

# A central role for the mammalian target of rapamycin in LPS-induced anorexia in mice

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## Abstract

Bacterial lipopolysaccharide (LPS), also known as endotoxin, induces profound anorexia. However, the LPS-provoked pro-inflammatory signaling cascades and the neural mechanisms underlying the development of anorexia are not clear. Mammalian target of rapamycin (mTOR) is a key regulator of metabolism, cell growth, and protein synthesis. This study aimed to determine whether the mTOR pathway is involved in LPS-induced anorexia. Effects of LPS on hypothalamic gene/protein expression in mice were measured by RT-PCR or western blotting analysis. To determine whether inhibition of mTOR signaling could attenuate LPS-induced anorexia, we administered an i.c.v. injection of rapamycin, an mTOR inhibitor, on LPS-treated male mice. In this study, we showed that LPS stimulates the mTOR signaling pathway through the enhanced phosphorylation of mTOR<sup>Ser2448</sup> and p70S6K<sup>Thr389</sup>. We also showed that LPS administration increased the phosphorylation of FOXO1<sup>Ser256</sup>, the p65 subunit of nuclear factor kappa B ( $P < 0.05$ ), and FOXO1/3a<sup>Thr24/32</sup> ( $P < 0.01$ ). Blocking the mTOR pathway significantly attenuated the LPS-induced anorexia by decreasing the phosphorylation of p70S6K<sup>Thr389</sup>, FOXO1<sup>Ser256</sup>, and FOXO1/3a<sup>Thr24/32</sup>. These results suggest promising approaches for the prevention and treatment of LPS-induced anorexia.

## Key Words

- ▶ anorexia
- ▶ FoxO1
- ▶ hypothalamus
- ▶ LPS
- ▶ mTOR
- ▶ NFκB
- ▶ rapamycin

*Journal of Endocrinology*  
(2015) **224**, 37–47

## Introduction

Overwhelming evidence has suggested that infectious and non-infectious pathogens stimulate the production of pro-inflammatory cytokines and induce weight loss and anorexia (Socher *et al.* 1988, Tracey *et al.* 1988, Grunfeld *et al.* 1989). Transcription factor nuclear factor kappa B (NFκB) is a key regulator of genes encoding cytokines, cytokine receptors, and cell adhesion molecules that stimulate inflammatory responses (Hayden & Ghosh 2008). Peripherally, lipopolysaccharide (LPS) activates NFκB, which results in the production of pro-inflammatory cytokines, such as interleukin 1 (IL1), tumor necrosis factor alpha (TNFα), and IL6 (Johnson 1997,

Segreti *et al.* 1997, Finck *et al.* 1998). NFκB normally remains inactive in the cytoplasm, as it is bound to IκBα, and translocates into the nucleus in response to LPS stimulation (Li & Karin 1999). In addition, LPS modulates the transcriptional activity of NFκB through the phosphorylation of the NFκB p65 subunit (Kim *et al.* 2011). It has been reported that cytokines have direct action on the central response to inflammatory stimuli, especially LPS (Laye *et al.* 2000, Sergeev *et al.* 2001, Ogimoto *et al.* 2006). In the hypothalamus, however, the mechanisms by which NFκB integrates the LPS signals to regulate food intake remain unclear.

The mammalian target of rapamycin (mTOR), an evolutionarily conserved serine/threonine kinase, regulates critical aspects of the regulation of cell growth, including transcription, translation initiation and elongation, and cell cycle progression, by responding to changes in energy status (Wullschleger *et al.* 2006). In addition, mTOR is necessary to control food intake by integrating the signals that monitor the nutritional and hormonal signals in the hypothalamus (Cota *et al.* 2006, Ropelle *et al.* 2008a,b). An active hypothalamic mTOR signaling pathway suppresses food intake; however, hypothalamic mTOR inhibition by i.c.v. injection of rapamycin significantly increased the short-term food intake in pre-satiated rats (Cota *et al.* 2006). mTOR is downstream of the phosphatidylinositol 3-kinase (PI3K)/AKT pathways; therefore, both insulin- and leptin-induced anorectic effects can be blocked by the inhibition of PI3K (Niswender *et al.* 2001, 2003). Cota *et al.* (2006) observed that the inactivation of mTOR with rapamycin apparently attenuated the anorexia and body weight loss induced by leptin.

Importantly, recent studies have indicated that the serine/threonine kinase AKT regulated the activation of NFκB following exposure to LPS in human macrophages (Monick *et al.* 2001). This finding suggests that the mTOR signaling pathway plays an important role in the activation of NFκB in immune cells. However, whether the mTOR pathway is involved in the activation of NFκB in the hypothalamus remains unknown.

The forkhead transcriptional factor subfamily forkhead box O1 (FoxO1) is another important downstream target of the PI3K/AKT pathways (Tang *et al.* 1999). Activated AKT phosphorylates FOXO1, leading to its nuclear exclusion and proteasomal degradation (Matsuzaki *et al.* 2003, Aoki *et al.* 2004). FOXO1 is a shared component that regulates peripheral metabolism and food intake (Kim *et al.* 2006). Previous studies have shown that FOXO1 protein plays essential roles in the transcriptional cascades that are responsible for metabolism in the muscle, liver, brain, pancreas, and adipose tissues (Nakae *et al.* 2002, Kamei *et al.* 2004, Kim *et al.* 2006, 2009). In the CNS, leptin decreases the expression of FOXO1 in the hypothalamus and induces a reduction in food intake and body weight (Kim *et al.* 2006, Kitamura *et al.* 2006). Activation of FOXO1 promotes coactivator–corepressor exchange and, therefore, activates agouti-related peptide (AGRP) expression and inhibits pro-opiomelanocortin (POMC) expression (Accili & Arden 2004). Therefore, it is important to understand how these pathways in the hypothalamus respond to LPS-induced anorexia.

