Cell Metabolism
Short Article

Duration of Rapamycin Treatment Has Differential Effects on Metabolism in Mice

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SUMMARY

The evolutionarily conserved target of rapamycin (TOR) signaling controls growth, metabolism, and aging. In the first robust demonstration of pharmacologically-induced life extension in mammals, longevity was extended in mice treated with rapamycin, an inhibitor of mechanistic TOR (mTOR). However, detrimental metabolic effects of rapamycin treatment were also reported, presenting a paradox of improved survival despite metabolic impairment. How rapamycin extended lifespan in mice with such paradoxical effects was unclear. Here we show that detrimental effects of rapamycin treatment were only observed during the early stages of treatment. These effects were reversed or diminished in mice treated for 20 weeks, with better metabolic profiles, increased oxygen consumption and ketogenesis, and markedly enhanced insulin sensitivity. Thus, prolonged rapamycin treatment lead to beneficial metabolic alterations, consistent with life extension previously observed. Our findings provide a likely explanation of the "rapamycin paradox" and support the potential causal importance of these metabolic alterations in longevity.

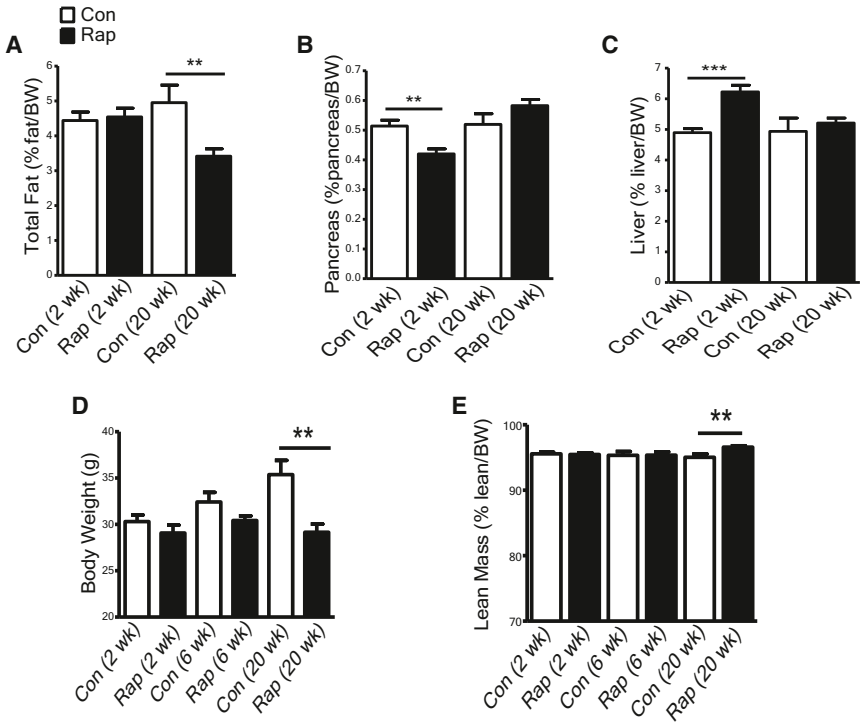
INTRODUCTION

mTOR (mechanistic target of rapamycin) is a master regulator of growth and metabolism. It senses upstream inputs of growth factors (such as insulin), nutrients, and energy status to regulate downstream events by its complex 1 (mTORC1) and/or its complex 2 (mTORC2) (Wullschleger et al., 2006). Rapamycin inhibits mTORC1, but longer rapamycin treatment also affects mTORC2 (Sarbassov et al., 2006). With various genetic backgrounds, extension of longevity was reported in mice with knockout of S6K1, a target of mTOR (Selman et al., 2009), and in mice fed (Harrison et al., 2009) or injected (Chen et al., 2009; Anisimov et al., 2011) with rapamycin, but rapamycin treatment was repeatedly reported to produce detrimental metabolic changes usually associated with reduced rather than extended longevity, including insulin resistance, hyperlipidemia, and glucose intolerance (Houde et al., 2010; Chang et al., 2009; Fraenkel et al., 2008). Thus, it remains unclear how rapamycin extends the lifespan of mice. In various studies, the length of rapamycin treatment ranged from 2 (Houde et al., 2010; Fraenkel et al., 2008) or 6 weeks (Chen et al., 2009; Chang et al., 2009) to 1.5–2 years (Harrison et al., 2009; Anisimov et al., 2011), when rapamycin led to longer survivorship. We hypothesized that these paradoxical findings might be due to differences in the duration of treatment and that longer rapamycin treatment might change the metabolic parameters controlled by insulin signaling toward a beneficial profile. To test this hypothesis, we compared the effects of injecting adult male mice with rapamycin for 2, 6, or 20 weeks. Indeed, the mice experienced negative effects of rapamycin treatment, including insulin resistance with short duration, but insulin signaling changed from an insulin resistant to an insulin-sensitive state after 20 weeks of rapamycin treatment.

