Rapamycin, but Not FK-506, Increases Endothelial Tissue Factor Expression
Implications for Drug-Eluting Stent Design

Jan Steffel, MD*; Roberto A. Latini, MD*; Alexander Akhmedov, PhD; Dorothee Zimmermann, BSc; Pamela Zimmerling, BSc; Thomas F. Lüscher, MD; Felix C. Tanner, MD

Background—Drugs released from stents affect the biology of vascular cells. We examined the effect of rapamycin and FK-506 on tissue factor (TF) expression in human aortic endothelial cells (HAECs) and vascular smooth muscle cells (HAVSMCs).

Methods and Results—Rapamycin enhanced thrombin- and tumor necrosis factor (TNF)-α–induced endothelial TF expression in a concentration-dependent manner. The maximal increase was 2.5-fold more pronounced than that by thrombin or TNF-α alone and was paralleled by a 1.4-fold higher TF surface activity compared with thrombin alone. Rapamycin by itself increased basal TF levels by 40%. In HAVSMCs, rapamycin did not affect thrombin- or TNF-α–induced TF expression. In contrast to rapamycin, FK-506 did not enhance thrombin- or TNF-α–induced endothelial TF expression. Thrombin induced a transient dephosphorylation of the mammalian target of rapamycin downstream target p70S6 kinase. Rapamycin completely abrogated p70S6 kinase phosphorylation, but FK-506 did not. FK-506 antagonized the effect of rapamycin on thrombin-induced TF expression. Rapamycin did not alter the pattern of p38, extracellular signal–regulated kinase, or c-Jun NH₂-terminal kinase phosphorylation. Real-time polymerase chain reaction analysis revealed that rapamycin had no influence on thrombin-induced TF mRNA levels for up to 2 hours but led to an additional increase after 3 and 5 hours.

Conclusions—Rapamycin, but not FK-506, enhances TF expression in HAECs but not in HAVSMCs. This effect requires binding to FK binding protein-12, is mediated through inhibition of the mammalian target of rapamycin, and partly occurs at the posttranscriptional level. These findings may be clinically relevant for patients receiving drug-eluting stents, particularly when antithrombotic drugs are withdrawn or ineffective, and may open novel perspectives for the design of such stents. (Circulation. 2005;112:2002-2011.)

Key Words: endothelium ▪ myocardial infarction ▪ signal transduction ▪ stents ▪ thrombosis

Percutaneous coronary intervention with stenting of the culprit lesion is the preferred treatment for patients with acute coronary syndromes.1–3 Several clinical trials have demonstrated that drug-eluting stents (DESs) are superior to bare-metal stents (BMSs) by decreasing the restenosis rates as well as major adverse cardiac events.4–6 Rapamycin (sirolimus), a macrolide lactone, is used on DESs because the drug inhibits proliferation and migration of vascular smooth muscle cells (VSMCs).7 FK-506 (tacrolimus), a macrolide immunosuppressant, is an alternative drug used with DESs.8,9 Despite reduced restenosis rates, however, stent thromboses have not decreased with DESs compared with BMSs.6,10–12 Indeed, several hundred cases of in-stent thrombosis have been reported with rapamycin-coated stents,13 and results from a recent multicenter registry imply that thrombosis rates with DESs may be higher in “real world” patients than reported in previous clinical trials.14 The reason for the discrepancy between reduced restenosis rates and unaltered or even enhanced thrombosis rates with DESs compared with BMSs is not known.6,12

Several factors are involved in the pathogenesis of in-stent thrombosis. These include procedure-related factors such as mechanical vessel injury or incomplete stent apposition, patient-related factors such as vessel size or coagulation activity, and finally, the thrombogenicity of the stent itself.15 It has not yet been explored, however, whether the drugs used for stent coating could be involved in the development of in-stent thrombosis.15

Tissue factor (TF), a 263-residue, membrane-bound glycoprotein, is a key enzyme in the initiation of coagulation; it
activates factor X (FX) by binding activated factor VII (FVIIa), which ultimately leads to thrombin formation. Initiation of coagulation is a key event in the pathogenesis of thrombosis and acute coronary syndromes. Not surprisingly, atheromatous plaques contain a variety of cells expressing TF, including endothelial cells (ECs) and VSMCs. Moreover, TF levels are elevated in the plasma and atherectomy samples from patients with unstable angina.14 Therefore, TF seems to be involved in the development of atherosclerosis and restenosis after percutaneous coronary intervention.17–19 TF may indeed play a major role in stent thrombosis as well. However, the effect of neither rapamycin nor FK-506 on TF expression has been investigated so far. Moreover, the role of the mammalian target of rapamycin (mTOR) in regulating TF expression is also not known. Thus, the present study was designed to investigate the influence of rapamycin and FK-506 on TF expression in human aortic endothelial cells (HAECs) and vascular smooth muscle cells (HAVSMCs).

