Gas6-Axl Pathway
The Role of Redox-Dependent Association of Axl With Nonmuscle Myosin IIB

Megan E. Cavet, Elaine M. Smolock, Prashanthi Menon, Atsushi Konishi, Vyacheslav A. Korshunov, Bradford C. Berk

Abstract—In vascular smooth muscle cells, Axl is a key receptor tyrosine kinase, because it is upregulated in injury, increases migration and neointima formation, and is activated by reactive oxygen species. Reaction of glutathione with cysteine residues (termed “glutathiolation”) is an important posttranslational redox modification that may alter protein activity and protein-protein interactions. To investigate the mechanisms by which reactive oxygen species increase Axl-dependent vascular smooth muscle cell function we assayed for glutathiolated proteins that associated with Axl in a redox-dependent manner. We identified glutathiolated nonmuscle myosin heavy chain (MHC)-IIB as a novel Axl interacting protein. This interaction was specific in that other myosins did not interact with Axl. The endogenous ligand for Axl, Gas6, increased production of reactive oxygen species in vascular smooth muscle cells and also increased the association of Axl with MHC-IIB. Antioxidants ebselein and N-acetylcysteine decreased the association of Axl with MHC-IIB in response to both Gas6 and reactive oxygen species. Blocking the Axl–MHC-IIB interaction with the specific myosin II inhibitor blebbistatin decreased phosphorylation of Axl and activation of extracellular signal–regulated kinase 1/2 and Akt. Association of MHC-IIB with Axl was increased in balloon-injured rat carotid vessels. Finally, expression of MHC-IIB was upregulated in the neointima of the carotid artery after balloon injury similar to upregulation of Axl protein expression, as shown in our previous studies. These results demonstrate a novel interaction between Axl and MHC-IIB in response to reactive oxygen species. This interaction provides a direct link between Axl and molecular motors crucial for directed cell migration, which may mediate increased migration in vascular dysfunction. (Hypertension. 2010;56:00-00.)

Key Words: vascular smooth muscle ■ receptor protein tyrosine kinase ■ myosin heavy chains ■ reactive oxygen species ■ vascular disease

The receptor tyrosine kinase Axl is a 140-kDa protein expressed in many cell types, including vascular smooth muscle cells (VSMCs), and is activated by growth arrest gene 6 (Gas6), a homologue of protein S.2,3 This leads to activation of downstream signaling cascades, including the phosphatidylinositol 3-kinase–Akt pathway, extracellular signal–regulated kinase 1/2, and phospholipase C-γ. Gas6 activation of Axl in VSMCs stimulates migration and inhibits apoptosis.4,5 Axl is a key VSMC receptor tyrosine kinase, because it is upregulated by injury, activated by reactive oxygen species (ROS), and increases neointima formation.1,6,7 This indicates a role for Axl in the pathogenesis of vascular diseases.1,5–9

Recent evidence indicates that ROS act as signaling molecules by causing glutathiolation of redox-sensitive proteins that contain cysteine thiols. On exposure to oxidants, the cysteine can be reversibly oxidized to sulfenic acid, which can form a disulfide bond with glutathione. Because this process is rapidly reversible by glutaredoxin, it is a regulatory mechanism to prevent further oxidation, protecting proteins against irreversible oxidative damage.10,11 Glutathiolation can alter both enzyme activity and protein-protein interactions. Because Axl is activated by ROS in VSMCs, we hypothesized that ROS will modulate Axl signal transduction by altering interaction with unknown glutathiolated proteins. Therefore, in this study we used glutathiolation as a means to assay for novel redox-sensitive Axl binding partners. We found a redox-induced interaction between Axl and glutathiolated nonmuscle myosin heavy chain (MHC)-IIB in VSMCs. MHC-IIB is involved in directed cell migration.12–14 Expression of MHC-IIB is increased in atherosclerotic lesions, balloon-injured carotid vessels, and in hypertensive arteries.15–18 This strongly suggests that increased expression of MHC-IIB contributes to the increased migratory response in vascular pathology. The Axl–MHC-IIB interaction occurs upon stimulation of VSMCs with both ROS and Gas6. This
interaction is important for Axl signaling in that inhibition of the interaction with the nonmuscle myosin II inhibitor blebbistatin decreases Axl phosphorylation and phosphorylation of downstream kinases ERK1/2 and Akt. Increased interaction between Axl and MHC-IIB in injured arteries suggests that this is important in response to vascular injury.

