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Metformin inhibits hepatic mTORC1 signaling via dose-dependent mechanisms involving AMPK and the TSC complex

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Abstract

Metformin is the most widely prescribed drug for the treatment of type-2 diabetes. However, knowledge of the full effects of metformin on biochemical pathways and processes in its primary target tissue, the liver, is limited. One established effect of metformin is to decrease cellular energy levels. The AMP-activated protein kinase (AMPK) and mechanistic target of rapamycin (mTOR) complex 1 (mTORC1) are key regulators of metabolism that are respectively activated and inhibited in acute response to cellular energy depletion. Here we show that metformin robustly inhibits mTORC1 in mouse liver tissue and primary hepatocytes. Using mouse genetics, we find that at the lowest concentrations of metformin that inhibit hepatic mTORC1 signaling, this inhibition is dependent on AMPK and the tuberous sclerosis complex (TSC) protein complex (TSC complex). Finally, we show that metformin profoundly inhibits hepatocyte protein synthesis in a manner that is largely dependent on its ability to suppress mTORC1 signaling.

Graphical Abstract

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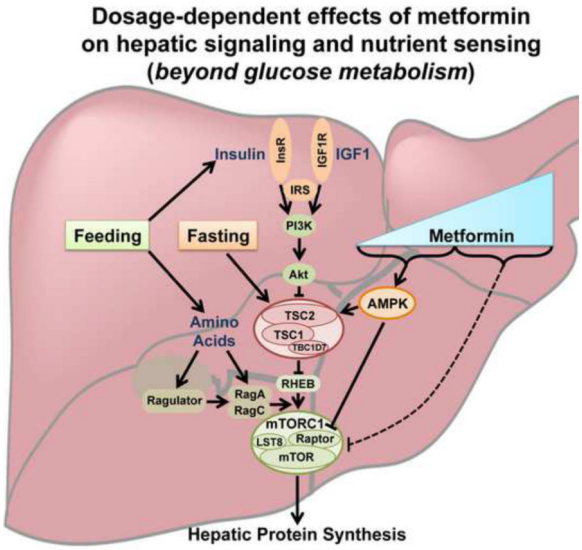
SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and one figure.

AUTHOR CONTRIBUTIONS

J.J.H. and K.H. guided and performed most experiments, with assistance from M.T., G.T., D.V.R, and G.H.. M.J.K. and A.S. measured plasma metformin via LC-MS. J.J.H., K.H., R.J.S., and B.D.M. conceived and designed the project, which was supervised by R.J.S. and B.D.M..



INTRODUCTION

Type-2 diabetes is a growing global epidemic affecting approximately 9% of the adult population worldwide (World Health Organization, 2016). The first-line treatment and most prescribed anti-diabetes drug is the biguanide metformin, which lowers blood glucose levels and reduces cardiovascular events in patients with type-2 diabetes (UK Prospective Diabetes Study Group, 1998; Inzucchi et al., 1998). Due to the well-established effects of metformin on hepatic glucose production (Johnson et al., 1993; Perriello et al., 1994) and the high expression level of the metformin transporter OCT1 in hepatocytes (Grundemann et al., 1994; Zhang et al., 1997), the liver is considered to be one of its primary target tissues. However, our understanding of the molecular and cellular targets influencing the physiological effects of metformin is far from complete.

One established direct target of metformin is mitochondrial complex I, which metformin inhibits, thereby decreasing cellular respiration and ATP levels (El-Mir et al., 2000; Owen et al., 2000; Zhou et al., 2001). Among other effects, metformin-induced energy depletion can stimulate the AMP-activated protein kinase (AMPK), and does so prominently in the liver (Zhou et al., 2001; Shaw et al. 2005). Through multiple downstream effectors, AMPK promotes ATP-producing catabolic processes while inhibiting ATP-consuming anabolic processes (Hardie and Ashford, 2014). Notably, compounds that activate AMPK inhibit the mechanistic target of rapamycin complex 1 (mTORC1) (Bolster et al., 2002; Krause et al., 2002; Dubbelhuis and Meijer, 2002), a major driver of anabolic metabolism that is aberrantly activated under conditions of hyperinsulinemia and obesity and is believed to contribute to the development of insulin resistance (Um et al., 2004; Khamzina et al., 2005, Howell and Manning, 2011). However, whether metformin treatment inhibits hepatic mTORC1 signaling, along with the mechanisms and consequences, is unknown.

mTORC1 is a nutrient-sensitive multiprotein complex whose core essential components include the protein kinase mTOR and scaffolding protein Raptor (Laplante and Sabatini,

2012). This protein kinase complex serves to link nutrient and energy signals, both local and systemic, to key anabolic processes that utilize nutrients and energy to produce macromolecules, including proteins, lipids, and nucleotides (Dibble and Manning, 2013; Howell et al., 2013). mTORC1 senses these upstream signals through two systems of small G proteins, the Rag and Rheb GTPases. Intracellular amino acids, and perhaps glucose, signal to mTORC1 through a pathway influencing engagement of the Rag proteins with mTORC1 (Bar-Peled and Sabatini, 2014; Efeyan et al., 2013). A second layer of regulation integrates multiple signals, including growth factor- and insulin-stimulated signaling pathways that influence the nucleotide binding state of Rheb, which in its GTP-bound state is an essential activator of mTORC1. These signals are largely perceived through a protein complex composed of the tuberous sclerosis complex proteins (TSC1 and TSC2) and the protein TBC1D7 (collectively referred to as the TSC complex), which functions as a GTPase-activating protein (GAP) for Rheb, thereby inhibiting the Rheb-dependent activation of mTORC1 (Dibble and Manning, 2013). Thus many signals that turn off mTORC1, such as growth factor withdrawal, do so by promoting Rheb inhibition through the TSC complex. As such, loss of function of any component of the TSC complex leads to sustained mTORC1 activity under conditions that would normally inhibit mTORC1 through this protein complex, such as in the liver during fasting (Sengupta et al., 2010; Yecies et al., 2011).

