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Chronic administration of theobromine inhibits mTOR signal in rats

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Running Title: Theobromine inhibits mTOR

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Abstract

Theobromine is a caffeine derivative and the primary methylxanthine in *Theobroma cacao*. We have shown previously that theobromine inhibits the Akt-mammalian target of rapamycin (mTOR) signal *in vitro*. In this study, we investigated whether orally administered theobromine could inhibit mTOR activity in rats. mTOR is phosphorylated by Akt. Thus, the level of phosphorylated mTOR was used as an index of mTOR activity.

Male Wistar rats were divided into two groups. The control group (CN) was fed a normal diet, while the theobromine group (TB) was fed a diet supplemented with 0.05% theobromine for 40 days. We measured body and tissue weights, food and water intake, blood count, concentrations of theobromine in the plasma, liver and brain, and the levels of phosphorylated mTOR in the liver and brain.

Orally administered theobromine did not affect the body and tissue weights, food and water intake, and blood count as determined by comparison with levels in rats that were fed standard chow. Theobromine was detected in the plasma, liver and brain obtained from TB rats, but was not detected in tissues obtained from CN rats. The phosphorylated mTOR levels in the liver and brain were significantly lower in TB rats than in CN rats. The results suggest that oral theobromine inhibits mTOR signalling *in vivo*.

Keywords:

Theobromine, mTOR, Akt, 4E-BP1, cerebral cortex, liver

Introduction

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that is activated by Akt. It regulates protein synthesis and degradation, cell survival, proliferation and longevity [1, 2]. Thus, dysregulation of mTOR may contribute to disease pathogenesis [2-5]. mTOR also plays a role in nutrient sensing, and is activated in the liver and other tissues in individuals with obesity [6, 7] and non-alcoholic fatty liver disease [8]. Chronic activation of mTOR in the liver exacerbates hepatocyte damage and hepatocellular carcinoma. Similarly, mTOR activity is important in maintaining normal cognition [9]. Hyperactivation of mTOR leads to cognitive deficits in several clinical cases, such as tuberous sclerosis and Alzheimer's disease (AD) [2-5, 10, 11]. Interestingly, the administration of

rapamycin, an inhibitor of mTOR, offsets cognitive deficits in mouse models of tuberous sclerosis and AD [12, 13]. Therefore, mTOR might be a target for the treatment of hepatocellular carcinoma and cognitive deficits.

Several studies in the last decade have indicated that cacao-containing foods, such as chocolate, may have beneficial effects on several aspects of human health, such as cognitive function [14, 15]. In particular, dark chocolate with high concentrations of cacao may have more beneficial effects, and has recently become a popular choice for consumption. Cacao contains many flavonoids that have pleiotropic anti-oxidant and anti-inflammatory effects. In addition, cacao contains the caffeine derivative theobromine [16].

Theobromine is the primary methylxanthine found in products made from cacao (*Theobroma cacao*) [17]. Theobromine is a phosphodiesterase (PDE) inhibitor and increases intracellular cyclic adenosine monophosphate (cAMP) [18]. cAMP signalling is fundamentally involved in neural wiring and in the brain mechanisms that mediate cognitive processes [19, 20]. cAMP activates the cAMP-response element-binding protein (CREB) which, in turn, induces the expression of the brain-derived neurotrophic factor (BDNF) that mediates neuronal functions like learning and memory. We previously confirmed that theobromine-fed mice performed better on learning tasks in a cAMP/CREB/BDNF pathway-dependent manner [21]. We also found that theobromine suppresses

mTOR activity *in vitro* [18]. However, the *in vivo* pharmacological actions of theobromine have not been fully elucidated. In this study, we examined whether orally administered theobromine regulates the mTOR signal in rats.

We found that theobromine suppressed mTOR signal *in vivo*. Our findings potentially provide a rationale for a nutritional approach in treating or preventing liver diseases and neurological disorders, such as hepatocellular carcinoma and cognitive deficits, respectively.

