PDGF-Induced Migration of Vascular Smooth Muscle Cells Is Inhibited by Heme Oxygenase-1 Via VEGFR2 Upregulation and Subsequent Assembly of Inactive VEGFR2/PDGFRβ Heterodimers


**Objective**—In cardiovascular regulation, heme oxygenase-1 (HO-1) activity has been shown to inhibit vascular smooth muscle cell (VSMC) proliferation by promoting cell cycle arrest at the G1/S phase. However, the effect of HO-1 on VSMC migration remains unclear. We aim to elucidate the mechanism by which HO-1 regulates PDGFBB-induced VSMC migration.

**Methods and Results**—Transduction of HO-1 cDNA adenoviral vector severely impeded human VSMC migration in a scratch, transmembrane, and directional migration assay in response to PDGFBB stimulation. Similarly, HO-1 overexpression in the remodeling process during murine retinal vasculature development attenuated VSMC coverage over the major arterial branches as compared with sham-vector-transduced eyes. HO-1 expression in VSMCs significantly upregulated VEGFA and VEGFR2 expression, which subsequently promoted the formation of inactive PDGFβR/VEGFR2 complexes. This compromised PDGFβR phosphorylation and impeded the downstream cascade of FAK-p38 signaling. siRNA-mediated silencing of VEGFA or VEGFR2 could reverse the inhibitory effect of HO-1 on VSMC migration.

**Conclusion**—These findings identify a potent antimigratory function of HO-1 in VSMCs, a mechanism that involves VEGFA and VEGFR2 upregulation, followed by assembly of inactive VEGFR2/PDGFRβ complexes that attenuates effective PDGFβR signaling. (Arterioscler Thromb Vasc Biol. 2012;32:1289-1298.)

**Key Words:** PDGF ■ heme oxygenase ■ migration ■ vascular smooth muscle cell

Heme oxygenase-1 (HO-1) exerts a vascular protective function under physiological conditions and disease. Low basic levels of HO-1 in, for example, endothelial cells (ECs) and vascular smooth muscle cells (VSMCs), are significantly upregulated after exposure to its substrate heme and various forms of physical and chemical stress. Heme degradation by HO-1 generates bioactive products that affect the vascular system, including iron, biliverdin, bilirubin, and carbon monoxide (CO). In ECs, the protective function of HO-1 is well studied; HO-1 activation has been shown to be required for both tube formation and directional migration of ECs, a process that is attributed to the bioactive function of CO. In cardiovascular disease, correlation studies in patients with atherosclerosis and knockout studies in murine atherosclerosis models have shown that HO-1 inhibits plaque growth. More importantly, HO-1 is a crucial factor for preventing destabilization of advanced atherosclerotic lesions into (rupture) vulnerable plaques by suppressing immune activation. These studies identify HO-1 as a potent atheroprotective enzyme.

In VSMCs, the HO-1 products, CO and bilirubin, have been implicated to inhibit cell proliferation by promoting cell cycle arrest at the G1/S phase. However, the role of HO-1 in VSMC PDGFBB-induced migration remains to be further elucidated. We demonstrate that HO-1 impedes PDGF-induced VSMC migration by promoting the formation of inactive VEGFR2/PDGFRβ heterodimer complexes that attenuate PDGFβR downstream signaling. We report a novel mechanism by which HO-1 regulates VSMCs activity.

**Methods**

**Primary VSMC Cultures**

Primary human aorta-derived VSMC (Lonza, Breda, NL) were cultured on gelatin-coated plates at 37°C/5% CO₂ in SmGM-2 medium
supplemented with a commercial bullet kit, 10% FCS, and penicillin/streptomycin (Lonza). Only cell cultures of passages 3 to 6 were used in the experiments.

Adenoviral Vector–Mediated Gene Transfer and Targeted siRNA Knockdown in Cell Culture

The commercial pAd/CMV/V5 Gateway system (Invitrogen, Breda, NL) was used to generate recombinant adenoviral vectors according to the manufacturer’s recommendations. Viral particles were harvested from 293 cell lysates, and the viral titer was determined by viral plaque assays. ΔE1/E3-sham adenovirus was used as a control adenovirus (adsham) in all studies. Primary human VSMCs were transduced by adHO1 or adsham (MOI 100) in SmGM-2 basal medium in serum low conditions (2% FCS, Lonza) for 2 hours. Additional targeted knockdown of VEGFR2 was achieved by transfer of a mix of 4 specific siRNAs sequences directed against VEGFR2 mRNA (Smartpool, Dharmacon, Etterleure, NL) in 50% to 60% subconfluent VSMC cultures, at 24 hours before adHO1 or adsham transfection. As a control, cells were transfected with a mix of 4 scrambled, nontargeting siRNAs (Dharmacon). Overexpression of the HO-1 was verified by quantitative (q)PCR and Western blot analysis at 1 and 2 days after adenoviral transduction, respectively. Efficient silencing of VEGFR2 was assessed by qPCR analysis at 3 days after transfection.

