Formin mDia1 Mediates Vascular Remodeling via
Integration of Oxidative and Signal Transduction Pathways

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Rationale: The mammalian diaphanous-related formin (mDia1), governs microtubule and microfilament dynamics while functioning as an effector for Rho small GTP-binding proteins during key cellular processes such as adhesion, cytokinesis, cell polarity, and morphogenesis. The cytoplasmic domain of the receptor for advanced glycation endproducts binds to the formin homology 1 domain of mDia1; mDia1 is required for receptor for advanced glycation endproducts ligand-induced cellular migration in transformed cells.

Objective: Because a key mechanism in vascular remodeling is the induction of smooth muscle cell migration, we tested the role of mDia1 in this process.

Methods and Results: We report that endothelial denudation injury to the murine femoral artery significantly upregulates mDia1 mRNA transcripts and protein in the injured vessel, particularly in vascular smooth muscle cells within the expanding neointima. Loss of mDia1 expression significantly reduces pathological neointimal expansion consequent to injury. In primary murine aortic smooth muscle cells, mDia1 is required for receptor for advanced glycation endproducts ligand-induced membrane translocation of c-Src, which leads to Rac1 activation, redox phosphorylation of AKT/glycogen synthase kinase 3β, and consequent smooth muscle cell migration.

Conclusions: We conclude that mDia1 integrates oxidative and signal transduction pathways triggered, at least in part, by receptor for advanced glycation endproducts ligands, therefore regulates pathological neointimal expansion. (Circ Res. 2012;110:00-00.)

Key Words: restenosis ■ vascular smooth muscle cell ■ signal transduction ■ formin ■ cellular migration

Mammalian diaphanous 1 (mDia1) is a member of the formin family, a group of multidomain proteins governing actin and microtubule remodeling.1,2 Formins are ubiquitously expressed and involved in a variety of fundamental cellular processes, such as adhesion, cytokinesis, polarity, and morphogenesis.3,4 GTP-bound Rho proteins both directly activate and may be modulated by the mDia family of formins.5,6 Release from autoinhibition allows the formin homology-2 domain to nucleate and progressively elongate linear actin filaments and to stabilize microtubules in support of polarized cell division, adhesion, and migration. Whereas the role of formins in cytoskeleton modification is well established, only limited reports are available concerning their implication in disease processes.7,8

We previously demonstrated that mDia1 binds to the intracellular domain of the receptor for advanced glycation endproducts (RAGE).9–10 mDia1 binds RAGE through its conserved poly-proline rich formin homology 1 domain. RAGE is a multiligand receptor of the immunoglobulin superfamily11 involved in a number of disease processes characterized by vascular pathology.12–17 Vascular stress leads to upregulation of RAGE ligands, including multiple members of the S100/calgranulin family such as S100B, advanced glycation endproducts, high mobility group box 1, amyloid-β peptide, and β-sheet fibrils, in the vessel wall, thereby facilitating their engagement of RAGE and induction of proinflammatory and tissue-damaging responses.17,18

The roles of RAGE and mDia1 in fundamental mechanisms governing cellular migration prompted us to test the role of mDia1 in regulation of vascular smooth muscle cell (SMC) signal transduction and migration. In vivo, we addressed these concepts by assessing the impact of mDia1 in neointimal expansion consequent to guide wire-induced endothelial denudation in a murine model. Our findings reveal
novel roles for mDia1 as a key regulator of signal transduction and oxidative stress events that control SMC migration and vascular remodeling.

Methods

An expanded Methods section is provided in the online-only Data Supplement.

Animals and Induction of Vascular Injury

Male C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and then bred in house for studies. Homozygous Drf1+/− mice were generated as previously described19,20 and were backcrossed >12 generations into C57BL/6 prior to any experimentation. All procedures were approved by the Institutional Animal Care and Use Committee of Columbia University and New York University Langone Medical Center. Male mice, age 8 to 12 weeks, were anesthetized and subjected to femoral artery endothelial denudation injury as previously described.21 All of the surgeries were performed by a single operator naive to the genotype of the animals.

Tissue Analyses

Harvesting of vessel segments and their analysis was performed as previously described.17 Representative sections from the vessels were stained with antibodies to detect mDia1, α-smooth muscle actin or control IgG.

Western Blotting

At least 8 arteries were snapfrozen in liquid nitrogen, pooled, and stored at −80°C. Tissue samples were ground to a fine powder under liquid nitrogen and lysates subjected to SDS-PAGE and Western blotting for detection of the following antigens: RAGE, mDia1, Nox1, Nox4, p47phox, phospho/total Akt, phospho/total glycogen synthase kinase (GSK) 3β ser 9, phospho/total Erk p44/42 mitogen activated protein kinase, and phospho/total c-Src.

Cell Culture and In Vitro Assays on Cultured SMCs

Mouse vascular SMCs were cultured from the aortas of 10-week-old male mice using a modification of the procedure of Tarvo and Barret.22 SMCs were cultured following an explant protocol in male mice using a modification of the procedure of Tarvo and Barret.22 SMCs were cultured following an explant protocol in male mice using a modification of the procedure of Tarvo and Barret.22 SMCs were cultured following an explant protocol in male mice using a modification of the procedure of Tarvo and Barret.22

Small Interference RNA to Knockdown mDia1, Nox1, and c-Src

Small interference RNA (siRNA) duplexes against mouse mDia1, Nox1, or c-Src were synthesized by Invitrogen ( Stealth RNAi®). Details of sequences and electroporation into primary SMCs are found in the Online Supplement.

Cell Transfection for Dominant Negative Rac and Constitutively Active GSK3

Dominant negative constructs of Rac1 (N17, Millipore) and constitutively active GSK3β and corresponding control vectors were purified following the manufacturer’s instructions and then transfected into the primary SMCs as described above (Nucleofactor). The plasmid pcDNA3-GSK3-β (S9A) was obtained from Addgene (plasmid no. 14754) and originally created by Dr. James Woodgett’s laboratory.

Cell Migration

Migration in response to RAGE ligands: S100B (10 μg/mL), generically provided by Dr. Guenter Fritz, carboxymethyllysine (human serum albumin, 10 μg/mL), generically provided by Dr. Eric Boulanger, or general effectors (platelet-derived growth factor [PDGF], 50 ng/mL; epidermal growth factor [EGF], 100 ng/mL; R& D systems) was assessed with wounding assay and chemotaxis assays as described in the Online Supplement.

Reactive Oxygen Species Measurement: Assessment of Nicotinamide Adenine Dinucleotide Phosphate Activity

Nicotinamide Adenine Dinucleotide Phosphate Oxidase Activity

Nicotinamide adenine dinucleotide phosphate (NADPH) dependent superoxide production was assessed by lucigenin-enhanced chemiluminescence as previously described.23

Dihydroethidium

Cells (1×10^6) were seeded and after overnight serum starvation in DMEM without red phenol, cells were reincubated with 10 μmol/L of dihydroethidium for 10 minutes then stimulated with RAGE ligands (S100B, 10 μg/mL). Fluorescence was evaluated with a fluorescence plate reader excitation/emission: 370/630 at the indicated times. Results are expressed as the difference between unstimulated and stimulated conditions.

p47phox and c-Src Translocation

Unstimulated and stimulated cells were used and manipulated following the recommendations of the manufacturer. After membrane preparations were prepared, detergent was removed and samples were subjected to immunoblotting for p47phox and c-Src (phospho- and total). Where indicated, immunoprecipitation experiments in SMCs were performed using rabbit polyclonal mDia1 antibody or the appropriate nonimmune IgG (Pgtlab), followed by Western blotting using mouse monoclonal c-Src antibody (Cell Signaling).

