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Anorexic response to rapamycin does not appear to involve a central mechanism

Hale Z. Toklu^{1,2}, Erin B. Bruce², Yasemin Sakarya^{1,2}, Christy S. Carter³, Drake Morgan⁴, Michael K. Matheny², Nataliya Kirichenko^{1,2}, Philip J. Scarpance², and Nihal Türner^{1,2}

¹Veterans Affairs Geriatric Research Education & Clinical Center, Gainesville, FL, USA

²Department of Pharmacology & Therapeutics, University of Florida, Gainesville, FL, USA

³Department of Aging and Geriatric Research, University of Florida, Gainesville, FL, USA

⁴Department of Psychiatry, University of Florida, Gainesville, FL, USA

Summary

The authors have previously demonstrated that a low and intermittent peripheral dose of rapamycin (1 mg/kg three times/week) to rats inhibited mTORC1 signalling, but avoided the hyperlipidemia and diabetes-like syndrome associated with higher doses of rapamycin. The dosing regimen reduced food intake, body weight, adiposity, serum leptin and triglycerides. mTORC1 signalling was inhibited in both liver and hypothalamus, suggesting some of the actions, in particular the decrease in food intake, may be the results of a central mechanism. To test this hypothesis, rapamycin (30 µg/day for 4 weeks) was infused into 23–25-month-old F344×BN rats by intracerebroventricular (icv) mini pumps. Our results demonstrated that central infusion did not alter food intake or body weight, although there was a tendency for a decrease in body weight towards the end of the study. mTORC1 signalling, evidenced by decreased phosphorylation of S6 protein at end of 4 weeks, was not activated in liver, hypothalamus or hindbrain. Fat and lean mass, sum of white adipose tissues, brown adipose tissue, serum glucose, insulin and leptin levels remained unchanged. Thus, these data suggest that the anorexic and body weight responses evident with peripheral rapamycin are not the result of direct central action. The tendency for decreased body weight towards the end of study, suggests that there is either a slow transport of centrally administered rapamycin into the periphery, or that there is delayed action of rapamycin at sites in the brain.

Keywords

aging; brain; central; grip strength; HOMA-IR; hypothalamus; intracerebroventricular; leptin; locomotor; mTOR; pS6; rapamycin

1. Introduction

Rapamycin, also known as sirolimus, is a macrolide drug with immunosuppressant activity, which is mainly used to prevent organ rejection after transplantation.¹ The mode of action of rapamycin is to bind the cytosolic protein FK-binding protein 12 (FKBP12) in a manner similar to tacrolimus. Unlike the tacrolimus-FKBP12 complex, which inhibits calcineurin, rapamycin-FKBP12 complex inhibits mTOR (mammalian target of rapamycin) pathway by directly binding to mTOR Complex 1 (mTORC1).¹ There is an interaction between excess intake of nutrients and enhanced mTOR signalling; thus, the mTOR pathway is a potential therapeutic target for several diseases, such as obesity, hypertension and diabetes.^{2,3}

Rapamycin has been shown to increase life span in mice by regulating cellular activities such as growth and survival, nutrient sensing, protein synthesis and autophagy.⁴ Our previous studies have also demonstrated that a low intermittent dose of rapamycin decreased food intake and body weight and improved metabolic response in young and aged rats.^{5,6} This resulted in decreased adiposity, but a preservation of lean mass. Furthermore, there was a significant decrease in leptin synthesis in adipose tissue from rapamycin treated animals, leading to a normalization of leptin levels in aged-obese rats compared with those of young controls.⁶ In addition, there was no increase in blood glucose and a decrease in triglycerides in the blood serum, suggesting a preservation of metabolic function.⁵ The latter is potentially due to the low intermittent rapamycin dose that is sufficient to inhibit the mTORC1, but apparently does not inhibit the mTORC2 pathway.⁶

