Hic-5 Mediates TGFβ–Induced Adhesion in Vascular Smooth Muscle Cells by a Nox4-Dependent Mechanism


Objective—Focal adhesions (FAs) link the cytoskeleton to the extracellular matrix and as such play important roles in growth, migration, and contractile properties of vascular smooth muscle cells. Recently, it has been shown that downregulation of Nox4, a transforming growth factor (TGF) β-inducible, hydrogen peroxide (H₂O₂)–producing enzyme, affects the number of FAs. However, the effectors downstream of Nox4 that mediate FA regulation are unknown. The FA resident protein H₂O₂-inducible clone (Hic)-5 is H₂O₂ and TGFβ inducible, and a binding partner of the heat shock protein (Hsp) 27. The objective of this study was to elucidate the mechanism, by which Hic-5 and Hsp27 participate in TGFβ-induced, Nox4-mediated vascular smooth muscle cell adhesion and migration.

Approach and Results—Through a combination of molecular biology and biochemistry techniques, we found that TGFβ, by a Nox4-dependent mechanism, induces the expression and interaction of Hic-5 and Hsp27, which is essential for Hic-5 localization to FAs. Importantly, we found that Hic-5 expression is required for the TGFβ-mediated increase in FA number, adhesive forces and migration. Mechanistically, Nox4 downregulation impedes Smad (small body size and mothers against decapentaplegic) signaling by TGFβ, and Hsp27 and Hic-5 upregulation by TGFβ is blocked in small body size and mothers against decapentaplegic 4–deficient cells.

Conclusions—Hic-5 and Hsp27 are effectors of Nox4 required for TGFβ-stimulated FA formation, adhesion strength and migration in vascular smooth muscle cell. (Arterioscler Thromb Vasc Biol. 2015;35:1198-1206. DOI: 10.1161/ATVBAHA.114.305185.)

Key Words: cell migration assay □ focal adhesions □ Hic5 protein □ Hsp27 protein □ Nox4 protein □ vascular smooth muscle

Nox proteins are the catalytic subunits of a family of nicotinamide adenine dinucleotide phosphate oxidases.¹ Nox homologs are primary sources of reactive oxygen species in vascular tissues that participate in numerous physiological and pathophysiological processes.³ In particular, in vascular smooth muscle cells (VSMCs) from rodent conductance arteries, 2 of these homologs are expressed: Nox1 and Nox4.² Nox4 localizes to focal adhesions (FAs), stress fibers, and the nucleus³ and is upregulated by transforming growth factor (TGF) β. Its hydrogen peroxide (H₂O₂)–producing activity is both causal for, and a consequence of, small body size and mothers against decapentaplegic (Smad)–dependent pathways.⁴⁵ Importantly, Nox4 has profound effects on the VSMC cytoskeleton and FAs. In fact, Nox4 is required for the expression of VSMC contractile apparatus proteins⁶ and is essential for FA formation.² However, the downstream effectors responsible for the regulation of cytoskeleton dynamics by Nox4, especially those that regulate synthesis and degradation of FA-related proteins, have yet to be identified.

FAs are specialized sites through which the cytoskeleton connects to the extracellular matrix. They are highly organized dynamic structures with rich signaling machinery capable of executing decisions about the cellular responses to a variety of stimuli. For instance, in VSMCs, FA turnover regulates migratory⁴ and biomechanical⁵ properties and thus is likely to affect vascular diseases. The components of FAs are diverse and

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include signaling proteins, as well as scaffolding molecules such as Paxillin\textsuperscript{10} and the \( \text{H}_2\text{O}_2 \)-inducible clone (Hic)-5\textsuperscript{11} that interact with Vinculin in a Rho GTPase-regulated manner.\textsuperscript{12} Hic-5, first identified as an \( \text{H}_2\text{O}_2 \) and TGF\( \beta \)-inducible gene,\textsuperscript{13} is now recognized as a member of the Paxillin family of proteins and functions as an adapter in FAs.\textsuperscript{11} In VSMCs, Hic-5 expression has been functionally implicated in the regulation of migration and injury-induced neointima formation\textsuperscript{14} and in the cellular responses to uni-axial stretch.\textsuperscript{15,16}

The heat shock protein (Hsp) 27 is a chaperone protein that confers cellular tolerance to a variety of stresses including heat and oxidative stress\textsuperscript{17} and its expression is increased by \( \text{H}_2\text{O}_2 \).\textsuperscript{18} Importantly, Hsp27 interacts with the actin cytoskeleton and has been shown to participate in the stabilization of FAs in response to heat shock stress.\textsuperscript{19} Interestingly, it has also been identified as a Hic-5-binding protein.\textsuperscript{20}

In this study, we hypothesized that Hic-5 and Hsp27 are downstream effectors of Nox4 that are required for proper FA assembly in VSMCs. We found that TGF\( \beta \) induces the expression of both Hic-5 and Hsp27 via a Nox4- and Smad-dependent mechanism, Hsp27 helps Hic-5 translocate to the FA, and Hic-5 expression is absolutely required for TGF\( \beta \)-induced, Nox4-mediated FA formation.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

