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Localized cysteine sulfenic acid formation by vascular endothelial growth factor: role in endothelial cell migration and angiogenesis

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Abstract

Reactive oxygen species (ROS) are important mediators for VEGF receptor 2 (VEGFR2) signalling involved in angiogenesis. The initial product of Cys oxidation, cysteine sulfenic acid (Cys-OH), is a key intermediate in redox signal transduction; however, its role in VEGF signalling is unknown. We have previously demonstrated IQGAP1 as a VEGFR2 binding scaffold protein involved in ROS-dependent EC migration and post-ischemic angiogenesis. Using a biotin-labelled Cys-OH trapping reagent, we show that VEGF increases protein-Cys-OH formation at the lamellipodial leading edge where it co-localizes with NADPH oxidase and IQGAP1 in migrating ECs, which is prevented by IQGAP1 siRNA or trapping of Cys-OH with dimedone. VEGF increases IQGAP1-Cys-OH formation, which is prevented by N-acetyl cysteine or dimedone, which inhibits VEGF-induced EC migration and capillary network formation. *In vivo*, hindlimb ischemia in mice increases Cys-OH formation in small vessels and IQGAP1 in ischemic tissues. In summary, VEGF stimulates localized formation of Cys-OH-IQGAP1 at the leading edge, thereby promoting directional EC migration, which may contribute to post-natal angiogenesis *in vivo*. Thus, targeting Cys-oxidized proteins at specific compartments may be the potential therapeutic strategy for various angiogenesis-dependent diseases.

Keywords: *reactive oxygen species (ROS), vascular endothelial growth factor, angiogenesis, sulfenic acid, redox signalling, cell motility, NADPH oxidase, endothelial cells*

Introduction

Reactive oxygen species (ROS) are generated in response to various stimuli, including growth factors, cytokines and G protein-coupled receptor agonist, and function as signalling molecules to mediate various biological responses, including cell growth and gene expression [1–4]. However, it remains unclear how highly diffusible ROS can activate a specific signalling, the so-called 'redox signalling'. Indeed, detecting ROS with high degree of spatial and temporal resolution by the currently available ROS reacting reagent remains a challenge [5–7]. Thus, development of a new technology to detect and analyze molecular targets of ROS as chemical footprint of endogenous ROS is essential. Reactive (low pKa) cysteine (Cys) residues in proteins become the primary targets of oxidative modifications [8,9]. Emerging evidence suggests that cysteine sulfenic

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acid (Cys-OH), the initial product of ROS-mediated reactive Cys oxidation, is a key intermediate in the functional modulation of enzymes and proteins involved in redox signalling [9–11]. Therefore, trapping Cys-OH upon its formation in cellular proteins using a detectable reagent is critically important for identifying redox-responsive proteins in cell signalling [10,12–16]. This may lead to understanding the mechanisms through which ROS regulate biological responses, including angiogenesis.

Vascular endothelial growth factor (VEGF) induces angiogenesis by stimulating migration and proliferation of endothelial cells (ECs) primarily through the VEGF receptor type 2 (VEGFR2, KDR/Flk1) [17]. We have previously demonstrated that ROS derived from NADPH oxidase play an important role in VEGFR2 signalling and angiogenic responses in ECs as well as post-natal angiogenesis *in vivo* [4,18–22]. Further, we discovered IQGAP1 as a novel VEGFR2 binding protein involved in ROS-dependent VEGFR2 signalling linked to EC migration [18,23] as well as post-ischemic neovascularization [24]. IQGAP1 is a scaffolding protein that plays a pivotal role in regulating actin cytoskeleton, cell adhesion and cell migration by interacting directly with actin, active Rac1/Cdc42, β-catenin, E-cadherin and the microtubule plus end-binding protein, CLIP-170 [25–27]. We found that in actively migrating ECs, IQGAP1 accumulates at the leading edge where it recruits NADPH oxidase and activated VEGFR2, facilitating localized ROS production, which may contribute to directional EC migration [18,23]. However, the role of Cys-OH formation in VEGF redox signalling and post-natal angiogenesis is poorly understood.

Recently, several dimedone-based chemoselective reagents capable of specific labelling of Cys-OH-formed proteins were developed, which allow for specific enrichment and identification of oxidized proteins [10,12–16,28]. In this study, using a cell permeable biotin-tagged derivative of dimedone, DCP-Bio1 [12,29–31], we demonstrate that VEGF stimulation of cultured ECs increases various Cys-OH-formed proteins. Immunofluorescence analysis reveals that Cys-OH-containing proteins are accumulated at the leading edge where they co-localize with NADPH oxidase, F-actin and IQGAP1 during directional EC migration. VEGF stimulation increases IQGAP1-Cys-OH formation at leading edge, which may contribute to directional EC migration and capillary network formation. *In vivo*, Cys-OH formation is increased in ischemic tissues in response to hindlimb ischemia. Our results suggest that Cys-OH formation may play an important role in redox regulation of VEGF signalling linked to endothelial migration and angiogenesis.

Methods

Cell culture conditions

Human umbilical vein ECs (HUVECs) were from VEC Technologies (Rensselaer, NY) and were grown in Endothelial Basal Medium and EGM-2 BulletKit (EGM2 media, Lonza) containing 5% fetal bovine serum (FBS) in 0.1% gelatin coated plastic or glass dishes. Cells between passages 4 and 7 were used.

Immunoprecipitation, immunoblotting and Cys-OH labelling

HUVECs were growth-arrested in EGM2 media containing 0.5% FBS overnight before stimulating with human recombinant $VEGF^{165}$ (R&D systems) at 37°C. Then, cells were rinsed and lysed in 500 µl of ice-cold lysis buffer, pH 7.4 (50 mM HEPES, 5mM EDTA, 50mM NaCl, 50mM sodium fluoride, 1mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1% Triton X-100, 10 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin). For the labelling of Cys-OH, HUVECs were lysed in de-oxygenized ice-cold lysis buffer (pH5.5) containing 0.1mM DCP-Bio1 [12,28], 200 units/ml Catalase, 100µM Diethylene triamine pentaacetic acid, and 5mM iodoacetamide (IAA). The cell lysates were nutated for an hour at 4°C, then centrifuged at 12,000g at 4°C for 10 min and the supernatant was collected. For immunoprecipitation, cell lysates (400-700 µg) were precipitated with antibody overnight at 4°C and then incubated with 25 µl of protein A/G-agarose beads for 2 hr at 4°C on a nutator. In order to affinity-capture biotin-linked proteins incorporating the Cys-OH trapping probe, lysates were incubated overnight with 20 µl of streptavidin beads (Thermo Scientific). Cell lysates (25 µg) or immunoprecipitates were separated using SDS-PAGE electrophoresis and transferred to nitrocellulose membranes, blocked for 1 hr in Tris-buffered saline (10 mM Tris-HCl, pH 7.4, 140mM NaCl) with 0.1% Tween 20 containing 5% non-fat dry milk and incubated overnight with primary antibodies. After incubation with secondary antibodies coupled to horseradish peroxidase for 1 hr, proteins were detected by Enhanced chemiluminescence (ECL).