Materials and Methods

Animals

Male Wistar rats (5 weeks of age) were housed individually in transparent plastic cages with wood chippings used as the bedding. They were maintained at 24.0 ± 0.1°C with 54 ± 5% relative humidity under a 12:12-hr light/dark cycle. The rats had access to food and water *ad libitum*. At the end of the administration period, the rats were anaesthetised using isoflurane, and the blood, liver and brain were sampled. The time frame was kept very similar for all rats. The study was conducted in accordance with the BCPT policy for experimental and clinical studies [22]. In addition, all animal experiments were performed in accordance with the Guidelines for Animal Experimentation of the Shimane University Faculty of Medicine, which complied with the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science.

Materials

Theobromine and caffeine-d9 were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Anti-mTOR, anti-phospho-mTOR (Ser2448), anti-Akt, anti-phospho-Akt (Ser473), anti-eIF-4E binding protein 1 (4E-BP1), anti-phospho-4E-BP1 (Thr37/46), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and horseradish peroxidase (HRP)-linked anti-rabbit IgG antibodies were purchased from Cell Signalling Technology, Inc. (Danvers, MA, USA). EzWestBlue was purchased from ATTO Corp. (Tokyo, Japan). The chow supplemented with 0.05% w/w theobromine was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan).

Experiment schedules

The experiment schedules are summarized in Fig. 1. All rats (n=48) had free access to standard chow (CRF-1, Oriental Yeast Co., Ltd.) for 10 days after admission. On day 0, the rats were divided into two groups. The first group consisted of control rats (CN, n=24) who were fed the CRF-1 chow for a maximum of 40 days. The second group of rats (TB, n=24) was fed the standard CRF-1 chow supplemented with 0.05% w/w theobromine (Oriental Yeast Co., Ltd.) for a maximum of 40 days. The concentration of theobromine was selected based on our previous report [21]. We confirmed that this concentration of theobromine had no adverse effects on the rodents. Body weights were measured every 10 days after admission. Tissue weights, food and water intake, and blood count were measured on days 10, 20, 30 and 40. Theobromine concentrations in the plasma, liver and brain were

analysed on the same days (n=6 in each group). The levels of phosphorylated mTOR, Akt, and 4E-BP1 in the liver were measured on day 30. The levels of phosphorylated mTOR in the cerebral cortex were measured on days 30 and 40 (n=6 in each group).

Measurements of theobromine contents

Theobromine was measured in the biological samples as previously described [21]. The liver and brain tissues were homogenised in phosphate-buffered saline (pH 7.4). Plasma, liver and brain samples were mixed with caffeine-d9 in acetonitrile as an internal standard. The samples were kept at −30°C for 30 min. and then centrifuged at 5,000 × *g* for 10 min. at 4°C. The supernatants were analysed using high-performance liquid chromatography in combination with electrospray ionisation-mass spectrometry that was performed with a TSQ quantum mass spectrometer (Thermo Fisher Scientific K.K., Tokyo, Japan).

Levels of phosphorylated mTOR, Akt, and 4E-BP1

Western blotting was performed as previously described [23]. The proteins in the liver and cerebral cortex were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The resolved proteins were transferred to polyvinylidene fluoride membranes and incubated with primary antibodies (1:1,000) and then with HRP-linked secondary antibodies (1:2,000). The blots were developed with EzWestBlue.

Statistical analyses

The data are expressed as mean ± standard error of the mean. Statistical significance was evaluated using two-way analysis of variance (ANOVA) or Student's t-test. P-values < 0.05 were considered statistically significant.

Results

Body and tissue weights and food and water intake

Body and tissue weights and food and water intake were determined every 10 days. The body weights of the rats that were fed theobromine-supplemented chow (TB rats) did not differ from those of the rats that were fed the standard chow (CN rats) (Fig. 2).

The weights of the heart, kidney, epididymal adipose and adeps renis tissues were not different between the CN and TB rats (Table 1). Food and water intake also did not differ between the two groups (Fig. 2). These results indicate that theobromine did not affect the feeding behaviour and body composition of the rats.

