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RESEARCH PAPER

Rapamycin impairs HPD-induced beneficial effects on glucose homeostasis

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BACKGROUND AND PURPOSE

Rapamycin, which is used clinically to treat graft rejection, has also been proposed to have an effect on metabolic syndrome; however, very little information is available on its effects in lean animals/humans. The purpose of this study was to characterize further the effects of the continuous use of rapamycin on glucose homeostasis in lean C57BL6/J mice.

EXPERIMENTAL APPROACH

Mice were fed a high-protein diet (HPD) for 12 weeks to develop a lean model and then were treated daily with rapamycin for 5 weeks while remaining on a HPD. Metabolic parameters, endocrine profiles, glucose tolerance tests, insulin sensitivity index, the expression of the glucose transporter GLUT4 and chromium distribution were measured *in vivo*.

KEY RESULTS

Lower body weight gain as well as a decreased caloric intake, fat pads, fatty liver scores, adipocyte size and glucose tolerance test values were observed in HPD-fed mice compared with mice fed a high-fat or standard diet. Despite these beneficial effects, rapamycin-treated lean mice showed greater glucose intolerance, reduced insulin sensitivity, lower muscle GLUT4 expression and changes in chromium levels in tissues even with high insulin levels.

CONCLUSION AND IMPLICATIONS

Our findings demonstrate that continuous rapamycin administration may lead to the development of diabetes syndrome, as it was found to induce hyperglycaemia and glucose intolerance in a lean animal model.

Abbreviations

EWAT, epididymal white adipose tissue; HFD, high-fat diet; HPD, high-protein diet; IPGTT, i.p. glucose tolerance test; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; mTORC2, mTOR complex 2; RWAT, retroperitoneal white adipose tissue; SD, standard diet

Tables of Links

TARGETS	
Transporters[a]	**Enzymes**[b]
GLUT4	Akt (PKB)
	mTOR
	S6K1

LIGANDS
Insulin
Rapamycin

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b]Alexander *et al.*, 2013a,b).

Introduction

Type 2 diabetes is a recognized nutrient overload disorder that occurs in obese subjects when the release of insulin by dysfunctioning pancreatic beta cells fails to control blood glucose levels in response to insulin resistance (Kahn *et al.*, 2006). Energy restriction, especially by more than 50% of energy as protein, is a common and successful alternative treatment for obesity, which is usually effective in achieving body weight loss and preventing further onset of diabetes (St Jeor *et al.*, 2001). Moreover, people with type 2 diabetes fed a high-protein diet (HPD) have lowered postprandial blood glucose levels and improved overall glucose control (Gannon *et al.*, 2003). Nevertheless, high-protein supplementation has been correlated with promotion of wound healing, protection against shock, malnutrition, control of anaemia, development of oedema and enhancement of immune defence after surgery and burn injury (Spence *et al.*, 1946).

Rapamycin (also known as sirolimus), a macrocyclic triene antibiotic, belongs to a class of macrocyclic-immunosuppressive agents with an anti-proliferative ability. Rapamycin inhibits the mammalian target of rapamycin (mTOR) kinase. The mTOR kinase exists in two physically and functionally distinct multiprotein complexes: mTOR complex 1 (mTORC1), which is rapamycin-sensitive and phosphorylates S6K1, and mTOR complex 2 (mTORC2), which is rapamycin- insensitive and phosphorylates PKB (Dann *et al.*, 2007; Dowling *et al.*, 2010). mTORC1-S6K1 signalling is related to the cellular integration centre for controlling cell growth and proliferation, and is known to play key roles in cancer, diabetes, obesity, cardiovascular diseases and neurological disorders (Dann *et al.*, 2007; Tsang *et al.*, 2007). Moreover, the control of food intake affects energy homeostasis, thereby extending the median and maximal lifespan, which is also affected by mTORC1-S6K1 signalling activity (McCormick *et al.*, 2011; Lamming *et al.*, 2012). Thus, treatment with rapamycin and its derivatives could have a broad impact in human disease. An important mechanism in the regulation of rapamycin involves the inhibition of the progression of cell cycle from the G1 to S phase in T cells (Terada *et al.*, 1993). Rapamycin's potent immunosuppressive capacity has led to its approval for use in clinical applications, including solid organ transplantation, by the Food and Drug Administration and European Medicines Evaluation Agency, and it has the potential for application in other areas in which cell proliferation plays a key role (Heemann and Viklicky, 2013).

In addition to evidence indicating that rapamycin-treated renal and liver transplant patients are at greater risk of developing dyslipidaemia, hypertriglyceridaemia and hyperglycaemia (Brattstrom *et al.*, 1998; Reuben, 2001), rapamycin has also been associated with cognitive impairment, and preclinical side effects, such as glucose intolerance, which is a significant risk factor for diabetes and cardiovascular diseases (Chang *et al.*, 2009b). However, there are conflicting reports on the effects of rapamycin, as it has been shown variously to inhibit the effects of mTOR, which may facilitate the maintenance of glucose homeostasis, provide resistance against diet-induced obesity and reduce the risk of diabetes (Um *et al.*, 2006; Tsang *et al.*, 2007). Consistent with this notion, these studies indicated that a diabetic side effect of rapamycin may exist in patients with cachexia, as well as in animal models of obesity and diabetes. Thus, the extent of its role has not been fully explored, especially in lean individuals and subjects with normal glucose tolerance. In this study, we sought to determine whether the action of rapamycin could contribute to the metabolic effects of transplant recipients given high-protein supplements in physical recovery and lean human/animal with normal blood glucose levels, or even with hypoglycaemia. We thus developed a lean animal model by feeding mice a diet rich in protein. The effects of rapamycin treatment on HPD-induced lean animals were evaluated in terms of blood glucose, endocrine profiles, insulin signalling expression and distribution of chromium, which is an essential element in the normal metabolism of carbohydrates, lipids and protein, and exerts beneficial effects in people with glucose intolerance, diabetes, obesity and neuropathy (Anderson, 1998; Ryan *et al.*, 2003).

Methods

Animals and drug treatment

Male C57BL/6J mice at 5 weeks of age, obtained from the Education Research Resource, National Laboratory Animal Centre, Taiwan, were used in accordance with the Guidelines for the Care and Use of Laboratory Animals as recommended by the Taiwan government. The protocol for the experimental mice was also reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the National Chung Hsing University (IACUC Approval No. 98-85). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving

animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 60 animals were used in these experiments. Animals were killed by an overdose of anaesthetic, urethane (1.2 mg kg^{-1}) combined carbon dioxide.