Confocal immunofluorescence microscopy

HUVECs were grown to confluence on a cover glass coated with 0.1% gelatin, and then a scratch was made to induce wound injury in the presence of VEGF (20 ng/ml). Cells labelled with 0.1 mM DCP-Bio1 for 30 min were fixed with 4% paraformaldehyde containing 5 mM IAA to prevent further oxidation and were permeabilized in 0.05% Triton X-100 in PBS for 10 min. In some experiments,

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DCP-Bio1 was incubated for 30 min with or without dimedone (10 mM) after permeabilization. After blocking, cells were incubated with FITC-conjugated anti-avidin for 1 hr. For F-Actin staining, cells were incubated with Alexa-568 conjugated anti-phalloidin (Invitrogen) for 20 min at room temperature. In order to detect the co-localization of p47phox with Cys-OH probe, HUVECs were transiently transfected with DsRed-p47phox (from Dr. Lance Terada at University of Texas Southwestern). Images were taken using the confocal laser scanning imaging system (Zeiss LSM510 META). Controls without primary antibody (control) showed no fluorescence labelling.

siRNA transfection

Control small-interference RNA (siRNA) was purchased from Ambion Inc. The sequences of specific siRNA against IQGAP1 were as described before [18]. HUVECs were grown to 50 % confluence in 100 mm dishes and transfected with 50 nM siRNA using Oligofectamine (Invitrogen), as described previously [32]. Cells were used for experiments at 48 hr after transfection.

Modified Boyden chamber assay

HUVECs were plated at 1×10^5 cell suspension/chamber in low serum media with 10 mM dimedone or the vehicle onto the upper chamber of Transwell chambers inserts containing 8 μm pore (BD-Biosciences). Chemotaxis was achieved by the presence of 50 ng/ml VEGF with 10 mM dimedone or vehicle in 37°C culture incubator. After 6 hr, the Transwell chambers were rinsed with PBS, and the cells on top were removed with a cotton tip applicator. Cells on the bottom of the Transwell chambers were fixed in 100% methanol and stained with Diff-Quick Stain kit from Imed Inc. Eight random fields per Transwell chambers were imaged with a Nikon digital camera, and the number of cells was counted by using NIH Image J software.

Wound scratch assay

HUVECs were grown to confluence in 6-well plates. They were pretreated with 10 mM dimedone or the vehicle for 30 min before a scratch was applied with a plastic pipette tip to mimic wound injury. After 24 hr at 37°C culture incubator in growth arrest medium containing 50 ng/ml VEGF, migrating cells were assessed by the closure of the wound area.

Capillary network formation assay

HUVECs were seeded on top of the thick matrigel-coated wells (BD Biosciences) at 4×10^4 per well in the presence of 10 mM dimedone or the vehicle in complete EGM2 growth media containing VEGF. After incubation at 37°C for 16–18hr, images were taken with a Nikon digital camera. Eight random fields per well were imaged.

Hindlimb ischemia model and in vivo labelling with DCP-Bio1

Study protocols were approved by the Animal Care and Institutional Biosafety Committee of University of Illinois at Chicago (ACC: 09-066). C57BL6 mice at 8 to 12 weeks old purchased from Jackson Laboratory were used for the experiments. The mice were subjected to unilateral hindlimb surgery under anaesthesia with intra-peritoneal administration of ketamine (100 mg/kg) and xylazine (10 mg/kg). We performed ligation and segmental resection of left femoral vessels as described previously [33]. We measured ischemic (left)/non-ischemic (right) limb blood flow ratio in lower limb using a laser Doppler blood flow (LDBF) analyzer (PeriScan PIM 3 System; Perimed) as described previously [22]. Three days after ischemia, 50 μl of 0.5 mM DCP-Bio1 was injected into the tibialis anterior (TA) muscles with 30G needles. The TA muscles were used because we observed that most of these muscles had signs of ischemic damage (irregular-shaped myofibers with expanded interstitial space and cell infiltration). After 15 min, the legs were harvested and immediately fixed in 4% paraformaldehyde with 5 mM IAA for 1 hr at room temperature. The TA muscles were excised and additionally fixed for 1 hr, dehydrated with sucrose and embedded in O.C.T. compound. The frozen sections with 5 μm thickness were incubated with avidin/biotin complex (ABC) reagent and developed with diaminobenzidine (DAB), following endogenous peroxidase quenching. The sections without ABC reagent were used as a negative control.

Statistical analyses

All values are expressed as mean ± SE. The significance of the difference between two groups was evaluated by an unpaired Student's *t*-test.

