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Opposing and uncoupling effects of mTOR and S6K1 in the regulation of endothelial tissue factor expression

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Rapamycin has been reported to enhance tissue factor (TF) expression. The present study investigated roles of mammalian target of rapamycin (mTOR) and its downstream S6K1 in this process. We showed here that, consistent with rapamycin, knocking-down mTOR enhanced thrombin-induced TF mRNA and protein levels, whereas silencing S6K1 mitigated up-regulation of TF protein but not TF mRNA level. The enhanced TF protein level upon mTOR-silencing was further augmented by over-expression of a constitutively active S6K1 mutant and reduced by blocking RhoA, $p38^{mapk}$ or NF-κB. The results reveal an opposing and uncoupling effect of mTOR and S6K1 in regulating TF expression.

1. Introduction

Tissue factor (TF), the key enzyme in extrinsic coagulation cascade, triggers the coagulation leading to local formation of thrombin at vascular injury sites [1]. In a positive feedback mechanism, thrombin up-regulates vascular TF expression [2,3], that plays a crucial role in acute thrombotic episodes [1]. Endothelial TF expression is up-regulated by multiple signaling pathways including RhoA, $p38^{mapk}$, $p44/42^{ERK}$, JNK, and NF-κB [2–4]. In contrast, PI3-K reduces endothelial TF expression independently of its downstream effectors Akt/eNOS [3]. Numerous studies including our own showed that rapamycin enhances endothelial TF expression, suggesting that mammalian target of rapamycin (mTOR) suppresses endothelial TF expression [5–7]. These findings raise some concern about the prothrombotic risk of rapamycin (sirolimus)-eluting stents in patients with coronary artery disease [8]. Despite the well documented effect of rapamycin on TF expression, it remains elusive whether the rapamycin's effect is indeed attributed to mTOR and through its downstream effector S6K1 [5–7].

S6K1 phosphorylates the 40S ribosomal protein S6, resulting in enhanced protein translation [9]. Activation of S6K1 relies on the direct phosphorylation of Thr389 and Thr229 by mTOR and PDK1, respectively [10,11]. mTOR–S6K1 pathway is involved in various cellular functions e.g. cell growth, differentiation, and motility [9]. Although many effects of mTOR are consistent with those of S6K1 [12], there is evidence showing that not all the functions of mTOR are mediated through S6K1. For example, mouse myocyte proliferation is blunted by inhibition of mTOR, but not affected by S6K1 deletion [13].

In the present study, we investigated roles of mTOR and S6K1 in endothelial TF expression by recombinant adenovirus (rAd)-based interfering RNA (RNAi) approach.

2. Materials and methods

2.1. Materials

See the Supplementary data.

2.2. Generation of rAds

Generation of rAd expressing short hairpin RNA (shRNA) targeting human mTOR or S6K1 driven by the U6 promoter (rAd/U6-$mTOR^{shRNA}$ or -$S6K1^{shRNA}$, respectively) was carried out as

Abbreviations: mTOR, mammalian target of rapamycin; TF, tissue factor; HUVEC, human umbilical vein endothelial cell; rAd, recombinant adenovirus; shRNA, short hairpin RNA

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described previously [14]. The control rAd/U6-LacZ^{shRNA} was from Invitrogen. The mTOR and S6K1 targeting sequences are CCGCATTGTCTCTATCAAGTT and GGACATGGCAGGAGTGTTTGA, respectively. rAd/CMV-HA-Rho19 (a dominant negative mutant of RhoA) was described previously [15]. rAd/CMV-HA-S6K1-ca (a constitutively active S6K1 mutant F5A-E389-R3A) [16] and -HA-IκBα-AA (the super-repressor IκBα) [17] was kindly provided by Dr. S. Huang (Louisiana State University, USA) and Dr. S. Giovanni (University of Fribourg, Switzerland), respectively.

