Pharmacology

Halofuginone Stimulates Adaptive Remodeling and Preserves Re-Endothelialization in Balloon-Injured Rat Carotid Arteries

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Background—Three major processes, constrictive vessel remodeling, intimal hyperplasia (IH), and retarded re-endothelialization, contribute to restenosis after vascular reconstructions. Clinically used drugs inhibit IH but delay re-endothelialization and also cause constrictive remodeling. Here we have examined halofuginone, an herbal derivative, for its beneficial effects on vessel remodeling and differential inhibition of IH versus re-endothelialization.

Methods and Results—Two weeks after perivascular application to balloon-injured rat common carotid arteries, halofuginone versus vehicle (n=6 animals) enlarged luminal area 2.14-fold by increasing vessel size (adaptive remodeling; 123%), reducing IH (74.3%) without inhibiting re-endothelialization. Consistent with its positive effect on vessel expansion, halofuginone reduced collagen type 1 (but not type 3) production in injured arteries as well as that from adventitial fibroblasts in vitro. In support of its differential effects on IH versus re-endothelialization, halofuginone produced greater inhibition of vascular smooth muscle cell versus endothelial cell proliferation at concentrations ≈50 nmol/L. Furthermore, halofuginone at 50 nmol/L effectively blocked Smad3 phosphorylation in smooth muscle cells, which is known to promote smooth muscle cell proliferation, migration, and IH, but halofuginone had no effect on phospho-Smad3 in endothelial cells.

Conclusions—Periadventitial delivery of halofuginone dramatically increased lumen patency via adaptive remodeling and selective inhibition of IH without affecting endothelium recovery. Halofuginone is the first reported small molecule that has favorable effects on all 3 major processes involved in restenosis. (Circ Cardiovasc Interv. 2014;7:594-601.)

Key Words: cell proliferation • collagen • halofuginone • Smad3 protein • vascular endothelium • vascular patency

Cardiovascular disease is the most common cause of death in the developed world. Atherosclerosis is the primary pathology underlying the great majority of cardiovascular morbidity including coronary artery and peripheral vascular disease.1 Vascular interventions such as angioplasty and bypass are commonly performed to restore circulation to vital organs. However, such treatments inflict injury to the native vessel wall, causing vessel renarrowing (or restenosis). The injury, including denudation of the endothelial layer and mechanical stretching of the vessel, evokes an excessive wound-healing response, including the release of various cytokines and growth factors at the injury site from monocytes and macrophages.2 There are at least 3 major processes contributing to restenosis: (1) constrictive remodeling or overall constriction of the vessel wall, which has been proposed to be attributable to excessive matrix protein production (eg, collagen type 1 [Col-1]) in the adventitia;3 (2) intimal hyperplasia (IH), which is overgrowth of cells and matrix in the subintimal layer; and (3) delayed recovery of the protective endothelial inner lining leading to thrombosis and also exacerbation of IH and constrictive remodeling.2

In contrast to our knowledge about IH, the mechanisms of arterial remodeling are much less understood.1 Arterial remodeling results in a permanent change in vessel diameter; this might be through vessel expansion (termed adaptive remodeling) or shrinkage (termed constrictive remodeling).3 As has been observed in patients and in animal models of restenosis, constrictive remodeling accounts for a considerable portion of the lumen loss and in many cases can be a greater contributor to restenosis than IH. Either prevention of constrictive remodeling or alternatively induction of adaptive remodeling has been shown to attenuate postangioplasty lumen loss.3 It is thought that deposition of extracellular matrix in the adventitia might be contributory to constrictive remodeling after angioplasty.
WHAT IS KNOWN

- Drugs currently used in stents to prevent restenosis, such as Rapamycin, inhibit intimal hyperplasia but cause delayed re-endothelialization and thrombosis and also produce constrictive vessel remodeling.
- Halofuginone, a derivative of herbal medicine, is an extracellular matrix modulator that is in a phase II clinical trial for Kaposi sarcoma and a phase I clinical trial for cancer.

WHAT THE STUDY ADDS

- Halofuginone compared with vehicle control is able to produce a 2-fold lumen enlargement via expansive (or adaptive) vessel remodeling and selective inhibition of intimal hyperplasia with preserved re-endothelialization in a rat angioplasty model.
- Halofuginone-induced adaptive remodeling is associated with greater inhibition of collagen type I production versus type 3 by adventitial fibroblasts; selective mitigation of intimal hyperplasia versus re-endothelialization involves a mechanism of preferential halofuginone inhibition of Smad3 activation and cell proliferation in smooth muscle cells versus endothelial cells.
- Halofuginone is the first small molecule that is shown to produce favorable effects on all 3 major processes that are involved in restenosis.

