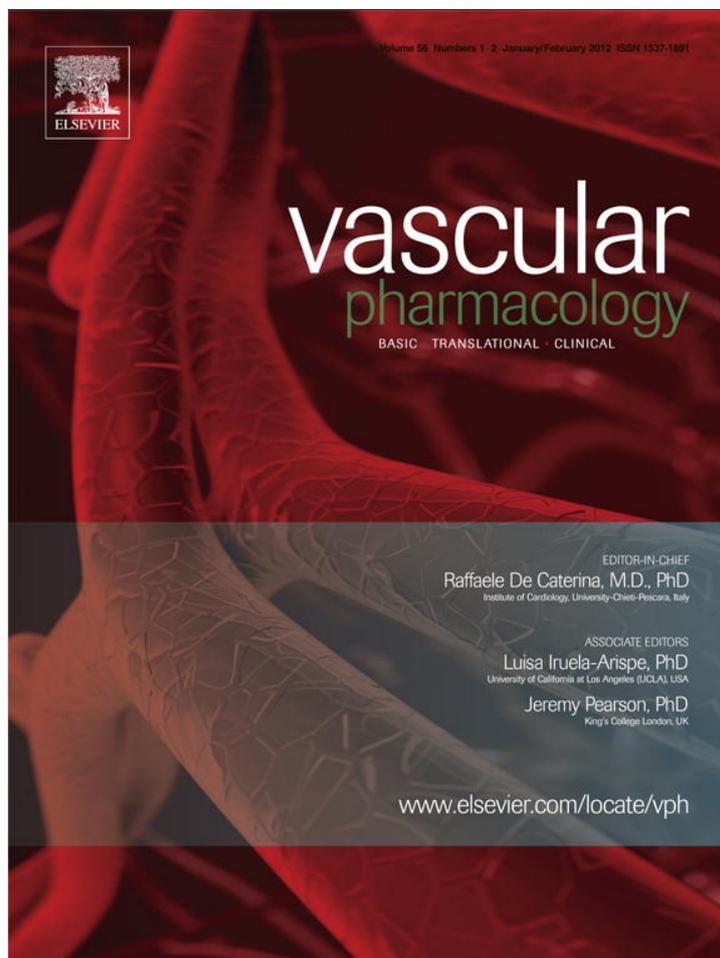


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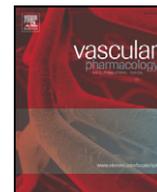
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Inhibition of Mitogen Activated Protein Kinase Activated Protein Kinase II with MMI-0100 reduces intimal hyperplasia ex vivo and in vivo

Akihito Muto^{a,b}, Alyssa Panitch^d, Namho Kim^d, Kinam Park^d, Padmini Komalavilas^{e,f}, Colleen M. Brophy^{e,f}, Alan Dardik^{a,b,c,*}

^a Department of Surgery, Yale University School of Medicine, New Haven, CT, United States

^b Department of Vascular Biology and Therapeutics Program, Yale University School of Medicine, New Haven, CT, United States

^c VA Connecticut Healthcare System, West Haven, CT, United States

^d Weldon School of Biomedical Engineering, Purdue University, 206 S. Martin Jischke Drive, West Lafayette, IN 47907, United States

^e Department of Surgery, Vanderbilt University, Nashville, TN 37232, United States

^f VA Tennessee Valley Healthcare System, Nashville TN, United States

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ABSTRACT

Vein graft intimal hyperplasia remains the leading cause of graft failure, despite many pharmacological approaches that have failed to translate to human therapy. We investigated whether local suppression of inflammation and fibrosis with MMI-0100, a novel peptide inhibitor of Mitogen Activated Protein Kinase II (MK2), would be an alternative strategy to reduce cell proliferation and intimal hyperplasia. The cell permeant peptide MMI-0100 was synthesized using standard Fmoc chemistry. Pharmacological doses of MMI-0100 induced minimal human endothelial and smooth muscle cell proliferation (30% and 12% respectively). MMI-0100 suppressed IL-6 expression to control levels, without effect on IL-8 expression. MMI-0100 caused sodium nitroprusside induced smooth muscle cell relaxation and inhibited intimal thickening in human saphenous vein rings in a dose-dependent fashion. In a murine aortic bypass model, MMI-0100 reduced intimal thickness in vein grafts by 72%, and there were fewer F4/80-reactive cells in vein grafts treated with MMI-0100. MMI-0100 prevents vein graft intimal thickening ex vivo and in vivo. These results suggest that inhibition of MK2 with the cell-permeant peptide MMI-0100 may be a novel strategy to suppress fibrotic processes such as vein graft disease.

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1. Introduction

There are > 1 million coronary bypass procedures a year worldwide, with human greater saphenous vein remaining the most commonly used conduit. However, less than half of these grafts remain patent after 12 years (Motwani and Topol, 1998), with more recent data from the PREVENT IV trial demonstrating 42% graft occlusion within 18 months (Alexander et al., 2005). Graft failure typically leads to myocardial infarction and death, the need for repeated

coronary bypass procedures and, consequently, substantial costs to the healthcare system. Thus, approaches to decrease vein graft failure rates would improve outcomes after arterial bypass procedures, yielding significant clinical and health economic benefits.

The leading cause of bypass graft failure is intimal hyperplasia of the vein conduit (Clowes and Reidy, 1991). While its causes are as yet incompletely understood, intimal hyperplasia likely results from a cascade of events triggered by the tissue response to mechanical injury associated with surgical vein harvest and conduit preparation; in addition, the damage induced by mechanical dilation used to “break” vessel spasm is refractory to current vasodilators and other pharmacologic approaches (Dashwood et al., 2004; Dashwood and Loesch, 2007).

On a cellular–molecular level, intimal hyperplasia is mediated by a sequence of events, including inflammatory processes in response to vessel trauma, resulting in vascular smooth muscle proliferation, migration, and extracellular matrix production (Allaire and Clowes, 1997). This is associated with a phenotypic modulation of smooth muscle cells from a contractile to a synthetic phenotype, with “synthetic” cells secreting extracellular matrix proteins (Mosse et al., 1985). Graft functional responses are also impaired, leading to abnormal vasorelaxation (Klyachkin et al., 1993; Lorusso et al., 2007). All of

Abbreviations: CaMKI, calcium/calmodulin-dependent protein kinase I; EC, endothelial cell; GA, gentamycin/amphotericin; HAEC, human aortic endothelial cells; HASMC, human aortic smooth muscle cells; HCAEC, human coronary artery endothelial cells; hnRNP A0, heterogeneous nuclear ribonucleoprotein A0; HSP27, heat shock protein 27; HSV, human saphenous vein; IL, interleukin; I:M, intima:media; MAPK, mitogen activated protein kinase; MK, mitogen activated protein kinase activated protein kinase; MLEC, mouse lung endothelial cells; PBS, phosphate-buffered saline; PE, phenylephrine; SMC, smooth muscle cell; SNP, sodium nitroprusside; TTP, tristetraprolin.