A detailed description of the rest of the methods can be found in the online Data Supplement.

Results

HO-1 Inhibits Directional Migration of VSMCs Under PDGFBB Stimulation

We examined the effect of HO-1 overexpression on PDGF-mediated migration of human aorta-derived VSMCs. Adenoviral vector-mediated transduction of human HO-1 resulted in a significant increase in HO-1 mRNA and protein levels, as validated by qPCR and Western blot analysis as compared with sham adenovirus-transduced VSMCs at 48 and 72 hours, respectively (Figure 1A and 1B). To exclude any confounding effect of cell proliferation, the data of the migration assays were acquired after 4 hours of VSMC migration.

In a standard scratch assay, HO-1 expression reduced the migratory distance in response to 10 ng/mL PDGFBB by 62% (Figure 1C). To assess the effect of HO-1 on VSMCs during injury-independent migration, a transwell migration assay was also conducted. HO-1 overexpression attenuated PDGFBB-induced transwell-movement of VSMCs by 43%, as compared with sham virus–treated cells (Figure 1D).

Likewise, in a directional-migration assay, HO-1 expression in VSMCs decreased the absolute migration distance by 34% as compared with sham-transduced VSMCs (Figure 1E). The absolute migration distance is the distance that each tracked VSMC has migrated from its starting point to its end point during 2 hours of chemotactic migration in response to PDGFBB. Decrease in VSMC migration after HO-1 overexpression was associated with loss of cell polarity, which is required for directional migration.13 HO-1 significantly reduced polarization of the microtubuli-organizing center (MTOC) toward the direction of the cell-clear area, as shown by γ-tubulin staining of the MTOC and acetylated α-tubulin staining of the microtubule cytoskeleton (Figure 1F). As previously reported, HO-1 inhibits cell cycle progression in VSMCs via a p21<sup>−/−</sup>-dependent pathway.4 To distinguish between the effect of HO-1 on migration and cell proliferation, we conducted experiments in mitomycin-c–treated VSMCs in which cell cycle progression was blocked in the early prophase. HO-1–induced inhibition in VSMC migration was independent from the gene’s effect on cell proliferation, as a decrease in VSMC migration was observed in the mitomycin-c–treated cultures in response to PDGFBB (Figure 2A) or VEGFA stimulation (Figure 2B).

To identify the effective HO-1 metabolite in VSMC-migration repression, directional migration response to PDGFBB was assessed during coincubation with 50 μM and 100 μM of bilirubin or CO using the potent CO donor tricarborylidenochlororuthenium (II) dimer (CO-releasing molecule [CORM2]). Bilirubin did not affect VSMC migration (Figure 2C and Figure 1A in the online-only Data Supplement), whereas CO treatment significantly decreased VSMCs migration compared with cells treated with equimolar ruthenium (II) chloride hydrate (CORM-2 control) as matched controls (Figure 2D and Figure 1B in the online-only Data Supplement). The HO-1–induced antimigratory effect in VSMCs was dependent on HO-1 enzymatic activity, as coincubation of adHO1–transduced cells with the HO-1 inhibitor ZnPPIX restored VSMC migration in a dose-dependent manner (Figure 2E and Figure IC in the online-only Data Supplement).

HO-1 Decreases VSMC Migration During Postnatal Arterial Differentiation of the Retinal Vasculature in Mice

Next, we validated the inhibitory effects of HO-1 in vivo. During the postnatal retinal vasculature development in mice, mural cells including VSMCs and pericytes migrate over the major blood vessels of the vascular tree that are destined to become arteries. Intravitreal injection of adenovirus with a vector encoding the full-length murine HO-1 cDNA at day 4 induced significant upregulation of HO-1 mRNA as compared with sham virus-injected controls (Figure 3A). HO-1 expression did not affect the progression of the vasculature toward the retinal borders, as indicated by isolecitin-B4 staining of endothelial cells. To assess VSMC migration in vivo, the length of the VSMC-covered arteries was divided by the total distance between neural plexus and the retinal borders to calculate relative length (X=[A/B]*100) (Figure II in the online-only Data Supplement). HO-1 reduced the relative length of the VSMC-covered arteries (% distance in X) that originated from the optical disk, as shown by VSMC α-actin staining: HO-1 adenovirus-transduced retinas showed a 35% decrease in the relative length of the VSMC covered arteries as compared with sham virus-transduced controls (Figure 3B). To validate that vascular expansion is not affected by HO-1, the length of the vascular network was divided by the total distance between neural plexus and the retinal borders to calculate relative length, using isolecitin-B4 staining (for ECs, Y=[A/B]*100). The relative length (% distance Y) of the vascular network was not affected by HO-1 overexpression as compared with adsham-transduced controls.