We have previously shown that downregulation of Nox4 or its activator Poldip2 significantly reduces the number of FAs in VSMCs.\textsuperscript{7} We first confirmed that, as previously reported for other cell types,\textsuperscript{14-16,21,22} Hic-5 localizes to FAs in VSMCs. Using an in situ proximity ligation assay, we found that in human aortic smooth muscle cells (HASMCs) Hic-5 colocalizes with Vinculin and p-FAK, as shown by the characteristic punctate staining indicative of protein interaction (Figure 1A and 1B, respectively). Hic-5 localization to FAs was increased more than 2× in the presence of TGF\( \beta \), a well-established Nox4 agonist\textsuperscript{23,24} and a positive regulator of FAs in VSMCs.\textsuperscript{24} Because Hic-5 is a FA localized protein and previous reports showed that Nox4 also localizes to the FA, we evaluated if Hic-5 colocalizes with Nox4. Indeed, we found that both the proteins colocalize in FA in an association that is increased after TGF\( \beta \) treatment (Figure 1C). For these studies, we used a custom made antibody against Nox4 (characterized in Figure 1A in the online-only Data Supplement).

Nox4 is activated by TGF\( \beta \) to produce \( \text{H}_2\text{O}_2 \). Because Hic-5 was first identified as a \( \text{H}_2\text{O}_2 \)-inducible clone also activated by TGF\( \beta \) treatment,\textsuperscript{13} we hypothesized that TGF\( \beta \) mediates upregulation of Hic-5 and that this mechanism may require \( \text{H}_2\text{O}_2 \) produced by Nox4. Indeed, TGF\( \beta \) significantly increases Hic-5 expression in HASMCs as early as 6 hours after the treatment and Hic-5 stays elevated during a period of 24 hours (Figure 1D). Importantly, transfection of HASMCs with siNox4 (which successfully downregulates Nox4 mRNA and protein without affecting Nox1 protein levels; Figure IA–IC in the online-only Data Supplement, respectively) abolished TGF\( \beta \)-induced Hic-5 protein expression (Figure 1E). This effect seems to be specific for Nox4 because Nox1 deficiency had no effect on the ability of TGF\( \beta \) to upregulate Hic-5 expression (Figure 1D in the online-only Data Supplement). To determine if this effect occurs at the mRNA level, we used reverse transcription quantitative polymerase chain reaction. We found that TGF\( \beta \) significantly increased Hic-5 mRNA, and that this increase is completely abolished when Nox4 is downregulated (Figure 1F). The regulation of Hic-5 by the TGF\( \beta \)/Nox4 pathway is specific because neither Nox4 nor TGF\( \beta \) had any effect on the expression of the close homolog Paxillin at the mRNA or protein level (Figure IIA and IIB in the online-only Data Supplement). Moreover, this effect is likely to be mediated by \( \text{H}_2\text{O}_2 \) derived from Nox4 because the upregulation of Hic-5 induced by TGF\( \beta \) was also blocked by pretreatment with the antioxidant N-acetyl cysteine (Figure 1G), and more importantly, exogenously added \( \text{H}_2\text{O}_2 \) (to a final concentration of 50 \( \mu \text{mol/L} \)) was able to partially recover TGF\( \beta \)-induced Hic-5 expression in Nox4-deficient cells (Figure 1H). Finally, it is important to note that downregulation of Hic-5 had no effect on Nox4 expression (Figure 1B in the online-only Data Supplement).

TGF\( \beta \) superfamily ligands bind to a TGF type II receptor, which recruits and phosphorylates a TGF type I receptor.\textsuperscript{25} The type I receptor then phosphorylates Smads2/3, which recruits and phosphorylates a TGF type I receptor.\textsuperscript{25} Given that Figure 1 clearly shows that Nox4 is required for Hic-5 expression, we sought to determine if the Smad pathway is required for TGF\( \beta \)-induced upregulation of Hic-5 in HASMCs. Our results (Figure 2A and 2B) show that Hic-5 mRNA and protein upregulation was significantly blunted in Smad-deficient cells. Moreover, downregulation of Nox4 significantly inhibited TGF\( \beta \)-induced Smad2 phosphorylation (Figure 2C), suggesting that Smad pathways are downstream of Nox4 in Hic-5 mRNA regulation. This is likely to be a direct effect of Nox4-derived \( \text{H}_2\text{O}_2 \) because Smad2/3 can be phosphorylated by exogenously added \( \text{H}_2\text{O}_2 \) (Figure IIIA in the online-only Data Supplement), but the absence of Nox1 does not impair the ability of TGF\( \beta \) to induce Smad phosphorylation in HASMCs (Figure IIIB in the online-only Data Supplement). Together, these data demonstrate that TGF\( \beta \) induces Hic-5 mRNA expression by a Nox4- and Smad-dependent mechanism.