* Corresponding author at: Yale University School of Medicine, Vascular Biology and Therapeutics, 10 Amistad Street, Room 437D, PO Box 208089, New Haven, CT 06520-8089, USA. Tel.: +1 203 785 7991; fax: +1 203 737 2290.

E-mail address: alan.dardik@yale.edu (A. Dardik).

these processes lead to pathologic narrowing of the vessel lumen, graft stenosis, and ultimately graft failure (LoGerfo et al., 1983).

Although a number of drugs aiming to reduce development of intimal hyperplasia have been tested in clinical trials, these products have failed. Antithrombotic and antiplatelet agents such as warfarin, clopidogrel and aspirin have little or no effect on intimal hyperplasia (Kent and Liu, 2004). Two large clinical trials for the prevention of coronary and peripheral vascular vein graft failure using an E2F decoy to prevent smooth muscle proliferation also failed in their primary endpoint (Alexander et al., 2005; Conte et al., 2006). Accordingly, availability of novel therapeutic approaches to improve graft patency remains an unmet need.

Recently, Epstein, et al. demonstrated that suppression of the innate immune response in the context of vascular injury dramatically down-regulated the degree of intimal hyperplasia (Danenberg et al., 2002; Epstein et al., 2008). These results suggest that inflammation plays a major role in intimal thickening and that peri-procedural suppression of inflammation could decrease intimal hyperplasia by a clinically meaningful degree. However, immune suppression on a systemic level during surgical procedures and the post-operative recovery period can increase infection risk, and as such is not clinically feasible. Therefore, we investigated whether local suppression of inflammation, via *ex vivo* vein graft treatment with MMI-0100, a peptide inhibitor of MAPKAP kinase II (MK2), would be a novel alternative strategy to reduce intimal thickening following vein bypass surgery.

Mitogen Activated Protein Kinase Activated Protein Kinase II (MAPKAP Kinase II, MK2) is an intracellular kinase activated by the p38 Mitogen Activated Protein Kinase (MAPK) (Rouse et al., 1994) that, in turn, phosphorylates transcription factors tristetraprolin (TTP) (Sandler and Stoecklin, 2008) and hnRNP A0 (Rousseau et al., 2002). TTP and hnRNP A0 are known to interact with AU-rich regions of mRNA to control mRNA stability and expression. Importantly, studies show that suppression of MK2 activity results in down-regulation of inflammatory cytokine expression, including TNF- α , IL-1 β , and IL-6 (Kotlyarov et al., 1999; Winzen et al., 1999; Lehner et al., 2002; Neininger et al., 2002; Wang et al., 2002; Thomas et al., 2008; Funding et al., 2009).

We recently developed a cell-permeant MK2 inhibitor peptide (Lopes et al., 2009) that was based on a peptide designed by Hayess and Bendorff (Hayess and Bendorff, 1997). However, further work with this peptide demonstrated that it was relatively nonselective and toxic, which led to development of significantly more specific inhibitor peptides, including MMI-0100 (Ward et al., 2009). In an animal model of abdominal adhesions, i.e. rat bowel anastomosis, we reported that a single dose of MMI-0100 applied locally at the time of surgery reduces both number and severity of abdominal adhesions without impairing normal intestinal healing, as determined by hydroxyproline content and burst pressure of the colonic anastomosis (Ward et al., 2011).

These results suggest that inhibition of MK2 with MMI-0100 inhibits inflammatory responses leading to excess extracellular matrix deposition and formation of scars and adhesions. Given the role of inflammation in the development of intimal hyperplasia, we investigated whether MMI-0100 could similarly reduce this clinically relevant vascular process and perhaps ultimately vein graft failure. Therefore, we tested whether MMI-0100 affected vascular cell proliferation and reduced intimal hyperplasia *ex vivo* and *in vivo*.

2. Material and methods

2.1. Cell culture

Primary human aortic endothelial cells (HAEC) were obtained from Invitrogen; HAECs were cultured in Medium 200 supplemented with LSGS (Low Serum Growth Supplement), containing FBS (2% v/v), hydrocortisone (1 μ g/ml), human epidermal growth factor (EGF,

10 ng/ml), Basic Fibroblast Growth Factor (bFGF, 3 ng/ml), gentamycin/amphotericin (GA) and heparin (10 μ g/ml). Primary human aortic smooth muscle cells (HASMC) were obtained from Invitrogen; HASMC were cultured in EGM Bullet Kit – EBM-2 Endothelial Basal Medium 2 supplemented with hEGF (10 ng/ml), hydrocortisone (1.0 μ g/ml), GA (50 μ g/ml), FBS (5%), VEGF, hFGF-B, R³-IGF-1, and ascorbic acid. Primary human coronary artery endothelial cells (HCAEC) were obtained from Lonza; HCAECs were cultured in Medium 231 supplemented with SMGS (Smooth Muscle Growth Supplement), containing FBS (4.9% v/v), bFGF (2 ng/ml), hEGF (0.5 ng/ml), heparin (5 ng/ml), insulin (5 μ g/ml), BSA (0.2 μ g/ml), and GA.

All cultures were maintained in 25 cm² polystyrene tissue culture flasks in a 37 °C, 5% CO₂/95% air environment, with cell culture media refreshed every other day. All cells were seeded at a density of 20,000–30,000 cells/cm², as required by the specific experiment, and allowed to grow to 80–90% confluence before being harvested/passaged. Only cells from early passages (numbers 2–8) were utilized in experiments.

Primary cultures of mouse lung endothelial cells (MLEC) were isolated as previously described (Ackah et al., 2005; Muto et al., 2011). After immunoselection with magnetic beads, endothelial cells were immortalized with polyoma middle T-antigen. Isolated MLECs were maintained with EBM-2/EGM-2 MV SingleQuot Kit Supplement & Growth Factors (Lonza) containing 15% fetal bovine serum. Cell proliferation in MLEC was measured at 24 and 72 h after MMI-0100 treatment by direct cell counting after trypsin treatment.

2.2. MMI-0100 reconstitution/dilution

MMI-0100 was synthesized using standard Fmoc chemistry as previously described, with the peptide sequence YARAAARQARAKALARQLGVAA (Ward et al., 2009). 114 mg of MMI-0100 (MW = 2283.67 g/mol; Moerae Matrix, Inc.) was dissolved in 5 ml of phosphate-buffered saline (PBS) to yield a 0.01 M stock solution, which was divided into 500 μ l aliquots and stored at –20 °C. Serial dilutions of stock solution were made to achieve appropriate drug concentrations for each study.

2.3. MTS cell proliferation assay

The CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega) was used to assess drug effects on cell proliferation according to the manufacturer's instructions. Briefly, HAECs and HASMCs from early passages were grown to 80–90% confluence in 25 cm² tissue culture flasks in a 37 °C/5% CO₂ incubator prior to harvest. 200 μ l of each type of cell suspension (at 20,000 cells/cm²) was seeded onto separate 96-well plates to yield an approximate 60% confluence per well. Cells were allowed to adhere to the plate surface overnight, followed by addition of 20 ng/ml of TNF- α to stimulate production of inflammatory agents. After a 4–6 h incubation period, MMI-0100 peptide drug was added and cells were incubated for another 20–24 h. Each well was then supplemented with 100 μ l of fresh medium and 20 μ l of CellTiter 96® AQ_{ueous} One Solution Reagent and incubated for an additional 1.5–2 h prior to measuring absorbance of each well at 490 nm with a SoftMax-equipped plate reader.