RESULTS

Duration of Rapamycin Treatment Changed Body Features

Starting at an age of 3 months, the male mice were injected with rapamycin for 2, 6, or 20 weeks according to the protocol by Chen et al. (2009) and were then sacrificed when rapamycin treatments were completed. With 2 or 6 weeks of rapamycin treatment, adiposity, body weight (BW), and food consumption were not altered; however, after 20 weeks of treatment, they were reduced dramatically (Figures 1A, 1D, 1F, and 1G and Figure S1A available online) without significant changes in lean body mass (Figures 1E and S1B). Prolonged rapamycin treatment prevented normal body weight gain (Figures 1D and 1F) mainly due to decreased adiposity (Figure 1A, S1A, 1E, and S1B and data not shown). Pancreas mass was reduced after 2 weeks of rapamycin treatment but was restored with 20 weeks of treatment (Figures 1B and S1C). Liver mass was increased after 2 weeks of rapamycin treatment, but it no longer differed from that of controls after 20 weeks of treatment (Figures 1C and S1D). Thus, body features associated with metabolic syndrome, including smaller pancreas and enlarged liver, appeared in the mice with 2 weeks of rapamycin treatment, but with continued treatment these features returned to normal levels, and adiposity, body weight, and food consumption were decreased. The most striking differences between the effects of short versus prolonged rapamycin treatment concern insulin signaling, glucose and lipid homeostasis, and metabolism.



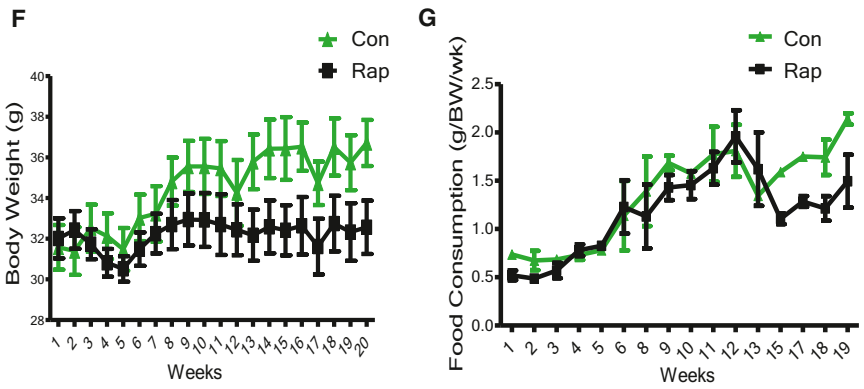


Figure 1. Body Characteristics Alter with Duration of Rapamycin Treatment

(A) Relative total fat mass [absolute value (g) / body weight (g)] × 100. Total fat includes subcutaneous fat from the thighs, a pair of perigonadal (visceral) depots, a pair of perinephric depots, and interscapular brown fat.

(B) Relative mass of the pancreas.

(C) Relative mass of the liver.

(D) Body weight.

(E) Relative total lean mass [absolute value (g)/body weight (g)] × 100. Total lean mass is estimated as follows: lean mass = body weight – total fat mass.

(F) Time course of body weight measured over 20 weeks of rapamycin treatment.

(G) Time course of food consumption over 19 weeks of rapamycin treatment.

Data are means ± SEM (n = 8–16 for control, n = 9–16 for rapamycin). p values were calculated between the rapamycin-treated and control groups at each time point independently. The error bars represent the SEM. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. See also Figure S1.

Prolonged Rapamycin Treatment Increased Insulin Sensitivity

Insulin signaling is important in the control of longevity in both mice and humans, although the specific mechanisms are not completely understood and some findings are controversial (Barzilai et al., 2012). Normally, insulin inhibits hepatic gluconeogenesis and increases lipogenesis. In individuals exhibiting insulin resistance, excess insulin is produced as a compensatory mechanism. When insulin resistance develops, insulin initially loses its ability to inhibit hepatic gluconeogenesis but maintains the ability to enhance lipogenesis. As a result, hyperglycemia and hypertriglyceridemia occur (Matsumoto et al., 2006). mTORC1 is required for insulin-induced stimulation of lipogenesis but not for inhibition of gluconeogenesis (Li et al., 2010), and 2 weeks of rapamycin treatment inhibited lipogenesis and upregulated gluconeogenesis (Houde et al., 2010). Similarly, 2 weeks of rapamycin treatment in our study increased insulin levels 2.5-fold compared with controls (Figure 2A). The mice became glucose intolerant (Figure 2D) and insulin resistant (Figure 2E), with increased glucose (Figure 2B and Table S1) and HOMA-IR (homeostatic model for assessment of insulin resistance) levels (Figure 2C). However, with 6 weeks of rapamycin treatment, the mice experienced a transition from insulin resistance toward an improved metabolic state (Figure 2A–2C, 2F, and 2G and Table S1). Strikingly, with 20 weeks of rapamycin treatment, both insulin levels and insulin sensitivity were altered in a direction opposite to that observed with 2 weeks of treatment: insulin levels decreased (Figure 2A), while insulin sensitivity increased (Figure 2I) drastically compared with the controls. Although glucose intolerance remained, especially at the early stage of the glucose tolerance test (GTT; Figure 2H), HOMA-IR values derived from both insulin and glucose levels were significantly reduced (Figure 2C). These findings indicate that with prolonged rapamycin treatment, the mice progressed from being insulin resistant to having improved insulin sensitivity.