The biosynthetic processes stimulated by mTORC1, such as protein synthesis, are heavily dependent on ATP, and multiple mechanisms have evolved to ensure mTORC1 is only active under energy charged conditions. Energy stress-mediated activation of AMPK provides one such mechanism of mTORC1 regulation. AMPK can directly phosphorylate TSC2 on S1387, thereby promoting its inhibition of Rheb and mTORC1 (Inoki et al., 2003; Shaw et al., 2004). In parallel, AMPK also exerts a direct inhibitory signal on mTORC1 through the phosphorylation of S722 and S792 on Raptor (Gwinn et al., 2008). Additional mechanisms of mTORC1 inhibition in response to perturbations in cellular energy levels have been suggested in various studies (Dibble and Manning, 2013), including those that are independent of AMPK or the TSC complex (Ben-Sahra et al., 2011; Dennis et al., 2001; Kalender et al., 2010; Kim et al., 2013; Liu et al., 2014; Sofer et al., 2005; Zheng et al., 2011). However, the relative importance of different mechanisms inhibiting mTORC1 is likely influenced by the particular form, degree and duration of energy stress, as well as the cell or tissue type.

Conflicting studies exist regarding how metformin inhibits mTORC1 signaling, with some concluding that the drug acts through AMPK and the TSC complex (Dowling et al., 2007) and others stating that the regulation is entirely independent of this mechanism (Ben-Sahra et al., 2011; Kalender et al., 2010). However, these and other studies have been restricted to cancer cells and mouse embryo fibroblasts and have often used high doses (e.g., ≥10 mM) and durations (e.g., ≥24 hours) of metformin treatment. Surprisingly, the effects of metformin on mTORC1 signaling, including the mechanism of regulation and downstream consequences, have not been studied in the physiological context of the liver – the target organ of metformin treatment for its primary indication, type-2 diabetes. Here we demonstrate that metformin strongly inhibits mTORC1 signaling in mouse liver and primary mouse and human hepatocytes. Using mouse genetics, we reveal a biphasic response to

metformin for its suppression of mTORC1. At the lowest doses that inhibit mTORC1, metformin requires AMPK and the TSC complex for this effect, whereas at higher doses AMPK and TSC complex-independent mechanisms are revealed. Finally, we demonstrate that metformin strongly attenuates hepatic protein synthesis through a mechanism requiring its inhibitory effects on mTORC1.

RESULTS AND DISCUSSION

Hepatic mTORC1 signaling is inhibited by metformin, which at the lowest inhibitory dose acts through AMPK

Hepatic mTORC1 signaling is acutely activated by feeding (Figure 1A) (Yecies et al., 2011). Therefore, to determine the pharmacological effects of metformin on mTORC1 activation in the mouse liver, we used an established fasting-refeeding paradigm to obtain consistently elevated levels of mTORC1 activity prior to treatment. Mice were fasted overnight followed by refeeding prior to a 1 h administration of metformin or vehicle. It has been suggested that the standard dose of metformin used in humans for the treatment of type-2 diabetes (~20 mg/kg) is similar to the 250 mg/kg dose typically used in mice, with both giving plasma concentrations in the 10 μM range (Foretz et al., 2014; Memmott et al., 2010). We found that plasma metformin concentrations with dosing of 200 or 250 mg/kg in our experimental paradigm were ~35 μM (Figure S1A), which is consistent with previous studies using the same delivery method and acute treatment (1 to 2 hours) with doses ranging from 125 to 350 mg/kg (Dowling et al., 2016; Memmott et al., 2010; Chandel et al., 2016). In these previous studies, metformin concentrations rapidly decreased after the first hour to levels well below the plasma concentration of 10 μM measured in humans. Thus, the mice in our study experienced a pharmacological range of metformin similar to humans. At both 200 and 250 mg/kg doses of metformin, liver mTORC1 activity was strongly suppressed, as measured by phosphorylation of the mTORC1-specific substrate ribosomal S6 kinase 1 (S6K1) and its downstream target S6 (Figure 1A).

To determine the mechanism of metformin's inhibition of hepatic mTORC1 signaling, we compared the response of mice with tamoxifen-inducible, liver-specific deletion of both isoforms of the AMPK catalytic subunit, AMPKα1 and AMPKα2, to littermate controls. Control mice (AMPKα1$^{fl/fl}$/α2$^{fl/fl}$) or those lacking AMPK (L-AMPKα1/α2-DKO) were fasted overnight, refed for 2 h and treated with saline or metformin (250 mg/kg) for the last hour. In control mice, metformin treatment strongly induced hepatic AMPK activation, as scored by the activating phosphorylation on AMPK-T172 and the AMPK-dependent phosphorylation sites on Raptor, S722 and S792 (Figure 1B). As above, metformin inhibited multiple markers of mTORC1 signaling in the livers of control mice, including phosphorylation of S6K1, S6 and eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1), as indicated by phosphorylation of 4E-BP1-S65 and corresponding mobility shifts. However, the L-AMPKα1/α2-DKO mice exhibited sustained liver mTORC1 signaling upon metformin treatment, with a corresponding loss of induction of AMPK and Raptor phosphorylation (Figure 1B). It should be noted that some dampening of mTORC1 activity can be detected in metformin-treated L-AMPKα1/α2-DKO livers, as observed in lighter immunoblot exposures for phosphorylation of S6K1, which is the direct substrate

most sensitive to mTORC1 inhibition (Kang et al., 2013), and mobility shifting of 4E-BP1 (Figure S1B). However, mTORC1 signaling in AMPK-deficient livers is consistently resistant to metformin relative to controls.

