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N-Formyl-3,4-methylenedioxy-benzylidene-γ-butyrolaetam, KNK437 induces caspase-3 activation through inhibition of mTORC1 activity in Cos-1 cells

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ABSTRACT

The mammalian target of rapamycin complex 1 (mTORC1: mTOR–raptor interaction) and heat shock protein 70 (Hsp70) regulate various cellular processes and are crucial for the progression of many cancers and metabolic diseases. In the recent study, we reported that interaction of Hsp70 with tuberous sclerosis complex 1 (TSC1) regulated apoptosis. This study was designed to elucidate the underlying mechanism in Cos-1 cells. Here, we show that *N*-formyl-3,4-methylenedioxy-benzylidene-γ-butyrolaetam (KNK437), which inhibits the expression level of Hsp70, abrogated phosphorylation of mTOR and S6K in response to insulin, and inhibited mTORC1 activity *via* disruption of an interaction between mTOR and raptor. In addition, KNK437 did not alter TSC1/2 complex formation. Furthermore, KNK437 inhibited the mTOR–raptor interaction on the outer membrane of the mitochondria and triggered caspase-3 activation. A reduction in the level of Hsp70 could result in the inhibition of the mTORC1 signaling pathway, thereby inducing apoptosis.

1. Introduction

The mammalian target of rapamycin complex 1 (mTORC1) pathway is a major regulator of cell growth. mTOR is a serine/threonine kinase of the PIKK (phosphoinositide 3-kinase-related kinase) family and plays a role in cellular metabolism and cell growth [1,2]. It is known mTOR possesses two different complexes, mTORC1 and mTORC2, which is well conserved from yeast to human [3,4]. mTORC1 is composed of mTOR, mLST8 and raptor, and consequently promotes phosphorylation of its effectors S6K and 4E-BP1 [5–7]. Rapamycin in complex with FK506-binding protein FKBP12 interacts with mTOR to block phosphorylation of S6K by mTOR [8]. Thus, this interaction of mTOR with raptor (mTORC1), which is sensitive to rapamycin, is an important tool in studying cancer and diabetes [9–11]. On the other hand, mTORC2 interacts with rictor, Protor and mLST8/GβL and these interactions are insensitive to rapamycin [12].

Recent studies reveal that protein synthesis through mTORC1 signaling is highly regulated by TSC1/2 complex activity [13]. The formation of the TSC1/2 complex regulates both its function and stability. In response to stimulation by insulin or growth factors, TSC2 is phosphorylated at Thr1462, thereby disrupting the dissociation of TSC1 from TSC2 [14]. In fact, the mTORC1 signal is inhibited by TSC1/2 complex and activated in cell by phosphorylation or loss-of-function of TSC1 or TSC2 [15–17]. Consequently, aberrant mTORC1 signaling, which is a direct result of mutations in TSC1 or TSC2, has resulted in tumor formation [18]. The possible association of mTORC1 activity with growth factors is therefore crucial in studying the signaling pathway and cell tumorigenicity.

mTOR has been shown to act as an intracellular regulator of cell growth and is regulated by a large number of signals including nutrients and growth factors, such as glucose, amino acids and insulin [19,20]. Recent reports have demonstrated that inhibition of mTOR by rapamycin not only attenuates insulin-resistance but also induces autophagy and apoptosis [21–23]. Therefore, rapamycin is used as an anti-cancer agent under clinical trials in an mTORC1-dependent manner.

Like Quercetin, KNK437 has been reported as an anti-cancer therapeutic and inhibits the expression of a chemically-induced *Hsp70* gene [24–26]. In fact, over-expression of Hsp70 induced a strong acceleration of cell growth and facilitated cell tumorigenicity [27,28]. Thus, increasing data now point toward Hsp70 being a key player in cell survival and apoptosis. We also reported that interaction of TSC1 with Hsp70 regulated apoptosis [29]. Thus, the association of Hsp70 with mTORC1 activity may be linked to regulation of tumorigenesis or apoptosis. However, we could not investigate whether KNK437 affected the mTORC1 signal activity. The aim of the present study was to investigate the possibility of a link between KNK437 and mTORC1 in the regulation of tumorigenesis/apoptosis.