Moreover, in normal retinal development, the proximal section of the side branches of the major retinal arteries are covered by VSMCs. HO-1 expression significantly reduced VSMC coverage of these side branches at junctional sites (Figure 3C), whereas the EC vessel density was not affected. To further validate these findings, Western blot analysis of the
Figure 1. HO-1 inhibits PDGFBB-mediated VSMC migration of cultured human VSMCs. A, qPCR analysis of HO-1 mRNA levels in human VSMCs treated with either adHO1 or adsham adenovirus, 48 hours after transduction. B, Western blot analysis for HO-1 of whole lysates of adHO1 or adsham-transduced human VSMCs, 72 hours after transduction. C, Scratch assay quantifying VSMCs migration in response to FCS (10% and 20%) or PDGFBB (10 ng/mL) from the scratch edge (indicated by the solid black line) at 12 hours. Bar graphs show the migration distance of the different groups. Data represent mean±SD of 3 different experiments. *P<0.05 versus control and adsham. D, Transwell migration assay using VSMCs. The number of cells migrated to the bottom of an 8-µm pore membrane in response to PDGFBB (10 ng/mL) were assessed after 6 hours, using calcein acetoxymethyl ester uptake. The bar graph shows the area of cell coverage on the membrane after migration. Data represent mean±SD of 4 separate experiments. *P<0.05 versus control and adsham. E, Live imaging for 2 hours in a directional migration assay of VSMCs identified the migration distance of individually tracked cells into the cell-cleared area. Data represent mean±SD of 4 separate experiments. A total of 20 cells were tracked in each experiment. *P<0.05 versus control and adsham. F, Cell polarization assessment in VSMCs, as identified by the microtubule-organizing center (MTOC) γ-tubulin (green fluorescent) and DAPI (blue) staining in the directional migration assay. Bar graph indicates the percentage of cells correctly polarized toward the cell-cleared area. White arrow indicates the direction of migration. Data represent mean±SD of 4 separate experiments. A minimum number of 40 cells were assessed in each experiment. *P<0.05 versus adsham.
endothelial marker CD31 and VSMC marker VSMC α-actin was performed. In line with the whole mount analysis, retinal protein levels of CD31 were not affected by HO-1 overexpression, whereas a significant increase in VSMC α-actin level was observed as compared with adsham-injected mice (Figure 3D). Assessment of β-actin showed no difference in loading/blotting efficiency. These data demonstrate that in the murine retinal vascular development model, HO-1 overexpression mainly affects VSMCs.

**HO-1 Attenuates PDGFRβ Signaling by Promoting VEGFR2/PDGFRβ Complex Formation**

To test whether the decline in migration efficiency in response to HO-1 could be attributed to poor PDGFRβ chemotactic signaling, we assessed PDGFRβ phosphorylation in primary human VSMCs in response to PDGFBB stimulation in vitro. Western blot analysis shows that PDGFBB stimulates PDGFRβ phosphorylation at the activation site Tyr1021 in sham-transduced VSMC cultures, whereas HO-1 overexpression diminishes the PDGFBB-induced response (Figure 4A). Immunoblotting for β-actin showed no differences between the 2 groups. PDGFRβ deactivation was associated with a significant upregulation in VEGFA and VEGFR2 mRNA levels in HO-1–expressing cells, as demonstrated by qPCR (Figure 4B). PDGFBB/VEGFA stimulation resulted in VEGFR2/PDGFRβ complex formation in primary human VSMCs, as detected by coimmunoprecipitation (co-IPP) (Figure 4C). HO-1 expression in VSMCs significantly increased VEGFR2 binding to PDGFRβ (Figure 4C), indicating that HO-1 promotes the formation of the inactive
VEGFR2/PDGFRβ complex. We validated these findings by conducting the Co-IPP with a VEGFR2-pulldown followed by PDGFRβ immunoblotting (Figure 4D). In line with our qPCR findings, HO-1 overexpression in VSMCs increased VEGFR2 protein levels, resulting in more capture of VEGFR2 protein on the Co-IPP beads (Figure 4D and 4E). VEGFR2/PDGFRβ complex formation could be confirmed in both adsham-treated and adHO-1–treated samples. Quantification of the Co-IPP PDGFRβ/VEGFR2 ratio showed no difference, due to an increase in VEGFR2 levels as a result of HO-1 expression (Figure 4E). However, a significantly higher level of PDGFRβ phosphorylation was detected in the PDGFRβ/VEGFR2 complexes formed in the adHO-1–treated group, as indicated by an increase in phospho-PDGFRβ/PDGFRβ ratio in the adHO-1 Co-IPP samples versus the Co-IPP adsham samples (Figure 4E). These data clearly indicate that
PDGFRβ/VEGFR2 complex formation is indeed increased in response to HO-1 expression.