Rac1 Activity

Rac1 activity was assessed with a pull-down assay using Pak1 as a specific ligand for activated Rac1 followed by SDS-PAGE electrophoresis and immunoblotting with Rac1 monoclonal antibody.

mDia1 Rescue Experiment in Drf1+/− SMCs

Plasmid pEFN.mDia1, corresponding to the murine myc-tagged derivative of mDia1 was a generous gift from Dr. John Copeland (University of Ottawa).

RNA Extraction and RT-PCR

Total RNA was extracted from at least 8 vessels prepared for immunoblotting as above using the micro scale RNA isolation kit. Reverse transcription was performed following the protocol suggested by the manufacturer. The primers used are indicated in the Online Supplement.

Statistical Analysis

In all experiments, unless otherwise indicated, data are reported as the mean±SEM in at least 3 replicates per group. Data were
Results
mDia1 Is Upregulated in Vessels Consequent to Guide Wire Injury and Deletion of mDia1 in Mice Reduces Neointimal Expansion

To test the role of mDia1 in vascular SMC migration and signal transduction, we employed a murine model of guide wire-induced femoral artery endothelial denudation to stimulate neointimal formation. Wild-type (WT) C57BL/6 mice and control or sham mice were subjected to the identical procedure, the latter without guide wire injury. Pathological neointimal expansion 21 days after injury was observed by Van Gieson’s elastic staining only in the guide wire-injured mice but not the sham controls (Figure 1D versus 1A, respectively). Immunostaining with antimDia1 IgG versus isotype control IgG revealed expression of mDia1 in all the vessels studied, consistent with the ubiquitous expression of formin proteins (Figure 1B and 1E, respectively). However, injured arteries displayed enhancement of mDia1 expression particularly in cells within the expanding neointima (Figure 1E). As vascular SMCs are a chief component of the neointima, we analyzed mDia1 expression in the vessels and found that mDia1 antigen colocalized in part with α-smooth muscle actin within the neointima (Figure 1G, 1H, and 1I).

To further study regulation of mDia1 in vascular injury, we harvested femoral arteries of WT animals on day 0, 4, 10, 21, or 28 after injury and analyzed mDia1 transcripts and protein levels by quantitative real-time PCR and Western blot. mDia1 mRNA levels were significantly increased by day 4 after injury (P<0.01) compared with day 0 (Figure 1J). Consistent with this finding, compared to day 0 (no injury), mDia1 protein levels were also increased by day 4 after injury (P<0.001), and peaked at day 10 (P<0.001) (Figure 1K). An analogous pattern of expression was found for that of RAGE antigen in the vessel tissues over this time course (Figure 1L).

We determined the fate of mDia1 expression in mice devoid of RAGE that were subjected to identical degrees of femoral artery injury. Interestingly, Western blotting revealed that although mDia1 was present in RAGE−/− mice vessels, its expression was not significantly altered over the same course from 0 to 28 days after guide wire injury (Figure 1 mol/L). Taken together, these results revealed that mDia1 expression is upregulated in a time- and RAGE-dependent manner in injured arterial vessels.

Prompted by these observations, we sought to establish if mDia1 contributes to injury-provoked vascular remodeling. We subjected Drf1−/− mice deficient in mDia1, and their age- and gender-matched male WT mice to femoral artery endothelial denudation. We first analyzed RAGE expression patterns in the femoral arteries in sham and injury states and found that RAGE upregulation occurred by day 4 and day 10 in both WT and Drf1−/− mice (Figure 1N). Hence, deletion of mDia1 had no impact on RAGE expression. We performed quantitative morphometric analysis of the vessels postinjury; at 3 weeks, none of the sham-operated arteries displayed neointimal formation (data not shown), but significant neointima was observed in WT mice. Compared to wt mice, the intima/media ratio was ∼4.8-fold lower in mice devoid of mDia1 (Drf1−/−), 0.77±0.4 versus 0.16±0.1, respectively; P<0.001 (Figure 1O). Consistent with this result, intimal area measurements were also highly reduced in the Drf1−/− mice and luminal area was significantly higher compared to the WT group. No significant changes were found between the 2 groups in the media area measurements or overall vessel size (internal elastic lamina and external elastic lamina) (Online Table I). These morphometric measurements are consistent with a reduced pathological inward remodeling in the Drf1−/− mice after guide wire injury.

mDia1 Deletion Blocks RAGE Induced Lamellipodia Formation and Migration in Primary Murine Aortic SMCs

Neointimal expansion in the injured vessel wall depends on a number of factors but critically on vascular SMC migration. Actin polymerization and consequent lamellipodia formation are the initial events leading to cell migration; mDia1 has been implicated in these processes. We isolated primary SMCs from WT, RAGE+/− or Drf1−/− murine aortas and analyzed mDia1 expression. Although mDia1 protein was expressed in WT cells, as well as cells devoid of RAGE, mDia1 protein was not detected in vascular SMC isolated from Drf1−/− animals (Figure 2A).

To probe the mechanisms by which mDia1 regulated vascular SMC signal transduction and migration, cells were treated with the prototypic RAGE ligand, S100B, to stimulate cell surface-anchored RAGE before analysis of actin content. Unstimulated cells revealed a high content of stress fibers as evidenced by phalloidin staining. Compared to unstimulated cells, incubation of WT SMCs with S100B induced disappearance of the stress fibers, change of shape with induction of cell polarity, and the appearance of cytoplasmic protrusions enriched in both F-actin and in mDia1 (lamellipodia) (Figure 2B and 2D). S100B did not induce lamellipodia formation in RAGE−/− or Drf1−/− SMCs, suggesting that mDia1 was required for the effects of RAGE ligand S100B. In contrast, stimulation of WT, RAGE+/−, and Drf1−/− SMCs with a non-RAGE ligand, PDGF, induced lamellipodia formation comparably in SMCs from the 3 different genotypes of mice (Figure 2C and 2D). These data indicated that RAGE and mDia1 were required for S100B- but not for PDGF-stimulated lamellipodia formation.