2.4. Cell apoptosis analysis

The apoptotic effect of MMI-0100 on MLEC was measured at 24 h after MMI-0100 treatment. MLECs were removed from the tissue culture plate by trypsin, and re-suspended at 1.0 \times 10⁶/ml concentration. Apoptotic cells were detected by AlexaFluor 488 annexin V/Dead Cell Apoptosis Kit (Invitrogen) using flow cytometry sorting analysis.

2.5. ELISA for IL-6, IL-8, and MCP-1 detection

HCAECs were cultured and seeded onto a 96-well plate, using methods described in the MTS proliferation assay, above. Cells were again stimulated with 20 ng/ml of TNF- α for 6 h and then treated with MMI-0100 for approximately 24 h. Supernatants were then collected and analyzed for drug effect on inflammatory cytokines.

IL-6 (cat #: 900-K16; lot #: 0909016) and IL-8 (cat #: 900-K18; lot #: 0209018) ELISA kits (Peprotech; Rocky Hill, NJ) were used to measure levels of these cytokines from HCAEC supernatants following treatment with MMI-0100. 9 standards were prepared by following the manufacturer's protocol. 10 μ l of supernatant was diluted with 90 μ l of diluent; 3 replicates of each sample were used. Data were collected at 405 nm with correction at 650 nm on a plate reader. Each plate was monitored for 1 h with readings taken every 5 min. Concentrations of IL-6 and IL-8 in test samples were determined by extrapolating from a standard curve. Data are expressed as mean \pm SEM.

MCP-1 production from MLEC was analyzed using conditioned culture medium by Quantikine Mouse CCL/JE/MCP-1 Immunoassay (R&D Systems) following the manufacturer's instructions.

2.6. NO analysis

To measure nitric oxide production, conditioned medium from MLEC was examined at 24 h after treatment with MMI-0100. The medium was processed for the measurement of nitrite (NO $_2^-$) by a NO-specific chemiluminescence analyzer (Sievers) as previously described (Muto et al., 2011).

2.7. Human saphenous vein

Following approval by Vanderbilt Medical Center's Institutional Review Board, de-identified, discarded segments of human saphenous vein (HSV) were collected from consented patients undergoing coronary artery or peripheral vascular bypass surgeries. HSV segments were stored in a saline solution until the end of the surgical procedure, at which time they were placed in cold transplant harvest buffer (100 mM potassium lactobionate, 25 mM KH $_2$ PO $_4$, 5 mM MgSO $_4$, 30 mM Raffinose, 5 mM Adenosine, 3 mM Glutathione, 1 mM Allopurinol, 50 g/l Hydroxyethyl starch, pH 7.4). The vessels were used within 24 h of harvest. Using sterile technique, HSV segments were transferred to a 60-mm Petri dish under a sterile hood. The edges (0.5 mm) of each segment were removed with a blade and excess adventitial tissue and fat removed with minimal manipulation. HSV segments were cut into consecutive rings of approximately 1.0 mm in width to be utilized for organ culture or muscle bath experiments. Two rings from each segment were immediately fixed in 10% formalin at 37 $^{\circ}$ C for 30 min to obtain pre-culture intimal thickening measurements.

2.8. Effect of MMI-0100 on smooth muscle physiology

In preparation for testing vein segment functional viability, HSV rings were weighed and their lengths recorded. To focus on smooth muscle responses, the endothelium was mechanically denuded by rolling the luminal surface of each ring at the tip of a fine vascular forceps before suspension in a muscle bath containing a bicarbonate buffer (120 mM NaCl, 4.7 mM KCl, 1.0 mM MgSO $_4$, 1.0 mM NaH $_2$ PO $_4$, 10 mM glucose, 1.5 mM CaCl $_2$, and 25 mM Na $_2$ HCO $_3$, pH 7.4) equilibrated with 95% O $_2$ and 5% CO $_2$ at 37 $^{\circ}$ C. The rings were stretched and the length progressively adjusted until maximal tension was obtained. Normalized reactivity was obtained by determining the passive length–tension relationship for each vessel segment. Rings were maintained at a resting tension of 1 g, which produces maximal responses to contractile agonists as previously determined, and equilibrated for 2 h in buffer. Force measurements were obtained using a Radnoti Glass

Technology (Monrovia, CA) force transducer (159901A) interfaced with a Powerlab data acquisition system and Chart software (AD Instruments, Colorado Springs, CO).

HSV rings were first contracted with 110 mM KCl (with equimolar replacement of NaCl in bicarbonate buffer) and the force generated was measured. 110 mM KCl causes membrane depolarization, leading to contraction of vessels containing functionally viable smooth muscle. After multiple KCl challenges, rings were washed and allowed to equilibrate in bicarbonate solution for 30 min, and then contracted with phenylephrine (PE, 10 $^{-7}$ –10 $^{-6}$ M). Rings were relaxed with a cumulative log dose of sodium nitroprusside (SNP), a nitric oxide donor, and force generated was recorded. All rings were again washed and equilibrated in buffer for 15 min. Rings were then incubated with either buffer alone or buffer plus 100 μ M MMI-0100 for 2 h, followed by treatment with the same doses of PE and SNP, and the forces generated again recorded. Measured force was normalized for ring weight and length and percent relaxation was calculated; force generated with 10 $^{-6}$ M PE was set as 0% relaxation.

2.9. Organ culture

After viability was determined in the muscle bath, additional rings were cut and placed in 8-well chamber slides and maintained in RPMI 1640 medium supplemented with 30% FBS, 1% L-glutamine and 1% penicillin/streptomycin for 14 days at 37 $^{\circ}$ C in an atmosphere of 5% CO $_2$ in air. The rings were either untreated or treated with MMI-0100 peptide (10, 50 or 100 μ M). The culture medium with treatments was replaced every 2–3 days.

2.10. Vessel morphometry

After 14 days of organ culture, vein segments were fixed in 0.5 ml of 10% formalin at 37 $^{\circ}$ C for 30 min and embedded in paraffin for sectioning. Beginning at the midportion of each ring, 5 transverse sections, spaced 5 μ m apart, were cut for each specimen. Sections were then stained with Verhoeff–van Gieson stain. Each section was examined using light microscopy (Carl Zeiss, Thornwood, NY) and 6 radially parallel measurements of intimal and medial thickness were randomly taken from each section (total of 6–12 measurements per ring). Intima was defined as tissue on the luminal side of the internal elastic lamina or the chaotic organization of the cells contained within it, whereas the medial layer was contained between the intimal layer and the external elastic lamina. Intimal and medial thickening was measured for each section at 5 \times magnification with the microscope's computerized image analysis system.