Abbreviations: TSC2, tuberous sclerosis complex 2; KNK437, *N*-formyl-3,4-methylenedioxy-benzylidene-γ-butyrolaetam

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2. Materials and methods

2.1. Cell culture

Cos-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Equitech-Bio, TX), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL/Invitrogen, Carlsbad, CA) at 37 °C in 5% CO_2. EEF4 (a TSC2-positive embryonic fibroblast cell line derived from the Eker rat) and EEF8 (a TSC2-negative embryonic fibroblast cell line derived from the Eker rat) [30] were cultured in DMEM/Nutrient Mixture F-12 Ham (Sigma) supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in 5% CO_2.

2.2. Inhibitor experiment

N-formyl-3,4-methylenedioxy-benzylidene-γ-butyrolaetam (KNK437) was dissolved in dimethyl sulfoxide (DMSO). DMSO was also added to the DMEM medium instead KNK437 for the control experiment [24,29].

2.3. Antibodies

Antibodies for immunoblotting against TSC2 (C-20), Mcl-1 and GRIM-19 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody against mouse TSC1 was purchased from ZYMED Laboratories (San Francisco, CA). Antibodies against β-actin and Hsp70 were purchased from Sigma and Stressgen (Ann Arbor, MI), respectively. Antibodies against cleaved Caspase-3, cleaved Caspase-9, mTOR and S6K were from Cell Signaling Technology, Inc. (Danver, MA). All primary antibodies were diluted in Tris-buffered saline with 0.05% (v/v) Tween20 (TBST) at 1:1000.

2.4. Isolation of inner or outer membrane of mitochondria

Mitochondrial fraction was isolated from Cos-1 cells using a mitochondria purification kit (BioChain Institute, Inc., Hayward, CA) for cultured cells, according to the supplier's instruction. Mcl-1 and GRIM-19 were used as mitochondrial markers [29].

2.5. Immunoprecipitation, immunoblotting

Cells were washed twice with PBS (−) and lysed with 100 μl lysis buffer (40 mM Hepes (pH 7.6), 120 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 0.3% CHAPS, 0.5 μg/ml leupeptin, 2 μg/ml aprotinin and 10 μg/ml PMSF (pH 7.6)). Lysates were incubated with a specific antibody in the presence of protein A-agarose beads. The beads were washed with IP (immunoprecipitation) buffer (40 mM Hepes (pH 7.6), 120 mM NaCl, 0.3% CHAPS, 1 mM EDTA, 0.5 μg/ml leupeptin, 2 μg/ml aprotinin and 10 μg/ml PMSF (pH 7.6)) and the immunoprecipitates were separated by SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis), transferred to PVDF (Immobilon-P, Millipore Co. Billerica. MA) membrane, and incubated with specific antibodies (each diluted 1:1000). The immunoblotting was developed using HRP-conjugated anti-rabbit/mouse IgG antibodies (each diluted 1:5000) as a secondary antibody and detected using the GE Healthcare ECL System (Piscataway, NJ).

3. Results

3.1. KNK437 suppressed phosphorylation and activity of mTORC1 signal

To test whether KNK437 inhibited phosphorylation of mTOR (Ser2448) and S6K (Thr389) in Cos-1 cells, we analyzed the effects of KNK437 treatment at various time periods (0, 2, 4 h) and at various concentrations (0–100 μM) on mTOR and S6K activation in Cos-1 cells. This was done by measuring changes in phosphorylation-dependent activity. KNK437 inhibited the phosphorylation of mTOR (Ser2448) and S6K (Thr389) in a time dependent manner (Fig. 1A). We also observed that KNK437 inhibited the phosphorylation of these sites in a concentration-dependent manner after a 4 hour treatment (Fig. 1B), thereby interfering with the mTORC1 signaling activity.

In general, mTOR is phosphorylated at Ser2448 in response to insulin stimulation [19]. We therefore examined whether KNK437 affected the phosphorylation of mTOR (Ser2448) and S6K (Thr389) during insulin stimulation. While mTOR and S6K were indeed phosphorylated by insulin, KNK437 inhibited the phosphorylation of mTOR and S6K in insulin-treated cells (Fig. 1C). These experiments showed that KNK437 inhibited phosphorylation of mTOR and S6K in response to insulin stimulation and further confirmed the suppressive nature of KNK437 on mTORC1 signaling.