In addition to mRNA regulation, TGF\( \beta \) may control the subcellular localization of Hic-5. In this regard, more than a decade ago, Jia et al\textsuperscript{20} identified Hic-5 as an Hsp27-binding protein using 2-hybrid screening. Because Hsp27 is a chaperone protein known to modulate FAs, we hypothesized that Hsp27 may participate in the TGF\( \beta \)/Smad/Nox4 pathway by increasing Hic-5 protein stabilization and aiding its localization to FAs. Because HASMCs have a low basal expression of
Hsp27, we first evaluated the ability of TGFβ to induce Hsp27 expression. As shown in Figure 3A, TGFβ was not only capable of significantly inducing Hsp27 expression, but also it did so in a Nox4-dependent manner that requires H2O2 production, as shown by the ability of siNox4 and N-acetyl cysteine to inhibit the Hsp27 upregulation by TGFβ (Figure 3A and 3B).

**Figure 1.** The focal adhesion protein hydrogen peroxide (H2O2)–inducible clone (Hic)-5 is regulated by transforming growth factor (TGF) β via a Nox4-dependent mechanism. A and B, Hic-5 localization to focal adhesions increases with TGFβ treatment. HASMCs were plated and maintained in serum-free medium for 2 days before treatment with TGFβ (2 ng/mL). Colocalization (red label) between Hic-5 and Vinculin (A), and Hic-5 and phospho-focal adhesion kinase (pFAK) (B) was determined by in situ proximity ligation assay (Duolink). C, Colocalization of Hic-5 and Nox4 in HASMCs is increased with TGFβ treatment. HASMCs were plated and serum deprived for 2 days and then treated with TGFβ for 24 hours. Cells were stained for Hic-5 (red) and Nox4 (green), and nuclei were detected with 4′,6-diamidino-2-phenylindole (blue). Images were captured using confocal microscopy. Colocalization is evident in yellow in the right panel. D–H, TGFβ increases Hic-5 expression via Nox4-produced H2O2. After 2 days of serum deprivation, HASMCs were treated with TGFβ (2 ng/mL) for the indicated times and Hic-5 was detected by Western blot (WB) (D). HASMC were transfected with nonsilencing RNA (siNeg) or siNox4 and treated with TGFβ (2 ng/mL) for 24 hours to analyze Hic-5 protein (E) and mRNA (F) by WB and reverse transcription quantitative polymerase chain reaction, respectively. G, Cells incubated with or without N-acetyl cysteine (10 mmol/L) for 2 hours before addition of TGFβ for 24 hours were analyzed by western blotting. H, HASMCs transfected with siNeg or siNox4 were treated with either TGFβ (2 ng/mL) or TGFβ (2 ng/mL)+H2O2 (50 μg/mL) and the expression of Hic-5 was analyzed by WB. All experiments were performed at least 3 independent times. Data are presented as average±SEM of three independent experiments.
Similar to what we found for Hic-5, TGFβ-induced Hsp27 expression was not affected by the absence of Nox1 (Figure IV in the online-only Data Supplement). Together, these results prompted us to evaluate the Smad-dependence of Hsp27 expression, and we observed that siRNA against Smad4 completely blocked the increase in Hsp27 observed after TGFβ treatment (Figure 3C). These data indicate that, similar to Hic-5, Hsp27 is upregulated in HASMCs after TGFβ treatment by a Nox4-dependent mechanism that requires Smad pathways.

As a chaperone protein, Hsp27 is likely to affect Hic-5 in 2 possible ways: increasing its protein stability or affecting its subcellular localization. In agreement with previous reports, we found that Hic-5 and Hsp27 physically interact after TGFβ treatment as they communoprecipitate (Figure 4A). However, their interaction has no consequence on Hic-5 protein stability or expression because downregulation of Hsp27 does not affect Hic-5 mRNA or protein levels (Figure VA and VB in the online-only Data Supplement, respectively). Significantly, we found that Hsp27 was required for proper localization of Hic-5 to FAs after TGFβ treatment (Figure 4B). Interestingly, this effect seems to be specific for Hic-5 because the other closely related member of the family, Paxillin, localizes to FAs even in Hsp27-deficient cells (Figure 4B).

Our results demonstrate that Hic-5 is regulated by a Nox4-dependent mechanism and that association with Hsp27 is required for its proper localization to the FAs. Once we established the upstream signaling pathway that regulates Hic-5, we focused on examining the downstream consequence of Hic-5 expression on FA formation and cell adhesion in HASMC. We found that, as measured by Vinculin staining, downregulation of Hic-5 resulted in significantly reduced numbers of FAs (Figure 5A and 5B), resembling the loss of FAs observed when either Nox4 or Hsp27 is downregulated (Figure 4B). This effect was more evident when the cells were stimulated with TGFβ, and was not due to Hic-5–mediated downregulation of Vinculin expression (Figure VI in the online-only Data Supplement).

Finally, we investigated the physiological relevance of this pathway. We reasoned that the role of this pathway to modulate FA composition and size may affect the ability of the cell to engage extracellular matrix components, and accordingly, it is likely to affect cell adhesion and as a consequence of impaired adhesion, cell migration.