Experiment I. The animals were divided into three groups to establish the lean animal model: (i) control group: mice were fed a standard diet (SD; diet 5008, 24% protein-enriched diet, 49.4% carbohydrates; PMI Nutrition International, Brentwood, MO, USA); (ii) high-fat diet (HFD) group: mice were fed a HFD (diet 592Z, 20.4% protein-enriched diet and modified laboratory with 35.5% lard; PMI Nutrition International Inc); (iii) HPD group: mice were fed a HPD (diet 5787, 60.0% protein-enriched diet, PMI Nutrition International Inc) (Supporting Information Table S1) group. All groups were fed *ad libitum* for 12 weeks. Mice were housed individually in standard plastic rodent cages in animal quarters with controlled temperature (22 ± 1°C), humidity (55 ± 5%) and a 12:12 h light–dark cycle. The body weights of all mice were measured for 12 weeks and then analysed.

Experiment II. Five-week-old male C57BL/6J mice were fed a HPD for 12 weeks to develop a lean animal with normal glucose levels, following our preliminary investigation described in experiment I. Mice were then randomly divided into two groups at 17 weeks of age. One group of mice was injected with i.p. rapamycin (2 mg·kg^{-1} body weight) once a day for 35 days (HPD-fed, rapamycin-treated mice; HPDR). The second group of mice, the HPD control, received a corresponding volume of vehicle (sterile 10% PEG400/8% ethanol followed by an equal volume of sterile 10% Tween 80) (Eshleman *et al.*, 2001; Chang *et al.*, 2009a). This dosage of rapamycin in mice is equivalent to a dosage of 0.17 mg·kg^{-1}·day^{-1} in humans when normalized to body surface area (Chodera and Feller, 1978; Chang *et al.*, 2009b) to mimic a typical therapeutic concentration in clinical human studies (Saunders *et al.*, 2001).

Measurement of body weight, food/caloric intake and hormone concentration

The body weights 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. In addition, at the end of the study period, animals were anaesthetized and various tissues as well as serum were harvested for subsequent analysis. Serum leptin and insulin concentrations were measured by use of the mouse leptin ELISA kit and rat insulin ELISA kit (#90030 and #INSKR020; Crystal Chem Inc, Downers Grove, IL, USA).

Histological and morphometric analysis of tissues

We measured heart, liver, spleen, kidney, retroperitoneal and epididymal fat pad weights; determined as a percentage of total body weight. Fat infiltration in the liver was identified by haematoxylin 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 (Chang *et al.*, 2009b).

Numerous sections were obtained from retroperitoneal and epididymal adipose tissue, and analysed systematically with respect to adipocyte size and number. Staining of the sections was performed with haematoxylin and eosin. For each sample, at least 10 fields (representing ~100 adipocytes) per slide were analysed (Bluher *et al.*, 2004). Images were acquired using a high-resolution digital microscope (Moticam 2300, Motic Instruments, Canada) and adipocyte size distribution was analysed using Motic Images Plus 2.0 software. Correlation of adipocyte size and its distribution (%) was determined for HFD control, HPD control and rapamycin-treated mice on a HPD.

Glucose tolerance test

At the end of experiment I, an i.p. glucose tolerance test (IPGTT) was performed at a dose of 1 g·glucose·kg^{-1} body weight in animals that had been fasted overnight with free access to water. Blood glucose levels were determined at 0, 30, 60 and 120 min after the i.p. injection of glucose. Throughout the aforementioned experiment, blood glucose was measured in blood taken from the tail vein at the times indicated earlier using a One Touch™ glucose meter (LifeScan Inc, CA, USA). To examine glucose tolerance, the area under the glucose tolerance curve (0–120 min) was calculated. Likewise, in experiment II, after 28 days of treatment with vehicle or rapamycin, an IPGTT was also performed in animals fed the HPD.

Western blot analysis

At the end of each experiment, animals were killed by an overdose of anaesthetic and gastrocnemius muscles were quickly removed, minced coarsely and then immediately homogenized. A Western blot was performed as previously described (Chang *et al.*, 2009b). The antibodies used for the Western blot analysis were as follows: anti-GLUT4, anti-phospho-S6K (phosphothreonine 389-specific), anti-S6K and anti-actin antibodies. Immunoreactive signals were detected using enhanced chemiluminescence reagents and the membranes were exposed on X-ray films. Quantification of protein expression and phosphorylation was performed on films using the NIH programme Scion image (Scion Corporation, Frederick, MD, USA).

Analysis of chromium concentrations

Whole blood and tissue samples of liver, muscle, epididymal fat pad and femur were removed immediately the animals had been killed and used for determinations of chromium levels. After being washed with saline, the tissue samples were blotted dry and weighed. Following overnight digestion of the blood/tissue samples with nitric acid at 100°C, the solutions were prepared for analysis of chromium concentration using a flame atomic absorption spectrophotometer (Z-2000 Polarized Zeeman Atomic Absorption Spectrophotometer, Hitachi, Tokyo, Japan). Analysis lines used were 359.3 for chromium. The following thermal treatment programme was used: drying from 80 to 120°C for 40 s, ashing at 700°C for 20 s, atomization at 2600°C for 5 s, cleaning at 2700°C for 4 s and cooling for 10 s. The argon gas was used at a flow rate of 200 mL·min^{-1} during the drying and ashing cycles and at 30 mL·min^{-1} during atomization. The signal intensity was calibrated with standard solutions of chromium. We meas-