Prolonged Rapamycin Treatment Improved Lipid Profile and Metabolism

Lipid homeostasis showed similar but more complex responses. Lipolysis breaks down triglycerides into glycerol and free fatty acids (measured as nonesterified fatty acids [NEFAs]), and lipogenesis generates triglycerides. Insulin inhibits lipolysis but enhances lipogenesis. Consequently, when insulin levels are high, less glycerol and NEFAs and more triglycerides are generated (Prentki and Madiraju, 2012). In our study, after 2 weeks of rapamycin treatment, the mice had increased insulin levels (Figure 2A), likely explaining lower glycerol (Figure 3A) and higher triglycerides (Figure 3C). After 6 weeks of rapamycin treatment,

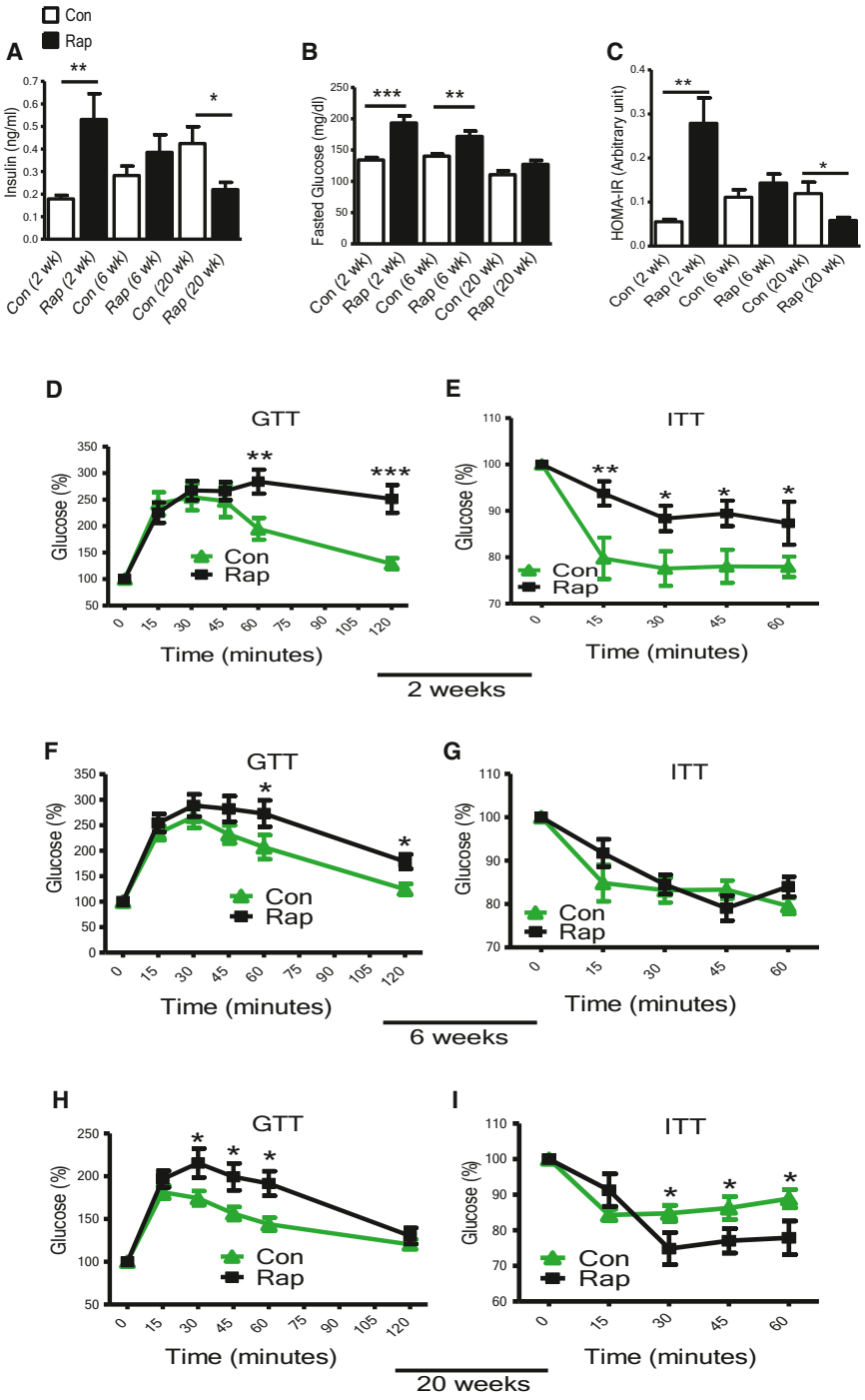


Figure 2. Insulin Signaling and Glucose Homeostasis Switch from Insulin Resistant State with Short Rapamycin Treatment to Insulin Sensitive State with Prolonged Rapamycin Treatment

(A) Plasma insulin levels.

(B) Fasted glucose levels.

(C) Homeostatic model for assessment of insulin resistance (HOMA-IR). HOMA-IR = (FPI × FPG) / 22.5, where FPI is fasted plasma insulin and FPG is fasted plasma glucose. Plasma and fasted glucose values were collected at sacrifice.

(D, F, and H) Glucose tolerance test (GTT). Sixteen-hour-fasted mice underwent GTT by intraperitoneal (i.p.) injection with 1 g glucose per kg of BW.

(E, G, and I) Insulin tolerance test (ITT). Mice were i.p. injected with 1 IU porcine insulin per kg of BW. Data are mean ± SEM (n = 8–16 for control, n = 9–16 for rapamycin). p values were calculated between the rapamycin-treated and control groups at each time point independently. The error bars represent the SEM.*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. See also Figure S2 and Table S1.

