Inhibition of Orai1-mediated Ca\(^{2+}\) entry is a key mechanism of the antiproliferative action of sirolimus in human arterial smooth muscle

Sarah König,\(^1\) Sara Browne,\(^1\) Bernhard Doleschal,\(^1\) Michaela Schernthaner,\(^1\) Michael Poteser,\(^1\) Heinrich Mächler,\(^2\) Eric Wittchow,\(^3\) Marlen Braune,\(^3\) Martin Muik,\(^4\) Christoph Romanin,\(^4\) and Klaus Groshner\(^4\)

\(^1\)Institute of Biophysics, Medical University of Graz, Graz, Austria; \(^2\)Department of Cardiac Surgery, Medical University of Graz, Graz, Austria; \(^3\)Vascular Interventions Research and Development Group, Biotronik Societas Europaea and Compagnie Kommanditgesellschaft, Erlangen, Germany; and \(^4\)Institute of Biophysics, University of Linz, Linz, Austria

Submitted 3 May 2013; accepted in final form 4 September 2013

König S, Browne S, Doleschal B, Schernthaner M, Poteser M, Mächler H, Wittchow E, Braune M, Muik M, Romanin C, Groshner K. Inhibition of Orai1-mediated Ca\(^{2+}\) entry is a key mechanism of the antiproliferative action of sirolimus in human arterial smooth muscle. Am J Physiol Heart Circ Physiol 305: H1646–H1657, 2013. First published September 20, 2013; doi:10.1152/ajpheart.00365.2013.—Sirolimus (rapamycin) is used in drug-eluting stent strategies and proven capable of reducing in-stent restenosis down to <10\% (3, 46). Current development of DES is mainly focused on immunomodulatory compounds, which are referred to as “limus” drugs (43), including structural relatives of FK506 (tacrolimus) such as everolimus, pimecrolimus, zotarolimus, and sirolimus (11). These drugs interact with FK506 binding proteins (FKBPs), thereby suppressing cellular proliferative programs by divergent mechanisms. The primary interaction partners are immunophilins such as FKBP12 but also its relatives FKBP12–6 and FKBP52 (15, 31, 35). Drug-associated immunophilins are known to inhibit distinct pathways of transcriptional and translational control such as nuclear factor of activated T-cell (NFAT) dephosphorylation or mammalian target of rapamycin (mTOR) phosphorylation, which is considered the main mechanism of action for sirolimus (37). In addition, binding of “limus” drugs to their FKBP targets prevents the function of these molecules as prolyl-peptidyl isomerases (14, 38, 40) and modifies the interaction of the FKBPs with other signaling molecules including ion channels (7, 41). Specifically, in smooth muscle, “limus” drugs appear to exert a rather complex pattern of cellular actions, resulting in either suppression or promotion of proliferation, depending on tissue origin and state (8, 13, 52). This complexity is a likely reason for the recently recognized discrepancy in the efficacy of “limus” drugs in DES strategies. Sirolimus effectively reduced restenosis in humans with complex coronary lesions (23, 24, 28), whereas pimecrolimus displayed modest efficacy to prevent in-stent restenosis in clinical studies. Indeed, in clinical use, pimecrolimus even seems to modestly promote proliferation (2, 42, 47). The molecular basis of the superior efficacy of sirolimus to prevent proliferation in the human coronary system is still elusive. Here we set out to analyze the cellular mechanisms of the antiproliferative action of sirolimus and pimecrolimus in cultured human smooth muscle. Our results provide evidence for sirolimus-induced suppression of smooth muscle Ca\(^{2+}\) signaling, specifically of store-operated Ca\(^{2+}\) entry (SOCE) based on Orai channels and downstream Ca\(^{2+}\)-dependent transcriptional control specifically of cAMP response element binding protein (CREB) activation, representing an as-yet unrecognized action of sirolimus. These effects were not observed for pimecrolimus. Inhibition of SOCE and CREB activation is suggested as an antiproliferative mecha-

Address for reprint requests and other correspondence: K. Groshner, Inst. of Biophysics, Harrachgasse 21 8010 Graz, Austria (e-mail: klaus.groshner@medunigraz.at).