To evaluate the role of Hic-5 in the regulation of TGFβ-mediated HASMC adhesion strength, we measured the force required to detach the cell from the extracellular matrix, which correlates with FA function, number, and size. To measure cell adhesion strength, we used the spinning disk system, which exposes an adherent cell population to a range of hydrodynamic shear forces that increase linearly with radial distance from the disk center. Subsequently, the number of adherent cells was counted at different radial positions, corresponding to known shear stress values. The adherent cell fraction decreases nonlinearly with respect to fluid shear stress, and cell adhesion
strength is defined as the fluid shear stress that produces 50% cell detachment. We observed that TGFβ treatment for 24 hours significantly increases HASMC adhesion strength, and that this effect is abrogated by Hic-5 downregulation (Figure 6A and 6B). Cell adhesion, together with motor-clutch–mediated force transmission, is necessary to generate traction that moves the cell forward during cell motility. Therefore, we evaluated if Hic-5-deficient cells exhibit impaired cell migration. Indeed, cells where Hic-5 has been downregulated using siRNA display significantly lower motility toward a chemoattractant stimulus than control cells (Figure 6C), similar to results previously obtained with knockdown of Nox4.

**Discussion**

Our previous work showed that Nox4 is a critical component of FAs. Indeed, not only is Nox4 an FA-resident protein but also its absence significantly reduces the number of FAs in the cell. However, and despite the striking nature of this effect, little is known about the means by which Nox4 affects FA. In this work, we identify 2 important downstream effectors of Nox4, such as Hic-5 and the chaperone protein Hsp27. We describe a mechanism by which Nox4 increases Hsp27 and Hic-5 expression and promotes their physical interaction, which is necessary for the proper localization of Hic-5 to FA, especially after TGFβ treatment (Figure 6C). Most importantly, the localization of Hic-5 to the FAs is functionally relevant because it mediates the TGFβ-induced increase in adhesion strength (Figure 6A and 6B) and cell migration (Figure 6C).

Manipulation of Nox4 and Hsp27 had no effect on Paxillin expression (Figure IIA and IIB in the online-only Data Supplement) or localization (Figure 4B). Because Paxillin
and Hic-5, both members of the Paxillin family of proteins, share highly conserved structural domains and exhibit extensive cross-talk and synergy, it is particularly interesting that the TGFβ/Nox4 pathway uses Hic-5 to strengthen FAs. This specificity seems to lie in 2 factors: the ability of the TGFβ/Nox4 pathway to induce Hic-5 expression (Figures 1D–1H) and the unique association of Hic-5 with Hsp27 (Figure 4A).

The fact that Hic-5 is positively regulated by a Nox4-dependent mechanism is not surprising considering that Hic-5 has been identified as a marker for smooth muscle contractile phenotype and differentiation in this cell type is a Nox4-dependent process. However, it was recently reported that in a lung myofibroblast cell line, Hic-5 functions as a negative regulator of myofibroblast differentiation, raising the interesting possibility that the role of Hic-5 is cell type dependent.

Furthermore, our data clearly indicate that it is Nox4-derived H$_2$O$_2$ that is responsible for the TGFβ-mediated increased of Hic-5 expression. The ability of exogenous H$_2$O$_2$ to reverse the effect of the siNox4 (Figure 1H) demonstrates that Nox4-produced H$_2$O$_2$ participates in the signaling pathways initiated by TGFβ in VSMC. This conclusion agrees with previous reports showing that in VSMCs, TGFβ induces Nox4-derived H$_2$O$_2$ starting 4 hours after treatment and continues during a period of 24 hours. Although the specific targets of H$_2$O$_2$ are unclear, we speculate that inactivation of Smad phosphatases is 1 probable possibility. Furthermore, even though the colocalization of Hic-5 and Nox4 is suggestive of a role for Nox4 in the FA, the local production of H$_2$O$_2$ is unlikely to participate in the Smad-mediated transcriptional mechanism described in this work and additional studies are required to test a possible role of local reactive oxygen species production in FA or adhesive forces.

Figure 4. Heat shock protein (Hsp) 27 associates with hydrogen peroxide–inducible clone (Hic)-5 and aids its localization to focal adhesion. HASMCs were serum deprived for 48 hours before treatment with or without transforming growth factor (TGFβ) (2 ng/mL) for 24 hours. A, Co-immunoprecipitation was performed using an unrelated IgG or primary antibody against Hic-5. The membrane was immunoblotted using anti-Hsp27 and Hic-5 antibodies. Bars represent the average of 3 independent experiments±SEM. B, HASMCs were transfected with siRNA against Hsp27 (siHsp27) and then serum deprived for 48 hours before treatment with TGFβ (2 ng/mL) for 24 hours. Cells were fixed and stained for Paxillin (green), Hic-5 (red). Scale bar, 20 μm. The images are representative for 4 independent experiments.