Methods

Cell Culture

HAECs and HAVSMCs were cultured as described.20,21 Cells were grown to confluence in 6-cm culture dishes and rendered quiescent for 24 hours before stimulation with thrombin or tumor necrosis factor (TNF)-α (Sigma). Rapamycin, wortmannin (both from Sigma), FK-506 (Alexis), and LY294002 (Cell Signaling) were added to the dishes 60 minutes before stimulation. Cytotoxicity was assessed with a colorimetric assay to detect lactate dehydrogenase release according to the manufacturer’s recommendations (Roche).

Western Blot Analysis and ELISA

Protein expression was determined by Western blot analysis as described.22,23 Cells were lysed in 50 mmol/L Tris buffer, and 30-μg samples were loaded and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred to a polyvinylidene difluoride membrane (Millipore) by semidry transfer. Antibody to human TF (American Diagnostica) was used at 1:2000 dilution; antibodies against the phosphorylated Thr-389 residue of p70 S6 kinase (S6K), phosphorylated p38 mitogen-activated protein (MAP) kinase (p38), phosphorylated p44/42 MAP kinase (extracellular signal–regulated kinase [ERK]), and phosphorylated c-Jun NH2-terminal kinase (JNK; all from Cell Signaling) were used at 1:2000 dilution; antibodies against total S6K, total p38, total ERK, and total JNK (all from Cell Signaling) were used at 1:3000, 1:1000, 1:500, and 1:1000 dilution, respectively. Antibodies against total β-tubulin (aT) expression (1:20 000 dilution, Sigma). Endothelial TF expression was also measured with a commercially available ELISA (American Diagnostica) according to the supplier’s recommendations.

Real-Time PCR Analysis

RNA was extracted and converted to cDNA as described.22 Real-time polymerase chain reaction (PCR) was performed in an MX3000P PCR cycler (Stratagene). All PCR experiments were performed with the SYBR Green JumpStart kit (Sigma). Each reaction (25 μL) contained 2 μL cDNA, 1 pmol of each primer, 0.25 μL of internal reference dye, and 12.5 μL of JumpStart Taq ReadyMix (containing buffer, dNTPs, stabilizers, SYBR Green, Taq polymerase, and JumpStart Taq antibody). Primers for human TF (L28) mRNA were used as described.22,24 Expression of the ribosomal protein L28 (L28) mRNA was used as a loading control; primers for human L28 were designed as follows: sense primer, 5′-GGACTCTGCAATTGGATGTT-3′ and antisense primer, 5′-TGTCTTCTGCGGATCATGTT-3′. The amplification program consisted of 1 cycle at 95°C for 10 minutes; followed by 40 cycles with a denaturing phase at 95°C for 30 seconds, an annealing phase at 60°C for 1 minute, and an elongation phase at 72°C for 1 minute. A melting curve analysis was performed after amplification to verify the homogeneity of the amplicon. For verification of amplicon size, PCR products were analyzed on an ethidium bromide–stained 1% agarose gel. In each real-time PCR run for TF and L28, a calibration curve generated from serial dilutions of a known TF and L28 standard, respectively, was included, and for each sample, the target values were corrected by those for L28.

TF Surface Activity

A colorimetric assay (American Diagnostica) was used to analyze TF surface activity according to the manufacturer’s recommendations, with some modifications as described.22,23 Cells were grown in 6-well plates; after stimulation, cells were washed twice with phosphate-buffered saline and incubated with human FVIIa and FX at 37°C, resulting in the formation of a TF/FVIIa complex at the cell surface. The TF/FVIIa complex converted human FX to FXa, which was subsequently measured by its ability to cleave a chromogenic substrate. Different concentrations of lipidated human TF were used as positive controls to confirm that the obtained results were in the linear range of detection (data not shown).