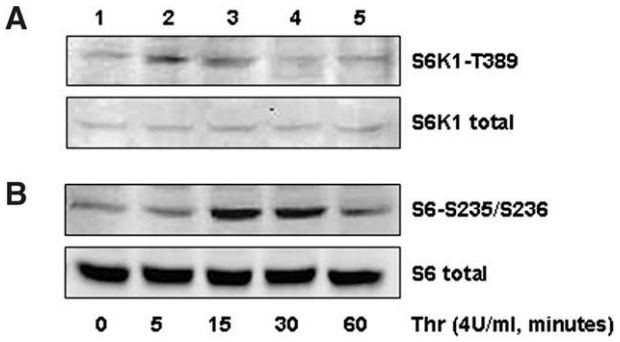

Fig. 1. Time course of mammalian target of rapamycin (mTOR)-S6K1 activation by thrombin. Human umbilical vein endothelial cells (HUVECs) were stimulated with thrombin for the indicated time after overnight serum-starvation. Cell lysates were subjected to immunoblotting analysis of S6K1- and S6-phosphorylation to assess mTOR and S6K1 activation, respectively. Shown is one of three independent experiments.

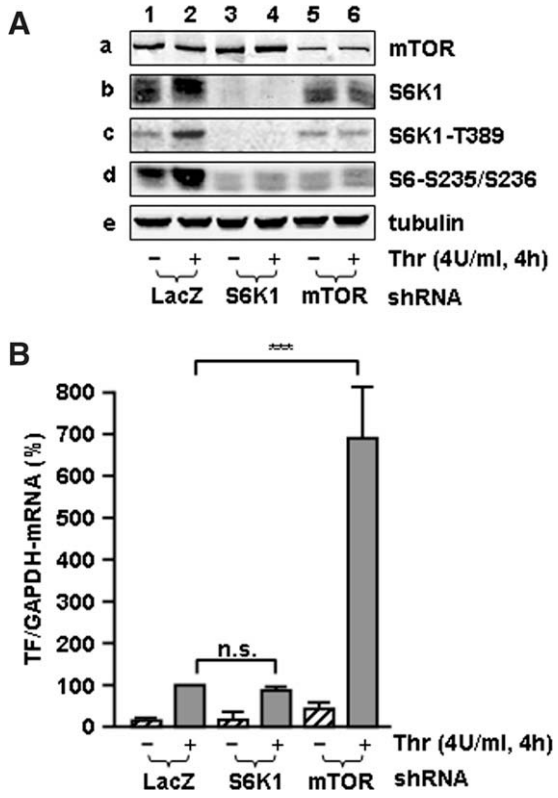

Fig. 2. Effects of silencing S6K1 and mTOR on thrombin-induced tissue factor (TF) mRNA expression. (A) Immunoblotting analysis of (a) mTOR expression; (b) S6K1 expression with mobility shift; (c) mTOR-mediated phosphorylation of S6K1-Thr389; (d) S6K1-mediated phosphorylation of S6-Ser235/236 and (e) tubulin in LacZ^{shRNA}-, S6K1^{shRNA}- or mTOR^{shRNA}-expressing cells. Shown are representative blots from three independent experiments. (B) TF mRNA expression by real-time qRT-PCR. Data from three independent experiments are reported as mean ± S.E.M.

2.3. Cultivation and adenoviral transduction of human umbilical vein endothelial cells (HUVECs)

Cultivation and transduction of HUVECs were performed as previously described [15]. For all experiments, cells were stimulated with thrombin (4 U/mL) or TNFα (10 ng/mL) at day 4 of post-transduction after overnight serum-starvation in 0.2% FCS-RPMI. Cells were treated for 4 h or indicated time for TF expression or signaling activation, respectively.

2.4. Activation of mTOR, S6K1, p38^{mapk}, and NF-κB pathway

Activation of mTOR, S6K1 or p38^{mapk} was assessed by monitoring phosphorylation of S6K1-Thr389, S6-Ser235/236 or p38^{mapk} – Thr180/Tyr185, respectively. Activation of NF-κB pathway was analysed by IκBα phosphorylation and degradation by immunoblotting. Cell lysate preparation, immunoblotting and quantification of the signals were performed as previously described [14].

2.5. RhoA activation

The RhoA activation was assessed by the pull-down assay as previously described [15].