In particular, an increase in Col-1 is often associated with constrictive remodeling, as is the case with other fibrotic diseases (eg, in the liver, pancreas, and skin). Inhibition of Col-1 production has been used as a general strategy for treating fibrosis. Nevertheless, arterial remodeling is a complex process. It is likely that not only collagen content but also crosslinking and collagen subtype play important roles. We have recently shown that an increase in the ratio of Col-3 (an elastic subtype of collagen) to Col-1 (a more rigid subtype) is associated with adaptive remodeling.8

Although both IH and constrictive remodeling result in narrowing of the vessel lumen, rapid re-endothelialization after arterial injury can prevent both thrombosis and IH.2 In normal arteries, endothelial cells (ECs) form a protective inner lining that functions as a barrier between the circulating blood and the vessel wall.2 However, after interventions for atherosclerosis, ECs are denuded and smooth muscle cells (SMCs) are left exposed to cytokines and growth factors and thus transformed into active contributors to intimal growth and constrictive remodeling, resulting in restenosis. Therefore, the more rapidly the endothelial layer can be recovered, the greater the inhibitory effect on restenosis. Thus, a drug that selectively inhibits SMC proliferation with less impairment of EC growth compared with SMCs. Probably for this reason very few agents have been reported to selectively inhibit the growth of SMCs versus ECs.5

Currently the only clinically used antirestenosis drugs are Rapamycin and Paclitaxel. Although these 2 widely used drugs are effective in reducing IH, there is evidence that they cause constrictive remodeling.6 Moreover, Rapamycin and Paclitaxel have been reported to inhibit re-endothelialization, causing late-stent thrombosis and sudden death.2,7 Therefore, a therapeutic agent that can in a favorable manner manipulate each of the 3 processes that lead to restenosis would theoretically be an extremely effective tool for preventing restenosis versus a simple inhibitor of IH. To the best of our knowledge, no such agent has yet been reported.

Halofuginone is a derivative of febrifugine, 1 of the 50 fundamental herbs of traditional Chinese medicine. This ancient drug is attracting tremendous interest for its potent extracellular matrix modulating, antifibrotic, antimetastatic, and antistress effects.8 In a recently completed phase II clinical trial, halofuginone was efficacious in reducing the lesions associated with Kaposi sarcoma. This drug has also entered phase I clinical trials for cancer.9 A positive effect of halofuginone on injury-induced IH was observed in 2 prior studies using less relevant models.10,11 However, whether halofuginone affects postinjury arterial remodeling and re-endothelialization has not been previously reported. Of particular relevance to the process of vascular remodeling, halofuginone is a specific inhibitor of Col-1 synthesis and thus has an antifibrotic effect in various tissues.8 Based on the previous observations that excessive Col-1 deposition leads to constrictive remodeling,2 we hypothesized that halofuginone may promote adaptive remodeling. Indeed, we found that halofuginone not only reduced IH but importantly also produced adaptive remodeling. Moreover, halofuginone had the added benefit of preserving luminal re-endothelialization. Our findings suggest that when administered locally halofuginone is a favorable antirestenosis agent with significant potential clinical utility.

Methods

An expanded Methods section is available in the Data Supplement.

Rat Carotid Artery Balloon Angioplasty Model, Perivascular Halofuginone Delivery, and Morphometry

All animal studies conform to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication No. 85-23, 1996 revision) and protocols approved by the Institutional Animal Care and Use Committee at the University of Wisconsin. Institutional review board approval has been obtained for use of cells derived from human samples. Carotid artery balloon angioplasty was performed in male Sprague–Dawley rats (Charles River; 300–330 g) as previously described.12 Halofuginone was then applied to the outside of the injured segment of the carotid artery by using F-127 pluronic gel (Sigma-Aldrich).12 This perivascular approach proved to be effective in the delivery of small-molecule drugs (such as rapamycin) or even nanoparticles into the injured rat carotid arterial wall.13 Before administration, 30 μg halofuginone from a DMSO stock was first dissolved in 30 μL of 10% DMSO and then mixed with 270 μL of 25% pluronic gel that was maintained on ice. We chose a concentration in vivo that was substantially higher than what has been shown to be effective in vitro because of the potential for halofuginone to diffuse away from the arterial wall. In the control group, equal volume of vehicle (30 μL of 20% DMSO) mixed with pluronic gel was applied. Animals were euthanized at 14 days postangioplasty, a time point at which significant remodeling occurred in our previous...
study. Adaptive remodeling was defined as an increase of external elastic lamina area. IH was quantified as a ratio of intima area versus media area. Re-endothelialization was assessed using Evans Blue assay as well as CD31 immunostaining.

Cell Culture and In Vitro Halofuginone Treatment
Rat aortic vascular SMCs and fibroblasts were isolated from the thoracoabdominal aorta of male Sprague–Dawley rats (anesthetized under 2.5% isoflurane) based on a protocol using an enzymatic dissociation method. Rat aortic ECs were purchased from Genlantis. For halofuginone treatment, cells were first starved for 12 or 24 hours in Dulbecco modified Eagle medium containing 0.5% FBS; the medium was then replaced with fresh Dulbecco modified Eagle medium supplemented with 10% FBS in which halofuginone was added to a desired concentration. For control, equal volume of DMSO was added to a final concentration of 0.1% (v/v versus medium). Proliferation of cultured SMCs was determined using cell number as a surrogate, which Alamar Blue (Invitrogen) assay was performed as previously described.