Proliferation

To examine the effect of rapamycin and FK-506 on EC proliferation, HAECs were seeded on 6-cm dishes at 7000 cells/cm2. After 24 hours, when cells had reached ~50% confluence, they were serum-starved for 24 hours before incubation with rapamycin (10−5 mol/L), FK-506 (10−7 mol/L), or carrier (0.1% dimethyl sulfoxide) in endothelial basal medium (EBM, Clonetics) containing 10% fetal calf serum (FCS). At the indicated times, cells were gently trypsinized and counted in a hemacytometer. Each analysis was performed in duplicate; results are representative of 3 independent experiments.

Apoptosis

To assess induction of apoptosis by rapamycin and FK-506, cells were cultured in chamber slides (Nunc) at 20 000 cells/well for 24 hours before serum-starvation for 24 hours. Cells were then incubated in EBM with 10% FCS containing rapamycin (10−6 mol/L), FK-506 (10−7 mol/L), or carrier (0.1% dimethyl sulfoxide). At the indicated times, cells were fixed with 4% paraformaldehyde and processed for terminal deoxynucleotidyl nick end-labeling (TUNEL) staining with a commercially available kit (Roche) according to the manufacturer’s recommendations. Afterward, cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Vector) and counted under a fluorescence microscope. Two hundred cells per time point and condition were counted, and the number of TUNEL-positive cells was assessed.

Statistics

Data are presented as mean±SEM. Unpaired Student t test was used for statistical analysis. A probability value <0.05 was considered significant.

Results

Rapamycin Enhances TF Expression in HAECs but Not HAVSMCs

Stimulation of HAECs with thrombin (1 U/mL) induced TF expression 23-fold as assessed by Western blotting analysis (Figure 1A). Incubation with rapamycin (10−8 to 10−7 mol/L) before stimulation with thrombin resulted in a concentration-dependent enhancement of TF expression (Figure 1A). The maximal increase was observed after 5 hours and was 2.3-fold compared with stimulation with thrombin alone and 51-fold compared with the basal level. Similarly, rapamycin (10−8 to 10−7 mol/L) enhanced TF expression in response to TF-α (5 ng/mL); this increase was 2.5-fold, resulting in a
35-fold induction compared with the basal level (Figure 1B). ECs express TF only at very low levels under basal conditions, and stimulation of HAECs with rapamycin alone increased basal TF expression by 40%, as assessed by Western blotting analysis (Figure 1C), or 25% as assessed by ELISA (Figure 1D). Expression of TF was 45/1000 pg per 500 000 cells for control, 57/1000 pg per 500 000 cells for rapamycin (10^-7 mol/L) alone, 468±29 pg per 500 000 cells for thrombin stimulation, and 702±30 pg/500 000 cells for thrombin stimulation in the presence of rapamycin (Figure 1D). The rapamycin-enhanced increase in thrombin-induced TF expression was paralleled by an increase of TF surface activity, which reached 1.4 times the level induced by thrombin alone (Figure 1E).

Similar to HAECs, thrombin (1 U/mL) and TNF-α (5 ng/mL) induced TF expression in HAVSMCs. In contrast to HAECs, however, rapamycin did not affect TF expression in response to either mediator in HAVSMCs. No cytotoxic effect of rapamycin was observed for any of the concentrations used (n=4, P=NS; data not shown).

**Figure 1.** Rapamycin enhances TF expression in HAECs. A, Rapamycin enhances thrombin-induced TF expression in a concentration-dependent manner. Values are given as a percentage of stimulation with thrombin alone. *P<0.0001, **P<0.001 vs thrombin alone. B, Rapamycin enhances TNF-α-induced TF expression in a concentration-dependent manner. Values are given as a percentage of stimulation with TNF-α alone. *P<0.02, **P<0.01 vs TNF-α alone. C, Rapamycin increases basal TF expression. Values are given as a percentage of unstimulated control. *P<0.01 vs unstimulated control. Values are representative of at least 3 different experiments; all blots were normalized to aT expression. D, TF ELISA confirms that rapamycin increases both basal (*P<0.05 vs unstimulated control) and thrombin-induced (***P<0.01 vs thrombin alone) TF expression. E, Rapamycin enhances thrombin-induced TF surface activity in a concentration-dependent manner. Values are given as a percentage of stimulation with thrombin alone. *P<0.01 vs thrombin alone.