Primary hepatocytes were isolated to determine whether the effects of metformin on liver mTORC1 signaling were cell autonomous, and to more closely examine the dose and time-dependence of the response. While the underlying cause remains a point of debate, it has been established that cells grown in culture, under supraphysiological levels of nutrients and oxygen, require much higher concentrations of metformin to elicit responses similar to those seen in vivo (He and Wondisford, 2015; Dowling et al., 2016; Memmott et al., 2010; Chandel et al., 2016). To this end, neither AMPK activation nor mTORC1 inhibition was observed in hepatocyte cultures treated with metformin doses of 0.2 mM or less. However, 0.5 mM metformin resulted in robust activation of AMPK at 2 h, which was concomitant with attenuation of mTORC1 signaling (Figure 1C,D). Next, primary hepatocytes from control and L-AMPKα1/α2-DKO mice were isolated and compared for their response to this range of doses and durations of metformin treatment. Hepatocytes from control mice responded to metformin in a dose-dependent manner, with all markers of mTORC1 activity decreasing as AMPK activity increased (Figure 1E). Similar to the *in vivo* observations, primary hepatocytes isolated from L-AMPK α1/α2-DKO mice were resistant to metformin-induced suppression of mTORC1 at all doses after 2 h treatment. However, at 5 h, L-AMPKα1/α2-DKO hepatocytes remained resistant to metformin for suppression of mTORC1 signaling only at lower doses, with mTORC1 being fully inhibited at higher doses of metformin, even in the absence of AMPK (Figure 1E). These data demonstrate that at the lowest doses and durations of metformin that inhibit mTORC1 signaling in hepatocytes, the compound acts through AMPK activation. However, AMPK-independent mechanisms of metformin action on mTORC1 also exist, which are revealed at higher doses and prolonged treatment.

Lower doses of metformin inhibit hepatic mTORC1 signaling in a manner that is dependent on the TSC complex

One established mechanism whereby AMPK can inhibit mTORC1 is through activation of the TSC complex (Inoki et al., 2003). To directly address a potential role for the TSC complex in the metformin-mediated inhibition of mTORC1 in the liver, we utilized L*Tsc1*KO mice with hepatocyte-specific deletion of *Tsc1*, encoding an essential core component of the complex. As previously demonstrated (Yecies et al., 2011), the *LTsc1*KO mice displayed sustained hepatic mTORC1 signaling under fasting conditions, at levels similar to refed littermate $Tsc1^{fl/fl}$ controls (Figure 2A). Interestingly, in this cohort of male mice, metformin (200 mg/kg) acutely suppressed mTORC1 signaling in livers of control mice, but mTORC1 activity was largely resistant to metformin in the livers of L*Tsc1*KO mice (Figure 2A). Unlike the TSC complex, AMPK is not required for suppression of mTORC1 signaling in the fasted state (Figure 2B), but L-AMPKα1/α2-DKO livers again showed sustained mTORC1 signaling with metformin treatment. Comparison of a cohort of female mice treated with increasing doses of metformin revealed suppression of mTORC1 signaling at doses as low as 100 mg/kg in controls, while the LTsc1KO levels showed no reduction in mTORC1 signaling at this dose and only a modest reduction at 200 mg/kg

(Figure 2C). An additional experiment treating a female cohort with 250 mg/kg further demonstrated that mTORC1 signaling was resistant to metformin in the livers of L*Tsc1*KO mice, but a partial decrease was observed (Figure 2D). Importantly, the L*Tsc1*KO mice responded similarly to control mice with respect to metformin-induced activation of AMPK and its phosphorylation of Raptor. This result is consistent with the TSC complex functioning downstream of AMPK in the suppression of mTORC1 signaling by metformin, with Raptor phosphorylation providing an additional mechanism of mTORC1 inhibition in the absence of the TSC complex.

To more closely investigate potential dose-dependent effects of metformin on mTORC1 signaling in a cell-autonomous manner, we compared the response of primary hepatocytes isolated from control or L*Tsc1*KO mice. To control for variations in response stemming from culture conditions, these comparisons were performed under serum-starved (Figure 3A), insulin-stimulated (Figure 3B), and serum-stimulated (Figure 3C) conditions. Although the pharmacodynamics varied somewhat, all three conditions showed similar results. Primary hepatocytes from control mice responded to metformin in a dose-dependent manner, with mTORC1 activity decreasing concomitantly with increased AMPK activity (Figure 3A–C). Similar to the *in vivo* findings and results with L-AMPK α1/α2-DKO hepatocytes, mTORC1 signaling was resistant to metformin in hepatocytes derived from L*Tsc1*KO mice at lower doses of metformin (0.5–1 mM). However, at 2 mM metformin mTORC1 was inhibited in both control and L*Tsc1*KO hepatocytes, supporting the existence of a TSC complex-independent mechanism at higher concentrations (Figure 3A–C). The effects of metformin on hepatocyte mTORC1 signaling were also time-dependent, with shorter durations (<5 h at 1 mM) having very little effect in L*Tsc1*KO hepatocytes compared to controls, while at 10 h of treatment mTORC1 was inhibited even in the absence of the TSC complex (Figure 3D). These data indicate that AMPK and the TSC complex are required to inhibit hepatic mTORC1 signaling acutely and at the lowest effective inhibitory doses of metformin, while more potent treatments engage additional mechanisms that bypass this first line of response.

