostasis, a dysfunction in the AMPK signaling pathway could result in metabolic disorders. In the presence of excess amino acids, suppression of AMPK was accompanied by activation of the mTORC1/STAT3/Notch1 axis and improved insulin sensitivity, supporting the idea that inactivation of AMPK is an essential mechanism underlying excess amino acid-induced insulin resistance. AMPK has been reported to inhibit mTORC1 through either direct phosphorylation of mTOR (6) or phosphorylation and activation of tuberin (21). In the current study, amino acid-inactivated AMPK stimulated the phosphorylation of mTOR and its downstream effectors S6K1 and 4EBP1, leading to inhibition of insulin sensitivity. Inhibition of mTOR by rapamycin prevented amino acid-inhibited insulin signaling but had no effect on AMPK activity. In addition, metformin could not prevent the upregulation of Notch1 in...
TSC2<sup>−/−</sup> MEF. Thus, activation of AMPK improves insulin sensitivity through inhibition of mTOR/STAT3/Notch1 signaling. This conclusion is also supported by our in vivo observations that chronic metformin therapy inhibited hyperactive mTORC1-activated STAT3, reduced Notch1 expression, and improved insulin sensitivity in mice maintained on a HPD.

Dephosphorylation of GSK-3β stimulates glycogen synthesis, which is a key function of insulin in lowering blood glucose. Earlier studies have shown that activation of mTORC1/
S6K1 signaling mediated amino acid-activated glycogen synthesis in muscle cells (2, 42). However, in TSC1/2-deficient MEFs, activation of mTORC1/S6K1 signaling leads to constitutive phosphorylation and inhibition of GSK-3 (56). Under conditions of insulin resistance, mTORC1/S6K1 signaling mediates inhibition of GSK3 in HepG2 cells (56). In the presence of HPD, hepatic glycogen synthesis was suppressed (31). Our data also indicate that amino acid-activated mTORC1 signaling inhibited GSK-3β activity and attenuated insulin-stimulated glycogen synthesis in HepG2 cells. Taken together, these findings suggest that excess amino acids may inhibit glycogen synthesis in the liver.

Activation of mTORC1 and its downstream molecule S6K1 is involved in the development of insulin resistance (16, 26). In the present study, activation of mTORC1 increased STAT3 phosphorylation, leading to upregulation of Notch1 and inhibition of insulin signaling. Conversely, inhibition of mTORC1 inactivated STAT3, reduced Notch1 protein expression, and improved insulin sensitivity. Moreover, inhibition of STAT3 prevented the hyperactive mTORC1-induced Notch1 expression and insulin resistance in TSC2−/− MEFs and HepG2 cells, suggesting that sustained activation of mTORC1 signaling in the presence of excess amino acids upregulated Notch1 expression and inhibited insulin signaling by activating STAT3.

Mammalian Notch receptors (Notch1–4), a family of transmembrane proteins, have traditionally been thought to play an important role in the regulation of cellular development, differentiation, and apoptosis (12). However, a recent study demonstrated that the inhibition of Notch markedly increases insulin sensitivity in diet-induced hepatic insulin-resistant animals, whereas Notch gain of function promotes insulin resistance (41), suggesting that Notch mediates diet-induced hepatic insulin resistance. Our study provides experimental support for the importance of Notch1 in mediating excess amino acid-induced insulin resistance. In the presence of excess amino acids, high expression of Notch1 was associated with impaired insulin signaling. Both administration of DAPT, a γ-secretase inhibitor that prevents Notch1 activation, and gene silencing inhibit Notch1; this inhibition attenuated excess amino acid-induced insulin resistance.