Western blot analysis of whole lysates further showed that HO-1 prevented PDGFBB-induced FAK Y397-phosphorylation (Figure 5A). In line with a decline in PDGFRβ signaling, HO-1 overexpression in VSMCs decreased p38 phosphorylation as compared with sham-treated VSMCs (Figure 5B), whereas total p38, total ERK1/2, and
phospho-ERK1/2 protein levels remained unaffected (Figure 5A through 5C). Immunoblotting for β-actin showed no differences between the 2 groups.

**VEGFR2 Silencing Rescues HO-1–Induced Antimigratory Effects on VSMCs**

To validate whether the deleterious effects of HO-1 on VSMC migration is caused by VEGFR2/ PDGFRβ heterodimer complex formation, VEGFR2 was silenced using siRNA technology in HO-1–expressing VSMCs in a transwell migration assay. Efficient silencing of VEGFR2 was validated by qPCR analysis (Figure 6A). Transgenic HO-1 expression in VSMCs inhibited cell migration as compared with nontransduced and sham adenovirus-transduced VSMCs (Figure 6B). VSMCs treated with both adHO1 and nontargeting scrambled siRNA (sisham) showed a similar reduction in migration capacity in response to PDGFBB. However, transfection with siRNA targeting VEGFR2 rescued the attenuating effect of HO-1 on VSMC migration. Similarly, in a directional migration assay, the inhibitory effects of HO-1 on VSMCs migration could be reversed by VEGFR2 silencing (Figure 6C). Likewise, knockdown of VEGFA in HO-1 overexpressing VSMCs attenuated the inhibitory effect of HO-1 on directional migration (Figure 6D). Taken together, these data indicate that HO-1 impairs VSMC migration via VEGFA and VEGFR2 regulation. To verify that HO-1 interferes with VSMC migration is indeed mediated via p38 MAPK signaling, directional migration assays were conducted with VSMCs that were double-transduced with adHO-1 and a cDNA plasmid that expresses constitutively active MAPKAPK2, a direct downstream target of p38. Indeed, migration of adHO-1 treated VSMCs was successfully rescued by restoration of the p38 signaling cascade (Figure 6E). Knockdown efficiency was validated by qPCR analysis and showed ≈66% effective silencing of VEGFA and ≈2-fold induction of MAPKAPK2 as compared with the appropriate controls (sisham- or adsham-treated). These additional studies further provide proof for the involvement of p38 signaling and VEGFA in the observed HO-1–mediated effects on migration.

**Discussion**

This study provides evidence for HO-1–mediated interference of PDGFBB-induced migration of VSMCs, demonstrated in vitro using standard migration assays, and in vivo, using a murine model for retinal vasculature development. The data in the retina model identified HO-1 as a potent regulator of arterial vessel differentiation by modulating VSMC coverage.