The observations that rapamycin reduced food intake and that mTORC activity was inhibited in both the liver and hypothalamus suggested that a central mechanism might be involved. This is supported by a recent report that a single intracerebroventricular (icv) injection of rapamycin decreased food intake and body weight in rats.⁷ In contrast, our previous finding indicated that central infusion of rapamycin for 28 days had no effect on locomotor activity or food consumption and initially did not decrease body weight.⁶ In the latter phase of the experiment, after day 17, body weight diminished, but it is not clear if this was due to a central mechanism or transport of rapamycin into the periphery. The present study examines this controversy in more detail in order to determine if the rapamycin-mediated reduction in food consumption is due to a central mechanism, and employs aged Fischer 344 × Brown Norway (F344×BN) rats, an animal model with demonstrated heightened sensitivity to rapamycin compared to young rats.

2. Results

The results indicated that central infusion of rapamycin did not alter food intake or delta body weight, although there was a tendency for a decrease in body weight towards the end of the study (Fig. 1). Consistently, fat and lean mass (expressed as a percentage of body weight) were unchanged throughout the 4 weeks of icv rapamycin infusion (Table 1). Moreover, central rapamycin treatment did not have a significant effect on the distribution of body fats (Table 1). Although serum leptin tended to be lower in the rapamycin group, it was not statistically significant. Similarly, fasting blood glucose and insulin resistance index did not significantly change with icv rapamycin treatment (Table 2).

Decreased phosphorylation of S6 protein is one measure of inhibition of mTORC1 signalling. However, in our study, pS6 levels (normalized to total S6 or GAPDH) remained unchanged in the mediobasolateral hypothalamus (MBH), and hindbrain (HB) regions of the brain as well as in the liver (Fig. 2).

Physical performance was evaluated by two tests, i.e. locomotor activity and grip strength in half of the rats. The total distance travelled and time spent in the center area during the locomotor activity test session were not significantly different among control and 4 weeks icv rapamycin infused rats (Fig. 3a, b). In addition, grip strength between control and rapamycin treated rats were not different (Fig. 3c).

3. Discussion

We previously demonstrated that a low and intermittent peripheral dose of rapamycin (1 mg/kg intraperitoneally (i.p.) three times/week) to young rats inhibited mTORC1 signalling and reduced leptin synthesis, but avoided the hyperlipidemia and diabetes-like syndrome associated with administration of higher doses of rapamycin.^{6,8} This dosing regimen reduced food intake, body weight, adiposity, serum leptin, and triglycerides. Moreover, we observed a transient decrease in food consumption and a persistent decrease in body weight. These data suggest there may be more than one mechanism in operation. mTORC1 signalling was inhibited in both liver and hypothalamus, suggesting some of the actions, in particular, the decrease in food intake may be the result of a central mechanism. Other evidence also supports a central role for rapamycin. The experimental mTOR activating compound, MHY1485 was shown to inhibit autophagy by the suppression of lysosomal fusion.⁹ The icv administration of the glucocorticoid, dexamethasone inhibited the hypothalamic target of rapamycin in high fat diet-fed chicks, supporting the involvement of a hypothalamic mTOR pathway for the regulation of appetite.¹⁰ Furthermore, hypothalamic mTORC1 signalling was shown to mediate the anorectic, weight- reducing, and sympathetic effects of central insulin action in mice.¹¹

Consistent with our previous studies, Hebert et al. reported that i. p. rapamycin decreased food intake and weight gain for several days.⁷ Moreover, they demonstrated a long-term reduction in body weight which lasted at least 10 weeks without additional rapamycin injections, and without any signs of malaise or glucose intolerance. Two rapamycin injections 2 weeks apart had additive effects on weight loss and adiposity. Decreased food intake and body weight effects were also observed with single icv injection of rapamycin,⁷ suggesting that this effect is either partially mediated by the brain or is secondary to the peripheral action of rapamycin transferred out of the brain.