Calcium signaling; sirolimus; CREB; NFAT; coronary restenosis

IMMUNOMODULATORY DRUGS are successfully employed in percutaneous transluminal coronary angioplasty (PTCA) to prevent injury-induced restenosis (11, 42). This complication involves vascular smooth muscle cell (VSMC) phenotype switching from contractile to the synthetic form (25, 50) as a pivotal pathophysiological event. Stent implantation promotes proliferation and migration of VSMCs to form a neointima and thereby the basis of a restenotic lesion (9). Strategies to minimize or eliminate in-stent restenosis include modifications of stent-design and controlled release of immunosuppressive, antineoplastic, and anti-inflammatory substances from the stent surface (30, 42). Drug-eluting stent (DES) technology has proven capable of reducing in-stent restenosis down to <10\% (3, 46). Current development of DES is mainly focused on immunomodulatory compounds, which are referred to as “limus” drugs (43), including structural relatives of FK506 (tacrolimus) such as everolimus, pimecrolimus, zotarolimus, and sirolimus (11). These drugs interact with FK506 binding proteins (FKBPs), thereby suppressing cellular proliferative programs by divergent mechanisms. The primary interaction partners are immunophilins such as FKBP12 but also its relatives FKBP12–6 and FKBP52 (15, 31, 35). Drug-associated immunophilins are known to inhibit distinct pathways of transcriptional and translational control such as nuclear factor of activated T-cell (NFAT) dephosphorylation or mammalian target of rapamycin (mTOR) phosphorylation, which is considered the main mechanism of action for sirolimus (37). In addition, binding of “limus” drugs to their FKBP targets prevents the function of these molecules as prolyl-peptidyl isomerases (14, 38, 40) and modifies the interaction of the FKBPs with other signaling molecules including ion channels (7, 41). Specifically, in smooth muscle, “limus” drugs appear to exert a rather complex pattern of cellular actions, resulting in either suppression or promotion of proliferation, depending on tissue origin and state (8, 13, 52). This complexity is a likely reason for the recently recognized discrepancy in the efficacy of “limus” drugs in DES strategies. Sirolimus effectively reduced restenosis in humans with complex coronary lesions (23, 24, 28), whereas pimecrolimus displayed modest efficacy to prevent in-stent restenosis in clinical studies. Indeed, in clinical use, pimecrolimus even seems to modestly promote proliferation (2, 42, 47). The molecular basis of the superior efficacy of sirolimus to prevent proliferation in the human coronary system is still elusive. Here we set out to analyze the cellular mechanisms of the antiproliferative action of sirolimus and pimecrolimus in cultured human smooth muscle. Our results provide evidence for sirolimus-induced suppression of smooth muscle Ca\(^{2+}\) signaling, specifically of store-operated Ca\(^{2+}\) entry (SOCE) based on Orai channels and downstream Ca\(^{2+}\)-dependent transcriptional control specifically of cAMP response element binding protein (CREB) activation, representing an as-yet unrecognized action of sirolimus. These effects were not observed for pimecrolimus. Inhibition of SOCE and CREB activation is suggested as an antiproliferative mecha-

http://www.ajpheart.org

H1646 0363-6135/13 Copyright © 2013 the American Physiological Society
nism by which sirolimus prevents neointima formation in human arteries.

MATERIALS AND METHODS

Cell isolation, cultivation, and transfection. Human coronary artery smooth muscle cells (hCASM) were kindly provided by Dr. I. Wakabayashi (Yamagata University School of Medicine, Yamagata, JP). hCASM and embryonic kidney cells (HEK293) were cultured using standard conditions (37°C and 5% CO2) and Medium 231 (Invitrogen, Vienna, AT), supplied with smooth muscle growth supplement (Invitrogen) for hCASM, and DMEM (Invitrogen), supplied with 10% FCS for HEK293 cells.

For transfection, hCASM or HEK293 cells were seeded at 10^5 cells per well into 30-mm dishes. After ~12–18 h, adherent cells were transfected using either Lipofectamin LTX (Invitrogen; for hCASM) or FuGENE (Promega, Mannheim, Germany; for HEK293 cells) in accordance with the manufacturer’s instructions. For NFAT translocation experiments, hCASM were transfected with 4 µg green fluorescent protein (GFP)-NFAT DNA. Double transfections using 2 µg yellow fluorescent protein-Orai1 + 2 µg cyan fluorescent protein-stromal interaction molecule 1 (Stim1) were done to reconstitute the Ca2+ release-activated Ca2+ current (CRAC) pore in HEK293 cells in electrophysiological experiments.

Chemicals. Sirolimus (rapamycin, Sigma-Aldrich, Vienna, AT) and pimecrolimus (Elidel, Selleck Chemicals, Vienna, Austria) were applied at a concentration of 10 µmol/l, based on previous analyses of compounds’ tissue concentrations (19, 48).

Human aortic organ culture model. Tissue of ascending aorta one centimeter distally of left main artery was obtained from routine aortic aneurysmectomy. All patients had provided their informed consent to use the removed tissue for the current study. All procedures have been approved by the local Ethics Committee (reference number 24–104 ex 11/12) and in accordance with the Declaration of Helsinki. Aortic segments were washed with sterile PBS containing antibiotics and transferred to the laboratory in cold PBS. For organ culture and generation of vessel structures of coronary-like dimensions, adventitia was removed and segments of 15–20 mm in length and 10 mm in width were sewn to tubes of approximately 2–2.5 mm in diameter to fit to coronary stent size.

Test devices. Vessels were randomly allocated to two different groups, which were dilated by different balloon expandable coronary stents systems at sizes of 3.0/15 mm. The cobalt-chromium bare metal stent (BMS; n = 6) consisted of the PRO-Kinetic Energy (BIONETRONIK) BMS with a strut thickness of 60 µm. The sirolimus-eluting stent (n = 6) was based on the PRO-Kinetic Energy BMS and coated as previously described (19). All stents were implanted for 14 days.

Ex vivo stent implantation and culture conditions. Balloon-expandable coronary stent systems were used as outlined above. For the detailed stent implantation procedure, see Koenig et al. (18).