To further evaluate the impact of mDia1 in RAGE-induced SMC migration, we performed chemotaxis experiments using Transwell migration chambers. Cells placed in the upper chamber migrated toward the chemotacticant placed in the bottom chamber (vehicle, PDGF 50 ng/mL, or S100B 10 μg/mL). WT cells demonstrated a significant chemotactic response to PDGF and S100B (Figure 2E). However, cells retrieved from Drf1−/− mice revealed significantly less S100B-induced migration, although chemotaxis in response to PDGF was not impaired in SMCs devoid of mDia1 (Figure 2E). These results were confirmed by an in vitro scratch wounding assay in cultured monolayers of SMCs, where stimulation with RAGE ligands, S100B or carboxymethyllysine-AGE human serum...
Increased expression and impact of mammalian diaphanous-related formin (mDia1) after endothelial denudation injury. Wild-type (WT), Drf1<sup>−/−</sup>, and receptor for advanced glycation endproducts (RAGE)<sup>−/−</sup> mice were subjected to femoral artery endothelial denudation or sham, and tissues analyzed at the indicated times. A and D, Assessment of neointimal expansion by Van Gieson's Elastic (EVG) staining on day 21 after injury in WT mice (A, sham, and D, injury). B, C, E, and F, Immunostaining for mDia1 or isotype IgG control in WT mice on day 21 after injury (E, F) or sham (B, C). G–I, Colocalization studies: sections of injured vessels were stained for mDia1 and α-smooth muscle cell actin (SMA). Immunofluorescence studies revealed a colocalization of the 2 molecules in the neointima on day 21. J, qRT-PCR analysis of mDia1 expression. Sham and injured WT vessels (n=5/group) were harvested and subjected to RNA isolation and quantitative real-time PCR analysis for detection of mDia1 transcripts. Results are expressed in mean±SEM (K, L, Western blot analysis of mDia1 and RAGE in injured vessels. WT mice (n=4 per time point and per group) were subjected to femoral artery injury. At the indicated time, arteries were retrieved and pooled for preparation of lysates. These lysates were used for Western blot analysis. Each Western blot was subjected to densitometric quantification and results are expressed as the mean±SEM of at least 3 Western blots per condition. A representative Western blot is shown. M, mDia1 expression in RAGE<sup>−/−</sup> mice subjected to femoral artery injury was detected by Western blot analysis at the indicated times. Histogram represents the mean±SEM of at least 3 Western blots per condition. A representative picture is shown. N, Quantitative analysis of RAGE expression after injury in WT and Drf1<sup>−/−</sup> mice: Lysates from n=4 mice per time point and per group were prepared as in K and L, and subjected to at least 3 independent Western blot analyses for RAGE expression. Densitometric quantification was done and data are expressed as mean±SEM. A representative Western blot is shown. O, Intima/media ratio measurement based on morphometric analysis of the vessels of WT and aged-sex-matched Drf1<sup>−/−</sup> mice (n=11/group) was performed 21 days after guide wire-induced femoral artery denudation. Representative images are shown. *P<0.05, **P<0.01, ***P<0.001.
albumin, induced migration selectively of WT cells, but not Drf1−/− cells (Figure 2F).

EGF, which, like PDGF, is not a RAGE ligand, has been reported as a chemotactic factor requiring mDia1 cooperation in formation of lamellipodia in carcinoma cells.28 To confirm the role of mDia1 in EGF-dependent migration of SMCs, we stimulated WT SMCs with EGF, 100 ng/mL, and found that EGF induces significant migration in WT but not Drf1−/− cells (Figure 2F). These data indicate that mDia1-mediated induction of lamellipodia formation and migration of SMCs is both RAGE-dependent and RAGE-independent.

mDia1 Deletion Blocks Inhibitory Phosphorylation (Ser 9) of GSK3β

We next sought to identify the signaling pathways regulated by mDia1 in vascular SMCs. It was previously shown in cultured fibroblasts that mDia1 modulated GSK3β serine 9 (ser 9) phosphorylation,29 and that GSK3β was implicated in
SMC migration.\textsuperscript{30} We tested ser 9 GSK3β phosphorylation in injured arteries of WT mice and found a time-dependent increase in phospho-GSK3β ser 9 in WT arteries (Figure 3A). In contrast, in arteries retrieved from Drf1\textsuperscript{−/−} mice, phospho-GSK3β ser 9 did not increase from baseline in response to injury over the same time course (Figure 3A). Of note, we previously demonstrated that guide wire-induced femoral artery injury resulted in activation of the mitogen activated protein kinase ERK1/2 signaling pathway in WT mice, and that this mechanism was independent of RAGE.\textsuperscript{17} Here we found that mitogen activated protein kinase ERK1/2 phosphorylation is induced equivalently at day 4 and 10 after injury in both WT and Drf1\textsuperscript{−/−} animals (Figure 3B), thereby suggesting that the effects of both RAGE\textsuperscript{17} and mDia1 were likely not mediated via ERK1/2 mitogen activated protein kinases.

We next probed the role of mDia1 in RAGE ligand-induced GSK3β ser 9 phosphorylation in primary vascular SMCs. Deletion of mDia1 inhibited phosphorylation of AKT (a principal kinase mediating GSK3β ser 9 phosphorylation) and GSK3β (Figure 3C and D) in response to S100B stimulation. These results were confirmed by downregulation experiments in which transfection of WT cells with mDia1 siRNA significantly inhibited AKT and GSK3β ser 9 phosphorylation in response to S100B, whereas scramble-transfected cells displayed a time-dependent phosphorylation of AKT and GSK3β ser 9 (Online Figure I).

To confirm the role of mDia1 in RAGE ligand-induced GSK3β ser 9 phosphorylation, SMCs retrieved from Drf1\textsuperscript{−/−} animals were transfected with full-length murine mDia1 construct to restore mDia1 expression, as assessed by Western blot (Figure 3E). Compared to vector (mock)-transfected SMCs, transfection of Drf1\textsuperscript{−/−} cells with cDNA expressing mDia1 restored phosphorylation of AKT and ser 9 GSK3β in response to S100B (Figure 3F and 3G, respectively).

Lastly, we tested the effect of a non-RAGE ligand, PDGF,\textsuperscript{17} on cellular signaling. We found that phosphorylation of AKT and GSK3β was induced similarly in wild type and Drf1\textsuperscript{−/−} cells in response to PDGF (Figure 3H), supporting that these effects of PDGF, as well as formation of lamellipodia (Figure 2), do not require mDia1.

**GSK3 β Ser 9 Inhibitory Phosphorylation Is Required for RAGE-Induced Vascular SMC Migration**

Our next step was to analyze the role of RAGE in GSK3β ser 9 phosphorylation in SMC migration. We stimulated primary WT SMCs with S100B and found a dose- and time-dependent phosphorylation of GSK3β at serine 9 (Figure 4A), which did not occur in RAGE\textsuperscript{−/−} SMCs (Figure 4B). To definitively implicate GSK3β phosphorylation in S100B-induced SMC migration, we transfected WT cells with a mutated form of GSK3β (constitutively active GSK3βGSK3ββ) that is unable to be phosphorylated at serine 9. On stimulation with S100B, migration was highly significantly reduced in constitutively active GSK3β–transfected cells compared to control-transfected cells (Figure 4C). These data support the role of GSK3β ser 9 phosphorylation in S100B-RAGE-mediated SMC migration.

