0022-3565/09/3293-959–966$20.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
JPET 329:959–966, 2009

Vol. 329, No. 3
150367/3472054
Printed in U.S.A.

The Journal of PHARMACOLOGY And Experimental Therapeutics

Inhibition of Vascular Smooth-Muscle Cell Proliferation and Arterial Restenosis by HO-3867, a Novel Synthetic Curcuminoid, through Up-Regulation of PTEN Expression

Karuppaiyah Selvendiran, M. Lakshmi Kuppusamy, Anna Bratasz, Liyue Tong, Brian K. Rivera, Cameron Rink, Chandan K. Sen, Tamás Kálai, Kálmán Hideg, and Periannan Kuppusamy

Davis Heart and Lung Research Institute, Division of Cardiovascular Medicine, Departments of Internal Medicine (K.S., M.L.K., A.B., L.T., B.K.R., P.K.) and Surgery (C.R., C.K.S.), Ohio State University, Columbus, Ohio; and Institute of Organic and Medicinal Chemistry (T.K., K.H.), University Pécs, Pécs, Hungary

Received December 26, 2008; accepted March 9, 2009

ABSTRACT

Phosphatase and tensin homolog (PTEN), a tumor suppressor gene, has been shown to play a vital role in vascular smooth muscle cell (SMC) proliferation and hence is a potential therapeutic target to inhibit vascular remodeling. The goal of this study was to evaluate the efficacy and mechanism of HO-3867 [((3*E*,5*E*)-3,5-bis[(4-fluorophenyl)methylidene]-1-[(1-hydroxy-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-yl)methyl]piperidin-4-one)], a new synthetic curcuminoid, in the inhibition of vascular SMC proliferation and restenosis. Experiments were performed using human aortic SMCs and a rat carotid artery balloon injury model. HO-3867 (10 μM) significantly inhibited the proliferation of serum-stimulated SMCs by inducing cell cycle arrest at the G_1 phase (72% at 24 h) and apoptosis (at 48 h). HO-3867 significantly increased the phosphorylated and total levels of PTEN in SMCs. Suppression of PTEN expression by PTEN-small interfering RNA transfection reduced p53 and p21 levels and increased extracellular signal-regulated kinase 1/2 phosphorylation, resulting in decreased apoptosis. Conversely, overexpression of PTEN by cDNA transfection activated caspase-3 and increased apoptosis. Furthermore, HO-3867 significantly down-regulated matrix metalloproteinase (MMP)-2, MMP-9, and nuclear factor (NF)-κB expressions in SMCs. Finally, HO-3867 inhibited arterial neointimal hyperplasia through overexpression of PTEN and down-regulation of MMPs and NF-κB proteins. HO-3867 is a potent drug, capable of overexpressing PTEN, which is a key target in the prevention of vascular remodeling, including restenosis.

Restenosis after angioplasty procedures is a significant clinical problem (Choy et al., 2001; Coolong and Kuntz, 2007). A dominant cellular event in the renarrowing of the vascular lumen after angioplasty is smooth muscle cell (SMC) proliferation and migration. After vascular injury, the SMCs start to proliferate and then migrate into the developing neointima, thus becoming the major cellular substrate of the restenotic tissue. The rough-surfaced endoplasmic reticulum in SMCs can express a large number of growth-regulatory molecules and extracellular matrix components, which are involved in the regulation of cell proliferation and migration (Jackson and Schwartz, 1992). The control of SMC proliferation by activation of tumor suppressor genes such as p53, phosphatase and tensin homolog (PTEN), and blocking the genes involved in cell cycle progression has been shown to be useful in many clinical applications (Chen et al., 2004; Kim et al., 2004; Koide et al., 2007; Wassmann et al., 2007).

PTEN plays a vital role in cells of the cardiovascular system (Oudit et al., 2004). PTEN regulates cardiac myocyte hypertrophy and survival (Schwartzbauer and Robbins, 2001; Oudit et al., 2004; McKinsey and Kass, 2007). Overexpression of PTEN has been shown to inhibit growth factor-induced proliferation, migration, and survival of vascular SMCs (Huang and Kontos, 2002; Moon et al., 2004; Huang et

The synthesis of HO-3867 was supported by the Hungarian Research Fund [Grant OTKA T048334].
Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
doi:10.1124/jpet.108.150367.

ABBREVIATIONS: SMC, smooth muscle cell; PTEN, phosphatase and tensin homolog deleted on chromosome 10; HO-3867, (3*E*,5*E*)-3,5-bis[(4-fluorophenyl)methylidene]-1-[(1-hydroxy-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-yl)methyl]piperidin-4-one; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; ERK, extracellular signal-regulated kinase; p, phosphorylated; NF, nuclear factor; siRNA, small interfering RNA; MMP, matrix metalloproteinase; FAK, focal adhesion kinase; PCR, polymerase chain reaction; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FBS, fetal bovine serum; DAPI, 4,6-diamidino-2-phenylindole; ATF, activating transcription factor; BI, balloon injury.

al., 2005). The inhibition of SMC proliferation by activation of PTEN has attracted much attention as a pharmacologic target and may prove to be an attractive therapeutic strategy for treating neointimal hyperplasia (Chen et al., 2004; Garl et al., 2004; McKinsey and Kass, 2007). We identified a new class of diarylidenyl piperidones, capable of activating PTEN leading to inhibition of cancer cell proliferation (Selvendiran et al., 2007). The activation of PTEN was principally due to inhibition of proteasomal degradation of PTEN. In the present study, we hypothesized that PTEN would be suppressed after vascular injury and that compounds capable of activating PTEN expression would have an inhibitory effect on neointimal formation. In the present work, we have tested the effect of HO-3867 (Fig. 1A), a member of the diarylidenyl piperidone class, on SMC proliferation (Selvendiran et al., 2008b). We observed that HO-3867 inhibited SMC proliferation and neointima formation in a rat carotid artery injury model through up-regulation of PTEN expression. The results suggested that up-regulation of PTEN expression by HO-3867, or similar compounds, could be a potential approach for the treatment of SMC proliferation-related diseases, such as postangioplasty restenosis.

Materials and Methods

Reagents. Dimethyl sulfoxide (DMSO) and antibodies directed against actin and FLAG were obtained from Sigma-Aldrich (St. Louis, MO). Clonetics smooth muscle cell basal medium, fetal bovine serum, antibiotics, sodium pyruvate, trypsin, and phosphate-buffered saline (PBS) were obtained from Invitrogen (Carlsbad, CA). Polyvinylidene fluoride membrane and molecular weight markers were obtained from Bio-Rad (Hercules, CA). Antibodies directed against Akt, pAkt (Ser473), ERK1/2, phosphorylated (p) ERK1/2, PTEN, pPTEN (Ser380 and Thr381/382), NF-κB, cleaved caspase-3, and PTEN siRNA kit were purchased from Cell Signaling Technology Inc. (Danvers, MA). PTEN plasmid (plasmid 10786; 1437 pSG5L Flag HA PTEN) was obtained from Addgene Inc. (Cambridge, MA). Antibodies directed against cyclin D1, p53, p21, p27, matrix metalloproteinase (MMP)-2, MMP-9, and focal adhesion kinase (FAK)