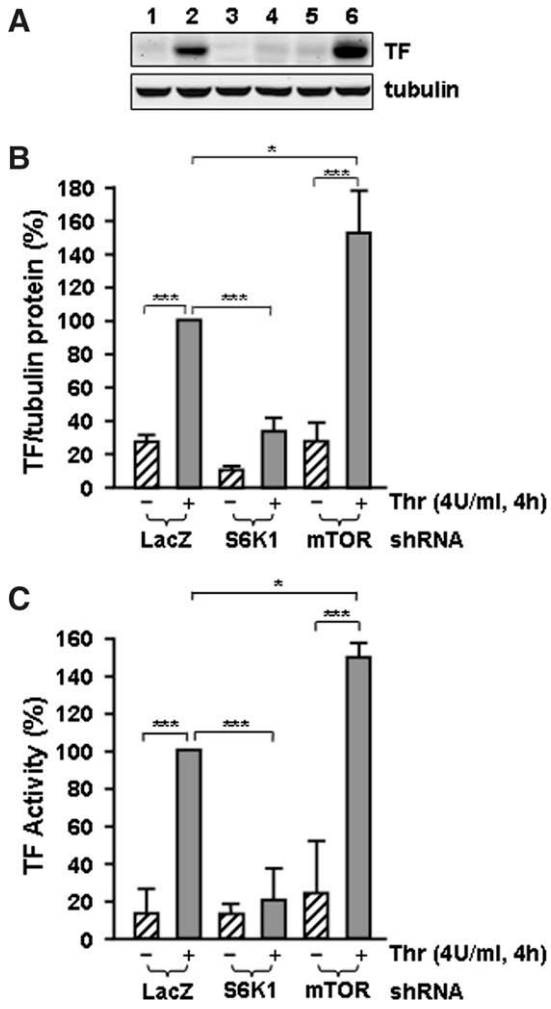

Fig. 3. Silencing S6K1 abrogated thrombin-induced TF protein level and TF activity. Cells were treated as in Fig. 2 and TF protein level was examined by (A) immunoblotting. (B) Quantification of TF/tubulin signals from five independent experiments. (C) TF activity (n = 3).

2.6. TF activity assay

After treatment, cells were extracted using 50 mmol/L Tris-buffered saline (pH 8.0) with 1% Triton-X-100 and centrifuged at 14 000×*g* for 20 min. The supernatant containing same amount of protein was subjected to TF activity measurement using a chromogenic activity assay kit (AssayPro, #CT1002b) according to manufacturer's instruction.

2.7. Quantitative real-time reverse transcription PCR (qRT-PCR)

TF mRNA expression was evaluated by two-step qRT-PCR. Total RNA was extracted with SV total RNA isolation system (Promega). First-stranded cDNA was synthesized from 500 ng total RNA with a random primer. Real-time PCR was performed with the iQ™ SYBR Green Supermix and iCycler system (Bio-Rad). TF mRNA expressions were normalized to the reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

2.8. Statistical analysis

The effects of S6K1^{shRNA}, mTOR^{shRNA} or other treatments were calculated as % relative to thrombin-induced TF expression/activity in LacZ^{shRNA}-control cells which served as 100%. All data were given as mean ± S.E.M. The ANOVA with Bonferroni post hoc test was used for statistical analysis. A two-tailed value of $P < 0.05$ was considered as statistically significant difference. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ between the indicated groups.

3. Results

3.1. Thrombin activates mTOR and S6K1 in endothelial cells

Activation of mTOR as monitored by phosphorylation of S6K1 at Thr389 was observed at 5–15 min upon thrombin stimulation in HUVECs (Fig. 1A), while activation of S6K1 i.e. phosphorylation of S6 at Ser235/236 was detectable at 15–30 min (Fig. 1B).

3.2. Silencing mTOR, but not S6K1, enhances thrombin-induced TF mRNA expression

Knock-down of mTOR or S6K1 reduced their expression (Fig. 2Aa or Ab, lanes 5 and 6 or lanes 3 and 4, respectively) and phosphorylation of their substrates (Fig. 2Ac or Ad, lane 6 or 4 compared with lane 2) in parallel. Of note, there was a basal phosphorylation of S6K1 and S6 (Fig. 2Ac and Ad, lane 1), and the activation of mTOR–S6K1 pathway could also be appreciated by the mobility shift of S6K1 upon thrombin stimulation (Fig. 2Ab, lane 2). Thrombin-induced TF mRNA expression was not significantly affected by silencing S6K1, but remarkably enhanced by knocking-down mTOR when compared with LacZ^{shRNA}-control cells (Fig. 2B, $n = 3$; $P < 0.001$ vs. LacZ^{shRNA}).