**Figure 2.** Rapamycin does not affect TF expression in HAVSMCs. Rapamycin does not affect thrombin- (A) or TNF-α- (B) induced TF expression in HAVSMCs. Values are given as a percentage of stimulation with thrombin (1 U/mL, Figure 3A) or TNF-α (5 ng/mL, Figure 3B) did not alter TF expression. No cytotoxic effect of FK-506 was observed for any of the concentrations used (n=4, P=NS; data not shown).

**FK-506 Does Not Affect TF Expression**

Incubation with FK-506 (10^-8 to 10^-7 mol/L) before stimulation with thrombin (1 U/mL, Figure 3A) or TNF-α (5 ng/mL, Figure 3B) did not alter TF expression. No cytotoxic effect of FK-506 was observed for any of the concentrations used (n=4, P=NS; data not shown).
Rapamycin Enhances TF Expression by Inhibiting mTOR Activity

Phosphorylation of S6K, a downstream target of the mTOR, is frequently used to assess mTOR inhibition by rapamycin. When stimulated with thrombin (1 U/mL), S6K phosphorylation was transiently decreased after 30 minutes to a minimum of 19% of the basal level (Figure 4A, left). Rapamycin (10⁻⁷ mol/L) completely abrogated S6K phosphorylation, in both the presence and absence of thrombin (Figure 4A, right). Similarly, inhibition of phosphatidylinositol 3-kinase with LY294002 or wortmannin almost completely abrogated S6K phosphorylation, again independent of thrombin stimulation (Figure 4C). In contrast, FK-506 (10⁻⁷ mol/L) did not affect phosphorylation of S6K in either the presence or absence of thrombin (Figure 4B).

Rapamycin Enhances TF Expression by Binding to FKBP-12

Rapamycin and FK-506 bind to the same intracellular receptor, FK binding protein-12 (FKBP-12). When HAECs were treated with increasing concentrations of FK-506 for 30 minutes before incubation with rapamycin, FK-506 reduced the effect of rapamycin on thrombin-induced TF expression (Figure 5). Indeed, when incubated with the highest concentration of FK-506 (10⁻⁷ mol/L), the increase in TF expression elicited by rapamycin with respect to stimulation with thrombin alone was reduced by 41% (P<0.05).

Effect of Rapamycin on Thrombin-Induced TF mRNA Levels

Real-time PCR revealed that thrombin induced TF mRNA expression in a time-dependent manner (Figure 6A). Rapamycin did not alter thrombin-induced mRNA expression compared with stimulation by thrombin alone after 0.5, 1, and 2 hours. However, after 3 and 5 hours of stimulation, rapamycin significantly augmented thrombin-induced TF mRNA levels (Figure 6A and 6B). Rapamycin significantly increased thrombin-induced TF protein expression after 3, 5, and 7 hours compared with stimulation by thrombin alone (Figure 6C).

Rapamycin did not affect the pattern of MAP kinase activation observed after thrombin stimulation. Indeed, phosphorylation of p38 (Figure 7A), ERK (Figure 7B), and JNK (Figure 7C) remained unaltered after pretreatment with rapamycin compared with stimulation by thrombin alone.

Rapamycin, but Not FK-506, Inhibits EC Proliferation

EC proliferation was induced by incubation with EBM containing 10% FCS (Figure 8A, control). Rapamycin (10⁻⁷ mol/L) prevented FCS-induced EC proliferation. In contrast, FK-506 (10⁻⁷ mol/L) did not significantly inhibit EC proliferation (Figure 8A).

TUNEL staining was used to examine whether rapamycin (10⁻⁷ mol/L) or FK-506 (10⁻⁷ mol/L) induced apoptosis in HAECs (Figure 8B). Representative sections are shown. After 24 hours, TUNEL-positive cells accounted for 5.3±0.7% of cells in the control group, 4.7±1.7% for rapamycin (P=NS versus control), and 4.8±1.5% for FK-506 (P=NS versus control). After 48 hours, 4.2±1.7% of control cells, 3.5±0.6% of rapamycin-treated cells (P=NS versus control), and 4.6±2.3% of FK-506-treated cells (P=NS versus control) were TUNEL-positive. Cells incubated with H₂O₂ (1 mmol/L) for 6 hours as well as serum withdrawal for 48 hours served as positive controls and resulted in a significant increase in apoptotic cells (data not shown). Thus, neither rapamycin (10⁻⁷ mol/L) nor FK-506 (10⁻⁷ mol/L) led to an increase in apoptotic cells compared with control conditions.

