

A TRPC3 blocker, ethyl-1-(4-(2,3,3-trichloroacrylamide)phenyl)-5-(trifluoromethyl)-1H-pyrazole-4-carboxylate (Pyr3), prevents stent-induced arterial remodeling

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Abbreviations: TRPC, canonical transient receptor potential; BrdU, 5-bromo-2'-deoxyuridine; Pyr3, ethyl-1-(4-(2,3,3-trichloroacrylamide)phenyl)-5 (trifluoro-methyl)-1H-pyrazole-4-carboxylate; PCNA, Proliferating Cell Nuclear Antigen; Stim, stromal interaction molecule; HCASM, human coronary artery smooth muscle cells; HMEC-1, human microvascular endothelial cells; AOC, aortic organ culture; BMS, bare metal stent; DES, drug-eluting stent; Fura-2AM, fura-2 acetoxymethyl ester; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

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Abstract

TRPC-mediated Ca^{2+} entry has been implicated in the control of smooth muscle proliferation, and might represent a pivotal mechanism underlying in-stent restenosis. As we have observed significant expression of TRPC3 in human smooth muscle from coronary as well as aorta, we tested the efficiency of a recently discovered TRPC3 selective Ca^{2+} entry blocker Pyr3 to prevent vascular smooth muscle proliferation and stent implantation-induced hyperplasia of human aorta. The effect of Pyr3 on proliferation was measured by detection of BrdU incorporation and PCNA expression in human coronary smooth muscle and microvascular endothelium, which displays significantly smaller expression levels of TRPC as compared to smooth muscle. Pyr3 inhibited smooth muscle proliferation but lacked detectable effects on endothelial proliferation. Measurements of ATP-induced Ca^{2+} signals revealed that Pyr3 suppressed agonist-induced Ca^{2+} entry more effectively in vascular smooth muscle as compared to endothelial cells. Inhibitory effects of Pyr3 on stent implantation-induced arterial injury was tested using a novel in vitro model of in-stent hyperplasia in human arteries based on organ typical culture of human aortic constructs. Pyr3 effectively prevented increases in tissue levels of PCNA and Ki67 at 2 weeks after stent implantation into human aortae. Similarly, proliferation markers were significantly suppressed when implanting a Pyr3 releasing stent prototype as compared to a bare metal stent control. Our results suggest TRPC3 as a potential target for pharmacological control of smooth muscle proliferation. Selectively inhibition of TRPC Ca^{2+} entry channels in vascular smooth muscle is suggested as a promising strategy for in-stent restenosis prevention.

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Introduction

Alterations in Ca^{2+} signaling and Ca^{2+} transcription coupling of smooth muscle are crucially involved in arterial restenosis after angioplasty (Magnier-Gaubil et al., 1996; Munaron et al., 2004). Various Ca^{2+} signaling mechanisms have been implicated in the promotion of neointima hyperplasia (Sweeney et al., 2002; Watanabe et al., 2009; Ogawa et al., 2012) including TRPC-mediated Ca^{2+} entry. Since modified expression of TRPC proteins was observed in response to angioplasty (Bergdahl et al., 2005), these signaling molecules were proposed to contribute to disease progression involving angioplasty-induced remodeling of Ca^{2+} signaling pathways. Consequently, receptor/phospholipase C and store-operated Ca^{2+} entry mechanisms have emerged as attractive targets for pharmacological prevention of in-stent restenosis, which occurs in 13-36% of cases (Yutani et al., 1999). In-stent restenosis is the result of a healing reaction, which includes vascular smooth muscle phenotype switching from contractile to a synthetic form (Moses et al., 2001; Yang et al., 2001; Costa and Simon, 2005). Strategies to eliminate or minimize in-stent restenosis include the controlled release of antiproliferative, immunosuppressive and anti-inflammatory drugs from the stent surface. Current drug-eluting stent (DES) technology is mainly based on antimetabolic drugs such as paclitaxel (Park et al., 2003) and immunomodulatory compounds such as sirolimus (Rensing et al., 2001; Serruys et al., 2002). One major disadvantage of this type of drugs is the inevitable disturbance of endothelial functions and impairment of re-endothelialization of the dilated vessel segments (Matter et al., 2006). Thrombosis is significantly promoted as a consequence of incomplete recovery of the endothelium, and late DES thrombosis has been recognized as the major life threatening complication (Finn et al., 2007). Selective pharmacological targeting of smooth muscle Ca^{2+} entry channels may enable a muscle-selective antiproliferative intervention, which preserves endothelial functions. This concept is based on the hypothesis of different signaling pathways being involved in smooth muscle and

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endothelial Ca^{2+} -transcription coupling. Store-operated Ca^{2+} entry mediated by Stim/Orai complexes has been suggested as an important mechanism for the control of proliferation in both endothelial and smooth muscle cells (Abdullaev et al., 2008; Peel et al., 2008; Ogawa et al., 2012). Similarly, TRPC proteins have been implicated in the control of smooth muscle proliferation and have also been proposed as pivotal Ca^{2+} signaling molecules in the endothelium (Remillard and Yuan, 2006; Groschner, 2010). Tissue specific differences appear to exist in the TRPC expression pattern and in TRPC Ca^{2+} signaling. Moreover, TRPC-mediated Ca^{2+} entry pathways in endothelial as well as smooth muscle show striking plasticity along with phenotype switching (Bergdahl et al., 2005; Berra-Romani et al., 2008; Graziani et al., 2010). While TRPC4 along with TRPC1 and TRPC6 appear highly expressed and of functional significance in endothelial cells (Freichel et al., 2004; Paria et al., 2004; Graziani et al., 2010), TRPC6 and TRPC3 have been identified as important players in diseased states of vascular smooth muscle (Thippogowda et al.; Yao and Garland, 2005; Liu et al., 2010; Park et al., 2011). As TRPC3 represents a Ca^{2+} entry channel that is tightly linked to transcriptional control (Poteser et al., 2011) and is expressed at significant level in human arterial smooth muscle, we set out to evaluate the therapeutic potential of selective inhibition of this Ca^{2+} entry channel. Utilizing a recently identified selective inhibitor of TRPC3 (Glasnov et al., 2009; Kiyonaka et al., 2009) and a novel in vitro model of stent-induced smooth muscle hyperplasia, we provide the first evidence for the suitability of isoform selective suppression of TRPC signaling as an effective DES strategy.

