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In human endothelial cells rapamycin causes mTORC2 inhibition and impairs cell viability and function

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Aim Drug-eluting stents are widely used to prevent restenosis but are associated with late endothelial damage. To understand the basis for this effect, we have studied the consequences of a prolonged incubation with rapamycin on the viability and functions of endothelial cells.
Methods and results Human umbilical vein or aorta endothelial cells were exposed to rapamycin in the absence or in the presence of tumour necrosis factor α (TNFα). After a 24 h-incubation, rapamycin (100 nM) caused a significant cell loss associated with the increase of both apoptosis and necrosis, as quantified by propidium iodide staining, caspase 3 activity, and lactate dehydrogenase release. Rapamycin also impaired cell mobility, as assessed by a wound test, and promoted the formation of actin stress fibres, as determined with confocal microscopy. Moreover, the inhibitor prolonged TNFα-dependent E-selectin induction, inhibited endothelial nitric oxide synthase expression at both mRNA (quantitative real-time polymerase chain reaction) and protein level (enzyme-linked immunosorbent assay and western blot), and lowered bioactive nitric oxide output (RFL-6 reporter cell assay). Under the conditions adopted, rapamycin inhibited both mammalian target-of-rapamycin complexes (mTORC1 and mTORC2), as indicated by the reduced amount of raptor and rictor bound to mTOR in immunoprecipitates and by the marked hypophosphorylation of protein S6 kinase I (p70S6K) and Akt, determined by western blotting. The selective inhibition of mTORC1 by AICAR did not affect endothelial viability.
Conclusion A prolonged treatment with rapamycin impairs endothelial function and hinders cell viability. Endothelial damage seems dependent on mTORC2 inhibition.

1. Introduction

Drug-eluting stents (DES), coated with vehicles that slowly elute antiproliferative agents, like paclitaxel, rapamycin (sirolimus), or FK506 (tacrolimus),[1,2] were developed to hamper the hyperplastic response that follows the application of bare metal stents. The use of DES has reduced the risk of restenosis after angioplasty to levels below 10%.[3,4] In particular, rapamycin, the most widely employed drug in DES, suppresses the growth of neointimal vascular smooth muscle cells by inhibiting the mammalian target of rapamycin (mTOR), a serine/threonine kinase that plays a pivotal role in the regulation of cell growth and proliferation (for review see Wullschleger *et al.*[5]).

Recent evidences have demonstrated an increased incidence of late thrombosis of rapamycin-eluting stents after the discontinuation of dual antiplatelet therapy.[6] These effects may be caused by the long-term endothelial dysfunction that is now well documented after the implantation of rapamycin-eluting stents.[7,8] The molecular basis for the endothelial defect has not been fully elucidated yet, although the intracellular targets of rapamycin are well known. Once bound with the protein FKBP12, rapamycin prevents the formation of the multiprotein active complex mTORC1 that controls translational efficiency and cell cycle progression.[5] Moreover, mTOR is also known to form the mTORC2 complex, which was believed to be rapamycin-insensitive and has different targets, such as the actin cytoskeleton.[9] Interestingly, the activity of mTORC1 and mTORC2 have different effects on Akt, with the former working as an inhibitor and the latter as an activator of Akt kinase.[10] Also FK506 binds FKBP12, but this

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complex interacts with calcineurin and inhibits the de-phosphorylation of the nuclear factor of activated T cells without any interaction with mTORC1 or mTORC2.[11]

The levels of pro-inflammatory cytokines are particularly increased in sites of arterial injury[12] and, consistently, higher concentrations of inflammatory markers are detected after coronary stenting.[13] Tumour necrosis factor α (TNFα) appears of peculiar interest in this context because it activates endothelial cells affecting the expression of many genes. Interestingly, rapamycin, but not FK506, synergizes TNFα stimulation of tissue factor expression in human endothelial cells.[14] Most recently, our group demonstrated that rapamycin also enhances TNFα-dependent induction of CAT2A and CAT2B arginine transporters, two indicators of endothelial activation.[15] Surprisingly, we found that the mTOR inhibitor induces the transporters even in the absence of TNFα, suggesting that rapamycin may alter endothelial function even in the absence of pro-inflammatory stimuli. To verify this hypothesis, we have studied here the effects of a prolonged exposure to rapamycin on endothelial cell viability and functions.

2. Methods

2.1 Cell culture and experimental treatment

Human umbilical vein endothelial cells (HUVEC) were obtained as described previously;[16] human aortic endothelial cells (HAEC) were purchased by Cambrex Bio Science, Walkersville, Inc.. Both cell types were routinely grown in collagen-coated, 10 cm-diameter dishes in medium 199 (M199), with glutamine raised to 2 mM. The culture medium was supplemented with 20% fetal bovine serum (FBS), endothelial cell growth supplement (ECGS 37.5 μg/mL), and heparin (75 U/mL). Cells were kept at 37°C, pH 7.4, in an atmosphere of 5% CO_2.The investigation conformed with the principles outlined in the Declaration of Helsinki (*Cardiovascular Research* 1997;35:2–4). Unless otherwise stated, cells were used 2–3 days after the passage when cultures were almost confluent (18 000 $\pm$ 2000 cells/cm^2 for HUVECs and 23 000 $\pm$ 2000 cells/cm^2 for HAECs). AICAR (5-aminoimidazole-4-carboxamide-1-b-d-ribofuranosyl 3′-5′-cyclic-monophosphate), FK506, rapamycin, TNFα, and wortmannin were added to complete, serum supplemented growth medium from 100$\times$ stock solutions in M199.

2.2 Cell number and viability

Cells were grown on 2-cm^2 wells of disposable Falcon™ 24-well cell culture plates. Cell number was measured with a Cell Counter ZM (Coulter Electronics Ltd, Luton, U.K.) after culture trypsinization. Cell viability was tested incubating cells for 2 h with fresh growth medium supplemented with 44 μM resazurin;[17] fluorescence was then measured at 572 nm with a Wallac 1420 Victor^2 Multilabel Counter (Perkin Elmer, Monza, Italy).