We hypothesized that the mTOR pathway is involved in LPS-induced anorexia. In this study, the interactions between the inflammatory signals induced by LPS and the neuronal regulatory network related to appetite control were investigated during the development of anorexia. The results indicated that the mTOR signaling pathway is involved in LPS-induced anorexia, and the inhibition of mTOR signaling provides an attractive way to alleviate LPS-induced anorexia.

## Materials and methods

### Animals

Kunming male mice (7–10 weeks old) were housed in a controlled environment of 24 °C with a 12 h light:12 h darkness cycle. All mice were fed in individual cages and had free access to standard chow and water before the experiments started. The experimental procedures were approved by the Institutional Animal Care and Use Committees in accordance with the criteria outlined in the Guide for the Care and Use of Laboratory Animals (Beijing, People's Republic of China).

### Food intake and body weight measurements after LPS injection

Mice were randomly divided into four groups, with six mice in each group, and all mice were fasted for 24 h before the experiment. At the start of the dark cycle, the mice were injected intraperitoneally with LPS (*Escherichia coli* 055:B5, Sigma) at dosages of 0, 10, 100, or 1000 µg/kg BW and were then given immediate access to food. Food intake was recorded after 1, 2, 4, 6, 8, 12, 18, and 24 h. Body weight was recorded at 0, 24, 48, and 72 h after injection. Time course was used to determine the response to LPS treatment.

### Implantation of i.c.v. cannulae

After an overnight fast, the mice were anesthetized by i.p. injection of 3% pentobarbital sodium (Merck) at a dose of 40 mg/kg body weight. The mice were mounted using a stereotaxic apparatus (Huai Bei Zheng Hua, Anhui, China), and a small incision was made in the flesh along the midline. The cannula was fixed to the skull with dental cement. The coordinates of the third cerebral ventricle from the bregma was 1.82 mm posterior to the bregma and 5 mm below the surface of the skull. The mice were allowed to recover for 1 week before the experiment.

### I.c.v. injection

After a 24-h fast, four groups of mice were subjected to two treatments: i.c.v. injected with rapamycin (R0395-1MG, Sigma) or the vehicle (DMSO in artificial cerebrospinal fluid) 2 h before the start of the dark cycle (Cota *et al.* 2006, Verhulst *et al.* 2012); rapamycin was dissolved in DMSO at a concentration of 25 mg/ml and was used at a final concentration of 20 µg/2 µl. Following the infusion, the guide cannula remained inserted for ~30 s to allow the drug to diffuse away from the cannula tip. One hour later, the two groups of mice in each treatment were given an i.p. injection of either 500 µg/kg LPS or saline. The mice were then returned to their cages, and food was returned 2 h later. The cumulative food intake was recorded at 1, 2, 4, 6, and 12 h from food reoffered.

### Nuclear and cytoplasmic protein extract

Twelve mice were randomly divided into two groups ( $n=6$  in each group) and were fasted for 20 h prior to the experiment. At the start of the dark cycle, the mice were injected intraperitoneally with 500 µg/kg (BW) LPS or saline. Two hours later, the mice were killed by decapitation, and the hypothalami were promptly removed according to Kim *et al.* (2004) to extract the nuclear and cytoplasmic proteins. The nuclear and cytoplasmic protein extractions were conducted on ice with the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, Jiangsu, People's Republic of China) according to the

manufacturer's instructions. Proteins were then stored at  $-80^{\circ}\text{C}$  until further analysis.

### Gene expression analysis

According to the effects of LPS administration on food intake and body weight, the 100 or/and 500 µg/kg BW doses of LPS were chosen for the subsequent experiments. The mice were fasted for 24 h and received pretreatment with rapamycin or vehicle; the mice were then injected intraperitoneally with 500 µg/kg (BW) LPS or saline. Two hours later, the mice were killed and the hypothalami were promptly removed. The samples were rapidly frozen in liquid nitrogen and then stored at  $-80^{\circ}\text{C}$  until further analysis.

Total RNA was isolated from the hypothalami using TRIzol reagent (Invitrogen). The integrity and concentration of the RNA were assessed by measuring the optical density at 260–280 nm using a biophotometer (Eppendorf, Hamburg, Germany). Total RNA (1 µg) was reverse transcribed using the PrimeScript RT Reagent Kit (Perfect Real Time, TaKaRa, Dalian, China) according to the manufacturer's procedures. Quantitative real-time RT-PCR (qRT-PCR) was conducted using SYBR Green I Dye (TaKaRa) on an Applied Biosystems Real-time PCR System 7500 (Applied Biosystems). The qRT-PCR included a 95 °C denaturation step for 10 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 40 s. The primer sequences used in the qRT-PCR analysis are listed in Table 1. The housekeeping gene used for correction was  $\beta$ -actin. Relative mRNA abundance was calculated according to the established method (Livak & Schmittgen 2001).