increase in insulin levels was much less pronounced, but insulin was still higher than in controls (Figure 2A); glycerol and NEFA levels were suppressed (Figures 3A and 3B), while triglycerides remained elevated (Figure 3C). In contrast, after 20 weeks of rapamycin treatment, insulin levels were reduced significantly (Figure 2A) and triglycerides declined to control levels (Figure 3C). Thus, also with regard to lipid homeostasis, the duration of rapamycin treatment determined the direction of the changes. Similar to the findings in human renal transplant patients, who received rapamycin as an immunosuppressant for 12 months (Blum, 2002), hypertriglyceridemia detected after short rapamycin treatment was normalized when the treatment was continued for 20 weeks. Although the mice treated with rapamycin for 20 weeks no longer had hypertriglyceridemia, they could have had elevated levels of free fatty acids, reflecting enhanced lipolysis and reduced lipogenesis. Elevation of free fatty acids is associated with the metabolic syndrome (Prentki and Madiraju, 2012). Interestingly, NEFA levels were not increased by 20 weeks of rapamycin treatment (Figure 3B). What other lipid metabolic changes could have developed with 20 weeks of rapamycin treatment to produce a decrease of free fatty acids? Adipose-specific knockout of Raptor results in mice being lean due to enhanced oxygen consumption (VO_2) without affecting respiratory quotient (RQ) (Polak et al., 2008). Could the mice treated with rapamycin for 20 weeks in our study have higher oxygen consumption (VO_2) to burn more fatty acids? Similar to the previous report (Cunningham et al., 2007), the mice with 2 weeks of rapamycin treatment had reduced VO_2 (Figure 3D), suggesting that they expend less energy. An intermediate state was observed in mice treated with rapamycin for 6 weeks, such that VO_2 was not significantly different from the controls (Figure 3E). In contrast, after 20 weeks of rapamycin treatment, the mice had higher VO_2 than the controls (Figure 3F), suggesting that they expend more energy, which was consistent with the findings in