2.3 Propidium iodide staining

After experimental treatments, floating and adherent cells, grown on 10 cm-diameter dishes, were collected and fixed in ice-cold methanol for 30 min at 4°C. After washing with phosphate-buffered saline (PBS), cells were incubated for 1 h at 37°C in the dark in 1 mL of PBS containing propidium iodide (PI) (20 μg/mL), Triton X-100 (0.1%), and RNAse A (10 μg/mL). Propidium staining of at least 10^5 cells was acquired by FACSCalibur Flow Cytometer (Becton Dickinson, Milano, Italy); the analysis was performed using Cell Quest software. Cells undergoing apoptosis were expressed as the percentage of hypodiploid cells (sub-G1 population) on total cells.

2.4 Caspase 3 activity

The activity of caspase 3 was measured in cell cultures, grown on 10 cm-diameter dishes, with Caspase 3 Colorimetric Assay Kit (Sigma Aldrich, Milano, Italy) following manufacturer's instructions. The chromophore *p*-nitroaniline (pNa), the product of caspase-mediated cleavage, was quantified at 405 nm with a Victor^2 Multilabel Counter. Caspase 3 activity was expressed as nmol pNA/min/mg of protein.

2.5 Lactate dehydrogenase release

Lactate dehydrogenase (LDH) released from necrotic cells into the medium was assessed in 24-well plates cultures with a CytoTox 96® Non-Radioactive Cytotoxicity assay (Promega, Milano, Italy). The amount of LDH released was expressed as percent of the total LDH released from untreated cells lysed by the buffer contained in the kit (% of cytotoxicity).

2.6 Detection of endothelium-derived nitric oxide using RFL-6 reporter cell assay

To measure bio-active nitric oxide (NO) released by endothelial cells, a RFL-6 reporter cell assay[18] was performed on cells seeded on 6-well plates in Locke's solution supplemented with 1 mM Arg, 20 U/mL superoxide dismutase (Roche, Mannheim, Germany), and 0.6 mM 3-isobutyl-1-methylxanthine.

2.7 Endothelial wound healing assay

The method of Weber *et al.*[19] was used with minor modifications. Confluent HUVEC cultures, grown on 6-well plates, were pretreated for 12 h with rapamycin, TNFα, or both, and wounded with two parallel strokes with a sterile 0.4-mm-200 μL Gilson style extension length tip. After 12 and 24 h, images of the same fields were acquired with a Nikon Eclipse Inverted Microscope (Nikon Italia, Firenze, Italy) equipped with a Nikon DS Cooled Camera Head DS-5Mc using a Proximo 32 Image Analysis software. The number of cells migrated into 1 mm^2 of wound area was quantified.

2.8 Quantitative real-time polymerase chain reaction

One microgram of total RNA, isolated with RNeasy Mini Kit® (Qiagen, Milano, Italy), was reverse transcribed and 40 ng of cDNA amplified as described previously.[15] The following forward and reverse primers (5 pmol each) were used: 5′ ACC AGC CCA GGT TGA ATG 3′ and 5′ TAA CGG AAA CTG CCA GAA GC 3′ for E-selectin; 5′ AGC CTC AAG ATC ATC AGC AAT G 3′ and 5′ CAC GAT ACC AAA GTT GTC ATG GA 3′ for GAPDH. For endothelial nitric oxide synthase (eNOS) detection cDNA was amplified with 2$\times$ Platinum® qPCR SuperMix-UDG according to LUX chemistry (Invitrogen, Milano, Italy), along with the primers 5′ CAC TGT CGC TCG GCC ATC ACA G[FAM]G 3′ (forward) and 5′ GGC TGT TCC AGA TTC GGA AGT 3′(reverse). Data were expressed as the ratio between proband mRNA and GAPDH mRNA.

2.9 Western analysis and immunoprecipitation

Cells, grown on 10 cm-diameter dishes, were washed and scraped in ice-cold PBS then collected by low speed centrifugation. After lysis in RIPA buffer, followed by sonication and centrifugation, supernatants were quantified by a modified micro Lowry protein assay; 30 μg of total protein were electrophoresed on an 8% SDS–polyacrylamide gel and immunoblotted as described previously.[15] The membranes were probed with anti-eNOS primary antibody (1:200) overnight at 4°C and then exposed to horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:10 000). GAPDH, detected with a monoclonal antibody (1:500), was employed for standardization. The expressions of total and phospho-p70S6 and of total and phospho-Akt kinases were determined with, respectively, the

PhosphoPlus® p70S6 Kinase Antibody kit and the anti-Akt or anti-phospho-Akt (Ser473) antibodies from Cell Signaling (Celbio, Milano, Italy), following manufacturer's instructions.

For co-immunoprecipitation experiments, the immunoprecipitation protocol (for analysis by western blotting) by Cell Signaling (Celbio) was followed. Briefly, 650 μg of cell lysate were immunoprecipitated with anti-mTOR antibody (1:100); the expression of mTOR, raptor, and rictor were then analysed in the immunoprecipitates and in whole-cell lysates by western blotting according to manufacturer's instructions. All the antibodies, purchased by Cell Signaling, were diluted 1:1000.

2.10 Enzyme-linked immunosorbent assay

Cells, seeded on 96-well plates, were washed with PBS and fixed with 2% paraformaldehyde for 15 min. The monolayers, after an overnight incubation in 3% bovine serum albumin in PBS at 4°C, were incubated for 1 h at 37°C with anti-E-selectin polyclonal antibody (1:50). After washing in PBS and a 30 min-incubation with biotin-conjugated mouse anti-rabbit antibody (1:300), cells were exposed to the Streptavidin-HRP conjugate (1:100). Optical density was measured at 490 nm with a Victor^2 Multilabel Counter and referred to protein content.

2.11 Visualization of actin

After the experimental treatments, HUVECs seeded on coverslips were stained with AlexaFluor®488 phalloidin (Molecular Probes, Eugene, OR), as already described.[20] Actin cytoskeleton was visualized with a confocal system (LSM 510 Meta, Carl Zeiss, Stuttgart, Germany). Phalloidin signal was acquired in a 16-bit scale (ranging from 0 to 4096) at 488 nm and the emission recorded through a 510 nm primary beamsplitter and a 530 nm dichroic filter. The Image Examiner software was used to analyse the images.

2.12 Statistics

Statistical analysis was performed using one way ANOVA with the Bonferroni *post hoc* test. *P*-values >0.05 were considered not significant.