2.11. Mouse vein graft model

All procedures, protocols, and medications were approved by the Institutional Animal Care and Use Committee and were performed and administered within NIH and ethical guidelines. 12-week-old C57Bl/6 wild type mice (Harlan) were used for all experiments, as previously described (Muto et al., 2011). To obtain veins, an approximately 2.0 mm segment of the intrathoracic inferior vena cava was isolated and excised. Prior to implantation, the vein was treated *ex vivo* with 100 μ M MMI-0100 peptide solution, or control PBS solution, for 20 min at room temperature.

To implant the vein graft, a midline incision was made in the abdomen of a recipient mouse and the infrarenal abdominal aorta was exposed. The abdominal aorta was temporarily occluded with atraumatic micro-clamps and a segment corresponding to the length of the vein graft was excised. The vein was sutured into the arterial circulation using 10-0 nylon in continuous fashion.

Vein grafts were followed postoperatively using the Vevo770 High-Resolution Imaging System (VisualSonics, Toronto, Canada), with weekly measurements of graft wall thickness. At 28 days after

surgery, mice were sacrificed to allow explantation of the vein graft. Tissue was either frozen with RNA stabilization reagent (Qiagen) or explanted for paraffin embedding after circulatory flushing with ice-cold PBS followed by 4% paraformaldehyde perfusion–fixation. Vein graft wall thickness, lumen diameter, and outer wall diameter (elastic lamina) were measured in elastin-stained sections using computer morphometry (ImageJ).

2.12. Histology and immunohistochemistry

Vein graft samples were fixed as noted above and harvested for histology. Specimens were embedded in paraffin and cut in cross section (5 μm). Hematoxylin & Eosin, Masson trichrome, and van Gieson elastin staining were performed for all samples. Cells were cultured on gelatin-coated cover slips and fixed with methanol.

All sections analyzed with immunohistochemistry were first treated for antigen retrieval using 10 mmol/l citrate buffer (pH 6.0) prior to boiling or proteinase K (20 $\mu\text{g}/\text{ml}$) treatment, at room temperature, for 10–min. Immunohistochemical detection was performed using a primary antibody to F4/80 (AbD Serotec) according to the manufacturer's instructions, and then secondary detection was performed using DAB as well as NovaRED substrate (Vector). Sections were counterstained with Mayer's Hematoxylin. Images were captured with an Axioimager A1 (Carl Zeiss) and density was analyzed by Image J (NIH).

2.13. Statistics

Statistical analysis was performed with one-way ANOVA followed by Tukey test to compare experimental groups. Analyses were done with OriginPro 8 software (Originlab, Northampton, MA) or GraphPad software (La Jolla, CA). Statistical significance was accepted within a 95% confidence limit. Results are presented as arithmetic mean \pm SEM graphically.

3. Results

3.1. MMI-0100 induces minimal cell proliferation

To determine the effect of MMI-0100 on human endothelial cell (EC) and smooth muscle cell (SMC) proliferation under stress conditions, such as what occurs during surgical vein graft harvest and handling, human EC and SMC cultures were treated with three concentrations of MMI-0100 (0.25 mM, 0.5 mM, and 1 mM) following pre-treatment with TNF- α , a cytokine that stimulates cellular inflammation and stress as well as activates MK2. Both 0.25 mM and 0.5 mM concentrations of MMI-0100 slightly increased cell proliferation in both cell types compared to control cells treated with 20 ng/ml TNF- α alone (maximal with 0.5 mM: 30% and 12% increases in EC and SMC cultures, respectively; Fig. 1). However, while the 1 mM MMI-0100 treatment also increased both EC (11%) and SMC (7%) proliferation as compared to control, this response was not as robust as that induced by treatment with 0.5 mM MMI-0100 (Fig. 1). Phase contrast images of EC and SMC treated with MMI-0100 for 24 h showed no obvious morphological changes as compared to control cells (Fig. 1C). These data suggest that MMI-0100 has no major negative effects on vascular cell proliferation or morphology during stress conditions.

3.2. MMI-0100 reduces Interleukin-6 expression in endothelial cells

Since MMI-0100 has no effects on TNF- α -stimulated proliferation, we investigated the anti-inflammatory effect of MMI-0100 by assaying expression of Interleukin 6 (IL-6) and Interleukin 8 (IL-8) secreted by human coronary endothelial cells (HCAEC) following TNF- α stimulation. HCAECs were seeded on a multi-well plate at a density of approximately 25,000 cells/cm². After a 6 h incubation with TNF- α , which activates MK2

and stimulates IL-6 production, MMI-0100 (0.5 mM) was added to the culture medium. After 24 h of drug treatment, supernatant from each well was collected and assayed for cytokine expression. MMI-0100 treatment reduced the level of TNF- α -induced IL-6 expression to that of the untreated control (Fig. 2A). However, since IL-8 is not under the control of MK2, its expression levels should not be affected by addition of an MK2 inhibitor (Coxon et al., 2003); consistent with this expectation, MMI-0100 had no effect on the level of TNF- α -induced IL-8 expression (Fig. 2B). These data suggest specificity of MMI-0100 on suppressing TNF- α -induced IL-6 production.

3.3. MMI-0100 enhances human saphenous vein relaxation

To examine the direct role of MMI-0100 on smooth muscle relaxation, human saphenous vein (HSV) rings were pre-treated with buffer or MMI-0100 (100 μM) and then rings were contracted with phenylephrine (10⁻⁶ M) and relaxed with sodium nitroprusside (10⁻⁸ and 10⁻⁷ M; SNP). Pretreatment of HSV rings with MMI-0100 led to a significant increase in relaxation (25.622 \pm 6.38 and 92.54 \pm 3.09 for 10⁻⁸ and 10⁻⁷ M SNP, respectively) when compared to untreated control (10.825 \pm 5.62 and 72.768 \pm 6.99 for 10⁻⁸ and 10⁻⁷ M SNP, respectively) (Fig. 3). There was no significant difference in relaxation response when HSV rings were pretreated with the control peptide (transduction domain, PTD peptide, 50 or 100 μM) when compared to the untreated control (data not shown). In addition, MMI-0100 did not induce relaxation in the absence of SNP, with no reduction of basal tension and no reduction of phenylephrine-induced force (data not shown).

3.4. MMI-0100 reduces intimal hyperplasia in a human saphenous vein organ culture model

To examine the effect of MMI-0100 on development of intimal hyperplasia, we measured intimal thickness of HSV in a human organ culture model in the presence of high serum and different concentrations (10–100 μM) of MMI-0100. HSVs were cultured for 14 days in 30% serum. All veins were deemed viable at the time of culture by adequate contraction with a phenylephrine challenge in a muscle bath. The average intimal thickness of pre-cultured vein segments was 43.7 \pm 7.8 μm . After culture, the average intimal thickness of the control was 81.6 \pm 17.3 μm . The average intimal thickness in the presence of 50 μM and 100 μM MMI-0100 was 42.7 \pm 6.0 μm and 50.4 \pm 10.7 μm , respectively, with a significant reduction in intimal thickness (Figs. 4A and 5). Measurement of the intima:media (I:M) ratio showed a greater reduction of the I:M ratio at the 100 μM concentration of MMI-0100 (Fig. 4B).

