

Full Paper

Rapamycin Protects Against High Fat Diet–Induced Obesity in C57BL/6J MiceGeng-Ruei Chang¹, Yi-Shin Chiu¹, Ying-Ying Wu¹, Wen-Ying Chen¹, Jiunn-Wang Liao², Te-Hsin Chao^{1,3}, and Frank Chiahung Mao^{1,*}¹Department of Veterinary Medicine, and ²Graduate Institute of Veterinary Pathobiology, National Chung Hsing University, 250, Kuo Kuang Road, Taichung, Taiwan, 40227³Department of Colorectal Surgery, Taichung Veterans General hospital, 160, Chung-Kang Road, Taichung, Taiwan, 40705

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Abstract. Rapamycin (RAPA), an immunosuppressive drug used extensively to prevent graft rejection in transplant patients, has been reported to inhibit adipogenesis *in vitro*. In this study, we investigated the anti-obesity effects of RAPA in C57BL/6J mice on a high-fat diet (HFD). Mice treated with RAPA (2 mg/kg per week for 16 weeks) had reduced body weight and epididymal fat pads/body weight, reduced daily food efficiency, and lower serum leptin and insulin levels compared with the HFD control mice. However, RAPA-treated mice were hyperphagic, demonstrating an increase in food intake. Dissection of RAPA-treated mice revealed a marked reduction in fatty liver scores, average fat cell size, and percentage of large adipocytes of retroperitoneal and epididymal white adipose tissue (RWAT and EWAT), compared to the HFD control mice. These results suggest that RAPA prevented the effect of the high-fat diet on the rate of accretion in body weight via reducing lipid accumulation, despite greater food intake. It is likely that RAPA may serve as a potential strategy for body weight control and/or anti-obesity therapy.

[Supplementary Tables: available only at <http://dx.doi.org/10.1254/jphs.08215FP>]**Keywords:** rapamycin, obesity, food intake, adipose tissue, leptin**Introduction**

Obesity is a global health problem and is an important risk factor for metabolic syndromes such as insulin resistance, hypertension, hyperlipidemia and potentially, type 2 diabetes, cardiovascular diseases, and nonalcoholic fatty liver diseases (1–3). When an imbalance occurs between energy intake and expenditure, obesity may result due to increased adipose mass (4). On the other hand, the mammalian target of the rapamycin (mTOR) pathway is an evolutionarily conserved signaling network that plays critical roles in eukaryotic cell growth, survival, and translation (5). Moreover, the mTOR pathway integrates input from multiple upstream pathways, including those involving insulin, growth factors, nutrients, mitogens, and energy (6). Furthermore,

increased activation of the mTOR pathway has been linked to obesity and diabetes (7, 8).

The anti-fungal macrolide rapamycin (RAPA, also known as sirolimus), is an mTOR kinase inhibitor isolated from the soil bacterium *Streptomyces hygroscopicus* (9). RAPA produces inhibiting effects by binding to the immunophilin FK506 binding protein (FKBP12) to form a complex, which then binds directly to mTOR, inhibiting mTOR and the mTOR-mediated signaling network (10). Subsequently, RAPA was shown to have potent immunosuppressive and antiproliferative effects (11). As an immunosuppressant drug, it is used extensively following kidney, liver, and heart transplants to prevent acute graft rejection (12, 13). In addition, because of its antiproliferative action, RAPA holds promise as a novel anticancer agent (14). Recently, it has been reported to inhibit human adipocyte differentiation *in vitro* (15) and evidence suggests a role for RAPA in controlling intermediary metabolism (16).

To date, the full extent of the role of RAPA in treating

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human obesity has not been fully explored, but it has been observed that renal and liver transplant patients treated with RAPA have developed dyslipidemia, hypertriglyceridemia, and hyperglycemia (17–20). In this study, we attempted to determine its effects on body weight control and food intake and used histological methods to analyze fatty liver scores and adipocyte content of fat pads in an animal model treated with RAPA. We dosed diet-induced obese mice intraperitoneally with 2 mg/kg of RAPA once per week for 16 weeks. This treatment course reduced the adverse effects that may occur with a stronger or more frequent RAPA-treatment.