**mDia1 Deletion Blocks Reactive Oxygen Species Formation In Vivo and In Vascular SMCs**

It is well established that oxidative stress is a key process implicated in vascular SMC migration and neointimal formation,\textsuperscript{31} yet; the proximal signals triggering reactive oxygen species (ROS) formation and consequent SMC migration have yet to be fully identified. To test the hypothesis that mDia1 is required for oxidative stress in vascular SMCs, we measured NADPH oxidase activity in lysates prepared from the injured arteries of WT and Drf1\textsuperscript{−/−} mice. Compared to sham-operated animals, WT arteries displayed a significant increase in NADPH oxidase activity 12 hours following the injury, \(P<0.001\), whereas no significant increases were observed in arteries retrieved from the Drf1\textsuperscript{−/−} mice (Figure 5A). We next determined NADPH oxidase activity in SMCs. NADPH oxidase activity was increased by \(\sim 2\) fold in response to S100B in WT cells at 15 minutes and 30 minutes stimulation, but no significant increase was found in RAGE\textsuperscript{−/−} or Drf1\textsuperscript{−/−} cells (Figure 5B). We employed dihydroethidium to measure superoxide production in response to S100B stimulation in SMCs, and tested the effect of siRNA-mediated reduction of mDia1 expression (Figure 5C) and deletion of mDia1. WT cells exhibited a time-dependent increase in superoxide production in response to S100B, which was maximal at 60 minutes (\(\sim 4\)-fold increase compared to time zero, \(P<0.01\)) (Figure 5D). S100B stimulation required RAGE, as RAGE\textsuperscript{−/−} cells demonstrated no response to S100B (Figure 5D). Furthermore, SMCs transfected with mDia1 siRNA (Figure 5D) and Drf1\textsuperscript{−/−} cells revealed a significant inhibition of S100B-induced generation of superoxide compared to scramble-transfected cells at 60 minutes stimulation (Figure 5D).

We next examined p47phox, Nox1, and Nox4, key molecules of the NADPH oxidase complex, and found that S100B stimulation of WT SMCs significantly increased the amount of p47phox at the cell membrane, whereas no translocation was found in RAGE\textsuperscript{−/−} or Drf1\textsuperscript{−/−} cells (Figure 5E). Further, increased expression of Nox1 was found in WT cells treated with S100B, but no increases were noted in SMCs devoid of RAGE or mDia1 (Figure 5F). In contrast, the expression of Nox4 was not modified by S100B stimulation in WT, RAGE\textsuperscript{−/−} or Drf1\textsuperscript{−/−} cells (Figure 5G). Note that previous studies established that upregulation of Nox1 in SMCs was accounted for by increased translation of Nox1 protein; our results are completely in line with this observation.\textsuperscript{32}

**mDia1, Rac1, and Nox1 Are Required for RAGE Ligand-Induced Generation of ROS and Consequent Cellular Signaling in Vascular SMCs**

Rac1 is a major component of activated NADPH oxidase. To test if mDia1 impacts Rac1 activation in vascular SMCs, cells were incubated with S100B. WT but not Drf1\textsuperscript{−/−} SMCs revealed activation of Rac1 at 15 minutes post-S100B stimulation (Figure 6A). Our next goal was to establish the hierarchy of vascular SMC signaling events following S100B stimulation. We first tested the relationship between RAGE induced oxidative stress and kinase phosphorylation, and examined S100B-induced GSK3 β ser 9 phosphorylation in SMCs in the presence or absence of the ROS scavenger, tyron...
Figure 3. Mammalian diaphanous-related formin (mDia1) deletion blocks inhibitory phosphorylation (ser 9) of GSK3β stimulated by receptor for advanced glycation endproducts (RAGE) ligands. A and B, Glycogen synthase kinase (GSK3β) serine 9 (ser 9) phosphorylation and mitogen activated protein (MAP) kinase ERK1/2 phosphorylation were tested in lysates from wild type (WT) or age-matched Drf1+/− mice arteries after injury. Mice (n=4 per time point and per group) were subjected to femoral artery injury and followed until the indicated day. Then, arteries were retrieved and pooled for preparation of lysates. These lysates were used for Western blot analysis. Each Western blot was subjected to densitometric quantification and results are expressed as the mean±SEM of at least 3 Western blots per condition. A representative picture of these Western blots is shown. C and D, Western blot analysis of AKT/GSK3β ser 9 phosphorylation in WT and Drf1−/− primary smooth muscle cells (SMCs) stimulated with RAGE ligand S100B (10 μg/mL). Deletion of mDia1 inhibits GSK3β serine 9 and AKT phosphorylation by S100B. Results are represented as mean±SEM of 3 independent experiments. E–G, Transfection of murine mDia1 into Drf1−/− primary SMCs rescues AKT phosphorylation and ser 9 phosphorylation of GSK3β by S100B. In E, Western blot for mDia1 is shown in mDia1-transfected (lane 2) and mock vector control transfected (lane 3) Drf1−/− SMCs. Ln 1 represents mDia1 expression in WT SMCs. In F, mDia1- or vector-transfected SMCs were treated with S100B (10 μg/mL) and AKT (F) and ser 9 phosphorylation of GSK3β (G) was determined at the indicated time. Results are represented as mean±SEM of 3 independent experiments. (H) Western blot analysis of AKT and GSK3β ser 9 phosphorylation in WT and Drf1−/− primary SMCs stimulated with non-RAGE ligand platelet derived growth factor (PDGF) (50 ng/mL). *P<0.05, **P<0.01, ***P<0.001.
Incubation of SMCs with tyron inhibited S100B-stimulated GSK3β ser 9 phosphorylation (Figure 6B); similar findings were observed with N-Acetyl cysteine (25 mmol/L) (data not shown). These results suggest that ROS generation was required for RAGE-mediated ser 9 GSK3β phosphorylation.

To explore the specific mechanisms linking RAGE and mDia1 to ROS generation, we hypothesized that Rac1 and Nox1 activation were essential for both S100B-stimulated ROS generation and kinase activation in vascular SMCs. We employed dominant negative Rac1 and Nox1 siRNA to test these concepts. Compared to cells transfected with the control vector, cells transfected with dominant negative Rac revealed significantly less generation of superoxide and migration in response to S100B (Figure 6C and D). Dominant negative Rac inhibited AKT (Online Figure II) and GSK3β on ser 9 is required for RAGE-induced SMCs’ migration. WT primary SMCs were transfected with an empty vector or with constitutively active GSK3β plasmid (CA-GSK3β), before analysis of S100B (10 μg/mL)-induced migration by wound healing assay. Results are presented as mean ± SEM of 3 independent experiments. *P<0.05, **P<0.01, ***P<0.001.

(10 mmol/L). Incubation of SMCs with tyron inhibited S100B-stimulated GSK3β ser 9 phosphorylation (Figure 6B); similar findings were observed with N-Acetyl cysteine (25 mmol/L) (data not shown). These results suggest that ROS generation was required for RAGE-mediated ser 9 GSK3β phosphorylation.

mDia1 Is Critical for RAGE-Induced c-Src Translocation to the Membrane and Consequent Redox Signaling

It has been shown that RAGE activates c-Src kinases in SMCs33 and that c-Src kinases bind to mDia1 and act as downstream effectors of diaphanous proteins.34–35 We tested RAGE-induced c-Src phosphorylation in WT and in Drfl−/− SMCs. Analysis of total lysates found that stimulation with S100B induced phosphorylation of c-Src kinases in both WT and Drfl−/− cells (Figure 7A). However, analysis of the membrane fractions revealed that membrane translocation of phospho-c-Src in response to S100B was observed in WT cells, but not RAGE−/− or Drfl−/− SMCs (Figure 7B). To further confirm the interaction of c-Src with mDia1 we performed coimmunoprecipitation studies. Immunoprecipitation using antimDia1 IgG, but not nonimmune IgG, from S100B-stimulated SMC lysates resulted in immunoprecipitation of phospho-c-Src (Figure 7C). AntimDia1 antibody immunoprecipitated mDia1 by Western blot as well (data not shown).