Figure 5. Hydrogen peroxide–inducible clone (Hic)-5 mediates transforming growth factor (TGFβ)–induced focal adhesions. Quiescent HASMCs were treated with nonsilencing RNA (siNeg) or siRNA against Hic-5 (siHic-5) for 2 days. After treatment with TGFβ (2 ng/mL) for 24 hours, cells were fixed and stained for Vinculin (red), and nuclei were detected with 4′,6-diamidino-2-phenylindole (blue). Scale bar, 20 μm. A, The image represents the average of the focal adhesion number per cell, n=25. B, Four independent experiments were performed with consistent results.
The lack of suitable commercial Nox4 antibodies has been a limiting factor for the field of Nox biology. In this study, we used a custom made antibody (kindly provided by Dr David Lambeth) originally characterized in the supplemental material of Hilenski et al.\(^3\) This study was performed with the last remaining vial of the antibody, which is unfortunately no longer available. However, the additional experiments shown in Figure IA in the online-only Data Supplement clearly demonstrate the specificity of this antibody because the immunofluorescence signal is attenuated by siRNA against Nox4 (Figure IA i in the online-only Data Supplement), and the 65 and 80 kDa bands are no longer detected after preincubation of the antibody with blocking peptide (Figure IA ii in the online-only Data Supplement). The 80 kDa band is the predominant form that we detect in VSMC, as well as in tissues of wild-type mice, but not their Nox4 knockout littermates (Figure IA iii in the online-only Data Supplement). The reason for the apparent increase in molecular weight is unclear, but may be related to glycosylation (R.E. Clempus and K.K. Griendling, unpublished observations, 2007).

Figure 6. Hydrogen peroxide–inducible clone (Hic)-5 mediates transforming growth factor (TGF) \(\beta\)–induced cell adhesion strength and platelet-derived growth factor (PDGF)–induced migration in HASMCs. Immediately after siRNA electroporation, HASMCs were seeded onto fibronectin-coated glass coverslips. One day postseeding, the cells were serum-starved for 48 hours, stimulated with 2 ng/mL of TGF\(\beta\) for 24 hours, and spun inside the spinning disk device to quantify adhesion strength, which is defined as the shear stress at which 50% of cell detachment occurs. Representative adhesion profiles for each of the each condition (A) and bar graph is the average of \(n=7\) to 10 (B). C, Hic-5-deficient cells have impaired cells migration. After transfections, HASMCs were starved for 48 hours, trypsinized, and reseeded in a collagen-coated transwell insert. After stimulation for 3 hours with PDGF (10 ng/mL), the cells migrating through the membrane were stained with 4′,6-diamidino-2-phenylindole and counted. The graph represents mean±SEM of migrated cells per field from 3 independent experiments. \(*P<0.01.\)
Nox4-produced reactive oxygen species (Figure 1) and Smads (Figure 2A and 2B). The fact that N-acetyl cysteine completely reverses TGFβ-induced Hic-5 expression (Figure 1G) supports the idea that Hic-5 expression is downstream of Nox4-induced Smad activation (Figure 2C). It is also possible that Smad activation may be necessary, but not sufficient, to drive Hic-5 expression. It was recently demonstrated that the serum response factor is essential for TGFβ-mediated induction of Hic-5 expression in VSMCs.28 Our own and other studies have linked serum response factor expression to TGFβ-induced H2O2,5,30 and directly to Nox4-derived H2O2.6,8 On the basis of these observations, it is possible that the activation of serum response factor by a Nox4-produced H2O2-mediated pathway may also contribute to Hic-5 upregulation after TGFβ treatment.

The small GTPase RhoA seems to be required for the cytoskeletal effects of Nox4. In fact, a constitutively active form of RhoA rescues the loss of FAs induced by Poldip2/Nox4 deficiency.2 It is noteworthy that inhibition of Hic-5 reduces RhoA activation in TGFβ-stimulated epithelial cells34 and that overexpression of Hic-5 in these cells leads to increased formation of stress fibers in a Rho kinase–dependent manner. These observations thus raise the possibility that Hic-5 might modulate Nox4-mediated Rho activity, perhaps in a scaffolding capacity. Independently of the mechanism, the presence of Hic-5 in the FA has profound effects on the function of FAs because the knockdown of this protein was sufficient to completely abrogate the increase in adhesion strength generated by TGFβ treatment in HASMC (Figure 6A and 6B), which exactly resembles the reduction in the number of FAs observed when Hic-5 is downregulated (Figure 5A and 5B).

After vascular injury or during advanced stages of atherosclerosis, medial VSMCs proliferate and then migrate into the intimal layer.32 The process of VSMC migration involves a series of well-orchestrated steps where reactive oxygen species play a central role.8 Indeed, we and others have demonstrated that migration of VSMC is blocked in vivo and in vitro by antioxidants or after reducing the expression of Nox4-based enzymatic complexes.33,35 Our data further support this notion and offer new targets for H2O2-mediated signaling pathways during migration (Figure 6C).

In summary, we have shown that Hic-5 is a novel effector of Nox4 that is upregulated in VSMCs after TGFβ treatment by a Smad-dependent mechanism that requires H2O2, produced by Nox4. In addition, we found that the correct subcellular localization of Hic-5 within FAs requires the chaperone protein Hsp27, also a target of Nox4-mediated gene transcription. Our results provide a molecular explanation for the striking effects of Nox4 on FA dynamics and identify 2 important mediators that act jointly. This pathway seems to be active not only in smooth muscle cells derived from humans but also from rat (Figure 1C) and mouse (Figures I, III, and IV in the online-only Data Supplement). These findings shed light on the redox-sensitive pathways that lead to cell attachment and may be potentially important in understanding those functions of VSMCs associated with actin reorganization, such as migration and contractility.