Statistical Analysis
Data are presented as means±SEM. Statistical analysis was conducted using 2-tailed unpaired Student t test and ANOVA. Statistical significance was validated by P<0.05.

Results
Halofuginone Produces Adaptive Remodeling and Reduces IH in Balloon-Injured Rat Carotid Arteries
Excessive Col-1 deposition has been suggested to be a cause of constrictive remodeling after arterial injury, and halofuginone is a specific inhibitor of Col-1 synthesis, in cell lines including fibroblasts. These reports prompted us to test whether halofuginone, because of its Col-1–inhibiting properties, might prevent constrictive arterial remodeling after injury.

We performed rat carotid balloon angioplasty, which is a widely accepted model to mimic angioplasty in humans, and then administered halofuginone locally by dissolving halofuginone in pluronic gel and then applying the gel around the injured artery. The results of morphometric analyses using carotid sections collected on day 14 postangioplasty are shown in Figure 1. We found that compared with vehicle (DMSO) control, halofuginone treatment produced a 123% increase in external elastic lamina area (Figure 1C), indicating that halofuginone induced adaptive remodeling after balloon injury of rat carotid arteries. Further analysis revealed that the intima/media ratio, a measure of IH, decreased by 74.3% (Figure 1D), and luminal area increased by 2.14-fold (Figure 1E).

Halofuginone Inhibits the Production of Type 1 But Not Type 3 Collagen From Adventitial Fibroblasts In Vivo and In Vitro
An increase in the ratio of Col-3/Col-1, the critical adventitial collagen subtypes, is thought to contribute to favorable arterial remodeling. However, the effect of halofuginone on Col-1 and Col-3 protein levels in arteries treated with angioplasty has not been previously evaluated. To assess whether halofuginone might facilitate adaptive remodeling by affecting collagen homeostasis, we examined the effect of halofuginone on the production of Col-1 and Col-3 in vivo and in vitro. Through immunostaining of carotid sections collected 14 days after angioplasty, we found that Col-1 was significantly reduced in the adventitia in halofuginone-treated balloon-injured arteries compared with vehicle control (Figure 2C). In contrast, Col-3 levels were not significantly changed although there was a numeric increase (Figure 2F). We further tested the halofuginone effects on these 2 collagen subtypes in vitro using primary rat aortic adventitial fibroblasts. We found that although Col-1 secretion, measured by Western blotting, from fibroblasts decreased by 30% (relative to control) in the presence of 50 nmol/L halofuginone, Col-3 secretion did not change significantly (Figure 2G and 2H). The combined evidence from these in vivo and in vitro experiments indicates that halofuginone specifically inhibits the production of Col-1 versus Col-3 by adventitial fibroblasts, thus providing a potential mechanism for this drug’s favorable effect on adaptive remodeling.

Halofuginone Inhibits Rat SMC Proliferation in Balloon-Injured Arteries as Well as in Cell Culture
SMC proliferation and migration are critical contributors to IH. We investigated whether halofuginone inhibited intimal growth by altering these SMC behaviors. We first addressed this question in vivo by immunostaining rat carotid sections for proliferating cell nuclear antigen, a marker for proliferating cells. Indeed, proliferating cell nuclear antigen–positive cells were greatly reduced (by 66.2%) in the halofuginone-treated arteries relative to that of vehicle-treated controls.
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Halofuginone (HF) treatment reduces proliferating cell nuclear antigen (PCNA)-positive cells in vivo and smooth muscle cell (SMC) proliferation and migration in vitro. A, PCNA immunostaining of carotid arteries treated periadventitialy with either vehicle (DMSO) or HF. Sections were collected 14 d after angioplasty. Shown are representative cross-sections with quantification of PCNA-positive cells. HPF indicates high-power field. Each bar represents a mean (±SEM) of 36 sections from 4 animals; *P<0.05. B, Effect of HF on the proliferation of cultured rat SMCs. Cells were starved for 24 h and then stimulated with 10% FBS in the presence of vehicle or HF (50 nmol/L). A scratch was made before FBS stimulation. Quantification of migration is expressed as a relative rate of recovery of the scratch wound. Each bar represents a mean (±SEM) of 3 separate experiments; *P<0.05.