Although the effect of HO-1 on VSMC proliferation via modulation of cell cycle progression regulatory proteins such as p21<sup>cip1</sup> and p27<sup>kip1</sup> has been well described, the effect of HO-1 on migration and subsequent regulatory mechanism must be further elucidated. Our studies identified HO-1 as a potent inhibitor of VSMC migration. Previously, Rodriguez et al have shown in vivo studies that HO-1 could impede VSMC migration via inhibition of Nox1 enzymatic activity. In the current study, we describe a novel molecular mechanism for HO-1 in VSMC regulation, providing first-time evidence that HO-1 impedes VSMC migration by interference of PDGFRβ signaling via VEGFR2 and VEGFA upregulation. It has been reported that HO-1 could promote tube formation and vessel sprouting in ECs. In the murine postnatal retinal vasculature development model, vascular network expansion occurs during the first 7 days after birth over the retinal surface from the neural plexus site toward the
Figure 6. VEGFR2 and VEGFA silencing rescues HO-1 inhibitory effects on PDGFBB-mediated VSMC migration. A, qPCR analysis of VEGFR2 mRNA levels of cell lysates derived from adsham-treated or adHO1 adenoviral vector–treated VSMCs. Data shown as mean±SD of 3 different experiments; *P<0.05 versus adsham and adHO1+siVEGFR2. B, PDGFBB stimulated transwell migration of human VSMCs. Cells migrated through an 8-µm-pore membrane were assessed after 6 hours of migration in response to PDGFBB. The bar graph shows the area of cell coverage on transwell membrane for the different groups. C, PDGFBB stimulated directional migration of human VSMCs. The white circle indicates the original cell-free area before migration. Cells were assessed after 4 hours of migration using Calcein-AM staining. Bar graph indicates the percentage of cell coverage of the cell-cleared area for the different groups. SiRNA-mediated knockdown of VEGFR2 in HO-1 expressing VSMCs restores cell migration efficiency in both assays. Data are presented as mean±SD of 3 separate experiments; *P<0.05 versus control, adsham, and adHO1/siVEGFR2. D, PDGFBB stimulated directional migration of human VSMCs. The white circle indicates the original cell-free area before migration. Cells were assessed after 12 hours of migration using Calcein-AM staining. Bar graph indicates the percentage of cell coverage of the cell-cleared area for the different groups. SiRNA-mediated knockdown of VEGFA in HO-1 expressing VSMCs successfully restores cell migration efficiency. Data are presented in mean±SD of 3 separate experiments; *P<0.05 versus control, adsham, sisham/adsham, and adHO1/siVEGFA. E, PDGFBB stimulated directional migration of human VSMCs. The white circle indicates the original cell-free area before migration. Cells were assessed after 12 hours of migration using Calcein-AM staining. Bar graph indicates the percentage of cell coverage of the cell-cleared area for the different groups. Adenovirus-mediated transduction of a cDNA plasmid that expresses constitutively active MAPKAPK2 in adHO-1-transduced VSMCs restores cell migration efficiency. Double adsham-treated VSMCs were used as controls. Data are presented as mean±SD of 3 separate experiments; *P<0.05 versus adsham+adsham and adHO1+adMAPKAPK2.
retinal boundaries. The lack of difference at day 8 between adHO1-treated and adsham-treated retinas in relative length of the vasculature indicate that at least in our model, vascular network expansion was not in vivo affected by HO-1 overexpression. In contrast, HO-1 impeded vascular coverage by VSMCs, both shown by reduction in the distance achieved by VSMC migration over the predeveloped vascular structures (Figure 2B), and a decrease in the numbers of vessel junctions that are covered by these mural cells (Figure 2C) in adHO1-transduced eyes. It was previously demonstrated that PDGFBB and VEGFA costimulation inhibited VSMC coverage of the EC vasculature in a matrigel angiogenesis model in mice, pointing toward an antagonistic relation between PDGF and VEGFA during neovascularization. In our study, HO-1 induced upregulation of VEGFR2 and VEGFA, which coincided with increased formation of inactive VEGFR2/PDGFRβ heterodimers. Indeed, it has been reported that PDGFBB/VEGFA coactivation could lead to competitive binding of VEGFR2 to PDGFRβ, resulting in inactive VEGFR2/PDGFRβ heterodimers. Previous studies reported that HO-1 could enhance VSMC production of VEGFA, whereas HO-1–derived CO release by VSMCs could downregulate PDGFBB production by neighboring endothelial cells. Therefore, HO-1 could induce a shift in VEGFR2 and PDGFRβ ratio, thereby promoting the formation of these heterodimers. In vitro migration assays comparing CO with bilirubin stimulation indeed show that CO is the main metabolic product of HO-1 involved in the inhibitory effect of the enzyme in line with previous reports of the cytostatic function of CO on VSMC during hypoxia. In our study, HO-1 significantly increased VEGFR2 and VEGFA levels, whereas PDGFRβ expression remained unaffected (data not shown), indicating that in the case of HO-1 upregulation in VSMCs, the shift in this VEGFR2/PDGFRβ ratio favoring heterodimer formation was mainly attributed to the rise in VEGFR2 signaling. Further evidence that VEGFR2 and VEGFA upregulation by HO-1 was responsible for its antimigratory function was provided by our additional in vitro migration experiments, which clearly showed that VEGFR2 or VEGFA silencing could rescue the HO-1–induced phenotype in VSMCs, indicating that inhibition of PDGFBB-induced VSMC migration by HO-1 is indeed mediated via a VEGFR2/VEGFA-dependent pathway.