NFAT translocation. hCASM were transfected to express an NH2-terminally GFP-tagged NFATc1 fusion protein (16) and cultured on coverslips. The measurements were performed at room temperature beginning in a nominally Ca2+-free buffer containing (in mM) 140 NaCl, 5.4 KCl, 1 MgCl2, 10 glucose, and 10 HEPES at pH 7.4. After 5 min incubation, cells were challenged with 500 µM ATP, and after another 5 min, 2 mM Ca2+ was readded and incubated for 15 min.

Agents (sirolimus, pimecrolimus) were continuously present in all buffers during experiments and the agonist (ATP) remained present after administration. GFP-NFAT translocation was monitored (488 nm excitation) with standard fluorescence microscopy (Zeiss Axiosvert equipped with a Coolsnap HQ camera). GFP-NFAT fluorescence was distinguished by specific cellular localization. Nuclear/cytoplasmic fluorescence intensity ratios of cells were analyzed and calculated using ImageJ software (National Institutes of Health).

Measurement of intracellular Ca2+ signaling. hCASM were grown on coverslips and loaded with the calcium indicator 2’-naphthofluorescein diacetate (Invitrogen) for 45 min in Opti-MEM medium (Invitrogen), washed with PBS, and continuously perfused throughout the experiment at 2°C.

Fig. 1. Sirolimus (Sir) is more potent than pimecrolimus (Pim) in inhibiting proliferation of human vascular smooth muscle cells. Human coronary artery smooth muscle cells (hCASM) were cultured in the absence and presence of 10, 50, and 100 µM Pim and 10 µM Sir for 3 days, respectively. A: Western blot analysis of proliferating cell nuclear antigen (PCNA) in whole cell lysates of hCASM. A, top: quantitative analysis of PCNA protein expression normalized to β-actin expression and expressed as fold of control (CTRL; dashed line) (means ± SE, n = 4); **P < 0.01 vs. PCNA expression in CTRL cells; *P < 0.05 and **P < 0.01 vs. PCNA expression in Pim-treated cells. Statistical significance was determined based on ANOVA. A, bottom: total cell lysates were immunoblotted with anti-PCNA and anti-β-actin antibody. B: divergent effects of Pim from 5 bromodeoxyuridine (BrDU) assays and Sir from 4 BrDU assays upon BrDU incorporation into DNA of human vascular smooth muscle cells. BrDU incorporation was determined after 12 h and expressed as percentage of CTRL. *P < 0.05 vs. BrDU incorporation in Pim-treated cells and CTRL. cells. Statistical significance was determined based on ANOVA.
room temperature with nominally calcium-free buffer and challenged acutely with 500 μM ATP (protocol and buffers equal to NFAT translocation studies) or preincubated with 1 μM thapsigargin for 5 min to initiate SOCE. Agents (sirolimus, pimecrolimus) were continuously present in all buffers during experiments, and the agonist (ATP) remained present after administration. For calcium readdition, 2 mM extracellular CaCl2 was added. Excitation light was supplied via a Polychrome II polychromator (TILL Photonics, Oberhausen, DE), and emission was detected by a Sensicam CCD camera (PCO Computer Optics, Kelheim, DE) attached to a Nikon Diaphot 300 microscope and using a UV transmission-capable objective (Nikon Plan Fluor 20×/0.50). Ca2+-sensitive fura-2 AM fluorescence was measured ratiometrically at 340/380-nm wavelength with an emission at 510 nm. Recordings were analyzed by using Axon Imaging Workbench (Axon Instruments, Ismaning, DE). Ratios for Ca2+-free (minimum) and Ca2+-saturated (maximum) fura-2 were determined as 0.8 ± 0.002 and 1.2 ± 0.041, respectively, in hCASM cells permeabilized with 10 μM ionomycin.

**Electrophysiology.** Patch pipettes were pulled from borosilicate glass capillaries (Harvard Apparatus, resistance 3–5 MΩ). Signals were low-pass filtered at 1 kHz and digitized at 5 kHz. Membrane potentials were controlled with an Axopatch 200B (Axon Instrument) voltage-clamp amplifier using pClamp8 (Axon Instrument) software and acquired with a Digidata 1322A analog to digital converter (Axon Instruments). The data were analyzed with Clampfit 8 software (Axon Instruments). For HEK293 cells, voltage-clamp protocols (voltage ramps from -90 to +90 mV, and holding potential 0 mV) were controlled by pClamp software (Axon Instruments). HEK-293 cells expressing STIM1 and Orai1 to reconstitute the CRAC pore were generated as described in Muik et al. (26). Extracellular solution contained (in mM) 145 NaCl, 5 CsCl, 1 MgCl2, 10 HEPES, 10 glucose, and 10 CaCl2 at pH 7.4. Intracellular solution consisted of (in mM) 3.5 MgCl2, 145 cesium methanesulphonate, 8 NaCl, 10 HEPES, and 20 EGTA at pH 7.2.