Halofuginone Preserves Re-Endothelialization in Balloon-Injured Arteries

Encouraged by the dramatic reduction of IH by halofuginone and its favorable effect on adaptive remodeling, we next determined whether halofuginone affects re-endothelialization, another important factor involved in the development of restenosis. Two weeks after angioplasty, we incised longitudinally the carotid arteries that were stained in the lumen with Evans Blue, a widely used method to measure re-endothelialization. Our data reveal that the ratio of re-endothelialized area (not stained by Evans Blue) versus total area was greater in

(Figure 3A), indicating a strong inhibitory effect of halofuginone on SMC proliferation in injured arteries. We then confirmed the inhibitory effect of halofuginone on proliferation of cultured SMCs. As shown in Figure 3B, 10% serum produced a 2.2-fold increase in rat aortic medial SMC proliferation. Although halofuginone at 1 nmol/L had no effect on SMC proliferation, 50 nmol/L halofuginone reduced serum-stimulated proliferation by 70.6% and 100 nmol/L halofuginone reduced by 82.3%.

Moreover, using an in vitro scratch model of migration, we found that 50 nmol/L halofuginone inhibited rat aortic SMC migration by 73.6% 48 hours after treatment (Figure 3C). Taken together, our data indicate that halofuginone potently inhibits proliferation as well as migration of vascular SMCs.
Halofuginone-treated arteries versus vehicle-treated controls (Figure 4A). To confirm this finding, we performed another commonly used assay for re-endothelialization, immunostaining of CD31 (a marker of ECs). Similarly, we found that re-endothelialization was not reduced but rather significantly increased in halofuginone-treated vessels compared with vehicle-treated vessels subjected to angioplasty (Figure 4B). Thus, our data indicate that halofuginone applied periadventitiously preserved re-endothelialization of rat carotid arteries after balloon angioplasty.

**Halofuginone Preferentially Inhibits SMC Versus EC Proliferation In Vitro**

A highly desirable property of drugs designed to inhibit restenosis is selective inhibition of SMC versus EC proliferation, leading to the suppression of intimal growth but not re-endothelialization in arteries injured by vascular reconstruction.

Very few agents have been shown to possess this beneficial feature. The observed in vivo inhibitory effect of halofuginone on IH but not re-endothelialization prompted us to examine differential effects of halofuginone on the in vitro proliferation of primary aortic SMCs and aortic ECs both at passage 5. To observe the effect of halofuginone for a range of concentrations, we created dose–response curves of both cell types. At high concentrations (>100 nmol/L), halofuginone equally inhibited the growth of both cell types. However, at some lower concentrations ≤50 nmol/L, halofuginone preferentially inhibited SMC versus EC proliferation (Figure 5). These results indicate that halofuginone is a preferential inhibitor of SMC versus EC proliferation in a certain range of concentrations.

**Halofuginone Inhibits Phosphorylation of Smad3 in Balloon-Injured Arteries as Well as in Cultured Rat Aortic SMCs But Not Aortic ECs**

We further explored the molecular mechanism by which halofuginone differentially inhibits SMC versus EC proliferation by assessing its effect on the canonical transforming growth factor-β (TGF-β)/Smad3 pathway. Our group has previously shown that TGF-β and its signaling effector Smad3 are upregulated after arterial injury and contribute to neointima formation by stimulating proliferation of vascular SMCs. Knocking down Smad3 inhibits SMC proliferation and IH. Interestingly, an inhibitory effect of halofuginone on Smad3 activation (phosphorylation) was observed in some other cell types but has not been examined in vascular SMCs. We, therefore, investigated whether halofuginone might inhibit Smad3 signaling differentially in SMCs versus ECs.

We first evaluated the effect of halofuginone treatment on phospo-Smad3 in the injured arterial wall, providing a mechanism for its inhibitory effect on SMC proliferation and IH. We found that pSmad3 (Figure I in the Data Supplement) was greatly increased in rat carotid arteries 14 days after angioplasty compared with uninjured arteries. Cells stained positively for pSmad3/Smad3 were located in the neointimal and medial layers (Figure 6A and 6D). In particular, these Smad3-expressing cells were enriched in the region adjacent to the lumen. Remarkably, treatment of injured arteries with halofuginone substantially reduced pSmad3 (by 54.9%; Figure 6C).

**Figure 4.** Halofuginone (HF) treatment preserves re-endothelialization in rat carotid arteries after angioplasty. **A,** Evans Blue staining. Arteries were treated at the time of balloon angioplasty with either vehicle (DMSO) or HF and stained with Evans Blue on d 14 after angioplasty. Shown are representative stained arteries; uninjured arteries were used as positive control. The areas denuded of endothelium are blue; re-endothelialized areas are unstained. Re-endothelialization was quantified and expressed as the ratio of unstained versus total area. Each bar represents a mean (±SEM) of 6 animals. **B,** Immunostaining for CD31. Arteries were treated at the time of balloon angioplasty with either vehicle (a) or HF (b) and collected on d 14 after angioplasty for CD31 immunostaining. Inset in a shows the distinction of CD31 staining (brown) and nuclei counterstaining with hematoxylin (blue). Uninjured artery serves as a positive control for CD31 staining (c). Arrow (blue) indicates external elastic lamina; arrow head (red) marks internal elastic lamina. Quantification (d) was performed as described in Methods section. Each bar represents a mean (±SEM) of 4 animals.