We next delineated the role of c-Src in S100B-mediated downstream events. Reduction of c-Src expression with siRNA (Figure 7D) significantly inhibited S100B-induced Rac 1 activation (Figure 7E), and ROS production in SMCs (Figure 7F), whereas scramble siRNA had no effect. Finally,
down regulation of c-Src expression significantly inhibited RAGE ligand-induced AKT phosphorylation (Online Figure III) and GSK3β ser 9 phosphorylation (Figure 7G).

Taken together, these results establish a critical role for mDia1 in transducing the effects of RAGE ligands on c-Src, Rac1, and Nox1 activation in consequent phosphorylation of AKT and GSK3β ser 9, processes essential for RAGE ligand-induced vascular SMC migration. As the initial signaling events are dependent on RAGE and mDia1, these findings integrate for the first time the role of this axis in regulating oxidative and cell signaling mechanisms controlling SMC migration and neointimal formation (Figure 8).
Formins are effectors and modulators of Rho-GTPase signal transduction involved in regulation of actin and microtubule rearrangements during cell migration and cytokinesis.1,3 mDia1 is one of the best characterized members of this family, but only limited reports have implicated mDia1 directly in disease processes.7,8,36,37 As neointimal expansion triggered by denuding arterial injury is a well-established...
Figure 7. Mammalian diaphanous-related formin (mDia1) regulates receptor for advanced glycation endproducts (RAGE)-induced c-Src translocation to the membrane and consequent Rac1 activation, reactive oxygen species (ROS) generation and cellular signaling in smooth muscle cells (SMCs). (A), Western blot analysis of S100B induced phosphorylation of c-Src in total lysates of wild type (WT) and Drf1−/− SMCS reveals identical degrees of c-Src phosphorylation in both cell types. B, Western blot analysis of S100B (10 μg/mL)-induced c-Src translocation to the membrane in WT, RAGE−/−, and Drf1−/− primary SMCs. Membrane fractions were prepared from unstimulated and stimulated SMC and then used for Western blots for Phospho-c-Src and total c-Src. Histograms represent mean±SEM of the densitometric quantification of 3 independent experiments. A representative picture is shown for each Western blot. (C), Immunoprecipitation studies. Lysates obtained from WT SMCs were stimulated with S100B and subjected to immunoprecipitation using rabbit polyclonal antimDia1 IgG or isotype nonimmune control. Immunoprecipitated products were subjected to Western blot analysis of c-Src. C-Src immunoprecipitates with mDia1 antibody, but not with the isotype control. Input is Western blot for total c-Src. (D), c-Src protein expression after transfection with c-Src small interference RNA (siRNA) vs scramble siRNA. E, c-Src is involved in RAGE induced Rac activation. Cells were transfected with c-Src siRNA prior to stimulation with S100B (10 μg/mL) and pulldown of activated rac1 was performed at the indicated time. Results are expressed as mean±SEM of 3 independent experiments; **P<0.01. F and G, Effect of c-Src downregulation in S100B (10 μg/mL)-induced superoxide production and glycogen synthase kinase (GSK3β) ser 9 phosphorylation is shown. In G, histograms represent mean±SEM of the densitometric quantification of 3 independent experiments. A representative picture is shown for each Western blot. *P<0.05, **P<0.01, ***P<0.001.
stress used to identify factors involved in acute vascular remodeling, we tested the role of mDia1 and report for the first time significant upregulation of mDia1 mRNA and protein levels in the neointima of injured arterial vessels compared to sham-treated arteries. Moreover, genetic deletion of mDia1 protected animals against neointimal hyperplasia. As invading and proliferating SMCs express mDia1 and are the main constituents of the neointima, our data link mDia1 to fundamental SMC responses to injury.

RAGE and RAGE ligands are upregulated in the vessel wall after guide wire injury, leading not only to SMC activation, migration, and proliferation, but also to extracellular matrix accumulation. Blockade of RAGE by use of soluble truncated receptor or genetic deletion significantly reduces neointimal hyperplasia in murine and rat models of injury. mDia1 is an intracellular binding partner for RAGE, critical for RAGE-induced C6 glioma cell migration and RAGE-induced Egr1 expression in hypoxic macrophages. In the latter case, we showed that hypoxia results in rapid release of RAGE ligand advanced glycation endproducts into the supernatants of cultured cells, hence linking hypoxia directly to activation of RAGE signaling via these advanced glycation endproducts ligands. Here we show that in injured vessels, deletion of mDia1 mimics the protective effect of RAGE blockade, suggesting that reduction of the neointimal hypertrophy in mice is at least in part through blockade of the impact of RAGE. In line with this hypothesis, we found that mDia1 deletion inhibits RAGE ligand-induced signaling and cell activation in isolated SMCs.

Ligand binding to RAGE induces production of ROS largely by the NADPH oxidase system, but the proximate mechanisms involved in this process are not fully identified. Our data reveal the novel finding that mDia1 is required for RAGE-induced oxidative stress generation and consequent signaling events. Furthermore, our findings highlight seminal insights into the important role of RAGE-dependent NADPH oxidase activation as the initial key event leading to superoxide production, AKT and GSK3β serine 9 phosphorylation, and SMC migration. Activation of NADPH oxidase is an ordered multistep process, including protein phosphorylation, GTPase activation, and translocation of certain cytosolic proteins to the plasma membrane. Our data reveal that in mDia1 deficient cells, membrane translocation of c-Src and p47phox, activation of Rac1, and upregulation of Nox1 on RAGE ligand stimulation are inhibited, suggesting that RAGE-induced NADPH oxidase activation requires the cooperation of mDia1.

As underlying mechanisms included a possible role for mDia1 in Rac1 activation, we investigated the role of c-Src kinase. Src family kinases are potent activators of Rac1. They are also important effectors of the formin family of proteins. Additionally, recent reports highlighted the role of mDia1 in v-Src kinase membrane translocation and in c-Src mediated activation of Rac1 on EGF stimulation. In our studies, RAGE ligand-induced c-Src translocation to the membrane was defective in primary aortic SMCs isolated from mice. Our data also demonstrate key roles for c-Src in RAGE-dependent ROS generation and signaling, as siRNA-mediated reduction of c-Src expression inhibited Rac1 activation, superoxide production and consequent AKT and GSK3β phosphorylation. These results are strengthened by previous reports suggesting a critical role for Src kinases in RAGE-dependent ROS production. Moreover, here we identified mDia1 as a key molecule regulating RAGE dependent c-Src translocation to the membrane and consequent redox signaling.