Methods and Materials
Antibodies to Axl and ERK1/2 were from Santa Cruz Biotechnology; antibodies to phospho-ERK, phospho-Akt (Ser-473), and Akt were from Cell Signaling; MHC-IIB antibody was from Covance, and smooth muscle actin was from DAKO. LiCor fluorescent secondary antibodies were from Molecular Probes. Blebbistatin was from Sigma. LY83583 was from RBI. Gas6 and anti-Axl antibody for immunofluorescence were kindly provided by Brian Varnum (Amgen). Immunohistochemistry reagents were from Covance. All of the other reagents and chemicals were obtained from Sigma.

Cell Culture
Cultured VSMCs were obtained from rat aorta as described. VSMCs were grown in DMEM supplemented with 25 mmol/L of NaHCO3, 10 mmol/L of HEPES (pH 7.4), 50 IU/mL of penicillin, 50 µg/mL of streptomycin, and 10% FBS containing 5.5 mmol/L of glucose in a 5% CO2/95% O2 incubator at 37°C.

Biotinylated Glutathione Ethyl Ester Labeling
Biotinylated glutathione ethyl ester (BioGEE), a membrane-permeable analogue of glutathione, labeling was performed as described by Sullivan et al. Briefly, biotinylated glutathione ester was prepared by mixing 25 mmol/L of sulfo-N-hydroxysuccinimide–biotin with 25 mmol/L of glutathione ethyl ester in 50 mmol/L of NaHCO3 at pH 8.5 for 2 hours followed by the addition of 1.25 mol/L of glycine at pH 8.5 for 5 minutes. Cells were incubated in 250 µmol/L of BioGEE for 1 hour. Cells were then stimulated and rinsed in PBS, and then immunoprecipitation was performed as described below. BioGEE-labeled proteins were detected using horseradish peroxidase (HRP)-streptavidin (Pierce).

Preparation of Cell Lysates and Immunoprecipitations
Cell monolayers were rinsed with ice-cold PBS (150 mmol/L of NaCl, 20 mmol/L of NaPO4 [pH 7.4]) and then scraped in 1 mL of PBS. After a brief centrifugation, the cells were solubilized in 1 mL of cell lysis buffer (10 mmol/L of HEPES [pH 7.4], 50 mmol/L of Na pyrophosphate, 50 mmol/L of NaF, 50 µmol/L of NaCl, 5 mmol/L of EDTA, 5 mmol/L of EGTA, 1 mmol/L of NaVO4, and 0.5% Triton plus 1:1000 protease inhibitor mixture). Cells were sonicated for 20 seconds, agitated on a rotating rocker at 4°C for 30 minutes, and centrifuged at 12,000 g for 30 minutes to remove insoluble cellular debris.

For immunoprecipitation studies, lysates were precleared for 1 hour with protein G agarose (Invitrogen), followed by incubation with anti-Axl antibody for 3 hours and protein G agarose for an additional 1 hour. Immunoprecipitates were then washed 4 times with 1 mL of cell lysis buffer before the addition of Laemmli sample buffer. After heating at 95°C for 3 minutes, proteins were resolved on SDS-PAGE and transferred to nitrocellulose membranes for Western analysis. Immunoreactive bands were detected with LiCor fluorescent secondary antibodies and the LiCor Odyssey Infrared Imaging System. Analysis of blot was performed using the LiCor densitometry software.

Measurement of ROS Production
Hydroethidium and 2’,7’-dichlorodihydrofluorescein (DCF) diacetate was used to measure ROS in VSMCs. VSMCs were loaded with DCF-diacetate (5 µmol/L for 30 minutes), medium was aspirated, and VSMCs were stimulated with Gas6 (100 ng/mL) or H2O2 (300 µmol/L) for 3 minutes in a light-protected humidified chamber at 37°C. Cells were rinsed and images obtained for 1 minute at 10-second intervals with an Olympus BX51 epifluorescence microscope equipped with a ×40 water immersion lens, with excitation 485 nm and emission 535 nm.

