IQGAP1 Regulates Reactive Oxygen Species–Dependent Endothelial Cell Migration Through Interacting With Nox2

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Objective—Endothelial cell (EC) migration is a key event for repair process after vascular injury and angiogenesis. EC migration is regulated by reorganization of the actin cytoskeleton at the leading edge and localized production of reactive oxygen species (ROS) at the site of injury. However, underlying mechanisms are unclear. We reported that IQGAP1, an actin binding scaffold protein, mediates VEGF-induced activation of gp91phox (Nox2)-dependent NAD(P)H oxidase and EC migration. We thus hypothesized that Nox2 and IQGAP1 may play important roles in ROS-dependent EC migration in response to injury.

Methods and Results—Using a monolayer scratch assay with confluent ECs, we show that ROS production is increased at the margin of scratch area and Nox2 translocates to the leading edge, where it colocalizes and associates with both actin and IQGAP1 in migrating ECs. Knockdown of IQGAP1 using siRNA and inhibition of the actin cytoskeleton blocked scratch injury-induced H2O2 production, Nox2 translocation and its interaction with actin, and EC migration toward the injured site.

Conclusions—These suggest that IQGAP1 may function to link Nox2 to actin at the leading edge, thereby facilitating ROS production at the site of injury, which may contribute to EC migration. (Arterioscler Thromb Vasc Biol. 2005;25:2295-2300.)

Key Words: actin cytoskeleton ■ endothelial cell migration ■ IQGAP1 ■ NAD(P)H oxidase ■ reactive oxygen species

Endothelial migration is a key event during the repair of damaged vessels after vascular injury and angiogenesis, and this may contribute to limiting the development of atherosclerosis.1,2 Cell migration is regulated by the dynamic reorganization of the actin cytoskeleton, protrusion at the front of the cell, and retraction at the rear. It is a highly localized event, involving the generation of spatially and temporally restricted signaling molecules, including the small GTPase Rac1 and phosphatidylinositol 3,4,5 trisphosphate [PI(3,4,5)P3],4 the product of PI 3-kinase, at the site of the new leading edge. Although excess amounts of reactive oxygen species (ROS) are toxic, physiological levels of ROS serve as signaling molecules to regulate many growth and migratory responses.5,6 ROS are also necessary for reparative angiogenesis in the ischemic heart7 and hindlimb8 as well as wound-healing in vivo.9 The PI 3kinase-Rac pathway is also involved in ROS production.10 In endothelial cells (ECs), endogenous H2O2 accumulates in actively migrating cells at the site of injury, which is required for cytoskeletal reorganization and cell migration.11 However, underlying regulatory mechanisms are unclear.

In ECs, NAD(P)H oxidase is a major source of ROS.12 ECs express NAD(P)H oxidase subunits that are identical to those found in phagocytes, including the membrane-bound gp91[phox] (now known as Nox2) and p22[phox], the cytosolic components p47[phox] and p67[phox], and Rac1.12 On stimulation, cytosolic components translocate to the membrane to form a multimeric protein complex, leading to production of ROS.12 Recently, 4 homologues of Nox213 have been identified in nonphagocytic cells. Among them, Nox4 is abundantly expressed in ECs and involved in basal superoxide production.14 We reported that Nox2-derived ROS play an essential role in vascular endothelial growth factor (VEGF)-stimulated signaling linked to EC migration15 as well as neovascularization in vivo in response to VEGF15 and hindlimb ischemia.8 We have recently identified IQGAP1 as a novel VEGF receptor type2 (VEGFR2) binding protein16 and found that it is a critical regulator for VEGF-induced ROS production and EC migration. IQGAP1 is a scaffold protein that plays a pivotal role in regulating actin cytoskeleton, cell adhesion, and cell migration17,18 by interacting directly with calmodulin, actin, active Rac1/Cdc42, β-catenin, E-cadherin, the microtubule plus end-binding protein, CLIP-170, and a tumor suppressor protein, adenomatous polyposis coli (activated protein C [APC]).19 In actively migrating cells, IQGAP1

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accumulates at the leading edge and cross-links actin filaments. Recent evidence shows that IQGAP1 links active Rac1 to APC and CLIP170 to form a multi-molecular complex at the leading edge, thereby connecting actin cytoskeleton and microtubule dynamics during directional migration. We previously reported that IQGAP1 expression is dramatically increased in the luminal regenerating EC layers induced H2O2 production, Nox2 translocation and its interaction with actin, and EC migration toward injured site. These results suggest that IQGAP1 may function as a scaffold to link Nox2 to actin at the leading edge, thereby facilitating ROS production at the site of injury, which may contribute to EC migration.