3.5. MMI-0100 inhibits intimal hyperplasia in a mouse vein graft model

To confirm the inhibitory effects of MMI-0100 on intimal hyperplasia development in an ex vivo model, we examined the role of MMI-0100 in an in vivo model of intimal hyperplasia, using a mouse model of vein graft adaptation. Vein grafts were treated with PBS or MMI-0100 (100 μM) for 20 min prior to implantation and then followed weekly with ultrasound. Weekly ultrasound examination of the vein graft wall thickness showed diminished wall thickness at all postoperative time points in vein grafts treated with MMI-0100, with a ratio of 2.6-fold thicker at 4 weeks, compared to 4.7-fold thicker at 4 weeks in control grafts (Fig. 6A, B). Histological staining of the grafts confirmed 72% reduced wall thickness with MMI-0100 treatment compared to control grafts, as seen in vivo with ultrasound (Fig. 6C, D). Examination of the grafts for F4/80 immunohistochemical reactivity demonstrated fewer F4/80-positive cells infiltrating into vein grafts treated with MMI-0100, consistent with fewer infiltrating macrophages in grafts treated with MMI-0100 (Fig. 6E, F).

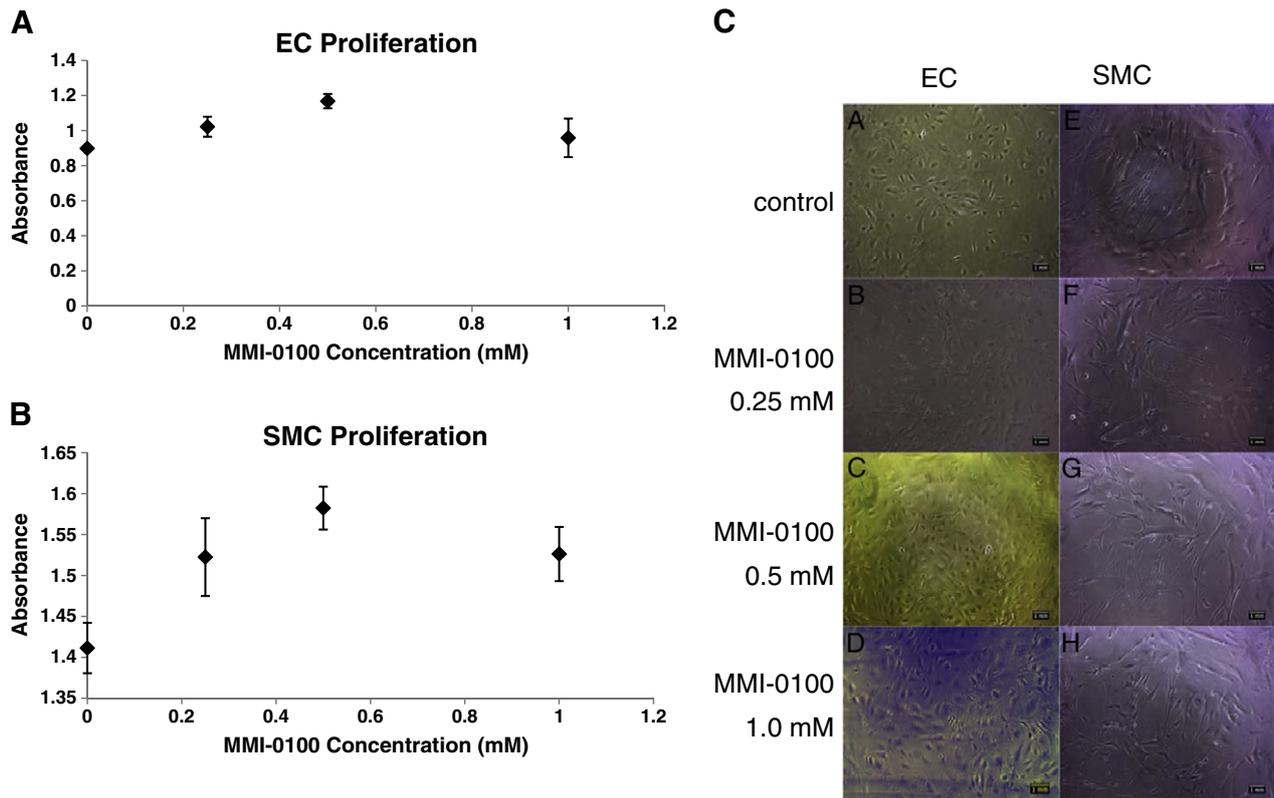


Fig. 1. A, B) Effect of three concentrations of MMI-0100 on HAEC (A) and HASMC (B) proliferation. C) Microscopic images of ECs (A–D) and SMCs (E–H) before and after 24 h of MMI-0100 treatments. From top to bottom, the concentrations of MMI-0100 are (A, E) no treatment, (B, F) 0.25 mM, (C, G) 0.5 mM, and (D, H) 1 mM.

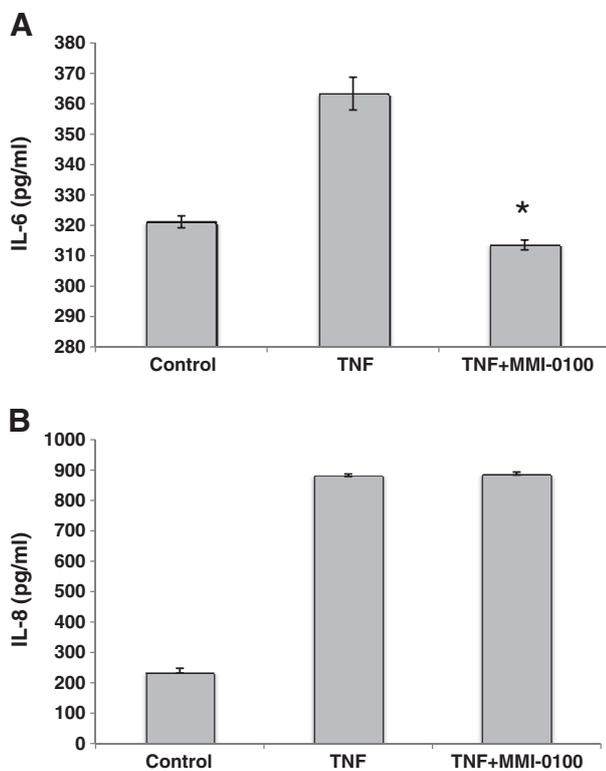


Fig. 2. Comparison of IL-6 (A) and IL-8 (B) levels secreted by endothelial cells, without and with MMI-0100. IL6 and IL-8 were measured in pg/ml. Data shown as mean \pm SEM from 3 replicates. * $p < 0.05$ compared to the TNF group.