Carotid Balloon Injury
Balloon injury of the rat left carotid was performed exactly as described using male Sprague-Dawley rats (300 to 400 g; Charles River Laboratories, Wilmington, MA). At the end of the experiment, the injured and uninjured contralateral vessels were removed and snap frozen in liquid nitrogen. In a separate experiment, animals were perfusion fixed, and carotid arteries were paraffin embedded as described. All of the procedures were carried out in a specific pathogen-free animal care facility at the University of Rochester and were approved by the university’s committee on animal resource.

Immunohistochemistry
After balloon injury, carotid arteries were dissected and paraffin embedded. Cross-sections of injured and contralateral uninjured carotid arteries were deparaffinized and incubated in 3% hydrogen peroxide for 10 minutes, followed by antigen retrieval in citrate buffer (pH 6.0) for 20 minutes. Sections were stained according to protocol proved by Covance. Polyclonal MHC-IIB antibody was diluted in DAKO antibody dilution buffer 1:5000. Negative control goat IgG was used at the corresponding dilution. Cross-sections were counterstained with hematoxylin.

Statistical Analysis
All of the experiments were carried out ≥3 times. Differences were assessed by ANOVA, and P<0.05 was considered significant in all of the experiments.

Results
Redox-Sensitive Interaction Between Axl and MHC-IIB
To detect proteins that associated with Axl in a redox-dependent manner we used BioGEE labeling, as described by Sullivan et al. Rat aortic VSMCs incubated with BioGEE were stimulated with H2O2 (0 to 1000 µmol/L), and Axl was immunoprecipitated. Glutathiolated proteins that interacted with Axl were identified using streptavidin-HRP after nonreducing SDS-PAGE. A 225-kDa protein communoprecipitated with Axl (Figure 1). This protein was no longer present upon preabsorption of the H2O2-stimulated cell lysate with streptavidin (data not shown). In addition, samples were treated with dithiothreitol to disrupt disulfide bonds. This abolished the detection of the immunoprecipitated band upon H2O2 stimulation (Figure S1A, available in the online Data Supplement at http://hyper.ahajournals.org). The 225-kDa protein was identified by mass spectrometry as MHC-IIB. To confirm that Axl and MHC-IIB do interact, cells lysates were immunoprecipitated with Axl, and MHC-IIB was immunoblotted. The interaction increased in response to H2O2 (Figure S1B).

To demonstrate that MHC-IIB interacted with Axl in a redox-sensitive manner, VSMCs were stimulated with H2O2 (300 µmol/L) for the indicated times (0 to 20 minutes), after which Axl was immunoprecipitated and MHC-IIB was immunoblotted (Figure 2). MHC-IIB interaction with Axl increased upon stimulation with H2O2 in a time-dependent, manner peaking between 3 and 10 minutes. (Figure 2A). To investigate the specificity of this interaction, the ability of smooth muscle MHCs (SM1 and SM2) to associate with Axl was assessed by ANOVA, and P<0.05 was considered significant in all of the experiments.

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Gas6, the endogenous ligand for Axl, increases Rac activity, which is upstream of NADPH oxidase in neuronal cells, suggesting that Gas6-Axl should increase ROS-mediated effects in VSMCs. Therefore, we measured H$_2$O$_2$ generation in VSMCs using the H$_2$O$_2$-sensitive fluorophore DCF-diacetate. Gas6 (100 ng/mL) increased DCF fluorescence by 4-fold (~50%) of the increase with H$_2$O$_2$; Figure 4A).

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was determined. Neither SM1 nor SM2 coimmunoprecipitated with Axl after stimulation with H$_2$O$_2$ (Figure 2B). Diamide (30 µmol/L, a thiol oxidizer) and LY83583 (1 µmol/L, a superoxide generator) also increased the interaction between Axl and MHC-IIB, further demonstrating the importance of glutathiolation and ROS in the association between the 2 proteins (Figure 2C and 2D).