**Figure 5.** Differential inhibitory effect of halofuginone (HF) on the proliferation of smooth muscle cells (SMCs) versus endothelial cells (ECs). Dose–response experiments using human aortic SMCs and human aortic ECs are described in detail in Methods section (see the Data Supplement). Shown is a representative of 3 similar experiments. Each data point is a mean (±SD) of triplicates; *P<0.05 compared with SMCs at the same concentration (50 nmol/L).
We then compared in vitro the effect of halofuginone on Smad3 activation in SMCs and ECs, both primary cells from rat aorta (Figure 6G and 6H). The Western blot data demonstrate that whereas in SMCs halofuginone greatly reduced serum-stimulated Smad3 phosphorylation, pSmad3 levels in ECs were not changed by halofuginone treatment. We further determined whether the halofuginone effect on pSmad3 was mediated through the canonical TGF-β/Smad3 signaling pathway. Indeed, we found that in SMCs halofuginone inhibited TGF-β-activated Smad3 signaling but did not affect pSmad3 in ECs. Thus, the combined in vivo and in vitro data indicate that halofuginone effectively blocked phosphorylation of Smad3 in SMCs without altering Smad3 activation in ECs. These results suggest that halofuginone may preferably inhibit SMC versus EC proliferation through a mechanism that involves differential inhibition of Smad3 signaling in these 2 cell types.

**Discussion**

In this study we have made a novel finding that when locally administered to balloon-injured rat carotid arteries, halofuginone effectively induces adaptive remodeling and selectively inhibits neointima growth without impeding re-endothelialization. The sum of halofuginone’s effects is a dramatic 2-fold increase in lumen patency in treated arteries compared with controls. These favorable features of halofuginone suggest that this small molecule might have an important clinical role in the treatment of stenotic diseases.

An inhibitory effect of halofuginone on IH has been examined in 2 prior studies. However, there are several distinct and novel differences between our findings and those of the previous studies. First, in the prior studies, the models used were less relevant to restenosis (ear crush in 1 and carotid anastomosis in the other). Moreover, the effect of halofuginone in our study was substantial and much greater than what has been previously observed; we found a dramatic 2-fold increase of luminal area in arteries treated with halofuginone. Our findings show that the effect of halofuginone is largely related to its ability to influence adaptive remodeling. Lastly, we found that halofuginone has a selective effect on SMC proliferation and IH but spares re-endothelialization. Thus we think that halofuginone is the first small molecule that has been shown to produce a beneficial effect on all 3 major processes critical to restenosis (IH, adaptive remodeling, and re-endothelialization).

Although the effect of halofuginone on IH has been explored, how halofuginone affects arterial remodeling and endothelial recovery was not known. We tackled this question through rat carotid balloon angioplasty, a widely accepted model recapitulating restenosis despite the absence of an atherosclerotic plaque. The critical role of adaptive remodeling in the prevention of restenosis has been highlighted in several recent reports (for review, see Goel et al). Studies in the clinical setting and animal models have shown that constrictive remodeling can be an even more important contributor to
restenosis than IH. Geometrically, relatively small changes in overall vessel diameter produced by adaptive remodeling can result in major changes in luminal area. For example, a 10% increase in vessel diameter without a change in plaque area can result in a 150% increase in luminal area. In a similar manner, but conversely, constrictive remodeling can cancel the gain of luminal area that is made by effective inhibition of IH. Our own studies in the rat carotid injury model reveal that rapamycin, which has a substantial inhibitory effect on IH, is also associated with constrictive remodeling (K.C. Kent et al, unpublished data).

Although an intraluminally implanted stent can prevent constrictive remodeling, this approach is not applicable to open vascular reconstructions (>300,000 cases per year in the United States) including bypass and endarterectomy. Moreover, stents are costly; they have the ability to incite in-stent IH and stent-edge constrictive remodeling, making reintervention much more challenging. Thus, stent-free approaches are becoming highly desirable for treating stenotic arteries and veins. Manipulating arterial remodeling from constrictive to adaptive seems to be a promising strategy that can profoundly attenuate restenosis after open surgery or angioplasty without the need to implant a stent. There are only a limited number of drugs or molecular interventions that have been shown to produce adaptive remodeling. As well, the mechanisms that influence arterial remodeling remain poorly understood.