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Methods

Cell culture and transfection

HCASM (human coronary artery smooth muscle cells) were kindly provided by Prof. Dr. I. Wakabayashi (Yamagata University School of Medicine, Yamagata, JP) and cultured in Medium 231 (Invitrogen, Vienna, AT) supplied with Smooth Muscle Growth Supplement (SMGS, Invitrogen) and antibiotics. HMEC-1 (human microvascular endothelial cells) were cultured in MCDB131-Medium (Gibco®, BRL Life Technologies, Carlsbad, CA) supplemented with 10 ng/ml epidermal growth factor (EGF, BD Biosciences, San Diego, CA), 15% Fetal Bovine Serum (PAA, Pasching, AT) and 1 µg/ml Hydrocortisone (Sigma Aldrich, Steinheim, DE). Both cell lines were cultured under standard conditions at 37°C and 5% CO₂. For transfections, hCASM were seeded at 10⁵ cells per well into 30 mm dishes. After ~12-18 h, adherent cells were transfected using 4 µg DNA was used per dish and Lipofectamin LTX (Invitrogen) in accordance with the manufacturer's instructions. For functional knock-down of TRPC3, the dominant negative pore mutant YFP-hTRPC3 E630K and the N-terminal fragment of hTRPC3, eYFP-NTRPC3 (aa 1-103 of TRPC3) were used. Control transfections were performed with peYFP-C1 vector.

Pyrazole 3 (Pyr3)

The TRPC3 pore blocker ethyl-1-(4-(2,3,3-trichloroacrylamide)phenyl)-5 (trifluoro-methyl)-1H-pyrazole-4-carboxylate (Pyr3) was synthesized as described recently (Glasnov et al., 2009; Obermayer et al., 2011). When Pyr3 was introduced at a single concentration, we used 10 µM, since this concentration has been shown repeatedly to exhibit maximum inhibitory action on TRPC3 without significant effects on other TRPC channel proteins (Kiyonaka et al., 2009).

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Human aortic organ culture model (AOC-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 conformed 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 three different groups, which were dilated by different balloon expandable coronary stents systems at sizes of 3.0/15 mm. The cobalt-chromium BMS (L605, n=4) consisted of the PRO-Kinetic Energy® (BIOTRONIK AG, Switzerland) BMS with a strut thickness of 60 µm and a second control BMS group laser-cutted with the identical design but made of stainless steel (316L, n=7). The Pyr3 eluting stent (P3-S; n=4) was based on the PRO-Kinetic Energy® BMS with a spray-generated biodegradable polymeric stent coating, poly(D,L-lactide) (PDLLA) interspersed with Pyr3 at 12 µg/mm. All stents were implanted for 14 days.

Ex vivo stent implantation and culture conditions

Balloon-expandable coronary stent systems based on the PRO-Kinetic Energy® (BIOTRONIK AG, Switzerland) were used as outlined above. Balloon catheters with mounted stents were inserted into aortic segments and inflated with 12 bar for 5 s to achieve

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overexpansion of the construct resulting in about 0,5 mm increase in diameter. Subsequently pressure was reduced to 10 bar for a period of 60 s. Unstretched aortic tissue served as controls. After stent placement, aortic segments were transferred to 60 mm dishes and cultured in Dulbecco's modified Eagle's medium (Invitrogen) at 37°C in 5% CO₂ for up to 2 weeks. Culture medium was changed every second day. After certain durations in culture, stents were removed from the aortic segments by unseaming. Samples used for biochemical and gene expression studies were quickly frozen in liquid nitrogen and stored at -80°C.

Measurement of intracellular Ca²⁺ signaling

HCASM and HMEC were grown on coverslips and loaded with the calcium indicator Fura-2AM (2 μM, Invitrogen) for 45 min in Opti-MEM medium (Invitrogen), washed with PBS and continuously perfused throughout the experiment at room temperature with nominally calcium-free buffer and challenged with ATP (protocol and buffers equal to NFAT translocation studies). Agonist (ATP) and inhibitor (Pyr3) remained continuously 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 CCDcamera (PCO Computer Optics, Kelheim, DE). Ca²⁺-sensitive Fura-2AM 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).

Morphology

Tissues from the AOC were fixed in 4% paraformaldehyde (Sigma-Aldrich) and embedded in paraffin. Consecutive sections (5 μm) were cut and processed for staining. Hematoxylin and eosin (HE) staining was performed to investigate tissue viability and cell morphology and

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Elastica van Gieson's staining (EvG) to differentially stain collagen and smooth muscle. Microscopic images were taken using a Nikon Eclipse E600 equipped with a Nikon Digital Sight DS-U1 unit (SPACH OPTICS INC. New York, US).

Proliferation Assay

Cell proliferation was determined by bromodeoxyuridine (BrdU) incorporation into newly synthesized DNA of actively proliferating cells. A colorimetric BrdU Cell Proliferation Assay (Merck, Vienna, AT) was employed according to the manufacturer's instructions. HCASM and HMEC were cultured in 100 mm dishes and incubated for 2 days with 1, 3, 5 and 10 μ M Pyr3, respectively. Cells were trypsinized and hCASM were seeded at a density of 5×10^4 cells/ml and HMEC were seeded at a density of 2×10^4 cells/ml onto 96-well plate in their respective medium applied with supplemental, antibiotics and Pyr3 in the mentioned concentrations. After sedimentation (~ 6 hours) BrdU-label was administrated and incubated overnight. Absorbance was measured using a spectrophotometric plate reader at dual wavelengths of 450-550 nm.

RNA isolation and qRT-PCR

For RNA extraction AOC tissue was first incubated with RNAlater[®]-ICE (Applied Biosystems) over night to avoid RNA degradation and subsequently pulverized with a micro-dismembrator (B. Braun Biotech International, Melsungen, DE). Total RNA was extracted using the TRIzol[®] Plus RNA Purification System (Invitrogen) and reverse-transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Vienna, AT) according to the manufacturer's protocol. Aliquots of 10 ng of each cDNA were amplified by qRT-PCR disposing Power SYBR[®] Green Mastermix (Applied Biosystems). 6 pmol of the primers (Sigma-Aldrich) for human Ki-67 (5'-CTACTCCAAGAAGCCTGTG-3'

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(forward), 5'-AATGAAGTTGTTGAGCACTCTG-3' (reversed)), human TRPC3 (5'-CATTCTGGCCATTGGCTACT-3' (forward), 5'-GCAGACCCAGGAAGATGATGAA-3' (reversed)), human PCNA (5'-ACACTAAGGGCCGAAGATAACG-3' (forward), 5'-ACAGCATCTCCAATATGGCTGA-3' (reversed)) and human GAPDH (5'-ATGGGGAAGGTGAAGGTTCG-3' (forward), 5'-GGGGTCATTGATGGCAACAATA-3' (reversed)) were constituted. The PCR reaction was directly monitored by the 7300 Real-Time PCR System with the appropriate software SDS v1.4 (Applied Biosystems). Results were expressed relative to control (unstretched tissue) and normalized to GAPDH expression levels.