Materials and Methods

Animals and diets

Male C57BL/6J mice at 4 weeks of age were obtained from the Education Research Resource, National Laboratory Animal Center (NLAC), Taiwan. Mice were fed ad libitum a high-fat diet (HFD diet #592Z, modified Lab w/35.5% lard; PMI Nutrition International Inc., Brentwood, MO, USA; 67% of calories provided by fat and metabolizable energy as 4.5 kcal/g) for 20 weeks prior to the start of the experiment to induce obesity. Animals were housed individually in standard plastic rodent cages in animal quarters maintained at 22°C. They were maintained on a 12-h light/dark cycle and, unless otherwise specified, had ad libitum access to pelleted mice chow and water. Procedures and conditions were reviewed and approved by the Institutional Animal Care and Use Committees (NCHU IACUC number 94-51) at NCHU. In addition, all procedures adhered to the Guidelines for the Care and Use of Laboratory Animals recommended by the Taiwanese government.

Drug treatment

Male C57BL/6J mice on a HFD at 24 weeks ages were randomly divided into 2 groups. No significant weight differences were observed between these two groups (28.76 ± 0.49 and 28.63 ± 0.69 g). One group of mice was injected intraperitoneally with RAPA (2 mg/kg body weight; LC Laboratories, Woburn, MA, USA) once a week for 16 weeks. The second group of mice, the control, received a corresponding volume of a vehicle (sterile 10% PEG400 / 8% ethanol, followed by an equal volume of sterile 10% Tween 80) (21). This dosage of RAPA was based on previous studies involving immunosuppression in mice (22–24).

Determination of body weight, food intake, and measurement of blood glucose and hormone concentration

Body weight and food intake of mice were recorded and measured weekly. To estimate food consumption, food intake was assessed by weighing the food in each cage dispenser, including the food that was spilled on the floor of the cage. Blood samples were collected from the tail venipuncture from mice at 40 weeks of age for analysis of plasma glucose concentration using a One Touch™ II glucose meter (Lifescan Inc., Milpitas, CA, USA). In addition, at the end of the study period, animals were anesthetized; various tissues and the serum were harvested for subsequent analysis. Serum leptin and insulin concentrations were measured by a mouse leptin ELISA kit and rat insulin ELISA kit (Crystal Chem Inc., Downers Grove, IL, USA).

Histological and morphometric analysis of tissues

We measured liver, retroperitoneal, and epididymal weights according to body weight. Fatty infiltration in the liver was classified by hematoxylin and eosin staining as follows: no visible fat: score 0; <5% of liver surface infiltrated by fat: score 1; 5%–25% fat: score 2; 25%–50% fat: score 3; and >50% fat: score 4 (25).

Multiple sections were obtained from retroperitoneal and epididymal adipose tissue and analyzed them systematically with respect to adipocyte size and number. Staining of the sections was performed with hematoxylin and eosin. For each sample, at least 10 fields (representing approximately 100 adipocytes)/slide were analyzed (26). Images were acquired using a digital microscope (Nikon, Tokyo) and were analyzed using Motic Images Plus 2.0 software. Correlation of adipocyte size and number was expressed in both vehicle and RAPA-treated mice.

Statistical analyses

Each reported result is a mean \pm S.E.M. The differences between 2 groups were analyzed by the *t*-test for comparison. A *P* value of less than 0.05 was considered significant.

Results

Effects of RAPA on body weight, food intake, and leptin in HFD-induced mice

In our preliminary studies, there were no significant differences in anti-obesity effects, food intake, or serum leptin levels between RAPA-treated mice maintained on a standard diet (SD) and SD control mice (Supplementary Table 1: available in the online version only). However, surprisingly, RAPA-treated mice increased body weight on the HFD at a significantly lower rate compared to the