The effect of antioxidants on the interaction of MHC-IIB with Axl was studied by pretreatment of VSMCs for 30 minutes with the antioxidants N-acetylcysteine (1 mmol/L), which reduces protein thiols, and ebselen (40 µmol/L), which is a glutathione peroxidase mimetic (Figure 3A and 3B). Cells were then stimulated with H$_2$O$_2$ for the indicated times, and Axl was immunoprecipitated (Figure 3). Antioxidants completely abolished the interaction between Axl and MHC-IIB. Therefore, ROS cause glutathiolation of MHC-IIB and induce association of MHC-IIB with Axl.

**Interaction Between Axl and MHC-IIB Is Induced by Gas6**

Gas6, the endogenous ligand for Axl, increases Rac activity, which is upstream of NADPH oxidase in neuronal cells, suggesting that Gas6-Axl should increase ROS-mediated effects in VSMCs. Therefore, we measured H$_2$O$_2$ generation in VSMCs using the H$_2$O$_2$-sensitive fluorophore DCF-diacetate. Gas6 (100 ng/mL) increased DCF fluorescence by 4-fold (~50%) of the increase with H$_2$O$_2$; Figure 4A), demonstrating that Gas6 stimulates production of H$_2$O$_2$. Because Gas6 is a ligand for Axl and is upregulated in vascular injury, we studied the ability of Gas6 to increase the interaction between Axl and MHC-IIB, as described for H$_2$O$_2$ above. Gas6 increased the Axl–MHC-IIB interaction with a similar time course to H$_2$O$_2$ (Figure 4B). We further demonstrated that Gas6 increased association of glutathiolated MHC-IIB with Axl by labeling VSMCs with BioGEE, and immunoprecipitating with Axl antibody as was done for Figure 1. Immunoblotting with HRP-streptavidin, followed by MHC-IIB antibody, demonstrated that glutathiolated MHC-IIB interacted with Axl after treatment with Gas6 at 3 and 5 minutes (Figure 4C).

The effect of antioxidants on the interaction of MHC-IIB with Axl mediated by Gas6 was studied by pretreating VSMCs for 30 minutes with N-acetylcysteine (1 mmol/L) and ebselen (40 µmol/L). Cells were then stimulated with H$_2$O$_2$ (300 µmol/L). To examine interaction between Axl and SM1 and SM2, cell lysates were immunoprecipitated with anti-Axl antibody and immunoblotted with SM1 and SM2 antibody (top). Lysates show expression of SM1 and SM2 in VSMCs (top). Equal loading was confirmed using anti-Axl antibody (bottom).

**Inhibition of MHC-IIB Activity Inhibits MHC–IIB-Axl Interaction and Axl Signaling**

To investigate the role of the interaction between MHC-IIB and Axl on Axl-dependent signaling we pretreated VSMCs with the myosin II inhibitor blebbistatin (10 µmol/L) for 30 minutes, followed by stimulation with Gas6 (100 ng/mL) for varying times. Axl was then immunoprecipitated, and inter-
action with MHC-IIB was determined by Western blotting. As shown in Figure 6A, treatment of cells with blebbistatin inhibited the interaction between Axl and MHC-IIB. We then determined the effect of blebbistatin on phosphorylation of Axl and Axl downstream targets. Blebbistatin inhibited tyrosine phosphorylation of Axl (and, hence, activation) by 50% at 5, 10, and 20 minutes of Gas6 stimulation (Figure 6B).

Activation of both ERK1/2 and Akt by Gas6 was inhibited by blebbistatin treatment in a time-dependent manner (Figure 6C and 6D). ERK phosphorylation in the presence of blebbistatin was decreased by ~2-fold compared with control at all of the time points, with maximal inhibition achieved at 20 minutes, when ERK phosphorylation was inhibited by 35% (P<0.05; n=3; Figure 6C). Inhibition of Akt activation (~1.3-fold) in the presence of blebbistatin compared with control was evident at 10 minutes and greatest after 20 minutes of Gas6 stimulation, where Akt phosphorylation was inhibited by 20% (P<0.05; n=3; Figure 6D). These data indicate that the interaction of MHC-IIB with Axl augments Gas6-Axl signaling in VSMCs.