**Proliferation assay.** Cell proliferation was determined by bromodeoxyuridine (BrdU) incorporation into newly synthesized DNA of actively proliferating hCASM. A colorimetric BrdU Cell Proliferation Assay (Merck, Vienna, Austria) was performed according to the manufacturer’s instructions. Briefly, hCASM were treated with agents (sirolimus, pimecrolimus) for 2 days and remained continuously present during the experiment. Cells were seeded at a density of 105 cells/ml onto 96-well plate in Medium 231 including smooth muscle growth supplement and antibiotics. After sedimentation (~6 h), BrdU label was administrated and incubated overnight. Absorbance was measured using a spectrophotometric plate reader at dual wavelengths of 450–550 nm.

**Western blot analysis.** For Western blot analysis, tissue from aortic organ culture was pulverized (micro-dismembrator, B. Braun International) and hCASM were trypsinized, washed with ice-cold PBS, and centrifuged. Tissue powder and cell pellets were resuspended in...
ice-cold lysis buffer using the Oproteome Mammalian Protein Prep Kit (Qiagen, Hilden, DE) according to the manufacturer’s protocol. After determination of protein concentrations of hole cell lysates by BCA Protein Assay (Fisher Scientific, Vienna, AT), samples were administered with 5× Laemml buffer (Sigma-Aldrich) and denatured at 95°C for 5 min. Equal amounts of protein were separated by SDS-PAGE and subsequently transferred to nitrocellulose membranes. Transferred proteins were probed with mouse anti-proliferating cell nuclear antigen (anti-PCNA; 1:1,000; BD Biosciences, Schwechat, Austria), rabbit monoclonal anti-phospho-CREB (1:500; New England Biolabs, Frankfurt, Germany), and mouse anti-β-actin (Sigma-Aldrich). Chemoluminescent protein detection was carried out using horseradish peroxidase-conjugated secondary antibody and Chemi Glow Chemiluminescence Substrate Sample Kit (Biozym Biotech Trading, Vienna, Austria). Band intensities were quantified using Herolab RH-5.2 dark room hood, equipped with an E.A.S.Y 1.3 HC camera (Herolab, Wiesloch, Germany). Results were expressed relative to control and normalized to β-actin band intensity.

**Immunocytochemistry and microscopy.** Staining procedure and microscopy were performed as described in Schernthaner et al. (36). For short, fixed cells were incubated with rabbit anti-Ki-67 antibody 1:200 (Abcam, Cambridge, UK) overnight at 4°C. As a secondary antibody, anti-rabbit tetramethylrhodamine isothiocyanate (1:300; Sigma-Aldrich) was used. Immunostaining was performed on a Zeiss microscope (Zeiss) in conjunction with two diode lasers (445 nm, 515 nm) and a dual-port adapter (dichroic: 505lp; cyan emission filter: 485/30; yellow emission filter: 535/50; Chroma Technology). This system was attached to an Axiovert 200 M microscope (Zeiss) and controlled with a VISIVIEW 2.1.1 software (Visitron Systems). Image correction due to cross talk and cross excitation were performed before the calculation. Therefore, appropriate cross-talk calibration factors were determined for each construct on each day of the FRET experiment. After threshold determination and background subtraction, the corrected FRET image (E_{FRET}) was calculated on a pixel-to-pixel basis with a custom-made software (9a) integrated in MatLab 7.0.4 according to the method published by Zal and Gascoigne, 2004 with a microscope specific constant G value of 2.0. All experiments were performed at room temperature.

**Statistical analysis.** Numerical data are presented as means ± SE with n representing the number of cells measured in Ca{sup 2+}-imaging experiments or of independent Western blot experiments. For imaging experiments, numbers of coverslips and cell preparations used for analysis are given in the legends. Continuous variables were checked for normal distribution by the Kolmogorov-Smirnov test and compared by Student’s t-test or ANOVA with Bonferroni pairwise post hoc correction or Kruskal-Wallis test for multiple comparisons as appropriate. Asterisks (*) were used to indicate significance in comparisons of individual data groups; hash mark (#), to indicate a significant difference versus baseline. A P value < 0.05 was considered statistically significant.