We then further hypothesized that HO-1–induced VEGFR2/PDGFRβ heterodimer formation would diminish overall PDGF signaling by competing with active PDGFRβ homodimer formation. Indeed, PDGFRβ phosphorylation at the Tyr1021 activation site was significantly decreased by HO-1 in VSMCs, whereas total PDGFRβ remained unaffected. In addition, phosphorylation of PDGFRβ in the PDGFRβ/VEGFR2 complex was diminished by HO-1 overexpression. The PDGF signaling cascade further involves binding of the functional PDGFRβ homodimer complex to FAK, which subsequently triggers FAK activation via phosphorylation of its SH2-binding site Y397 by cSrc family members. Further downstream, PDGFBB signaling triggers activation of the mitogen activated protein (MAP) kinase family members, including ERK1/2 and p38. Whereas ERK1/2 activation is mainly required for cell proliferation, p38 activation is crucial for PDGFBB-induced actin-cytoskeleton reorganization during cell migration. In our experiments, HO-1 expression in VSMCs mainly impeded activation of the FAK-p38 signaling cascade, implying that the earlier reported inhibitory effects of HO-1 on VSMC proliferation is regulated via a PDGFBB independent pathway. It has been demonstrated that the antiproliferative effect of HO-1 on VSMCs was mediated via increased bioavailability of cyclic guanosine monophosphate (cGMP) activation, which subsequently leads to upregulation of p21^CIP1. The bioactive product of HO-1 CO converts via activation of soluble guanylyl cyclase, guanosine triphosphate (GTP), into cGMP. Therefore, HO-1 inhibition of VSMC proliferation could be attributed to a rise in intracellular CO levels.

Previously, we have shown that heme oxygenase 1 could inhibit cell cycle progression by inducing G1 cell cycle arrest. This additional function of the gene could complicate the interpretation of the data on migration. To exclude potential off-target effects of HO-1 on VSMC, we acquired the data in the different migration assays within 4 hours of incubation time. Because most primary human cells in culture including VSMCs go through the G1/G2 cell cycle once per 12 hours, it is very unlikely that cell division would have a profound effect on the migration results, as the division time for both the HO-1–silenced group and the controls are close to nonsignificant and therefore comparable. More importantly, live imaging of single cells followed during 2 hours of migration rules out the effect of HO-1 as a confounding factor and clearly shows that HO-1 impeded migration of VSMCs. In addition, migration experiments with the cell cycle inhibitor mitomycin-c have further validated the specific antimigratory function of HO-1.

Our findings imply that increased VEGFR2 activation and subsequent VEGFR2/PDGFRβ heterodimer formation could be involved in the protective effect of HO-1 overexpression that was previously observed in an animal model of arterial restenosis. Clinical restenosis, as observed in cardiovascular patients, is characterized by VSMC proliferation and migration in response to vascular injury during treatment, leading to pathological neointimal growth and lumen occlusion. New experiments are currently set up to assess the role of HO-1 in PDGFBB-induced migration of VSMCs in this disease.

In conclusion, this study identified HO-1 as an important regulator in the process of VSMC migration and described for the first time a novel molecular mechanism in which HO-1 induces VSMC insensitivity to PDGF chemotaxis, by compromising PDGFRβ signaling via VEGFR2 and VEGFA upregulation.

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Disclosures

None.

References


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Supplement Material
Material and Methods

VSMC migration assays

Scratch assay: VSMCs at 24 hours post transduction were grown to 80% confluency and serum-starved overnight in serum low conditions. The monolayer was scratched to generate a cell free area. VSMCs were stimulated with either SmGM-2 medium (Cambrex, NL) with 10 - 20% FCS or with PDGFBB 10 ng/ml. The cells were allowed to migrate for 4 hours before analysis.

Transwell migration assay: Human VSMCs at 24 hours post transduction were serum-starved overnight in serum low conditions before harvesting with Accutase (Lonza, NL). Cells were washed and 150000 cells/0.5 ml were seeded in the upper chamber of a 8 µm pore insert (BD Biosciences, NL) in a 24 wells plate. In the lower chamber, 1.5 ml of SmGM-2 medium supplemented with 2% FCS and PDGFBB 20 ng/ml was added. After 4 hours of migration, viable cells were visualized by Calcein-AM (BD Biosciences, NL). Quantification of the migrated cells was conducted using a commercial image analysis system (Impak C, Clemex Technologies, Canada).

Directional migration assay: Cell free areas were created by inserting flexible micro-plugs (AMS Biotechnology, Germany) into a gelatine coated 96 plate-wells. Human VSMCs at 24 hours post transduction were harvested with Accutase and 50000 cells were seeded per well in 100 µl complete SmGM-2 medium. After 8 hours of incubation, the VSMCs were serum-starved overnight in SmGM-2 medium with 2% FCS. The microplug barriers were removed the following day before addition of 100 µl SmGM-2 basal medium supplemented with 2% FCS and PDGFBB 20 ng/ml per well unless stated otherwise. After 4-12 hours of migration, viable cells were visualized by Calcein-AM uptake (BD Biosciences, NL). For cell-cycle inhibition studies, VSMCs were treated with mitomycin-c (Sigma, NL), 2 hours prior assay initiation. For co-incubation studies,
bilirubin (Sigma, NL), tricarbonyldichlororuthenium (II) dimer (CO-releasing molecule (CORM2, Sigma, NL) and ruthenium (II) chloride hydrate (CORM-2 control, Sigma, NL), or Zn(II) Protoporphyrin IX (ZnPPIX, Scientific Frontier, UK) were added to the culture during assay progression. For time lapse imaging of VSMC migration, the above described protocol was carried out in an Attofluor incubation chamber and image acquisition was performed on an Axiovert 100 M microscope equipped with an Axiocam MRC digital camera (Carl Zeiss, NL). During the timelapse experiments, cells in the incubation chamber were maintained at 37°C in a constant humidified atmosphere, with controlled and heated CO₂ flow. Imaging took place every 12 min for four hours. Migratory distance was determined using Axio Vision software (Carl Zeiss, NL).