Although MMI-0100 induces minimal proliferation of human EC and SMC (Fig. 1), we confirmed the effect using physiological doses of MMI-0100 on murine EC. Murine ECs were positive for Eph-B4, the marker of venous identity (Fig. 7A). MMI-0100 did not induce significant murine EC proliferation at physiological doses (Fig. 7B). Similarly, MMI-0100 did not induce EC apoptosis at any dose (Fig. 7C). MMI-0100 did not stimulate MCP-1 production, even at high doses (Fig. 7D), consistent with reduced number of macrophages in vein grafts treated with MMI-0100 (Fig. 6E, F). Interestingly, nitric oxide

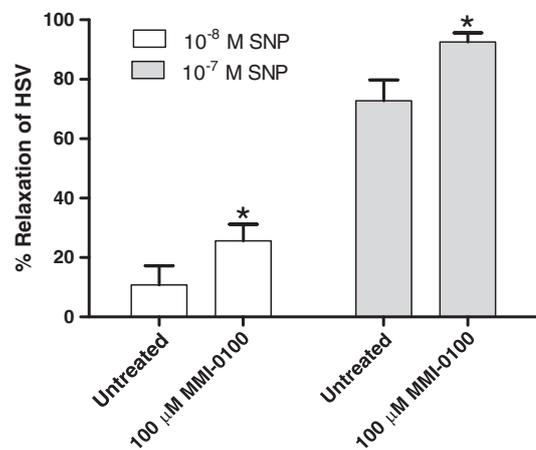


Fig. 3. MMI-0100 peptide enhances sodium nitroprusside (SNP)-induced relaxation of human saphenous vein (HSV). * $p < 0.05$ compared to untreated, $n = 4$.

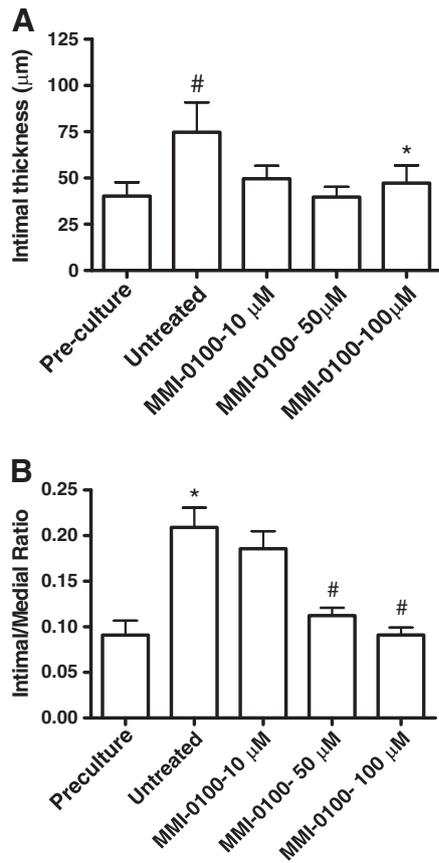


Fig. 4. Effect of MMI-0100 peptide treatment on (A) intimal layer thickening, and (B) intimal-to-medial ratio, in human saphenous vein (HSV). A) # $p < 0.05$ compared to pre-culture. * $p < 0.05$ compared to untreated, $n = 4-5$. A representative graph is shown. B) * $p < 0.05$ compared to pre-culture. # $p < 0.05$ compared to untreated. $n = 5$. Graph shows the cumulative data.

(NO) production was not suppressed, and was even enhanced at physiological doses of MMI-0100 (Fig. 7E), suggesting perhaps an additional mechanism of action on endothelial cells.

4. Discussion

Recent successes demonstrating that suppression of monocytes prior to vascular injury inhibits intimal hyperplasia (Danenberg et al., 2002; Epstein et al., 2008) led us to test the efficacy of a potent anti-inflammatory compound, MMI-0100, in inhibiting development of intimal hyperplasia. Additional motivation for these studies came from our previous work demonstrating that MMI-0100 suppressed inflammatory cytokine production in human plural mesothelial cells after stimulation with IL-1 β or TNF α and also suppressed surgically induced adhesions following bowel anastomosis procedures in rats (Ward et al., 2011). Together, these data suggest that MMI-0100 inhibits fibrosis as well as inflammation and may also effectively inhibit intimal hyperplasia in conjunction with vascular graft surgeries.

In the current study, consistent with studies in human mesothelial cells, pharmacological MMI-0100 treatment of vascular cells induced minimal effects on cell proliferation or morphology and reduced TNF- α -induced IL-6, but not IL-8, secretion in cultured human vascular cells. Similarly, physiological doses of MMI-0100 did not significantly stimulate proliferation or apoptosis, or suppress NO production, in murine EC. These studies also demonstrate enhanced saphenous vein relaxation and reduced intimal hyperplasia in human saphenous vein rings ex vivo, as well as reduced vein graft intimal hyperplasia in an in vivo mouse model. Taken together, these results show that MMI-0100 prevents vein graft intimal thickening, possibly via reduced inflammatory processes in response to surgical vein graft harvest and during subsequent vein graft adaptation.

A single ex vivo exposure of the vein graft to MMI-0100 at the time of surgery inhibits intimal hyperplasia development in an animal vein graft model for several weeks post-surgery (Fig. 6). Since these effects on vein graft adaptation occur over an extended period of time, it is likely that MMI-0100 induces alterations in gene transcription. We have shown previously that MMI-0100 suppressed heterogeneous nuclear ribonucleoprotein A0 (hnRNP A0) phosphorylation (Ward et al., 2011). Rousseau et al. showed that hnRNP A0 is phosphorylated by MK2 and its phosphorylated form is released from the AU-rich 3' untranslated region of IL-6 mRNA to stimulate protein expression (Rousseau et al., 2002). MK2 is also known to phosphorylate tristetraprolin (TTP), another transcription factor that regulates TNF α and COX2 production (Mahtani et al., 2001; Streicher et al., 2010). Thus, inhibition of MK2 will down regulate inflammatory cytokine production that can lead to

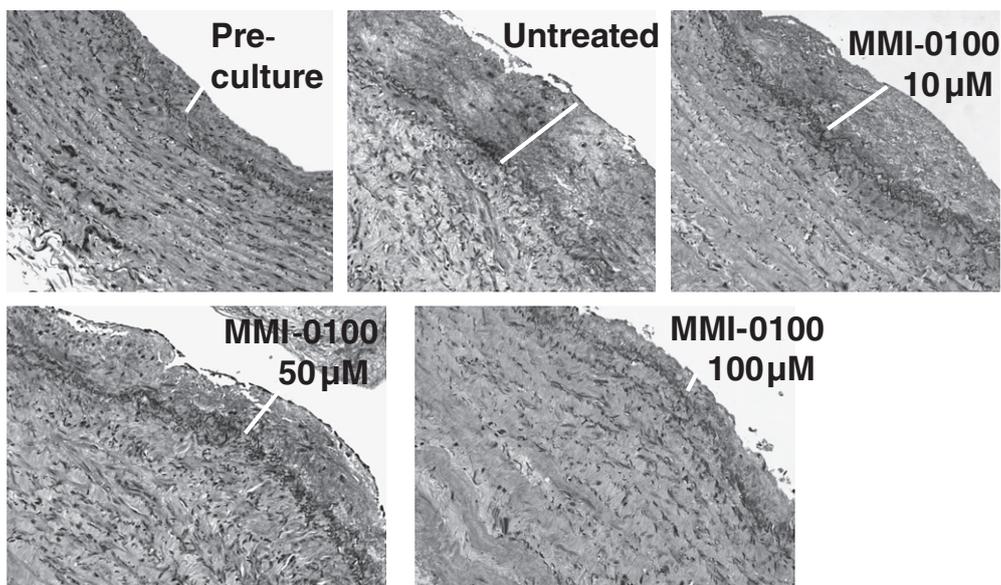


Fig. 5. Effect of MMI-0100 peptide treatment on intimal layer thickening in human saphenous vein. Representative sections for each condition are shown at 20 \times magnification. White bar indicates intimal thickening.