Blood counts and theobromine concentrations in the plasma, liver and brain

Blood count and theobromine concentration were determined in tissues every 10 days. Red blood cell count (RBC), white blood cell count (WBC), platelet count (PLT) and haemoglobin concentration (Hb) in TB rats did not differ from those in CN rats (Table 2).

Theobromine was detected in the plasma, liver and cerebral cortex of TB rats, but not in those of CN rats on days 10, 20, 30 or 40 (Table 3). The theobromine concentrations in plasma and liver homogenates were 14.99 ± 1.01 μg/mL and 1.72 ± 0.30 μg/mL, respectively, on day 30. The respective levels on day 40 (17.02 ± 1.37 μg/mL and 1.51 ± 0.04 μg/mL) were similar. Changes in the theobromine concentration in the cerebral cortex differed from those in the plasma or liver. The theobromine concentration in cerebral cortex homogenates was 0.87 ± 0.03 μg/mL on day 30 and was twofold greater on day 40 (1.94 ± 0.16 μg/mL).

Levels of phosphorylated mTOR, Akt and 4E-BP1 in the liver

We examined whether orally administered theobromine suppressed the mTOR signal in the liver in CN and TB rats on day 30. The levels of phosphorylated Akt and mTOR proteins in the liver were significantly lower in the TB rats than in the CN rats (Fig. 3), which indicated that mTOR activity was suppressed in the TB rats. In the liver, the level of phosphorylated 4E-BP1, a molecule downstream of mTOR, was significantly lower in the TB rats than in the CN rats (Fig. 3). These results suggested that orally administered theobromine inhibited the mTOR signal in the liver.

Levels of phosphorylated mTOR in the brain

We examined whether orally administered theobromine suppressed the mTOR pathways in the cerebral cortex in CN and TB rats on days 30 and 40. Although the levels of phosphorylated

mTOR proteins in the cerebral cortex were not significantly different between the TB rats and the CN rats on day 30 (Fig. 4A), the levels in the TB rats were significantly lower than those in the CN rats on day 40 (Fig. 4B). These results suggested that orally administered theobromine might inhibit the mTOR signal in the brain depending on the levels of theobromine present.

Discussion

This study reveals that the oral administration of theobromine through theobromine-supplemented chow inhibited the mTOR signal in the liver and brain. Theobromine was detected in the plasma, liver and brain of theobromine-fed rats, but was not detected in the tissues of standard chow-fed rats. In addition, chronically administered theobromine did not affect the body and tissue weights, food and water intake, and blood count of rats, as determined by comparison with rats that were fed standard chow.

Theobromine was detected in the plasma, liver and brain of the rats at least 10 days after initiation of the theobromine-supplemented diet. Subsequently, the concentrations of theobromine in the plasma, liver and brain of the rats gradually increased in a time-dependent manner (Table 3). The submaximal concentrations of theobromine in the plasma (14.99 ± 1.01 μg/mL) and liver homogenates (1.72 ± 0.30 μg/mL) were reached 30 days after initiation of the theobromine-supplemented diet, while the submaximal concentration of theobromine in brain homogenates (1.94 ± 0.16 μg/mL) was reached on day 40 (Table 3). The submaximal concentrations

of theobromine in the liver (day 30) and brain (day 40) might have been sufficient to produce pharmacological effects, as has been described [16, 17, 21]. To enter the brain, theobromine must cross the blood-brain barrier, which constitutes endothelial cells, pericytes and glial cells. Consequently, the duration needed to reach submaximal levels in the brain after the initiation of the theobromine-supplemented diet might be longer than that in the liver. Since only one concentration (0.05% w/w) of theobromine was administered for up to 40 days, further study may be required at different concentrations and for prolonged periods.

The presence of theobromine in the liver and brain might influence the signalling pathways in these organs through its pharmacological actions. As expected, the present study demonstrated that oral theobromine inhibited the mTOR signal in the liver and brain. Theobromine is a recognized inhibitor of phosphodiesterase (PDE), and so is expected to increase the concentration of cAMP by preventing its hydrolysis [18]. Recently, we demonstrated that the levels of phosphorylated vasodilator-stimulated phosphoprotein (VASP) in the brain are increased in theobromine-fed mice [21]. VASP is an established substrate of protein kinase A (PKA) [24, 25]. The increased levels of phosphorylated VASP in the brains of theobromine-fed mice indicate an increase in cAMP levels in the brain. Similarly, orally administered theobromine may act as a PDE inhibitor *in vivo* and increase the cAMP levels in the livers and brains of theobromine-fed rats.