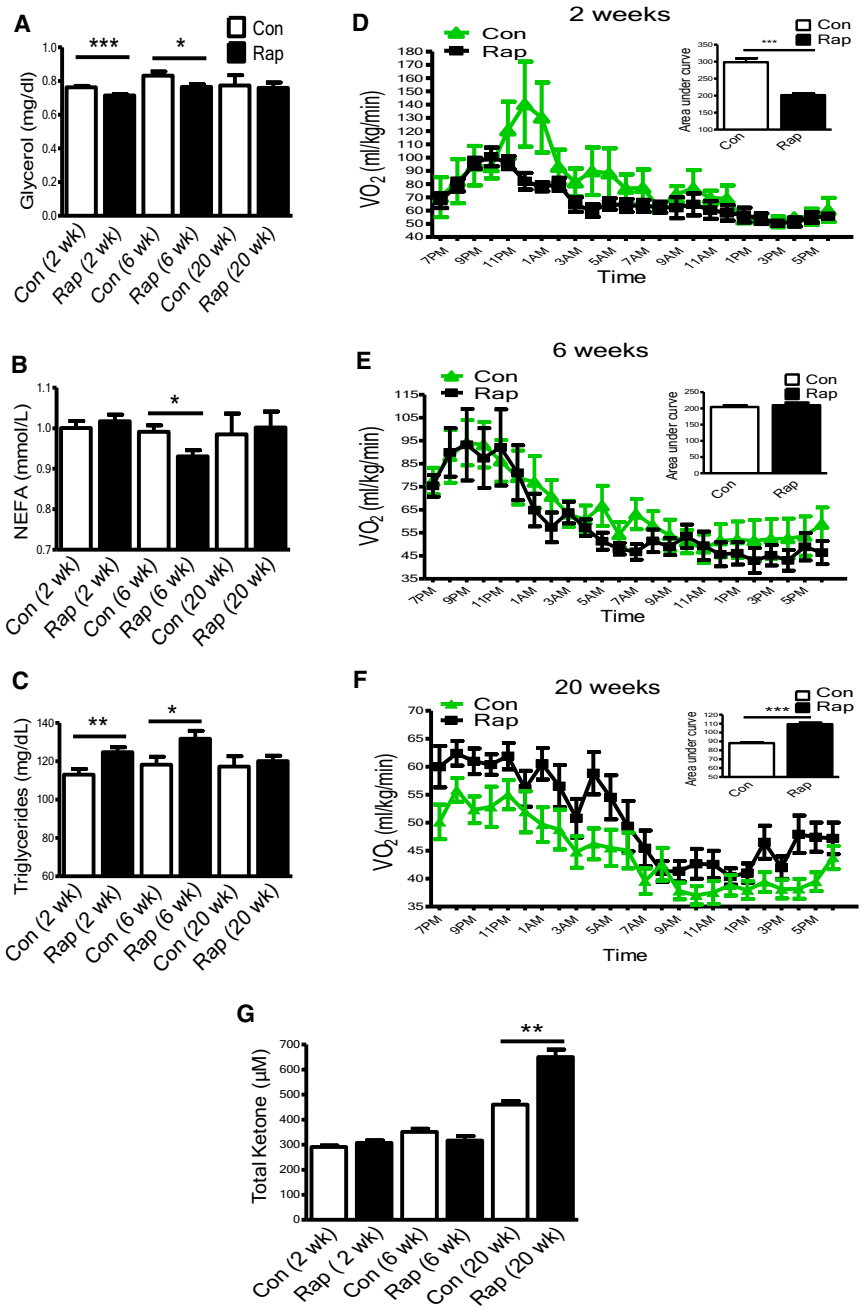


Figure 3. Lipid Profile, Oxygen Consumption, and Total Ketone Body Production Chang after Different Lengths of Rapamycin Treatment

(A) Plasma glycerol levels.

(B) Plasma NEFA levels.

(C) Plasma triglycerides levels. For plasma chemical analysis, mice were fasted for 16 hr, and thusly collected plasma was subjected to the assays.

(D–F) Oxygen consumption VO_2 (ml/kg/min) measured via indirect calorimetry (AccuScan Instruments, Columbus, OH) after 2 weeks (D), 6 weeks (E), or 20 weeks (F) of rapamycin treatment. Data are mean ± SEM (n = 8 for control or rapamycin).

(G) Plasma total ketone bodies. Mice were fasted for 16 hr, and plasma total ketone bodies were measured with total ketone body kits (Wako, Richmond, VA). Data are mean ± SEM (n = 8–16 for control, n = 9–16 for rapamycin).

The error bars represent the SEM.*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

mice with adipose-specific knockout of Raptor (Polak et al., 2008). Duration of rapamycin treatment did not alter RQ (Figure S2) or spontaneous locomotor activity (data not shown). The present findings indicate that as the length of rapamycin treatment increased from 2 and 6 to 20 weeks, the energy metabolism was switched from lower energy expenditure and reliance on carbohydrates to higher energy expenditure and increased reliance on burning fatty acids. Fatty acid breakdown is also related to ketogenesis. mTOR was reported to control fasting-induced ketogenesis, and this process was linked to modulation of aging (Sengupta et al., 2010). Could it be possible that with 20 weeks of rapamycin treatment the mice also have enhanced ketogenesis in our study? Indeed, the levels of total ketone bodies were not altered after 2 or 6 weeks of rapamycin treatment but were significantly increased when the treatment was continued for 20 weeks (Figure 3G).