**RESULTS**

**Impact of sirolimus and pimecrolimus on proliferation of human VSMCs.** The two prototypical members of the “limus” drug family, sirolimus and pimecrolimus, were compared for their ability to suppress proliferation of hCASMs. In a first series of experiments, we compared the effects of pimecrolimus and sirolimus on the proliferation status of cells during a 3-day culture period. Levels of the proliferation marker PCNA and incorporation of BrdU were determined. With pimecrolimus, a significant inhibition of PCNA immunoreactivity required concentrations > 50 μM (Fig. 1A) and even a slight increase in PCNA levels was observed at 10 μM. By contrast, incubation with 10 μM sirolimus (Fig. 1A) clearly inhibited PCNA immunoreactivity down to a level even slightly lower than that obtained with 100 μM pimecrolimus. Similarly, cell proliferation measured as BrdU incorporation was suppressed at 10 μM concentrations only by sirolimus but not by pimecrolimus (Fig. 1B). These results confirm the divergent (~10-fold difference in potency) antiproliferative activity of these “limus” drugs on human arterial smooth muscle proliferation using an in vitro cell culture model (18).
NFAT-activation in human arterial smooth muscle is inhibited by both pimecrolimus and sirolimus. As a next step, we analyzed the effect of pimecrolimus and sirolimus on NFAT translocation in hCASMs using a GFP fusion protein of NFAT to monitor its translocation to the nucleus. As sirolimus, in contrast to pimecrolimus, lacks inhibitory effects on calcineurin, suppression of NFAT translocation was predominantly expected for pimecrolimus. Surprisingly, ATP-induced NFAT nuclear translocation was strongly suppressed by 30-min preincubation with either pimecrolimus (10 μM; Fig. 2A) or sirolimus (10 μM; Fig. 2B). The extent of inhibition observed in the presence of pimecrolimus and sirolimus was similar (66 and 65%, respectively). Thus the antiproliferative activities of the two “limus” drugs are similar when estimated at the level of NFAT signaling. Moreover, the observed suppression of NFAT translocation by sirolimus indicates that a calcineurin-independent mechanism as the target of sirolimus in human smooth muscle. Recently, Ca²⁺ signaling mechanisms have been suggested as targets of sirolimus (29, 47). Consequently, a specific impact of sirolimus on Ca²⁺-dependent transcriptional control was considered as a potential basis of its superior antiproliferative action in human arterial cells.

CREB activation in human arterial smooth muscle cells is inhibited effectively by sirolimus but not by pimecrolimus. Another important transcription factor involved in Ca²⁺-transcription coupling of vascular smooth muscle is CREB. Therefore, we compared the effects of the tested “limus” drugs on CREB activation. Figure 3 compares the phosphorylation of CREB in ATP-stimulated cells preincubated with pimecrolimus and sirolimus, respectively. CREB phosphorylation was effectively prevented by sirolimus (10 μM) but remained unaffected by treatment with pimecrolimus (10 μM).

As Ca²⁺ signaling is the key upstream event that governs both NFAT as well as CREB phosphorylation and activity, we hypothesized that sirolimus affects smooth muscle proliferation by interference with a Ca²⁺ entry pathway linked to

---

**Fig. 4. Sir but not Pim inhibits agonist-induced Ca²⁺ signaling in human arterial smooth muscle cells.** hCASMs were incubated with 10 μM Pim (n = 77, 9 coverslips; A) and 10 μM Sir (Sir; n = 80, 9 coverslips; B) for 30 min. **Left:** time courses of Ca²⁺-sensitive fura-2 fluorescence ratio are indicated at basal conditions (unstimulated + Ca²⁺ readjustment; n = 35, 6 coverslips) and stimulated with 500 μM ATP (n = 63, 10 coverslips) in the absence and presence of Pim and Sir. Ca²⁺ readjustment (from nominally free to 2 mM Ca²⁺) is illustrated (means ± SE from 4 fura-2 experiments). **Middle:** arithmetic mean values of the slope of the intracellular Ca²⁺ signals during reentry (±SE). #P < 0.001 vs. CTRL cells under basal conditions; *P < 0.001 vs. ATP-stimulated CTRL cells. Statistical significance was determined based on ANOVA. **Right:** representative single traces of the Ca²⁺ entry of fura-2 experiments at stimulated conditions (500 μM ATP) in the absence and presence of Pim and Sir.
control of gene expression. Consequently, we compared the effects of both drugs on Ca\(^{2+}\) signaling.

Pimecrolimus and sirolimus differently affect agonist-induced Ca\(^{2+}\) entry and STIM/Orai-mediated Ca\(^{2+}\) currents. Analysis of the impact of “limus” drugs on ATP-stimulated Ca\(^{2+}\) entry into hCASMs revealed a clear difference in the inhibitory action. As shown in Fig. 4A, pimecrolimus (10 \(\mu\)M) did not change the rate of rise in Ca\(^{2+}\)-sensitive fluorescence during Ca\(^{2+}\) readdition protocols in ATP-stimulated hCASMs, whereas sirolimus (10 \(\mu\)M) effectively inhibited Ca\(^{2+}\) entry signal in these cells (Fig. 4B).

In parallel, we delineated drug sensitivity of SOCE in hCASMs, generating store depletion by use of thapsigargin (Fig. 5). Again, sirolimus but not pimecrolimus was found to suppress Ca\(^{2+}\) entry into Ca\(^{2+}\) store-depleted human smooth muscle cells. As illustrated in Fig. 5, Ca\(^{2+}\) entry induced by Ca\(^{2+}\) readdition to cells treated by thapsigargin was inhibited by 38% in the presence of sirolimus.