Previous reports have linked mDia1 and c-Src interaction to actin polymerization and cell protrusion. It was shown that mDia1 interaction with c-Src and mDia interacting protein on EGF stimulation and Vav2 activation led ultimately to Rac1 activation. Our results are in complete agreement with these reports and illustrate this relationship in vascular SMCs. Indeed, the potential participation of Vav2 in RAGE-induced Rac1 activation is a subject of future investigation.

It is well established that RAGE engagement potentially activates numerous signaling pathways depending on the cell type stimulated. Here we show that RAGE ligand S100B

![Figure 8. Proposed mechanism of the role of mammalian diaphanos-related formin (mDia1) in receptor for advanced glycation endproducts (RAGE)-induced redox signaling smooth muscle cell migration and neointimal expansion. Collectively, the findings in this manuscript establish a critical role for mDia1 in transducing the effects of RAGE ligands on c-Src, Rac1, and Nox1 activation in consequent phosphorylation of AKT/glycogen synthase kinase (GSK)3β ser 9, processes essential for RAGE ligand-induced vascular smooth muscle cell migration.](image-url)
impacts the GSK3β phosphorylation state of serine 9. Unlike most kinases, GSK3β is constitutively active in its unphosphorylated form. Phosphorylation of GSK3β at serine 9 inhibits its activity and consequently induces glycosyn-
thesis, mitochondrial protection, and inhibition of the ubiquitin-proteasome system leading to cell survival and protein stability. Increased vascular SMC survival mediated by GSK3β ser 9 phosphorylation has been reported by Park and colleagues as a mechanism leading to neointimal expansion after balloon injury of the carotid artery in rats. Our data reveal that RAGE activation induces GSK3β ser 9 mediated migration of vascular SMCs, therefore suggesting that the phosphorylation status of GSK3β is important for 2 processes critically involved in neointimal expansion, SMC survival, and migration.

Importantly, we found that mDia1 deletion prevented RAGE induced phosphorylation of GSK3β and consequent SMC migration. mDia1 was previously linked to GSK3β phosphorylation during lysophosphatidic acid induced microtubule stabilization, in a manner dependent on a novel PKC isoform which was required to phosphorylate and inhibit GSK3β. We report that mDia1 is essential for RAGE induced c-Src translocation to the membrane and Rac1 activation. These signaling events account for RAGE-induced ROS production and consequent downstream AKT and GSK3β phosphorylation; however, a direct activation of PI3Kinasce pathway by membrane bound Src cannot be excluded.

It is essential to note that although mDia1 is required for the actions of RAGE ligands and EGF on lamellipodia formation, mDia1 was not required for PDGF-mediated formation of lamellipodia nor for PDGF-stimulated phosphorylation of AKT and ser 9 GSK3β. We report that mDia1 is essential for RAGE induced c-Src membrane translocation and Rac1 activation. The key molecular events leading to redox phosphorylation of AKT and GSK3β and SMC migration. As deletion of mDia1 protected against aberrant vascular remodeling, blockade of RAGE-mDia1 may be a newly discovered therapeutic approach to limit pathological inward remodeling in human vascular diseases.

Acknowledgments
The authors gratefully acknowledge Dr. John W. Copeland (University of Ottawa) for sharing cDNA to express murine mDia1 and Ms. Latoya Woods for her expert assistance in the preparation of the manuscript.

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Disclosures
None.

References

**Novelty and Significance**

**What is Known?**

- Ligand engagement by the receptor for advanced glycation endproducts (RAGE) triggers oxidative stress in vascular smooth muscle cells.
- Deletion of RAGE is highly protective in a murine model of femoral artery endothelial denudation injury.
- The cytoplasmic domain of RAGE binds to the formin mDia1; in transformed cells, mDia1 is required for RAGE-activated cell migration and signaling.

**What is New?**

- mDia1 is essential for RAGE ligand-mediated generation of oxidative stress.
- Expression of the formin mDia1 is elevated in the arterial tissue of wild-type, but not RAGE-null, mice after femoral artery endothelial denudation injury.
- mDia1 is required for RAGE ligand-stimulated generation of Rac and lamellipodia, the recruitment of c-Src to the plasma membrane, activation of Akt and GSK3β serine 9, and the in vivo migration of vascular smooth muscle cells following femoral artery endothelial denudation injury.

The transmembrane RAGE molecule lacks intrinsic signaling activity despite strong evidence for its ability to induce tyrosine signaling and cell migration in response to stress. Previous studies identified the formin mDia1 as a binding partner that interacts directly with the cytoplasmic tail of RAGE. mDia1 is a canonical Rho GTPase member of the diaphanous-related formin family. mDia1 generates linear actin filaments and stabilizes microtubule dynamics and is known to dock with Src family nonreceptor tyrosine kinases. Using genetically deficient mice or cells lacking either RAGE or mDia1, we tested the hypothesis that mDia1 is a key intermediate in RAGE signaling. We report that injury to the femoral artery significantly upregulates mDia1 gene and protein expression and is RAGE-dependent. Loss of mDia1 expression significantly impairs pathological neointimal expansion consequent to injury that is known to activate RAGE. In primary murine aortic smooth muscle cells, mDia1 is required for RAGE ligand-induced membrane translocation of c-Src, Rac1 activation, redox phosphorylation of Akt and GSK3β, and migration. Hence, for the first time we have illustrated key roles for mDia1 in smooth muscle cell migration and signal transduction. Taken together, these data illuminate a novel RAGE-mDia1 signaling axis that mediates pathological neointimal formation in the vasculature in response to injury.
Formin mDia1 Mediates Vascular Remodeling via Integration of Oxidative and Signal Transduction Pathways
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I. Detailed Methods

Animals and induction of vascular injury.
Male mice, age 8-12 weeks, were anesthetized with intraperitoneal injections of ketamine (50 mg/kg) and xylazine (5 mg/kg). A groin incision was made, and the common femoral artery was clamped at the level of the inguinal ligament; an arteriotomy was made distal to the epigastric branch. An angioplasty guide wire (0.25-mm diameter; Advanced Cardiovascular Systems Inc., Santa Clara, California, USA) (1) was inserted and placed in the femoral artery distal to the inferior epigastric artery. The clamp was removed, and the endoluminal denudation was induced by three passages of the guide wire (1). In this model, denudation was limited to the intima. In instrumented arteries, vessels that had been subjected to excessive injury (beyond the intima) were readily apparent because the internal elastic lamina (IEL) was not visible. These arteries were immediately excluded from consideration. After removal of the wire, the arteriotomy site was ligated. Post-operative management included fluid treatments and pain medication (buprenorphine). All of the surgeries were performed by a single investigator blinded to the genotype of the animals.

Tissue analyses
At the indicated times after surgery, femoral artery specimens were retrieved and fixed for 24 h in formalin (10%); segments were cut transversely at 3-mm intervals from the inguinal ligament to the epigastric artery, and tissues were embedded in paraffin. The section (5 µm) at the midportion of each femoral artery was treated with Van Gieson’s elastic staining kit (Sigma-Aldrich), and the degree of intimal thickening was analyzed quantitatively using a Zeiss microscope and image analysis system (Media Cybernetics Inc., Silver Spring, Maryland, USA) by one of the investigators blinded to the experimental conditions. Three types of measurements, made by a single investigator blinded to the experimental conditions, included assessment of luminal area, the area encircled by the IEL, and the area encircled by the external elastic lamina (EEL). Intimal area was calculated by subtracting the area encircled by IEL from that encircled by the EEL (1).