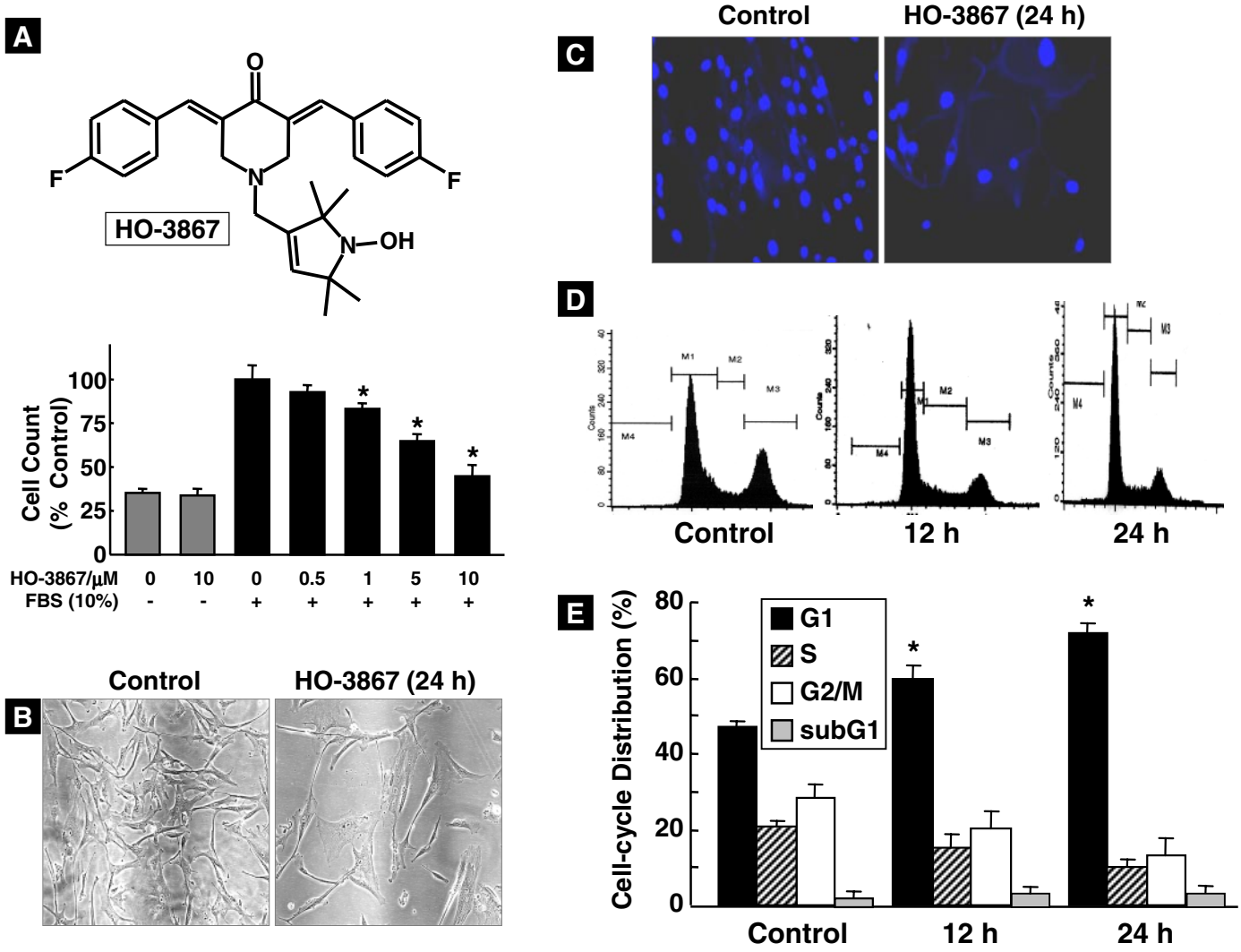

Fig. 1. Effect of HO-3867 on cell survival, cell cycle arrest, and apoptosis in human aortic SMCs. A, effect of HO-3867 on the survival of serum (FBS)-stimulated SMCs. *, $p < 0.05$ versus untreated cells. B, phase-contrast microscopy images (100×) of SMCs incubated with vehicle only (Control) or with 10 μM HO-3867 for 24 h. C, fluorescence microscopy images (200×) of DAPI-stained (blue) SMCs treated with vehicle (Control) or with 10 μM HO-3867 for 24 h. D, representative (of $n = 6$) flow cytometric data showing cell cycle (DNA content) distribution in SMCs incubated with vehicle or with 10 μM HO-3867 for 12 or 24 h. E, cell cycle (DNA content) distribution (percentage of total) in the treatment groups shown in D. Data represent mean± S.E.M. ($n = 6$). *, $p < 0.05$ versus control.

were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). RNase was obtained from Promega (Madison, WI). Enhanced chemiluminescence reagents were obtained from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). HO-3867 was synthesized in our laboratories. The synthesis of HO-3867 and related compounds will be published separately. HO-3867 was prepared in 0.5% DMSO for in vitro studies.

Cell Culture. Human aortic SMCs were obtained at passage 3 (AoSMC; Lonza Walkersville, Inc., Walkersville, MD). The cells were maintained in culture in a humidified incubator at 37°C and 5% CO_2 in smooth muscle cell growth medium (SmGM-2). Cells were trypsinized and passaged at 95% confluence. Studies were performed using cells at passages 4 to 6 at 60 to 90% confluence. Cells were counted using an automated cell counter (NucleoCounter; New Brunswick Scientific, Edison, NJ).

Cell Cycle Analysis. Flow cytometry was performed as reported previously (Selvendiran et al., 2008a).

Western Blotting. Western blotting was performed as reported previously (Selvendiran et al., 2007).

Gelatin Zymography. MMP-2 and MMP-9 was determined by gelatin zymography as reported previously (Cho and Reidy, 2002).

Immunofluorescence. SMCs, grown on Lab-Tek Chamber slides (Nalge Nunc International, Rochester, NY), were fixed with 4% paraformaldehyde for 10 min at room temperature and then washed in PBS containing 0.05% Tween 20. Nonspecific reactions were blocked with Protein Block Serum-Free (DAKO, Kyoto, Japan) and then incubated with an anti-PTEN, MMP-2, or MMP-9 antibody at 4°C overnight. After washing in PBS containing 0.05% Tween 20, the specimens were treated with Alexa Fluor goat anti-mouse IgG (H+L) antibody (Invitrogen, Carlsbad, CA) for 45 min at room temperature and then counterstained by propidium iodide after digestion of RNA by RNase (Invitrogen). The immunostaining of PTEN, MMP-2, and MMP-9 proteins was visualized using a fluorescence microscope (Nikon, Tokyo, Japan).

PTEN siRNA and PTEN cDNA Transfection. The PTEN siRNA and cDNA transfection experiments were performed as reported (Selvendiran et al., 2007).