2.13 Materials

Endotoxin-free FBS and M199 (Euroclone) were purchased from Celbio and TNFα (Alexis) from Vinci-Biochem (Firenze, Italy). All the antibodies (Santa Cruz Biotechnology) were purchased from DBA Italia (Milano, Italy). Biotin-conjugated mouse anti-rabbit and Streptavidin-HRP were from DAKO (Milano, Italy). Sigma was the source of rapamycin, FK506, AICAR, wortmannin, as well as of all the other chemicals.

3. Results

3.1 Rapamycin but not FK506 causes cell loss in endothelial cell cultures

The effects of different concentrations of rapamycin or FK506 (1–100 nM) on HUVEC cell number and viability are shown in *Figure 1A* and *B*. The experiment was performed both in the absence and in the presence of TNFα. Rapamycin caused a significant decrease in cell number at concentrations higher than 1 nM when employed alone, while, in the presence of TNFα, the mTOR inhibitor provoked a significant cell loss even at this concentration. Cell viability, measured with the fluorescent indicator resazurin, was significantly affected at rapamycin concentrations higher than 1 nM both in the absence and in the presence of TNFα. In contrast, FK506 (tacrolimus) did not affect either endothelial cell number or viability at any concentration

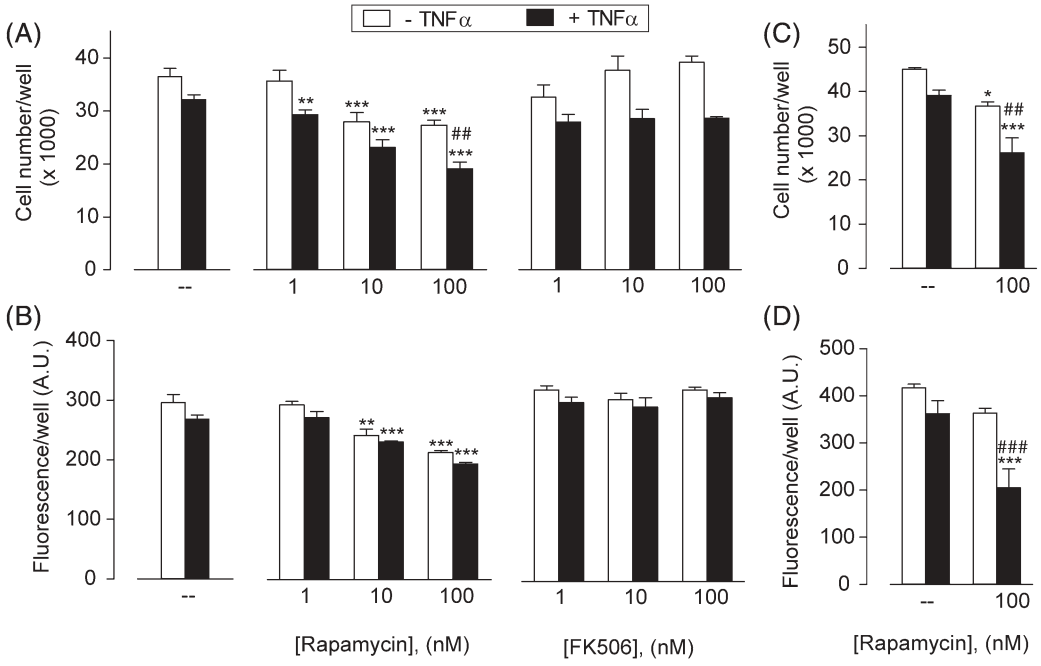

Figure 1 Dose-dependent effects of rapamycin and FK506 on endothelial cell number and viability. (*A* and *B*) Human umbilical vein endothelial cells were incubated with the indicated concentrations of rapamycin or FK506, in the absence or in the presence of TNFα (10 ng/mL), as indicated. After 24 h, cells were trypsinized and counted (*A*); cell viability was assessed in parallel cultures with the fluorescent indicator resazurin (*B*) (see Methods). Bars represent means (with SEM indicated) of six independent determinations obtained in three experiments. (*C* and *D*) Effects of mTOR inhibition on human aortic endothelial cells cultures. Cells number (*C*) and viability (*D*) were assessed after 24 h treatment with 100 nM rapamycin, in the absence or in the presence of TNFα (10 ng/mL), as indicated. Bars represent means (with SEM indicated) of six independent determinations obtained in two experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ treated vs. control, untreated cells; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ cells treated with rapamycin and TNFα vs. cells treated with TNFα alone.

used, even in the presence of TNFα. Also in cultures of HAECs rapamycin (100 nM) produced a significant decrease of cell number (*Figure 1C*) and viability (*Figure 1D*) in the presence of TNFα. A significant decrease in cell number was observed also after treatment with the inhibitor alone.

3.2 Rapamycin promotes endothelial cell death

To characterize further rapamycin-dependent cell loss, we have studied indicators of apoptosis and necrosis in both HUVECs and HAECs treated with the drug (*Figure 2*). Rapamycin, either alone or used together with TNFα (*Figure 2A*), produced a significant activation of caspase 3, whereas the cytokine alone had no significant effect. The quantification of apoptosis with PI staining yielded consistent results in HUVECs, whereas a significant increase in the percentage of apoptotic cells was obtained in HAEC cultures only when rapamycin and TNFα were simultaneously present (*Figure 2B*). Both in HUVECs and in HAECs, the combined treatment with TNFα and rapamycin produced an increase in the release of LDH, an indicator of necrotic death (*Figure 2C*).