Tissue sections were stained with rabbit polyclonal anti-mDia1 (Abcam, Cambridge, MA, USA) monoclonal anti-smooth muscle α-actin IgG (1:100; Sigma-Aldrich), or rabbit control IgG (DakoCytomation, Glostrup Denmark). Sections were deparaffinized and blocked with hydrogen peroxide (3%) in methanol for 30 minutes. Blocking was performed with CAS blocking solution (Zymed laboratories, San Francisco, CA, USA). Primary antibodies were added to slides and incubated overnight at 4°C. Secondary antibodies (1:100 affinity-purified biotinylated rabbit IgG; Sigma-Aldrich) were added for 30 minutes at room temperature. Sections were reacted with HRP-conjugated streptavidin (1:100; Sigma-Aldrich) for 30 minutes at room temperature and developed with 3,3′-diaminobenzidine (DAB) substrate kit (Vector Laboratories, Burlingame, California, USA). Counterstaining was performed with Mayer’s hematoxylin solution (Sigma-Aldrich). Slides were then progressively dehydrated with increasing concentration of ethanol before mounting.

Western blotting
At least eight arteries were snap-frozen in liquid nitrogen, pooled, and stored at –80°C. Tissue samples were ground to a fine powder under liquid nitrogen and incubated in ice-cold lysis buffer (Cell Signaling Technology, Beverly, Massachusetts, USA). Protein extracts of cultured cells were prepared with the same lysis buffer. Particulate material was removed by
centrifugation, and protein concentration was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, California, USA). Equal amounts of total protein (10–30 µg/sample) were subjected to SDS-PAGE (7.5–12%) followed by electrophoretic transfer to nitrocellulose membranes. Nonspecific binding was blocked by incubation of membranes with nonfat dry milk (5%) for 1 hour. Blots were incubated with rabbit anti-human RAGE IgG (Genetex, Irvine, CA, USA), rabbit anti-Nox1 (Novus Biologicals, Littleton, CO USA), rabbit anti-Nox4 (Novus Biologicals, Littleton, CO USA), or with the following antibodies (for total or phospho-form), from Cell Signaling Technology: Rabbit anti-AKT, anti-GSK3β ser9, ERK1/2 (p44/p42 MAP kinase), and anti-c-Src (Y416); dilution of 1:1,000, overnight at 4°C. HRP-conjugated donkey anti-rabbit IgG secondary antibody (1:5,000, GE Healthcare) was used to identify sites of binding of the primary antibodies.

Cell culture and in vitro assays on cultured SMCs.

Mouse vascular SMCs were cultured from the aortas using a modification of the procedure of Tarvo and Barret (2). Mouse vascular SMCs were cultured from the aortas of male 10-week-old mice by following an explant protocol in accordance with institutional guidelines. Mice were anesthetized intraperitoneally with a mixture of Ketamine (50mg/kg) and xylazine (5mg/kg). Aortas from the subclavian branch to the renal bifurcation were excised aseptically from the animal and placed into ice-cold PBS. After removal of the peri-aortic fat, the vessels were incubated at 37°C for 25 minutes in a cocktail of DMEM + Antibiotics + 1% Collagenase + 1% soybean trypsin inhibitor. The digested vessels were carefully cut into small pieces and plated in 6 well plates for at least 7 days in DMEM with 10% FBS, antibiotic/antimycotic and 2 mM of glutamine to induce smooth muscle cell proliferation. Experiments were conducted on SMCs after five to seven passages in culture. Cultures were composed of more than 95% SM-α-actin based on immunostaining. Human recombinant S100B was prepared as previously described (3) Endotoxin was removed following previously described protocols (4).

Co-Immunoprecipitation experiments.

Primary smooth muscle cells were seeded at 1x10^6 cell/100 mm tissue culture dish in complete medium and grown for 24 hours prior to overnight starvation in serum-free medium. Cells were then stimulated with S100B, 10 µg/ml, for 30 minutes and cell lysis was performed as follows: cell were rinsed with ice-cold phosphate-buffered saline and lysed using MPER lysis buffer (Pierce) containing 1 mM phenylmethylsulfonyl fluoride and Complete Protease Inhibitors (Roche Applied Science). After scraping cells from the dish, cellular debris was pelleted and immunoprecipitation was performed with 2 µg of polyclonal rabbit anti-mDia1 IgG (Ptlglab) overnight at 4°C, followed by the addition of protein A/G agarose (Pierce) for 4 hrs at 4°C. Protein A/G agarose was pelleted and washed three times using MPER lysis buffer followed by resuspension in NUPAGE sample buffer and reducing reagent (Invitrogen) for electrophoresis. Western blots were prepared and blotted with monoclonal mouse anti-c-Src IgG (Cell Signaling).

Small interference RNA to knockdown mDia1, Nox1 and c-Src. Small interference RNA (siRNA) duplexes against mouse mDia1, Nox1 or c-Src were synthesized by Invitrogen (Stealth RNAi®). Sequences were the following: mDia1: sense 5’UACAGAGGAAGACUAAUUGAAGCC 3’ & anti sense 3’GGCUUCAUAUCAGCUUCUCUGUA 5’
c-Src: Sense 5’UUGGAUGUGGGACAUACGGUAGUA 3’ & anti-sense 3’UCACUACCGUAUGUCCCAACAUCAA 5’.
The siRNA duplexes against mDia1, Nox1, c-Src or scramble duplexes (negative control) were electroporated into primary smooth muscle cells using the Nucleofector device according to the manufacturer’s protocol (Lonza, program P 24). After 24 hours, cells were stimulated with RAGE ligands and subjected to oxidative stress assessment, migration or phosphorylation studies.

**Cell Migration.**

Migration in response to RAGE ligands: S100B (10µg/ml) and carboxymethyllysine (CML, 10 µg/ml) or a general effector (PDGF, 50ng/ml; EGF 100ng/ml, R&D systems, Minneapolis, MN, USA) was assessed with wounding assay. Cells were grown to confluence in 6-well plates and wounded with a P200 pipette tip. T0 pictures were immediately taken for a baseline width of each scratch. Effectors were then added and cells were maintained at 37°C and 5% CO2 to allow migration. Wound width was measured in selected pictures at 0 and 12 hours and the number of cells migrating below the T0 wound were quantified as effective migrating cells. In some experiments, migration was also assessed with Chemotaxis assays using the QCM Colorimetric Cell Migration Assay (Chemicon). Cells were seeded per upper chamber fitted with a lower 8-µm porous polycarbonate membrane and the insert was placed in the lower chamber of a 24-well dish containing Dulbecco's modified Eagle's medium containing RAGE ligands (S100B), positive control (PDGF) or vehicle and incubated at 37 °C for 8 hours according to the manufacturer's instructions (Chemicon). Cells migrated through the porous membrane were incubated with Cell Stain Solution (Chemicon) followed extraction and optical density measurement at 560 nm.