Reverse Transcription-PCR. Total RNA was isolated from SMCs using the RNeasy system according to the instructions of the manufacturer. RNA quantification was done using spectrophotometry. Reverse transcription (RT)-PCR analysis for the mRNA expressions in PTEN and the internal control GAPDH was carried out using a GeneAmp PCR System Veriti thermo cycler (Applied Biosystems, Foster City, CA) under the following conditions: initial denaturation at 94°C for 2 min, 35 cycles of amplification (denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 30 s), and extension at 72°C for 5 min. The sequences (5′-3′) for the primer pairs of PTEN and GAPDH, respectively, were as follows: PTEN, GCCATCATCAAAGAGATCGT (forward) and GGATCAGAGTCAGTGG (reverse); and GAPDH, GTCAACGGATTTGGTCGTATT (forward) and AGTCTTCTGGGTGGCAGTGAT (reverse). The PCR products were electrophoresed on 1.5% agarose gel and stained with ethidium bromide.

Carotid Artery Balloon Injury. All experiments involving animals were performed in accordance with the relevant guidelines and regulations approved by the Internal Animal Care and Use Committee of The Ohio State University. Sprague-Dawley rats weighing 300 to 350 g were anesthetized by an intraperitoneal injection of ketamine (60 mg/kg) and xylazine (5 mg/kg). Under a stereomicroscope, the right common, external, and internal carotid arteries were exposed by a longitudinal midline cervical incision. Blood flow was temporarily interrupted by ligation of the common and internal carotid arteries using vessel clips. The external carotid artery was ligated permanently using 6-0 silk suture. A 2F Fogarty arterial embolectomy catheter (Edwards Lifesciences, Irvine, CA) was introduced through an arteriotomy in the external carotid artery just below the ligature and advanced to the common carotid artery. To produce the injury, the balloon was inflated with saline and passed six times with rotation from just under the proximal edge of the omohyoid muscle to the carotid bifurcation. The balloon was then deflated, and the catheter was withdrawn. Blood flow was restored by removing the clips on the common and internal carotid arteries. After visual inspection to ascertain adequate pulsation of the common carotid artery, the surgical incision was closed, and the rats were allowed to recover from anesthesia in a humidified and warmed chamber for 2 to 4 h. Immediately after completion of the carotid injury procedure, subcutaneous administration of HO-3867 (in 10% DMSO + 40% polyethylene glycol + 50% PBS) commenced at a dose of 5 or 10 mg/kg/day, and continued for 2 weeks ($n = 5$ for each group). The control group received the vehicle only. After treatment for 14 days, the animals were sacrificed with an overdose of pentobarbital (200 mg/kg), and the carotid arteries were collected for morphometric analysis and isolation of proteins for analysis by Western blotting. For morphometric analysis, carotid arteries were fixed in 10% formalin, dehydrated, and embedded in paraffin. Sections of 5-μm thickness were obtained at equally spaced intervals in the middle of the injured (experimental) and uninjured (control) common carotid artery segments. The samples were stained with hematoxylin and eosin. The intimal and medial areas were measured using MetaMorph software (Molecular Devices, Sunnyvale, CA), and intimal/medial ratios were calculated.

Statistical Analysis. Data were expressed as mean ± S.E.M. Comparisons among groups were performed using a Student's t test. The significance level was set at $p < 0.05$.

Results

HO-3867 Inhibits Human Aortic SMC Proliferation through G_1 Cell Cycle Arrest. We first studied the effect of HO-3867 on serum-stimulated proliferation of human aortic SMCs, in vitro (Fig. 1A). SMC proliferation was significantly increased upon treatment with 10% FBS for 24 h. The cells were then treated with 0, 0.5, 1, 5, and 10 μM HO-3867 for 24 h, after which the cells were trypsinized and counted for viability. The viable cell count decreased in a concentration-dependent manner after treatment with HO-3867. Significantly reduced cell-counts were observed at 1, 5, and 10 μM concentrations, the latter of which was used in all successive in vitro experiments. We determined the EC_{50} value to be 3.82 μM. We observed inhibition of SMC proliferation by both phase-contrast microscopy (Fig. 1B) and by nuclear staining with DAPI (Fig. 1C). Next, we determined whether the growth inhibition of SMC by HO-3867 was due to cell cycle arrest or apoptosis. SMCs were treated with 10 μM HO-3867 for 12 or 24 h. The cells were then fixed, and cell cycle populations were determined by flow cytometry. The results showed that HO-3867 induced G_1 cell cycle arrest in SMCs in a time-dependent manner (Fig. 1, D and E). There was no effect on the sub-G_1 population, indicating that HO-3867 did not induce apoptosis in SMCs at 12 or 24 h of treatment. The results suggested that HO-3867 inhibited the proliferation of human aortic SMCs through G_1 cell cycle arrest.

HO-3867 Induces Increased PTEN Expression and Activity in SMCs. The tumor suppressor gene PTEN recently has been found to play a vital role in vascular SMC proliferation and migration (Moon et al., 2004; Huang et al., 2005). Hence, we investigated whether HO-3687 can increase the PTEN expression and/or activity in SMCs in vitro. As shown in Fig. 2A, HO-3867 (10 μM) induced PTEN expression and PTEN activation (pPTEN) in cultured SMCs at as early as 1 h of treatment. This observation was further confirmed by immunofluorescence microscopy (Fig. 2B). To con-