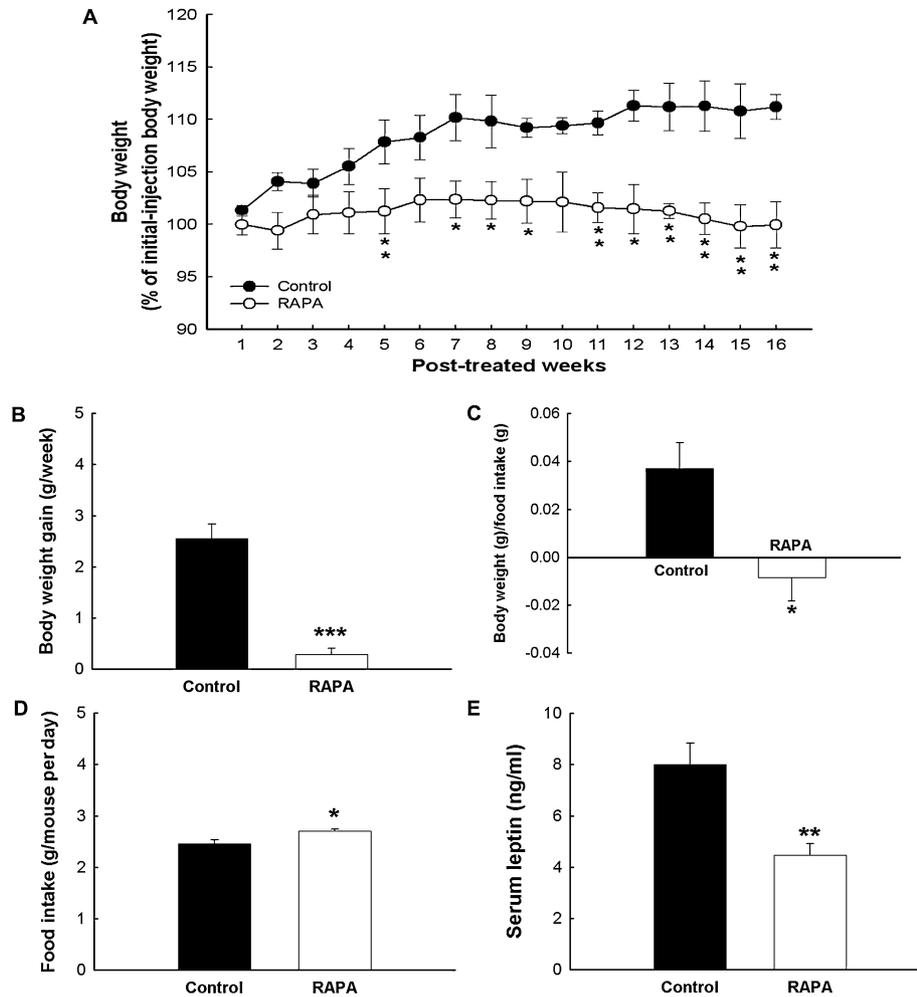


Fig. 1. Effect of RAPA treatment on growth curves (A), body weight gain (B), daily food efficiency (C), food intake per mouse per day measured (D), and serum leptin levels (E) in high fat diet-induced obesity mice. All values are given as means \pm S.E.M. $n = 7$ for all groups. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$: RAPA-treated animals vs. vehicle-treated group.

HFD control mice. The most marked difference was observed after 16 weeks of RAPA-treatment when a 12% difference in body weight was measured (Fig. 1A). RAPA also significantly reduced body weight gain compared to HFD control mice (0.29 ± 0.12 vs. 2.56 ± 0.28 g/week, $P < 0.001$) (Fig. 1B). Moreover, RAPA administration decreased the daily food efficiency of mice feed on the HFD (-0.0085 ± 0.0178 vs. 0.0369 ± 0.0107 g bw/g food, $P < 0.05$) (Fig. 1C). Furthermore, RAPA-treated mice were hyperphagic by an estimated 10% increase in food intake when food consumption was adjusted per mouse (Fig. 1D). The lower body weight in RAPA-treated mice can therefore not be explained by a reduced food intake. The expression of serum leptin levels, known to be involved in regulating food intake, was significantly lower with RAPA treatment (Fig. 1E). This result is consistent with the food intake data.

Table 1. Effects of rapamycin on liver and fat pad weights in C57BL/6J mice fed the HFD

	Control	RAPA
Absolute weight (g)		
Liver	1.02 ± 0.04	0.96 ± 0.03
RWAT	0.14 ± 0.03	0.14 ± 0.04
EWAT	1.37 ± 0.09	$0.94 \pm 0.08^{**}$
Percent of body weight		
Liver	3.00 ± 0.06	3.18 ± 0.09
RWAT	0.38 ± 0.09	0.46 ± 0.15
EWAT	4.04 ± 0.18	$3.12 \pm 0.19^{**}$

Data are presented as means \pm S.E.M. Highly significant difference at $P < 0.01$ (**). For all groups, $n = 7$. RWAT, retroperitoneal white adipose tissue; EWAT, epididyma white adipose tissue.