Adventitial extracellular matrix proteins, especially collagen, are thought to contribute to the mechanism of arterial remodeling. Constrictive remodeling resembles the fibrotic process that contributes to multiple diseases; excessive Col-1 accumulation leads to scarring and arterial constriction. As a specific inhibitor of Col-1 synthesis, halofuginone has shown efficacy in several conditions that have fibrosis as their cause. Thus, in a similar manner, by inhibiting Col-1, halofuginone may prevent constrictive remodeling. There are at least 2 mechanisms that may contribute to halofuginone-induced adaptive remodeling. First, inhibition of Col-1 production by halofuginone likely prevents excessive Col-1 deposition in the adventitia, thus mitigating the constrictive band that produces arterial narrowing. Second, the decrease of Col-1 content may alter the Col-1 to Col-3 ratio in the adventitia. Col-1 and Col-3 are the 2 major subtypes in the extracellular matrix of vasculature. While Col-1 confers rigidity and tensile strength, Col-3 provides elasticity to the vessel wall. Studies in non-human primates as well as humans reveal that an increased ratio of Col-3/Col-1 is beneficial for normal vascular function and adaptive remodeling. Moreover, we have recently found in balloon-injured rat carotid arteries that adaptive remodeling induced by perivascular application of connective tissue growth factor is associated with enhanced production of Col-3 versus Col-1. In the current study, a decrease in Col-1 (but not Col-3) content in halofuginone-treated arteries favorably increased the Col-3/Col-1 ratio and thus may have contributed to adaptive remodeling.

It is well recognized that both SMC proliferation and migration play critical roles in the formation of IH. Although an inhibitory effect of halofuginone on bovine arterial SMC proliferation has been previously observed, we provide herewith the first in vivo evidence of halofuginone inhibition of cell proliferation in the injured arterial wall. Moreover, we found that migration of rat SMCs is impeded by halofuginone as well. An effect of halofuginone on SMC migration has not been previously reported. This impairment of proliferation as well as migration of SMCs may to a great extent account for the mitigation of IH in halofuginone-treated arteries.

One of the major drawbacks of antirestenotic drugs that inhibit SMC proliferation is their similar negative impairment of EC proliferation. In fact, ECs are usually more sensitive to inhibitors compared with SMCs. Vessels undergoing reconstruction or angioplasty are denuded of the endothelial layer, and these drugs impair the process of re-endothelialization. The consequence is the potential for acute thrombosis because ECs provide the protective antithrombotic luminal lining. Well-known examples are the 2 currently clinically applied drugs, Rapamycin and Paclitaxel. In addition to their therapeutic effect of inhibiting SMC proliferation and migration, these drugs also suppress the growth, mobility, and survival of ECs, thus causing retarded re-endothelialization, thrombosis, and enhanced restenosis. Obviously, the high likelihood that drugs will inhibit both SMC and EC proliferation imposes a major challenge for current and future interventions to treat restenosis. Drugs that selectively inhibit SMC proliferation but not EC growth are highly desirable. Our data show that within a relatively large window of concentration (5–100 nmol/L), halofuginone preferentially inhibits SMC versus EC proliferation. Indeed, consistent with these in vitro findings, halofuginone treatment greatly inhibited injury-induced SMC neointimal proliferation but did not impair re-endothelialization. In contrast to our findings with halofuginone, periadventitial administration of Rapamycin has been shown to significantly inhibit re-endothelialization. Only very few agents have been reported to differentially inhibit SMC versus EC proliferation, and the molecular mechanisms for the selectivity are poorly understood. Halofuginone may provide a valuable tool for uncovering the mechanisms that differentially control SMC versus EC proliferation.

We investigated the mechanism underlying differential halofuginone inhibition of SMC versus EC proliferation through the TGF-β/Smad3 pathway, which is a critical player in both cell types. Both TGF-β and Smad3 are upregulated after arterial injury. Studies from our laboratory and others have demonstrated that the expression of Smad3 in injured arteries exacerbates IH, whereas the inhibition of Smad3 blocks SMC proliferation and IH. Although halofuginone has been previously shown to inhibit phosphorylation of Smad3 in pancreatic stellate cells and fibroblasts, our data show that halofuginone attenuates Smad3 phosphorylation in rat aortic SMCs as well. However in contrast, an inhibitory effect of halofuginone on Smad3 phosphorylation was not observed in rat aortic ECs. This differential inhibitory effect of halofuginone on the activation of Smad3 signaling in SMCs versus ECs is consonant with our observation that halofuginone effectively attenuated SMC proliferation and IH but did not inhibit regrowth of the endothelium after arterial injury. Thus, this novel information of halofuginone influence on Smad3 signaling provides interesting mechanistic insight into a preferential inhibition of SMC versus EC proliferation.
Study Limitations
The effect of halofuginone in healthy rat carotid arteries may be different from that in diseased human vessels, which may limit the ability to extrapolate the current findings to outcomes in humans. It should also be noted that we have limited ability to translate the concentrations that were effective in vitro to concentrations in vivo. Thus, in vivo studies, we applied higher concentrations of halofuginone assuming there would be substantial loss of the drug to the surrounding tissues because of diffusion. We were unable to measure the precise concentration of halofuginone present within the arterial wall. Nonetheless, we observed substantial inhibition of cell proliferation and IH by halofuginone in balloon-injured arterial wall, suggesting that the concentration we used in vivo recapitulated halofuginone’s in vitro effects.

Conclusions
We identified halofuginone as a drug that beneficially influences all the 3 major processes that contribute to restenosis in a rat angioplasty model. Halofuginone stimulates adaptive remodeling while suppressing intimal growth and has no inhibitory effect on the recovery of the endothelial layer. There are no other small molecules reported that possess all 3 of these favorable properties.