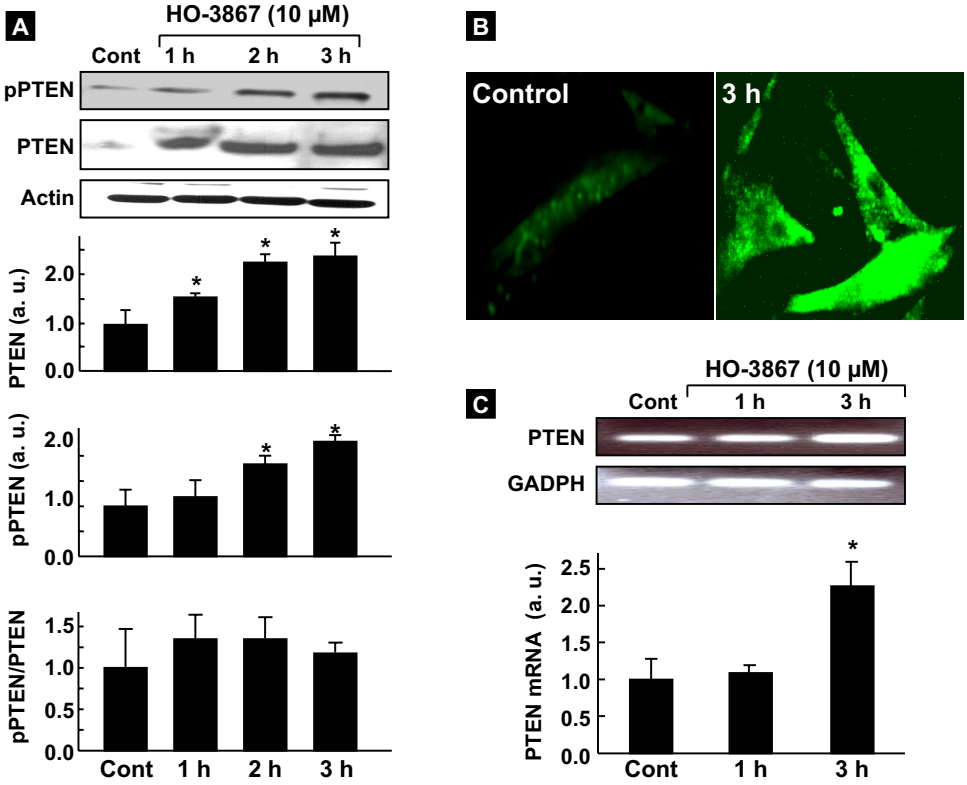

Fig. 2. HO-3867 up-regulates PTEN expression in human SMCs. A, immunoblots of phosphorylated PTEN (pPTEN) and total PTEN expressions in SMCs treated with HO-3867 for 1, 2, or 3 h. Also shown are quantitative results of densitometry analysis of bands and pPTEN/PTEN ratio. Data represent the mean ± S.E.M. ($n = 6$). *, $p < 0.05$ versus control. B, immunofluorescence images (400×, obtained using Alexa Fluor 488-conjugated anti-PTEN antibody) of PTEN expression in SMCs treated with 10 μM HO-3867. C, RT-PCR analysis of PTEN mRNA expression in SMCs treated with 10 μM HO-3867. *, $p < 0.05$ versus control.

firm whether the increase in the expression of PTEN was due to its transcriptional induction by HO-3867, we performed RT-PCR analysis for PTEN mRNA. Cells treated with HO-3867 clearly showed an increase in PTEN mRNA expression in 3 h (Fig. 2C).

HO-3867 Activates ATF-2 in SMCs. ATF-2 is a transcription factor that is involved in PTEN expression (Shen et al., 2006). Western blot analysis showed that ATF-2 was activated by HO-3867 within 15 min (Fig. 3). We next wanted to check the involvement of reactive oxygen species-mediated p38 mitogen-activated protein kinase pathways in the activation of ATF-2. However, we did not detect the production of reactive oxygen species or activation of p38 mitogen-activated protein kinase in cultured SMCs (data not shown). The results seemed to suggest that HO-3867 directly activated ATF-2, thereby inducing PTEN expression in SMCs.

HO-3867-Induces G_1 Arrest, Which Is Partially Blocked by PTEN siRNA in SMCs. To determine whether the HO-3867-induced growth inhibition in SMCs was attributable to PTEN, we treated PTEN siRNA-transfected SMCs with HO-3867 and analyzed cell cycle distribution by flow cytometry. As shown in Fig. 4A, the level of PTEN, induced by HO-3867, was repressed in cells transfected with siRNA targeting PTEN compared with HO-3867-treated cells lacking PTEN siRNA or HO-3867-treated cells transfected with negative control siRNA. The suppression of PTEN using siRNA in HO-3867-treated cells resulted in an increase of pERK1/2 and decreased p53 and p21 levels. Cyclin D1 was not altered in the siRNA-treated group. In addition, suppression of PTEN increased cell survival (Fig. 4B) and partially blocked the HO-3867-induced cell cycle arrest (Fig. 4C) and apoptosis (Fig. 4D). The results indicated that PTEN plays a role in the G_1 cell cycle arrest and apoptosis caused by HO-3867.

HO-3867 Down-Regulates MMP-2, MMP-9, NF-κB, and FAK. MMP-2 and MMP-9 are known to be involved in the control of SMC proliferation and migration into the in-

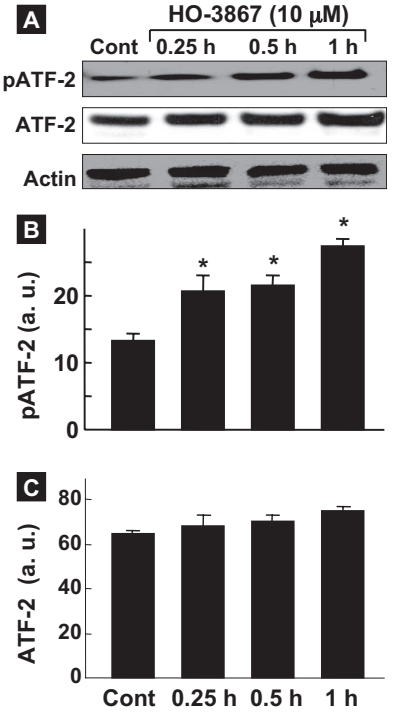

Fig. 3. HO-3867 up-regulates ATF-2 activation in human SMCs. A, immunoblots of pATF-2 and total ATF-2 expressions in SMCs treated with 10 μM HO-3867. Also shown are quantitative results of densitometry analysis of pATF-2 (B) and ATF-2 (C) bands. Data represent the mean± S.E.M. ($n = 6$).

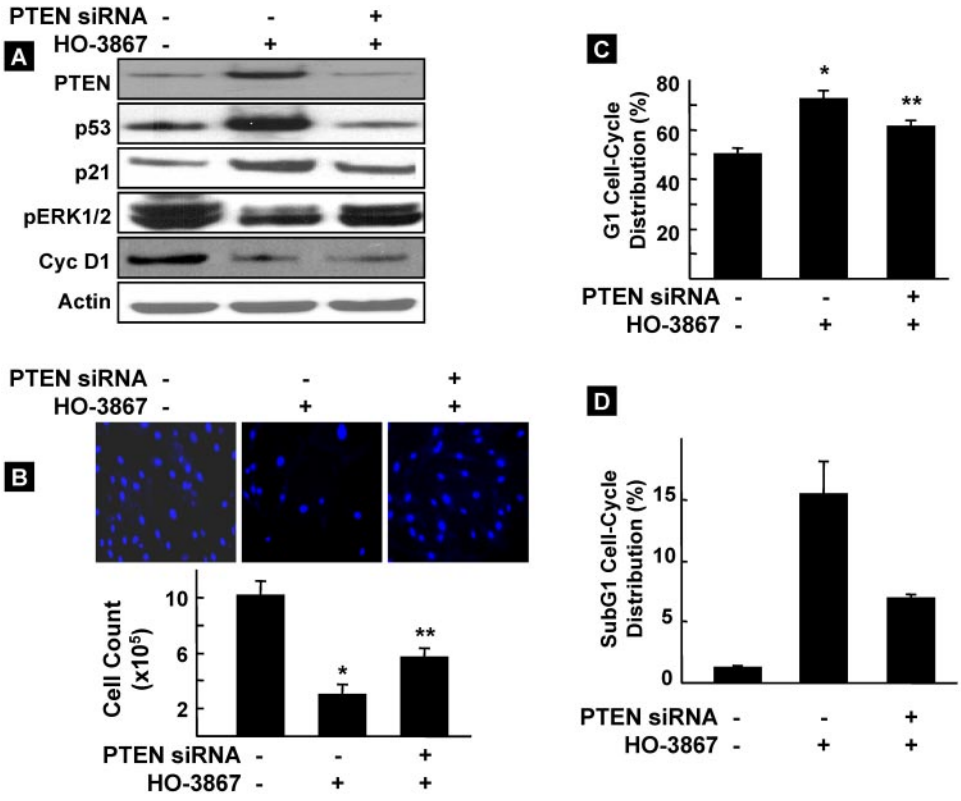

Fig. 4. PTEN siRNA inhibits HO-3867-induced cell cycle arrest. A, effect of PTEN siRNA on HO-3867-induced up-regulation of PTEN expression. Representative Western blot images from PTEN siRNA-transfected SMCs treated with 10 μM HO-3867 for 24 h are shown. B, fluorescence microscopy images (200×) of DAPI-stained (blue) PTEN siRNA-transfected SMCs treated with 10 μM HO-3867 for 24 h. Bar graph, cell count; expressed as mean ± S.E.M. (n = 3). *, $p < 0.05$ versus control (−/−); **, $p < 0.05$ versus HO-3867 group (−/+). C, G_1 cell cycle (DNA content) distribution (percentage of total) in PTEN siRNA-transfected SMCs treated with 10 μM HO-3867 for 24 h. Data represent mean± S.E.M. (n = 6). *, $p < 0.05$ versus control (−/−); **, $p < 0.05$ versus HO-3867 group (−/+). D, quantification of sub-G_1 cell cycle population by flow cytometry in PTEN siRNA-transfected cells treated with 10 μM HO-3867 for 48 h.