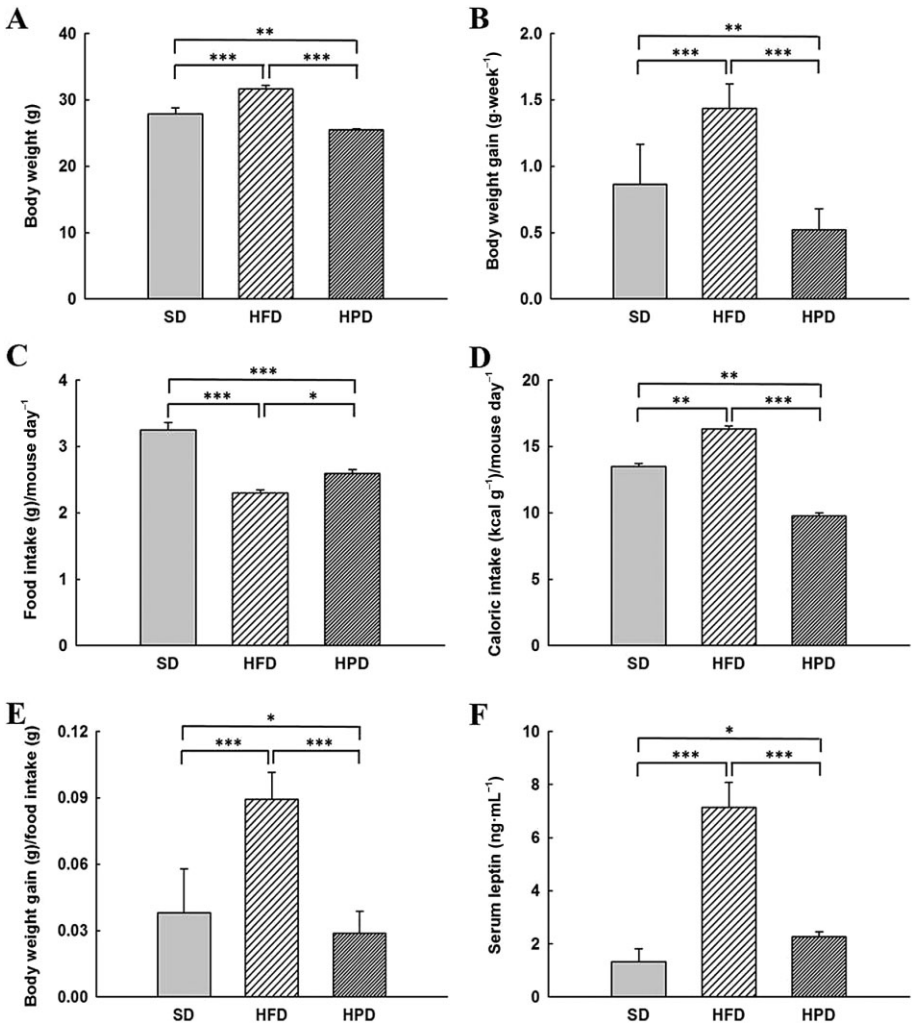

Figure 1

Effects of diet on (A) body weight, (B) body weight gain, (C) food intake per mouse day measured, (D) caloric intake per mouse per day measured, (E) daily food efficiency and (F) serum leptin levels in mice after 12 weeks *ad libitum* consumption of either SD (23.5% protein), HFD (16% protein) or HPD (60% protein). All values are given as mean ± SEM, $n = 10$ for all groups. Statistically significant at $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

ured each sample in triplicate and the mean value was employed for analysis. Concentrations are expressed as $\mu g \cdot L^{-1}$ (ppb) for each sample indicating the quantity of chromium in dry weight. In addition, the relative recovery rate of chromium was determined at 5 ppb by 94% ($n = 5$). The total levels of chromium in the samples were determined at $R^2 > 0.995$ by regression analysis of the sample absorption data on a standard curve.

Statistical analysis

Results are shown as mean ± SEM. The differences between the two groups were analysed by *t*-test for comparison. An ANOVA test followed by *post hoc* Bonferroni test was used to determine the differences when more than two groups were analysed. A *P*-value of less than 0.05 was considered significant. Fisher's exact test was also used to evaluate the significance of contingency data.

Materials

Rapamycin was from LC Laboratories (Woburn, MA, USA). Anti-GLUT4, anti-phospho-S6K, anti-S6K and anti-actin antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Enhanced chemiluminescence reagents were from Pierce Chemical Co (Rockford, IL, USA).

Results

HPD effects on body weight indices, food/caloric intake, food efficiency and serum leptin levels

We fed male C57BL6/J mice a SD, HFD or HPD for 12 weeks. The different dietary intakes led to differences in body metabolic parameters and serum leptin levels (Figure 1). The weights of HPD-fed mice after 12 weeks were 9 and 19%

lower, respectively, compared with the SD- and HFD-fed mice, which shows a statistically significant difference (Figure 1A). In addition, body weight (bwt) gain was significantly attenuated in HPD-fed mice by 40 and 64%, respectively, paralleling the SD and HFD group (Figure 1B). However, HPD-fed mice had significantly increased mean daily food intake (2.59 ± 0.06 vs 2.29 ± 0.04 g per mouse day^{-1}, respectively, $P < 0.05$) (Figure 1C), but the daily caloric intake of HPD group was decreased (9.76 ± 0.22 vs 13.49 ± 0.24 kcal per mouse day^{-1}, respectively, $P < 0.001$), compared with the corresponding values for the HFD group (Figure 1D). However, the daily food intake and daily caloric intake of the HPD group was significantly lower ($P < 0.001$ and $P < 0.01$, respectively) than those of the SD group. Furthermore, the daily food efficiency of HPD-fed mice was lower than that of mice fed a SD (0.028 ± 0.007 vs 0.037 ± 0.018 g bwt g^{-1} food, respectively, $P < 0.05$) and was lower than that of mice in the HFD group (0.028 ± 0.007 vs 0.089 ± 0.012 g bwt g^{-1} food, $P < 0.001$) (Figure 1E). Leptin is involved in regulating food intake. HPD-fed mice demonstrated a significant 3.2-fold decrease ($P < 0.001$) in serum leptin levels compared with HFD-fed mice (Figure 1F). This result is consistent with the food intake data. In addition, the serum leptin levels of HPD-fed mice showed a 1.7-fold increase ($P < 0.05$) compared with the SD group, which was significantly different. Consistent with this result, we observed a decrease in body weight gain in HPD mice that could not be attributed to an increase in food intake; it may have been due to decreased caloric intake. Thus, mice fed a HPD for 12 weeks became lean, indicating that there was a reduced increase in weight in mice fed a HPD compared with those fed a SD or HFD.

HPD reduces organ/fat pad weight

Next, we assessed whether these differences in weight were related to alterations in body composition or adiposity. After 12 weeks of the experimental diet, the body compositions marked significant differences in heart, liver, spleen, retroperitoneal white adipose tissue (RWAT) and epididymal white adipose tissue (EWAT), but not kidneys, between the HPD and HFD groups (Figure 2). With the exception of the hearts, spleen and kidneys, the weights of the liver and epididymal fat pads of HPD-fed mice were also significantly lower than those of the SD group. Relative to the HFD group, the weights of the liver, spleen, RWAT and EWAT in HPD-fed mice were significantly lower, by 18, 27, 36 and 68%, respectively, when normalized for body weight (Figure 2). In addition, liver and EWAT as percentages of body weight in mice fed a HPD were significantly decreased, by 13 and 31%, respectively, when compared with the respective weights of the SD group. However, no differences were found for the heart and kidney as percentage of body weight among the three groups.