Immunoblotting

Pulverized (micro-dismembrator, B. Braun International) tissue from AOC was resuspended in ice-cold lysis buffer using the Qproteome Mammalian Protein Prep Kit (Qiagen, Hilden, DE) according to the manufacturer's protocol. Protein concentrations of whole-cell lysates were determined by BCA Protein Assay (Fisher Scientific, Vienna, AT). Samples were suspended in 5x Laemmli 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. Results were expressed relative to control (unstretched tissue) and normalized to β -actin signal intensity. Transferred proteins were probed with mouse anti-PCNA, 1:1000 (BD Biosciences, Schwechat, AT), and mouse anti- β -actin (Sigma-Aldrich) and custom made rabbit anti-TRPC3 (Eder et al., 2007). Chemoluminescent protein detection was carried out using horseradish peroxidase-conjugated secondary antibodies anti-mouse, 1:5000 and anti-rabbit, 1:3000 (both from Sigma-Aldrich) and Chemi Glow Chemiluminescence Substrate Sample Kit (Biozym Biotech Trading GmbH, Vienna, AT).

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Bands intensity were quantified using Herolab RH-5.2 dark room hood, equipped with an E.A.S.Y 1.3 HC camera (Herolab GmbH, Wiesloch, DE).

Immunochemistry and microscopy

Staining procedure and microscopy were performed similar to (Scherthaner et al., 2012). For short, fixed cells were incubated with rabbit anti-Ki67 antibody 1:200 (Abcam, Cambridge, UK) or with custom made rabbit anti-TRPC3 antibody 1:300 (Eder et al., 2007) in PBS overnight at 4° C. As a secondary antibody anti-rabbit-TRITC 1:300 (Sigma Aldrich) in PBS for 1h at 37° C was used.

Statistical Analysis

Numerical data are presented as mean \pm S.E.M. Number of experiments (N) is given for human aortic segments and cell preparations. 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. A p-value $< 0,05$ was considered statistically significant.

Results

TRPC3 expression and sensitivity to the TRPC3 inhibitor Pyr3 in human vascular smooth muscle and endothelial cells.

TRPCs are considered of importance for the control of vascular functions by playing a pivotal role in Ca²⁺ homeostasis of vascular smooth muscle as well as endothelial cells (Groschner, 2010). Expression as well as cellular function of a particular TRPC species may differ between smooth muscle and endothelium, opening the view on selective targeting of vascular functions by selective modulation of TRPC isoforms. Here, we set out to explore potential

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this concept with respect to TRPC3, for which a selective blocker has recently become available. In an initial set of experiments we determined at which levels TRPC3 is expressed in human arterial smooth muscle and endothelial cell lines. We detected significantly higher TRPC3 protein expression in human coronary smooth muscle (hCASM) as compared to human microvascular endothelium (HMEC) (Figure 1). We confirmed expression and demonstrated plasma membrane localization of TRPC3 by immunostaining of hCASM, demonstrating TRPC3 immunoreactivity in the plasma membrane (supplemental information, Figure S1).

Based on the proposed crucial role of TRPC3 in cellular Ca^{2+} homeostasis, specifically in receptor/phospholipase C-dependent Ca^{2+} signaling of vascular cells (Kamouchi et al., 1999; Lin et al., 2004), we expected a TRPC3 expression-dependent impact of Pyr3 on Ca^{2+} homeostasis in these cells. As both cell preparations express P2Y receptors (Strobaek et al., 1996; Mortensen et al., 2009) we compared the action of Pyr3 in a setting of ATP-stimulated phospholipid metabolism. Figure 2 illustrates modulation of ATP-stimulated Ca^{2+} entry into hCASM and HMEC by Pyr3. The TRPC3 inhibitor was either administered acutely after activation of Ca^{2+} entry or cells were preincubated with Pyr3 (10 μM). Acute administration of Pyr3 (10 μM) resulted in inhibition of Ca^{2+} entry into both cell types, which was more pronounced in hCASM (70%; Figure 2, upper) than in HMEC (50%; Figure 2, lower). Preincubation with Pyr3 revealed an even stronger difference in the pyrazol sensitivity of Ca^{2+} signaling in these cells. Pyr3 (10 μM) eliminated the rise in Ca^{2+} sensitive fluorescence during Ca^{2+} re-addition in ATP-stimulated hCASM, while exhibiting a moderate inhibitory effect on Ca^{2+} entry in HMEC. Moreover, the profound inhibitory effect of Pyr3 on smooth muscle Ca^{2+} homeostasis was evident in addition by a lack of detectable Ca^{2+} mobilization by ATP in myocytes after preincubation with Pyr3 in Ca^{2+} free medium. Pyr3 is likely to promote passive depletion of Ca^{2+} stores in hCASM as indicated by experiments in which Ca^{2+} was

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mobilized using ionomycin (data not shown). Our results demonstrate a divergent Pyr3 sensitivity of Ca²⁺ homeostasis in these cell lines, which corresponds to the observed relative TRPC3 expression levels.

Pyr3 inhibits proliferation of human smooth muscle cells (hCASM) but not of human endothelial cells (HMEC).

The observed higher sensitivity of agonist-induced Ca²⁺ entry in smooth muscle as compared to endothelial cells prompted us to test for differences in the antiproliferative action of Pyr3 between these cell types and for a potential smooth muscle selective suppression of cell proliferation. We first measured cell proliferation by BrdU incorporation. The results show dose-dependent antiproliferative activity of Pyr3 in hCASM, while antiproliferative effects of Pyr3 were lacking up to concentrations of 10 μM (Figure 3A). In addition, we confirmed the antiproliferative effect by monitoring the proliferation marker PCNA at the mRNA level (supplemental information, Figure S2) and protein expression level of both cell types (Figure 3B). Pyr3 was administered at concentrations of 1 μM, 3 μM and 5 μM, which caused concentration-dependent inhibition of PCNA expression in hCASM, but were without significant effects in HMEC. As our results indicated a crucial impact of TRPC on smooth muscle proliferation, we tested this concept by a genetic approach. Knock-down of TRPC3 function was performed by expression of the dominant negative TRPC3 constructs E360K and n-TRPC3, respectively. Both dominant negative proteins strongly suppressed smooth muscle proliferation as monitored by Ki-67 expression (supplemental information, Figure S3).