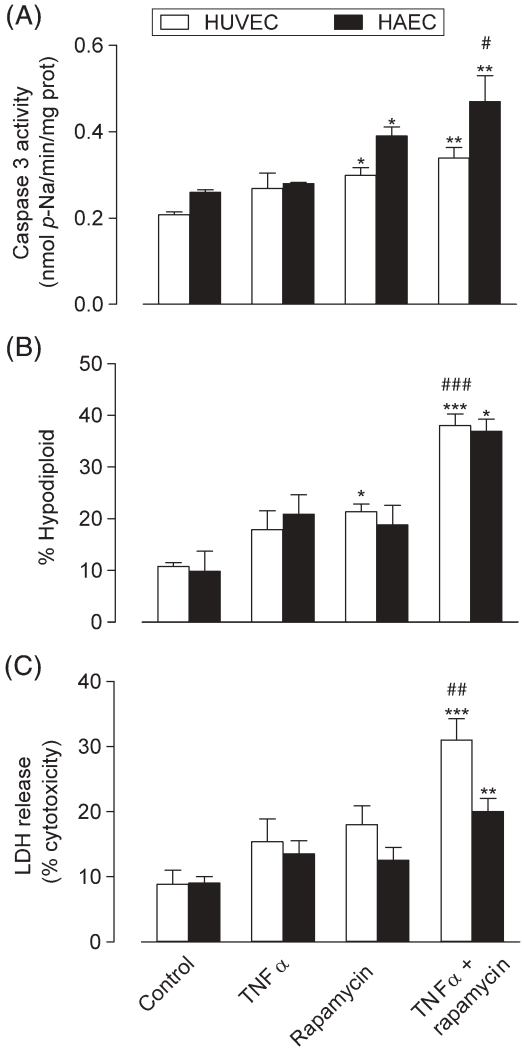

Figure 2 Effects of rapamycin on human umbilical vein endothelial cells and human aortic endothelial cells viability. (*A* and *B*) Endothelial apoptosis after a 24 h-incubation in the absence (control) or in the presence of 10 ng/mL TNFα, 100 nM rapamycin, or both, was assessed by determining the activity of caspase 3 in cell extracts (*A*) or the percentage of hypodiploid, sub-G1 population in PI stained cultures (*B*) (see Methods). Data are means ± SEM of three different experiments. (*C*) The amount of lactate dehydrogenase released in the culture medium was measured under the same conditions described in (*A* and *B*). Data, expressed as the percent of cytotoxicity (see Methods), are means ± SEM of six determinations in three (human umbilical vein endothelial cells) or two (human aortic endothelial cells) different experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ treated vs. control, untreated cells; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ cells treated with rapamycin and TNFα vs. cells treated with TNFα alone.

3.3 Rapamycin impairs endothelial functions

The results presented above demonstrate that rapamycin significantly hinders cell viability, particularly in the presence of TNFα. To test if comparable effects were detectable on endothelial functions, we assessed cell mobility, the expression of E-selectin, and NO output in HUVECs incubated with rapamycin, TNFα, or both compounds.

Cell mobility was assessed with a wound test *in vitro* (*Figure 3*). The results show that, while the wound was almost completely repaired after 12 h in both control and TNFα-pretreated HUVECs, wound borders were still clearly detectable in cells preincubated with either rapamycin or rapamycin and TNFα. After 24 h, wound borders were no more detectable even under these conditions, although endothelial cultures exhibited evident changes in morphology (loss of cobblestone organization, presence of frankly apoptotic cells, enhanced cell pleomorphism). The estimation of the endothelial re-colonization of the wounded area (*Figure 3B*) indicated that, both at 12 h and at 24 h, fewer cells had migrated into the wounded area of cultures pretreated with rapamycin or with rapamycin and TNFα, compared with either untreated cultures or cultures treated with the cytokine alone.

As expected,[21] TNFα induced a strong and transient stimulation of E-selectin at either mRNA or protein level (*Figure 4A* and *B*), with maximal effects at 4 h and values substantially returned to basal levels within 24 h. At 4 or 8 h, rapamycin did not modify E-selectin expression either in the absence or in the presence of TNFα. In contrast, after 24 h of treatment, E-selectin mRNA was much higher in endothelial cells incubated with both compounds compared with cells treated with each compound alone. As a consequence, under this condition E-selectin protein level was still significantly higher than in control cells.

It is well known that TNFα lowers eNOS mRNA through transcriptional and post-transcriptional mechanisms.[22] The results reported in *Figure 5A*, indicate that, after a 24 h-treatment of HUVECs, rapamycin produced a significant decrease of eNOS mRNA, comparable to that caused by TNFα. Moreover, the combined treatment caused the largest decrease in eNOS mRNA both at 8 h and at 24 h of incubation. Changes in eNOS protein level were even more marked, with the maximal decrease detected in cells treated with both rapamycin and TNFα (*Figure 5B*). As expected from eNOS inhibition, all the experimental treatments produced a fall in bioactive NO production, although the change reached statistical significance only in cells treated with rapamycin alone (*Figure 5C*).

3.4 Rapamycin inhibits both mTORC1 and mTORC2

Rapamycin is a well-established inhibitor of mTORC1 complex,[5] although recent evidence suggests that prolonged