Results

VEGF stimulation increases protein Cys-OH formation in HUVEC

Since VEGF stimulation increases ROS production in ECs, we examined the Cys-OH formation in response to VEGF in HUVECs using a biotin-conjugated dimedone-based Cys-OH detecting probe, DCP-Bio1 [12,28–31]. VEGF-stimulated lysates extracted in the presence of DCP-Bio1 are affinity-captured by streptavidin-linked agarose beads, and then biotinylated Cys-OH-modified proteins were separated by SDS-PAGE, followed by immunoblotted with anti-biotin antibody. Figure 1 shows that VEGF stimulation increases Cys-OH in

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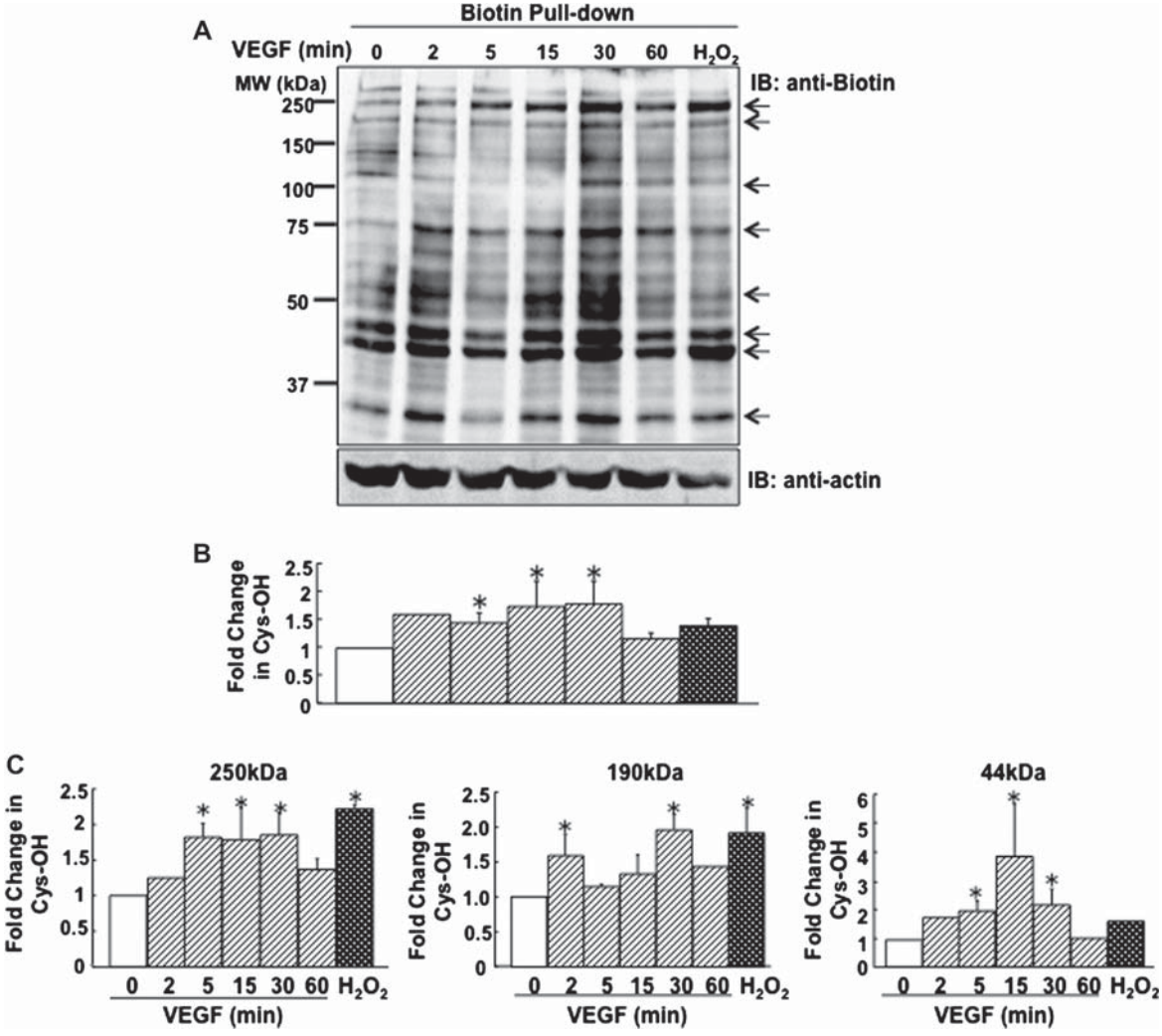

Figure 1. VEGF stimulation increases protein Cys-OH formation in HUVECs. Growth-arrested HUVECs were stimulated with VEGF (50 ng/ml) for indicated times, or H_2O_2 (0.5 mM for 15 min), and cells were extracted in lysis buffer containing DCP-Bio1. Lysates were pulled down with streptavidin beads, followed by immunoblotting with anti-biotin antibody to detect biotin-bound Cys-OH-formed proteins. NC indicates 'negative control', in which beads were added in lysis buffer without lysates. **A**, Representative blot for VEGF-induced DCP-Bio1-labelled Cys-OH-formed proteins. Arrows indicate various increased SOH-formed proteins in response to VEGF. **B** and **C**, Bar graph represents averaged data for VEGF-induced total **(B)**; or 250kDa, 190kDa and 44kDa **(C)** Cys-OH-formed proteins over the unstimulated cells (control), expressed as fold change (n = 3). $^*p < 0.05$ vs. without VEGF.

various proteins, with molecular weights between 35 kDa and 250 kDa (see arrows), in a time-dependent manner. Similarly, exogenous H_2O_2 (0.5 mM) application for 15 min also increases widespread protein Cys-OH formation with similar molecular weight in HUVECs. Western analysis or immunoprecipitation with individual antibodies suggests Cys-OH-formed proteins with around 42-44 kDa as ERK1/2, 50 kDa as actin, 55 kDa as PTP1B, 190 kDa as IQGAP1 and 250 kDa as VEGFR2 (data not shown).

Protein Cys-OH formation increases at the leading edge where it co-localizes with F-actin and p47phox during directional EC migration

Since ROS production is increased at the site of directional EC migration after wound injury of confluent ECs [23,34], we next examined sub-cellular localization of Cys-OH formation in migrating ECs. For this purpose, confluent HUVECs after wound scratch in the presence of VEGF are labelled with DCP-Bio1, and the oxidized proteins with Cys-OH groups are visualized by immunofluorescence analysis. Figure 2A shows that basal DCP-Bio1 staining is found predominantly in the cytosol, perinuclear and nuclear regions in confluent ECs before scratch without VEGF. In actively migrating ECs after wounding in the presence of VEGF, Cys-OH-labelled proteins co-localize with F-actin, as visualized by phalloidin staining, at the leading edge (about 95%) (Figure 2A). Furthermore, Cys-OH-positive fluorescence co-localizes with p47phox, a cytosolic component of NADPH oxidase at the leading edge (Figure 2B). These results suggest that the ROS generating system