Materials and Methods

Mouse and rabbit anti-IQGAP1 antibodies were form BD Bioscience (for immunofluorescence) and Santa Cruz (for immunoprecipitation). Rabbit anti-Nox2 (gp91phox) antibody is highly selective for human Nox2 protein, which has been demonstrated by peptide competition studies.

Other materials are in the online data supplement. Cell culture, measurements of intracellular H2O2 levels, a monolayer scratch assay, immunoprecipitation and immunoblotting, confocal immunofluorescence microscopy, synthetic siRNA and its transfection, and statical analysis are described in the Material and Methods section in the online data supplement (http://atvb.ahajournals.org).

Results

Nox2 Accumulates at the Leading Edge After Scratch Injury

We performed a monolayer scratch assay, which primarily measures migration toward the injured sites. Confluent monolayers of HUVECs were scratched, loaded with dichlorofluorescein diacetate (DCF-DA), and the change in dichlorofluorescein (DCF) fluorescence was monitored using immunofluorescence confocal microscopy. Scratch injury induced a significant increase in DCF fluorescence within 1 hour, peaked at 4 hours, which continued at least for 8 hours (Figure 1A) and gradually decreased to the basal levels within 12 hours (data not shown). We confirmed that DCF signal was abolished by preincubation with polyethylene glycol (PEG)-catalase, suggesting that it mainly detects H2O2 production, as reported previously. At 4 hours after the scratch, cells at the margin of the scratched area produced significantly more H2O2 than did cells away from the injured sites in migrating ECs (Figure 1B).

To determine whether an increase in H2O2 production at the margin of scratched area was caused by the spatially restricted localization of Nox2, a critical component of endothelial NAD(P)H oxidase, we examined the subcellular localization of Nox2 in actively migrating ECs. Nox2 accumulated at the leading edge of the cell membrane in actively migrating ECs (Figure 1B). Furthermore, nonimmune IgG (control) showed no staining (Figure IC). In contrast, in confluent HUVEC monolayers before scratch or in migrating ECs away from the scratched area, Nox2 was found predominantly in perinuclear and nuclear regions (Figure IB), which is consistent with the previous report. We then examined the functional role of ROS and Nox2 in EC migration induced by scratch injury. Pretreatment of HUVECs with antioxidants (N-acetylcysteine [NAC], a thiol antioxidant, and PEG-catalase) or Nox2 siRNA, which knockdowns Nox2 protein (Figure ID) markedly inhibited scratch-induced cell migration toward the injured sites (Figure IC; Figure IIA, available online at http://atvb.ahajournals.org).
Role of Actin Cytoskeleton in Nox2 Localization at the Leading Edge and ROS Production After Scratch Injury

To determine the role of actin cytoskeleton in localization of Nox2 at the leading edge during active EC migration, we examined the effects of inhibition of actin depolymerization with latrunculin A or actin stabilization with jasplakinolide. Both latrunculin A and jasplakinolide inhibited the localization of Nox2 at the leading edge (Figure 2B) without significant effect on microtubule structure stained with α-tubulin (Figure IIIC). Furthermore, both inhibitors reduced the scratch-induced increase in DCF fluorescence without affecting basal levels (Figure IIB).

IQGAP1 Colocalizes and Associates With Actin and Nox2 at the Leading Edge After Scratch Injury

IQGAP1 is an actin-binding scaffolding protein involved in regulating the actin cytoskeleton. Thus, we examined whether IQGAP1 colocalizes and associates with Nox2 at the leading edge after the injury. In confluent monolayers of HUVECs immediately after the scratch, IQGAP1 was mainly localized at the cell margin (Figure 3A). However, in actively migrating ECs at 4 hours after scratch, IQGAP1 accumulated at the leading edge as well as at the side opposite to the leading edge (Figure 3A). Of note, IQGAP1 predominantly colocalized with F-actin (Figure 3B) and Nox2 (Figure 4A) at the leading edge in migrating ECs. Moreover, immunoprecipitation analysis demonstrated that scratch injury promoted association of IQGAP1 with actin (Figure 3B) and Nox2 (Figure 4B), which correlated with their colocalization demonstrated by immunofluorescence study. Their association returned to basal levels when the ECs have completed migration (data not shown).