**Table 1** Gene-specific primers used for the analysis of gene expression

| Gene           | GenBank accession no. | Primer sequences (5'–3')                                  | Product size (bp) |
|----------------|-----------------------|---|-------------------|
| $\beta$ -actin | NM_007393.3           | F: ACCACACCTTCTACAATGAG<br>R: ACGACCAGAGGCATACAG          | 182               |
| POMC           | NM_008895.3           | F: CGGGAGGCGACGGAAGAGAAAA<br>R: AACAAAGATTGGAGGGACCCCTGT  | 112               |
| NPY            | NM_023456.2           | F: CCGCCACGATGCTAGGTAACAAG<br>R: CCCTCAGCCAGAATGCCCAAAC   | 91                |
| AGRP           | NM_007427.2           | F: GCGGAGGTGCTAGATCCACAGAA<br>R: AAGGCATTGAAGAAGCGGCAGTAG | 137               |
| TNF $\alpha$   | NM_013693.2           | F: AAGCCTGTAGCCCACGTCGTA<br>R: GGCACCACTAGTTGGTTGCTTTG    | 122               |
| IL6            | NM_031168.1           | F: GAGGATACCACTCCCAACAGACC<br>R: AAGTGCATCATCGTTGTTCATACA | 141               |
| IL1 $\alpha$   | NM_010554.4           | F: TCGGGAGGAGACGACTCTAA<br>R: AGGTCGGTCTACTACCTGTG        | 146               |
| IL1 $\beta$    | NM_008361.3           | F: GAAGAAGAGCCCATCTCTG<br>R: TCATCTCGGAGCCTGTAGTG         | 98                |

POMC, pro-opiomelanocortin; NPY, neuropeptide Y; AgRP, agouti-related peptide; TNF $\alpha$ , tumor necrosis factor alpha; IL6, interleukin 6; IL1 $\alpha$ , interleukin 1 alpha; IL1 $\beta$ , interleukin 1 beta.

## Western blotting analysis

The mice were fasted for 24 h and received pretreatment with rapamycin; the mice were then injected intraperitoneally with 500 µg/kg (BW) LPS or saline. Two hours later, the mice were killed by decapitation and the hypothalami were quickly removed. The samples were rapidly frozen in liquid nitrogen and then stored at  $-80^{\circ}\text{C}$  until further analysis. Total protein was extracted from the hypothalami, which were lysed and homogenized in 500 µl of precooled Radio Immuno-precipitation Assay buffer (Beyotime) containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, sodium orthovanadate, sodium fluoride, EDTA, and leupeptin and was supplemented with PhosStop Phosphatase Inhibitor (Roche) and 1 mM phenylmethanesulfonyl fluoride (Beyotime). Then, the samples were centrifuged at 15294 g for 5 min at  $4^{\circ}\text{C}$ . The protein concentration was measured using the BCA Protein Assay Kit (Beyotime). The protein lysates were denatured for 10 min at  $100^{\circ}\text{C}$  and were then electrophoresed in running buffer on a 7.5–12% Tris-glycine SDS-polyacrylamide gel. The lysates were transferred to a PVDF microporous membrane (Millipore, Billerica, MA, USA) at 80 V at  $4^{\circ}\text{C}$  for 2 h. After incubation for 1 h in block solution (5% BSA, 0.1% Tween-20, and 0.02% sodium azide in PBS, pH 7.6) at room temperature, the membranes were incubated at  $4^{\circ}\text{C}$  overnight in primary antibodies against phospho-p70S6 kinase (Thr389) (#9234, Cell Signaling Technology, Inc., Beverly, MA, USA), phospho-FOXO1 (Ser256) (#9461, Cell Signaling Technology, Inc.), phospho-FOXO1 (Thr24)/FOXO3a (Thr32) (#9464, Cell Signaling Technology, Inc.), phospho-NFκB p65 (Ser536) (93H1) rabbit mAb (#3033, Cell Signaling Technology, Inc.), phospho-mTOR (Ser2448) (#2971, Cell Signaling Technology, Inc.), p70S6 kinase (#2708, Cell Signaling Technology, Inc.), mTOR (#2983, Cell Signaling Technology, Inc.), FOXO1 (L27) (#9454, Cell Signaling Technology, Inc.), NFκB p65 (C22B4) rabbit mAb (#4764, Cell Signaling Technology, Inc.), and β-actin mouse monoclonal (AA128, Beyotime) respectively. The blots were then incubated with anti-rabbit or anti-mouse HRP-conjugated IgG (Bio-Rad Laboratories) for 1 h. The bands were detected using Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Pittsburgh, PA, USA) and were visualized by exposure to X-ray film (Kodak). Quantification was made using the Image J 1.43 Software (National Institutes of Health, Bethesda, MD, USA).

## Statistical analyses

All data were analyzed with the SAS Software (SAS, version 8e; SAS Institute, Cary, NC, USA). A one-way ANOVA

model was used to evaluate the means of the LPS treatment. Then, Duncan's multiple comparisons test was used to detect significant differences among the different groups. A two-way ANOVA was performed to evaluate the main effect of LPS administration and rapamycin treatment and their interaction. If a significant interaction was detected, differences among various groups were assessed with Duncan's multiple comparisons test. The data are shown as the mean  $\pm$  s.e.m.  $P < 0.05$  was regarded as statistically significant.

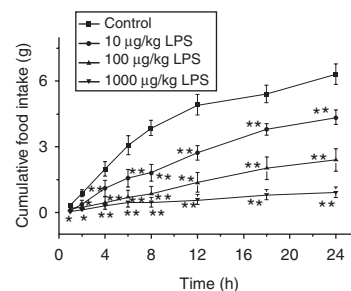
## Results

### Effects of LPS on food intake and body weight

Consistent with previous studies, we found that LPS treatment of 10–1000 µg/kg BW induced a significant decrease in food intake ( $P < 0.01$ , Fig. 1), with progressive bodyweight loss over the first 24 h (data not shown).