Much of the available evidence has shown that methylxanthines, including theobromine and caffeine, exert a pharmacological action by inhibiting Akt activation via cAMP *in vitro* [18, 26, 27]. PKA and exchange protein directly activated by cAMP (Epac) are molecular players located downstream of cAMP [28]. We have shown that Epac, but not PKA, is involved in cAMP-dependent Akt deactivation [29]. Akt is a molecular player upstream of mTOR that activates mTOR [30]. Both p70S6 kinase and 4E-BP1 are phosphorylated by activated mTOR and subsequently regulate the S6 ribosomal protein (S6) and eIF-4E, respectively [30]. The present finding of lower levels of phosphorylated Akt, mTOR and 4E-BP1 in theobromine-fed rats indicates that theobromine inhibits the Akt-mTOR pathway *in vivo*, as it does *in vitro*. Analysis of second downstream target of mTOR, such as the S6 Kinase activity assay, may be required in the future.

mTOR hyperactivation has been implicated in several pathologies that include autoimmune diseases, cognitive deficits, cancer and acquired immune deficiency syndrome [31-34]. Since the accumulation of theobromine in the brain has been clearly detected, the use of theobromine could also be of particular relevance for neuroinflammatory conditions, such as multiple sclerosis and the rodent counterpart experimental autoimmune encephalomyelitis (EAE). Rodent EAE benefits from the mTOR inhibitor, rapamycin and a Phase II study with rapamycin was recently conducted [33, 34]. Interestingly, this study demonstrated that orally administered theobromine suppressed mTOR activity in the liver and brain, but did not alter physiological parameters, such as body and tissue weights, food and water intake, and blood count. Therefore, our findings strongly suggest that cacao products,

including chocolate and cocoa, might be beneficial in the prevention of liver diseases and neurological disorders, such as hepatocellular carcinoma and cognitive deficits, respectively. Further studies using several disease model animals may be required.

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Conflicts of interest

The authors do not have any conflicts of interest to declare.

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Table 1

Heart, kidney, epididymal adipose and adeps renis tissue weights

	Day 10		Day 20		Day 30		Day 40	
	CN	TB	CN	TB	CN	TB	CN	TB
Heart	3.14 ± 0.11	3.12 ± 0.13	2.94 ± 0.08	2.75 ± 0.07	2.80 ± 0.06	2.93 ± 0.14	2.75 ± 0.08	2.69 ± 0.09
Kidney	6.95 ± 0.18	6.89 ± 0.19	7.04 ± 0.23	6.33 ± 0.20	6.40 ± 0.18	6.12 ± 0.11	6.06 ± 0.08	5.82 ± 0.08
Epididymal adipose	11.2 ± 0.3	12.7 ± 1.3	10.4 ± 1.7	12.9 ± 0.6	11.1 ± 0.9	12.5 ± 1.0	10.9 ± 0.5	12.8 ± 0.8
Adeps renis	3.86 ± 0.29	4.02 ± 0.24	4.07 ± 0.48	4.94 ± 0.38	4.17 ± 0.24	4.89 ± 0.48	4.40 ± 0.36	4.47 ± 0.28

Weight is expressed in g (mean ± standard error of the mean, SEM) in control (CN) and theobromine-fed (TB) rats

Table 2

White blood cell count (WBC), red blood cell count (RBC), platelet count (PLT) and haemoglobin concentration (Hb)