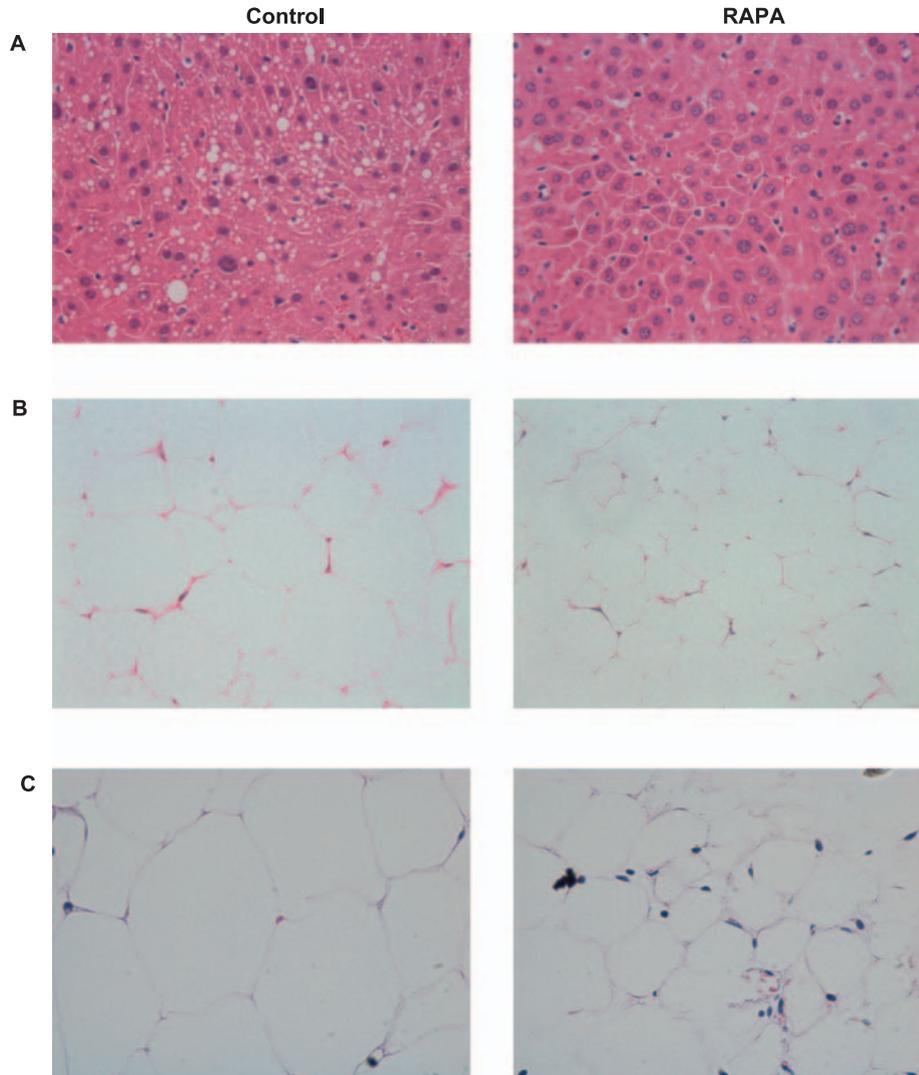


Fig. 2. Photomicrographs of liver (A), retroperitoneal (B), and epididymal (C) white adipose tissue samples stained with hematoxylin and eosin from vehicle- and RAPA-treated mice fed the HFD, illustrating greater differences for the RAPA group than the control group. All photomicrographs were determined as the magnification for histological analysis ($\times 400$).

Effects of RAPA on liver and fat pads mass on the HFD

The epididymal white adipose tissue (EWAT) weights of RAPA-treated mice were significantly lower (31%, $P < 0.01$) than that of control mice fed on the HFD (Table 1). There was no effect of RAPA on liver or retroperitoneal white adipose tissue (RWAT) weights. When normalized for body weight, RAPA significantly ($P < 0.01$) reduced EWAT by 23%. However, there was no difference in liver and RWAT between the 2 groups. These findings presented a reduction in fat pad weights and that RAPA prevented the effect of diet on the rate of accretion in fat pad weight.