Role of IQGAP1 in Localization of Nox2 and Its Interaction With Actin at the Leading Edge After Scratch Injury

To determine the role of IQGAP1 in localization of Nox2 at the leading edge and association of Nox2 with actin, we examined the effects of knockdown of IQGAP1 using siRNA. IQGAP1 siRNA, but not scrambled siRNA, inhibited scratch-induced increase in F-actin at the lamellipodia of protruding cell membrane, but not in stress fibers, as well as accumulation of Nox2 at the leading edge (Figure 5A) and Nox2-actin interaction (Figure 5B). Quantification of Nox2-positive cells at the leading edge further confirmed scratch-induced translocation of Nox2 is significantly inhibited by IQGAP1 depletion (Figure IIIA, available online at http://atvb.ahajournals.org). Of note, IQGAP1 siRNA almost completely knocked down IQGAP1 protein without affecting the expression of Nox2, actin, and α-tubulin (Figure 5B) or microtubule structure (Figure IIIB), confirming the specificity of IQGAP1 siRNA.

Role of Actin Cytoskeleton and IQGAP1 in ROS Production and EC Migration After Scratch Injury

To determine the functional significance of interaction of IQGAP1 with Nox2 and actin at the leading edge, we examined the effects of IQGAP1 siRNA on H2O2 production and EC migration in response to injury. IQGAP1 siRNA significantly inhibited scratch-induced increase in DCF fluorescence without affecting basal levels (Figure IIIIC), which is consistent with the results obtained with inhibition of actin cytoskeleton. Moreover, latrunculin A or jasplakinolide, as well as IQGAP1 siRNA, markedly inhibited scratch-induced cell migration (Figure 6A and 6B).

Figure 2. Role of actin cytoskeleton in Nox2 localization at the leading edge after scratch injury. A. Upper: at 4 hours after the scratch, cells were double-labeled with rabbit anti-Nox2 antibody (left), followed by anti-rabbit fluorescence isothiocyanate (FITC)-conjugated secondary antibody and Rhodamine Red X (RRX)-phalloidin (middle). Small white arrows point to the leading edge and large arrows point to direction toward the scratched area. Arrowheads in merged image indicate colocalization of Nox2 with F-actin at the leading edge. Images are representative of 4 independent experiments. A. Lower: Lysates from scratched HUVECs were immunoprecipitated with anti-Nox2 antibody and RRX-phalloidin.

Immunoblotted with anti-actin or Nox2 antibody. Nox2 blots as 2 bands of molecular weight between 75 to 100 kDa protein, which is consistent with the variably glycosylated protein reported previously. Graph shows averaged data of Nox2-actin interaction, expressed as fold change over basal (0 hour). *P<0.05 vs control (0 hour) (n=4). B. Effects of latrunculin A (10 nmol/L for 1 hour) or jasplakinolide (50 nmol/L for 1 hour) on scratch-induced Nox2 localization at the leading edge. At 4 hours after the scratch, cells were double-labeled with anti-Nox2 antibody and RRX-phalloidin.
Discussion

The present study demonstrates that EC scratch injury stimulates accumulation of Nox2 at the leading edge of the cell, where it colocalizes and associates with both IQGAP1 and actin during active migration of ECs. This response is associated with an increase in ROS production at the margin of scratched area. Our results are consistent with the notion that cell migration is a highly polarized event, which is dependent on the accumulation of active Rac1 and generation of PI(3,4,5)P3 at the leading edge.1,4 Of note, PI3-K-Rac1 pathway is an upstream mediator for activation of NAD(P)H oxidase.10 These suggest that scratch injury stimulates Nox2 accumulation to the leading edge where it promotes assembly of NAD(P)H oxidase, thereby increasing ROS production toward the injured area in migrating ECs. We also show that EC migration is significantly inhibited by NAC, PEG-catalase, or Nox2 siRNA, suggesting that Nox2-derived H2O2 plays an important role in EC migration. Of note, their inhibition is not complete, which may be attributable to the possibility that all the ROS involved in EC migration are not blocked completely by each intervention, or that ROS-independent pathways are also minimally involved.