	Day 10		Day 20		Day 30		Day 40	
	CN	TB	CN	TB	CN	TB	CN	TB
WBC ($\times 10^2/\mu L$)	85.6 ± 5.6	80.2 ± 5.9	75.8 ± 7.8	69.0 ± 11.0	83.3 ± 4.7	80.0 ± 6.3	75.7 ± 5.9	85.2 ± 5.6
RBC ($\times 10^4/\mu L$)	799.7 ± 18.6	784.0 ± 7.1	788.5 ± 16.6	762.0 ± 12.2	812.2 ± 12.9	804.8 ± 7.6	827.7 ± 13.8	821.2 ±15.3
PLT ($\times 10^4/\mu L$)	63.1 ± 10.5	70.5 ± 2.8	73.8 ± 4.8	74.5 ± 2.3	75.8 ± 4.5	74.5 ± 3.0	74.4 ± 1.7	74.7 ± 3.0
Hb (g/μL)	14.9 ± 0.28	14.6 ± 0.10	14.4 ± 0.30	14.2 ± 0.23	14.5 ± 0.14	14.8 ± 0.22	14.9 ± 0.19	14.7 ± 0.24

Results are expressed as mean ± standard error of the mean (SEM) in control (CN) and theobromine-fed (TB) rats

Table 3

Theobromine levels in plasma, liver and cerebral cortex

	Day 10		Day 20		Day 30		Day 40	
	CN	TB	CN	TB	CN	TB	CN	TB
Plasma (μg/mL)	N/D	7.67 ± 0.33	N/D	8.45 ± 0.50	N/D	14.99 ± 1.01	N/D	17.02 ± 1.37
Liver (μg/mL homogenate)	N/D	1.04 ± 0.12	N/D	1.40 ± 0.18	N/D	1.72 ± 0.30	N/D	1.51 ±0.04
Cortex (μg/mL homogenate)	N/D	0.68 ± 0.05	N/D	0.64 ± 0.03	N/D	0.87 ± 0.03	N/D	1.94 ±0.16

Results are expressed as mean ± standard error of the mean (SEM) in control (CN) and theobromine-fed (TB) rats. N/D; not detected

Figure legends

Figure 1

Feeding and experiment schedules. The rats (n=48) were divided into two groups. Those in the first group (control; CN, n=24) were fed the standard chow (CRF-1) for a maximum of 40 days. The second group (theobromine; TB, n=24) was fed chow supplemented with 0.05% w/w theobromine (CRF-1 with 0.05% theobromine) for a maximum of 40 days. Body weight and food and water intake were measured on days −10, 0, 10, 20, 30 and 40 (n=6 in each group). The tissue weight, blood count and theobromine concentrations in the plasma, liver and cerebral cortex were measured on days 10, 20, 30 and 40 (n=6 in each group). The phosphorylated mTOR, Akt and 4E-BP1 levels were measured on days 30 and 40 (n=6 in each group).

Figure 2

Body weights and food and water intake in control (CN;○) and theobromine-fed (TB;●) rats. Each data point represents the mean ± SEM (n=6 in each group).

Figure 3

Phosphorylated mTOR, Akt and 4E-BP1 levels on day 30 in the liver of rats. Theobromine inhibits mTOR, Akt and 4E-BP1 phosphorylation in the liver of the rats. Each data point represents the mean ± SEM (∗ $P < 0.05$, n=6 in each group).

Figure 4

Phosphorylated mTOR levels in the cerebral cortex of rats. Theobromine inhibits mTOR phosphorylation in the cerebral cortex of rats on day 40 (B), but not on day 30 (A). Each data point represents the mean ± SEM (∗∗ $P < 0.01$, n=6 in each group).