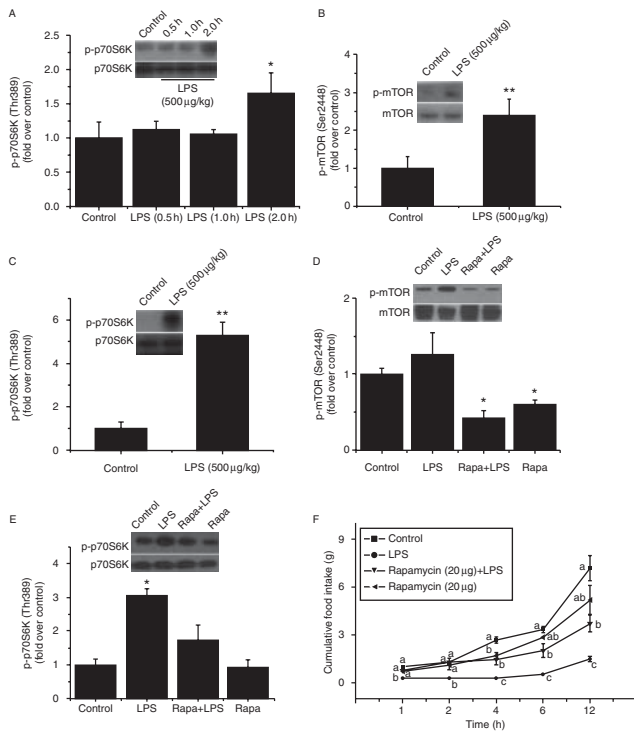
### Effect of LPS on the mTOR pathway

To investigate whether mTOR signaling is associated with LPS-induced anorexia, we conducted western blotting analysis on the hypothalami of mice to evaluate the phosphorylation levels of mTOR and p70S6K. We determined the phosphorylation level of p70S6K at Thr389 at different time points after LPS treatment (500 µg/kg BW). The results showed that the phosphorylation level of hypothalamic p70S6K was promoted after LPS administration ( $P < 0.05$ , Fig. 2A). We then measured the phosphorylation levels of mTOR and p70S6K 2 h after LPS treatment (500 µg/kg BW), and the results showed that phosphorylation levels of mTOR and p70S6K were significantly upregulated by LPS ( $P < 0.01$ , Fig. 2B and C).



**Figure 1**

Effect of LPS treatment on food intake. The daily food intake was measured in the mice i.p. injected with LPS at doses of 0, 10, 100, or 1000 µg/kg (BW). Values are the means  $\pm$  s.e.m. ( $n = 6$  per group,  $*P < 0.05$  and  $**P < 0.01$ ).

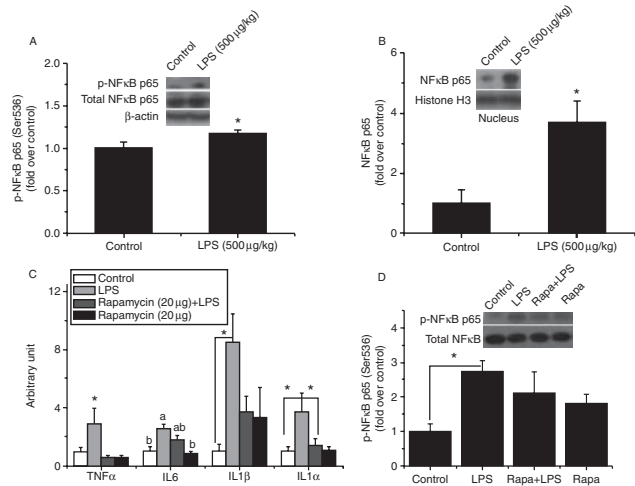
**Figure 2**

The mTOR plays a key role in LPS-induced anorexia. The phosphorylation of hypothalamic p70S6K (A) was determined at various time points after LPS (500 µg/kg BW) treatment. The levels of phospho-mTOR (Ser2448) (B) and phospho-p70S6 kinase (Thr389) (C) were determined in the hypothalami of mice 2 h after LPS (500 µg/kg) administration compared with the control (saline). Fasted mice were i.c.v. administered rapamycin (Rapa) or an equal volume of vehicle. One hour later, half of the mice were i.p. administered LPS (LPS and Rapa + LPS respectively). All measurements were determined 2 h after LPS treatment. (D) The level of hypothalamic phospho-mTOR (Ser2448). Two-way ANOVA showed a significant interaction between LPS and rapamycin treatment ( $P < 0.05$ ). (E) The level of hypothalamic phospho-p70S6K (Thr389). Two-way ANOVA showed a significant interaction between LPS and rapamycin treatment ( $P < 0.05$ ). (F) Cumulative food intake (g). Two-way ANOVA showed a significant interaction between LPS and rapamycin treatment at all time points ( $P < 0.05$ ); rapamycin was dissolved in DMSO at a concentration of 25 mg/ml and was used at a final concentration of 20 µg/2 µl. The values are the means  $\pm$  s.e.m. ( $n = 6$  mice per group). \* $P < 0.05$  and \*\* $P < 0.01$ , means without a common letter are different.