Pyr3 inhibits smooth muscle proliferation in a novel human ex vivo model of stent-induced arterial injury and neointimal hyperplasia

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Based on the observed antiproliferative activity, Pyr3 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. In an initial set of experiments, we tested suitability of this organ culture model. Preservation of tissue viability and architecture after a culture period of two weeks was demonstrated by microscopical evaluation of HE and EvG stained tissue sections (supplemental information, Figure S4). Employing this model system, we determined the impact of Pyr3 on stent implantation-induced hyperplasia in human arteries. In a first set of experiments we administered Pyr3 at 10 μ M via the organ culture medium and determined PCNA as proliferation marker. As illustrated in Figure 4A and 4B (*Left*) the implantation of 316L bare metal stents in the human aortic constructs caused a significant enhancement of PCNA mRNA levels as compared to unstretched controls (dashed line). Addition of Pyr3 (10 μ M) to the culture medium resulted in a significant reduction in PCNA mRNA levels measured 2 weeks after stent implantation. This antiproliferative effect was confirmed by measurement of PCNA protein levels (Figure 4A). As the continuous presence of an inhibitor in the culture medium does not represent the DES scenario, we further evaluated the suitability of Pyr3 as a drug for the prevention of in-stent restenosis by characterization of a standard bare metal stent coated by a Pyr3 containing polymer representing a Pyr3-DES prototype. Implantation of these stents into human aortic constructs resulted in a similar reduced level of proliferation markers as obtained with 10 μ M Pyr3 in the culture medium (Figure 4B). Additionally, we confirmed the antiproliferative impact of Pyr3, by qPCR experiments demonstrating that Pyr3 also reduces the proliferation marker Ki-67 at the mRNA levels (supplemental information, Figure S5). Our findings demonstrate that stent implantation into human aorta initiates neointima hyperplasia, which is suppressed by Pyr3 delivered via standard DES technology. These results clearly demonstrate a profound antiproliferative activity of Pyr3 in human arteries.

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Pyr3 inhibits stent implantation-induced upregulation of TRPC3 expression

As upregulation of TRPC expression and a positive “feed-forward” regulation of TRPC signaling have been reported for excessive TRPC activation in myocytes, we speculated that stent implantation may enhance TRPC3 expression in human aorta. Indeed, a significant increase in TRPC3 expression was observed in response to stent-induced injury of aortic constructs (Figure 5, *Left*) as compared to unstretched controls (dashed line). Implantation of Pyr3 releasing stents, by contrast, failed to induce significant changes in TRPC3 expression along with reduced in-stent hyperplasia. Stent-induced enhancement of TRPC3 mRNA expression was effectively prevented by Pyr3 delivery via the DES. These results were corroborated by quantification of TRPC3 protein levels (Figure 5, *Right*). These results indicate that selective TRPC3 inhibition prevents a positive “feed forward” regulation of TRPC3 signaling that is associated with stent-induced injury of human arteries.

Discussion

The success of angioplasty with BMS is typically hampered by a reactive smooth muscle hyperplasia. The need for new, efficient drugs to prevent in-stent restenosis motivated us to test the hypothesis of selective suppression of TRPC Ca^{2+} signaling as a suitable drug-eluting stent strategy. We present evidence for both antiproliferative activity of the selective TRPC3 channel inhibitor Pyr3 in human coronary smooth muscle and for a weak interference of this compound with proliferation of endothelial cells. Our results demonstrate that Pyr3 i) exerts a profound inhibitory effect on Ca^{2+} entry into human arterial smooth muscle, ii) suppresses proliferation of smooth muscle cells and iii) prevents stent-implantation-induced hyperplasia of human arterial smooth muscle.

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Cell type specific antiproliferative action of Pyr3

A particular challenge in drug eluting stent development is the identification of suitable antiproliferative compounds that do not suppress re-endothelialization, which is essential for the prevention of late thrombotic events after angioplasty (Finn et al., 2007). In line with profound TRPC3 expression in human arterial smooth muscle and its importance for control of transcriptional programs, as demonstrated by use of dominant negative TRPC3 pore mutations (Poteser et al., 2011) we observed inhibition of arterial proliferation by the TRPC3 inhibitor Pyr3. As the antiproliferative action of Pyr3 might be restricted to tissues with prominent TRPC3 expression, we tested its effect also in human endothelial cells which typically display low TRPC3 expression (Paria et al., 2004) but prominent expression of TRPC1, TRPC4 and TRPC6 (Cioffi and Stevens, 2006; Zhang and Gutterman, 2011; Cioffi et al., 2012). One example for this expression pattern is provided by endothelial cells of human microvascular origin (HMEC). We confirmed moderate expression of TRPC3 in HMEC and, indeed, observed insensitivity of cell proliferation to inhibition by Pyr3. Nonetheless, a role for TRPC3 expression in endothelial proliferation has been recently suggested from studies using siRNA knock-down strategy (Antigny et al., 2012). One explanation for the discrepant effects of siRNA knock-down and selective channel block might be the recently identified scaffold function of TRPC3 in Ca^{2+} transcription coupling (Poteser et al., 2011). Thus expression of the protein but not channel function might be important in endothelial cells. Proliferation of HMEC is likely governed by TRPC proteins other than TRPC3 as recently suggested (Fantozzi et al., 2003; Ge et al., 2009; Sundivakkam et al., 2009) or by STIM-Orai complexes (Abdullaev et al., 2008). Arterial smooth muscle Ca^{2+} signaling displayed a clearly higher sensitivity to Pyr3 than Ca^{2+} homeostasis in endothelial cells. Pyr3 (10 μM) virtually abolished Ca^{2+} readdition-induced Ca^{2+} entry and eliminated Ca^{2+} transients in hCASM induced by ATP in Ca^{2+} -free solutions, while no detectable change in Ca^{2+} transients were

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observed in HMEC. A similar inhibitory effect of Pyr3 was observed on smooth muscle Ca^{2+} mobilization by ionomycin. Indicating that TRPC3 is crucial to enable Ca^{2+} store filling a phenomenon that might be related to TRPC3 dependence of Ca^{2+} handling via NCX (Rosker et al., 2004; Eder et al., 2007; Poburko et al., 2008). Inhibitory effects of Pyr3 on endothelial Ca^{2+} entry were smaller than those on smooth muscle Ca^{2+} homeostasis. Due to the selectivity of Pyr3, these effects are unlike due to inhibition of TRPC channels but might be related to interaction of Pyr3 with other Ca^{2+} transport systems such as Orai1 (Schleifer et al., 2012). These effects were not associated with changes in proliferation. Thus, paucity of TRPC3 expression in HMEC may well explain the lack of antiproliferative effects of Pyr3. On the other hand suppression of smooth muscle proliferation in response to inhibition of TRPC3 function was also confirmed by dominant negative knock down (supplementary information, Figure S3) using well established TRPC3 constructs (Poteser et al., 2011).

Our results unequivocally strengthen the hypothesis of a tissue specific targeting of transcriptionally active Ca^{2+} entry pathways by pyrazole TRPC inhibitors. Thus, selective inhibition of arterial smooth muscle proliferation by Pyr3 may represent an attractive strategy for prevention on neointima formation, and in-stent restenosis. To test the principle efficiency and suitability of Pyr3 for this therapeutic application, we set out to investigate its effects in a simple ex-vivo model of stent-induced arterial injury.