STIM1/Orai1 complexes have been identified as key signaling elements of this Ca\(^{2+}\) entry into vascular smooth muscle (45). Nonetheless, these currents are essentially small in native human smooth muscle, and pharmacological characterization by electrophysiology is barely feasible. Therefore, we investigated the impact of sirolimus and pimecrolimus on the CRAC reconstituted by coexpression of Orai1 and STIM1 in HEK293 cells. CRAC currents were initiated by passive store depletion resulting from dialysis of the cells with a 20 mM EGTA-containing solution, and sirolimus or pimecrolimus (both 10 \(\mu\)M) was administered after full activation of the CRAC conductance (Fig. 6A). Results revealed a profound suppression of CRAC by sirolimus, whereas pimecrolimus was barely effective. To test whether sirolimus interferes with the Orai activating machinery in terms of STIM clustering, STIM1-STIM1 or STIM1-Orai1 interaction, we performed FRET experiments in cells overexpressing fluorescent STIM1 and Orai1 fusion proteins. As illustrated in Fig. 6B, sirolimus (10 \(\mu\)M) failed to suppress the enhanced FRET signal initiated by thapsigargin-induced store depletion for STIM1-STIM1 (left) as well as STIM1-Orai1 interactions (right). Moreover, clustering of STIM1 molecules was unaffected by sirolimus as

![Diagram A](image1)

![Diagram B](image2)

Fig. 5. Sir but not Pim inhibits store-operated Ca\(^{2+}\) entry. hCASMs were incubated with 10 \(\mu\)M Pim (\(n\) = 78, 8 coverslips; A) and 10 \(\mu\)M Sir (Sir; \(n\) = 85, 9 coverslips; B), respectively. Left: time courses of Ca\(^{2+}\)-sensitive fura-2 fluorescence ratio are indicated at basal conditions (unstimulated Ca\(^{2+}\) readdition; \(n\) = 38, 5 coverslips) and after stimulation with 1 \(\mu\)M thapsigargin (TG; 5 min preincubation; \(n\) = 83, 9 coverslips) in the absence and presence of Pim and Sir. Ca\(^{2+}\) readdition (from nominally free to 2 mM Ca\(^{2+}\)) is illustrated (means ± SE). Three different cell passages were used. Right: arithmetic mean values of the slope of intracellular Ca \(^{2+}\) signals during reentry (±SE). \#P < 0.001 vs. CTRL cells under basal conditions; *P < 0.001 vs. TG-stimulated CTRL cells. Statistical significance was determined based on ANOVA.
shown in Fig. 6C. These results point toward a rather direct action of sirolimus on the Orai pore complex.

The relevance of the Orai function in cell proliferation of smooth muscle cells was in addition tested by a genetic approach. Knockdown of Orai1 function was performed by expression of the dominant-negative Orai1 mutant E106Q. The dominant-negative protein strongly suppressed smooth muscle proliferation as monitored by Ki-67 expression (Fig. 7).

Sirolimus prevents stent implantation-induced activation of CREB in an in vitro model of stent-induced remodeling of human arteries. Sirolimus was tested for its ability to suppress in-stent neointimal hyperplasia in human arteries using a novel ex vivo organ culture model based on stent implantation into aortic constructs. The impact of stent implantation in the absence and presence of sirolimus released from the stent surface was determined using PCNA as proliferation marker, which was measured at protein expression levels. PCNA protein levels were enhanced in response to implantation of BMS into human aortic constructs. Sirolimus delivered via the DES suppressed PCNA levels below control, substantiating a prominent antiproliferative activity of sirolimus in human arteries (Fig. 8A).

To test whether this antiproliferative action involves inhibition of Ca$^{2+}$-dependent transcriptional signaling, we explored whether this antiproliferative effect of sirolimus is associated with the reduction of CREB phosphorylation in aortic organ culture model. Figure 8B compares the phosphorylation status of CREB in the ex vivo model of in-stent hyperplasia in the case of BMS and sirolimus-eluting stent implantation. CREB phosphorylation was effectively prevented by sirolimus in stented human aortic constructs.

**DISCUSSION**

This study demonstrates for the first time a profound inhibitory action of sirolimus (rapamycin) on the smooth muscle Ca$^{2+}$ signaling pathway formed by Orai1 and on downstream Ca$^{2+}$-dependent control mechanisms of gene expression. We
show that Ca$^{2+}$-dependent activation of NFAT and CREB is effectively suppressed by sirolimus in human arterial smooth muscle.

Sirolimus differs from pimecrolimus in its ability to inhibit Ca$^{2+}$ signaling and proliferation of human arterial smooth muscle cells. In line with clinical studies indicating a superior antiproliferative action of sirolimus in human arteries compared with pimecrolimus, another related immunomodulator (2, 24, 28, 42), we demonstrate a strikingly divergent action of these two “limus” drugs in a cell culture model of human coronary smooth muscle. Whereas pimecrolimus failed to inhibit proliferation up to 50 μM concentrations in this model, sirolimus at 10 μM clearly reduced expression of the proliferation marker PCNA. FKBP mediated inhibition of mTOR complex 1 (mTORC1) by sirolimus, representing a well-known mechanism of action, which is typically observed at low medium nanomolar concentrations (29). Consistently, translational repression by sirolimus, involving the mTOR/S6K pathway, is well documented for the arterial muscle (5, 10). Our present results demonstrate that sirolimus at micromolar concentrations exerts additional antiproliferative mechanisms, which are potentially involved in the superior action of sirolimus over its congener pimecrolimus. NFAT signaling represents the classical target of pimecrolimus action but is also a potential target of sirolimus. Interestingly, mTOR has also been reported as a regulator of NFAT nuclear translocation (52), and suppression of mTORC1 might therefore be considered as the basis of the observed sirolimus effects on NFAT translocation. Using expression and visualization of an NFAT-GFP fusion construct in cultured smooth muscle cells,