Discussion

This study demonstrates that rapamycin enhances endothelial TF expression in response to thrombin and TNF-α. The concentrations of rapamycin occurring in vivo compare well with those used in our study, as maximal systemic concentrations of rapamycin after deployment of 2 sirolimus-eluting...
stents are reported to be \(\approx 1 \, \text{ng/mL} \approx 1.15 \times 10^{-8} \, \text{mol/L}\); moreover, local concentrations, though difficult to assess, are likely to be significantly higher, partly because of rapamycin’s lipophilic properties, leading to accumulation of the drug in the vessel wall.\(^{11,30–32}\) Thus, the concentrations used in our study may be relevant for patients treated with DESs.

Reendothelialization is initiated soon after vascular injury; indeed, it has been observed to begin as early as 2 days after balloon dilation in animal models.\(^{33–35}\) In humans, partial reendothelialization has been documented 3 weeks after stent deployment.\(^{35–37}\) Sirolimus-eluting stents are designed in such a way that \(\approx 80\%\) of the rapamycin has eluted by 30

---

**Figure 4.** Rapamycin inhibits endothelial mTOR activity. A, Thrombin leads to a transient, time-dependent inhibition of S6K phosphorylation (left). Rapamycin (right) completely abrogates S6K phosphorylation in both the presence and absence of thrombin. Total levels of S6K remain unchanged. Values are presented as phosphorylated (Pho) S6K/total (Tot) S6K. \(P<0.0001\) vs unstimulated conditions. B, FK-506 affects neither basal phosphorylation levels nor thrombin-induced inhibition of S6K phosphorylation. Values are presented as phosphorylated S6K (Pho)/total (Tot) S6K. C, LY294002 (5 \(\times\) 10\(^{-6}\) mol/L, left) and wortmannin (10 \(\times\) 10\(^{-7}\) mol/L, right) almost completely abrogate S6K phosphorylation (Pho). Total (Tot) levels of S6K remain unchanged.
resistance. FK-506, which neither affects endothelial TF when clopidogrel is withdrawn or ineffective because of drug after deployment of sirolimus-eluting stents, particularly may indeed favor the development of in-stent thrombosis BMSs. Enhanced TF expression in the presence of rapamycin owing to its lipophilic properties, leading to chronic retention thrombus formation. Consequently, the use of platelet recep-

Figure 5. FK-506 antagonizes rapamycin-induced TF expression. Preincubation with FK-506 reduces rapamycin-enhanced TF expression. Values are presented as a percentage of stimulation with thrombin (1 U/mL) and rapamycin (10⁻⁷ mol/L). *P<0.05 and **P<0.02, compared with thrombin and rapamycin (10⁻⁷ mol/L). Blots are representative of at least 3 different experiments; all blots were normalized to aT expression.

days. Furthermore, rapamycin easily penetrates cell walls owing to its lipophilic properties, leading to chronic retention of the drug in arterial tissue. Thus, the time course of reendothelialization versus the kinetics of rapamycin release suggests that rapamycin-enhanced endothelial TF expression may be involved in the pathogenesis of in-stent thrombosis. In addition, inhibition of endothelial proliferation by rapamycin indicates that rapamycin delays reendothelialization, which may increase stent thrombogenicity even further.

Several hundred cases of acute and subacute in-stent thrombosis have been observed after deployment of rapamycin-eluting stents. In addition and in contrast to BMSs, late thrombosis has been reported after withdrawal of antithrombotic drugs with DESs. Most of these data originated from case reports or were collected in controlled clinical trials. Recent results from a large-scale, multicenter registry, however, indicate that in-stent thrombosis is likely underestimated under these circumstances and that it may occur at substantially higher rates in real world patients. The pathogenesis of in-stent thrombosis has not yet been fully explored; it is not known whether the pathogenic events leading to thromboses of DESs are similar to those of BMSs. Enhanced TF expression in the presence of rapamycin may indeed favor the development of in-stent thrombosis after deployment of sirolimus-eluting stents, particularly when clopidogrel is withdrawn or ineffective because of drug resistance. FK-506, which neither affects endothelial TF expression nor inhibits EC proliferation, may provide a more favorable environment for reducing thromboses of DESs. To assess the implications of these findings in vivo, however, further studies are needed to examine the degree as well as the spatiotemporal pattern of TF expression in the arterial wall after deployment of DESs.