**MTOC analysis by immunofluorescence microscopy**

Cells were washed twice with PBS and fixed in 4% paraformaldehyde at room temperature for 15 minutes and permeabilized using 0.15% Triton-X-100 for 10 min in blocking solution (1% BSA/0.05% Tween-20/PBS). Incubations with primary (1/200) and secondary (1/500) antibodies were performed for 1 hour at room temperature in blocking solution. Thereafter, cells were briefly washed in 70% and 100% ethanol, air-dried and mounted onto microscope slides using 10 μl of a 1:1 solution of VectaShield (Vector Laboratories, NL) and DAPI-DABCO (Molecular Probes, NL). The (microtubule-organizing center) MTOC was detected using a primary antibody against γ-tubulin (Sigma, NL), followed by a secondary Fitc-labeled antibody (Molecular Probes, NL) for detection. The microtubule network was counter-stained to ensure correct localization of the MTOC signal, using a primary antibody directed against acetylated tubulin (Sigma, NL). Immunofluorescent images were acquired using an Axiovert 100 M microscope (Carl Zeiss, NL) and an ORCA II ER camera (C4742-98, Hamamatsu
Photonics Systems, Japan). Image analysis of MTOC reorientation was performed using Openlab 3.1.5 software (Improvision, UK).

**Quantitative PCR, Western blot analysis and co-immunoprecipitation**

RNA was isolated using the RNAeasy kit (Qiagen, NL) and was checked for quality and quantity by capillary electrophoresis (Agilent 2100 Bioanalyzer, Agilent Technologies, NL), and reversed transcribed into cDNA. qPCR reactions were performed by real-time fluorescence assessment of the sybergreen signal using the iCycler iQ Detection System (Bio-Rad, NL) as described previously\(^1\). qPCR analysis was performed for transcripts of murine and human HO-1, VEGFA, and VEGFR2. Target mRNA expression levels are reported relative to the housekeeping genes; hypoxanthine guanine phosphoribosyl transferase (Hprt1) in murine samples, and β-actin in the human samples. For Western blot analysis, human VSMCs at 48 hours post transduction were overnight serum-starved and replenished with SmGM-2 basal medium supplemented with 2% FCS, VEGFA 40 ng/ml and PDGFBB 20 ng/ml for 30 minutes. The cells were lysed in NP40 buffer, and analysed on a 12.5% SDS-PAGE gel followed by immunoblotting using 1:1000 anti-p38, anti-p38 phospho-T180&Y182 antibody (Abcam, UK), 1:2000 anti-FAK, anti-FAK phospho-Y397 antibody and anti-ERK1/2 antibody (Abcam, UK), 1:1000 anti-ERK1/2 phospho-T105&Y187 antibody (Abcam, UK), and 1:1000 anti-HO-1 (OSA-110, Stressgen, NL) for protein analysis. 1:2000 β-actin was used as a loading control (Abcam, UK). Protein bands were visualized using the Li-Cor detection system (Westburg, NL). For PDGFRβ or VEGFR2 protein-complex co-immunoprecipitation, magnetic beads (Dynabeads, Invitrogen, NL) were coated and cross-linked using 2.5 µg PDGFRβ antibody (Bioscience, NL) or VEGFA antibody (Abcam, NL) before immunoprecipitation overnight at 4°C with cell lysates of transduced human VSMCs (50 µg total protein in 100 µl incubation buffer supplied by the Dynabeads system). Beads
were washed and protein samples were eluted with eluation buffer (Invitrogen, NL) before analysis on a 12.5% SDS-PAGE gel, followed by immunoblotting using 1:500 anti-PDGFRβ and anti-VEGFR2 (Abcam, UK), and 1:500 anti-PDGFRβ phospho-Tyr 1021 (Bioconnect, NL).