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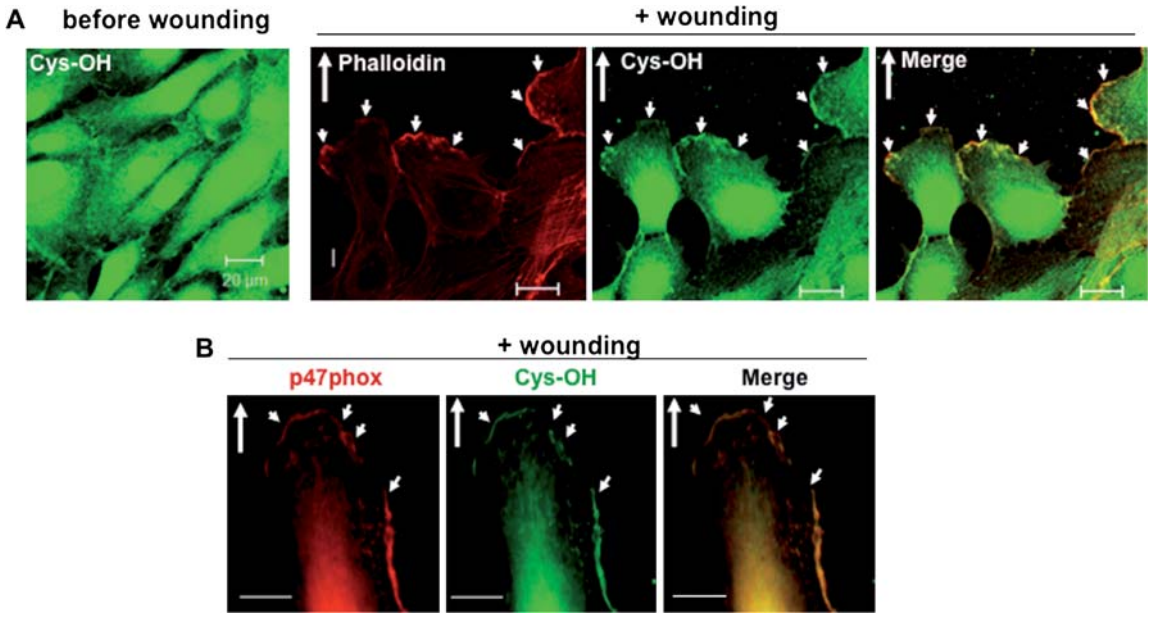

Figure 2. Protein Cys-OH formation increases at the leading edge where it co-localizes with F-actin and p47phox during directional EC migration. HUVECs before and after wound scratch in confluent ECs in the presence of VEGF were labelled with DCP-Bio1 to detect Cys-OH-formed proteins and Alexa Fluor-568 Phalloidin to detect F-actin **(A)** or dsRED-p47phox **(B)**. Small white arrows point to the leading edge and large arrows point towards the scratched area. Bar scale indicates 10 μm.

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and molecular targets of ROS co-localize at the specific plasma membrane compartments in active migrating ECs.

Protein Cys-OH formation co-localizes with IQGAP1 at the leading edge during VEGF-stimulated EC migration

We have previously demonstrated that IQGAP1, an actin- and Rac1-binding scaffolding protein, functions to recruit NADPH oxidase to the leading edge to promote localized ROS production and EC migration [18,23]. We thus examined the relationship between Cys-OH formation and IQGAP1 in VEGF-induced EC migration. Figure 3A shows that IQGAP1 co-localizes with Cys-OH formation, as visualized by DCP-Bio1-labelled fluorescence at the leading edge of migrating ECs. Of note, this DCP-Bio1-positive labelling at lamellipodia, but not IQGAP1 staining itself, is largely inhibited by Cys-OH trapping reagent dimedone. To further investigate the role of IQGAP1 in the Cys-OH formation at the leading edge, we examined the effects of IQGAP1 siRNA. Figure 3B shows that IQGAP1-depleted (−) cells, but not IQGAP1-positive (+) cells, in ECs transfected with IQGAP1 siRNA exhibit reduced DCP-Bio1 labelling at the lamellipodial leading edge, without affecting its cytosolic Cys-OH staining. IQGAP1 siRNA has no significant effects on basal Cys-OH staining (not shown). These results suggest that VEGF increases localized Cys-OH formation of IQGAP1 or its binding proteins at this specific plasma membrane compartment, which may contribute to directional EC migration.

VEGF stimulation increases Cys-OH formation of IQGAP1 in HUVECs

To determine whether VEGF stimulation increases Cys-OH formation in IQGAP1 in HUVECs, we performed immunoprecipitation of DCP-Bio1-labelled cell lysates with anti-IQGAP1 antibody, followed by immunoblotting with anti-biotin antibody. Figure 4 shows that IQGAP1 is basally oxidized, but VEGF stimulation slightly but significantly increases Cys-OH-formed IQGAP1 within 5 min with a peak at 15 min. Immunoblotting with specific IQGAP1 antibody for IQGAP1 immunoprecipitates indicates that the bands at around 190 kDa represent IQGAP1 (Figure 4). A separate experiment suggests that upper bands at around 250 kDa represent VEGFR2 (data not shown). Furthermore, antioxidant N-acetyl cysteine (NAC) or dimedone markedly reduced the bands for biotin-labelled proteins, confirming the ROS-dependent Cys-OH formation of IQGAP1 and its binding proteins after VEGF stimulation in ECs.

Trapping Cys-OH by dimedone inhibits VEGF-stimulated EC migration and capillary network formation

To determine the functional significance of Cys-OH formation, we examined the effects of Cys-OH trapping reagent dimedone on VEGF-induced EC migration as measured by wound scratch assay (Figure 5A) and modified Boyden chamber assay (Figure 5B). For the wound scratch assay, which resembles re-endothelialization and primarily measures migration of cells, confluent monolayers of