3.2. KNK437 disrupted mTOR-interaction with raptor

The activity of mTORC1 and interaction of mTOR with raptor is completely blocked by rapamycin [8]. We tested whether KNK437 exerted a similar effect to rapamycin on the expression levels as well as the interaction of mTOR and raptor. Our results showed that KNK437 specifically suppressed the expression of Hsp70, but not that of mTOR and raptor; however, it inhibited phosphorylation of mTOR as well as that of rapamycin (Fig. 2A). Furthermore, KNK437 induced dissociation of mTOR with raptor from a complex, in a similar manner to rapamycin (Fig. 2B). These results implied that KNK437 blocked mTORC1 activity *via* dissociation of mTOR from an mTOR–raptor complex.

3.3. KNK437 inhibited mTORC1 activity without affecting the TSC1/2 complex

The TSC1/2 complex negatively regulates mTOR–S6K signaling [17]. We hypothesized that KNK437 inhibited mTOR and S6K phosphorylation through an involvement with the TSC1/2 complex. To determine whether KNK437 affected the TSC1/2 complex formation, we carried out immunoprecipitation analyses using an antibody against TSC1. The results showed that KNK437 did not alter protein expression levels of TSC1 and TSC2 (Fig. 3A, upper). Moreover, there was no apparent change in the interaction of TSC1 with TSC2 in KNK437-treated cells (Fig. 3A, lower). This suggested that KNK437 did not affect TSC1/2 complex formation.

In general, $TSC1^{-/-}$ or $TSC2^{-/-}$ contributes to constitutive activation of mTOR signaling [13]. We therefore examined whether KNK437 directly inhibited mTOR activity in the EEF4 ($TSC2^{+/+}$) and EEF8 ($TSC2^{-/-}$) cells. KNK437 inhibited phosphorylation of mTOR and S6K in EEF8 cells but not in EEF4 cells (Fig. 3B). We therefore conclude that KNK437 directly inhibited phosphorylation and activation of mTOR and S6K.

3.4. KNK437 enhanced caspase-3 activity via inhibition of mTORC1 activity

mTOR localizes to membranes fractions, including the plasma membrane and mitochondrial fractions [31,32]. Here, we studied the possible effects of KNK437 on the mTOR–raptor complex and its localization on the mitochondrial fraction. An isolated mitochondrial fraction was incubated with proteinase K in the presence or absence of Triton X-100 [29]. This resulted in the degradation of mTOR and raptor by proteinase K (Fig. 4A), indicating that both these proteins localized to the outer membrane of the mitochon-