We observed similar results in hepatocytes with acute deletion of *Tsc1*. We isolated $Tsc1^{fl/fl}$ hepatocytes and treated them with either adenovirus expressing GFP or Cre to delete *Tsc1* in an isogenic setting. Here, even partial loss of TSC1 sustained mTORC1 signaling after metformin treatment (Figure 3E). One possible explanation for the apparent higher degree of metformin resistance for mTORC1 regulation in these hepatocytes with acute deletion of *Tsc1* is that adaptive mechanisms to cope with sustained mTORC1 signaling might be induced in the *LTsc1KO* livers. Importantly, in this experiment and the others presented (Figures 3A–D), the induction of AMPK activity by metformin was similar between *Tsc1*-deleted and control hepatocytes. Consistent with the similarity in AMPK activation, there was no significant difference in the effects of metformin on oxygen consumption rates between control and L*Tsc1*KO hepatocytes (Figure 3F). Therefore, hepatocytes lacking the TSC complex remain responsive to metformin but lose the inhibitory signal from low-dose metformin to mTORC1.

Metformin inhibits protein synthesis in hepatocytes through the TSC complex

Aside from the inhibition of hepatic gluconeogenesis, the physiological effects of metformin in the liver are poorly understood. The acute induction of cap-dependent translation and, ultimately, a global increase in protein synthesis are key functions of mTORC1 signaling. In cancer cells, metformin has been found to have similar effects to mTOR inhibitors for the suppression of protein synthesis (Larsson et al., 2012). Thus, we analyzed the effects of metformin on events controlling cap-dependent translation initiation and protein synthesis in hepatocytes. The 5'-end of mRNAs is capped with a 7-methylguanosine (m^7GTP) moiety that is bound by the translation initiation protein eIF4E, which nucleates a translation initiation complex. When mTORC1 signaling is inhibited, its direct downstream target 4E-BP1 is dephosphorylated and able to bind to eIF4E at the m^7GTP cap, thereby blocking translation initiation. Consistent with its suppression of mTORC1 signaling in hepatocytes, metformin treatment induced binding of 4E-BP1 to eIF4E on m^7GTP-agrose beads, which mimic the mRNA 5'-cap structure (Figure 4A). Importantly, this effect was dependent on the TSC complex and inhibition of mTORC1, as metformin failed to induce association of 4EBP1 with m^7GTP-bound eIF4E in L*Tsc1*KO hepatocytes (Figure 4B). This result is consistent with sustained phosphorylation of 4EBP1 and other markers of mTORC1 activity upon metformin treatment of these cells.

To more directly measure the relative effects of metformin on active protein synthesis in control and L*Tsc1*KO hepatocytes, the acute incorporation of ^{35}S-methionine into protein over 20 minutes was assessed. Metformin treatment had profound inhibitory effects on protein synthesis in control hepatocytes, resulting in a 75% decrease (Figure 4C,D). However, L*Tsc1*KO hepatocytes, with sustained mTORC1 signaling, displayed a more modest reduction in protein synthesis of about 25% in response to metformin. Primary human hepatocytes responded remarkably similar to mouse hepatocytes, with doses of metformin greater than 0.2 mM activating AMPK with a corresponding decrease in mTORC1 signaling, accompanied by a dose-dependent decrease in protein synthesis (Figure 4E). Thus, metformin has strong inhibitory effects on hepatocyte protein synthesis, in large part, through AMPK and TSC complex-dependent inhibition of mTORC1 signaling.

Conclusions

This study demonstrates that metformin is a potent inhibitor of mTORC1 signaling and its control of protein synthesis in the liver. Like other biological effects of metformin (Foretz et al., 2014; He and Wondisford, 2015), the mechanisms influencing hepatic mTORC1 activity vary with dose. Our data reveal a biphasic response to metformin, where low doses inhibit mTORC1 through AMPK and the TSC complex and higher doses act through alternative mechanisms (Figure 4F). Importantly, even at higher doses of metformin, the acute inhibition of mTORC1 requires AMPK and the TSC complex, indicating that these proteins represent the primary signaling mechanism involved. While loss of either AMPK or the TSC complex from the liver result in qualitatively similar responses, AMPK-deficient livers are somewhat more resistant to metformin for mTORC1 inhibition. This is consistent with AMPK suppressing mTORC1 activation through both the TSC complex (Inoki et al., 2003) and phosphorylation of Raptor (Gwinn et al., 2008). The delayed induction of AMPK- and TSC complex-independent mechanisms revealed by prolonged treatment with metformin,

suggests that the alternative regulation might involve induction of transcriptional control mechanisms. In addition to providing mechanistic insights into the effects of metformin on key pathways and functions in the liver, our findings regarding the differential dose-dependent effects of metformin uncover a likely explanation for the discrepancy between previous studies with conflicting conclusions regarding the mechanism of mTORC1 inhibition by metformin. In subsequent studies, it will be important to determine the physiological consequences of hepatic mTORC1 inhibition for the influence of pharmacological doses of metformin on systemic metabolism.

EXPERIMENTAL PROCEDURES

See Supplemental Experimental Procedures for extended methods.