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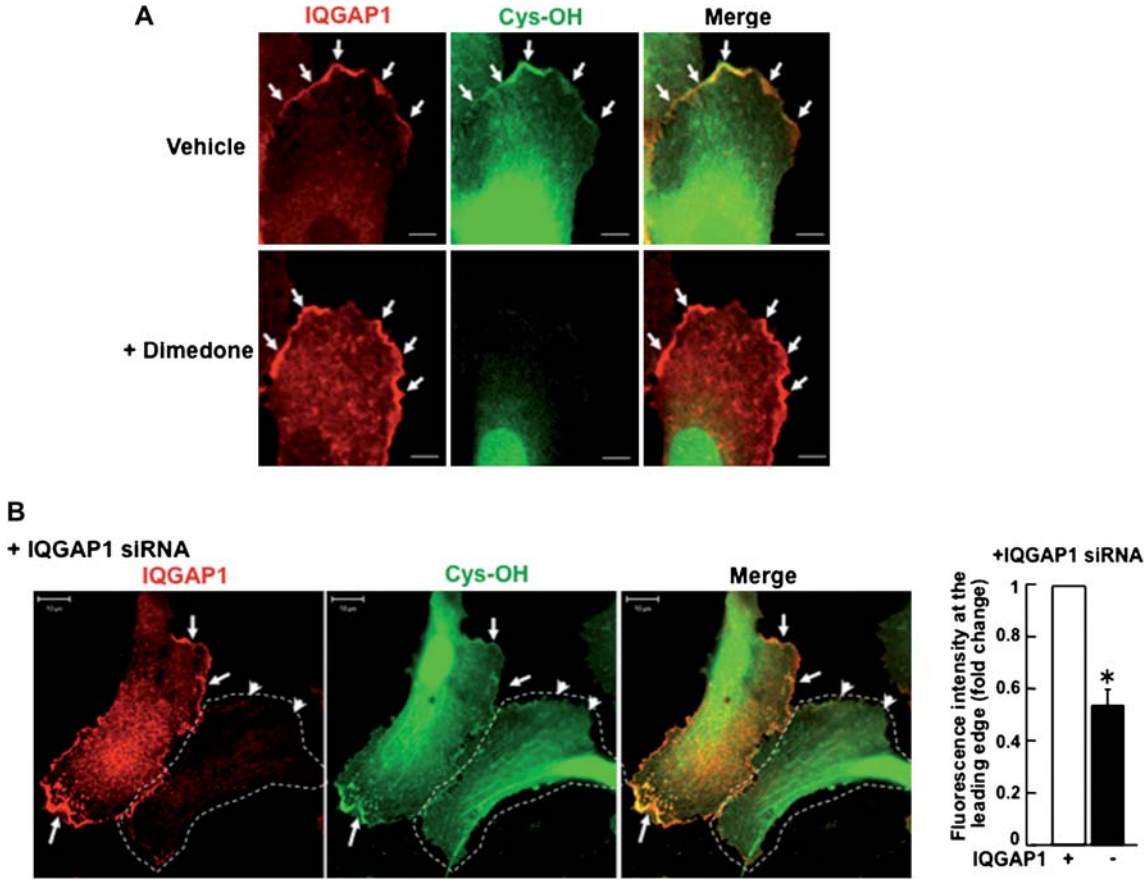

Figure 3. Protein Cys-OH formation co-localizes with IQGAP1 at the leading edge during VEGF-stimulated EC migration. **A** and **B**, HUVECs stimulated with VEGF were double-labelled with anti-IQGAP1 antibody (red) and Cys-OH probe DCP-Bio1 (green). White arrows point to the leading edge where Cys-OH and IQGAP1 localize. In **(A)** (lower panel), cells were incubated with 10mM dimedone together with the Cys-OH probe to show the specificity of DCP-Bio1 labelling. In **(B)**, IQGAP1-depleted cell (white dotted line) and IQGAP1-expressing cell in IQGAP1 siRNA-transfected HUVECs were shown. Small white arrows point that depletion of IQGAP1 using siRNA reduces Cys-OH formation at lamellipodial plasma membranes. Bar scale indicates 10 μm. Bar graph represents the DCP-Bio1 fluorescence intensity at the leading edge in IQGAP1-depleted (−) cells, expressed as fold change from that in IQGAP1-positive (+) cells set as 1.0 in HUVECs transfected with IQGAP1 siRNA. Total of 24 cells each were counted in 11 randomly selected high power fields from 2 independent experiments. $^{*}p < 0.05$.

HUVECs are wounded in the presence of VEGF, and the cells migrating into the wounded area are analyzed after 24 hr in the presence of dimedone or vehicle alone. We confirmed that knock-down of IQGAP1 with siRNA prevents VEGF-induced or wound scratch-induced EC migration (not shown) as we reported [18,23,35]. Figure 5A shows that dimedone almost completely inhibits cell migration towards the wound area. As shown in Figure 5B, modified Boyden chamber assays also demonstrate that dimedone inhibits VEGF-stimulated EC migration after 6 hr without affecting the basal state. Of note, dimedone has no effect on VEGF-induced actin cytoskeleton reorganization (Figure 6A), thereby eliminating toxic effects of this reagent. To determine the functional consequence of Cys-OH formation in EC migration, we examined the effect of dimedone on capillary network formation on Matrigel. HUVECs are placed on the top of a solid thick Matrigel in VEGF-containing media with dimedone or vehicle. Figure 6B shows that dimedone treatment markedly reduces the number of capillaries, suggesting that protein Cys-OH formation plays an important role in VEGF-induced angiogenic responses in ECs.

Cys-OH formation is increased in ischemic tissues in a mouse hindlimb ischemia model

To examine if Cys-OH formation is increased in angiogenesis model *in vivo*, we performed *in vivo* labelling with DCP-Bio1 by injecting it directly into the skeletal muscles before sacrificing the mice on day 3 after femoral artery ligation. Laser Doppler images of hindlimb ischemia model indicate a low perfusion signal (dark blue) and a high perfusion signal (yellow to red) detected immediately after operation (day 0) and on day 3 in the ischemic hindlimbs (Figure 7A). Positive DCP-Bio1 labelling is increased in small vessels (arrows) and skeletal myocytes in ischemic tissues (Figure 7B). By contrast,