RAPA reduced fatty liver score and adipocyte size

Morphometric analysis of RAPA-treated mice adipocytes in liver, retroperitoneal, and epididymal fat pads

by hematoxylin and eosin staining showed a lower fatty infiltration in the liver and a reduction in fat cell size of RWAT and EWAT, with some adipocytes being smaller in size, as compared to the HFD group (Fig. 2).

Furthermore, RAPA affected fatty liver scores and cell size in the RWAT and EWAT (Table 2). Results indicate that the adipocytes in RAPA-treated mice were consistently smaller compared with those in the HFD control mice, with an average of 50%, 65%, and 70% decrease in fatty liver size scores, retroperitoneal fat pads, and epididymal fat pads, respectively. Thus, changes in average cell diameter were commensurate with weight changes in the fatty pads. RAPA reduced fat mass primarily by decreasing the size of fat cells. At the

Table 2. Effects of rapamycin on fatty liver scores, adipocyte cellularity, and fat-cell size distribution in C57BL/6J mice fed the HFD

Variable	Control	RAPA
Fatty liver scores	1.71 ± 0.29	0.86 ± 0.26*
RWAT		
Adipocyte diameter (μm)	55.72 ± 2.99	19.73 ± 0.63***
Adipocyte diameter		
0–20 μm (%)	0 ± 0	57.41 ± 1.39***
20–40 μm (%)	13.73 ± 1.71	40.74 ± 1.21**
40–60 μm (%)	58.82 ± 0.88	1.85 ± 0.17***
60–80 μm (%)	23.53 ± 0.78	0 ± 0**
80–200 μm (%)	3.32 ± 0.26	0 ± 0*
EWAT		
Adipocyte diameter (μm)	84.57 ± 2.53	25.15 ± 2.10***
Adipocyte diameter		
0–20 μm (%)	0 ± 0	26.67 ± 1.57**
20–40 μm (%)	0 ± 0	60.00 ± 1.06***
40–60 μm (%)	1.88 ± 0.47	13.33 ± 1.43*
60–80 μm (%)	35.85 ± 3.39	0 ± 0**
80–200 μm (%)	62.27 ± 2.12	0 ± 0***

Data are presented as means ± S.E.M. Significantly different at $P < 0.05$ (*), highly significantly different at $P < 0.01$ (**), and very highly significantly different at $P < 0.01$ (***). $n = 7$ for all groups. RWAT, retroperitoneal white adipose tissue; EWAT, epididymal white adipose tissue.

same time, histological analysis of WAT showed that RAPA affected the cell size distribution in the fat pad. In general, the percentage of cells in the 0–40 and 0–60 μm diameter range was greater in RWAT and EWAT, respectively, from RAPA-treated HFD mice compared with HFD controls. In contrast, the proportion of cells in the 40–200 and 60–200 μm range was lower in RWAT and EWAT, respectively, for RAPA-treated mice compared to HFD controls. These results reveal that the HFD greatly increased the fatty liver score and adipocyte size and that RAPA may prevent these changes.

Effects of RAPA on fasting glucose levels and insulin concentrations

After 36 weeks on the HFD, we found no significant difference in plasma glucose levels between RAPA-treated mice fed the HFD and the HFD control mice (120.11 ± 6.92 mg/dl for the HFD control group and 122.63 ± 8.91 mg/dl for the RAPA treated group on the HFD) (Fig. 3A). Compared with the HFD controls, however, we observed that a 2-fold reduction in insulin was recorded for RAPA-treated mice (HFD control, 10.09 ± 2.05 ng/ml vs. RAPA, 4.97 ± 1.10 ng/ml; $P < 0.05$) (Fig. 3B). This suggests that RAPA treatment may reverse diet-induced hyperinsulinemia up to 40