Rapamycin was used to investigate the effect of mTOR inhibition on the LPS-induced anorexia. The mice were pretreated with rapamycin (20 µg/2 µl) for 1 h, followed by i.p. injection of 500 µg/kg BW LPS. Inactivation of the mTOR pathway significantly decreased the LPS-induced phosphorylation of mTOR and p70S6K ( $P_{\text{Rapa}} < 0.05$ , Fig. 2D and E). As shown in Fig. 2F, mTOR inhibition significantly increased food intake in the mice challenged with LPS, and at 2 h, the cumulative food intake was stimulated by  $\sim 4.3$ -fold compared with the consumption induced by a single injection of LPS ( $P_{\text{Rapa} \times \text{LPS}} < 0.05$ ).

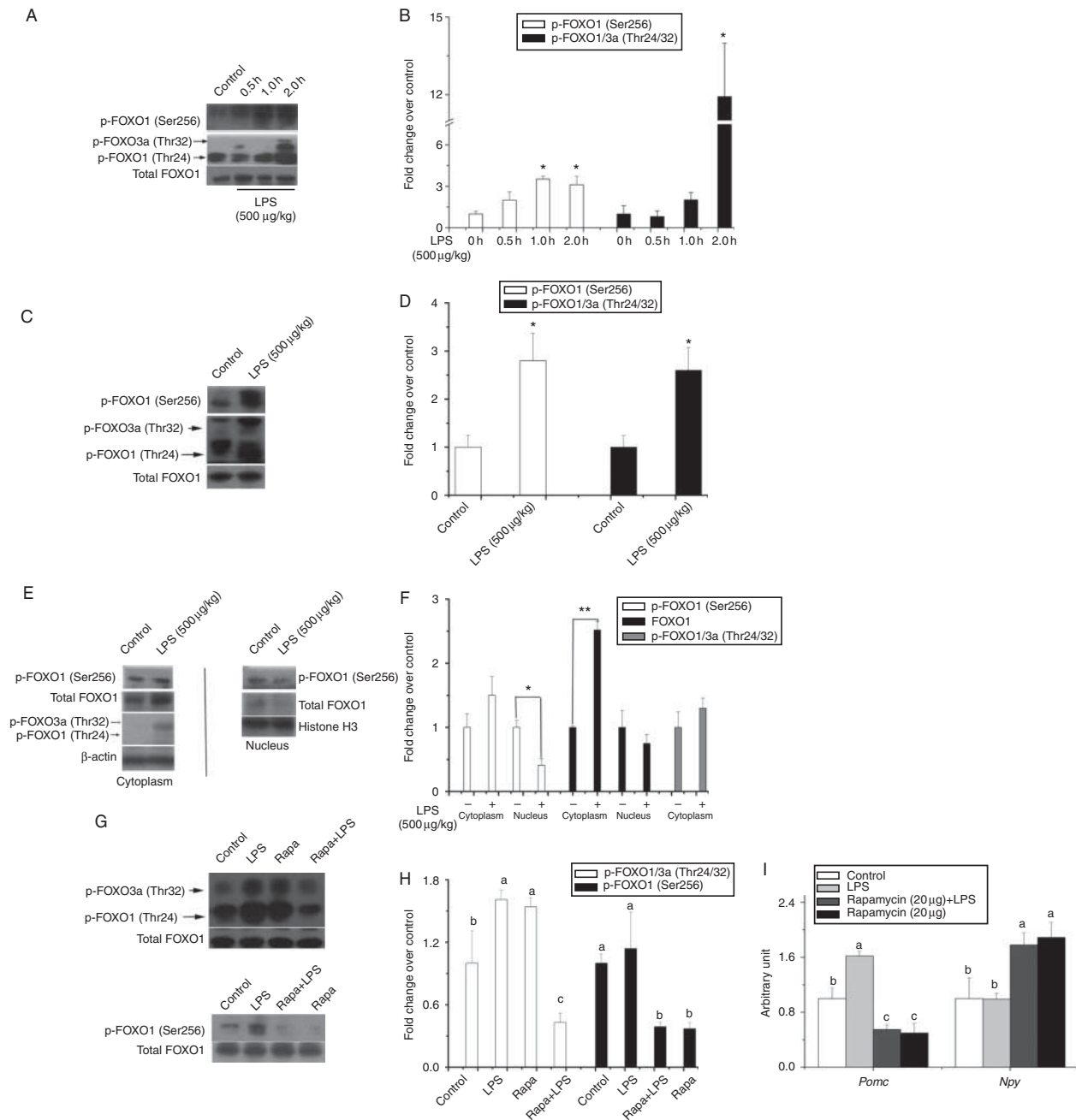
### Effects of LPS and mTOR inhibition on Ser536 phosphorylation of NFκB p65 and cytokine expression

The levels of the pro-inflammatory cytokine and appetite gene expression were determined in the 500 µg/kg BW LPS administration group. The results showed that the mRNA levels of *Il1α*, *Il1β*, *Il6*, and *Tnfα* were significantly increased compared with the control group ( $P < 0.05$ , Fig. 3C). The expression of the orexigenic appetite genes neuropeptide Y (*Npy*) showed no significant difference compared with the control group ( $P > 0.05$ , Fig. 4I). In contrast, the expression of *Pomc* was significantly upregulated following LPS injection relative to the control ( $P < 0.05$ , Fig. 4I). We further detected the activation of NFκB and the results showed that the phosphorylation of the NFκB p65 subunit on Ser536 was elevated  $\sim 1.2$ -fold following LPS administration ( $P < 0.05$ , Fig. 3A). The level of hypothalamic NFκB protein was significantly increased in the nuclear extracts ( $P < 0.05$ , Fig. 3B). In addition, as shown in Fig. 3C, after mTOR inactivation, the expression of the pro-inflammatory cytokines *Tnfα* and *Il1α*, which were induced by LPS, were significantly reduced ( $P_{\text{Rapa} \times \text{LPS}} < 0.05$ ), and the expression level of *Il1β* exhibited a downward trend ( $P_{\text{Rapa}} = 0.05$ ). However, the mRNA level of *Il6* showed