**Association of Axl and MHC-IIB Increases in Injured Vessels**

To determine whether the interaction between Axl and MHC-IIB was altered under pathological conditions, the left carotid arteries of Sprague-Dawley rats were balloon injured. At 7 days postinjury, total protein was extracted from vessels, and Axl was immunoprecipitated. Axl expression increased after injury (consistent with previously published findings)

We demonstrated previously that Axl expression increased in the neointima after balloon injury. Specifically, Axl was highly expressed in the subluminal neointima. Similarly, MHC-IIB expression in the carotid artery is increased in the neointima 14 days after injury (Figure 7C versus 7B). The latter suggests that, under pathophysiological conditions, there is an increase in MHC-IIB expression in the neointima. Axl and MHC-IIB expressions localize in the same region in the remodeled artery.

**Discussion**

The major finding of the present study is that ROS stimulate interaction between Axl and glutathiolated MHC-IIB, which augments Axl signaling. This interaction is induced by both ROS and Gas6, the endogenous ligand for Axl. Importantly, we show that Axl and MHC-IIB association regulates Axl signaling and occurs in the carotid artery after vascular injury. We propose that the interaction between MHC-IIB and Axl...
provides a direct link between receptor signaling and cytoskeletal molecular motors that are crucial for cell migration. This is the first study to demonstrate an interaction between receptor tyrosine kinases and nonmuscle myosin II. Interestingly, a constitutive interaction between the G proteincoupled receptor CXCR4 and MHC-IIA has been shown to increase β-arrestin–mediated receptor endocytosis, thus downregulating receptor signaling.23 In contrast, we have found an ROS-induced interaction between MHC-IIA and Axl that increases Axl signaling.

The exact mechanism by which stimulation of Axl increases intracellular ROS is unclear, although we propose that it is mediated through NADPH oxidase. In our system ROS increases intracellular ROS is unclear, although we propose that it is mediated through NADPH oxidase. In our system ROS

Figure 5. Gas6-dependent interaction between Axl and MHC-IIA is inhibited by antioxidants. Cells were pretreated with 1 mmol/L of N-acetylcysteine (A) or 40 μmol/L of ebselen (B) for 30 minutes. Cells were then stimulated with Gas6 (100 ng/mL) for the indicated times. Cell lysates were immunoprecipitated with anti-Axl antibody and interacting MHC-IIA detected by immunoblotting with MHC-IIA antibody. Equal loading was confirmed with anti-Axl antibody (bottom).

Figure 6. Disruption of the Axl–MHC-IIA interaction inhibits Axl signaling. Cells were pretreated with blebbistatin (10 μmol/L) for 30 minutes. Cells were then stimulated with Gas6 (100 ng/mL) for the indicated times. A, Cell lysates were immunoprecipitated with anti-Axl antibody and interacting MHC-IIA detected by immunoblotting with MHC-IIA antibody. Equal loading was confirmed with anti-Axl antibody (bottom). B, Cell lysates were immunoprecipitated with anti-Axl antibody and immunoblotted with phospho-tyrosine 4G10 antibody. Equal loading was confirmed with anti-Axl antibody (bottom). C, Lysates were immunoblotted with phosphospecific ERK1/2 antibody (above) and then reprobed with ERK1/2 antibody (bottom). D, Lysates were immunoblotted with phosphospecific Akt antibody (above) and then reprobed with Akt antibody (below).

signaling complex that includes myosin, Shc, GRB2, Nck, PAK, caldesmon, and myosin light chain kinase.27

Cell migration involves the coordination of signal transduction pathways and cytoskeletal modifications. Gas6 activation of Axl in VSMCs has been shown to stimulate ERK1/2 and Akt, which are required to promote migration and inhibit apoptosis, respectively.4,21 Several studies suggest that MHC-IIA has a role in directed migration of cells.12–14,28–29 MHC-IIA is required for directed cell migration by coordinating protrusive activities and stabilizing the cell periphery.12 Phosphorylation of MHC-IIA may regulate both its motor activity and its ability to assemble into filaments. In the aortic smooth muscle, angiotensin II stimulation results in abnormal cell shape, focal adhesions, and chemotaxis.29 Analysis of cell migration in MHC-IIA null fibroblasts demonstrated that MHC-IIA is required for directed cell movement by coordinating protrusive activities and stabilizing the cell periphery.12 Phosphorylation of MHC-IIA regulates both its motor activity and its ability to assemble into filaments. In the aortic smooth muscle, angiotensin II stimulation results in abnormal cell shape, focal adhesions, and chemotaxis.