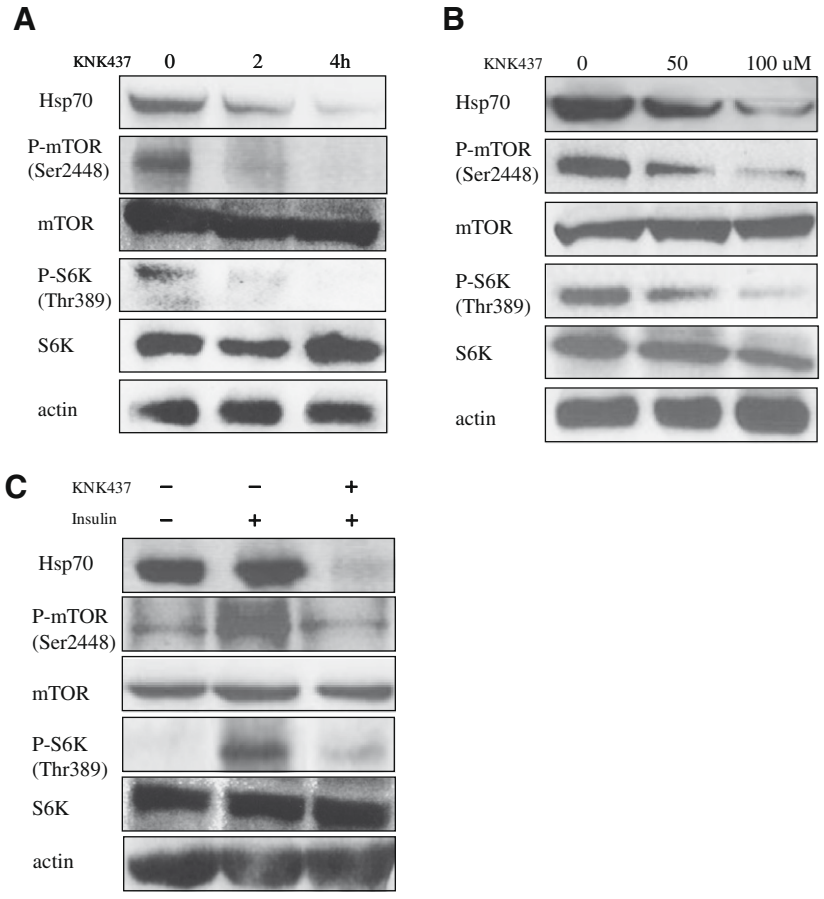

Fig. 1. Effects of KNK437 on phosphorylation of mTOR and S6K. (A) Serum-starved (2 h) Cos-1 cells were incubated with 100 μM KNK437 for 0, 2, 4 h. The effects on mTOR and S6K activation were examined in Cos-1 cells by measuring changes in phosphorylation-dependent activity. (B) Serum-starved (2 h) Cos-1 cells were incubated with KNK437 (0–100 μM) for 4 h. (C) Serum-starved (6 h) Cos-1 cells were exposed to KNK437 for 4 h, followed by stimulation with 100 nM insulin. Then, the cell lysates were immunoblotted with the indicated antibodies. Effect of KNK437 was determined by subjecting cell lysates to Western blot analysis using anti-Hsp70.

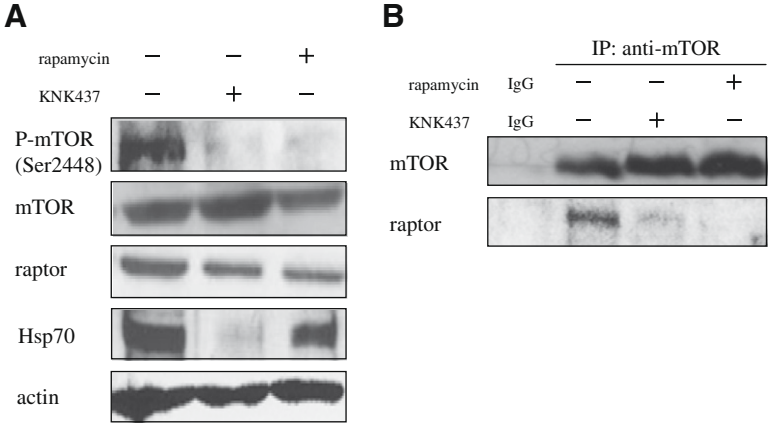

Fig. 2. Effects of KNK437 on the association between mTOR and raptor. (A) Serum-starved (2 h) Cos-1 cells were incubated in the presence of 100 μM KNK437 or 100 nM rapamycin. DMSO was used as the control. (B) After serum-starvation (2 h) Cos-1 cells were treated with KNK437 or rapamycin. Immunoprecipitation was carried out using anti-mTOR. Effect of KNK437 was determined by subjecting cell lysates to Western blot analysis using anti-Hsp70.

dria. Further, we tested whether KNK437 promoted the dissociation of raptor from mTOR on the mitochondria. The results demonstrated that KNK437 did not affect the localization of mTOR and raptor to the mitochondria, but inhibited mTOR–raptor complex formation on the mitochondria (Fig. 4B).

Hosoi et al. reported that rapamycin induced apoptosis in human rhabdomyosarcoma cells [33]. To test whether this apoptosis was mediated by the action of KNK437 and rapamycin on mTOR, we studied Cos-1 cells treated with KNK437 or rapamycin. KNK437 did not affect mTOR expression, but inhibited phosphorylation of mTOR (Fig. 4C). Like rapamycin, KNK437 activated caspase-9 and -3 (Fig. 4C). We therefore concluded that KNK437 induced apoptosis of Cos-1 cells through inhibition of mTORC1 activity.