Selective inhibition of TRPC3 is an efficient strategy to suppress stent-induced media hyperplasia

Effects of Pyr3 on arterial responses to stent implantation were investigated in an organotypical culture model of human aorta. Stent implantation into human aortic segments of coronary-like architecture initiated a substantial increase in proliferation markers. Pyr3, when administrated either simply via the culture medium or locally via a conventional drug

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eluting stent system, effectively prevented or ameliorated the injury response. Our results suggest that selective TRPC3 inhibition is suitable to prevent cell proliferation and tissue remodeling after by stent implantation. Effectiveness of Pyr3 as an inhibitor of tissue and organ responses to stress was initially suggested for hypertrophic remodeling of the heart (Kiyonaka et al., 2009) and is now demonstrated for stent-induced neointima formation. These processes of tissue remodeling appear to involve not only Ca^{2+} signaling due to stress-induced activation of TRPC channels but also a “feed forward”, vicious cycle of Ca^{2+} -mediated upregulation of TRPC expression. Consistently, we observed indeed enhanced TRPC3 expression in response to stent implantation into human aortic constructs that was prevented by Pyr3. Hence, selective block or inhibitory modulation of TRPC channels is proposed to prevent the stress-initiated vicious cycle and tissue remodeling processes.

Pharmacotherapeutic implications

TRPC signaling is likely involved in early steps of pathological remodeling processes, specifically in the cardiovascular system. This pathophysiological role is substantiated by the recently identified linkage of TRPC Ca^{2+} signals to control of gene transcription (Poteser et al., 2011). TRPC channels generate localized Ca^{2+} signals that govern transcription factors such as NFAT. Notably, Ca^{2+} -dependent transcriptional regulators are most likely linked to different upstream Ca^{2+} sources in different tissues. This opens the view on a tissue/cell type selective interference with the remodeling processes by selective inhibition of a defined set of Ca^{2+} entry channels. We suggest that selective interference with TRPC subtype signaling inhibits hyperplasia of injured arterial smooth muscle with little or no impairment of endothelial proliferation. In vivo studies are required to further explore the therapeutic benefit of a Pyr3 DES system in terms of reduced late stent thrombosis. Our results clearly support

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the concept of selective suppression of TRPC3 signaling as a promising pharmacological approach to improve the long-term success of angioplastic intervention.

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Authorship Contributions

Participated in research design: Koenig, Maechler, Wittchow, Braune, Groschner

Conducted experiments: Koenig, Hoefler, Schernthaner

Contributed new reagents or analytic tools: Kappe, Glasnov

Performed data analysis: Koenig

Wrote or contributed to the writing of the manuscript: Koenig, Wittchow, Groschner

Supplementary material

Supplementary material is available at *Journal of Pharmacology and Experimental Therapeutics* online.

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Footnotes

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Figure legends

Figure 1 TRPC3 protein expression levels in hCASM and HMEC. Western blot analysis of TRPC3 in whole cell-lysates of hCASM and HMEC. (*Right*) Representative immunoblot with

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anti-TRPC3 and anti- β -actin antibody. (*Left*) Quantitative analysis of TRPC3 protein expression (means \pm S.E.M., $n \geq 4$). Asterisk indicates statistically significant differences.

Figure 2 Pyr3 differently inhibits TRPC3-mediated Ca^{2+} entry in arterial smooth muscle and endothelial cells. HCASM (*Upper*) and HMEC (*Lower*) were incubated with 10 μM Pyr3 for 30 minutes or were acutely exposed to 10 μM Pyr3. Time courses of Ca^{2+} sensitive Fura-2 fluorescence ratio are displayed at basal conditions (unstimulated + Ca^{2+} re-addition) and stimulated with 500 μM ATP. Ca^{2+} re-addition (from nominally free to 2 mM Ca^{2+}) is illustrated (means \pm S.E.M., $n > 30$).

Figure 3 Pyr3 inhibits cell proliferation in arterial smooth muscle cells but not in endothelial cells. HCASM and HMEC were cultured in the absence and presence of 10 μM Pyr3 for 3 days. (A) Effect of Pyr3 on BrdU incorporation into DNA of hCASM and HMEC. BrdU incorporation was determined after 12 h and expressed as percent of control (mean \pm S.E.M., $n = 4$). (B) Western blot analysis of PCNA in whole cell-lysates of hCASM and HMEC. (*Upper*) Representative immunoblots with anti-PCNA and anti- β -actin antibody. (*Lower*) Quantitative analysis of PCNA protein expression normalized to β -actin expression and expressed as fold of control (dashed line) (mean \pm S.E.M., $n = 4$). Asterisks indicate statistically significant inhibition by Pyr3.

Figure 4 Pyr3 release from a stent system as well as Pyr3 in solution inhibits proliferation human arterial smooth muscle. QRT-PCR and western blot analysis of the proliferation marker PCNA in stented and unstretched (CTRL) human aortic arteries (hAorta). Stainless steel BMS (316L) were implanted and cultured in the absence and presence of 10 μM Pyr3 for 14 days (A) and cobalt-chromium BMS (L605), as well as Pyr3-eluting stents (P3-S) (B)

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were implanted and cultured for 14 days. Results were normalized to the unstretched control as 1 (dashed line) and referred to β -actin. (A, B) (*Left*) Quantitative qRT-PCR analysis of PCNA mRNA expression (means \pm S.E.M., $n \geq 4$). (*Right*) Representative immunoblot with anti-PCNA and anti- β -actin antibody (*Upper*) and quantitative analysis of PCNA protein expression (means \pm S.E.M., $n \geq 5$) (*Lower*). Asterisks indicate statistically significant inhibition by Pyr3.

Figure 5 Pyr3 inhibits TRPC3 expression in human arterial smooth muscle. QRT-PCR and western blot analysis of TRPC3 in stented and unstretched (CTRL) human aortic arteries (hAorta). Stainless steel BMS (316L) were implanted and cultured in the absence and presence of 10 μ M Pyr3 for 14 days. Results were normalized to the unstretched control as 1 (dashed line) and referred to β -actin. (A) Quantitative qRT-PCR analysis of TRPC3 mRNA expression (means \pm S.E.M., $n \geq 3$). (B) Representative immunoblot with anti-TRPC3 and anti- β -actin antibody (*Upper*). Quantitative analysis of TRPC3 protein expression (means \pm S.E.M., $n \geq 3$) (*Lower*). Asterisks indicate statistically significant inhibition by Pyr3.