**Figure 3**

Cross talk of the mTOR and NFκB in LPS-induced anorexia. Effects of LPS (500 µg/kg) administration on the phosphorylation of hypothalamic NFκB p65 at Ser536 (A) and hypothalamic NFκB (p65) nuclear accumulation compared with the control (B). Fasted mice were i.c.v. administered rapamycin (Rapa) or an equal volume of vehicle. One hour later, half of the mice were i.p. administered LPS (LPS and Rapa + LPS respectively). All measurements were determined 2 h after LPS treatment. (C) The mRNA expression of pro-inflammatory cytokines in the hypothalami of mice. (D) The level of hypothalamic phospho-NFκB p65 (Ser536). The values are the means  $\pm$  s.e.m. ( $n = 6$  mice per group). Means without a common letter differ significantly (\* $P < 0.05$ ).



**Figure 4**

Effects of the mTOR on FOXO pathway in LPS-induced anorexia. Fasted mice were i.p. administered LPS (500 µg/kg BW). The phosphorylation of hypothalamic FOXO1 at Ser256 and FOXO1/3a at Thr24/32 (A and B) was determined at various time points after LPS treatment. Fasted mice were i.p. administered LPS (500 µg/kg BW). Two hours later, the mice were killed by decapitation and the hypothalami were removed quickly. The phosphorylation of hypothalamic FOXO1 at Ser256 and FOXO1/3a at Thr24/32 (C and D) and the nuclear and cytoplasmic levels of these proteins were also determined 2 h after LPS (500 µg/kg) administration (E and F). Fasted mice were i.c.v. administered rapamycin (Rapa) (20 µg/2 µl) or an

equal volume of vehicle. One hour later, half of the mice were i.p. administered LPS (LPS and Rapa + LPS respectively). All measurements were determined 2 h after LPS treatment. The levels of hypothalamic phospho-FOXO1/3a Thr24/32 and phospho-FOXO1 (Ser256) (G and H). Two-way ANOVA showed a significant interaction between LPS and rapamycin treatment ( $P < 0.05$ ). The mRNA levels of the appetite genes *Pomc* and *Npy* (I). Two-way ANOVA showed a significant interaction between LPS and rapamycin treatment ( $P < 0.05$ ). The values are the means  $\pm$  s.e.m. ( $n = 6$  mice each group). Means without a common letter differ significantly ( $*P < 0.05$  and  $**P < 0.01$ ).

no significant differences compared with treatment with LPS only ( $P_{\text{Rapa} \times \text{LPS}} > 0.05$ ). Inhibition of mTOR did not induce a statistically significant change in the level of NF $\kappa$ B p65 phosphorylation at Ser536; however, this effect tended to be downregulated ( $P_{\text{Rapa}} = 0.086$ , Fig. 3D).

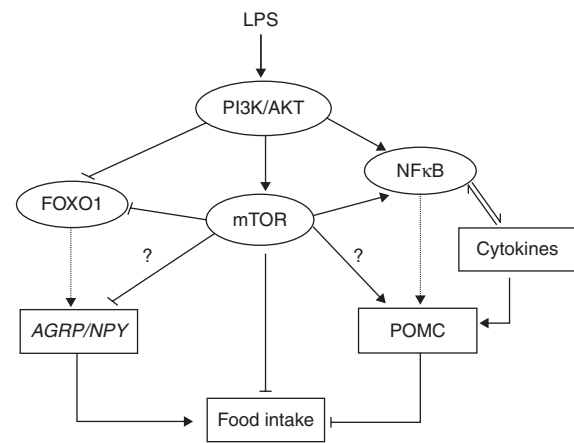
### Effects of LPS and mTOR inhibition on the phosphorylation of FOXO1 at Ser256 and Thr24 and FOXO3A at Thr32

To explore the effect of LPS administration on FOXO1, we determined the FOXO1 activity by examining the phosphorylation level of FOXO1 at Ser256 and Thr24 and the phosphorylation of FOXO3a at Thr32. The phosphorylation levels of FOXO1 at Ser256 and Thr24 and FOXO3a at Thr32 were measured in mice that were killed at various time points (0.5, 1.0, or 2.0 h) after LPS (500  $\mu\text{g}/\text{kg}$  BW) administration. The results revealed that the phosphorylation levels of hypothalamic FOXO1 were enhanced after LPS administration ( $P < 0.05$ , Fig. 4A and B). The phosphorylation levels of these proteins were then measured 2 h after LPS administration (500  $\mu\text{g}/\text{kg}$  BW). As shown in Fig. 4C and D, the phosphorylation levels of both FOXO1 and FOXO3a significantly increased ( $P < 0.05$ ) after LPS treatment, increasing by 2.8- and 1.9-fold respectively. After i.p. injection of LPS, the phosphorylation of cytoplasmic FOXO1 at Ser256 and of FOXO1/3a at Thr24/Thr32 showed no statistically significant changes ( $P > 0.05$ , Fig. 4E and F); however, the total protein level of FOXO1 was increased in the cytoplasm ( $P < 0.05$ , Fig. 4E and F) and decreased in the nucleus.