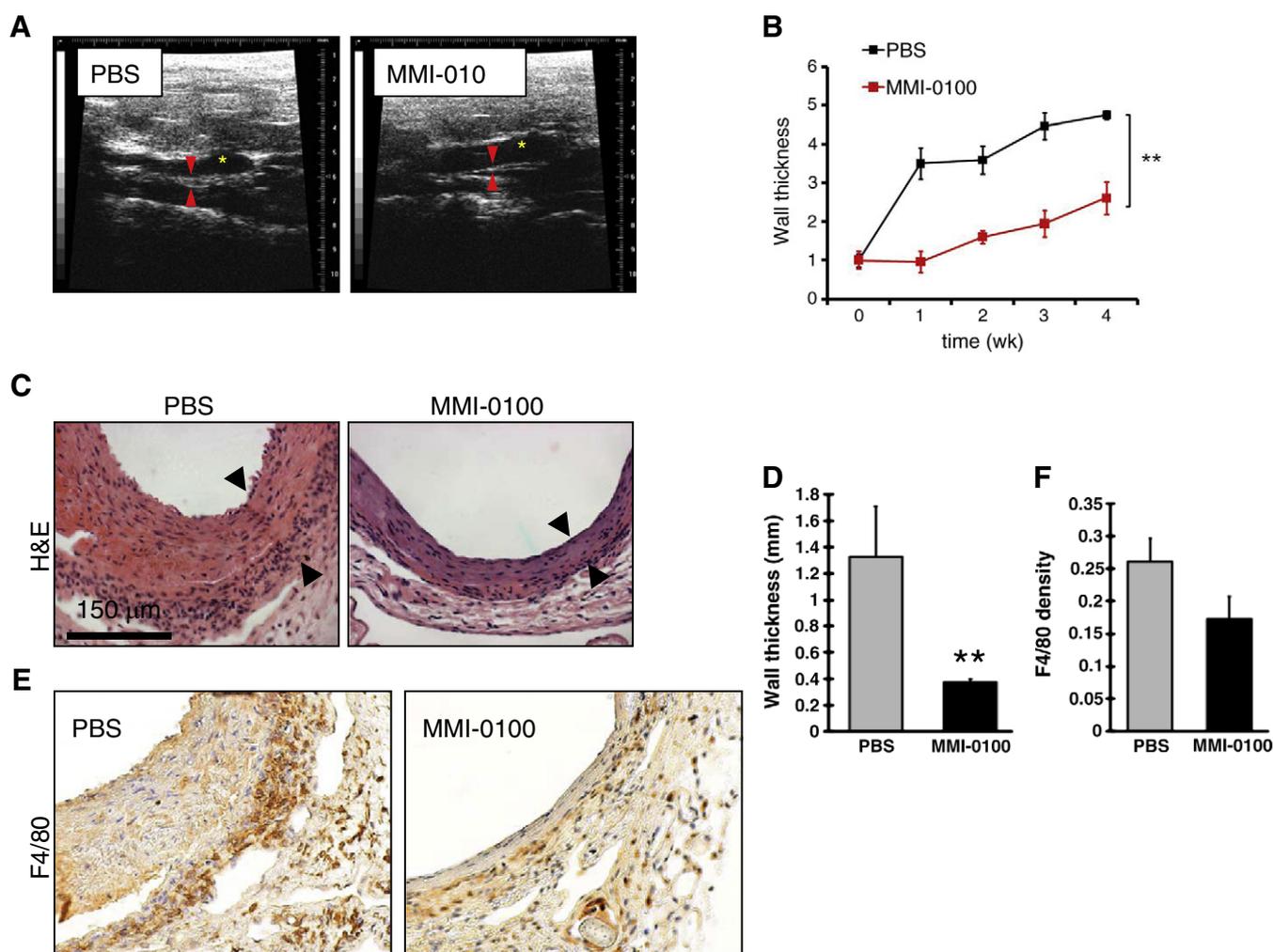


Fig. 6. Effect of MMI-0100 on vein graft adaptation. A) Mouse vein grafts were followed weekly with ultrasound; representative figures are shown. Yellow star shows the lumen of the graft; red arrows show the vein graft wall. B) Graph shows summary of ultrasound measurements of vein graft wall thickness, plotting the ratio of the wall thickness compared to the wall thickness at time of implantation. $**p < 0.0001$ at 4 weeks. C) Photomicrographs show representative portions of vein grafts treated with PBS or MMI-0100. Arrowheads show the intima-media layer. D) Bar graph shows summary of histological measurements of vein graft wall thickness. $n = 5$ or 6 at each time point. $**p = 0.016$. E) Mouse vein grafts were examined for F4/80 immunohistochemical reactivity; red-brown staining shows immunoreactive cells. F) Bar graph shows summary of F4/80 density showing reduced macrophages in the vein graft wall with MMI-0100 treatment.

both inflammation and intimal hyperplasia development. In addition to MK2 being required for cytokine production (Kotlyarov et al., 1999; Kotlyarov et al., 2002) as well as cyclooxygenase (COX)-2 protein synthesis (Streicher et al., 2010), MK2 has also been suggested to alter stability of α -actin mRNA (Sousa et al., 2007) and to modulate myofibroblast phenotype (Hagood and Olman, 2007). Thus, there are multiple mechanisms by which alteration of MK2 function might impact fibrotic processes such as vein graft intimal hyperplasia.

We have previously shown that inhibition of MK2 with a non-specific cell-permeable peptide inhibits heat shock protein 27 (HSP27) phosphorylation, TGF- β 1-induced intracellular HSP27 phosphorylation, as well as TGF- β 1-induced expression of connective tissue growth factor and collagen type I (Lopes et al., 2009). These results show that inhibition of MK2 may affect fibrotic cellular responses and are consistent with our previous study with the more specific MK2 inhibitor peptide, MMI-0100, showing reduced adhesion formation in a rat bowel anastomosis model (Kavalukas et al., 2009; Ward et al., 2011). These results are also consistent with reduced cellular turnover as well as effects on the TGF- β 1 pathway, both of which are associated with vein graft neointimal hyperplasia. Since TGF- β 1 can stimulate HSP27 phosphorylation, it is quite possible that the reduced intimal hyperplasia seen in vein grafts treated with MMI-0100 is associated with modulation of the TGF- β 1-HSP27

pathway. Inhibition of MK2 may also alter other downstream pathways that affect vein graft neointimal hyperplasia. For example, Nogo-B is phosphorylated at Serine-107 by MK2 or MK3, but not by other kinases that are activated by p38 (Rousseau et al., 2005). Although the function of Nogo-B is not currently understood, Nogo-B has a positive effect on vascular injury-induced remodeling and reduced neointimal development in both arterial and venous models of vascular injury (Kritz et al., 2008). Therefore MMI-0100 may alter Nogo-B function indirectly through downstream effects; however, exactly how phosphorylation of Nogo-B affects its function, or development of intimal hyperplasia, is not clear.