Mouse Studies

Tsc1$^{fl/fl}$ and L*Tsc1*KO mice on a C57BL/6J background were described previously (Yecies et al., 2011), with L*Tsc1*KO mice generated from crosses between *Tsc1*$^{fl/fl}$ and *Alb-Cre Tsc1*$^{fl/+}$ mice, through the albumin-Cre transgene (Postic and Magnuson, 2000; Kwiatkowski et al., 2002). Homozygous *AMPKα1*$^{fl/fl}$ and *AMPKα2*$^{fl/fl}$ mice previously described (Hasenour et al., 2014) on an FVB genetic background were bred with or without albumin-CreER^{T2} expression (Imai et al., 2000) were crossed to generate wild type controls (*AMPKα1*$^{fl/fl}$/*α2*$^{fl/fl}$) or inducible liver-specific deletion of both catalytic subunits of AMPK (*L-AMPKα1/α2-DKO*). The final cohorts were generated by tamoxifen (1 mg) treatment every other day for a total of 3 i.p. injections. Experiments, or primary hepatocyte isolation, were carried out approximately 2 weeks post-tamoxifen injection. Detailed methods are provided in Supplemental Experimental Procedures.

Primary Hepatocytes

Primary mouse hepatocytes were isolated as previously described (Yecies et al., 2011), and cryopreserved human hepatocytes were obtained from ThermoFisher (Lot # Hu8150). Oxygen consumption rates (OCR) were measured on an XF24 Seahorse extracellular flux analyzer (Seahorse Bioscience, 100867-100). Detailed methods are provided in Supplemental Experimental Procedures.

Protein Extraction, Immunoblotting, and Protein Synthesis Assays

Upon euthanization, liver tissue was harvested immediately and flash frozen in liquid nitrogen. 50–100 mg pieces of frozen liver tissue were dounce-homogenized in lysis buffer. Detailed methods regarding cell and tissue lysis, immunoblotting, antibodies used, and protein synthesis assays are provided in Supplemental Experimental Procedures

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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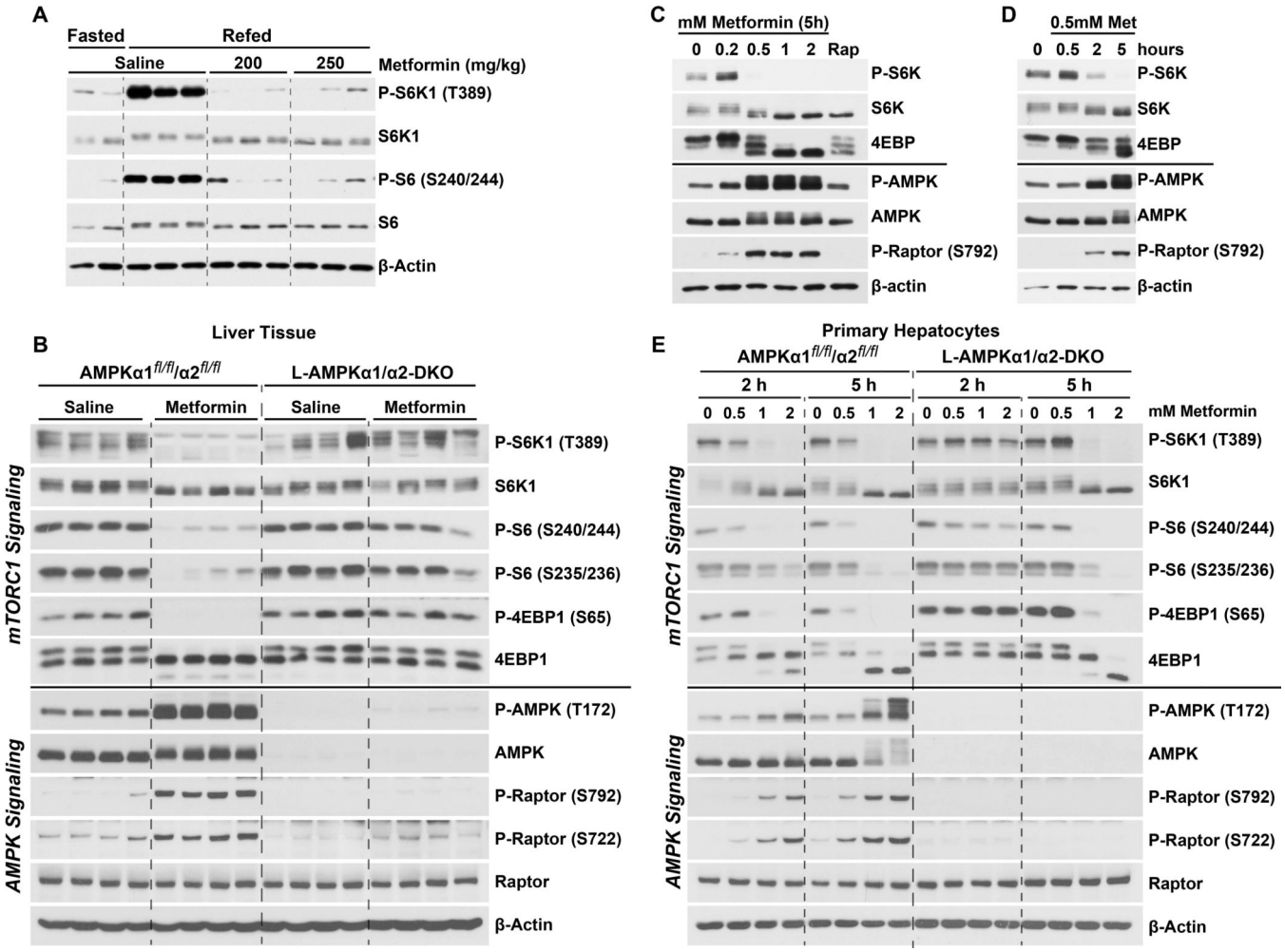

Figure 1. Metformin suppresses hepatic mTORC1 signaling in an AMPK-dependent manner

(A) Male mice (age 10 weeks) were fasted overnight then left unfed (Fast; n=2) or refed for 6 h, with saline, 200 or 250 mg/kg metformin treatment (n=3 per treatment) for the last 1 h. See Figure S1 for measurements of plasma metformin concentrations.