![Fig. 7. Suppression of Ki-67 expression by dominant-negative Orai proteins in arterial smooth muscle cells. Left: percentage of Ki-67 positive nuclei in vector-transfected hCASM cells (n = 119) compared with cells transfected with the dominant-negative Orai1 mutant E106Q (n = 128). Cells from 3 different passages were used. Cells were considered negative for Ki-67 immunoreactivity when showing mean nuclear fluorescence intensity < 15. ***P < 0.001 vs. vector-transfected cells. Statistical significance was determined by Student’s t-test. Right: representative confocal images of hCASM transfected with YFP-vector (top) or YFP-E106Q (green) and stained with an antibody against Ki-67/ TRITC (red) (bottom). Positions of nuclei are indicated by arrows. Note the Ki-67 positive nuclei in the nontransfected cell highlighted in circles at bottom. Scale bars represent 20 μm.](image)

![Fig. 8. Sir inhibits stent-induced arterial smooth muscle cell proliferation and pCREB. Western blot analysis of PCNA and pCREB in stented and unstretched (CTRL) human aortic arteries. Bare metal stents (BMS) and Sir-coated stents (SES) were implanted and cultured in the absence and presence of 10 μM Sir for 14 days. Results were normalized to the unstretched CTRL as 1 (dashed line) and referred to β-actin. Top: total smooth muscle cell lysates were immunoblotted with anti-β-actin and (A) anti-PCNA (A) and anti-pCREB antibody (B). A, bottom: quantitative analysis of PCNA (means ± SE, n = 6). #P < 0.05 vs. PCNA expression in CTRL cells; **P < 0.01 vs. PCNA expression in Pim-treated cells. Statistical significance was determined by ANOVA. B, bottom: quantitative analysis of pCREB protein expression (means ± SE, n = 4). #P < 0.05 vs. pCREB expression in CTRL cells; **P < 0.01 vs. pCREB expression in Pim-treated cells. Statistical significance was determined by ANOVA.](image)
we found that sirolimus indeed remarkably suppressed NFAT signaling in human arterial cells. The calcineurin inhibitor pimecrolimus and the mTOR inhibitor sirolimus exerted similar inhibitory effects on NFAT signaling in arterial smooth muscle cells. This is to our knowledge the first demonstration of an inhibitory interference of sirolimus with the NFAT pathway. NFAT signaling may be excluded as a critical determinant of human arterial smooth muscle proliferation, as inhibition of NFAT translocation by pimecrolimus was insufficient to suppress proliferation. Importantly, block of NFAT translocation by sirolimus required an about two to three orders of magnitude higher inhibitor concentration compared with that required for mTORC1 inhibition, indicating the existence of an alternative antiproliferative mechanism. This hypothesis was also supported by previous reports that demonstrated effects of sirolimus on G protein-coupled receptor function and phospholipase-C signaling (17, 22), which is tightly linked cellular Ca\(^{2+}\) homeostasis, as well as inhibitory effects of sirolimus on SOCE into human arterial smooth muscle (29). Inhibition of the Ca\(^{2+}\) entry was therefore considered as a potential mechanism involved in the antiproliferative action of sirolimus in human arteries. Such a modulation of Ca\(^{2+}\) signaling events was also indicated by observed suppression of NFAT nuclear translocation. Thus pimecrolimus and sirolimus suppress NFAT signaling by different mechanisms, suggesting a combination of these drugs may be highly efficient in preventing proliferation in certain tissues. Nonetheless, inhibition of Ca\(^{2+}\) signaling appeared as the prominent mechanism of sirolimus effects in human arteries. Consequently, we set out to investigate the interference of sirolimus with Ca\(^{2+}\) signaling in more detail and unraveled the existence of a sirolimus-sensitive Ca\(^{2+}\) entry pathway that is tightly linked to transcriptional control in human arterial muscle.