tima (Rouis, 2005). Activation of PTEN has been shown to suppress the MMP-2 and MMP-9 expression in SMCs (Park et al., 2002) and to inhibit the invasion of glioma cells (Park et al., 2002; Moon et al., 2004). Therefore, we investigated whether the HO-3867-induced PTEN expression could suppresses MMP expression in SMCs. The results (Fig. 5) revealed that HO-3867, through PTEN, down-regulated MMP-2 and MMP-9 expression in SMCs. In addition, HO-3867 inhibited NF-κB p65, one of the main regulator mechanisms in the inflammatory activation of MMP-9 in SMCs (Fig. 5A). We further observed that the expression of FAK, another known target of PTEN, was down-regulated by HO-3867. The results clearly suggested that HO-3867 inhibited SMC proliferation through PTEN-mediated down-regulation of MMP-2, MMP-9, NF-κB, and FAK expression.

Overexpression of PTEN by PTEN cDNA Enhances Apoptosis in SMCs. We next checked whether the PTEN overexpression was responsible for the inhibition of cell survival and proliferation in SMCs. We used wild-type PTEN cDNA transfection into SMCs for these experiments. Twenty-four hours after transfection, the cells were treated with 10 μM HO-3867 for 12 h. Cells transfected with PTEN cDNA showed a significantly reduced survival compared with non-transfected cells (Fig. 6A). Transfected cells treated with HO-3867 exhibited an even more significant reduction in their survival compared with untreated or nontransfected cells. Overexpression of PTEN by PTEN cDNA significantly increased the G_1 cell cycle arrest (Fig. 6B). Furthermore, the combination of PTEN cDNA overexpression and HO-3867 treatment significantly increased the apoptotic population (sub-G_1) compared with PTEN cDNA- or HO-3867-alone-treated groups (Fig. 6B). The combination treatment also showed a greater effect on the up-regulation of PTEN and inhibition of MMP-2, NF-κB, FAK, and survival proteins at 12 h (Fig. 6D). The results also showed a substantial increase in the cleaved caspase-3 levels in the PTEN cDNA + HO-3867-treated cells at 12 h, compared with untreated or non-transfected cells (Fig. 6D). The results established that

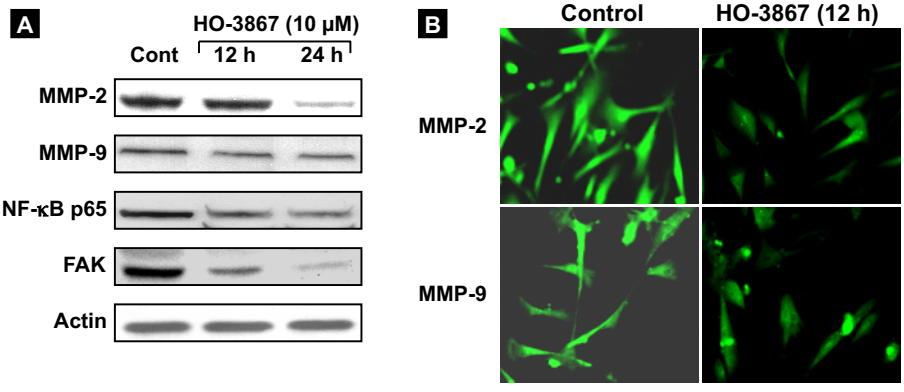

Fig. 5. HO-3867 down-regulates MMP-2, MMP-9, NF-κB, and FAK expression. SMCs were treated with 10 μM HO-3867 for 12 or 24 h. A, Western blot images showing down-regulation of SMC proliferation genes MMP-2, MMP-9, NF-κB, and FAK after 24-h exposure. B, immunofluorescence images of MMP-2 and MMP-9 expression (100×) at 12-h treatment.

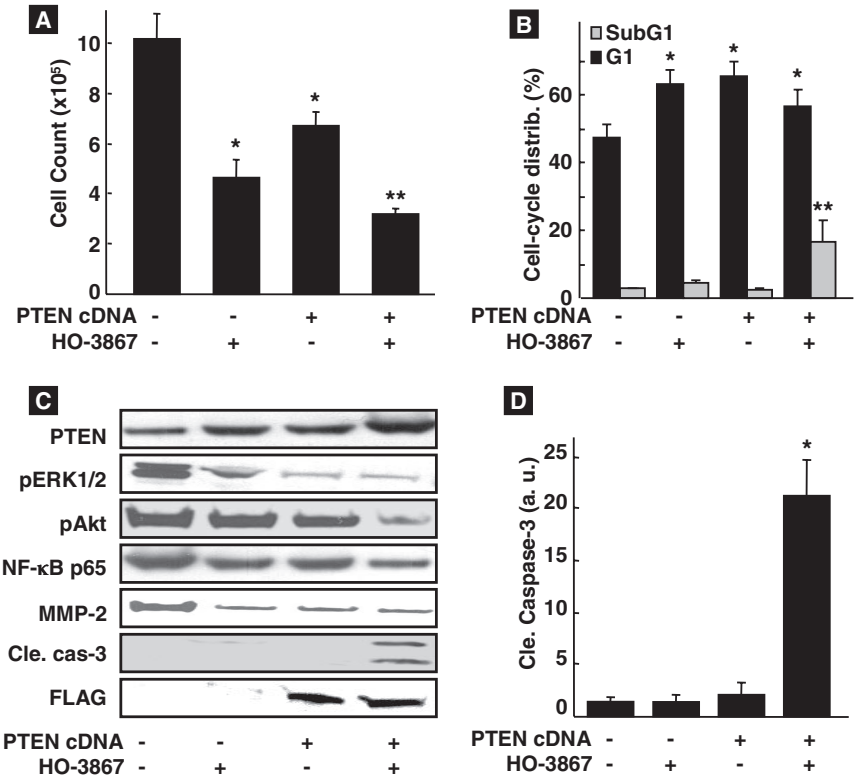

Fig. 6. PTEN overexpression induces apoptosis in SMCs. The PTEN/FLAG gene was transfected into SMCs, which were then treated with 10 μM HO-3867 for 12 or 24 h. A, effect of PTEN overexpression on HO-3867-induced inhibition of cell survival after 24 h. B, cell cycle distribution in PTEN cDNA-transfected cells treated with HO-3867 for 12 h. Data represent mean± S.E.M. ($n = 6$). *, $p < 0.05$ versus untreated control cells; **, $p < 0.05$ versus PTEN cDNA (+/−) or HO-3867 (−/+) group. Combination of PTEN cDNA and HO-3867 exposure had a significantly greater effect compared with the HO-3867- or cDNA alone-treated groups. Data represent mean ± S.E.M. ($n = 6$). *, $p < 0.05$ versus control group (−/−); **, $p < 0.05$ versus PTEN cDNA group ((+/−). C, Western blot images showing the effect of PTEN overexpression and 10 μM HO-3867 treatment (12 h) on pERK1/2, NF-κB, MMP-2, and MMP-9 protein levels. D, cleaved caspase-3 levels showing substantial increase in the combination treatment (+/+) group. Data represent mean ± S.E.M. ($n = 6$). *, $p < 0.05$ versus PTEN cDNA (+/−) or HO-3867 (−/+) group.