(B) Female *AMPKα1$^{fl/fl}$/α2$^{fl/fl}$* or *L-AMPKα1/α2-DKO* mice (age 6 mos) were fasted overnight, refed for 2 h and treated with saline or 250 mg/kg metformin for the last 1 h (n=4 per treatment). See Figure S1 for supporting data with a male cohort age 12 weeks.

(C,D) Primary hepatocytes were treated (C) for 5 h with indicated doses of metformin or 20 nM rapamycin (Rap) or (D) with 0.5 mM metformin for the indicated times.

(E) Primary hepatocytes from *AMPKα1$^{fl/fl}$/α2$^{fl/fl}$* or *L-AMPKα1/α2-DKO* mice were treated with the indicated concentrations of metformin for 2 or 5 h.

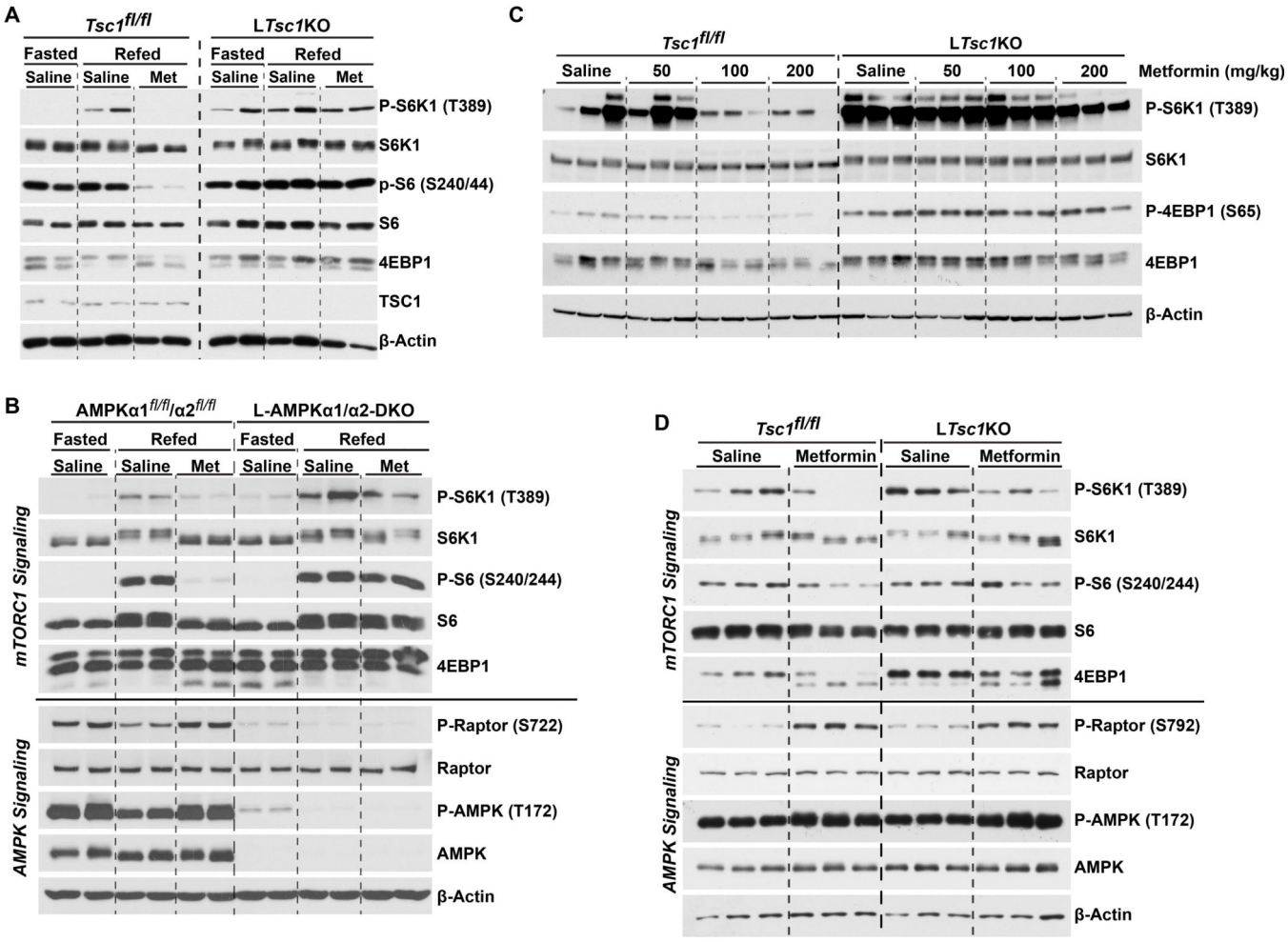

Figure 2. Mice with liver-specific disruption of the TSC complex display metformin-resistant mTORC1 signaling

(A) Male *TSC1*$^{fl/fl}$ or *LTsc1KO* mice (age 10 weeks) were fasted overnight then refed for 6 h and treated with saline or metformin for the last 1 h (n=2 per condition).