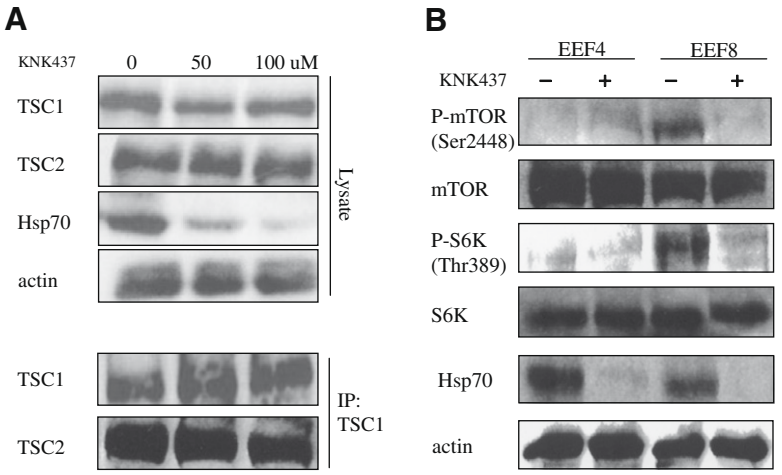

Fig. 3. KNK437 inhibits mTORC1 activity by a TSC2-independent mechanism. (A) Serum-starved (2 h) Cos-1 cells were exposed to 100 μM KNK437 for 4 h. Anti-TSC1 was used for immunoprecipitation from cells lysates, followed by immunoblotting with the indicated antibodies. (B) Serum-starved (6 h) EEF4 (*TSC2*$^{+/+}$) and EEF8 (*TSC2*$^{-/-}$) cells were incubated with 100 μM KNK437 for 4 h. Following this, the cell lysates were immunoblotted with the indicated antibodies. Effect of KNK437 was determined by subjecting cell lysates to Western blot analysis using anti-Hsp70.

4. Discussion

The mTORC1 signaling pathway promotes cellular survival and is constitutively activated in many cancers [9]. Hsp70, a chaperone protein, plays an important role in the survival of certain signaling proteins [27]. We have already reported that TSC1–Hsp70 complex regulated apoptosis [29]. Therefore, KNK437, which inhibit expression of Hsp70, was used in this study to test its effects on induction of apoptosis *via* the inhibition of mTORC1 activity.

In this study, we showed that KNK437 inhibited phosphorylation and activation of mTOR and S6K on sites Ser2448 and Thr389, respectively, in response to insulin in Cos-1 cells. We propose this to be a crucial stage in the KNK437-suppressed activation of the mTORC1 signaling pathway. Rapamycin, a well-known anticancer agent, binds to mTOR and induces the dissociation of the mTOR–raptor complex when coupled with FKBP12 [8]. As shown in Fig. 2B, KNK437 disrupted the mTOR–raptor complex and inhibited mTORC1 signal activity in a similar manner to rapamycin.

Next, we investigated whether KNK437 inhibited the mTOR signaling pathway through the TSC1/2 complex. The results showed that KNK437 did not alter TSC1/2 complex interaction. Although we previously reported that KNK437 suppressed phosphorylation of TSC2 (Thr1462) [34], this study demonstrated a direct inhibition of phosphorylation of mTOR and S6K through a TSC2-independent mechanism.

The mTOR activity regulates mitochondrial functions, including life extension and aging [35]. We therefore investigated the possible effects of KNK437 on interaction of mTOR and raptor on the mitochondrial fraction. As shown in Fig. 4A, although KNK437 did not affect the mitochondrial localization of mTOR and raptor, it disrupted the dissociation of raptor from the mTOR–raptor complex on the outer membrane of the mitochondria.

Mitochondria are known to play a key role in regulating cellular apoptosis. Activated mTORC1 negatively regulates apoptosis through S6K-dependent phosphorylation of BH3-only domain protein and rapamycin-induced apoptosis *via* inhibition of mTOR activity [21,36]. The results of this study revealed that KNK437 induced activation of caspase-9 and -3 (Fig. 4C). Therefore, KNK437 inhibited mTORC1 signaling and enhanced the apoptotic signal pathway. Recent studies have shown that combinations of rapamycin and other agents that target the mTORC1-dependent signaling pathways in many cancers are required to overcome resistance to treatment. Thus, our data suggest that a combination of KNK437 and rapamycin could be used as a valuable tool in studying the mechanisms of the mTORC1-related signal pathways.