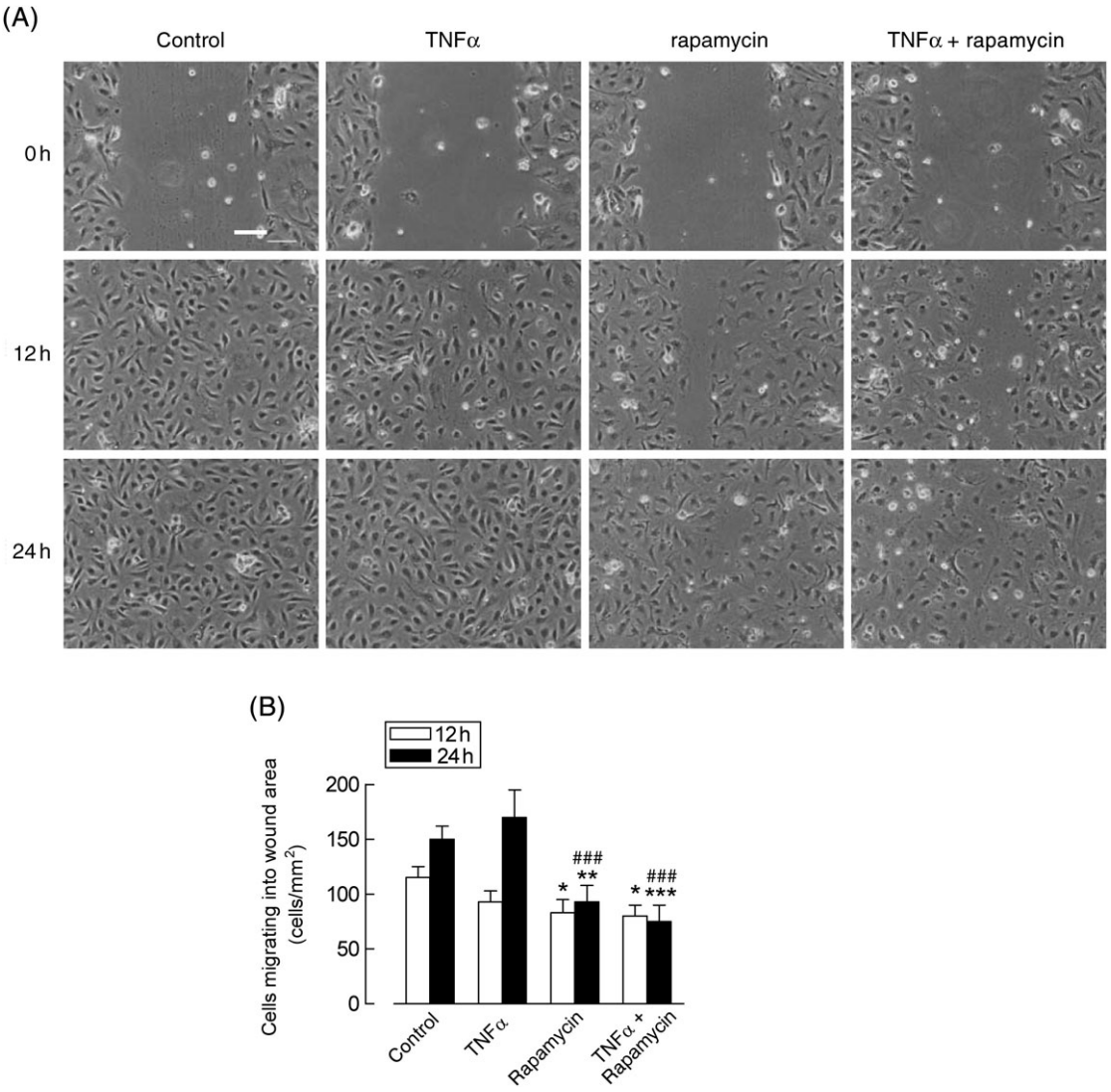

Figure 3 *In vitro* wound healing by human umbilical vein endothelial cells. Cells were preincubated for 12 h in the absence (control) or in the presence of 10 ng/mL TNFα, 100 nM rapamycin, or both, as indicated. At this time (0 h) cell monolayers were wounded with a tip and photographed. (*A*) Phase contrast images of the same representative field were taken 0, 12, and 24 h after wounding. Bar = 100 μm. (*B*) Cells migrated into the wound zone were counted within 3 different fields after 12 and 24 h. Bars represent means ± SD. The experiment was repeated three times with similar results. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ treated vs. control, untreated cells; $^{\#\#\#}P < 0.001$ cells treated with rapamycin and TNFα vs. cells treated with TNFα alone.

exposure to the drug may also inhibit mTORC2, which was supposed to be rapamycin-insensitive.[23] To assess the status of the two complexes under our experimental conditions, we adopted the approach used by Sarbassov *et al.*[23] in a non-endothelial model. The amount of raptor and rictor co-immunoprecipitated with mTOR was therefore determined in HUVECs incubated for 24 h in the absence or in the presence of rapamycin (*Figure 6A*). Rapamycin strongly reduced the amount of both raptor and rictor co-immunoprecipitated with mTOR, while it did not affect the expression of the three proteins in whole-cell lysates. These data demonstrate that a prolonged treatment with rapamycin hinders the formation of both mTORC1 and mTORC2 complexes in human endothelial cells. In support of these finding we have examined, under the same conditions, the phosphorylation status of Ser473-Akt, which is promoted by mTORC2 but inhibited by mTORC1,[10] and, in the same lysates, the phosphorylation of the specific mTORC1 substrate p70S6K. While TNFα did not modify either kinase, rapamycin produced a complete dephosphorylation of p70S6 kinase, as expected from mTORC1 inhibition (*Figure 6B*), associated with a marked hypophosphorylation of Akt, an effect only referable to mTORC2 inhibition. An almost complete suppression of p70S6 kinase phosphorylation was also obtained with AICAR, a compound that causes a rapamycin-independent inhibition of mTORC1 through the activation of AMP-activated protein kinase (AMPK).[24] However, in this case, no change in Akt phosphorylation was detectable. On the contrary, as expected, wortmannin, an inhibitor of PI3-kinase, markedly inhibited Akt phosphorylation. Interestingly, while AICAR had no effect on HUVEC viability, wortmannin caused a significant cell loss (*Figure 6C*).

It is known that mTORC2 modulates the organization of actin cytoskeleton and its inhibition promotes the formation of thick actin stress fibres[9]. Actin visualization in HUVECs (*Figure 7*) indicated that rapamycin, either in the presence or in the absence of TNFα (*Figure 7C* and *D*), markedly modified cytoskeletal organization, leading to the formation of

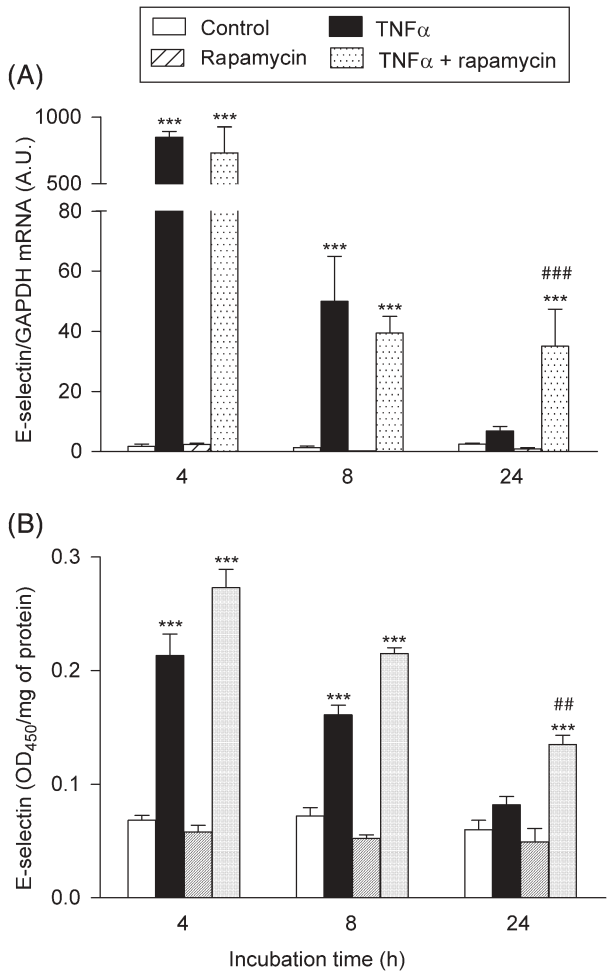

Figure 4 Effects of rapamycin on E-selectin mRNA and protein in human umbilical vein endothelial cells. Cells were incubated for 4, 8, or 24 h in the absence (control) or in the presence of 10 ng/mL TNFα, 100 nM rapamycin or both, as indicated. (*A*) E-selectin mRNA was analysed by quantitative real-time polymerase chain reaction and normalized to GAPDH mRNA. The results are means ± SEM of four separate experiments, each performed in duplicate. (*B*) In parallel cultures, the expression of E-selectin was evaluated by ELISA assay (see Methods). The results, normalized to protein content, are means ± SEM of six determinations within three separate experiments. $^{***}P < 0.001$ treated vs. control, untreated cells; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ cells treated with rapamycin and TNFα vs. cells treated with TNFα alone.