3.3. Opposing and uncoupling effects of mTOR and S6K1 on TF protein level and activity

Interestingly, the thrombin-enhanced TF protein level (Fig. 3A and B, lane 2) was prevented by silencing S6K1 (Fig. 3A and B, lane 4; $n = 5$, $P < 0.001$ vs. lane 2), but significantly enhanced by knocking-down mTOR (Fig. 3A and B, lane 6; $n = 5$, $P < 0.05$

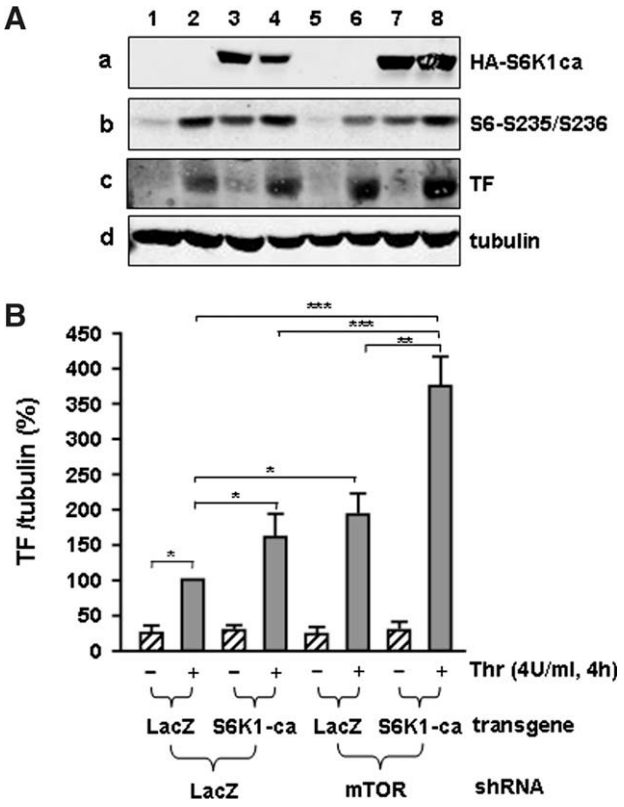

Fig. 4. Over-expression of a constitutively active S6K1 (S6K1-ca) augmented thrombin-induced TF protein level. Cells were first transduced with recombinant adenovirus (rAd)/U6-LacZ^{shRNA} or rAd/U6-mTOR^{shRNA} for 3 days followed by transduction with rAd/CMV-LacZ or rAd/CMV-HA-S6K1-ca for 1 day before treatment with thrombin. (A) Immunoblotting analysis of expression of HA-S6K1-ca with anti-HA antibody, S6-Ser235/236, TF and tubulin. (B) Quantification of TF/tubulin signals from three independent experiments.