In conclusion, KNK437 inhibits the mTORC1 signaling pathway through the direct dissociation of the mTOR–raptor complex, which is TSC1/2 complex independent. Our data suggest that inhibiting expression level of Hsp70 by KNK437 induces the activa-

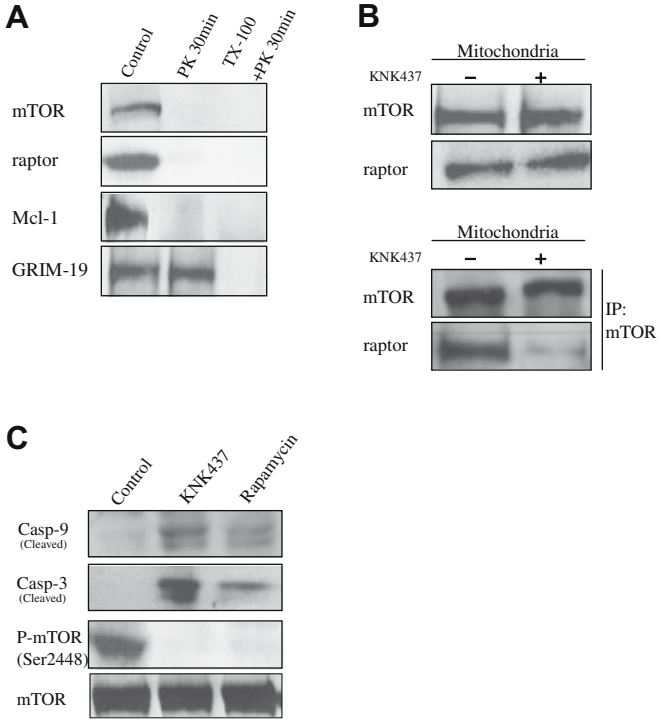

Fig. 4. KNK437 induces apoptosis *via* inhibition of mTORC1 activity. (A) Isolated mitochondrial fractions were incubated with or without proteinase K (PK). To disrupt mitochondrial integrity, Triton X-100 was added in the digestion buffer. GRIM-19 was used as a marker of the inner membrane of the mitochondria. (B) Mitochondrial fractions were isolated from Cos-1 cells, and immunoprecipitation was performed using anti-mTOR. (C) Serum-starved (2 h) Cos-1 cells was treated with DMSO (control), 100 μM KNK437 or 100 nM rapamycin. The cell lysates were immunoblotted with the indicated antibodies.

tion of caspase-3 through inhibition of the mitochondrial localization of mTORC1.