(B) Male *AMPKα1*$^{fl/fl}$*/α2*$^{fl/fl}$ or *L-AMPKα1/α2-DKO* (age 5 mos) mice were treated as in (A).

(C) Female *TSC1*$^{fl/fl}$ or *LTsc1KO* mice (age 12 week) were treated as in (A) with saline, 50, 100, or 200 mg/kg metformin (n=3 per condition).

(D) Female mice (age 14 weeks) treated as in (A) with saline or 250 mg/kg metformin (n=3 per condition).

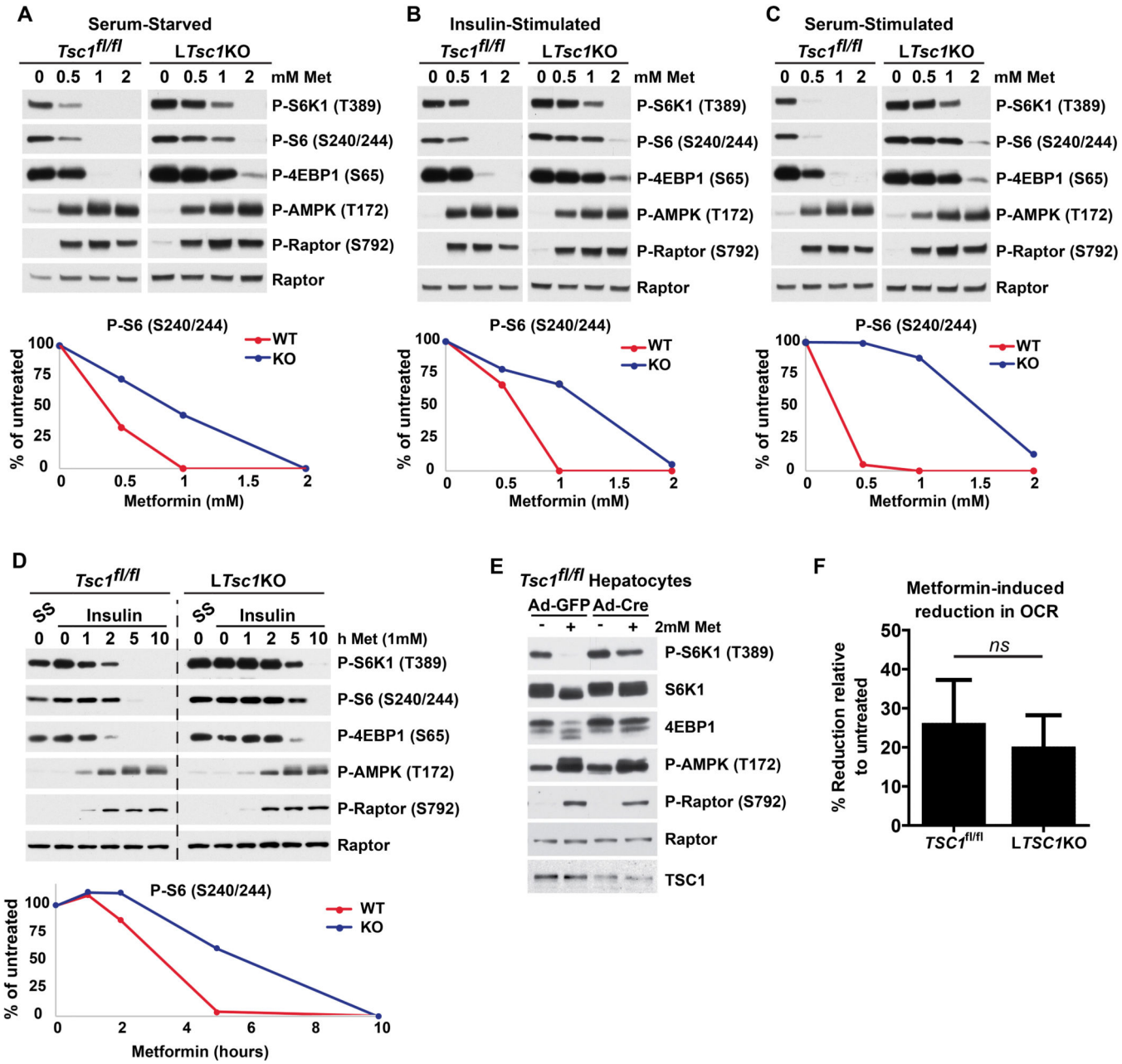

Figure 3. Low dose metformin suppresses hepatocyte mTORC1 signaling in a TSC complex-dependent manner

(A–D) Primary hepatocytes from *TSC1*$^{fl/fl}$ or *LTsc1KO* mice were serum-starved overnight and treated (A–C) for 5 h with increasing doses of metformin together with (A) no stimulation, (B) 100 nM insulin, or (C) 5% FBS, or (D) with a time course of treatment with 1 mM metformin following a 15-min pretreatment with 100 nM insulin. Quantitation of P-S6 band intensities via densitometry is shown in graphical format below each panel.

(E) Primary hepatocytes from *TSC1*$^{fl/fl}$ mice were infected with adenovirus expressing GFP or Cre 36 h prior to treatment with 2 mM metformin for 5 h.