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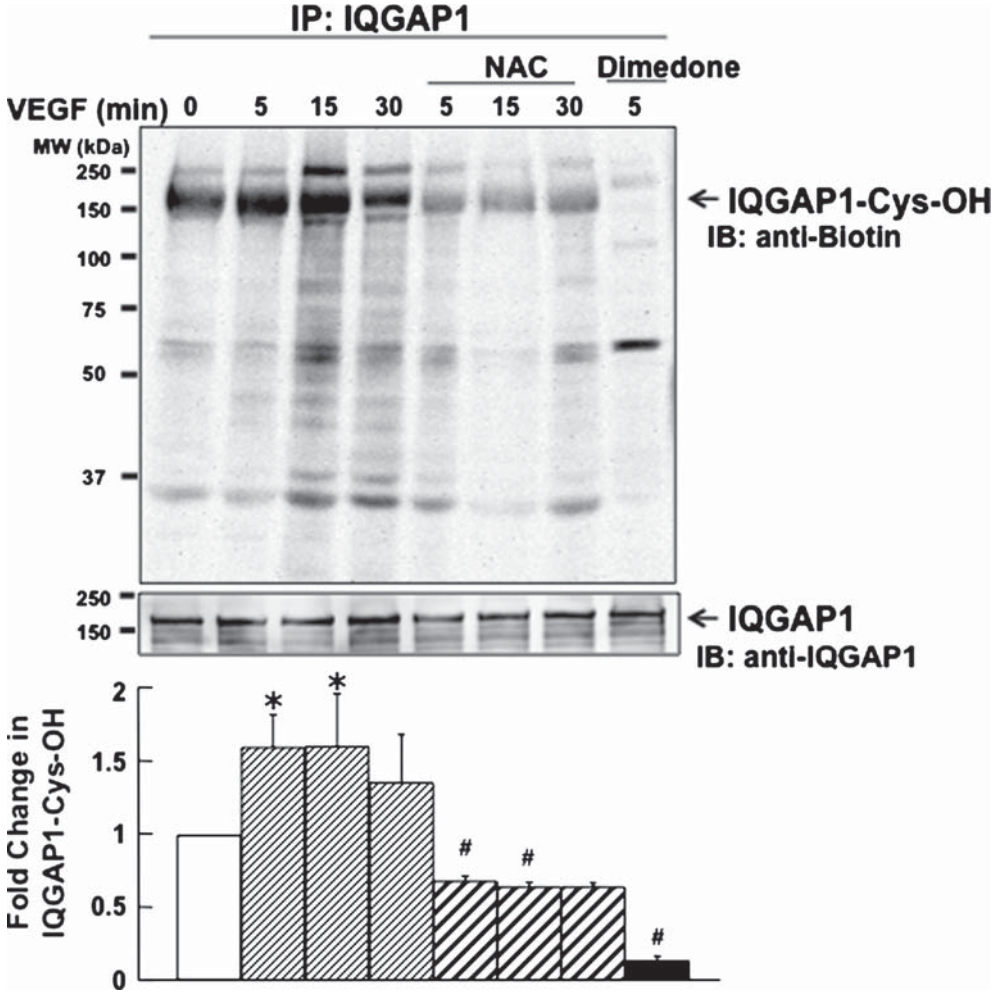

Figure 4. VEGF stimulation increases Cys-OH formation of IQGAP1 in HUVEC in a redox-dependent manner. HUVECs were pre-treated with or without thiol antioxidant N-acetyl cysteine (NAC, 20 mM for 1 hr) and then stimulated with VEGF (50 ng/ml) for indicated times. Lysates containing biotin-labelled Cys-OH trapping reagent were immunoprecipitated with anti-IQGAP1 antibody, followed by immunoblotting with anti-biotin antibody. Dimedone was added to the lysate to compete with the DCP-Bio1 binding to the Cys-OH-formed proteins. $^{*}p < 0.05$ vs. without VEGF, $p < 0.05$ vs. VEGF alone.

either ischemic tissues without ABC reagent (negative control) or non-ischemic tissues do not show significant labelling. Furthermore, hindlimb ischemia increases Cys-OH-formed IQGAP1 proteins in ischemic tissues (Figure 7C). These results suggest that *in vivo* DCP-Bio1 labelling is specific and that tissue ischemia increases Cys-OH-formed proteins including IQGAP1, possibly as a result of ROS production in ischemic tissue.

Discussion

ROS function as second messengers to mediate various biological responses through oxidation of Cys residues in signalling proteins. Within cells, endogenous ROS production is not ubiquitous and occurs at discrete intra-cellular compartments [3,36,37], which selectively induces oxidation of target proteins in a temporal-spatial-dependent manner. The initial product of Cys oxidation is Cys-OH, a key intermediate involved in redox signalling [9–11]. Thus, not only measuring ROS directly but also detecting Cys-OH formation in cellular proteins is critically important for understanding the mechanisms of redox signalling [11,15,16]. However, the role of Cys-OH formation in ROS-dependent VEGF signalling has not been demonstrated. Using a cell-permeable biotin-linked specific Cys-OH reactive probe, the present study shows that (1) VEGF stimulation increases Cys-OH formation of various proteins, in a time-dependent manner in ECs; (2) in actively migrating ECs, Cys-OH-containing proteins increase at the leading edge where it co-localizes with F-actin, NADPH oxidase and IQGAP1, which is inhibited by IQGAP1 siRNA or dimedone; (3) functionally, trapping Cys-OH with dimedone inhibits VEGF-induced EC migration and capillary network formation without affecting the actin cytoskeleton; (4) Cys-OH formation is markedly increased in a mouse hindlimb ischemia model. These data suggest