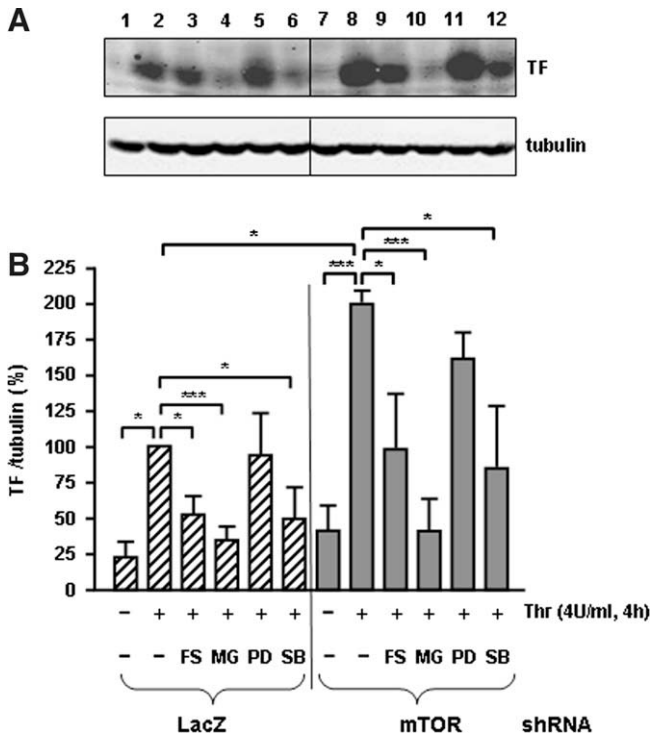

Fig. 5. Effects of inhibition of S6K1-independent pathways on TF protein level. Cells expressing LacZ^{shRNA} (lanes 1–6) or mTOR^{shRNA} (lanes 7–12) were pre-treated with various signaling inhibitors as indicated for 1 h before stimulation with thrombin: 10 μmol/L fluvastatin (FS), 10 μmol/L MG-132 (MG), 50 μmol/L PD98059 (PD), 10 μmol/L SB202190 (SB). Same amount of solvent was added to the control cells. (A) Immunoblotting analysis of TF and tubulin expression. (B) Quantification of the TF/tubulin signals from three independent experiments.

vs. lane 2). The effects of silencing mTOR or S6K1 on TF protein levels were well correlated with those on cellular TF activity (Fig. 3C, $n = 3$). Moreover, over-expression of a HA-tagged constitutively active S6K1 (HA-S6K1-ca) in LacZ^{shRNA}-control cells (Fig. 4Aa, lanes 3 and 4) augmented thrombin's effect on TF protein level (Fig. 4Ac and B, lane 4 vs. lane 2, $P < 0.05$). Of note, over-expression of HA-S6K1-ca alone (panel a, lane 3), despite the enhanced S6K1-mediated S6-phosphorylation (panel b, lane 3), was not sufficient to significantly increase TF protein level (Figs. 3Ac and 4B, lane 3). Under the condition of mTOR-silencing, whereby thrombin-induced up-regulation of TF protein level was augmented as compared with the control cells (Fig. 4Ac and B, lane 6 vs. lane 2), over-expression of HA-S6K1-ca further increased TF protein to the highest level (Fig. 4Ac and B, lane 8), reinforcing that mTOR and S6K1 exerts opposing and uncoupling effects on endothelial TF expression at mRNA and protein translational level, respectively. The requirement of S6K1 for TF protein up-regulation was further confirmed with TNFα. Like thrombin, TNFα activated S6K1 (Supplementary Fig. 1Ab, lane 2) and enhanced TF protein level (Supplementary Fig. 1Ac and B, lane 2 vs. lane 1) in LacZ^{shRNA}-control cells, which was reduced by silencing S6K1 (Supplementary Fig. 1Ac and B, lane 4 vs. lane 2).

3.4. Roles of mTOR–S6K1-independent signalings in TF up-regulation

Inhibition of RhoA, NF-κB, or p38^{mapk} with fluvastatin, MG-132, or SB202190, respectively, blunted thrombin-induced TF protein level in LacZ^{shRNA}-control cells, whereas blockade of p42/p44^{mapk} with PD98059 had no effect (Fig. 5, shaded bars, lanes 1–6). The augmented thrombin-induced TF protein level after knocking-down mTOR (lane 8 vs. lane 2) was also significantly reduced by fluvastatin, MG-132, or SB202190, but not by PD98059 (Fig. 5, gray bars, lanes 7–12). Similar results were obtained with over-expression of a dominant negative mutant of RhoA (Rho19) or the super-repressor IκBα (IκBα-AA) (Fig. 6).