(F) Oxygen consumption rates (OCR) of primary hepatocytes from *TSC1*$^{fl/fl}$ or *LTsc1KO* mice were measured for 1 h in the presence of 1 mM metformin and are graphed as the mean ± SEM OCR as a percent of untreated samples measured in parallel (n=4 experiments).

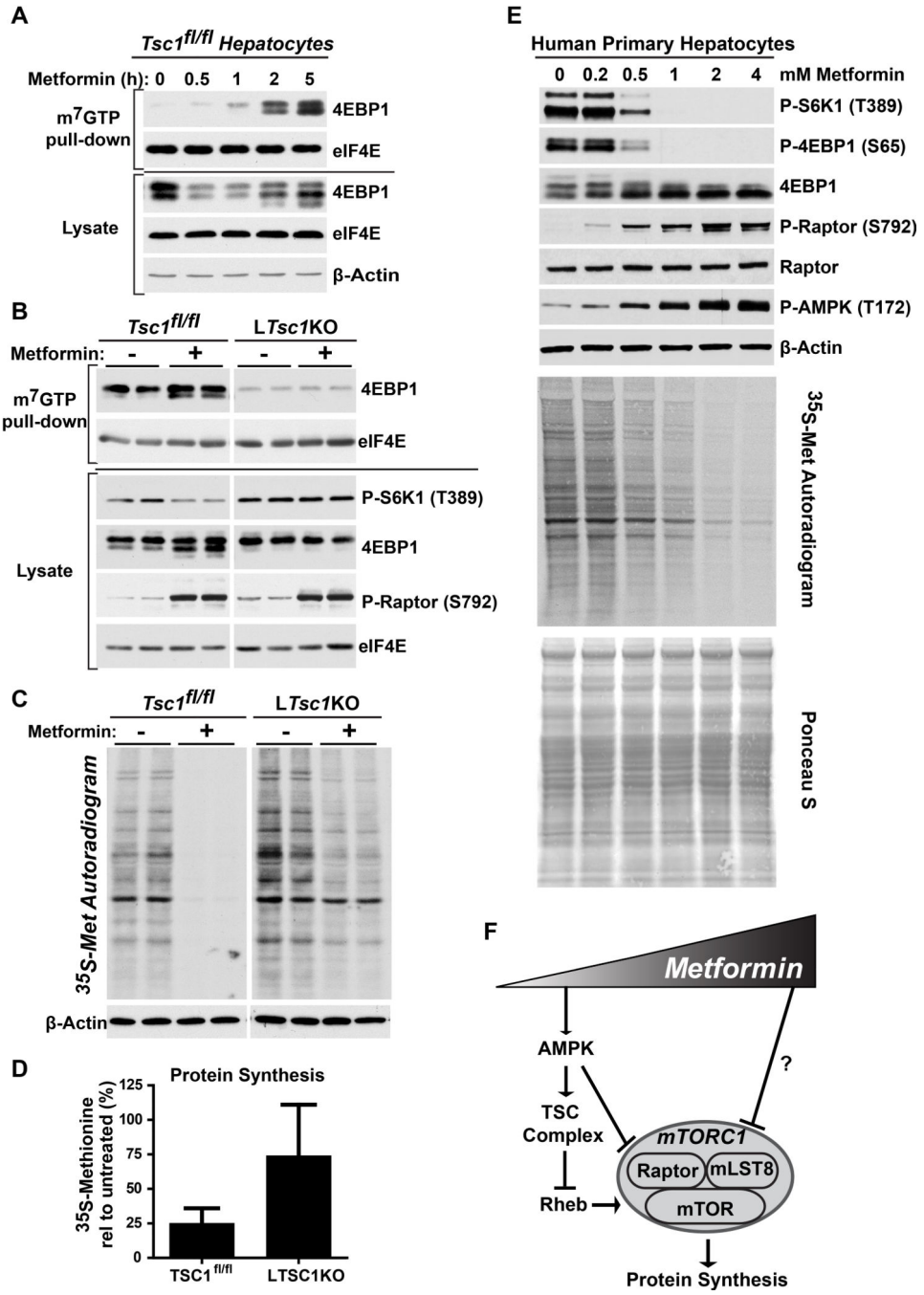

Figure 4. Metformin inhibits protein synthesis in hepatocytes through the TSC complex

(A) Primary hepatocytes from *TSC1*$^{fl/fl}$ mice were treated with 1 mM metformin for the indicated times, and mRNA 5'-cap-binding complexes were isolated from cell lysates with m^{7}GTP-agarose and analyzed by immunoblotting.

(B) Primary hepatocytes from *TSC1*$^{fl/fl}$ and *LTsc1KO* mice were treated with 1 mM metformin for 5 h and analyzed as in (A).

(C and D) Effects of metformin on hepatocyte protein synthesis. Primary hepatocytes from *TSC1*$^{fl/fl}$ and *LTsc1KO* mice were pretreated with insulin for 20 min followed by treatment

in the presence or absence of 1 mM metformin for 5 h, with a pulse label of [^{35}S]-methionine for the final 20 min. A representative autoradiogram from 5 independent experiments is shown. (D) Individual lanes from autoradiograms were quantified by densitometry and are graphed as mean ± SEM % incorporation relative to untreated cells (n=5 independent experiments). *P < 0.05 by Student's two-tailed t-test.

(E) Primary human hepatocytes were treated with the indicated doses of metformin for 5 h and, for assaying protein synthesis, were radiolabeled as in (C). Signaling was assessed by immunoblots, protein synthesis by autoradiogram, and total protein for this assay by Ponceau S staining.

(F) Model of dose-dependent, differential regulation of mTORC1 in response to increasing metformin concentrations.