Both mTORC1 and mTORC2 Were Involved in Metabolic Alterations by Duration of Rapamycin Treatment

Given the metabolic alterations we observed, it is crucial to know whether duration of the treatment changed the levels or effectiveness of rapamycin. With the same biochemical readout of rapamycin effectiveness used by Harrison et al. (2009), phosphorylation of ribosomal protein subunit S6 in liver was analyzed. The blood levels of rapamycin (Figure 4C) and inhibition of phosphorylation of S6 with longer rapamycin treatment (Figures 4A and 4B) were similar to those reported by Harrison et al. (2009). However, both the levels of rapamycin and inhibition of pS6 were not altered with duration of the treatments (Figure 4). Although S6 is a remote downstream target of mTORC1, the metabolic switch caused by duration of the treatments was not differentially regulated at the level of pS6. To test whether the regulation was upstream of S6 and whether mTORC2 was also involved, we measured mTOR, Raptor (a key component of mTORC1), Rictor (a key component of mTORC2), S6K1 and 4EBP1 (direct downstream targets of mTORC1), and AKT (a downstream target of mTORC2). Duration of rapamycin treatment did not change the levels of these proteins. Two weeks of rapamycin treatment reduced phosphorylation of mTOR, S6K1, 4EBP1, and AKT and 6 weeks of the treatment led to the transition stage, while 20 weeks of rapamycin treatment had reverse

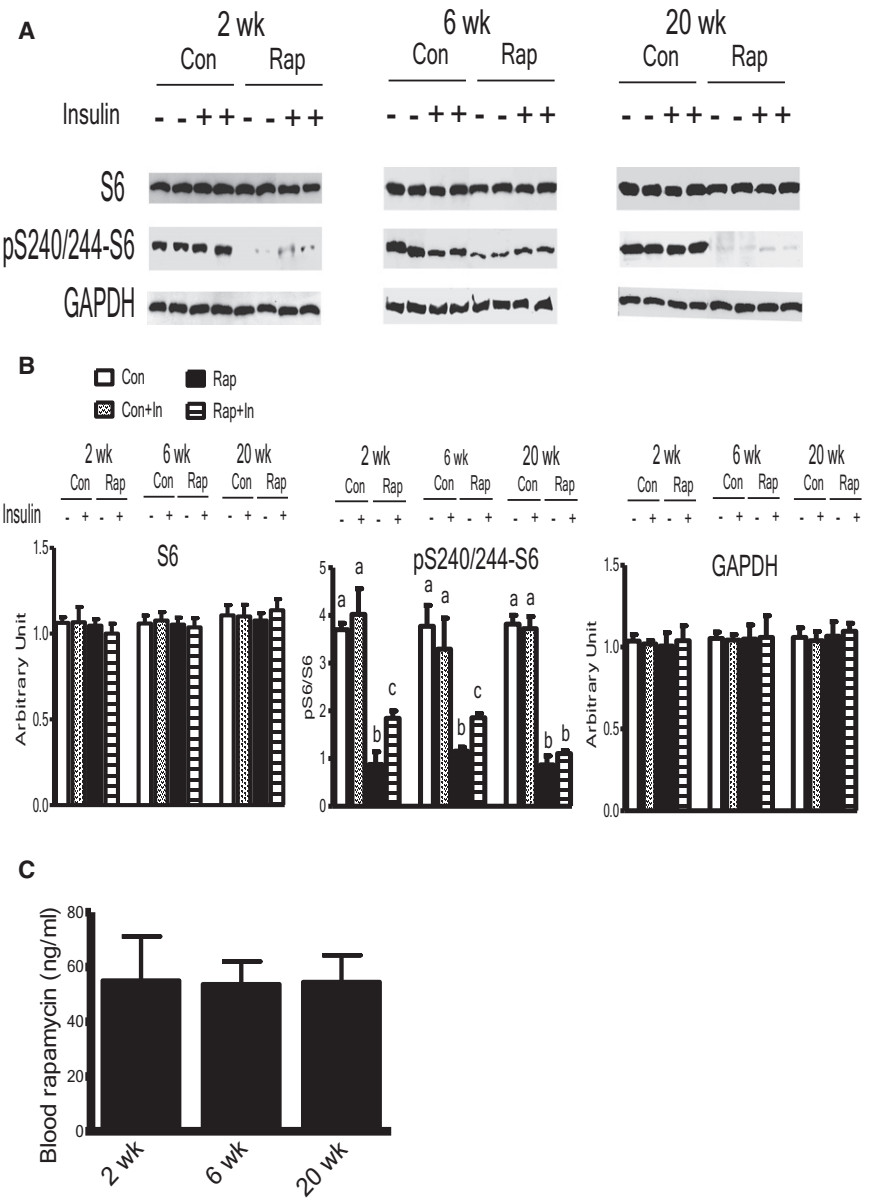


Figure 4. Effects of Different Duration of Rapamycin Treatment on the Blood Levels of Rapamycin and Phosphorylation of S6 in the Liver

(A) Western blots of S6 and phosphorylation of S6 at S240/244. Western blots are representative of three independent experiments. Each treatment includes six samples from six individual animals.

(B) Quantification of the western blots. Data are means ± SEM (n = 6).