PTEN overexpression is responsible for the inhibition of cell survival and proliferation in SMCs treated with HO-3867.

HO-3867 Inhibits Neointimal Formation after Balloon Injury in Rat Carotid Artery. We further performed in vivo experiments to evaluate the efficacy of HO-3867 in inhibiting neointimal formation after balloon injury in a rat carotid artery model of restenosis, as described under *Materials and Methods*. A significant intimal thickening was observed in the carotid arteries at 14 days after infliction of balloon injury (Fig. 7A). The thickening was significantly inhibited in rats receiving HO-3867 for 14 days. The intima/media ratio was significantly reduced in animals receiving HO-3867 (Fig. 7B). We then wanted to know whether the observed inhibitory effect of HO-3867 treatment on neointimal formation was associated with changes in the expression of regulatory proteins that we studied in the growth-stimulated SMCs in vitro. We observed that the PTEN expression was depressed drastically in rat carotid artery tissue after balloon injury compared with uninjured controls (Fig. 7C). Although treatment with 5 mg/kg HO-3867 restored PTEN to the control level, treatment with 10 mg/kg HO-3867 increased the PTEN level significantly above control level. Animals treated with 5 mg/kg did not show any significant inhibition of neointimal formation (data not shown). Western blot analysis of the tissues showed down-regulation of MMP-2, MMP-9, NF-κB p65, pERK1/2, pAkt, and cyclin D1 and up-regulation of p53 and cleaved caspase-3 (Fig. 7D). The activation of cleaved caspase-3 in the 10-mg/kg HO-3867 dose suggests that the treatment also induced apoptosis in vivo. We also confirmed the MMP-2 and MMP-9 activity using gelatin zymography. The MMP-2 and MMP-9 activities were significantly down-regulated in the treatment group compared with the injured/untreated group (Fig. 7E). The results strongly suggested that HO-3867 inhibited neointimal formation by up-regulation of PTEN expression, leading to down-regulation of MMP-2, MMP-9, and NF-κB expressions in the rat carotid artery model of restenosis.

Discussion

In this study, we have demonstrated the potential application of a novel synthetic compound, HO-3867, for inhibition of SMC proliferation through up-regulation of PTEN expression. The results showed pharmacologic inhibition of human aortic SMC proliferation in vitro and substantially reduced neointimal formation in vivo by HO-3867. The in vitro cell culture results showed that HO-3867 inhibited FBS-induced SMC proliferation through cell cycle arrest at the G_1 phase and by induction of apoptosis. This inhibitory effect was mediated by up-regulation of PTEN expression and several key growth-regulatory proteins including cyclin D1, MMPs, NF-κB, and ERK1/2. The in vitro studies using SMCs transfected with PTEN siRNA and PTEN cDNA confirmed that the HO-3867-induced overexpression of PTEN was responsible for the inhibition of SMC proliferation. The in vivo experiments in a rat carotid artery balloon injury model further showed a substantial depression of PTEN expression, which was restored far and beyond the control level after treatment with HO-3867.

Using a set of preliminary experiments, we have shown that HO-3867 specifically inhibits highly proliferating cells, such as stimulated SMCs or cancer cells, while sparing nonproliferating healthy cells (Tazi et al., 2008). We have observed that the EC_{50} value of HO-3867 in human SMCs was 3.82 μM. In the present study, we used the 10 μM concentration of HO-3867 to show its inhibitory effect on serum-stimulated SMCs. However, the same concentration of HO-3867 did not have any significant inhibitory effect on Chinese hamster ovary cells or human aortic endothelial cells (Tazi et