HPD reduces fat accumulation in the liver, adipocyte size and percentage of large adipocyte number

Morphometric analysis of mice fed a HPD indicated a marked decrease in fat content in the liver and smaller fat cells in RWAT and in EWAT, as observed by haematoxylin and eosin staining, relative to the SD and HFD group (Figure 3A). This

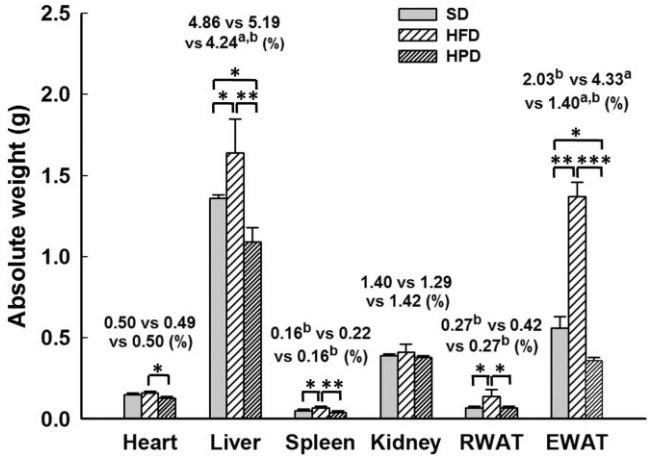

Figure 2

Effects of diet on absolute weight of organ and white adipose tissue mass, and weight of all organs and tissues normalized for body weight (%) in mice after 12 weeks *ad libitum* consumption of either SD (23.5% protein), HFD (16% protein) or HPD (60% protein). All values are given as mean ± SEM, $n = 10$ for all groups. Statistically significant at $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. $^{a,b}P < 0.05$, significantly different compared with (a) SD-fed animals and (b) HFD-fed animals.

finding was consistent with the lower fat pad weights observed in HPD-fed animals compared with the SD- and HFD-fed groups. These findings indicate that the HPD prevented fat accumulation in the liver and this may have reduced the hypertrophy of fat pads. Furthermore, to determine the effect of diet on fatty liver scores (Figure 3B), the sizes of fat cells in the RWAT (Figure 3C) and EWAT (Figure 3D) were assessed. These results indicate that there were significant differences among the three groups. We observed a 10-fold and threefold decrease in fatty liver scores of the HPD-fed mice, compared with the HFD- and SD-fed mice respectively. The expression levels for average adipocyte size revealed that the adipocytes in mice fed a HPD were consistently smaller compared with those in the HFD-fed group, with mean decreases in size of 86 and 85% for retroperitoneal and epididymal fat pads respectively. In addition, the sizes of adipocytes in RWAT and EWAT in the HPD-fed group were also smaller than those in the SD-fed group, with mean decreases of 53 and 33% respectively. Thus, the HPD had a greater preventive effect on fat cell hypertrophy than the SD.

HPD protected against glucose intolerance and decreased serum insulin levels

To examine the effect of diet on glucose homeostasis, glucose tolerance tests performed over a 2 h period showed marked differences among the three groups (Figure 4A). Remarkably, the glucose tolerance and blood glucose levels at 0, 30, 60 and 120 min were significantly ($P < 0.001$) lower in the HPD-fed animals than those in the HFD-fed groups. Furthermore, HPD-fed mice showed lower glucose tolerance test values than those of the SD-fed mice at 30 ($P < 0.001$), 60 ($P < 0.01$) and 120 min ($P < 0.01$). Likewise, the AUC for glucose levels