Although basic cell penetrating peptides may lead to nonspecific kinase inhibition or increased toxicity, we have previously shown that several novel domains lead to increased specificity; in particular, domains based on the antithrombin III-heparin binding domain lead to increased specificity of MK2 inhibition (Ward et al., 2009) compared to another, less-specific MK2 peptide inhibitor (Lopes et al., 2009). MMI-0100 is a relatively specific inhibitor of MK2, with preserved mitogen-activated protein kinase-activated protein kinase 5, p38, Protein kinase B beta, Protein kinase C delta, and Rho-associated coiled-coil containing protein kinase 1 activity at concentrations of MMI-0100 that completely inhibit MK2 activity (Ward et al., 2009). However, MMI-0100 can also inhibit calcium/

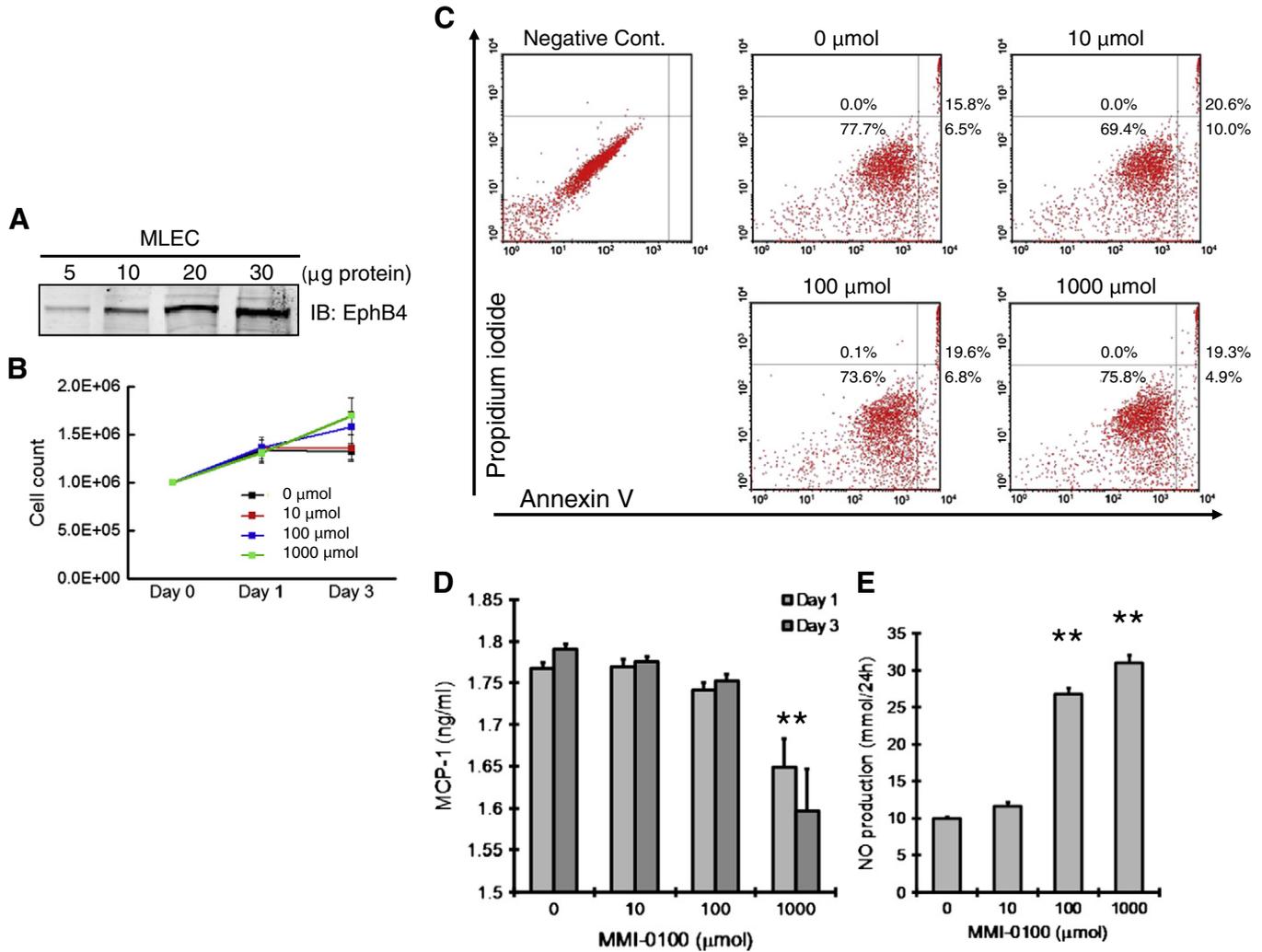


Fig. 7. Effect of MMI-0100 on murine EC. A) Murine EC analyzed for Eph-B4, by Western blot. Representative of n = 4 experiments. B) Graph shows mean of cell counts after treatment with MMI-0100. C) Representative analysis of MMI-0100 effects on EC apoptosis. n = 3 experiments. D) Bar graph shows MCP-1 production by murine EC after MMI-0100 treatment. * p < 0.0001. E) Bar graph shows NO production by murine EC after MMI-0100 treatment. ** p < 0.0001.

calmodulin-dependent protein kinase I (CaMKI) as well as Trk-B (Ward et al., 2009), both of which can alter smooth muscle function (Passier et al., 2000; Donovan et al., 1995), suggesting the possibility of selective gene expression mediating potential other effects of MMI-0100 (Wamhoff et al., 2006). However, it is likely that these other effects on smooth muscle cells would induce smooth muscle cell relaxation, augmenting MMI-0100 function. Furthermore, other peptide inhibitors of MK2 have similar inhibition of CaMKI, MK3, as well as other kinases, suggesting that MMI-0100 would have the fewest other effects of any tested MK2 peptide inhibitor (Ward et al., 2009). Therefore we believe that the inhibitory effects of MMI-0100 may be specific for fibrotic responses secondary to inflammation, such as vein graft intimal hyperplasia, and are likely to have few side effects if given clinically, particularly when locally-delivered compared to systemically-delivered.

We show, using the novel cell-permeant peptide inhibitor MMI-0100, that inhibition of MK2 inhibits intimal thickening in both ex vivo and in vivo models of intimal hyperplasia. Although there are several mechanisms by which MMI-0100 may inhibit intimal thickening, the sustained in vivo effects from a single ex vivo graft treatment at the time of graft surgery suggest clinical utility, especially in vein graft disease that is amenable to ex vivo treatment. As such, MMI-0100 may represent a novel strategy to inhibit fibrotic processes such as vein graft disease.

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