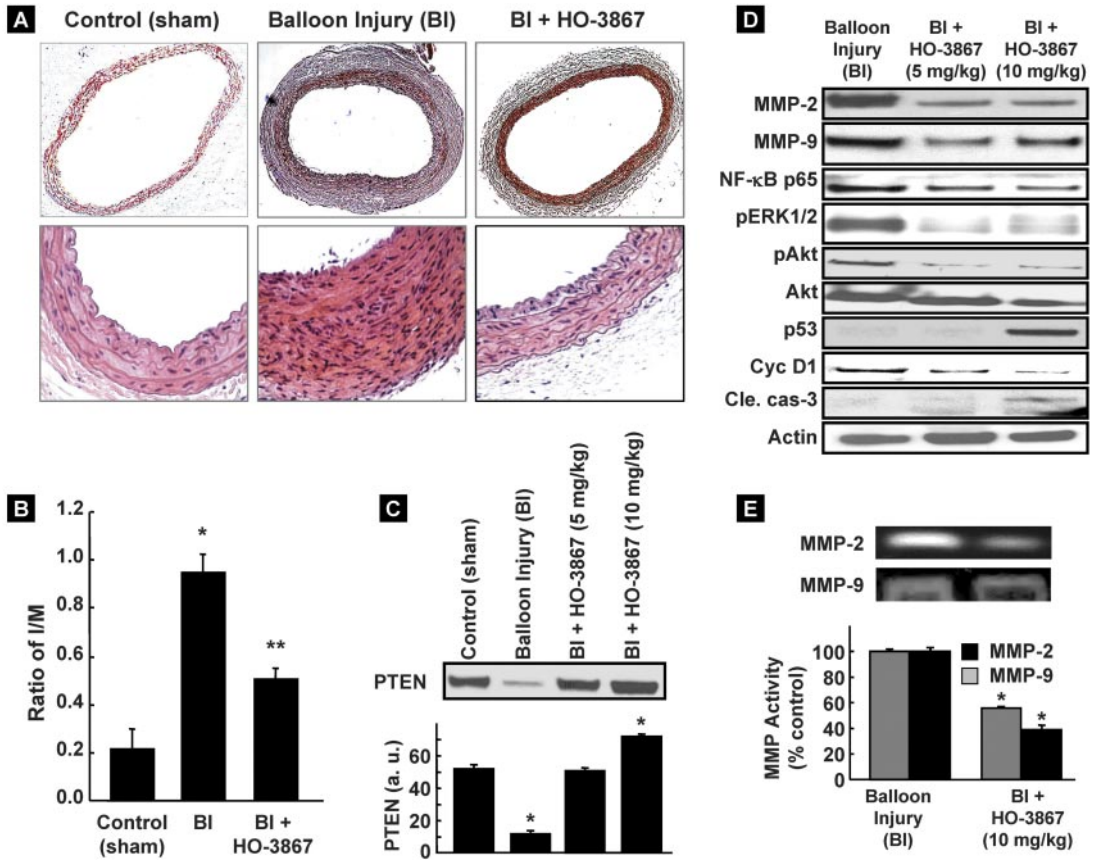

Fig. 7. HO-3867 inhibits neointima formation and up-regulates PTEN expression after rat carotid artery injury. A, representative cross-sections of uninjured (Control) and injured carotid arteries with (HO-3867) and without [balloon injury (BI)] HO-3867 treatment at 10 mg/kg. The cross sections were magnified and photographed at 10× and 40×. Cell proliferation is observed in both injured and treatment groups. B, morphometric measurements of neointima formation in uninjured or injured carotid arteries at 2 weeks after treatment. Data represent mean ± S.E.M. ($n = 5$). *, $p < 0.05$ versus control; **, $p < 0.05$ versus injured/untreated (BI). C, PTEN expression was drastically decreased in the BI group compared with the uninjured control group. HO-3867 treatment rescued PTEN levels in the group treated with 10 mg/kg. Densitometry analyses of visualized bands were performed for quantitation of PTEN expression. The data represent mean ± S.E.M. ($n = 3$). *, $p < 0.05$ versus control. D, protein expression of MMP-2, MMP-9, NF-κB, FAK, pERK1/2, pAkt, and cyclin D1 as analyzed by Western blotting. E, MMP-2 and MMP-9 activity. Data represent mean ± S.E.M. ($n = 5$). *, $p < 0.05$ versus untreated control.

al., 2008), suggesting that the cytotoxic effect of HO-3867 is specific to proliferating cells.

We reported recently that EF24, a synthetic curcuminoid having close structural similarity to HO-3867, increased PTEN expression and activity in human ovarian cancer cells (Selvendiran et al., 2007). The up-regulation of PTEN by EF24 was attributed to inhibition of ubiquitin-mediated PTEN degradation. The results of the present study showed that HO-3867 increased PTEN expression in SMCs by activation of ATF-2 phosphorylation and its binding to the PTEN promoter (Shen et al., 2006). The up-regulation of PTEN by HO-3867 in SMCs also could be augmented by inhibition of PTEN degradation, which we have not studied. Accumulating evidence suggests that activation of PTEN inhibits VSMC proliferation by blocking the phosphatidylinositol 3-kinase pathway and its downstream effector molecules, such as Akt/protein kinase B and ERK1/2 (Garl et al., 2004; Mourani et al., 2004). Our results clearly showed that the HO-3867-mediated activation of PTEN inhibited cell cycle progression by induction of p53 and p21 and down-regulation of ERK1/2 and cyclin D1, but without any change in Akt level. Recent studies showed the PTEN-mediated cell cycle arrest, possibly independent of Akt, was directly associated with increase in the stability, protein level, and transcriptional activity of p53 (Huang and Kontos, 2002; Freeman et al., 2003; Moon et al., 2004). Likewise, PTEN has been shown to regulate cell cycle arrest via protein phosphatase-dependent interaction with cyclin D1 (Weng et al., 2001). The oncogenic potential of PTEN is further highlighted by its roles in integrin signaling and ability to dephosphorylate FAK that can reduce cell adhesion and enhance migration (Tamura et al., 1999). Other studies have shown that, in addition to inhibition of proliferation, PTEN also can increase apoptosis induction concomitantly in SMCs (Huang and Kontos, 2002; Koide et al., 2007). Taken together, the results suggest that up-regulation of PTEN may play a role in the regulation of cell proliferation in SMCs.

The expression of MMPs plays a vital role in the pathogenesis of atherosclerosis and restenosis after vascular injury (Libby, 2002; Rouis, 2005). To be specific, MMP-2 and MMP-9 have been shown to be important for SMC proliferation and migration into the intima (Newby and Zaltsman, 2000). Activation of PTEN has been shown to suppress MMP-2 and MMP-9 expression in cultured cells (Moon et al., 2004). In the present study, we observed inhibition of MMP-2 and MMP-9 expressions in SMCs treated with HO-3867 (Fig. 5). We further observed that overexpression of PTEN using wild-type PTEN cDNA also inhibited the expression of MMP-2 at an early stage of treatment

(Fig. 6). Furthermore, silencing of PTEN expression (using PTEN siRNA) had no effect on the HO-3867-induced inhibition of MMP-2 levels (data not shown), suggesting that the HO-3867-induced inhibition of MMP-2 is a direct effect, independent of PTEN. In fact, HO-3867 did not have any effect on MMP-2 in PTEN-overexpressing (transfected with PTEN cDNA) cells, beyond that of HO-3867 alone or PTEN cDNA treatments. However, the overexpression of PTEN, in combination with HO-3867, induced a significant level of apoptosis by activation of cleaved caspase-3 (Fig. 6B). Our study, therefore, provides the first evidence that overexpression of PTEN, in combination with a synthetic compound, not only inhibits proliferation but also induces cell cycle arrest and apoptosis in SMCs. The up-regulation of PTEN expression and its role in cell cycle arrest and apoptotic signaling is not completely understood in the cardiovascular system. Our siRNA studies clearly established a link between PTEN expression and induction of cell cycle arrest and apoptosis. However, the complete inhibition of PTEN did not totally block SMC proliferation, so the actual mechanism by which HO-3867 contributes to the outcome of PTEN up-regulation and apoptosis requires further investigation.

The rat carotid artery balloon injury model that we have used in this study is characterized by a high degree of reproducibility, with the development of SMC-rich intimal lesions. The relatively short treatment period (2 weeks) resulted in a significantly diminished neointima formation after balloon injury. The rapid action of HO-3867 in vivo seems to suggest that the expressions of MMPs, ERK1/2, and NF-κB were more effectively suppressed in the tissue than in cultured cells. Thus, HO-3867 represents an attractive, potentially effective therapeutic agent for attenuating the vascular response to injury leading to restenosis. Our results strongly support the hypothesis that PTEN signaling may serve as a critical target for the inhibition of neointimal formation.

This is the first study to show that overexpression of PTEN by a synthetic compound effectively suppresses cell cycle progression in human SMCs and inhibits neointimal hyperplasia in a rat carotid artery injury model. The results suggest that PTEN could be a key target for the prevention of vascular proliferative disorders such as restenosis, and compounds such as HO-3867, which can up-regulate PTEN expression, may serve as useful therapeutic agents in this regard.

Acknowledgments

We thank Dr. William Sellers (Novartis Institute of Biomedical Research) for providing the PTEN plasmid.

References

Chen WJ, Lin KH, Lai YJ, Yang SH, and Pang JH (2004) Protective effect of propylthiouracil independent of its hypothyroid effect on atherogenesis in cholesterol-fed rabbits: PTEN induction and inhibition of vascular smooth muscle cell proliferation and migration. *Circulation* **110:**1313–1319.

Cho A and Reidy MA (2002) Matrix metalloproteinase-9 is necessary for the regulation of smooth muscle cell replication and migration after arterial injury. *Circ Res* **91:**845–851.