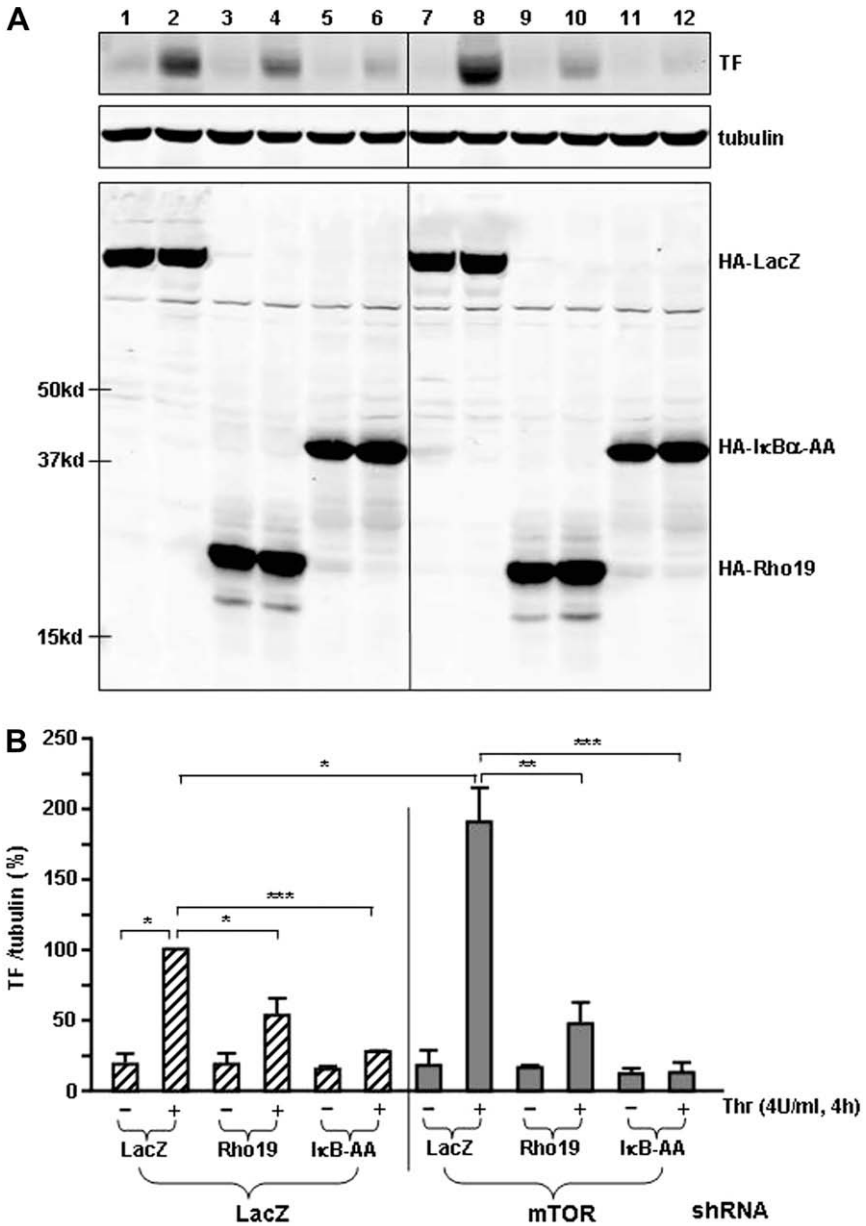

Fig. 6. Over-expression of dominant negative mutant of RhoA (Rho19) or super-repressor IκBα (IκBα-AA) blunted thrombin-induced TF protein level. (A) Immunoblotting analysis of expression of TF, tubulin, and HA-tagged transgenes with anti-HA antibody in cells transduced first with U6/LacZ^{shRNA} or U6/mTOR^{shRNA} followed by transduction with rAd/CMV-HA-LacZ, -HA-Rho19 or -HA-IκBα-AA as indicated. (B) Quantification of the TF/tubulin signals from three independent experiments.

3.5. mTOR and S6K1 do not interfere with activation of RhoA, NF-κB or $p38^{mapk}$

Stimulation of the cells with thrombin for indicated time activated RhoA, NF-κB, and $p38^{mapk}$ pathways (Supplementary Fig. 2, lane 2, $n = 3$). These effects of thrombin were however, not affected by silencing of either S6K1 or mTOR (lane 4 or 6, respectively, $n = 3$). Of note, despite induction of IκBα phosphorylation, thrombin did not cause significant degradation of IκBα in the cells.