The phosphorylation level of FOXO1 at Ser256 and FOXO1/3a at Thr24/Thr32 induced by LPS was decreased significantly after mTOR inhibition compared with LPS treatment alone ( $P_{\text{Rapa} \times \text{LPS}} < 0.05$ , Fig. 4G and H). When mTOR was blocked, the expression of the *Pomc* gene, which is induced by LPS, significantly decreased compared with LPS treatment alone; in contrast, there was an  $\sim 1.8$ -fold increase in the expression of NPY when mTOR was inhibited, compared with the LPS treatment alone ( $P_{\text{Rapa} \times \text{LPS}} < 0.05$ , Fig. 4I).

## Discussion

The role of mTOR signaling in the regulation of food intake and the response to nutrient availability has been widely studied (Cota *et al.* 2006, Wullschleger *et al.* 2006). In this study, we report that the mTOR signaling pathway is involved in LPS-induced anorexia. We demonstrated that LPS administration stimulated the mTOR signaling pathway and the central blockade of the mTOR pathway



**Figure 5**

Proposed model of hypothalamic mTOR actions on LPS-induced anorexia in mice. LPS administration activated PI3K/AKT pathway and then stimulated mTOR and NF $\kappa$ B signaling, increasing the expression of cytokines and POMC. In contrary, activated mTOR inhibits the activity of FOXO1 and decreases the expression of the orexigenic appetite genes *AGRP* and *NPY*. The pathways collaborated with each other and resulted in lower food intake ( $\rightarrow$ , stimulatory;  $\leftarrow$ , inhibitory;  $\rightleftarrows$ , might be stimulatory;  $?$ , need to be further studied; LPS, lipopolysaccharide; PI3K/AKT, phosphatidylinositol 3-kinase; FoxO1, forkhead box O1; mTOR, mammalian target of rapamycin; NF $\kappa$ B, nuclear factor kappa B; AgRP, agouti-related peptide; NPY, neuropeptide Y; POMC, pro-opiomelanocortin).

attenuated the LPS-induced anorexia. The result suggests that the NF $\kappa$ B and FoxO1 pathways may be associated with the regulation of mTOR in LPS-induced anorexia. The proposed model of mTOR actions on LPS-induced anorexia in mice is shown in Fig. 5.

### NF $\kappa$ B activation in the hypothalamus is involved in LPS-induced anorexia

In this study, a single i.p. injection of LPS 500  $\mu\text{g}/\text{kg}$  BW was used to induce peripheral inflammation (Bassi *et al.* 2012, Iwasa *et al.* 2014). LPS administration induced anorexia, which is in line with previous studies (Gautron *et al.* 2005, Ogimoto *et al.* 2006). LPS administration stimulates the release of pro-inflammatory cytokines such as IL1, TNF $\alpha$ , IL6, and so on (O'Reilly *et al.* 1988, Plata-Salaman 1999, Wong & Pinkney 2004, Bennani-Baiti & Walsh 2011). These cytokines may be associated with the induction of anorexia; however, hypothalamic inflammatory signals, rather than peripheral, have been identified as the major causes of LPS-induced anorexia (Laye *et al.* 2000, Plata-Salaman 2000, Wisse *et al.* 2007). In line with previous studies, the mRNA levels of cytokines such as *IL1 $\alpha$* , *IL1 $\beta$* , *TNF $\alpha$* , and *IL6* were significantly increased in the hypothalamus after LPS administration.

Although the critical role of the hypothalamic inflammatory signal in LPS-induced anorexia has been recognized, it is unclear how the central melanocortin system is regulated accordingly. A recent study has revealed that NF $\kappa$ B directly bound the *POMC* gene to promote transcription in response to LPS stimulation (Jang *et al.* 2010), and meanwhile, the transcriptional level of NF $\kappa$ B was upregulated by LPS treatment in the hypothalamus. Nuclear translocation of NF $\kappa$ B has been shown to be insufficient for the maximal activation of NF $\kappa$ B (Chen & Greene 2004). It has also been revealed that phosphorylation of the NF $\kappa$ B p65 subunit contributes to the transcriptional activation of NF $\kappa$ B (Sakurai *et al.* 1999). In this study, we found that i.p. administration of LPS activated hypothalamic NF $\kappa$ B by increasing the phosphorylation of the p65 subunit, the higher level of NF $\kappa$ B in the nucleus, and the upregulated expression of *POMC* may imply that the translocation of NF $\kappa$ B from cytoplasm into nucleus is related to the transcription of target genes such as *POMC* during LPS stimuli. These findings (Campo *et al.* 2010, Jang *et al.* 2010, Deshpande *et al.* 2011) collectively imply that NF $\kappa$ B activation in the hypothalamus plays a critical role in LPS-induced anorexia.

### The mTOR pathway plays a critical role in LPS-induced anorexia

Hypothalamic mTOR is an important molecule in energy homeostasis. Activation of the mTOR cascade has been linked with decreased food intake, and mTOR blockade attenuates anorexia and weight loss induced by insulin and leptin in the CNS (Cota *et al.* 2006, Morton *et al.* 2006). We observed that mTOR signaling was enhanced through the phosphorylation of mTOR<sup>Ser2448</sup> and p70S6K<sup>Thr389</sup> in the hypothalamus during the inflammatory process stimulated by LPS, which implies that the mTOR pathway may be associated with the suppressed food consumption induced by LPS. Therefore, we proposed that inhibition of hypothalamic mTOR could block the anorexigenic and weight-reducing effects of LPS. The suppressed food intake by LPS was partly restored by mTOR pathway blockade, suggesting that the mTOR signaling pathway plays an important role in LPS-induced anorexia.