This study aimed to test the hypothesis that rapamycin has a central mechanistic component with respect to the regulation of food intake and body weight. Our previous data with young rats that were infused with central rapamycin (icv with 4 week mini pumps) indicated that there were no significant decreases in food intake or body weight.⁶ Moreover, mTOR1 activity, as assessed by the increase in pS6 protein, was not inhibited in hypothalamus, although it was inhibited in liver (supplementary figure in Scarpace et al.⁶). In general, our previous studies indicated rapamycin is more efficacious in aged rats than young rats, thus

we considered aged rats to be better model to examine the effects of central rapamycin. The present data in old rats refuted our hypothesis, demonstrating that central rapamycin does not have a physiological effect on body weight, food intake or adiposity. In addition, locomotor activity and grip strength were unchanged with central rapamycin treatment. Finally, mTOR signalling was unchanged in either the brain (hypothalamus and HB) or periphery (liver) further supporting the idea that rapamycin has no central mode of action.

In both of our studies with central rapamycin infusion (previous study in young rats⁶ and the present study with old rats), body weight had a tendency to decrease towards the end of the experiment. It is likely that over time the central infusion of rapamycin leads to build up of rapamycin in the periphery, triggering a peripheral response. There is a well-established efflux mechanism for rapamycin via p-glycoproteins.¹² The inhibition of mTOR1 in the liver of young rats is consistent with the efflux of rapamycin. Efflux mechanisms are known to diminish with age.¹³ Decreased transport due to aging may result from either slowed transport rate or reduced number of transport proteins. This phenomenon may explain why liver mTOR1 was unchanged in aged rats (present study), but reduced in our previous study with central rapamycin infusion (supplementary figure in Scarpace et al.⁶). However, it remains a possibility that the tendency for weight loss at the end of the study period is a result of a delayed action of rapamycin at sites in the brain. In summary, the results of the present study suggest that there is no immediate response to central treatment with rapamycin. Towards the end of study, there was a tendency for a decrease in body weight, body fat and serum leptin. The latter is most likely the result of transport of the centrally administered rapamycin into the periphery. The complete lack of anorexic responses to central rapamycin treatment suggests that anorexic responses observed with peripheral rapamycin are not the result of direct central action.⁶⁻⁸ We speculate that the mechanism underlying these anorexic responses to peripheral rapamycin may be due to an initial peripheral action that communicates a signal to the hypothalamus that triggers an anorexic response to reduce food consumption.

4. Methods

Twenty-four male F344×BN rats, 23–25 months old were obtained from the National Institute on Aging Colony at Harlan Laboratories (Indianapolis, IN, USA). Animals were cared for in accordance with the principles of the Guide to the Care and Use of Experimental Animals; and protocol (#201203230) was approved by the University of Florida Institutional Animal Care and Use Committee. Rats were maintained on a 12:12 hour light:dark cycle and provided a standard rodent chow (17% kcal from fat, 25% from protein, no sucrose, 3.1 kcal/g, diet 7912; Harlan Teklad, Madison, WI, USA) and water ad libitum throughout the experimental protocol.

4.1 Surgical procedure and treatment

Rats were anaesthetized with isoflurane (2–3%) and their heads were prepared for surgery. Animals were placed into a stereotaxic frame and a small incision (1.5 cm) was made over the midline of the skull to expose the landmarks of the cranium. The following coordinates were used for injection into the third ventricle: 1.1 mm posterior to Bregma and 1.6 mm