(C) Blood levels of rapamycin. Data are means ± SEM (n = 4–6).

p values were calculated between the rapamycin-treated and control groups at each time point independently. Different letters represent $p \leq 0.05$. The error bars represent the SEM. See also Figures S3 and S4.

effects, compared with shorter rapamycin treatments (Figures S3 and S4). Thus, various measures of insulin signaling and its downstream metabolic effects changed drastically from unfavorable after 2 or 6 weeks of rapamycin treatment toward largely beneficial after 20 weeks of rapamycin treatment. In other words, duration of rapamycin treatment emerges as a key determinant of not only the magnitude but, importantly, also the direction of the responses.

DISCUSSION

The terms "prolonged" or "chronic" used to describe rapamycin treatment in various studies have been confusing. Several studies used a period of 2 to 6 weeks as prolonged or chronic rapamycin treatment (Houde et al., 2010; Chang et al., 2009; Fraenkel et al., 2008), while much longer periods of exposure (1.5–2 years) showed the ability of rapamycin to extend longevity (Harrison et al., 2009; Anisimov et al., 2011). Although various genetic backgrounds of mice, as well as various diets and methods of rapamycin administration, were used in the previous studies (Houde et al., 2010, Lamming et al., 2012), our 2 weeks of rapamycin treatment showed similar detrimental effects of short rapamycin treatment on insulin signaling and related downstream events. Results of 6 weeks of rapamycin treatment generally resembled the effects of 2 weeks of rapamycin treatment but appeared to represent a transitional state with regard to insulin sensitivity and HOMA-IR. In contrast, 20 weeks of rapamycin treatment had very different and generally "beneficial" effects, which may be a mechanism of extended longevity in mice by rapamycin (Harrison et al., 2009; Anisimov et al., 2011). The present study differed from the previous report by Harrison et al. (2009) in the genetic background of the mice, the source and composition of the diet, and the route of rapamycin administration; however, both studies involved long-term treatment with rapamycin and utilized a diet with a similar fat content (5.0% versus 4.6%), and both used genetically heterogeneous mice with partial commonality in the strains from which they were derived.

Rapamycin inhibition of mTOR signaling is primarily due to its actions on mTORC1, although prolonged rapamycin treatment also affects mTORC2. Hepatic mTORC2 was reported to mediate rapamycin-induced insulin resistance; however, short (2 to 4 weeks) rapamycin treatment in mice with hepatic Rictor deletion caused a further pronounced increase of insulin and glucose levels (Lamming et al., 2012). These data indicate that the actions of mTORC1 and mTORC2 might not be fully separate, and instead it may be their balance that determines the final impact on insulin signaling and longevity (Hughes and Kennedy,

2012). Our biochemical data showed that duration of rapamycin treatment affected not only levels of phosphorylation of mTOR, S6K1, 4EBP1, and AKT, but also direction of the responses. Based on our data, we speculated that both mTORC1 and mTORC2 played roles in these differential responses (Figures S3 and S4 and data not shown). Unexpectedly, considering the similar blood levels of rapamycin and inhibition of pS6 (Figure 4), prolonged rapamycin treatments in the mice no longer inhibited levels of pmTOR, pS6K1, p4EBP1, or pAKT, while it remained effective in inhibiting pS6 (Figures S3 and S4). The molecular mechanism for the disparate responses of pS6K1 and pS6 remains unclear. However, S6K2 could also play a significant role, as S6K2, not S6K1, is the predominant kinase for S6 phosphorylation (Pende et al., 2004). In addition, phosphorylation of S6K1 at T389 is not the only regulatory mechanism for phosphorylation of S6. Nevertheless, the striking difference in the insulin signaling responses might well underlie the distinct physiological effects and might potentially explain why prolonged rapamycin treatment is beneficial. The exact mechanisms of the counterintuitive signaling outcome upon prolonged rapamycin treatment will be investigated in future studies.

In the present study, alterations in insulin sensitivity induced by different durations of rapamycin treatment were closely associated with changes of glucose and lipid homeostasis and metabolism, as well as body composition. After 20 weeks of rapamycin treatment, the mice were lean with enhanced insulin sensitivity (measured by insulin tolerance test [ITT]; Figure 2I), increased oxygen consumption and ketogenesis, and improved serum lipid profile. The mice with prolonged rapamycin treatments showed a certain degree of glucose intolerance, especially at the early stages of GTT (Figure 2H), but those mice were able to clear glucose, albeit at a slower pace due to lower basal levels of insulin and higher insulin sensitivity (Figures 2A and 2I). Most of these phenotypic features are associated with extended longevity in several kinds of mutant mice (Bartke, 2005) and therefore can be viewed as candidate mechanisms of life extension in animals exposed to rapamycin (Harrison et al., 2009; Anisimov et al., 2011). In support of this suggestion, deletion of S6K1 attenuated age-related loss of insulin sensitivity and increased lifespan in mice (Selman et al., 2009). By showing differences between metabolic responses to short versus long-term rapamycin treatment, the present findings provide a likely explanation of the paradox of reported detrimental effects of rapamycin on insulin signaling and its ability to extend longevity.