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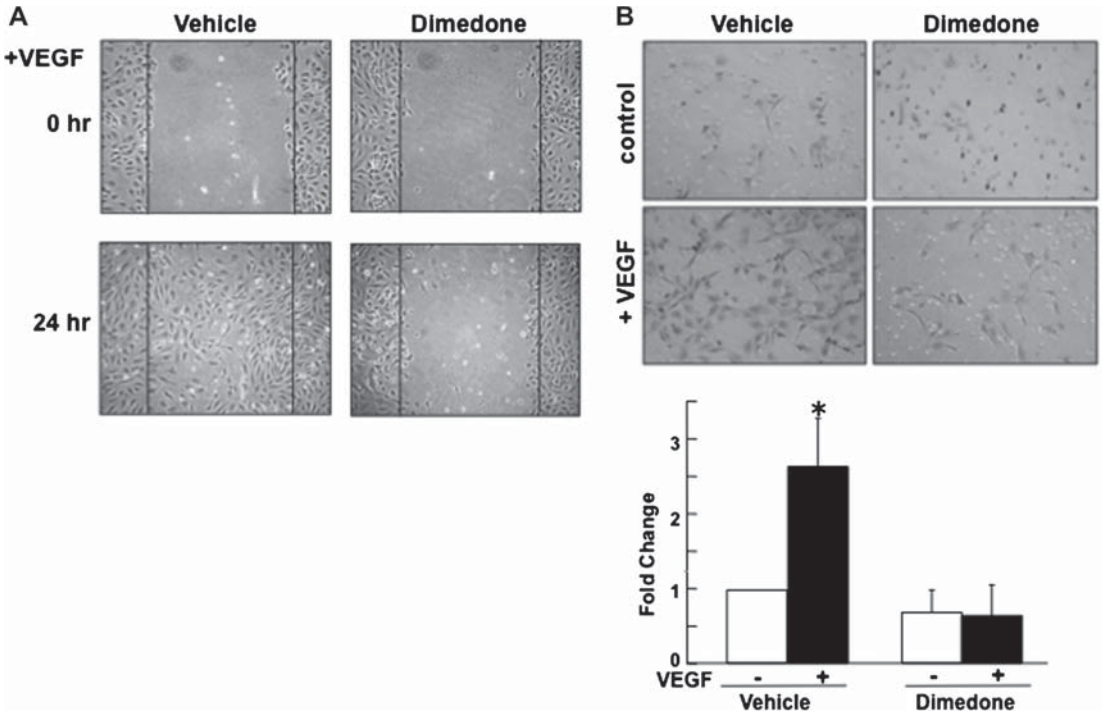

Figure 5. Trapping Cys-OH by dimedone inhibits VEGF-stimulated EC migration. Effects of dimedone on VEGF-stimulated EC migration using wound scratch assay **(A)** and modified Boyden chamber assay **(B)**. **A**, Confluent monolayer of HUVECs were pre-treated with dimedone (10 mM) or vehicle for 30 min, and scratch wound is induced in the presence of VEGF (50 ng/ml). Images were captured immediately (0 hr) and 24 hr after the wound scratch. The representative images were from three independent experiments. **B**, HUVECs were plated at 1×10^5 cell suspension in growth arrest media with dimedone (10 mM) or vehicle onto the upper chamber of Transwell chambers inserts. Chemotaxis was stimulated by 50ng/ml VEGF in the presence of 10mM dimedone or vehicle in the lower chamber. After 6 hr, migrated cells were measured. Bar graph represents averaged data, expressed as cell number counted per eight fields (×200) and fold change over that in unstimulated cells (control). $^*p < 0.05$ vs. vehicle without VEGF.

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that Cys-OH formation plays an important role in the redox regulation of VEGF signalling linked to EC migration and angiogenesis (Figure 8).

Using a biotin-conjugated dimedone analog, DCP-Bio1 [12, 29–31], to affinity-capture the Cys-OH-containing proteins, we demonstrate for the first time that various Cys-OH-formed proteins are increased following VEGF or exogenous H_2O_2 stimulation in cultured ECs. Western analysis or immunoprecipitation with individual antibodies identifies the Cys-OH-labelled proteins with molecular weights around 42-44 kDa as ERK1/2, 55 kDa as PTP1B, 190 kDa as IQGAP1 and 250 kDa as VEGFR2. We found that dimedone is able to block DCP-Bio1 labelling of proteins, thereby confirming that the reagent is causing staining within the cell due to its chemical reactivity and not because of non-specific association with cellular contents. The present study has also performed *in vivo* DCP-Bio1 injection to the animal to show that Cys-OH formation is increased in small vessels and skeletal myocytes in ischemic tissues, but not non-ischemic tissues, in a mice hindlimb ischemia model. Skeletal muscle secrets various proteins or factors involved in angiogenesis following hindlimb ischemia, which plays an important role in reparative neovascularization. Thus, DCP-Bio1 staining in ischemic skeletal muscle myocytes suggests that Cys-oxidized proteins involved in angiogenesis are increased or secreted from ischemic skeletal muscles in response to tissue ischemia. Moreover, we found that ischemia increases Cys-OH-formed IQGAP1 proteins in ischemic tissues after hindlimb ischemia. These findings support the important role of Cys-OH formation in post-natal angiogenesis *in vivo*. Consistent with our results, other studies using different Cys-OH-specific probes demonstrate the Cys-OH formation in tumor cells [14,15], isolated hearts subjected to H_2O_2 [10,38,39] and renal medulla of spontaneously hypertensive rat [40]. Detailed analysis to identify the ROS target proteins and their Cys oxidation sites using redox proteomics analysis and mass spectrometry in VEGF-stimulated ECs and ischemia hindlimb model *in vivo* is the objective of future studies.

Immunofluorescence analysis with DCP-Bio1 to visualize the sub-cellular localization of Cys-OH-formed proteins during EC migration reveals that DCP-Bio1 staining increases at the leading edge where it co-localizes with F-actin, p47phox, a cytosolic component of NADPH oxidase and IQGAP1 in VEGF-stimulated actively migrating ECs. This is consistent with the previous report that endogenous

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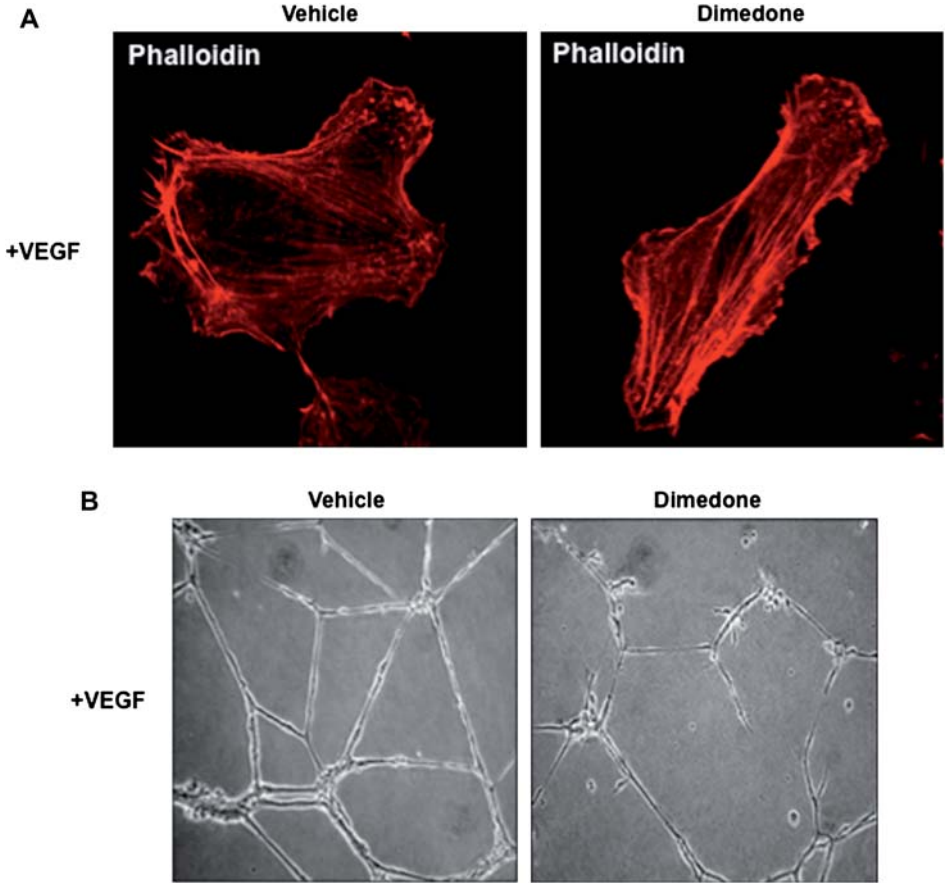