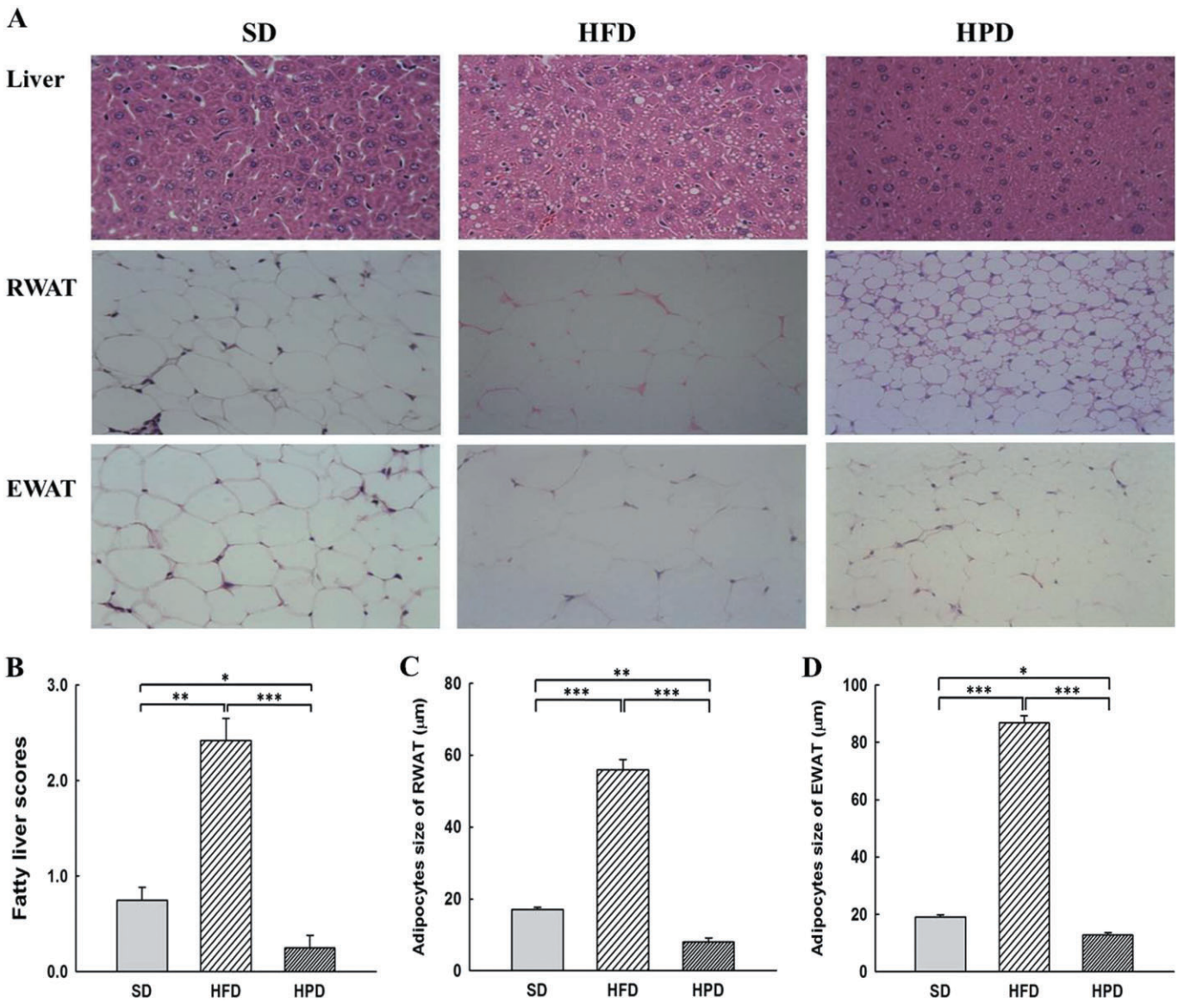

Figure 3

Photomicrographs of (A) liver, retroperitoneal and epididymal white adipose tissue samples stained with haematoxylin and eosin, (B) fatty liver scores, (C) adipocyte cellularity of RWAT and (D) adipocyte cellularity of EWAT from mice after 12 weeks *ad libitum* consumption of either SD (23.5% protein), HFD (16% protein) or HPD (60% protein). All values are given as mean ± SEM, $n = 9–11$ for all groups. All photomicrographs were determined as magnification for histological analysis (×400). Statistically significant at $^{*}P < 0.05$, $^{**}P < 0.01$and $^{***}P < 0.001$.

during the 120 min study period was significantly different and the HPD-fed mice had lower values than those of the HFD-fed ($P < 0.001$) and SD-fed ($P < 0.01$) animals (Figure 4B). Using the criterion of glucose intolerance as a blood glucose level above 900 mg·L^{-1} at 120 min post-i.p. glucose injection, we found that a significant fraction of mice on the HPD and SD exhibited lower glucose intolerance than the HFD-fed mice ($P < 0.001$; Figure 4C). Serum insulin concentrations showed a significant difference among the three groups. HPD-fed mice had lower insulin levels than the HFD-fed ($P < 0.001$) and SD-fed mice ($P < 0.05$; Figure 4D). Thus, mice fed a HPD showed normal glucose homeostasis with hypoinsulinaemia.

Rapamycin impaired glucose tolerance and reduced insulin content in HPD-induced lean mice

To assess the effect of rapamycin treatment on glucose homeostasis in lean mice fed a HPD, we next performed glucose tolerance tests in HPD-fed mice treated with rapamycin or vehicle for 28 days. Unfortunately, rapamycin raised fasting blood glucose ($P < 0.001$) and exacerbated glucose intolerance compared with that in the vehicle-treated mice, as evidenced by the IPGTT data (Figure 5A). Rapamycin administration resulted in a significant ($P < 0.001$) rise in fasting blood glucose levels at 30, 60 and 120 min after

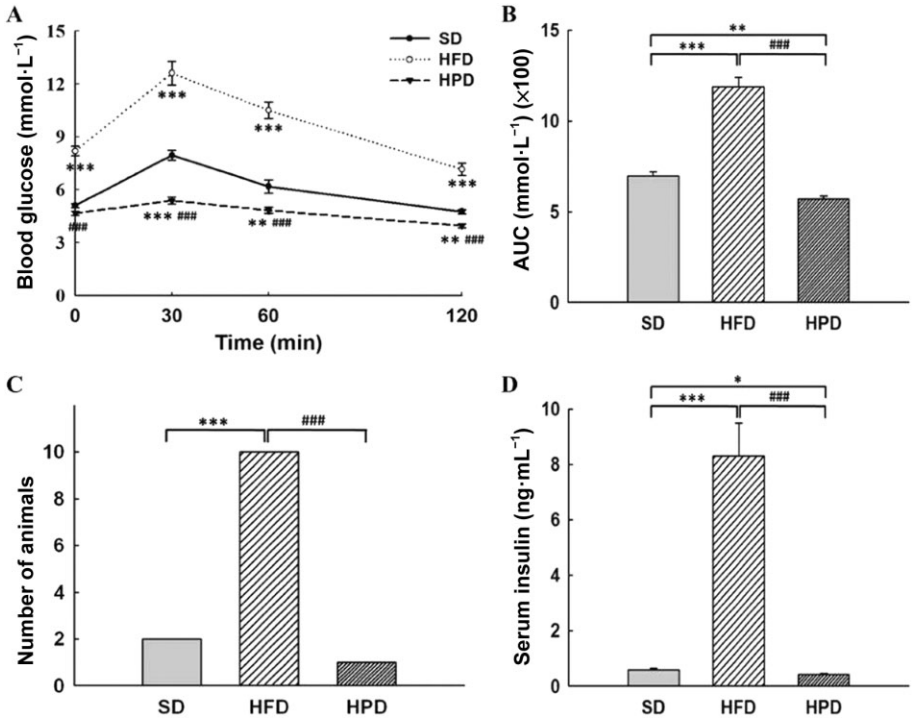

Figure 4

Effects of diet on (A) glucose tolerance test: 1 g·kg^{-1} of glucose, (B) AUC during the 120 min after glucose injection, (C) criterion of glucose intolerance (Fisher's exact test) and (D) serum insulin levels in mice after 12 weeks *ad libitum* consumption of either SD (23.5% protein), HFD (16% protein) or HPD (60% protein). All values are given as mean ± SEM, $n = 10$ for all groups. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with SD-fed animals; $^{###}P < 0.001$ compared with high-fat diet-fed animals.

glucose injection: blood glucose levels at the 120 min point of the test in the rapamycin group was 31.02% higher in response to the start of injection compared with −14.83% in the control group ($P < 0.001$). Furthermore, compared with the controls, the AUC for glucose levels during the 120 min study period increased twofold in rapamycin-treated mice, which was significant (Figure 5B). Using a criterion of glucose intolerance as a blood glucose level above 900 mg·L^{-1} at 120 min post-i.p. glucose injection, we found that a significant fraction of mice on rapamycin treatment showed glucose intolerance ($P < 0.001$, Figure 5C). However, rapamycin-treated mice had higher serum insulin levels ($P < 0.05$) compared with those in the control animals (Figure 5D). Thus, lean mice that received rapamycin developed symptoms of diabetes, with hyperglycaemia, hyperinsulinaemia and glucose intolerance.