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Disclosures

None.

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**Significance**

This work unveils downstream effectors of Nox4, a protein that is known to play an essential role in smooth muscle biology and differentiation by still unknown mechanisms. In addition, our data indicate a novel role for Hic-5 as a key mediator of TGFβ-induced vascular smooth muscle cell adhesion, which not only impairs cell migration but also is likely to affect vascular wall mechanical properties.
Hic-5 Mediates TGFβ–Induced Adhesion in Vascular Smooth Muscle Cells by a Nox4-Dependent Mechanism
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Materials and Methods

Recombinant human TGFβ1 was obtained from R&D systems and used at a final concentration of 2 ng/mL.

Cell Culture
Human aortic smooth muscle cells (HASMC) were obtained from Invitrogen. Cells were cultured in 231 media plus Smooth Muscle Growth Supplement (SMGS) as recommended by the company. Aortic SMCs were isolated from rat or wild type (wt) and Nox1−/− mouse by enzymatic dissociation and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum. All cultures were used between passages 6 and 12 for experiments. Cultures at 70% to 80% confluence were made quiescent by incubation in serum free media for 24 to 48 h before the experiments.

Western Blot
HASMCs were lysed in 1% Triton–containing lysis buffer and analyzed by western blotting. The following antibodies were used: β-actin (Sigma), Hic-5, Hsp27, and CDK4 (Santa Cruz, sc-28748, sc-1049, and sc-260 respectively), Hic-5 (BD Biosciences, #611164), and Nox4 (provided by Dr. David Lambeth, Emory University), α/β tubulin, p-smad2, smad2 and smad4 (Cell Signaling antibodies # 2148, 3101, 3122, and 9515 respectively). After incubation with horseradish peroxidase-conjugated secondary antibody, proteins were detected by chemiluminescence using a Kodak Imaging Station 4000mm Pro. Densitometry was performed using the Lane and Band analysis tool in the Carestream Molecular Imaging Software.

Small Interfering RNA Transfection Experiments
Cells were transfected by electroporation using a Nucleofector (Amaxa Biosystems) set to the U25 program or using RNAi max (Invitrogen) as recommended by the manufacturer. After transfection, cells were plated in serum containing media for 24 h and then serum deprived for 48 h prior to growth factor stimulation. We used predesigned sequences from Qiagen against human Smad4 (sense 5'-CAAGGUUGCACAUGGCAAd(TT)-3'), Nox4 (sense 5'-
GCAUCUGUUUCUAAACCUCAd(TT) -3’), Hsp27 (sense 5’-
GGACGAGCAUGGCUACAUd(TT)-3’) and Hic-5 (sense 5’-
GGACCGUCUGAAGAUAGGAd(TT)-3’).

Hic-5 Immunoprecipitation
Total cell lysates were prepared (Hepes pH 7.4 containing 1% deoxycholate and 0.5% Nonidet
P-40) and precleared with normal rabbit IgG (Santa Cruz, sc-2027) and sheep anti-rabbit M-280
dynabeads (magnetic beads, Invitrogen) for 1 hour. Hic-5 was immunoprecipitated with a
specific antibody (sc-28748) coupled to sheep anti-rabbit magnetic beads for 2 h at 4°C. Beads
were then washed 3 times with lysis buffer. Immunoprecipitated proteins were eluted from
magnetic beads, separated by SDS-PAGE and transferred to PVDF membranes for Western
blotting.

Quantitative Real-time PCR
Total RNA was purified from VSMC using the RNeasy kit (Qiagen, Chatsworth, CA), including
digestion with DNase I. Following reverse transcription using random primers and reverse
transcriptase (Superscript II, Invitrogen), cDNA was purified with the QIAquick kit (Qiagen).
Quantitative PCR (qPCR) of cDNA was carried out with a LightCycler instrument (Roche
Applied Science, Indianapolis, IN) in glass capillaries, using Platinum Taq DNA polymerase
(Invitrogen) and SYBR green (Invitrogen) dye. Predesigned human Hic-5 primers (QuantiTect
primer assay) were purchased from Qiagen (forward 5’-TATTGCTGGCAAGTGTTGTT-3’ and
reverse 5’-TGGAACAGCCTCCGCAAA-3’), used with 5 mM MgCl2 and an annealing
temperature of 55°C. Similarly, the following primer sequences were created for amplification of
Nox4 (forward 5’-CTGGAGGAGCTGGCTCGCAACGAAG-3’ and reverse 5’-
GTGACTCATGAGGAAGAGCCACCACCAGTGCAG-3’) used with 4 mM MgCl2 and an annealing
temperature of 58°C. Housekeeping primers amplifying human hypoxanthine
phospho-ribosyl-transferase 1 (HPRT1, Real Time Primers, Elkins Park, PA) (forward 5’-
TGACACTGGCAAAAACAGTGA-3’ and reverse 5’-GGTCTTTGCACCAGCAAGCT-3’)
and 18S (forward 5’-GAATTGAGGAGGGGCACCACCAG-3’ and reverse 5’-
GTGCAGCCCCGGACATCTAAGG-3’) were used with 4 mM MgCl2 and an annealing
temperature of 55°C for HPRT1 and 3 mM MgCl2 and 60°C for 18S. Data analysis was
performed using the mak3 module of the qPCR software library in the R environment\textsuperscript{1-3}. Results were normalized to the housekeeping gene and expressed in arbitrary units.