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Disclosures
None.

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Supplemental Materials

1. Supplemental Methods

Rat carotid artery balloon angioplasty model
Carotid artery balloon angioplasty was performed in male Sprague-Dawley rats (Charles River, 250-300g) as previously described\(^1\). Briefly, rats were anesthetized through inhalation of 2.5% isoflurane (the same throughout this study). The left common carotid artery was exposed through a midline cervical incision. A 2F Fogarty catheter (Edwards Lifesciences) was inserted into the common carotid artery via an arteriotomy in the external carotid artery. To produce arterial injury, the balloon was inflated and withdrew to the carotid bifurcation and this action was repeated three times. The external carotid artery was then permanently ligated, and blood flow was resumed. The animal studies conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, 1996 revision). All protocols were approved by the Institutional Animal Care and Use Committee at the University of Wisconsin.

Local perivascular HF delivery to injured arteries
In order to avoid potential systemic side effects, we treated balloon-injured arteries with HF using an established local drug delivery strategy as described in our previous report\(^1\). Briefly, to ensure complete solubility, 30 μg of HF from a DMSO stock was first dissolved in 30 μl of 10% DMSO and then mixed with 270 μl of 25% F-127 pluronic gel (Sigma-Aldrich) that was kept on ice. Immediately after balloon angioplasty in the left common carotid artery, the gel containing HF was applied to the outside of the injured segment of the carotid artery. In the control group, equal volume of vehicle (30 μl of 20% DMSO) mixed with pluronic gel was applied.
Morphometric analysis of adaptive remodeling, intimal hyperplasia, and restenosis

Two weeks after balloon angioplasty, common carotid arteries were collected from anesthetized animals (under 2.5% isoflurane) following perfusion fixation at a physiological pressure of 100 mmHg\(^1\), the animals were then euthanized in a CO\(_2\) chamber. Paraffin sections (5 \(\mu\)m thick) were excised at equally spaced intervals and stained with hematoxylin-eosin (H&E) for morphometric analysis, as described in our previous reports\(^1\).\(^2\). Nine sections from each of six either vehicle (DMSO) or HF-treated animals were used and an average parameter was calculated using total 54 sections. Planimetric parameters as follows were measured on the sections and calculated using Image J: the area inside external elastic lamina (EEL area) or internal elastic lamina (IEL area), lumen area, intima area (= IEL area- lumen area), and media area (= EEL area – IEL area).

Measurements were performed by a student blinded to the experimental conditions. In this report, adaptive remodeling is defined as an increase of EEL area; intimal hyperplasia is quantified as a ratio of intima area versus media area; restenosis is evaluated as a decrease in lumen area.

Immunostaining for assessment of collagen and pSmad3 levels and PCNA-positive proliferating cells in the arterial wall

Immunostaining was performed on carotid artery sections collected on day 14 after angioplasty following our published method\(^1\).\(^2\). Briefly, the sections were first incubated with each of the primary antibodies for 1h with a dilution ratio as follows: rabbit anti-Col-1 (Mybiosource), 1:100; rabbit anti-Col-3 (Fitzgerald), 1:750; rabbit anti-pSmad3 (Invitrogen), 1:100; rabbit anti-Smad3 (Invitrogen), 1:100; rabbit anti-PCNA (Santa Cruz), 1:200. The sections were then incubated with the ImmPRESS HRP-conjugated goat-anti-rabbit secondary antibody (Vector Laboratories, 1:200), followed by visualization with 3, 3-diaminobenzidine (DAB). The slides were counterstained with hematoxylin. Intensity of stained proteins on the sections was quantified using Image J. In each experimental group (DMSO control or HF treatment), at least 8 sections from each of 4 animals were used. For quantification of PCNA-positive cells, cell number was counted on 8-bit binary
images converted (by Image J) from the pictures of immunostained sections and normalized by the microscopic field. Cell counting was performed by a student blinded to experimental conditions.

**Assays for post-angioplasty re-endothelialization**

Re-endothelialization in balloon-injured arteries was evaluated on day 14 after angioplasty using Evans Blue assay according to the previously published method with minor modifications\(^3\). Briefly, 0.5 ml of 2% Evans Blue dye (Sigma-Aldrich) was injected into the saphenous vein of a rat anesthetized by 2.5% isoflurane. After 20 min, the rat was perfused with 20 ml of PBS buffer, the common carotid artery was then longitudinally opened and photographed on a white background. Remaining denuded areas were stained blue; unstained areas indicate re-endothelialization and were quantified using Image J. To further assess re-endothelialization, immunostaining of CD31 (an EC marker) was performed on carotid sections. Briefly, a goat anti-CD31 primary antibody (R&D Systems, 1:150) was incubated with the sections for 1h followed by an incubation with a biotinylated rabbit-anti-goat secondary antibody for 30 min. Immunostaining of CD31 was then visualized by using streptavidin-HRP and DAB. For quantification of re-endothelialization, we used Image J to measure the peri-luminal perimeter and the percentage of this perimeter that stained for CD31\(^4\).