Rapamycin reduced insulin sensitivity by altering the expression of GLUT4 transporters in HPD-induced lean mice

However, despite the beneficial effects of a HPD on body weight, fat pads, fasting glucose levels and glucose tolerance, chronic rapamycin administration further increased insulin resistance and exacerbated the insulin insensitivity in HPD-induced lean mice. This was supported by a drastic increase in the respective calculated HOMA-IR (Figure 6A) and insulin sensitivity index (Figure 6B) respectively. To further characterize the means by which glucose transport is reduced after mTOR inhibition, we evaluated the effect of rapamycin on glucose transporter expression. Compared with the control group, rapamycin treatment dramatically ($P < 0.01$) decreased phosphorylation of the mTOR downstream targets S6K1 (Thr^{389}), used as a specific marker of mTOR activity, that is pathologically characterized in diabetes development (Dann *et al.*, 2007; Chang *et al.*, 2009b). We also found that GLUT4 expression in muscle was significantly ($P < 0.05$) reduced in HFD-fed mice after 35 days of rapamycin treatment (Figure 6C).

Rapamycin affected chromium concentrations in tissues of HPD-induced lean mice

As observed previously in human and animal studies (Anderson, 1998), trivalent chromium may play an important role in glucose homeostasis by potentiating the effects of insulin. To evaluate whether lean rapamycin-treated mice exhibiting glucose intolerance would respond to changes in chromium concentrations, we measured the chromium content in tissues at the end of the study and the results are shown in Table 1. The concentrations of chromium in the blood and bone of rapamycin-treated lean mice fed a HPD were approximately 34 and 33% lower ($P < 0.05$), respectively, in comparison with vehicle-treated animals, while the chromium concentrations in liver and fat pads exhibited the reverse trend, with mean increases of 2.1- and

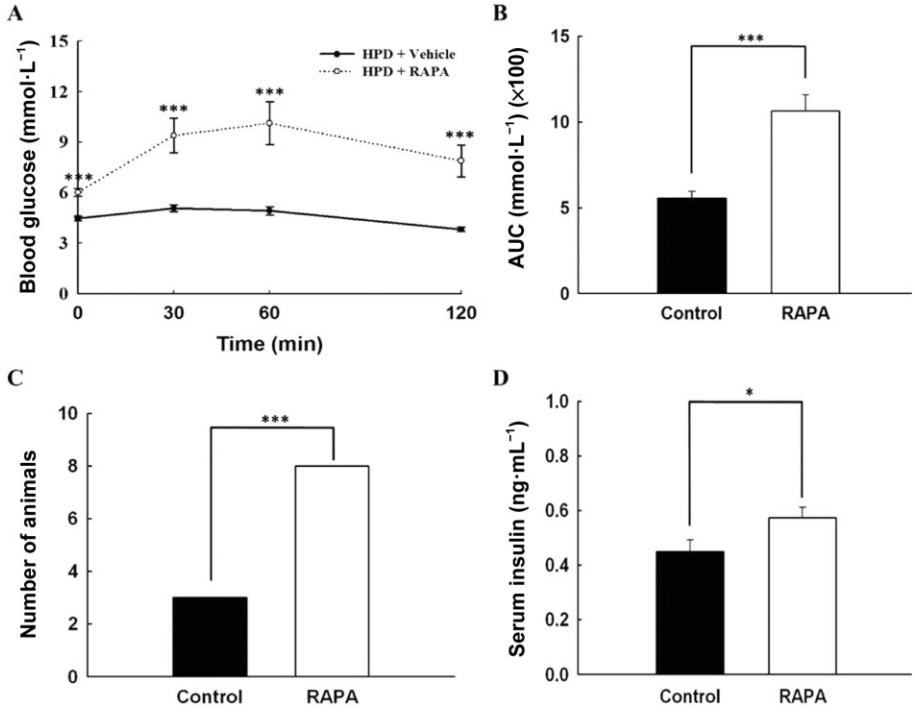

Figure 5

Effect of rapamycin on (A) glucose tolerance test: 1 g·kg^{-1} of glucose, (B) AUC during the 120 min after glucose injection, (C) criterion of glucose intolerance (Fisher's exact test) and (D) serum insulin levels were measured from vehicle or rapamycin-treated lean mice for 35 days. All values are given as mean ± SEM, $n = 12$ for all groups. *$P < 0.05$ and ***$P < 0.001$.

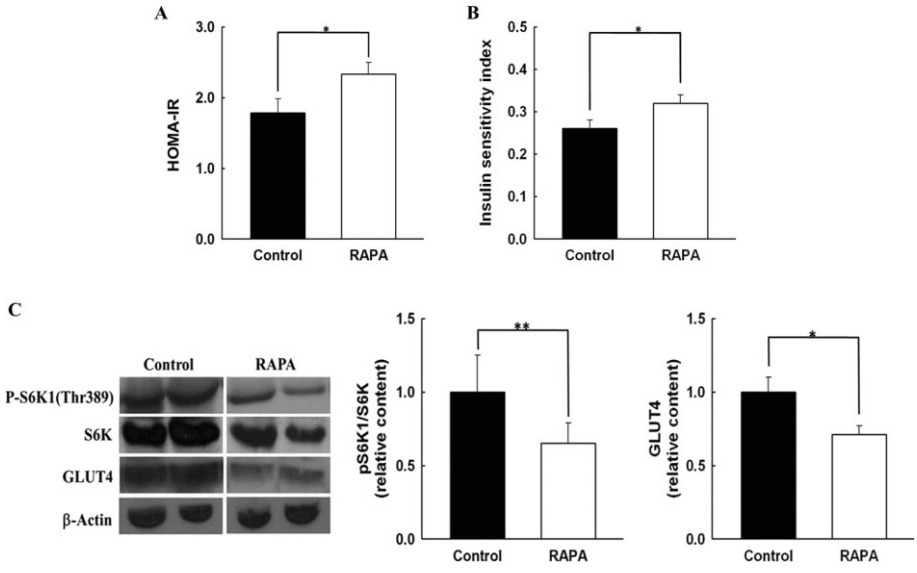

Figure 6

Effect of rapamycin on (A) HOMA–IR and (B) insulin sensitivity index and (C) expression of phospho-S6K1 and GLUT4 in gastrocnemius muscle; results were obtained from vehicle- or rapamycin-treated lean mice for 35 days. All values are given as mean ± SEM, $n = 12$ for all groups. *$P < 0.05$ and **$P < 0.01$.