Immunocytochemistry (ICC)
After transfection with siRNA using Amaxa, VSMCs were plated on MatTek dishes and serum deprived for 48 h. Following treatment with TGFβ (2 ng/ml) for 24 h, cells were rinsed quickly in ice-cold PBS and fixed in 4% paraformaldehyde for 10 minutes at room temperature. They were then permeabilized in 0.01% Triton X-100 in PBS for 7.5 minutes and rinsed with PBS. Aldehyde groups were quenched with 50 mM NH\textsubscript{4}Cl for 10 minutes at room temperature. Cells were then blocked using 3% bovine serum albumin in PBS for 1 hour and then incubated with the following antibodies: Vinculin (V4505; Sigma), Nox4, Hic-5, Hsp27 (2790; Abcam), or Paxillin (05-417; Millipore) and then incubated in either: Alexa 488-conjugated (A1001; Life Sciences), Alexa 568-conjugated (A10042; Life Sciences), AMCA-conjugated (115-155-003; Jackson ImmunoResearch), or Rhodamine Red X (RRX)-conjugated (111-025-144) secondary antibodies for 1 hour at room temperature. Cells were mounted in Vectashield containing DAPI (Vector Laboratories). Images were acquired with a Zeiss LSM 510 META Laser Scanning Confocal Microscope. For co-localization analysis, stacks of 12-bit files (Z step 0.3 μm) were captured. Some images were processed using the Imaris software (Bitplane AG, Zurich, Switzerland) using the volume rendering and colocalization module to generate a co-localization channel.

Proximity Ligation Assay (PLA)
The \textit{in situ} PLA assay Duolink (Axxora, Enzo Life Sciences) was used to detect protein-protein interactions. VSMC were plated on MatTek dishes and serum deprived for 48 h, and subsequently treated with TGFβ (2 ng/ml) for 24 h. Afterwards, cells were fixed, permeabilized and nonspecific reactivity blocked as described for ICC. The rest of the protocol was performed following the Duolink manufacturer’s recommendation. Antibodies raised in different species (Santa Cruz sc-287484 for Hic-5, Sigma V4505 for Vinculin and BD 611722 for the tyrosine 397 phosphorylated form of focal adhesion kinase, pFAK) and DNA-plus and minus probes (mouse plus probe, LNK920010030 and rabbit minus probe, LNK920050030) were combined. PLA DNA-probes in close proximity (<40 nm) can interact and, after enzymatic ligation, are
amplified using a DNA polymerase. Amplified DNA reacts with fluorescence-containing hybridization probes. When DNA is amplified, fluorescence in each single-molecule is visible as a distinct bright dot under the fluorescence microscope. Cells were mounted in Vectashield containing DAPI (Vector Laboratories) and images were acquired with a Zeiss LSM 510 META Laser Scanning Confocal Microscope. For co-localization analysis, stacks of 12-bit files (Z step 0.3 μm) were captured.

Adhesion Strength Quantification
Cell adhesion strength was measured using the spinning disk system as previously described. Twenty-five-mm diameter glass coverslips (Electron Microscopy Services) were first coated with 20 μg/mL human plasma fibronectin (Invitrogen) and subsequently blocked with 1% bovine serum albumin (Sigma-Aldrich). HASMCs were seeded onto the coverslips immediately after siRNA transfection. One day post-seeding, adherent cells were serum-starved for 48 h, stimulated with 2 ng/μL TGFβ for 24 h, and spun in PBS + 2 mM glucose (Sigma-Aldrich) for 5 minutes at a constant speed. The applied fluid shear stress is given by the formula: \( \tau = 0.8r \left( \frac{\rho \mu \omega^3}{12} \right)^{1/2} \), where \( r \) is the radial position from the center of the coverslip, and \( \rho, \mu, \) and \( \omega \) are the fluid density, viscosity, and rotational speed, respectively. After spinning, cells were fixed in 3.7% ice-cold paraformaldehyde (Electron Microscopy Services), permeabilized with 1% Triton X-100 (Sigma-Aldrich), stained with ethidium homodimer-1 (Invitrogen), and counted at specific radial positions using a 10x objective lens in a Nikon TE300 microscope equipped with a Ludl motorized stage, Spot-RT camera, and Image-Pro 6.3 analysis system. A total of 61 fields (80–100 cells per field before spinning) were analyzed, and cell counts were normalized to the number of cells in the center of the disk. The fraction of adherent cells (f) was then fitted to a sigmoid curve, \( f = 1 \left( 1 + \exp \left\{ b [\tau - \tau_{50}] \right\} \right) \) where \( b \) is the inflection slope, and \( \tau_{50} \) is defined as the shear stress for 50% cell detachment. \( \tau_{50} \) characterizes the mean adhesion strength of the cell population.