**Cell culture and in vitro HF treatment**

Rat aortic vascular smooth muscle cells (SMCs) and fibroblasts were isolated from the thoracoabdominal aorta of male Sprague-Dawley rats (anesthetized under 2.5% isoflurane) based on a protocol using an enzymatic dissociation method\(^2\). Cells were used at passages 5-7 for all experiments and were maintained in DMEM supplemented with 10% fetal bovine solution (FBS) at 37 °C with 5% CO\(_2\). Cell viability was > 95% as indicated by Trypan Blue exclusion assay. For HF treatment, cells were first starved for 24h in DMEM containing 0.5% FBS, the medium was then replaced with fresh DMEM supplemented with 10% FBS in which HF was added to a desired
concentration. For control, equal volume of DMSO was added to a final concentration of 0.1 % (v/v versus medium).

**Proliferation assay for cultured rat aortic SMCs**

Proliferation of cultured SMCs was determined using cell number as a surrogate, for which Alamar Blue (Invitrogen) assay was performed following manufacturer's instructions. Cells were grown to 60% confluence, starved with 0.5% FBS, and then stimulated with 10% FBS in the presence of vehicle (DMSO) or HF. After 48h, Alamar Blue was added to the cell culture (1/10 of the medium volume), incubated for 24h, and then fluorescence was read using a Safir2 plate reader (Tecan, excitation/fluorescence: 530nm/590nm, bandwidth: 15 nm). A background from cell-free medium was subtracted.

**Scratch assay for migration of cultured SMCs**

The effect of HF on SMC migration was determined using the scratch wound assay as described previously. Rat SMCs were grown to 90% confluence in 6-well plates and then starved for 24h in DMEM containing 0.5% FBS. A sterile pipette tip was used to generate an ~1 mm cell-free gap, and dislodged cells were washed away with serum free DMEM. The medium containing either vehicle (DMSO) or 50 nM HF was added to the wells and incubated for 48h. Photographs were taken before (0h) and after (48h) the incubation, and cell migration was quantified by Image J based on the width of remaining cell-free gaps.

**HF dose-response assay**

To compare the effects of HF on SMCs and ECs, we performed dose-response experiments using primary human aortic SMCs and human aortic ECs as described previously. Both cell types were purchased from Lonza simultaneously and then expanded in their respective optimal culture media (SMCs in SmGM-2 with 10% FBS and ECs in EGM-2 with 2% FBS, Lonza). Cells at passage 5
were seeded (2700 cells/200 μl medium/well) in a 96-well plate and incubated for 24h to allow cell attachment. Vehicle control (0.1 μl DMSO) or HF was added to generate different concentrations. After a 72 h incubation, Alamar Blue was added (1/10 of the medium volume) and incubated for another 24h, and then fluorescence was read as described above; a background from cell-free medium was subtracted. Curve fitting was performed with the Graph Pad Prizm software.

**Western blotting for in vitro evaluation of protein levels**

Rat SMCs were lysed in RIPA buffer containing protease inhibitors (50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, and 10 μg/ml aprotinin). Alternatively, for detection of collagen secretion, culture media from fibroblasts were collected and concentrated using a Centricon filter (Millipore). Protein concentrations of either cell lysates or concentrated media were determined by Bio-Rad DC™ Protein Assay kit. Thirty micrograms of proteins from each sample were separated by SDS-PAGE on 10% gels and transferred to nitrocellulose membranes. Proteins of interest were detected by immunoblotting using the following primary antibodies and dilution ratios: Rabbit anti-Col-3 (1:1000) and anti-Col-1 (1:1000) from Fitzgerald, rabbit anti-phospho-Smad3 (1:1000) and anti-Smad3 (1:1000) from Invitrogen, and mouse anti-β-actin from Sigma-Aldrich. After incubation of the blots with HRP-conjugated secondary antibodies (goat anti-rabbit or goat anti-mouse, 1:5000, BioRad), specific protein bands on the blots were visualized by using enhanced chemiluminescence reagents (Pierce) and recorded using a LAS-4000 Mini imager. Intensity of the bands was quantified by Image J.

**Statistical analysis**

Data are presented as mean ± standard error (SEM). Statistical analysis was conducted using two-tailed unpaired Student’s t-test. Data are considered statistically significant when a P value is < 0.05.
Figure S1. Smad3 and pSmad3 are elevated in rat carotid arteries after angioplasty

Balloon angioplasty was performed as described in Methods. Uninjured right carotid artery (A, B, and C) and injured left common carotid artery (D, E, and F) were collected from each rat on day 14 following angioplasty. Sections were prepared and stained with H&E (A and D), or immunostained for Smad3 (B and E) or pSmad3 (C and F). Shown are representative examples. Arrowhead marks IEL.
3. References