Table 1

Chromium levels in tissues from vehicle- or rapamycin-treated lean mice for 35 days

Variable	Control	Rapamycin
Blood (ppb)	7.48 ± 0.83	5.06 ± 0.45*
Bone (ppb)	211.15 ± 17.14	141.39 ± 15.83**
Liver (ppb)	18.31 ± 3.39	39.17 ± 9.72*
Epididymal fat pads (ppb)	41.39 ± 4.16	74.23 ± 9.47*
Muscle (ppb)	40.33 ± 4.29	41.69 ± 3.06
Kidney (ppb)	20.10 ± 2.21	20.81 ± 1.45

Data are presented as mean ± SEM. Statistically significant at *$P < 0.05$, **$P < 0.01$. $n = 12$ for all groups.

1.8-fold respectively. However, a similar concentration of chromium was found in the muscle and kidney in the HPD control and HPD rapamycin groups. Our results thus demonstrated marked changes in chromium metabolism with a net movement of chromium in hyperglycaemic mice.

Discussion

The aim of this study was to estimate the consequences of continuous administration of rapamycin in mice that received long-term intake of high-protein levels that induced leanness. The results showed that HPD markedly reduced body gain and caloric intake of mice fed a HPD was lower than that of mice fed a HFD or SD. Concomitantly, daily food efficiency, fat pad weights, fatty liver scores, adipocyte size, glucose intolerance and serum insulin levels were also lowered. The findings presented here show that mice fed a HPD for 12 weeks developed a lean body, had less visceral fat and achieved glucose homeostasis with hypoinsulinaemia. Subsequently, we showed that rapamycin treatment did not affect body weight change, food/caloric intake, serum leptin levels, fat pads and organ weights in HPD-induced lean mice compared with the control group (Supporting Information Table S2). However, more specifically, rapamycin-treated lean hypoglycaemic mice exhibited a sharp exacerbation of glucose intolerance and a reduction in insulin sensitivity, despite the greater serum insulin.

In accordance with our previous findings, as well as the results of others (Raz and Havivi, 1998; Reuben, 2001), we observed that a high-protein intake reduced body weight gain and improved weight control. Long-term (even exceeding 6 months) consumption of a HPD in rats had no deleterious effects on the hepatic system or kidneys (Lacroix *et al.*, 2004). However, the contribution of high-protein intake in facilitating weight loss by increasing thermogenesis is controversial (Brito *et al.*, 1992). The results of this study suggest that the lower gain in body weight in the HPD-fed mice may have been due to a decrease in caloric intake and daily food efficiency. This effect can prevent an increase in organ weight, fatty liver score and body fat mass. Furthermore, we observed that the decrease in weights of RWAT and EWAT in HPD-fed mice as compared with the HFD- and SD-fed mice were due to a decrease in average adipocyte size. This effect in HPD-fed mice may appear to cause a reduction in the number of large adipocytes, potentially due to the prevention of adipocyte hypertrophy by HPD. These results are in agreement with the notion that reducing the formation of new adipocytes from precursor cells (adipocyte differentiation) and limiting increases in adipocyte size due to fat storage (adipocyte hypertrophy) could result in decreased fat pad mass (Rosenbaum *et al.*, 1997).

Increased adiposity mass is associated with a decrease in oral glucose tolerance, as assessed by glucose tolerance test (Anderson *et al.*, 2001). We found that the body weights of mice fed a HPD were lower than those of mice fed a HFD and SD after 12 weeks. Weights of epididymal fat pads and measurements of fat mass expansion revealed marked differences among the three groups. This may be correlated with HPD intake leading to energy restriction, which induced leanness in mice as well as lower plasma glucose concentrations and glucose tolerance with hypoinsulinaemia. These results are in agreement with others (Parker *et al.*, 2002; Layman *et al.*, 2003), and can be explained by the improvement in insulin-mediated glucose uptake and the reduced postprandial insulin response achieved by preservation of lean mass in animals fed a HPD. As a consequence, high-protein intake had beneficial metabolic effects on insulin sensitivity through energy restriction and weight loss, both of which are associated with stabilization of lean mass and a decrease in fat tissue.

Typically, the antimicrobial agent rapamycin is used as an anti-rejection drug in transplant patients. Recently, a few reports have investigated the role of rapamycin in the mTOR pathway for nutrient regulation (Soliman, 2005). Moreover, administration of rapamycin may increase fatty acid oxidation (Sipula *et al.*, 2006) and inhibit adipocyte differentiation (Bell *et al.*, 2000) and adipocyte hypertrophy, leading to protection against obesity (Chang *et al.*, 2009a). However, the results from this study showed no specific effect of rapamycin in terms of body weight loss or gain in HPD-induced lean mice (Supporting Information Table S2). At the same time, rapamycin did not affect the weights of fat pads or organs in lean mice. However, rapamycin-treated mice had reduced food intake and the rats fed a SD had induced weight loss (Deblon *et al.*, 2012). Possibly, this was due to the relatively small difference in food/caloric intake in the rapamycin-treated group compared with the controls, which resulted in no significant differences in the levels of leptin, a hormone in the hypothalamus known to be involved in regulating food intake. On the other hand, the lean mice did not accumulate much body fat mass, which was provided for energy combustion by rapamycin administration. Thus, we suggest that rapamycin treatment of lean mice did not accelerate the development of more effective decreases in food intake and weight loss. Furthermore, the effects of rapamycin on food intake and energy homeostasis in lean animals consuming a restricted diet, such as HPD, may need to be confirmed by elevating the dosage. Differences between frequency and length of treatment are likely to have resulted in the variations in the observed effects of rapamycin.

Previous studies noted that rapamycin treatment, which inhibits the mTOR/S6K1 pathway, has beneficial effects in alleviating insulin resistance by enhancing insulin signalling and can also protect against the development of insulin-dependent diabetes mellitus in mice (Baeder *et al.*, 1992; Tzatsos and Kandror, 2006). However, other reports indicated that rapamycin inhibited the mechanistic target of mTORC1 and mTORC2, which may be relevant to the pathogenesis of type 2 diabetes (Dann *et al.*, 2007; Lamming *et al.*, 2012; Ye *et al.*, 2012). In addition, we previously found that the chronic effects of rapamycin on glucose metabolism under conditions of nutrition-induced hyperglycaemia were increased insulin resistance and exacerbated glucose intolerance in obese mice (Chang *et al.*, 2009b). Here, lean, glucose-tolerant mice fed a HPD were used to evaluate the effects of rapamycin during hypoglycaemia and our results showed that rapamycin treatment resulted in a dramatically worsened glucose tolerance, as shown by the 120 min AUC for plasma glucose levels and the reduced insulin sensitivity index. This observation may be explained by decreases in the expression of muscle glucose transporter protein, GLUT4, which were found in our analyses, and by decreases in insulin-stimulated Akt phosphorylation induced by long-term rapamycin administration, leading to a deterioration in insulin signalling (Fraenkel *et al.*, 2008; Lopes *et al.*, 2014). Our data suggest additional mechanisms by which muscle glucose uptake is impaired in response to chronic mTOR inhibition by rapamycin. This was consistent with decreases in phosphorylation of S6K1, which is the downstream effector of mTORC1/mTORC2 and is involved in signalling in obesity and diabetes.