Figure 1

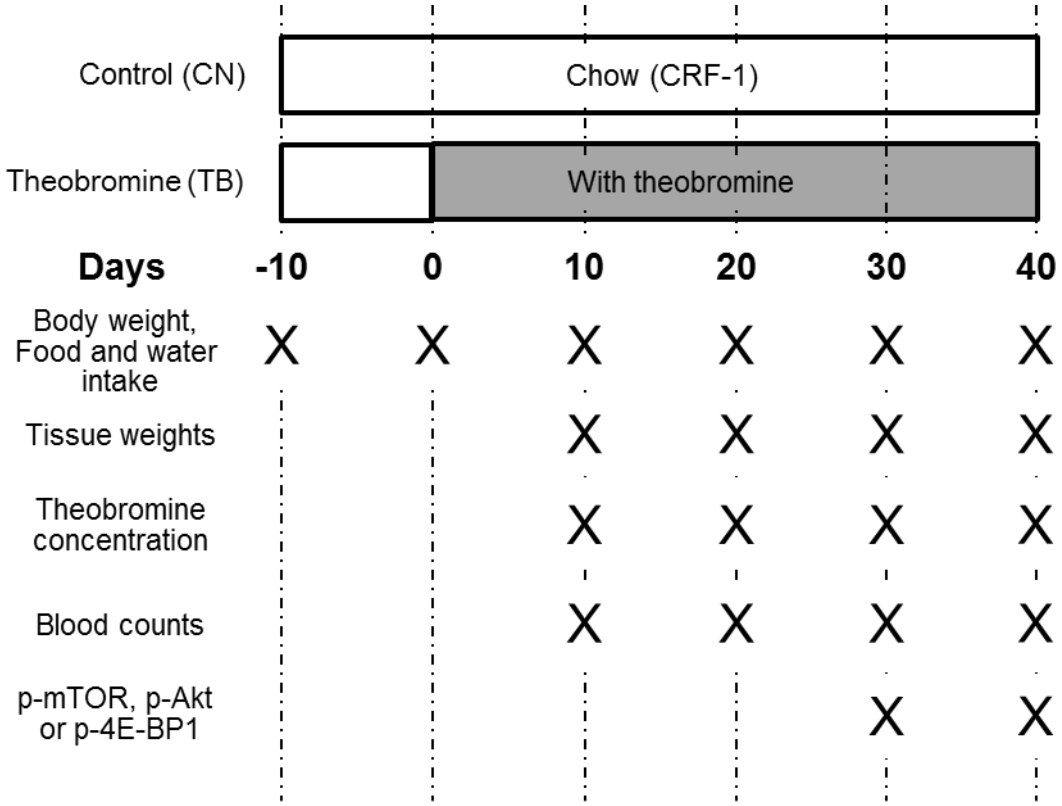


Figure 2

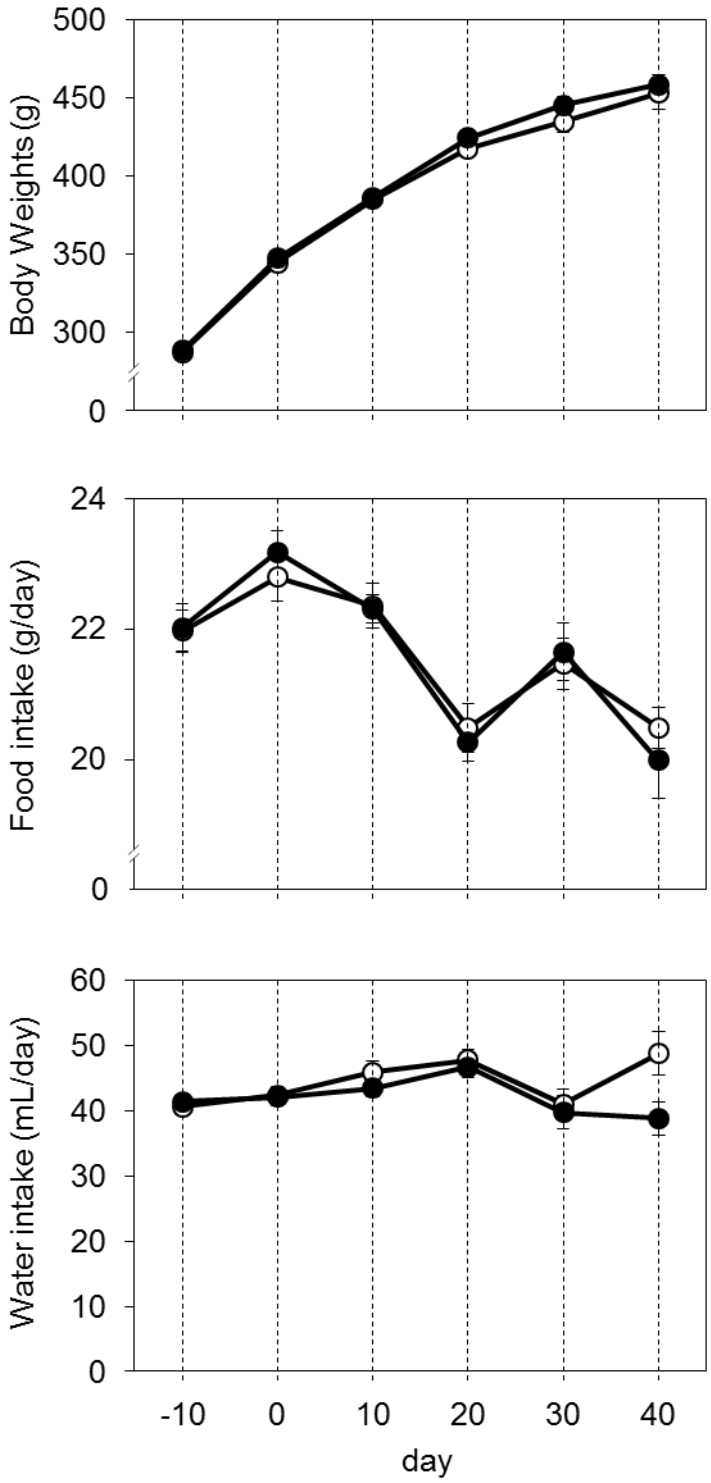
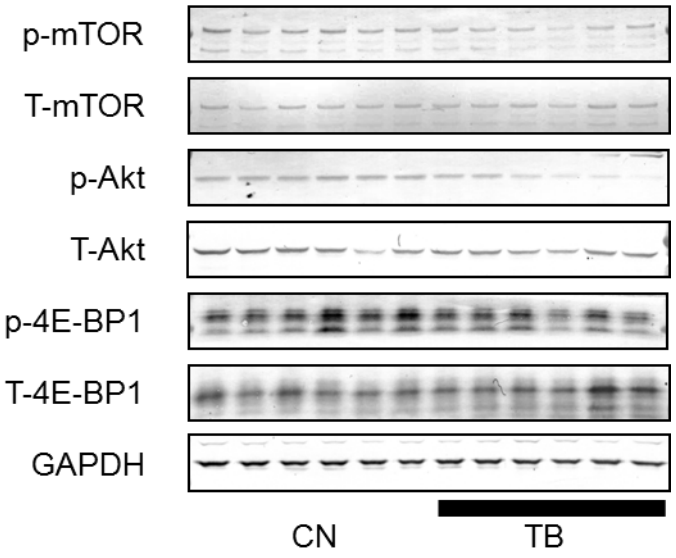
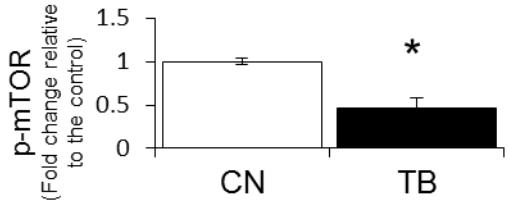
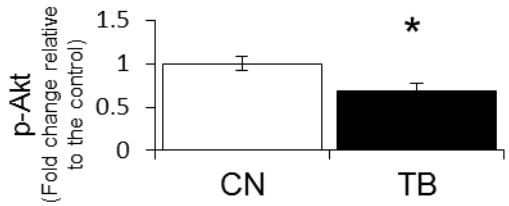


Figure 3







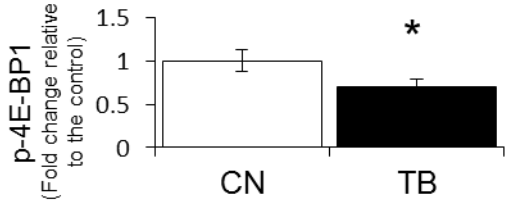


Figure 4