Choy JC, Granville DJ, Hunt DW, and McManus BM (2001) Endothelial cell apoptosis: biochemical characteristics and potential implications for atherosclerosis. *J Mol Cell Cardiol* **33:**1673–1690.

Coolong A and Kuntz RE (2007) Understanding the drug-eluting stent trials. *Am J Cardiol* **100:**17K–24K.

Freeman DJ, Li AG, Wei G, Li HH, Kertesz N, Lesche R, Whale AD, Martinez-Diaz H, Rozengurt N, Cardiff RD, et al. (2003) PTEN tumor suppressor regulates p53 protein levels and activity through phosphatase-dependent and -independent mechanisms. *Cancer Cell* **3:**117–130.

Garl PJ, Wenzlau JM, Walker HA, Whitelock JM, Costell M, and Weiser-Evans MC (2004) Perlecan-induced suppression of smooth muscle cell proliferation is mediated through increased activity of the tumor suppressor PTEN. *Circ Res* **94:**175–183.

Huang J and Kontos CD (2002) Inhibition of vascular smooth muscle cell proliferation, migration, and survival by the tumor suppressor protein PTEN. *Arterioscler Thromb Vasc Biol* **22:**745–751.

Huang J, Niu XL, Pippen AM, Annex BH, and Kontos CD (2005) Adenovirus-mediated intraarterial delivery of PTEN inhibits neointimal hyperplasia. *Arterioscler Thromb Vasc Biol* **25:**354–358.

Jackson CL and Schwartz SM (1992) Pharmacology of smooth muscle cell replication. *Hypertension* **20:**713–736.

Kim HS, Cho HJ, Cho HJ, Park SJ, Park KW, Chae IH, Oh BH, Park YB, and Lee MM (2004) The essential role of p21 in radiation-induced cell cycle arrest of vascular smooth muscle cell. *J Mol Cell Cardiol* **37:**871–880.

Koide S, Okazaki M, Tamura M, Ozumi K, Takatsu H, Kamezaki F, Tanimoto A, Tasaki H, Sasaguri Y, Nakashima Y, et al. (2007) PTEN reduces cuff-induced neointima formation and proinflammatory cytokines. *Am J Physiol Heart Circ Physiol* **292:**H2824–H2831.

Libby P (2002) Inflammation in atherosclerosis. *Nature* 420:868–874.

McKinsey TA and Kass DA (2007) Small-molecule therapies for cardiac hypertrophy: moving beneath the cell surface. *Nat Rev Drug Discov* **6:**617–635.

Moon SK, Kim HM, and Kim CH (2004) PTEN induces G1 cell cycle arrest and inhibits MMP-9 expression via the regulation of NF-kappaB and AP-1 in vascular smooth muscle cells. *Arch Biochem Biophys* **421:**267–276.

Mourani PM, Garl PJ, Wenzlau JM, Carpenter TC, Stenmark KR, and Weiser-Evans MC (2004) Unique, highly proliferative growth phenotype expressed by embryonic and neointimal smooth muscle cells is driven by constitutive Akt, mTOR, and p70S6K signaling and is actively repressed by PTEN. *Circulation* **109:**1299–1306.

Newby AC and Zaltsman AB (2000) Molecular mechanisms in intimal hyperplasia. *J Pathol* **190:**300–309.

Oudit GY, Sun H, Kerfant BG, Crackower MA, Penninger JM, and Backx PH (2004) The role of phosphoinositide-3 kinase and PTEN in cardiovascular physiology and disease. *J Mol Cell Cardiol* **37:**449–471.

Park MJ, Kim MS, Park IC, Kang HS, Yoo H, Park SH, Rhee CH, Hong SI, and Lee SH (2002) PTEN suppresses hyaluronic acid-induced matrix metalloproteinase-9 expression in U87MG glioblastoma cells through focal adhesion kinase dephosphorylation. *Cancer Res* **62:**6318–6322.

Rouis M (2005) Matrix metalloproteinases: a potential therapeutic target in atherosclerosis. *Curr Drug Targets Cardiovasc Haematol Disord* **5:**541–548.

Schwartzbauer G and Robbins J (2001) The tumor suppressor gene PTEN can regulate cardiac hypertrophy and survival. *J Biol Chem* **276:**35786–35793.

Selvendiran K, Bratasz A, Tong L, Ignarro LJ, and Kuppusamy P (2008a) NCX-4016, a nitro-derivative of aspirin, inhibits EGFR and STAT3 signaling and modulates Bcl-2 proteins in cisplatin-resistant human ovarian cancer cells and xenografts. *Cell Cycle* **7:**81–88.

Selvendiran K, Kuppusamy ML, Bratasz A, Rivera BK, Rink C, Sen CK, Hideg K, and Kuppusamy P (2008b) Abstract 5550: Inhibition of smooth muscle cell proliferation and balloon injury-induced nonintimal hyperplasia through activation of PTEN expression by H0–3867, a synthetic curcuminoid. *Circulation* **118 (18 Suppl):**S573.

Selvendiran K, Tong L, Vishwanath S, Bratasz A, Trigg NJ, Kutala VK, Hideg K, and Kuppusamy P (2007) EF24 induces G_2/M arrest and apoptosis in cisplatin-resistant human ovarian cancer cells by increasing PTEN expression. *J Biol Chem* **282:**28609–28618.

Shen YH, Zhang L, Gan Y, Wang X, Wang J, LeMaire SA, Coselli JS, and Wang XL (2006) Up-regulation of PTEN (phosphatase and tensin homolog deleted on chromosome ten) mediates p38 MAPK stress signal-induced inhibition of insulin signaling: a cross-talk between stress signaling and insulin signaling in resistin-treated human endothelial cells. *J Biol Chem* **281:**7727–7736.

Tamura M, Gu J, Danen EH, Takino T, Miyamoto S, and Yamada KM (1999) PTEN interactions with focal adhesion kinase and suppression of the extracellular matrix-dependent phosphatidylinositol 3-kinase/Akt cell survival pathway. *J Biol Chem* **274:**20693–20703.

Tazi MF, Selvendiran K, Kuppusamy ML, Tong L, Rivera BK, Hideg K, and Kuppusamy P (2008) Evaluation of a novel class of fluorinated curcumin analogs for safe and targeted anticancer therapy (STAT). *Free Rad Biol Med* **45 (Suppl 1):**S56–S57.

Wassmann S, Wassmann K, Jung A, Velten M, Knuefermann P, Petoumenos V, Becher U, Werner C, Mueller C, and Nickenig G (2007) Induction of p53 by GKLF is essential for inhibition of proliferation of vascular smooth muscle cells. *J Mol Cell Cardiol* **43:**301–307.

Weng LP, Brown JL, and Eng C (2001) PTEN coordinates G(1) arrest by down-regulating cyclin D1 via its protein phosphatase activity and up-regulating p27 via its lipid phosphatase activity in a breast cancer model. *Hum Mol Genet* **10:**599–604.

Address correspondence to: Dr. Periannan Kuppusamy, The Ohio State University, 420 West 12th Avenue, Room 114, Columbus, OH 43210. E-mail: kuppusamy.1@osu.edu