We demonstrate that Nox2 colocalizes with actin at the leading edge, but not with stress fibers in the cell body, in actively migrating ECs. Coimmunoprecipitation of Nox2 and actin suggests that Nox2 and actin physically associates in vivo directly or indirectly through the intermediate proteins. The functional significance of interaction of Nox2 and actin is demonstrated by the observation that disruption of actin assembly with latrunculin A or disassembly with jasplakolid inhibits scratch injury-stimulated accumulation of Nox2 at the leading edge, ROS production, and EC migration. Thus, an intact actin cytoskeleton may play an important role for proper localization of Nox2 at the leading edge, which is required for ROS production at the site of injury and EC migration.
migration. Molecular linkage between actin cytoskeleton and NAD(P)H oxidase has been demonstrated. In ECs, Wu et al. reported that VEGF promotes p47phox translocation to membrane ruffles via direct interaction with WAVE1, a promoter of the actin nucleation complex, which in turn activates NAD(P)H oxidase. Qian et al. showed that arsenic-induced NAD(P)H oxidase activation and EC migration are suppressed by cytochalasin D and jasplakinolide. In unstimulated ECs, Li et al. reported that NAD(P)H oxidase(s) exist in a predominantly perinuclear location and are associated with actin filaments. Similarly, the present study shows that Nox2 is found at the perinuclear region and nucleus area, and partially colocalizes with actin stress fibers in nonscratched, confluent monolayer of ECs. This may explain at least in part the basal interaction of Nox2 and actin as demonstrated by coimmunoprecipitation. It is possible that actin filaments may provide a structural basis to stabilize the NAD(P)H oxidase in static cells. On scratching monolayer of ECs, the reorganized actin cytoskeleton, which is the main driving force for cell migration, may enable Nox2 to localize to the leading edge where actin polymerization and actin cross-linking are increased. Whether other NAD(P)H oxidase components are assembled together with Nox2 at this specific compartment during active EC migration is currently under investigation.

To gain insight into the molecular mechanisms of how Nox2 and actin interact at the leading edge, we examined the role of IQGAP1, an F-actin binding scaffold protein involved in regulating actin cytoskeleton. It has been shown that IQGAP1 accumulates at the leading edge in actively migrating cells and directly binds and cross-links actin filaments, thereby regulating local actin assembly at the cell front. IQGAP1 also binds to active Rac1 through a GAP-related domain, thereby suppressing the intrinsic GTPase activity, which in turn increases active Rac1. Thus IQGAP1 promotes cell migration and invasion in a Rac1-dependent manner. We previously demonstrated that VEGF stimulation promotes recruitment of Rac1 to the IQGAP1 that associates with VEGFR2, which plays an essential role in VEGF-induced ROS production and EC migration. Here we show that IQGAP1 binds to and colocalizes with both Nox2 and actin at the leading edge during EC migration. This result suggests that IQGAP1 and Nox2 physically associate in vivo directly or indirectly through intermediate proteins such as F-actin which directly interacts with IQGAP1. The functional significance of interaction of IQGAP1 with Nox2 and actin is demonstrated by the observation that IQGAP1 siRNA inhibits scratch-
induced accumulation of Nox2 and actin at the leading edge, Nox2-actin interaction, 
H2O2 production, and EC migration. Although IQGAP1 has been shown to be involved in local 
control of microtubule stability through interacting with APC, 
active Rac1, and CLIP-170 at the leading edge in active 
migrating Vero cells,22 we found that IQGAP1 siRNA inhib-
its polarized actin assembly at the leading edge (Figure 5A) 
without significant effects on microtubule structure in 
HUVECs.

The present study suggests that IQGAP1 may function as 
an actin-binding scaffold protein to link/target Nox2 to actin 
cytoskeleton at the leading edge, thereby facilitating ROS 
production at the injured area, which may contribute to EC 
migration. Given that IQGAP1 expression is dramatically 
increased in the regenerating EC layers of balloon injured 
artery,16 it is tempting to speculate that IQGAP1 may con-
tribute to the repair process of ECs through an increase in 
Nox2-derived ROS in vivo. These observations provide 
insight into a role of IQGAP1 in temporally and spatially 
organized ROS-dependent EC migration as well as a novel 
mechanism by which ROS are involved in endothelial 
migration.

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Supplemental Figure I

A

ECs at the scratched area

+ PEG-Catalase

ECs away from the scratched area

B

Before scratch

+ scratch (0 h)

+ scratch (4 h)

Scrambled siRNA

Nox2 siRNA

C

Non-immune IgG

D

Scrambled siRNA

Nox2 siRNA

IP: Nox2

IB: Nox2

IB: α-tubulin

Supplemental Figure II

A

Migration of cells

control + NAC + PEG-Catalase

Scrambled siRNA + Nox2 siRNA

B

Before scratch

Merged phalloidin

α-tubulin

C

+ Vehicle

+ Latrunculin A

+ Jasplakinolide

D

DCF fluorescence

Vehicle

Latrunculin A

Jasplakinolide

Fold change
Supplemental Figure III

A

B

C

No. % No. %

Of cells beside

Scrambled

IQGAP1

siRNA

siRNA

*Fold change

Scrambled

IQGAP1

siRNA

Scramble

IQGAP1

siRNA

0

2

4

6

8

0

1

2

0.5

1

1.5

2

+ scratch

scramble

IQGAP1

siRNA

siRNA

0

4

(hr)