**ROS measurement: Assessment of NADPH activity.**

*NADPH oxidase activity.* NADPH dependant superoxide production was assessed by lucigenin enhanced chemiluminescence as previously described (5). Briefly, photon emission from the chromogenic substrate lucigenin as a function of acceptance of the electron/ O2- generated by the NADPH oxidase complex was measured every 15 seconds for 10 minutes in a 96 well plate luminometer. Twenty microliters of lysate (femoral arteries or cell lysates) containing 20 µg of proteins were used per well. Volumes were adjusted to 200 µl/well with NADPH oxidase assay buffer containing 250mM HEPES, 100µM of NADPH and 5µM of lucigenin. The plate was immediately introduced in the luminometer for reading. Results, average of at least triplicate wells, are expressed in relative lights units (CPS)/µg of proteins. DHE. Dihydroethidium (DHE) is a lipophilic cell-permeable dye that can undergo oxidation to ethidium bromide in the presence of superoxide, Ethidium then binds irreversibly to the double-stranded DNA, causing amplification of a red fluorescent signal. cells (1x10⁵) were seeded in a 48 well plate. After overnight serum starvation in DMEM without red phenol, cells were pre-incubated with 10µM of DHE for 10 minutes then stimulated with RAGE ligands (S100B 10 µg/ml). Fluorescence was evaluated with a fluorescent plate reader excitation/emission: 370/630 at T0, T5, 15, 30, 60, and 120 minutes. Results are expressed as the difference between unstimulated and stimulated condition.

*p47phox and c-Src translocation.*

Membrane preparations were prepared using Mem-PER Eukaryotic Membrane Protein Extraction Kit ® (Pierce-Thermoscientific). Unstimulated and stimulated cells (5x10⁶) were used and manipulated following the recommendations of the manufacturer. After membrane preparations were prepared, detergent was removed with SDS-PAGE-sample-prep ® (Pierce-
Thermoscientific), and samples were subjected to immunoblotting for p47phox (Abcam) and c-Src (phospho- and total, Cell signal technologies) and actin (BD Biosciences).

**Rac1 activity.**
Rac1 activity was assessed with a pull down assay using Pak1 as a specific ligand for activated Rac1 (Pierce-thermoscientific). Briefly 500 µg of proteins was used in each condition for affinity precipitation of activated Rac1 using human Pak1-PBD. After 1 hour incubation at 4°C, resin was washed extensively and activated Rac1 was eluted, then 25 µl of the eluted samples were used for SDS-PAGE electrophoresis and immunoblotting with Rac1 monoclonal antibody.

**mDia1 rescue experiment in Drf1−/− smooth muscle cells.**
Plasmid pEFN.mDia1, corresponding to the murine myc-tagged derivative of mDia1 was a generous gift from Dr. John Copeland (University of Ottawa). Plasmids were electroporated into primary Drf1−/− smooth muscle cells using the Nucleofector device according to the manufacturer’s protocol (Lonza, program P 24). After 24 hours, cells were harvested for Western blot assessment of mDia1 expression and for stimulation with RAGE ligand before phosphorylation studies.

**Immunoprecipitation experiments**
Primary smooth muscle cells were seeded at 1 x 10^6 cells/100-mm dish in complete media and grown for 24 h before starvation overnight in serum-free media. Cells were stimulated with S100B for 30 min, and cell lysis was performed as follows: cells were rinsed with ice-cold phosphate-buffered saline, and lysed using MPER lysis buffer (Pierce) containing 1 mM phenylmethylsulfonyl fluoride and Complete Protease Inhibitors (Roche Applied Science). After scraping, cellular debris was pelleted and Immunoprecipitation was performed with 2 µg of polyclonal rabbit anti-mDia1 (Ptglab) overnight at 4 °C, followed by the addition of protein A/G agarose (Pierce) for 4h at 4 °C. A/G-agarose was pelleted and washed three times using MPER lysis buffer followed by resuspension in NuPAGE sample buffer and reducing reagent (Invitrogen) for electrophoresis. Western blots were prepared and blotted with monoclonal mouse anti-c-Src (Cell Signaling). Total input material was blotted using antibody to total c-Src.

**RNA extraction and RT-PCR.**
Total RNA was extracted from at least eight vessels prepared as for immunoblotting above using the micro scale RNA isolation kit (Ambion). Reverse transcription was performed with Superscript II (Life Technologies Inc.), primed by oligo-dT, following the protocol suggested by the manufacturer. The following primers were used: for mouse mDia1 sense (5´-GCCAAGAATGAAATGGCTTCTC-3´); antisense (5´-TAACAGTGCCAGAGTCACCAGG-3´). To control for differences in total RNA, α-actin transcripts were visualized by RT-PCR using as primers sense (5´-GTGGGCCGCTCTAGGCACCAA-3´) and antisense (5´-CTCTTTGATGTCACGCACATTTC-3´).

**Statistical analysis.**
In all experiments, unless otherwise indicated, data are reported as the mean ± SEM. in at least three replicates per group. Data were analyzed by post hoc comparisons using 2-tailed t test, and a p value less than or equal to 0.05 was considered significant. Statistical comparisons among groups were determined using one-way analysis of variance (ANOVA).
II. Supplemental Figures and Figure Legends
Online Figure I: mDia1 down-regulation blocks AKT/GSK3β ser 9 phosphorylation.
Primary smooth muscle cells from WT animals were isolated and transfected with scramble or mDia1 siRNA. Cells were then stimulated with S100B (10 µg/ml) and kinetics of AKT and GSK3β ser 9 phosphorylation was then analyzed by Western blots before densitometric quantification. Histograms represent mean ± SEM of optical densitometry of at least 3 Western blots. A representative picture is shown. *p<0.05, ** p<0.01, *** p<0.001. Note that Western blots to verify the reduction of mDia1 protein expression post-siRNA mDia1 vs. scramble siRNA are shown in Figure 5C.
Online Figure II: Inhibition of Rac 1 blocks AKT phosphorylation.
Primary smooth muscle cells from WT animals were transfected with Dominant Negative Rac 1 construct or empty vector. Cells were then stimulated with S100B (10 µg/ml) and phosphorylation of AKT was analyzed by Western blot. Densitometric quantification of at least 3 independent Western blots is shown (mean ± SEM). *** p<0.001.
Online Figure III: Down-regulation of c-Src blocks AKT phosphorylation.
Primary smooth muscle cells from WT animals were transfected with scramble or c-Src siRNA. Cells were then stimulated with S100B (10 µg/ml) and phosphorylation of AKT was analyzed by Western blot. Densitometric quantification of 3 independent experiments (mean ± SEM) and a representative picture are shown. * p<0.05, ** p<0.01.
III. Supplemental Table
Online Table I: Morphometric analysis of the vessels of WT and aged-sex-matched Drf1−/− mice (n=11/group), 21 days after wire guide induced femoral artery denudation was performed. IEL indicates Internal Elastic Lamina and EEL indicates External Elastic Lamina. **p<0.01; *** p<0.001.

<table>
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<th>WT (µm²)</th>
<th>Drf1−/− (µm²)</th>
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<td>Luminal Area</td>
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<td>10160.2 ± 4094.3 **</td>
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** p < 0.01; *** p < 0.001
IV. Supplemental References