We demonstrated that H2O2 significantly increases intracellular oxidative stress. In addition, we have shown that
oxidative stress can activate Axl signaling, although the way by which this occurs is unclear. Our data suggest that one potential mechanism may be redox-dependent gluthiolation of MHC-IIB promoting its interaction with Axl. The finding that Axl stimulation leads to the activation of protein kinase C and Rac suggests that, under conditions of oxidative stress, an interaction between MHC-IIB and Axl provides a direct link between receptor signaling and cytoskeletal molecular motors that is crucial for migration. Therefore, it is conceivable that Axl and MHC-IIB could regulate spatial location and localized activity of MHC-IIB, thus providing a mechanism for localized and directed cell movement.

When grown in tissue culture, VSMCs undergo a phenotypic transition from the normal “contractile” phenotype observed in vivo to a synthetic type that resembles the cell type in atherosclerosis and restenosis. MHC-IIB is specifically expressed in VSMCs of the synthetic type. Increased MHC-IIB expression is also apparent in atherosclerotic lesions, media of balloon-injured vessels, and in hypertensive arteries. This strongly suggests that increased expression of MHC-IIB contributes to the increased migratory response in these pathological conditions.

Our data demonstrated that MHC-IIB is highly expressed in the subluminal neointima, a region consisting of highly proliferative cells, suggesting that MHC-IIB may play a role in cell proliferation under pathological conditions. In support of our finding, Takeda et al. showed that cardiac myocytes lacking MHC-IIB exhibited decreased proliferation, as well as cell hypertrophy. We have demonstrated that Axl expression is also increased in the subluminal neointima congruent with MHC-IIB expression. Specifically, Axl is highly up-regulated in balloon-injured carotid arteries with a time course paralleling that of neointima formation, and Axl expression is increased in VSMCs exposed to thrombin and angiotensin II. In addition, neointima formation is decreased in Axl knockout mice in response to injury or low flow. Furthermore, genetic deletion of Axl was shown to prevent vascular dysfunction and remodeling in salt-induced hypertension. Specifically, Axl knockout mice had reduced systolic blood pressure and improved vasorelaxation. There is also evidence suggesting an important role for Axl in the vascular response to injury mediated by ROS. Importantly, Axl is activated by H2O2, which is increased in vascular injury. Our results demonstrating an interaction between Axl and MHC-IIB provide a plausible mechanism for how Axl regulates the vascular response in pathological conditions.

**Perspectives**

We propose the following model (Figure S2): ligand-dependent (Gas6) and independent (H2O2) activations of Axl increase intracellular ROS that promote gluthiolation of MHC-IIB. This results in Axl and MHC-IIB interacting and activating ERK and promigratory signaling. Given the importance of cell oxidative stress and cell migration in vascular pathologies, it is highly likely that the Axl-MHC-IIB interaction increases VSMC migration relevant to the pathogenesis of vascular disease.

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**Disclosures**

None.

**References**


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Supplementary Data

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Running title: Redox sensitive Axl-MHC-IIB interaction

Supplementary Figures: 2
Figure S1.

a. Cells were harvested with 1mM DTT in the lysis buffer to disrupt disulphide bonds. Axl was immunoprecipitated. Normal SDS-PAGE gel was performed and BioGEE labeled proteins were detected with HRP-streptavidin. Note: MHC-IIB the 225 kDa was not detected; TCL = total cell lysate.

b. Cells were serum starved and stimulated with 600 µM H₂O₂ for 5 min. Cells were harvested and Axl was immunoprecipitated. Normal SDS-PAGE gel was performed and probed for MHC-IIB. Note: Interaction between Axl and MHC-IIB increases upon stimulation.
Figure S2. Proposed model: Upon Axl activation there is an increase in intracellular ROS. This results in glutathiolation of MHC-IIB, which promotes its interaction with Axl, activating Axl’s pro-migratory signaling through ERK activation.