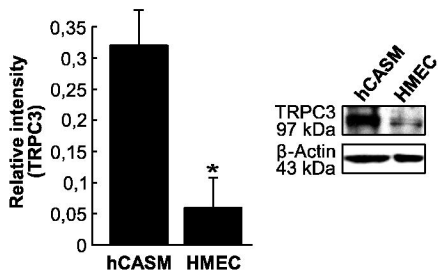


Figure 1

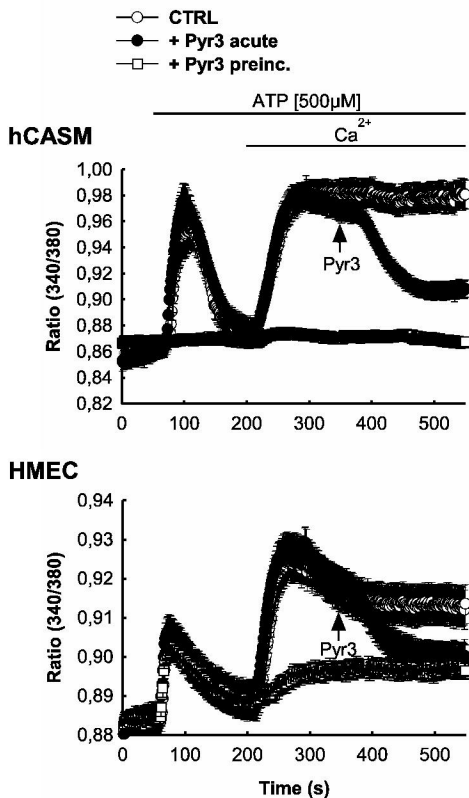


Figure 2

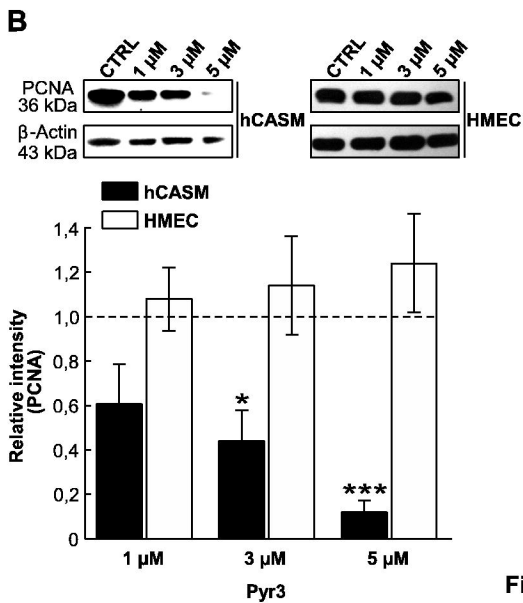
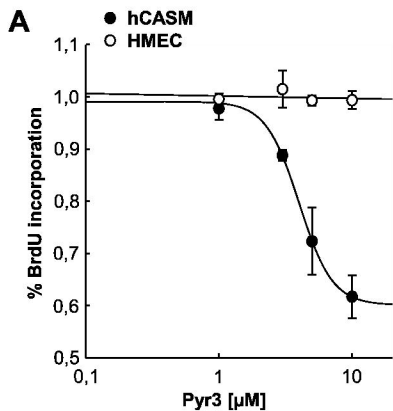
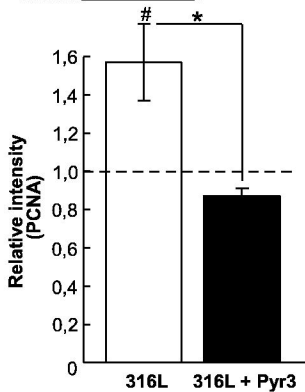
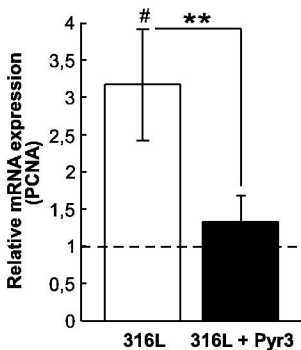
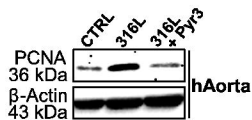
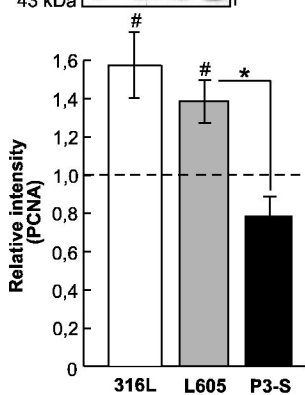
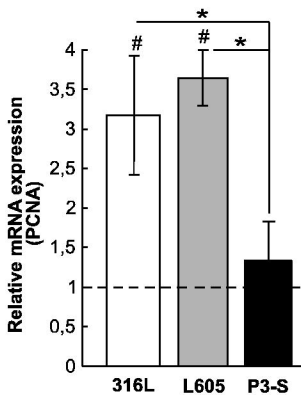
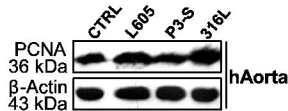


Figure 3

A**B**

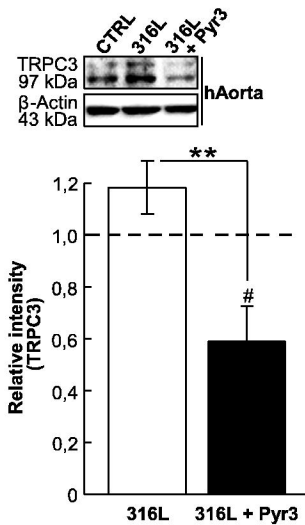
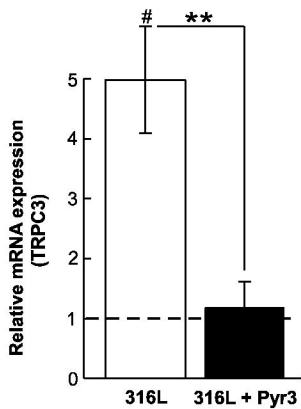


Figure 5

Supplemental Data

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A TRPC3 blocker, ethyl-1-(4-(2,3,3-trichloroacrylamide)phenyl)-5-(trifluoromethyl)-1H-pyrazole-4-carboxylate (Pyr3), prevents stent-induced arterial remodeling

Sarah Koenig, Michaela Schernthaner, Heinrich Maechler, C. Oliver Kappe, Toma N. Glasnov, Gerald Hoefler, Marlen Braune, Eric Wittchow and Klaus Groschner

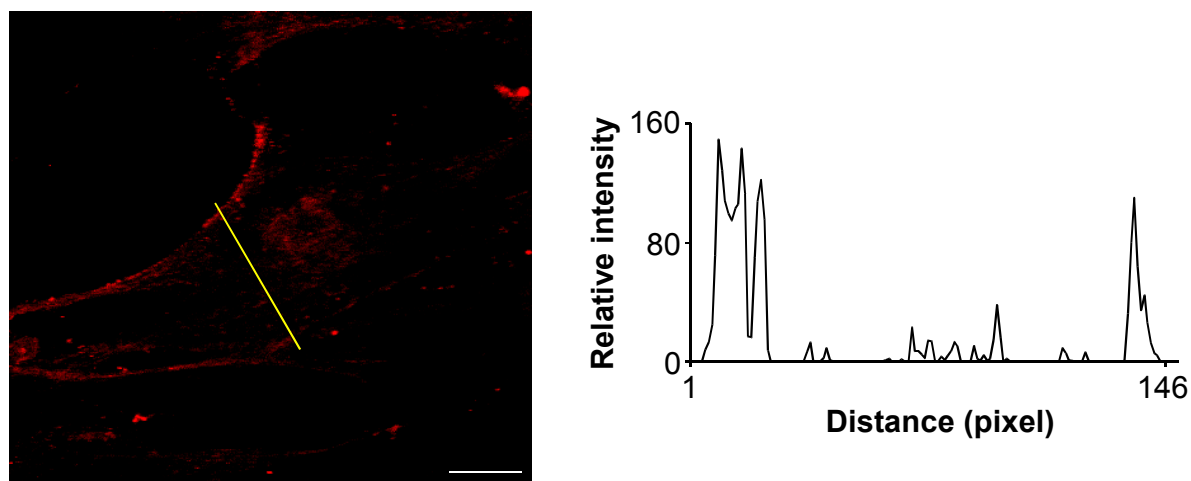


Figure S1 Endogenous expression of TRPC3 in arterial smooth muscle cells. (*Left*) Confocal image of hCASM immunostained with an antibody against TRPC3. (*Right*) The profile plot (yellow line) indicates the distribution of TRPC3. Scale bar represents 20 μm .