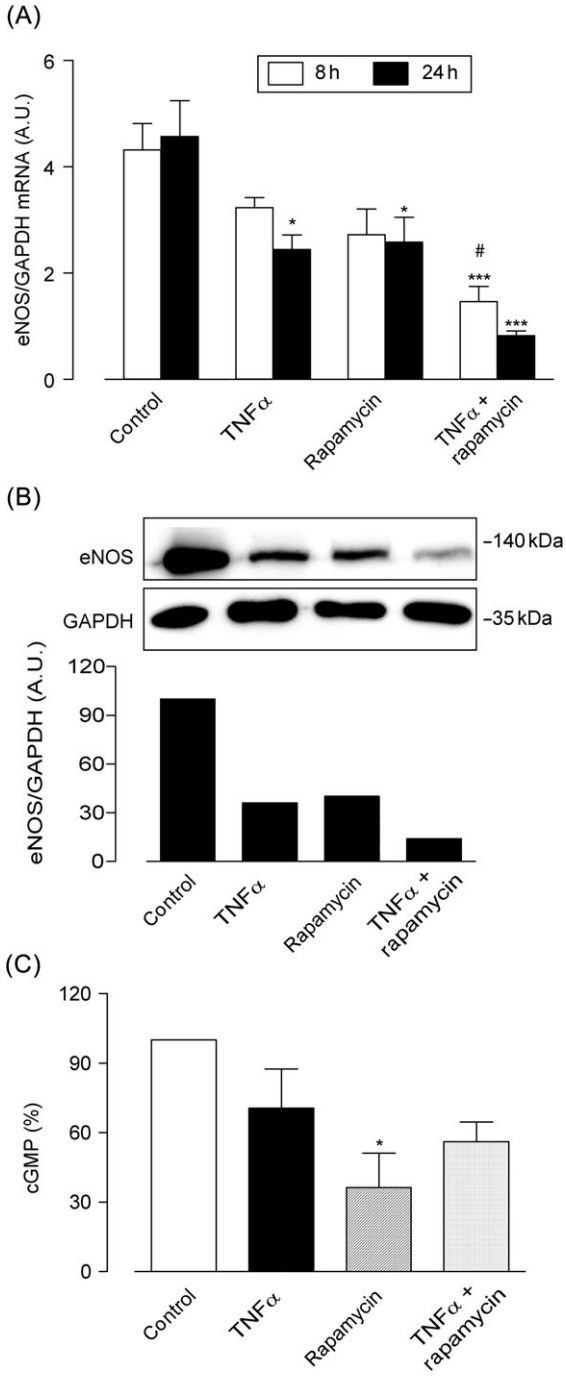

Figure 5 Effects of rapamycin on eNOS expression and nitric oxide release in human umbilical vein endothelial cells. Cells were incubated for 8 and 24 h in the absence (control) or in the presence of 10 ng/mL TNFα, 100 nM rapamycin, or both, as indicated. (*A*) eNOS mRNA was analysed by quantitative real-time polymerase chain reaction. The data, normalized to GAPDH mRNA, are means ± SEM of four separate experiments, each performed in duplicate. (*B*) In parallel cultures, the expression of eNOS was evaluated by western blot analysis after 24 h of treatment. A representative blot and the results of its densitometric analysis, normalized to GAPDH expression, are shown. The experiment was repeated three times with comparable results. (*C*) Bioactive nitric oxide produced by human umbilical vein endothelial cells was assessed as cGMP accumulation in RFL-6 reporter cells (see Methods). The results are expressed as percent of the control where 100% is equivalent to 0.43 ± 0.13 pmol of cGMP/10^6 cells. Each data point represents the mean ± SEM of six independent determinations in three different experiments. $^{*}P < 0.05$, $^{***}P < 0.001$ treated vs. control, untreated cells; $^{\#}P < 0.05$ cells treated with rapamycin and TNFα vs. cells treated with TNFα alone.

bundles of actin stress fibres much thicker than those observed in control cells or in cells treated with the cytokine alone (*Figure 7A* and *B*). As a result, the percentage of high-intensity, positive pixels (>2000 in a scale ranging from 50 to 4096), corresponding to stress fibres, was 4.2% for control, untreated cells; 8.5% for TNFα-treated cells; 18.9% for cultures treated with either rapamycin alone or with rapamycin and TNFα.

4. Discussion

Our data show that a prolonged exposure to the mTOR inhibitor rapamycin affects endothelial viability at concentrations equal or larger than 10 nM, increasing both necrotic and apoptotic parameters. Cell loss is even more pronounced in the presence of TNFα, when cell number decreases by almost 50% both in HUVECs and HAECs. At variance with rapamycin, FK506 did not affect HUVEC