**Sirolimus inhibits Orai1-mediated Ca\(^{2+}\) entry as well as CREB signaling in human arterial smooth muscle cells.** In our experiments designed to characterize sirolimus effects on NFAT signaling, we initiated NFAT nuclear translocation by purinergic receptor stimulation with ATP, which typically elicits intracellular Ca\(^{2+}\) mobilization and Ca\(^{2+}\) entry (34). Evaluation of sirolimus effects on ATP-stimulated Ca\(^{2+}\) signals revealed a profound inhibitory action of sirolimus on ATP-induced Ca\(^{2+}\) entry. The concurrent suppression of Ca\(^{2+}\) entry and NFAT signaling indicates tight linkage of the sirolimus-sensitive pathway to transcriptional control. Two Ca\(^{2+}\)-signaling mechanisms may be considered as potential candidates for this sirolimus-sensitive, transcriptionally relevant pathway. SOCE into arterial smooth muscle was reported to exhibit sirolimus sensitivity (29). Alternatively, a phospholipase C-controlled mechanism involving activation of Ca\(^{2+}\) entry via channels of the transient receptor potential canonical (TRPC) family may be involved. Both channel types have been implicated in transcriptional control (6, 33). The molecular nature of smooth muscle SOCE channels is still under debate, and the exact role of TRPC and Orai channel proteins has not yet been clearly delineated. It is important to note that agonist-induced Ca\(^{2+}\) entry most likely involves both a store-operated as well as a receptor/second messenger-mediated component, and a molecular cross talk between TRPC and Orai channel proteins has been suggested (21, 49). Moreover, a store-independent Ca\(^{2+}\) entry pathway formed by Orai1/3 channel complexes and activated in response to intracrine mechanism involving leukotriene C4 generation has been discovered as important for vascular remodeling (12). It appears important to note that paracrine and/or autocrine mechanisms are likely to contribute to the control of Ca\(^{2+}\) homeostasis, Ca\(^{2+}\)-dependent control of gene expression, and consequently of proliferation.
These mechanisms may affect both store-operated as well as store-independent Ca\(^{2+}\) signaling. Our experiments using a classical Ca\(^{2+}\) readaddition protocol and thapsigargin for store depletion clearly indicated inhibition of SOCE in human arterial smooth muscle by sirolimus as the primary mechanism of action. This effect was not observed with pimecrolimus and might therefore well explain the profound antiproliferative action of sirolimus at micromolar concentrations. As Orai1 has recently been suggested as a key player and crucial pore-forming subunit of store-operated Ca\(^{2+}\) channels in smooth muscle (1, 32, 45), we went on to test the hypothesis that sirolimus is able to affect store-operated Orai1 channels. Our attempt to analyze inhibition of SOCE in a model of heterologous expression of the classical CRAC channel generated by overexpression of STIM1 and Orai in HEK cells was successfully shown by administration of sirolimus. It is tempting to speculate that the mechanism by which sirolimus suppresses Ca\(^{2+}\) entry into human arterial cells is based on its binding to FKBP51. Immunosuppressant drugs such as tacrolimus have been reported to affect the function of Ca\(^{2+}\) entry channels composed of TRPC proteins (7, 38, 41). The inhibitory effects of these drugs on TRPC channels are likely related to a disruption of the peptide-pyrol isomerase function, which appears essential for TRPC channel function (38). Notably, FKBP51, specifically FKBP12.6, are as well of importance for ryanodine receptor Ca\(^{2+}\) channel function in the sarcoplasmic reticulum of smooth muscle (20, 39), and indirect effects of sirolimus on Ca\(^{2+}\) entry via modulation of intracellular channels cannot be excluded at this point. Nonetheless, inhibition of CRAC currents by sirolimus was rapid in onset and occurred within seconds after administration. This almost instantaneous inhibitory effect indicates a rather direct mechanism of action or even a block of the Orai1 channel pore. FRET experiments designed to test for interference with important upstream event in the Orai activation cascade revealed that sirolimus does not interfere with STIM1 clustering or its association with Orai1. Thus our results suggest sirolimus as a rather direct inhibitor of the Orai pore complex. This novel aspect of rapamycin pharmacology may be of particular clinical relevance in therapeutic situations, which generate high tissue concentrations of the “limus” drug such as DES strategies. It appears likely that structural relatives of sirolimus such as zotarolimus and everolimus (44), which appear of particular therapeutic value, exert part of their action via the same Ca\(^{2+}\)-dependent mechanism. Collectively, our results provide evidence for the existence of a sirolimus-sensitive, store-operated Ca\(^{2+}\) signaling pathway that plays a pivotal role for transcriptional control and phenotype switching in human smooth muscle. This mechanism impacts on transcription in human arterial smooth muscle via two Ca\(^{2+}\)-sensitive transcription factors NFAT and CREB. Specifically, CREB has recently been suggested as crucial player in smooth muscle phenotype switching (4, 27) and subsequent neointima formation (51). This mechanism is suggested as the basis of the superior activity of sirolimus in prevention of in-stent restenosis compared with other “limus” drugs specifically. A scheme illustrating the proposed key role of SOCE modulation in the vascular action of sirolimus is illustrated in Fig. 9.

The concept suggested by our findings in a cell culture model of human arterial smooth muscle was confirmed in an in vitro model of stent-induced arterial injury. We provide evidence for a novel mechanism of suppression of smooth muscle hyperplasia by local administration of sirolimus (DES) based on direct inhibition of STIM/Orai signaling and CREB phosphorylation.

We propose Orai-mediated Ca\(^{2+}\) entry as a sirolimus-sensitive signaling pathway crucially involved in the process of in-stent smooth muscle hyperplasia in humans.

ACKNOWLEDGMENTS

We thank Dr. R. Kehlenbach for providing the GFP-NFAT construct and Dr. I. Wakabayashi for providing the hCASM cell line.

GRANTS

This work was supported by the Austrian Science Fund Grants 21925-B19 and 22565-B (to K. Groschner) and P22565-B18 (to C. Romanin) as well as DK+ Metabolic and Cardiovascular Disease Grant W2126-B18.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