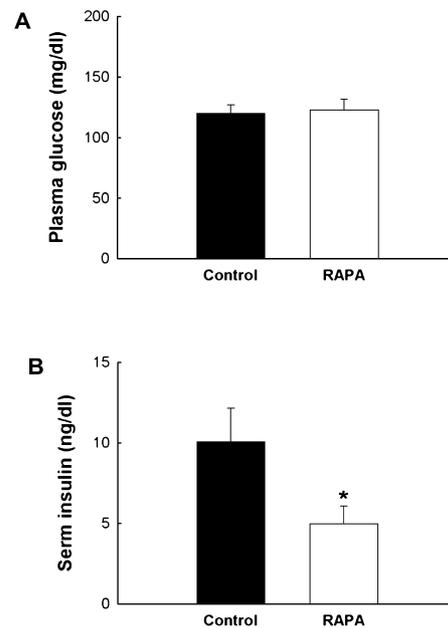


Fig. 3. Effect of RAPA on plasma glucose (A) and serum insulin (B) from vehicle- and RAPA-treated mice fed the HFD. Results are each expressed as the mean ± S.E.M. of 7 animals in each group. * $P < 0.05$: RAPA-treated animals vs. control group.

weeks of age. Thus, we propose that under the influence of extended RAPA treatment, RAPA may reduce serum insulin levels.

Discussion

In this study, we examined the effects of RAPA on the development of obesity in the C57BL/6J mouse strain fed on an HFD. Results support our prediction for RAPA as beneficial for decreasing body weight gain. The effect by RAPA was highly significant and persisted over time. Typically changes in body weight are associated with the amount of food intake. This was especially surprising because RAPA-treated mice showed hyperphagia relative to HFD control mice. Despite similar blood glucose levels, by the end of the 36 weeks on the HFD, however, RAPA treated mice exhibited significantly reduced insulin levels compared to the HFD group.

A high-fat diet significantly promotes the development of obesity (27, 28). Weight loss occurs when fat pad mass decreases as a result of reduced formation of new adipocytes from precursor cells (adipocyte differentiation) or by decreased adipocyte size due to fat storage (adipocyte hypertrophy) (29). Here, we suggest that the RAPA may prevent/reduce high fat diet-induced body weight, fat pad gain, and fat in the liver. The loss of body fat we observed in RAPA-treated mice may be due to a decrease in average adipocyte size and fatty liver scores.

Moreover, RAPA appeared to cause a reduction in the number of large adipocytes potentially due to its ability to promote fat combustion. This result is consistent with RAPA-reduced expressions of most adipogenic marker genes including PPAR γ , C/EBP α , FAS, aP2, adipon, and ADD1/SREBP1c, which affect lipogenesis, adipogenesis (30), and HFD-induced adipocyte hypertrophy (31). Thus, the ability of RAPA to prevent obesity that results from excess caloric intake suggests that RAPA might be a valuable tool in the search for key energy balance regulators.

Next we compared serum leptin concentrations because it is synthesized primarily in white adipose tissue and because it plays a role in the regulation of food intake in the hypothalamus (32). We suggest that greatly reduced leptin levels, due to decreased body fat, contributed to the increased food intake we observed in RAPA-treated mice. Since inhibition of the mTOR signaling pathway reduced leptin levels, we suggest that mTOR is a cellular fuel sensor that regulates energy intake. This result is similar to that found by Cota et al. (33). However, RAPA-treated mice lost rather than gained body weight, suggesting that RAPA increased energy combustion and lipid oxidation, thereby reducing adipocyte size and causing a reduction in body weight. These observations are consistent with RAPA-induced increases in fatty acid oxidation by increasing activities of carnitine palmitoyltransferases I and II (CPT I and CPT II), which is the primary intracellular regulatory enzyme of the fatty acid oxidation pathway (16). Moreover, decreased fatty liver scores are a result of RAPA inhibition of the mTOR pathway that may also be involved in the regulation of hepatic lipogenesis and lipid oxidation through the regulation of stearoyl-CoA desaturase (34). Thus, RAPA, an mTOR-pathway inhibitor, appears to have had a pronounced effect on body weight by reducing fat pad weight even with a significant increase in food intake, suggesting a specific effect on lipid metabolism.

Combined with the recent finding that RAPA reduced triacylglycerol accumulation and adipogenesis (35), but promoted catabolic processes (36), we suggest that administration of RAPA mimics a starvation-like signal. This result is similar to report by Peng et al. (37). When starvation signals occur, the physical response is an arousal of appetite for increased food intake, such as we observed in RAPA-treated mice. Concomitantly, fat accumulation in RAPA-treated mice was significantly reduced compared with control mice during the 36-week HFD-feeding period. Thus, the mechanism by which RAPA causes a decrease in body weight gain is mediated directly via increasing energy and fat combustion averting weight gain. This effect was confirmed

through the observation of reduced fat content in the liver, retroperitoneal, and epididymal adipose tissue in RAPA-treated mice. Analogous changes in feeding behavior have been observed in the S6K1 knockout mice (38).