**Mouse retinal angiogenesis model**

All experiments were conducted in compliance with institutional (Erasmus University Medical Center, Rotterdam, The Netherlands) and national guidelines. 4 days old C57bl/6 pups were anesthetized by placement on ice. 0.5 µl adHO1 (5x10⁷ pfu) was intra-vitreally injected in the left and 0.5 µl sham adenovirus (5x10⁷ pfu) in the right eye using a 33-gauge needle. At day 4 post-transduction, pups were sacrificed. The eyes were dissected to obtain the murine retinas, separating the retina from the choroid layer and taking care not to damage the borderzones that connect with the lens. After fixation in 4% PFA/PBS, the retinas were stained whole mount with rhodamin/Fitc isolectin-B4 (1:200) or anti-VSMC α-actin antibody conjugated to rhodamin (1:200, Sigma, NL) before assessment under the fluorescence microscope. A typical result of whole mount Immuno-fluorescence staining of the developing vasculature in the retinas of 8 days old mice is shown in supplemental data (Supplemented data figure II). The left micrograph shows the presence of α-actin+ VSMCs (red fluorescent signal) on developing arterial vessels that radiate from the neural plexus towards the edge of the retinal structure. The right micrograph shows the developing vasculature, identified by isolectin-B4+ ECs (green fluorescent signal). Note that although the vasculature has already reached the retinal borderzone, arterial specification is still expanding towards the retinal edges, with VSMCs actively migrating down the vascular tree away from the neural plexus. To assess VSMCs migration *in vivo*, the length of the VSMC-covered arteries was divided by the total distance between neural plexus and the retinal borders to calculate relative length,
using VSMC α-actin staining (for VSMCs: X=(A/B)*100). To validate that vascular expansion is not affected by HO-1, the length of the vascular network was divided by the total distance between neural plexus and the retinal borders to calculate relative length, using isolectin-B4 staining (for ECs Y= (A/B)*100). Adequate transgene expression was validated by qPCR analysis at day 2 post-injection from dissected retinas. For protein analysis, adsham and adHO1 treated retinas were harvested 4 days post-injection and were pooled per 4-5 mice per group. Samples were lysed in NP40 buffer, run on a 12.5% SDS-PAGE gel followed by immunoblotting using 1:1000 anti-CD31 (Abcam, UK), 1:400 anti-VSMC α-actin (Sigma, NL), and 1:2000 β-actin (Abcam, UK).

**Statistical analysis**

Statistical analysis was performed by Student’s *t*-test (2 samples comparison analysis), or by One-Way ANOVA (> 2 samples comparison analysis). Data are presented as mean ± STDEV. P values <0.05 were considered statistically significant.
Figures

A

control | Bilirubin 50µM | Bilirubin 100µM

B

CORM2 contr 50µM | CORM2 50µM

CORM2 contr 100µM | CORM2 100µM
Figure I: Effect of HO-1 metabolites on PDGFBB-stimulated directional migration of human VSMCs. White circles indicate the original cell-free area before migration. Cells were assessed after 12 hours of migration using Calcein-AM staining for visualization. (A) Co-incubation with 50μM or 100μM bilirubin are compared with non-
treated controls. No effect on VSMC migration was observed. (B) Co-incubation with the CO-donor CORM2 (100 µM and 100 µM) significantly decreased VMSC migration compared to co-incubation with CORM2 control compound (50 µM and 100 µM respectively). The anti-migratory effect of HO-1 overexpression could be reversed by inhibition of HO-1 activity by co-incubation with ZnPPIX (3 µM and 10 µM). (C) Typical results of control, adsham and adHO-1 treated VSMC migration in response to PDGFBB are shown. HO-1 overexpression significantly inhibited VSMC migration as compared to adsham-treated and non-treated controls. Co-incubation with the highest concentration ZnPPIX (10 µM) fully restored migratory capacity of adHO-1 treated VSMCs. Data represent mean ± STDEV of 3 separate experiments. A minimal number of 10 wells per group were assessed in each experiment. *P<0.05 versus adsham and control groups.
Figure II: A typical result of whole mount Immuno-fluorescence staining of the developing vasculature in the retinas of 8 days old mice

(A) The left micrograph shows the presence of α-actin+ VSMCs (red fluorescent signal) on developing arterial vessels that radiate from the neural plexus towards the edge of the retinal structure. The right micrograph shows the developing vasculature, identified by isoelectin-B4+ ECs (green fluorescent signal). Note that although the vasculature has already reached the retinal borderzone, arterial specification is still expanding towards the retinal edges, with VSMCs actively migrating down the vascular tree away from the neural plexus. To assess VSMCs migration in vivo, the length of the VSMC-covered arteries was divided by the total distance between neural plexus and the retinal borders to calculate relative length, using VSMC α-actin staining (for VSMCs: X=(A/B)∗100). To validate that vascular expansion is not affected by HO-1, the length of the vascular
network was divided by the total distance between neural plexus and the retinal borders to calculate relative length, using isolectin-B4 staining (for ECs $Y = (A/B) \times 100$).

References