In the in vivo study, HPD feeding led to high expression of Notch1 and impairment of insulin signaling. Chronic administration of either metformin or rapamycin reduced Notch1 expression and consequently improved insulin sensitivity. Mechanistically, in the presence of excess amino acids, we found that upregulation of Notch1 protein levels resulted in the high expression of Rbp-Jk and Hes1 proteins, thereby enhancing FoxO1 protein levels. FoxO1 is a mediator of insulin signaling and plays an important role in maintaining glucose homeostasis (36) through transcriptional regulation of G6Pase and PEPCK, which are rate-limiting enzymes in the regulation of hepatic gluconeogenesis (43). Thus, activation of Notch1 may mediate insulin resistance in a FoxO1-dependent manner. Activation of STAT3 by amino acids has been implicated in the development of insulin resistance by induction of SOCS3. However, the underlying mechanism responsible for STAT3-stimulated SOCS3 induction is not clear. Notch1 signaling has been reported to be required for the activation of SOCS3 expression in macrophages. Whether amino acid-activated STAT3 stimulates SOCS3 induction in hepatocytes through Notch1 warrants further investigation.

In summary, in the presence of excess amino acids, suppression of the AMPK signaling pathway led to insulin resistance. This effect could be attributed to the activation of the mTORC1/STAT3/Notch1 signaling pathway. Activation of AMPK normalized mTORC1/STAT3/Notch1 signaling, thereby preventing amino acid-induced insulin resistance (Fig. 7C). Our findings suggest that the components of the AMPK/mTORC1/STAT3/Notch1 axis may be attractive targets for the prevention and treatment of insulin resistance induced by excessive nutrient intake.

ACKNOWLEDGMENTS

We thank Kathy Kyler, University of Oklahoma Health Sciences Center, for help with editing the manuscript.

GRANTS

This study was supported by funding from the National Institutes of Health (HL-079584, HL-080499, HL-074399, HL-089920, and HL-096032 to M. H. Zou and 1P20-RR-024215–01 to Z. Xie and M. H. Zou), an American Heart
Association Scientist Development Grant (Z. Xie), the Juvenile Diabetes Research Foundation (M. H. Zou), the Oklahoma Center for the Advancement of Science and Technology (M. H. Zou and Z. Xie), and the American Diabetes Association (M. H. Zou). M. H. Zou is a recipient of the National Established Investigator Award of the American Heart Association.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
H.L., J.L., and C.H. performed the experiments; m.-h.Z. and Z.X. edited and revised the manuscript; m.-h.Z. and Z.X. approved the final version of the manuscript; Z.X. contributed to the conception and design of the research; Z.X. analyzed the data; Z.X. interpreted the results of the experiments; Z.X. prepared the figures; Z.X. drafted the manuscript.

REFERENCES
7. Cifuentes M, Garcia MA, Arrabal PM, Martinez F, Yanez MJ, Jara H.L., J.L., and C.H. performed the experiments; m.-h.Z. and Z.X. edited and revised the manuscript; m.-h.Z. and Z.X. approved the final version of the manuscript; Z.X. contributed to the conception and design of the research; Z.X. analyzed the data; Z.X. interpreted the results of the experiments; Z.X. prepared the figures; Z.X. drafted the manuscript.

REFERENCES
7. Cifuentes M, Garcia MA, Arrabal PM, Martinez F, Yanez MJ, Jara H.L., J.L., and C.H. performed the experiments; m.-h.Z. and Z.X. edited and revised the manuscript; m.-h.Z. and Z.X. approved the final version of the manuscript; Z.X. contributed to the conception and design of the research; Z.X. analyzed the data; Z.X. interpreted the results of the experiments; Z.X. prepared the figures; Z.X. drafted the manuscript.

REFERENCES
7. Cifuentes M, Garcia MA, Arrabal PM, Martinez F, Yanez MJ, Jara H.L., J.L., and C.H. performed the experiments; m.-h.Z. and Z.X. edited and revised the manuscript; m.-h.Z. and Z.X. approved the final version of the manuscript; Z.X. contributed to the conception and design of the research; Z.X. analyzed the data; Z.X. interpreted the results of the experiments; Z.X. prepared the figures; Z.X. drafted the manuscript.