### Crosstalk of the mTOR and NF $\kappa$ B pathways in LPS-induced anorexia

Peripherally, mTOR signaling is involved in the regulation of the cytokine-induced inflammatory response, and the inhibition of mTOR activation by rapamycin has been

shown to inhibit IKK $\beta$ -mediated tumorigenesis (Lee *et al.* 2007). It has also been reported that inhibition of mTOR decreases the production of proinflammatory cytokines, inhibits the phosphorylation of the NF $\kappa$ B p65 subunit, and attenuates acute lung injury after intratracheal LPS administration (Lorne *et al.* 2009). A previous report has shown that inhibition of NF $\kappa$ B attenuated the reductions in body weight and food intake induced by tumors (Kawamura *et al.* 1999). However, the relationship between mTOR and NF $\kappa$ B in the hypothalamus remains unclear. In this experiment, the decreased phosphorylation level of NF $\kappa$ B p65 subunit by rapamycin treatment indicated that mTOR pathway inhibition may attenuate the inflammatory response induced by LPS in hypothalamus. Furthermore, we observed that mTOR pathway inhibition attenuated LPS-stimulated transcriptional expression of *Tnf $\alpha$* , *Il1 $\alpha$* , and *Il1 $\beta$*  ( $P_{\text{Rapa}}=0.05$ ). These results are in accordance with Schmitz *et al.* (2008), who showed that serum TNF $\alpha$  was suppressed by rapamycin treatment in LPS challenged mice, whereas the level of IL6 essentially remained unchanged. However, in other cell/tissue types, such as monocytes, rapamycin exposure did reduce the LPS-induced IL6 release (Schaeffer *et al.* 2011), suggesting that the discrepancies among the studies investigating IL6 production in response to mTOR inhibition are dependent on the cell/tissue type and the environmental conditions. In line with previous observations, rapamycin treatment prevented an increase in IL1 $\alpha$  and tended to suppress mRNA expression of *Il1 $\beta$*  ( $P_{\text{Rapa}}=0.05$ ) in response to LPS treatment, indicating that mTOR pathway inhibition attenuated LPS-induced cytokine expression in the hypothalamus. Harris *et al.* (2011) demonstrated that rapamycin inhibited IL1 $\beta$  secretion by bone marrow-derived cells treated with LPS. The result suggests that, in the hypothalamus, the inhibition of mTOR pathway partially prevented the activation of NF $\kappa$ B and, in turn, the production of proinflammatory cytokines, leading to the attenuation of anorexia induced by LPS.

### Effects of the mTOR on FOXO pathway in LPS-induced anorexia

The FOXO transcription factors FOXO1, FOXO3a, and FOXO4 are the targets of PI3K signaling, which regulates their activity via phosphorylation (Tang *et al.* 1999). FOXO1 has been shown to be related to the regulation of food intake (Kim *et al.* 2006). Moreover, it is interesting that FOXO1 has also been associated with the production of certain cytokines, and knockdown of *FOXO1* attenuates the hyper-inflammatory phenotype in the peripheral system



(Crossland *et al.* 2008, Su *et al.* 2009, Brown *et al.* 2011). As downstream targets of PI3K, the interaction between FOXO and mTOR is complicated, and several reports have suggested that FOXO negatively regulates mTOR signaling under different physiological conditions (Gilley *et al.* 2003, Jia *et al.* 2004). In this study, the result indicated that the FOXO1 pathway in the hypothalamus is influenced by LPS treatment. It is interesting that our results also showed that mTOR inhibition by rapamycin alleviated the LPS-induced phosphorylation of FOXO1 at Thr24 (pThr24-FOXO1) and mTOR alone blocked the decrease in the phosphorylation level of FOXO1 (Ser256). These findings indicated that rapamycin could decrease the LPS-induced phosphorylation of FOXO1, partly through the inhibition of LPS-induced mTOR signaling, resulting in the reduced phosphorylation level of FOXO1/3a (Thr24/32), as well as through the direct suppression of FOXO1 Ser256 phosphorylation by mTOR; these findings were similar to the observed roles of rapamycin derivatives (Zeng *et al.* 2007). As FOXO1 has three phosphorylation sites (Thr24, Ser256, and Ser319), the influence of the different phosphorylation levels at Thr24 and Ser256 on the role of FOXO1 in mTOR-regulated appetite control needs to be investigated further.

The proposed model of mTOR actions on LPS-induced anorexia in mice is shown in Fig. 5. LPS administration activated PI3K/AKT pathway and then stimulated mTOR and NF $\kappa$ B signaling, increasing the expression of cytokines and POMC. In contrary, activated mTOR inhibits the activity of FOXO1 and decreases the expression of the orexigenic appetite genes *AGRP* and *NPY*. The pathways collaborated with each other and resulted in lower food intake.

In conclusion, blocking the mTOR pathway significantly attenuated LPS-induced anorexia. Thus, this result suggests an attractive option to alleviate the development of LPS-induced anorexia.

#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

#### Funding

This work was supported by grants from the Natural Science Foundation of China (grant numbers 31072045 and 31272467).

#### Acknowledgements

The authors acknowledge the excellent technical assistance provided by Xianyao Li and Jingpeng Zhao.

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Received in final form 7 October 2014

Accepted 27 October 2014

Accepted Preprint published online 27 October 2014