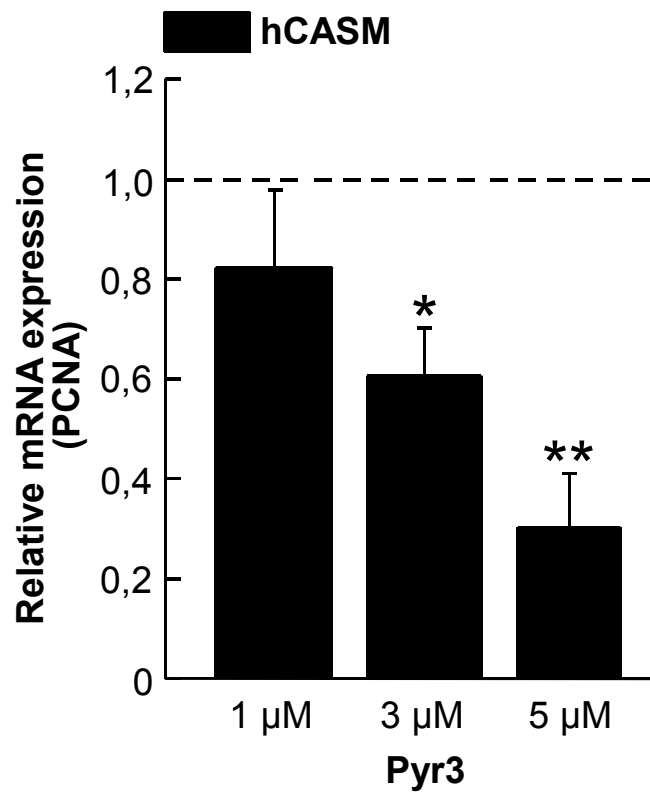


Figure S2 Pyr3 inhibits PCNA mRNA expression in arterial smooth muscle cells. HCASM were cultured in the absence and presence of 10 μM Pyr3 for 3 days. Quantitative qRT-PCR analysis of the proliferation marker PCNA mRNA expression (mean ± S.E.M., n = 3). Results were normalized to the unstretched control as 1 (dashed line) and referred to GAPDH. Asterisks indicate statistically significant inhibition by Pyr3.

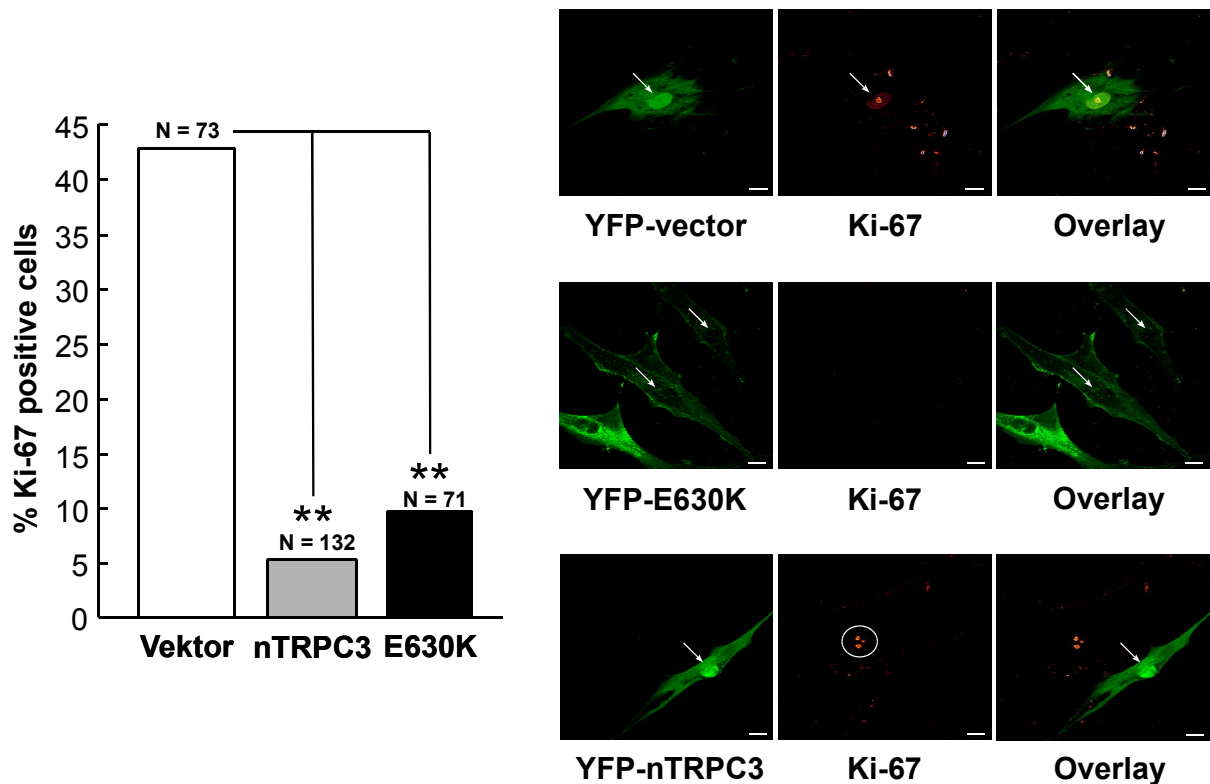


Figure S3 Suppression of Ki-67 expression by dominant negative TRPC3 proteins in arterial smooth muscle cells. (*Left*) Bar graph shows percentage of Ki-67 positive nuclei in vector-transfected hCASM cells compared to cells transfected with the dominant negative TRPC3 mutant E630K or with the N-terminal fragment of hTRPC3 (nTRPC3). Cells were considered negative for Ki-67 immunoreactivity when showing mean nuclear fluorescence intensity < 15. Asterisks indicate statistically significant differences. (*Right*) Representative confocal images of hCASM transfected with (*Upper*) YFP-vector, (*Middle*) YFP-E630K or (*Lower*) YFP-nTRPC3 (green) and stained with an antibody against Ki-67/TRITC (red). Positions of nuclei are indicated by arrows. Note the Ki-67 positive nucleus in the non-transfected cell highlighted in a circle in the lowest picture. Scale bars represent 20 μ m.