ventral from the skull surface on the midline (medial fissure), with the nose bar set at 4 mm below the ear bars (below zero). A small hole was drilled through the skull and a 23-gauge stainless steel guide cannula was placed into the third ventricle. The cannula was attached to the Alzet osmotic mini pumps (Alza, Palo Alto, CA, USA) by a polyethylene (PE)50 tubing long enough to implant on the back, between or slightly posterior to the scapulae. A small incision was made at the base of the neck and a subcutaneous pocket to receive the pump was created by blunt dissection. All the pumps were filled with artificial cerebrospinal fluid (ACSF). After a recovery and equilibrium period of a week, the dummy pumps are replaced with vehicle- or rapamycin-containing pumps and brain infusion was continued for 4 weeks more. Rapamycin solution was prepared by dissolving 16.8 mg rapamycin in 5 mL vehicle (10% DMSO in PEG 400). Rapamycin was infused by an osmotic mini pump that is inserted into the lateral ventricle via an implanted cannula at a rate of 0.25 $\mu\text{L}/\text{hour}$ resulting in a rapamycin dose of 30 $\mu\text{g}/\text{day}$. Rapamycin dose was determined based on our previous studies with young rats.⁶

4.2 Food intake, KCal intake and delta body weight

Rats were housed individually and food consumption and body weight were recorded in grams daily throughout the experiment. Changes in body weight (delta body weight) were reported from the start of drug treatment.

4.3 Body composition of fat and lean mass

Body composition was assessed using time-domain nuclear magnetic resonance (TD-NMR) in restrained but fully conscious rats (TD-NMR Minispec, Bruker Optics, The Woodlands, TX, USA) 2 days before (baseline) and 4 weeks after the initiation of rapamycin treatment.

4.4 Locomotor activity

Animals were placed in activity chambers (43×43 cm, Med Associates, Inc., St. Albans, VT, USA) for 5-minute sessions. Total distance travelled (cm) and spatial location (time in “center” or “margin”) were tracked with an overhead camera and Ethovision XT 7.0 software (Noldus Information Technology Inc., Wageningen, the Netherlands). Locomotor activity and grip strength tests were performed 2 days prior to sacrifice.

4.5 Grip strength

Forelimb grip strength was determined using an automated grip strength meter (Columbus Instruments, Columbus, OH, USA). The rat was grasped by the base of the tail and suspended above a grip ring. After approximately 3 seconds, the rat was gently lowered toward the grip ring and allowed to grasp the ring with its forepaws. The remainder of the rat's body was quickly lowered to a horizontal position, and the animal's tail was pulled until grasp of the ring was broken. The mean force in grams was determined with a computerized electronic pull strain gauge. Maximal force obtained from three trials was adjusted to body weight and was used as the dependent measure.

4.6 Serum leptin

Enzyme immunoassays were used to determine the levels of leptin (rat leptin ELISA kit, EZRL-83K; Milipore, Waltham, MA, USA). Leptin was assayed with the blood (fed state) that was collected during sacrifice.

4.7 Blood glucose, serum insulin and insulin resistance index

Fasting blood glucose was measured at 4 weeks post treatment. Prior to death, half of the rats (6/group) were fasted overnight, and glucose was measured by CardioCheck Blood Testing Device (Health Check Systems Inc, Brooklyn, NY, USA) with a drop of tail blood. Then 500 μ L of blood was collected and centrifuged at 4000 G for 15 minutes to separate serum. Serum insulin was assessed via ELISA (Mercodia, Winston Salem, NC, USA). Homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated using the HOMA2 Calculator, developed by the Diabetes Trial Unit, University of Oxford, Oxford, UK.¹⁴

4.8 Tissue harvesting and preparation

Rats were killed by thoracotomy under anaesthesia (5% isoflurane). The circulatory system was perfused with 20 mL of ice-cold saline. Perirenal and retroperitoneal white adipose tissues (PWAT and RTWAT, respectively), and brown adipose tissue (BAT) were excised. The hypothalamus was removed by making an incision medial to piriform lobes, caudal to the optic chiasm, and anterior to the cerebral crus, to a depth of 2–3 mm. Collected MBH, HB and liver samples were sonicated in 50 mmol/L Tris-HCl, pH 6.8, plus protease inhibitors. Protein concentrations were determined using the DC protein assay kit (Bio-Rad, Hercules, CA, USA).