Migration Assay
Migration was assayed using a modified Boyden chamber assay as previously described. Hic5 or control siRNA transfected HASMCs were grown to 85% confluence and then made quiescent in serum-free media for 24 h before migration. HASMCs (5×104 cells/well) were added to the
upper chamber of a Transwell dish on a 6.5-mm insert with a collagen-coated polycarbonate membrane containing 8-μm pores (Costar). VSMCs were then exposed to PDGF (10 ng/mL) in the lower chamber for 3 h, after which nonmigrated cells were removed from the upper chamber using a cotton swab. The cells remaining on the inserts were fluorescently stained with 4′,6-diamidino-2-phenylindole (DAPI) (1 μg/mL) and visualized using a Zeiss Axioskop microscope. Migrated cells per membrane were quantified using ImageJ software.

Statistical Analysis
Results were expressed as means ± SEM from at least three independent experiments. Statistical significance was assessed using analysis of variance (ANOVA), followed by Bonferroni’s multiple comparison post-hoc test. A value of P<0.05 was considered significant.

Supplement Figure I: Nox4 is upstream of Hic-5 and mediates TGFβ-induced Hic-5 expression.

(A) Nox4 antibody characterization. i) Control or siNox4 treated rat aortic smooth muscle cells (RASMCs) were processed for ICC; ii) Total lysates from RASMC or HEK cells were analyzed by western blot using Nox4 antibody with or without preincubation with blocking peptide; iii) protein samples from kidney or lung from wild type or Nox4 KO mice were analyzed by western blot using Nox4 antibody. (B-D) HASMCs were treated with non-silencing RNA (siNeg) or siNox4. After 2 days of serum deprivation, cells were treated with TGFβ (2 ng/mL) for 24 hrs. (B) Total RNA was prepared and Nox4 expression was evaluated as described in the Methods section. (C) Hic-5 and Nox4 and (D) Nox1 proteins were analyzed by western blot using specific antibodies. (E) Aortic VSMC derived from wild type and Nox1y−/− mouse were serum deprived for 2 days and treated with TGFβ (2 ng/mL) for 24h to detect Hic-5 expression. All experiments were performed at least three independent times. Data are presented as average ± SEM of three independent experiments.
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Supplemental Figure II: Nox4 downregulation does not affect Paxillin mRNA or protein expression.
HASMCs were transfected with siNeg or siNox4 and maintained in serum-free medium for 2 days before treatment with TGFβ (2 ng/ml) for 24 hours. Total mRNA and proteins were extracted and subjected to real time PCR (A) and western blot analysis (B) for Paxillin respectively. The image is representative of three independent experiments.
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Supplemental Figure III: TGFβ–mediated Smads phosphorylation is mediated by H₂O₂ but is independent of Nox1.

(A) HASMCs were maintained in serum-free medium for 2 days before treatment with H₂O₂ T (50 µM) for 30 min. (B) aortic VSMC derived from wild type and Nox1⁻/⁻ mouse were serum deprived for 2 days and treated with TGFβ (2 ng/mL) for 24h to detect Hic-5 expression. The image is representative of three independent experiments.
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(A) HASMCs were maintained in serum-free medium for 2 days before treatment with H₂O₂ T (50 µM) for 30 min. (B) aortic VSMC derived from wild type and Nox1<sup>y/-</sup> mouse were serum deprived for 2 days and treated with TGFβ (2 ng/mL) for 24h to detect Hic-5 expression. The image is representative of three independent experiments.
Supplemental Figure IV: TGFβ–mediated Hsp27 is independent of Nox1.
Aortic VSMC derived from wild type and Nox1<sup>y/-</sup> mouse were serum deprived for 2 days and treated with TGFβ (2 ng/mL) for 24h to detect Hsp27 expression. The image is representative of three independent experiments.
Supplemental Figure V: siHsp27 has no effect on Hic-5 mRNA or protein expression.
HASMCs were transfected with siNeg or siHsp27 and maintained in serum-free medium for 2 days prior to TGFβ treatment (2 ng/ml) for 24 hrs. (A) Hic-5 mRNA was measured by RT-qPCR. (B) Total proteins were extracted and subjected to western blot analysis for Hic-5. β-actin was used as a loading control. Bars represent the average of three independent experiments ± SEM.
Supplemental Figure V: siHsp27 has no effect on Hic-5 mRNA or protein expression.

HASMCs were transfected with siNeg or siHsp27 and maintained in serum-free medium for 2 days prior to TGFβ treatment (2 ng/ml) for 24 hrs. (A) Hic-5 mRNA was measured by RT-qPCR. (B) Total proteins were extracted and subjected to western blot analysis for Hic-5. β-actin was used as a loading control. Bars represent the average of three independent experiments ± SEM.
Supplemental Figure VI: Knockdown of Hic-5 protein has no effect on Vinculin protein expression.

HASMCs were transfected with siNeg or siRNA against Hic-5 (siHic-5) and then serum deprived for 48 hrs before treatment with TGFβ (2 ng/mL) for 24 hrs. Total proteins were extracted and subjected to western blot analysis for Vinculin. β-actin was used as a loading control. Bars represent the average of three independent experiments ± SEM.