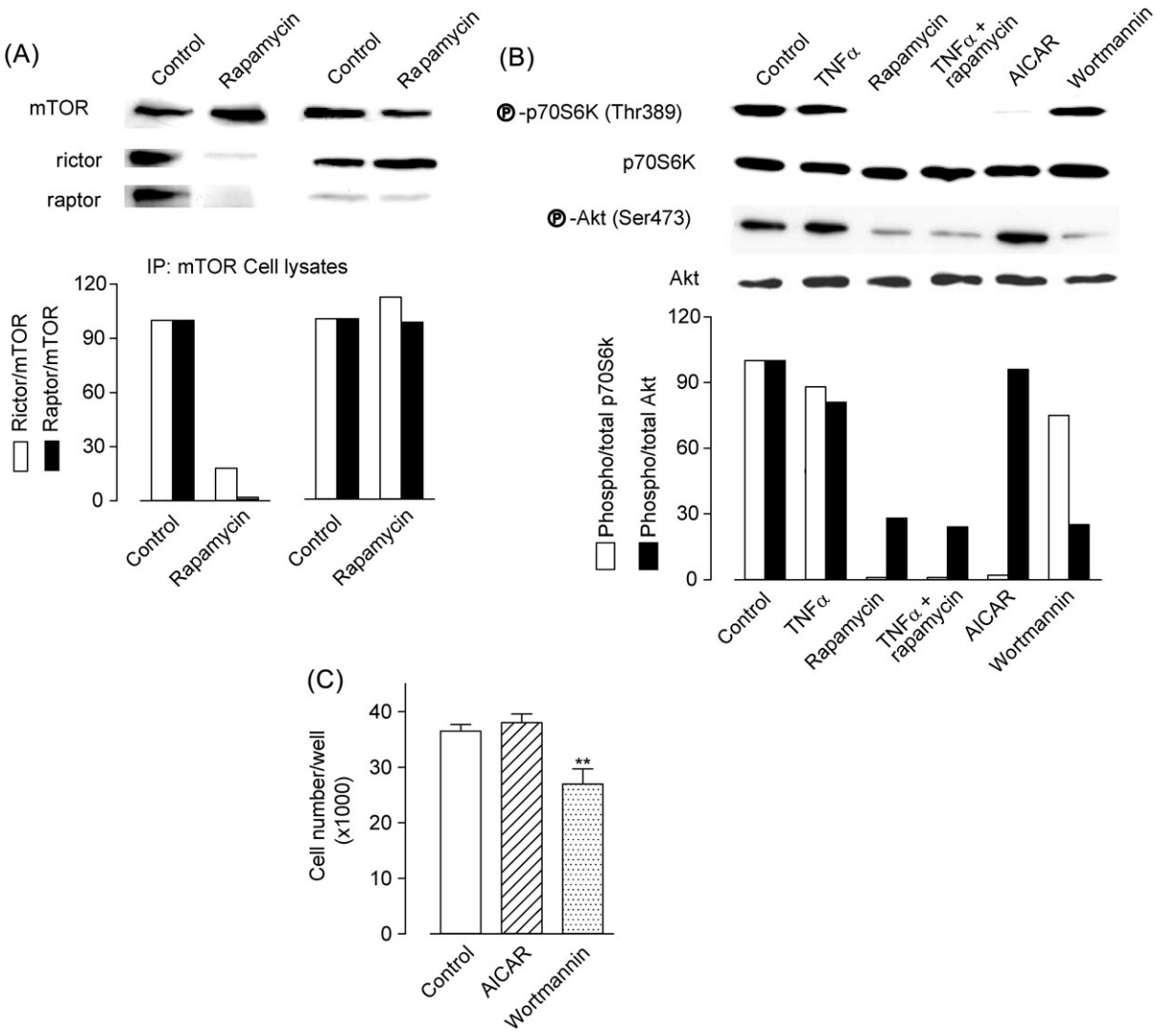

Figure 6 Effects of mTOR inhibition on p70S6K and Akt phosphorylation and cell viability. (*A*) Human umbilical vein endothelial cells were incubated for 24 h in the absence (control) or in the presence of 100 nM rapamycin. Whole-cell lysates and mTOR immunoprecipitates prepared from the lysates were analysed for mTOR, rictor, and raptor by western blot; the ratios between rictor or raptor and mTOR expression (% of control) are reported. A representative experiment, repeated twice with comparable results, is shown. (*B*) Human umbilical vein endothelial cells were incubated for 24 h in the absence (control) or in the presence of 10 ng/mL TNFα, 100 nM rapamycin, both compounds, 2 mM AICAR or 100 nM wortmannin, as indicated. Cell lysates were analysed for total and Ⓟ-p70S6K (Thr389) or total and Ⓟ-Akt (Ser473) by western blot; the ratios between total and Ⓟ-p70S6K or total and Ⓟ-Akt expression (% of control) are indicated. A representative experiment, repeated three times with comparable results, is shown. (*C*) Endothelial cells number was evaluated after 24 h treatment with 2 mM AICAR or 100 nM wortmannin. Bars represent means (with SEM indicated) of six independent determinations obtained in three experiments. $^{**}P < 0.01$ treated vs. control, untreated cells.

viability, even in the presence of the cytokine. Since both rapamycin and FK506 interact with FKBP12, the differences in their eventual effects must reside in the different intracellular targets, calcineurin for FK506 and mTOR for rapamycin.

mTOR is a component of two distinct multiprotein complexes: mTORC1, a recognized target of rapamycin, composed by mTOR, mLST8, and raptor; and mTORC2, previously considered rapamycin-insensitive, formed by mTOR, mLST8, and rictor.[5] However, prolonged exposure to rapamycin has been recently described to inhibit also mTORC2.[23] Through the two complexes, mTOR elicits different functions. In particular, mTORC1 is the major regulator of ribosomal biogenesis and protein synthesis through the phosphorylation of p70S6K and 4E-BP1, whereas mTORC2 has been shown to promote the phosphorylation of its downstream target Akt and the organization of cytoskeletal actin microfilaments.[10,25] In HUVEC, a 24 h-treatment with rapamycin causes a diminished expression of both raptor and rictor in mTOR-immunoprecipitates (*Figure 6A*), a finding consistent with the results obtained by Sarbassov *et al*. in HeLa cells,[23] which clearly demonstrates that the formation of both complexes mTORC1 and mTORC2 is hindered under the conditions in which rapamycin injuries endothelial cells. As a consequence, rapamycin-treated cells exhibit not only a complete suppression of p70S6K phosphorylation, as expected from mTORC1 inhibition, but also an increased formation of actin stress fibres and a decrease of Akt phosphorylation, two effects only referable to mTORC2 inhibition.[23,26] In particular, since Akt activation mediates pro-survival signals for vascular endothelium,[27,28] its inhibition via mTORC2 suppression could have effects comparable to those observed after its direct inhibition by wortmannin (*Figure 6*). Consistently, the AMPK-activator