Platelet activation is a crucial event in the pathogenesis of thrombus formation. Consequently, the use of platelet recep-
tor blockers such as clopidogrel have greatly reduced the incidence of stent thromboses, whereas withdrawal of antiplatelet therapy favors thrombus formation. Moreover, clopidogrel inhibits the release of TF from aggregating platelets, which is of particular interest, as platelet aggregation and secretion are increased in human platelets treated with rapamycin. Thus, effective antiplatelet therapy may account for the fact that thrombosis rates of sirolimus-eluting stents are not clearly higher than those of BMSs.

TF induction after deployment of rapamycin-eluting stents may also have a prothrombotic effect on ECs distal to the stented site. Indeed, remote effects of rapamycin have been demonstrated, with pronounced endothelial dysfunction in coronary arteries distal to sirolimus-eluting stents compared with BMSs. In addition, to the effect on ECs within the stented region, rapamycin may also increase TF expression in ECs in the distal coronary vasculature. Such an effect may also contribute to the no-reflow phenomenon after stent deployment.

Rapamycin did not enhance thrombin- or TNF-α-driven TF expression in HAVSMCs, indicating that rapamycin does not constitute an additional thrombogenic signal to the VSMC layer. Indeed, a much higher incidence of acute and subacute stent thromboses would be expected if rapamycin induced TF expression in VSMCs. Although the rapamycin-induced increase in endothelial TF expression may favor neointima formation via the release of growth factors from aggregating platelets, the inhibitory effect of rapamycin on the proliferation and migration of VSMC is very likely to prevent the vessel from such effects. Consistent with this interpretation, sirolimus-eluting stents reduce neointima formation despite inducing a procoagulative state owing to enhanced endothelial TF expression.

Both thrombin, a coagulation factor, and TNF-α, an inflammatory cytokine, are classic inducers of TF expression in vascular cells. Thrombin induced TF expression 27-fold when examined by Western blotting analysis and 10.3-fold by ELISA; similarly, rapamycin enhanced thrombin-induced TF expression by 2.3-fold in Western blot analysis and 1.5-fold by ELISA. This difference may be due to a different sensitivity and/or specificity of the 2 assays. In our study, rapamycin enhanced TF expression in response to both thrombin and TNF-α; it may thus upregulate TF expression in a prothrombotic as well as an inflammatory environment, both of which are encountered in the coronary vasculature after stent deployment.

Biologically active TF is located at the cell surface, and rapamycin-enhanced TF protein expression was indeed paralleled by an increase in TF surface activity. The increase in activity was not as pronounced as that of protein expression; this discrepancy has also been observed in response to thrombin alone. The distribution of TF in several cellular compartments and/or the expression of encrypted TF might account for this difference.

The inhibitory role of phosphatidyl inositol 3-kinase on TF expression is established, as its inhibition enhances TF expression in response to thrombin. The mTOR is a downstream target of phosphatidyl inositol 3-kinase. Binding of rapamycin to its intracellular receptor FKBP-12 leads
to formation of the rapamycin–FKBP-12 complex, which in turn inhibits mTOR activity. Phosphorylation of the downstream target of mTOR, S6K, is routinely used as a readout for the inhibitory effect of rapamycin on mTOR; indeed, mTOR-dependent phosphorylation of the Thr-389 residue of S6K is necessary for its activity. In the present study, we have shown that stimulation with thrombin leads to a transient inhibition of S6K phosphorylation. Rapamycin as well as the phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 abrogated S6K phosphorylation in both the presence and absence of thrombin. Because thrombin stimulation as well as preincubation with rapamycin led to inhibition of this pathway, resulting in disinhibition of TF expression, these observations are consistent with the interpretation that mTOR plays an inhibitory role in TF expression.

FK-506 competitively binds to the same intracellular receptor as rapamycin, ie, FKBP-12. In contrast to rapamycin, however, the FK-506–FKBP-12 complex inhibits the phosphatase calcineurin and has no effect on mTOR activity. Consistently, FK-506 did not alter thrombin- or TNF-α–induced TF expression. To assess the specificity of our observations, we coincubated FK-506 and rapamycin before thrombin stimulation. The enhancing effect of rapamycin on thrombin-induced TF expression could indeed be reduced by FK-506. These findings indicate that binding of rapamycin to FKBP-12 is necessary for inhibition of mTOR activity and enhancement of TF expression.