EXPERIMENTAL PROCEDURES

Mice Maintenance

The animal procedures were approved by the Laboratory Animal Care and Use Committee of Southern Illinois University School of Medicine. Mice were housed under temperature- and light-controlled conditions (22°C ± 1°C, 12 hr light/12 hr dark cycle) with ad libitum access to food (Chow 5001 with 23.4% protein, 5% fat, and 5.8% crude fiber; LabDiet PMI Feeds, St. Louis, MO). Our breeding colony was developed by mating of mice with 129 Ola/BALB/c background with mice derived from crossing of C57BL/6 and C3H strains and thereafter breeding of the resulting animals in a closed colony without brother × sister matings. The colony generated thus has a heterogeneous genetic background. Beginning at the same age (3 months), male mice were injected i.p. with 4 mg/kg BW rapamycin (LC labs, Woburn, MA) or vehicle every other day according to the protocol by Chen et al. (2009) and were then sacrificed after 2, 6, or 20 weeks of rapamycin treatment.

Glucose Tolerance Test and Insulin Tolerance Test

Sixteen-hour-fasted mice underwent GTT by i.p. injection with 1 g glucose per kg of BW. Blood glucose levels were measured at 0, 15, 30, 45, 60, and 120 min with a PRESTO glucometer (AgaMatrix, Salem, NH) for GTT. Nonfasted mice were injected i.p. with 1 IU porcine insulin (Sigma, St. Louis, MO) per kg of BW. Blood glucose levels were measured at 0, 15, 30, and 60 min for ITT. The data for both ITT and GTT are presented as a percentage of baseline glucose.

Indirect Calorimetry

Mice injected for 2, 6, or 20 weeks with rapamycin were subjected to indirect calorimetry (AccuScan Instruments, Columbus, OH). The system uses zirconia, infrared sensors and light beam arrays to monitor oxygen consumption (VO_2), carbon dioxide output (VCO_2), and spontaneous locomotor activity inside respiratory chambers in which individual mice were tested. After 24 hr acclimation, mice were monitored in the chambers for 24 hr with ad libitum access to food (Chow 5001; LabDiet PMI Feeds) and water. All comparisons are based on animals studied simultaneously in eight different chambers connected to the same O_2, CO_2, and light beam sensors. Gas samples were collected and analyzed every 5 min per animal, and the data were averaged for each hour.

Assessment of Blood Chemistry

After 2, 6, or 20 weeks of rapamycin treatment, mice were fasted for 16 hr and sacrificed. Plasma was collected at sacrifice. Per the manufacturer's protocol, insulin was measured with Mouse Insulin ELISA Kits (Crystal Chem, Downers Grove, IL), total ketone bodies and NEFAs were measured via colorimetric assays from Wako Chemicals (Richmond, VA), glycerol was measured with kits from Sigma (St. Louis, MO), and triglycerides were measured with kits from Pointe Scientific (Canton, MI). The whole-blood samples were used to measure rapamycin levels using HPLC-tandem MS as detailed in the Supplemental Experimental Procedures.

Western Blot Analysis

Mice were fasted for 16 hr. Plasma was collected, and then half of the animals from each experimental group were injected with insulin or saline through the liver portal vein to stimulate insulin signaling pathway with a previously described protocol (Bonkowski et al., 2009). Two minutes after insulin injection, mice were sacrificed and the liver tissues were collected. Approximately 500 mg liver samples were homogenized in 0.5 ml ice-cold T-PER tissue protein extraction buffer (Thermo Scientific, Rockford, IL) with protease and phosphatase inhibitors (Sigma). Total protein (40 μg) was separated by SDS-PAGE with Criterion XT Precast Gel (Bio-Rad, Hercules, CA) and blotted with the antibodies. Antibodies, including mTOR, phospho-mTOR (Ser2448), Raptor, phospho-Raptor (Ser792), Rictor, phospho-Rictor (Thr1135), S6K1, phospho-S6K1(Thr389), 4EBP1, phospho-4EBP1(Ser65), AKT, phospho-AKT(Ser473), S6, phospho-S6 (Ser240/244), and GAPDH, were obtained from Cell Signaling Technology (Beverly, MA). Western blots were quantified with Multi Gauge V3.0 software (Fujifilm North America, Edison, NJ).

Statistical Analysis

Data are presented as means ± SEM. The error bars represent SEM. Differences between two groups were assessed with unpaired two-tailed Student's t tests. All statistical analyses were conducted with SPSS Statistics 17.0 (SPSS Company, Quarry Bay, Hong Kong).

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2013.02.008.

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