Furthermore, our results indicate that mice treated with rapamycin had an increased insulin resistance index, which has been proposed as a method to assess insulin resistance and secretion (Haffner *et al.*, 1996). Generally, increased adipose tissue or hepatic steatosis has been suggested to represent one of the factors contributing to insulin resistance (Garg and Misra, 2002). However, in our study, fat pads and fatty liver scores in rapamycin-treated mice were not increased in HPD-induced lean mice, but rather reduced, when obese mice with rapamycin treatment remained on a HFD (Chang *et al.*, 2009a,b). In addition, we found increased serum insulin levels in rapamycin-treated mice, which is consistent with defects in hepatic insulin clearance (Houde *et al.*, 2010). The increased insulin levels did not correspond to enhanced insulin signalling, which is the major pathophysiological indicator of insulin resistance. Together, these data appeared to show that systemic mTOR inhibition may lead to alterations in insulin signalling and glucose homeostasis, which might be subsequent to insulin resistance as evidenced by the higher serum insulin levels. In view of this phenomenon, the effect of rapamycin on insulin resistance was to worsen the hypoglycaemia induced by long-term consumption of HPD as a deterioration in the insulin signalling results in a reversal of the metabolic state to exacerbate glucose homeostasis.

The exacerbation of impaired glucose tolerance and induction of hyperglycaemia after rapamycin treatment was pronounced in HPD-induced lean animals, despite the higher insulin levels. The movement of chromium was also shown to play a metabolic role, as there was an alteration in the distribution of chromium in the tissue, a condition that is known to alter glucose metabolism (Anderson, 1998; Clodfelder *et al.*, 2004). One major finding indicated that the chromium moved from the blood to the metabolic tissues, subsequently facilitating activation of insulin receptors and thereby contributing to increased insulin signalling to reduce blood glucose levels (Reuben, 2001). Interestingly, we found that rapamycin administration also caused alterations in chromium levels in tissues/organs as there was reduced chromium levels in the blood and bone but increased levels in the liver and fat pads of rapamycin-treated mice compared with those of the control mice. However, chromium supplementation did not obviously prevent mice treated with rapamycin from developing symptoms of diabetes, including hyperglycaemia and glucose intolerance.

In addition, we found that the concentration of chromium in the liver showed the largest increase, over twofold, compared with those of other tissues, paralleling the control group, which is similar to the result reported by Wu *et al.* (1997). However, this could be reversed by insulin injection (Clodfelder *et al.*, 2001). Moreover, Anderson and Polansky (1995) indicated that the highest amounts of chromium were found in kidneys, spleen and pancreas following oral chromium administration, and insulin had no effect on chromium retention in healthy and STZ-induced diabetic rats. This apparent discrepancy is likely to be due to differences among chromium absorption, chromium excretion and blood plasma chromium levels (Clodfelder *et al.*, 2004). In addition, markedly lower chromium levels in hyperglycaemic rapamycin-treated lean animals' bones were observed than those in the control group. Studies have shown that essential minerals whose physiological pool exists in the bone are released to overcome hyperglycaemia by enhancing insulin signalling (Nishimuta, 2000). These data suggest that long-term mTOR inhibition results in a compensatory mechanism that may trigger the release of essential metals from the reserve pool into insulin-sensitive tissues where they showed pathophysiological effects.

Rapamycin is currently an approved immunosuppressive and powerful anti-proliferative drug that helps to prevent organ rejection in transplant patients and is used as long-term therapy in the United States, Europe and other parts of the world. Our study has important clinical implications, given that long-term rapamycin exposure is required to treat organ allograft rejection in human clinical trials (Johnson *et al.*, 2001; Morales *et al.*, 2005). Here, our data on chronic use of rapamycin raised the concern that rapamycin has the potential to disrupt glucose homeostasis, thereby elevating the risk of glucose intolerance and insulin resistance, which could lead to diabetes. Taken together with our previous finding (Chang *et al.*, 2009b) and others (Brattstrom *et al.*, 1998; Reuben, 2001), and with the exception of body weight gain, rapamycin treatment, especially prolonged use, is associated with adverse side effects, such as glucose intolerance, hyperinsulinaemia and insulin resistance in transplant patients. Thus, caution is warranted in the administration of rapamycin in clinical practice and further studies are necessary to understand how to reduce the risk of rapamycin treatment in transplant and diabetes patients.

In conclusion, this study demonstrated that HPD can be used to develop a lean animal model with glucose tolerance

for animal/human research. However, continuous administration of rapamycin in lean mice ultimately led to excessive promotion of hyperglycaemia, impairment of glucose homeostasis and a reduction in insulin sensitivity. It is proposed that a reduction in the expression of skeletal muscle GLUT4 contributes to these alterations in glucose metabolism. In addition, rapamycin treatment also resulted in a redistribution of tissue chromium levels, but the effect of this essential metal failed to overcome hyperglycaemia. These data suggest that the effect of rapamycin obviously accelerates the development of diabetes symptoms in mice, whereas HPD allowed the mice to maintain glucose homeostasis. Thus, rapamycin may be effective in the treatment of organ rejection in transplant patients, but its long-term use may cause adverse effects associated with diabetes, even in lean humans.

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Author contributions

Y.-S. C., Y.-Y. W., Y.-C. L. and P.-H. H. carried out testing analysis and interpretation of data for this work. G.-R. C. contributed to the study design and advised on translational aspects of experiments. F. C. M. supervised all experiments, advised on statistical analysis and co-wrote the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Table S1 Composition of the experimental diets.
Table S2 Effects of rapamycin on body weight, metabolic effects, serum leptin levels, organ weight and fatty liver scores of lean mice treated for 35 days with vehicle or rapamycin.