TF expression in response to a variety of stimuli is mediated by MAP kinase activation, leading to increased transcription. Indeed, thrombin induced an increase in p38, ERK, and JNK phosphorylation as well as an increase in TF transcription. However, rapamycin did not alter the pattern of thrombin-induced p38, ERK, and JNK activation.

Figure 6. Effect of rapamycin on TF mRNA induction. A, Real-time PCR demonstrates a time-dependent induction of TF mRNA in response to thrombin. Rapamycin does not alter this pattern of induction after stimulation for 0.5, 1, and 2 hours. Values are given as a percentage of stimulation with thrombin alone for 2 hours. B, Analysis of ΔΔCt values comparing the effect of rapamycin on thrombin-induced mRNA levels for every time point reveals that rapamycin significantly increases thrombin-induced TF mRNA after stimulation for 3 and 5 hours. *P<0.0005, **P<0.005. All values are representative of 4 different experiments and were normalized to L28 mRNA expression. C, Rapamycin enhances thrombin-induced TF protein expression in a time-dependent manner. Values are given as a percentage of stimulation with thrombin alone for 3 hours. *P<0.0001, **P<0.01 vs thrombin alone. Values are representative of at least 3 different experiments. All blots were normalized to aT expression.
Consistent with this observation, thrombin-induced mRNA levels were unchanged by rapamycin for up to 2 hours after stimulation. However, after 3 and 5 hours of thrombin stimulation, rapamycin increased mRNA levels compared with stimulation by thrombin alone. Taken together, these data imply that the enhancing effect of rapamycin on thrombin-induced TF expression initially occurs at the post-transcriptional level and hence, is independent of MAP kinase activation.

Figure 7. Rapamycin does not affect MAP kinase activation. Stimulation with thrombin leads to phosphorylation (Pho) of the MAP kinases p38 (A), ERK (B), and JNK (C). Rapamycin does not alter this pattern of MAP kinase activation. Total (Tot) levels of p38, ERK, and JNK remain unchanged. Blots are representative of at least 3 different experiments.

Figure 8. Rapamycin, but not FK-506, inhibits EC proliferation. A, Rapamycin completely inhibits EC proliferation induced by 10% FCS; in contrast, FK-506 does not significantly affect EC proliferation. *P<0.05 vs control; **P<0.005 vs control; and ***P<0.002 vs FK-506. Three different experiments were performed in duplicate for each experimental condition. B, There was no increase in TUNEL-positive cells after incubation with rapamycin or FK-506 for 24 and 48 hours. Slides show representative TUNEL-positive cells with the corresponding DAPI staining after 24 hours of incubation with carrier (left), rapamycin (middle), and FK-506 (right).
activation, although a transcriptional effect of rapamycin cannot be ruled out at later time points. Indeed, mTOR is known to exert posttranscriptional effects, and TF expression can be regulated at both the transcriptional and posttranscriptional level.47,48

In summary, our study reveals that rapamycin, but not FK-506, enhances endothelial TF expression and reduces HAECl proliferation. These effects may favor the development of thrombus formation after deployment of sirolimus-eluting stents, particularly when antithrombotic drugs are withdrawn or ineffective, and may have interesting implications for the design of DESs.

Acknowledgments

This work was supported by the Swiss National Science Foundation (grant No. 3200B0-102232/1 to Dr Tanner and grant No. 3100-068118.02/1 to Dr Lüscher), the Bonizzi-Theler Foundation, the Hartmann-Müller Foundation, the Herzog-Egli Foundation, and the Olga Mayenfisch Foundation. The authors thank Dr F. Eberli and Dr W. Maier for discussion.

References


Rapamycin, but Not FK-506, Increases Endothelial Tissue Factor Expression: Implications for Drug-Eluting Stent Design

Jan Steffel, Roberto A. Latini, Alexander Akhmedov, Dorothee Zimmermann, Pamela Zimmerling, Thomas F. Lüscher and Felix C. Tanner

_Circulation_. 2005;112:2002-2011; originally published online September 19, 2005; doi: 10.1161/CIRCULATIONAHA.105.569129

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/112/13/2002

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org/subscriptions/