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References

[1] A. Soulard, M.N. Hall, Snapshot: mTOR signaling, Cell 129 (2007). 434.e431–432.e432.
[2] D.C. Fingar, J. Blenis, Target of rapamycin (TOR): an integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression, Oncogene 23 (2004) 3151–3171.
[3] S. Wullschleger, R. Loewith, M.N. Hall, TOR signaling in growth and metabolism, Cell 124 (2006) 471–484.
[4] J. Urano, T. Sato, Y. Otsubo, M. Yamamoto, F. Tamanoi, Point mutations in TOR confer Rheb-independent growth in fission yeast and nutrient-independent mammalian TOR signaling in mammalian cells, Proc. Natl. Acad. Sci. USA 27 (2007) 3514–3519.
[5] K. Hara, Y. Maruki, K. Yoshino, N. Oshiro, S. Hidayat, C. Tokunaga, J. Avruch, K. Yonezawa, Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action, Cell 26 (2002) 177–189.
[6] H. Nojima, C. Tokunaga, S. Eguchi, N. Hidayat, K. Yoshino, K. Hara, N. Tanaka, J. Avruch, K. Yonezawa, The mammalian target of rapamycin (mTOR) partner, raptor, binds the mTOR substrates p70S6 kinase and 4E-BP1 through their TOR signaling (TOS) motif, J. Biol. Chem. 278 (2003) 15461–15464.
[7] N. Oshiro, K. Yoshino, S. Hidayat, C. Tokunaga, K. Hara, S. Eguchi, J. Avruch, K. Yonezawa, Dissociation of raptor from mTOR is a mechanism of rapamycin-induced inhibition of mTOR function, Genes Cells 9 (2004) 359–366.
[8] G.G. Chiang, R.T. Abraham, Phosphorylation of mammalian target of rapamycin (mTOR) at Ser-2448 is mediated by p70S6 kinase, J. Biol. Chem. 280 (2005) 25485–25490.
[9] D.M. Sabatini, MTOR and cancer: insights into a complex relationship, Nat. Rev. Cancer 6 (2006) 729–734.
[10] D.J. Price, J.R. Grove, V. Calvo, J. Avruch, B.E. Bierer, Rapamycin-induced inhibition of the 70-kilodalton S6 protein kinase, Science 257 (1992) 973–977.
[11] J.K. Chen, J. Chen, G. Thomas, S.C. Kozma, R.C. Harris, S6 kinase 1 knockout inhibits uninephrectomy- or diabetes-induced renal hypertrophy, Am. J. Physiol. Renal Physiol. 297 (2009) 585–593.
[12] D.D. Sarbassov, S.M. Ali, D.H. Kim, D.A. Guertin, R.R. Latek, H. Erdjument-Bromage, P. Tempst, D.M. Sabatini, Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton, Curr. Biol. 27 (2004) 1296–1302.
[13] E.J. Unlmann, W. Li, D.K. Scheidenhelm, C.L. Gau, F. Tamanoi, D.H. Gutmann, Loss of tuberous sclerosis complex 1 (TSC1) expression results in increased Rheb/S6K pathway signaling important for astrocyte cell size regulation, Glia 47 (2004) 180–188.
[14] L.D. Aicher, J.S. Campbell, R.S. Yeung, Tuberin phosphorylation regulates its interaction with hamartin. Two proteins involved in tuberous sclerosis, J. Biol. Chem. 276 (2001) 21017–21021.
[15] H.C. Dan, M. Sun, L. Yang, R.I. Feldman, X.M. Sui, C.C. Ou, M. Nellist, R.S. Yeung, D.J. Halley, S.V. Nicosia, W.J. Pledger, J.Q. Cheng, Phosphatidylinositol 3-kinase/AKT pathway regulates tuberous sclerosis tumor suppressor complex by phosphorylation of tuberin, J. Biol. Chem. 277 (2002) 35364–35370.
[16] S.L. Cai, A.R. Tee, J.D. Short, J.M. Bergeron, J. Kim, J. Shen, R. Guo, C.L. Johnson, K. Kiguchi, C.L. Walker, Activity of TSC2 is inhibited by Akt-mediated phosphorylation and membrane partitioning, J. Cell Biol. 172 (2006) 279–289.
[17] M. Nellist, B. Verhaaf, M.A. Goedbloed, A.J. Reuser, A.M. van den Ouweland, D.J. Hally, TSC2 missense mutations inhibit tuberin phosphorylation and prevent formation of the tuberin–hamartin complex, Hum. Mol. Genet. 10 (2001) 2889–2898.
[18] J.R. Sampson, Therapeutic targeting of mTOR in tuberous sclerosis, Biochem. Soc. Trans. 37 (2009) 259–264.
[19] B.T. Nave, M. Ouwens, D.J. Withers, D.R. Alessi, P.R. Shepherd, Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation, Biochem. J. 344 (1999) 427–431.
[20] S.R. von Manteuffel, P.B. Dennis, N. Pullen, A.C. Gingras, N. Sonenberg, G. Thomas, The insulin-induced signalling pathway leading to S6 and initiation factor 4E binding protein 1 phosphorylation bifurcates at a rapamycin-sensitive point immediately upstream of p70s6k, Mol. Cell Biol. 17 (1997) 5426–5436.