Leptin signaling causes a decrease in food intake, resulting in weight loss (39, 40). To test the relationship between the anti-obesity effect, leptin levels, and RAPA, a genetic model of obesity was examined: male ob/ob mice (without leptin synthesis). There was no significant difference in food intake between ob/ob mice treated with RAPA on the HFD and HFD control ob/ob mice (Supplementary Table 2: available in the online version only). However, our supplementary data indicated an increase in food intake in ob/ob mice treated with RAPA compared to RAPA-treated C57BL/6J mice (3.72 ± 0.31 vs. 2.71 ± 0.04 g/mouse per day, $P < 0.05$). Therefore there is likely a cumulative effect on food intake occurring with simultaneous RAPA-treatment and an absence of leptin signaling. Moreover, the observation of greater body weight gain in RAPA-treated ob/ob mice was likely due to the dominant effect of increased food intake on body weight compared to losses that occur via fat combustion promotion by RAPA. Thus the anti-obesity effects of RAPA at clinical therapeutic concentrations, such as those used with congenital leptin deficiency or leptin-resistant humans/mice, will be diminished. Anti-obesity effects of RAPA may be restored by elevating the dosage of RAPA.

The mTOR signaling pathway has been widely studied in vivo to illustrate its involvement in the regulation of food intake, behaving similarly to other endogenous molecules, including neuropeptide Y (NPY), agouti-related peptide (AgRP), proopiomelanocortin (POMC), cocaine- and amphetamine-regulated transcript (CART) (41, 42), and forkhead box O1 (FOXO1) (43, 44) whose activities are regulated by leptin and linked to the regulation of energy balance. These endogenous molecules' association with food intake may work within the mTOR pathway or interact with each other in the hypothalamus (45). However, further investigation is necessary to determine the details of how these factors interact.

With short-term RAPA-pretreatment in vitro, RAPA acts as an enhancer of insulin-stimulated glucose uptake by preventing IRS-1 mass loss and IRS-1/PI 3-kinase complex decay and produces a maximum response to vanadate (46). Previous studies found that glucose uptake is reduced after prolonged treatment with RAPA in vitro (16, 47), potentially due to RAPA's involvement with inhibiting transcriptional/translational regulation of basal glucose transporters. However, our 16-week course of once per week treatment with RAPA produced

blood glucose levels that were not significantly different between the control and RAPA-treated groups. We suggest that differences in the length or frequency of RAPA-treatment may cause a variation in the effect of RAPA and therefore explain these contradictory results.

Although the cause for the decrease in insulin secretion remains to be proven conclusively, it may be due to RAPA's pharmacological inhibition of its target protein mTOR, which is a component of the IRS/PI3k/Akt/mTOR/PHAS-I signaling pathway involved in insulin secretion and biosynthesis (48, 49). In addition, our *in vivo* studies showed that inhibiting the mTOR pathway leads to a decrease in obesity. These novel observations however conflict with that of prolonged use of rapamycin in humans which is associated with excessive weight gain, severe hyperinsulinemia, insulin resistance, and hyperglycemia in organ transplant patients (45, 50, 51). Taken together, these observations indicate that a considerable adverse effect of RAPA may exist for patients in a cachexia condition. Furthermore, a phenomenon of RAPA resistance may exist among insulin-dependent diabetic mellitus (IDDM) patients (7). Further studies are needed to understand the complexities and how to reduce the risk of RAPA in transplant and diabetes patients.

In conclusion, our study demonstrates that treatment with RAPA, an immunosuppressant drug, significantly reduces body adiposity mass and reduces insulin levels. However, RAPA administration increased food intake in mice with high fat diet-induced obesity. These findings provide evidence that changes in the mTOR pathway affect food intake and body weight *in vivo* and that the mTOR pathway plays a role in the regulation of food intake and energy homeostasis. Understanding the mechanism for the balance/imbalance between food intake and energy homeostasis may lead to what predisposes some people to obesity and diabetes.

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