4. Discussion

Numerous studies including our own reported the inhibitory effect of mTOR on TF expression based on the observation that rapamycin enhances TF expression in vascular cells [5–7]. In the present study, we demonstrate that silencing mTOR, in agreement with rapamycin, also markedly enhanced thrombin-induced TF mRNA (Fig. 2). In contrast, silencing S6K1 reduced thrombin-induced TF protein level without affecting TF mRNA expression (Figs. 2 and 3A and B). The opposing effects of silencing mTOR and S6K1 on TF protein level (Fig. 3A and B) were also reflected by their opposing effects on the cellular TF activity (Fig. 3C). Moreover, TNFα-induced TF protein level was also inhibited by silencing S6K1 (Supplementary Fig. 1). These results demonstrate that S6K1 does not mediate mTOR's suppressing effect on TF mRNA expression but instead enhances TF protein translation. The positive regulatory effect of S6K1 on TF protein level was further confirmed by over-expression of a constitutively active S6K1 mutant which enhances thrombin's effect on TF protein level in both $LacZ^{shRNA}$-control and mTOR-silenced cells (Fig. 4). It is noteworthy that the enhanced TF protein level after mTOR-silencing was further augmented by expression of the active S6K1 mutant (Fig. 4). These results provide further convincing evidence for the opposing and uncoupling effect of mTOR and S6K1 in endothelial TF expression, and imply that the much weaker enhancement in TF protein level vs. TF mRNA after knocking-down mTOR (Fig. 3 vs. Fig. 2) could be explained by the concomitant inhibition of S6K1.

The fact that knocking-down mTOR enhances TF protein level despite concomitant inhibition of S6K1-mediated TF protein translation, suggest that other mTOR–S6K1-independent signalings must exist. Indeed, blockade of RhoA, NF-κB, and $p38^{mapk}$, but not $p44/42^{ERK}$ by pharmacological inhibitors and/or by over-expression of dominant negative mutants reduced up-regulation of TF protein level upon thrombin stimulation or mTOR-silencing (Figs. 5 and 6), demonstrating that mTOR and S6K1 in concert with those signalings regulate endothelial TF expression in a complex manner as depicted in the Fig. 7. It seems that silencing mTOR or S6K1 does not affect activation of these pathways in response to thrombin, since phosphorylation levels of the protein kinases or formation of RhoA-GTP were not affected under these conditions (Supplementary Fig. 2). The results suggest that the markedly enhanced TF mRNA expression upon mTOR-silencing is likely translated by these S6K1-independent pathways (Fig. 7). It is to point out that interactions between mTOR–S6K1 and these pathways at more downstream levels cannot be excluded.

In summary, this study demonstrates an opposing and uncoupling effect of mTOR and S6K1 in regulation of endothelial TF expression. While mTOR suppresses endothelial TF mRNA expression, S6K1 is required for TF protein translation, which works in concert with other signaling pathways such as RhoA, $p38^{mapk}$, and NF-κB (Fig. 7). Our findings may have potential clinical implications. Some clinical studies reported that rapamycin (sirolimus)-eluting stents, despite its property of preventing vascular restenosis in patients with coronary artery disease, may have increased thrombotic risk [8], which might be partly related to the increased TF expression/activity through inhibition of mTOR in the vascular cells. It would be interesting to test whether inhibition of S6K1 alone, rather than mTOR as achieved by rapamycin, proves sufficient to prevent vascular restenosis and be superior in reducing thrombotic propensity by developing specific S6K1 inhibitors.

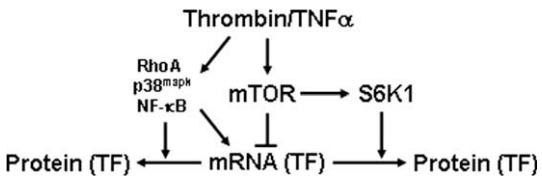

Fig. 7. Integrated model of signaling pathways in regulation of TF expression. The up-regulation of TF expression by thrombin represents the sum of the functions of many signaling pathways. The stimulating effect of S6K1 at the translational level and the functions of mTOR–S6K1-independent pathways at the transcriptional and/or translational levels overcome the suppressive effect of mTOR on TF mRNA expression, leading to an increase in TF mRNA and protein expression. Inhibition of mTOR removes the suppressive effect of mTOR on TF mRNA expression, results in markedly elevated TF mRNA available for protein translation by mTOR–S6K1-independent pathways that lead to increased TF protein level despite of concomitant inhibition of S6K.

Acknowledgements

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