[21] U. Ozcan, L. Ozcan, E. Yilmaz, K. Düvel, M. Sahin, B.D. Manning, G.S. Hotamisligil, Loss of the tuberous sclerosis complex tumor suppressors triggers the unfolded protein response to regulate insulin signaling and apoptosis, Mol. Cell 29 (2007) 541–551.
[22] T. Kanazawa, I. Taneike, R. Akaishi, F. Yoshizawa, N. Furuya, S. Fujimura, M. Kadowaki, Amino acids and insulin control autophagic proteolysis through different signaling pathways in relation to mTOR in isolated rat hepatocytes, J. Biol. Chem. 279 (2004) 8452–8459.
[23] L. Rachdi, N. Balcazar, F. Osorio-Duque, L. Elghazi, A. Weiss, A. Gould, K.J. Chang-Chen, M.J. Gambello, E. Bernal-Mizrachi, Disruption of Tsc2 in pancreatic beta cells induces beta cell mass expansion and improved glucose tolerance in a TORC1-dependent manner, Proc. Natl. Acad. Sci. USA 105 (2008) 9250–9255.
[24] M. Koishi, S. Yokota, T. Mae, Y. Nishimura, S. Kanamori, N. Horii, K. Shibuya, K. Sakai, M. Hiraoka, The effects of KNK437, a novel inhibitor of heat shock protein synthesis, on the acquisition of thermotolerance in murine transplantable tumor *in vivo*, Cancer Res. 7 (2001) 215–219.
[25] S. Yokota, M. Kitahara, K. Nagata, Benzylidene lactam compound, KNK437, a novel inhibitor of acquisition of thermotolerance and heat shock protein induction in human colon carcinoma cells, Cancer Res. 60 (2000) 2942–2948.
[26] R.K. Hansen, S. Oesterreich, P. Lemieux, K.D. Sarge, S.A. Fuqua, Quercetin inhibits heat shock protein induction but not heat shock factor DNA-binding in human breast carcinoma cells, Biochem. Biophys. Res. Commun. 239 (1997) 851–856.
[27] J.A. Barnes, D.J. Dix, B.W. Collins, C. Luft, J.W. Allen, Expression of inducible Hsp70 enhances the proliferation of MCF-7 breast cancer cells and protects against the cytotoxic effects of hyperthermia, Cell Stress Chaperones 6 (2001) 316–325.
[28] M. Jäättelä, Over-expression of hsp70 confers tumorigenicity to mouse fibrosarcoma cells, Int. J. Cancer 60 (1995) 689–693.
[29] H. Inoue, T. Uyama, T. Suzuki, M. Kazami, O. Hino, T. Kobayashi, K. Kobayashi, T. Tadokoro, Y. Yamamoto, Phosphorylated hamartin–Hsp70 complex regulates apoptosis via mitochondrial localization, Biochem. Biophys. Res. Commun. 391 (2010) 1148–1153.
[30] T. Kobayashi, O. Minowa, Y. Sugitani, S. Takai, H. Mitani, E. Kobayashi, T. Noda, O. Hino, A germ-line Tsc1 mutation causes tumor development and embryonic lethality that are similar, but not identical to, those caused by Tsc2 mutation in mice, Proc. Natl. Acad. Sci. USA 98 (2001) 8762–8767.
[31] S.M. Schieke, D. Phillips, J.P. McCoy Jr, A.M. Aponte, R.F. Shen, R.S. Balaban, T. Finkel, The mammalian target of rapamycin (mTOR) pathway regulates mitochondrial oxygen consumption and oxidative capacity, J. Biol. Chem. 281 (2006) 27643–27652.
[32] S. Paglin, N.Y. Lee, C. Nakar, M. Fitzgerald, J. Plotkin, B. Deuel, N. Hackett, M. McMahill, E. Sphicas, N. Lampen, J. Yahalom, Rapamycin-sensitive pathway regulates mitochondrial membrane potential, autophagy, and survival in irradiated MCF-7 cells, Cancer Res. 65 (2005) 11061–11070.
[33] H. Hosoi, M.B. Dilling, T. Shikata, L.N. Liu, L. Shu, R.A. Ashmun, G.S. Germain, R.T. Abraham, P.J. Houghton, Rapamycin causes poorly reversible inhibition of mTOR and induces p53-independent apoptosis in human rhabdomyosarcoma cells, Cancer Res. 59 (1999) 886–894.
[34] H. Inoue, M. Ndong, T. Suzuki, M. Kazami, T. Uyama, K. Kobayashi, T. Tadokoro, Y. Yamamoto, Hamartin–Hsp70 interaction is necessary for Akt-dependent tuberin phosphorylation during heat shock, Biosci. Biotech. Biochem. 73 (2009) 2488–2493.
[35] H.G. Wendel, R.L. Silva, A. Malina, J.R. Mills, H. Zhu, T. Ueda, R. Watanabe-Fukunaga, R. Fukunaga, J. Teruya-Feldstein, J. Pelletier, S.W. Lowe, Dissecting eIF4E action in tumorigenesis, Genes Dev. 21 (2007) 3232–3237.
[36] A. Freilinger, M. Rosner, G. Krupitza, M. Nishino, G. Lubec, S.J. Korsmeyer, M. Hengstschläger, Tuberin activates the proapoptotic molecule BAD, Oncogene 25 (2006) 6467–6479.