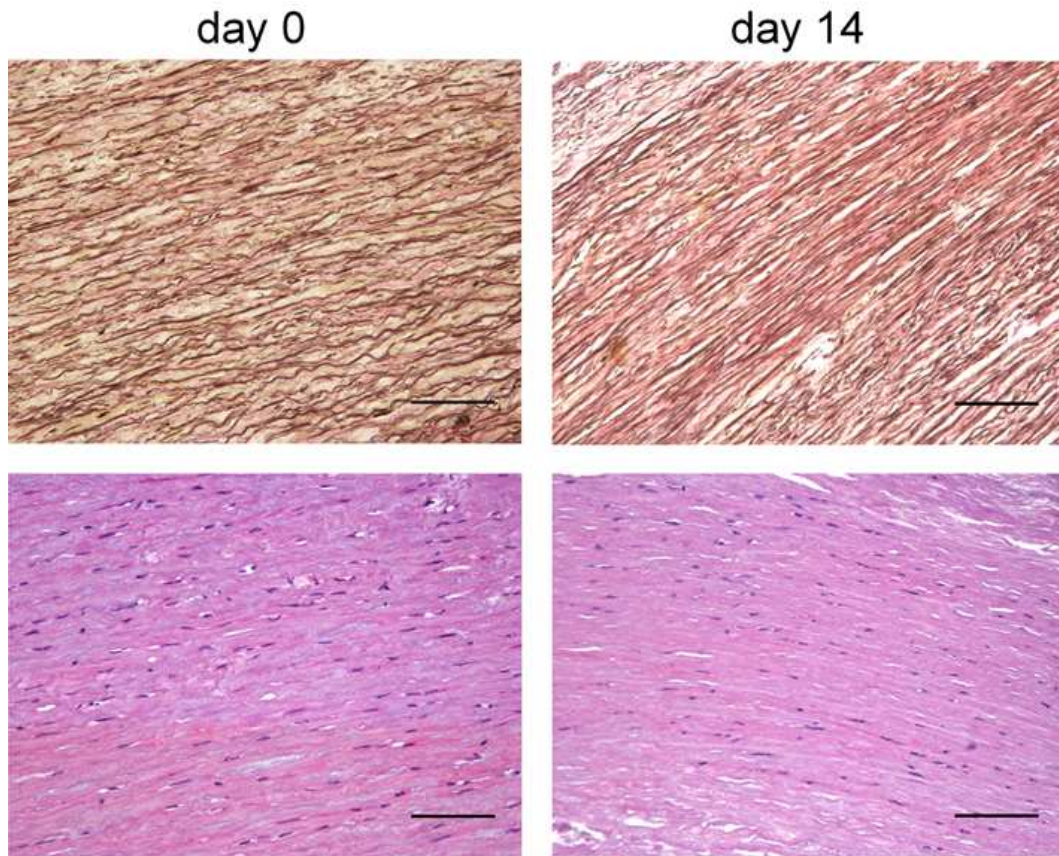


Figure S4 Tissue viability and architecture of stented human aortic constructs is preserved after two weeks in organ culture. Representative histological cross sections of human aortic tissue (*Left*) at day 0 and (*Right*) at day 14 after bare metal stent implantation were stained with (*Upper*) EvG and (*Lower*) HE. Scale bars represent 100 μm .

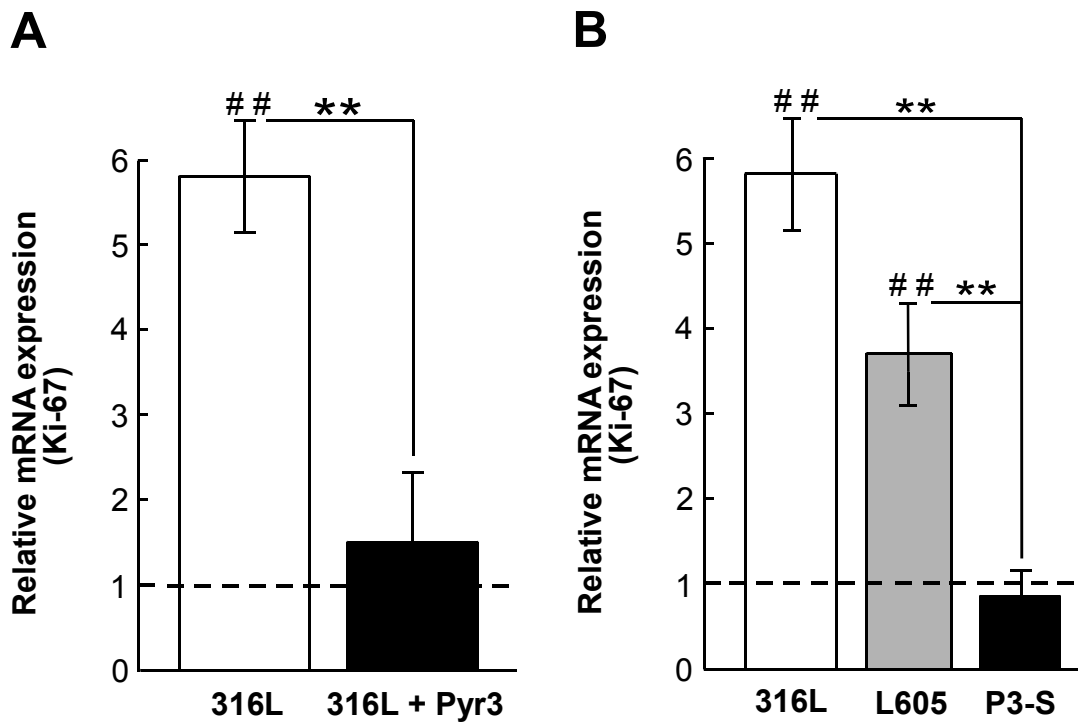


Figure S5 Pyr3 in solution as well as Pyr3 released from a stent system inhibits Ki-67 mRNA expression in human arterial smooth muscle. Quantitative qRT-PCR analysis of the proliferation marker Ki-67 mRNA expression (mean \pm S.E.M., $n \geq 4$) in stented and unstretched (CTRL) human aortic arteries. (A) Stainless steel BMS (316L) were implanted and cultured in the absence and presence of 10 μ M Pyr3 for 14 days and (B) 316L, cobalt-chromium BMS (L605), as well as Pyr3-eluting stents (P3-S) were implanted and cultured for 14 days. Results were normalized to the unstretched control as 1 (dashed line) and referred to GAPDH. Asterisks indicate statistically significant inhibition by Pyr3.