4.9 Western blot analysis

Briefly, an equal amount of protein for each sample was separated by 10–12.5% SDS-PAGE for 1 hour at 100 mA. After electrophoresis, the proteins were transferred to nitrocellulose membranes and blocked with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween 20. All membranes were incubated overnight at 4°C with primary antibody. Immunoreactivity was visualized by ECL Prime detection system (GE Healthcare, Piscataway, NJ, USA) and quantified by ImageQuant TL (GE Healthcare).

4.10 mTOR signalling

Inhibition of mTOR signalling was assessed by the amount of phosphorylated S6 (pS6) protein in the liver, MBH and HB by Western blot analysis. Respectively, 25, 26 and 32 μ g protein were loaded for the liver, MBH and HB. The pS6 antibody (4856S, Cell Signalling, Danvers, MA, USA) and total S6 antibody (2217S, Cell Signalling) were used in 1/1000; while glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (3683S, Cell Signalling) antibody was used in 1/10000 concentrations for normalization.

4.11 Statistical analysis

Statistical analysis was carried out using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). All data were expressed as mean $\pm$ standard error of the mean (SEM).

Groups were compared with one-way ANOVA followed by Tukey's multiple comparison post-hoc test or Student's *t* test. Values of $P < 0.05$ were considered statistically significant.

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Figure 1.

Delta body weight (BW) over time following central infusion of rapamycin (triangles) or vehicle (squares). Day 0: pump change from artificial cerebrospinal fluid (ACSF) to vehicle (□; control) or (▴) rapamycin. Inset: Cumulative food intake over the course of the experiment with vehicle (open bars), and rapamycin (solid bars) treated old rats. N=12 rats/group

Figure 2. Phosphorylated S6 (pS6), total S6 (tS6), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein derived from Western blots in brain and liver of intracerebroventricular (icv) vehicle (control; open bars) or rapamycin (solid bars) infused old rats. N=6 rats/group

Figure 3.

Locomotor activity. (a) Time spent in the centre; (b) total distance travelled; and (c) grip strength were not significantly different between vehicle (control; open bars) or rapamycin (solid bars) infusion for 4 weeks. N=6 rats/group

Table 1

Body weight, fat %, lean % and distribution of body fats (week 4) in intracerebroventricular (icv) vehicle (control) or rapamycin treated old rats. N=12 rats/group. Pre, week 0; Post, week 4

	Fat%		Lean %		BW (g)	RTWAT (g)	PWAT (g)	EWAT (g)	Sum of WATs (g)	bat (g)
	Pre	Post	Pre	Post						
Control	28.4±0.4	28.9±0.3	55.5±0.4	55.6±0.3	533.7±17	11.2±0.9	2.2±0.2	11.0±0.8	24.4±1.6	0.65±0.04
Rapamycin	28.3±0.3	28.8±0.3	55.9±0.5	56.2±0.3	510.2±13	8.1±0.6*	2.6±0.4	9.6±0.5	20.3±1.2	0.64±0.03

BW, body weight; BAT, brown adipose tissue; PWAT, perirenal white adipose tissue; RTWAT, retroperitoneal white adipose tissue; EWAT, Epididymal white adipose tissue; WAT, white adipose tissue.

Table 2

Serum glucose, insulin, homeostasis model assessment of insulin resistance (HOMA-IR) index and leptin levels in icv vehicle (control) or rapamycin treated old rats. N=6 rats/group

	Glucose (mg/dL)	Insulin (μ g/L)	HOMA-IR	Leptin (ng/ml)
Control	169.33 $\pm$ 15.0	1.14 $\pm$ 0.2	3.27 $\pm$ 0.5	14.33 $\pm$ 2.0
Rapamycin	140.17 $\pm$ 8.4	1.81 $\pm$ 0.3	4.82 $\pm$ 0.8	9.39 $\pm$ 1.1