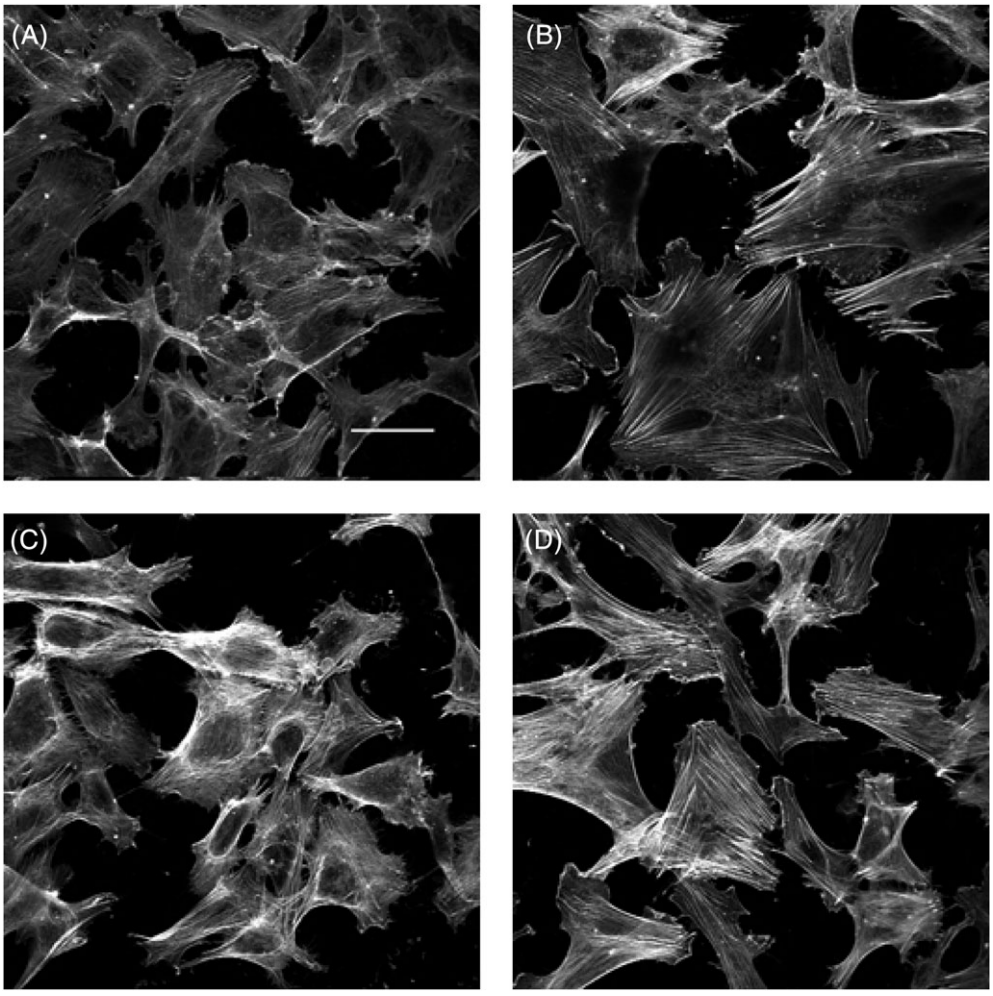

Figure 7 Effect of rapamycin on actin microfilaments. After incubation for 24 h in the absence (control, *A*) or in the presence of 10 ng/mL TNFα (*B*), 100 nM rapamycin (*C*) or both compounds (*D*), the monolayers were fixed and stained with phalloidin (see Methods). Single confocal sections of representative fields, taken at the maximal signal intensity, are shown. A representative experiment, repeated twice with comparable results, is shown. Bar = 50 μm.

AICAR, a compound that inhibits only mTORC1, as demonstrated by the observed hypophosphorylation of p70S6K but not of Akt (*Figure 6A*), has no effect on endothelial cell viability (*Figure 6C*). These findings are consistent with a recent paper by Dormond *et al.*,[29] which demonstrates the pro-death effect of Akt inhibition by rapamycin, taking advantage of an Akt dominant active construct.

Our experiments *in vitro* have also documented significant indicators of endothelial dysfunction associated with rapamycin treatment. The endothelial bioactive NO production is markedly decreased, an effect consistent with the fall of eNOS mRNA and protein expression. Rapamycin-induced hindrance of NO output supports the hypothesis, proposed by Fuke *et al.*, that the abnormal vasoconstrictive response to acetylcholine, observed in the peri-stent area after implantation of rapamycin-luting stents, is due to an impaired NO production.[8] Since Akt is known to activate eNOS by direct phosphorylation,[30] also the inhibition of NO output, observed in rapamycin-treated cells, may derive from Akt inhibition. In cells treated with TNFα in the absence of rapamycin, the inhibition of eNOS expression is not followed by a significant decrease of bioactive NO output. This result can be explained by the TNFα-dependent stimulation of eNOS activity mediated by Akt activation.[31] However, in cells treated with TNFα and rapamycin the lowest eNOS expression is not associated with a significant decrease in NO output, suggesting the existence of Akt-independent mechanisms of eNOS stimulation by TNFα.[32]

Rapamycin also prolongs the TNFα-dependent induction of E-selectin expression. Together with literature evidence,[14,15] this result suggests a sustained endothelial activation by the pro-inflammatory cytokine if mTOR is inhibited.

Interestingly, AICAR does not alter either eNOS or E-selectin expression (results not shown), indicating that the effects of rapamycin on these proteins are due to mTORC2 inhibition and, thus, possibly linked to rapamycin-dependent endothelial damage.

Defects or delays of stent re-endothelization has been proposed as one of the major mechanisms for stent thrombosis with DES, as documented in a recent autopsy study.[33] This *in situ* observation may be related to the reduced endothelial mobility obtained in the wound healing assay (*Figure 3*). Recently, rapamycin has been described to delay wound healing *in vivo*, an effect associated to a decrease of VEGF and NO in the wound tissues.[34] Interestingly, Akt-dependent

pathway plays a pivotal role in VEGF-mediated endothelial cell migration[35] and rapamycin hampers angiogenesis *in vivo* by inhibiting Akt phosphorylation.[36]

In summary, this report demonstrates that a prolonged treatment with rapamycin, alone or even more clearly in association with TNFα, impairs endothelial cell function and viability. These changes are observed under conditions associated with the inhibition of both mTORC2 and mTORC1 but not after the inhibition of mTORC1 alone, suggesting that mTORC2 activity plays an important role for endothelial welfare.

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