Figure 6. Trapping Cys-OH by dimedone inhibits VEGF-stimulated capillary network formation without affecting actin cytoskeleton. **A**, HUVECs were pre-treated with dimedone (10 mM) or vehicle for 30 min and then stimulated with 50ng/ml VEGF for 15 min. Cells were labelled with Alexa Fluor-568 Phalloidin to detect F-actin. **B**, HUVECs were seeded on Matrigel-coated plates in the presence of 10 mM dimedone or vehicle in complete EGM2 growth media containing VEGF (50 ng/ml). After 16–18 hr, eight random fields per well were imaged and the representative pictures were shown.

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ROS accumulates in actively migrating cells at the site of injury, which is required for EC migration [23,34]. In basal state before wounding without VEGF, as found for Nox2 staining [23], DCP-Bio1 fluorescence is found predominantly in the cytosol, perinuclear and nuclear regions. We have previously reported that IQGAP1 functions as a scaffold to recruit NADPH oxidase and activated VEGFR2 to the leading edge, thereby promoting localized ROS production and EC migration [18,23]. The present study demonstrates that IQGAP1 siRNA specifically reduces DCP-Bio1 labelling at the lamellipodial leading edge without affecting cytosolic Cys-OH staining and thus inhibiting VEGF-induced EC migration. Given that VEGF-induced increase in total amount of Cys-oxidized IQGAP1 protein is small, localized NADPH oxidase activation and resulting Cys-OH formation of IQGAP1/VEGFR2 complex at the leading edge may play an important role in VEGF-induced directional EC migration. Cytosolic Cys-OH staining may reflect basal constitutive ROS production from other sources such as mitochondria or other processes, which is not mediated through IQGAP1. Our finding is consistent with the notion that localization of ROS production and their targets at specific compartments is required for activation of specific redox signalling events involved in various biological responses [3].

Functional significance of VEGF-induced Cys-OH formation is demonstrated that trapping Cys-OH with dimedone inhibits VEGF-induced EC migration and capillary network formation. Of note, dimedone has no effects on phalloidin staining with or without VEGF stimulation or basal migration, thereby eliminating its toxic effects. Since VEGF increases Cys-OH formation of F-actin in ECs (Figure 1), it is possible that trapping Cys-OH on actin by dimedone may change the turnover of actin stress fibres, which may not be seen by phalloidin staining in fixed cells. Consistent with our data, dimedone has been shown to inhibit T-cell proliferation without killing the cells or apoptosis [41]. We acknowledge that many proteins including IQGAP1 are oxidized following VEGF stimulation in ECs and that inhibition of VEGF-induced EC migration by dimedone does not provide the direct evidence that IQGAP1-Cys-OH

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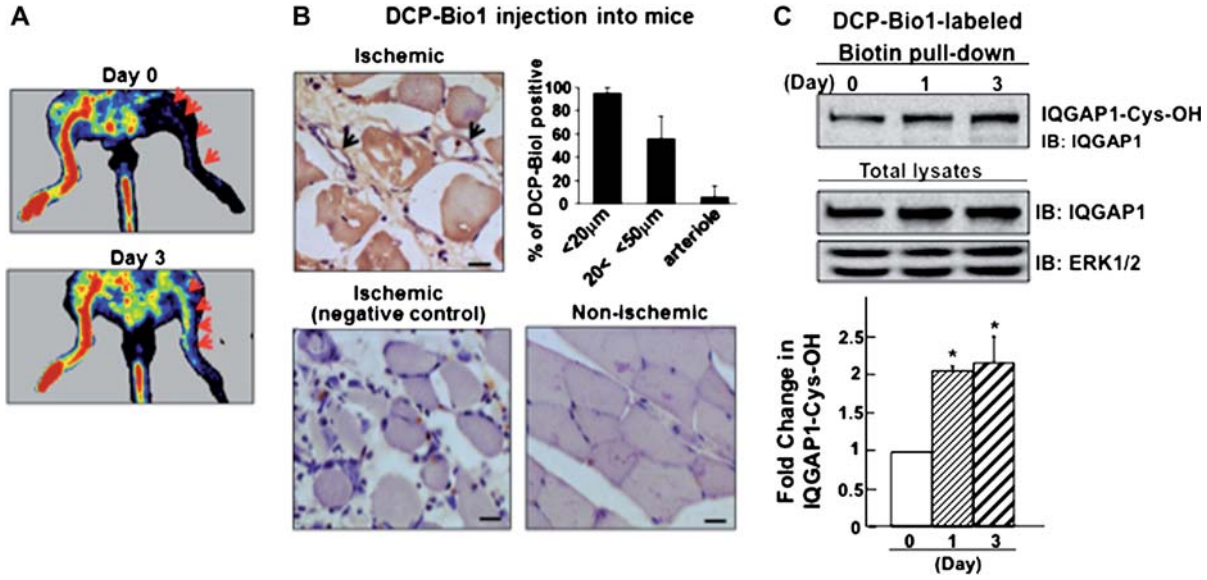

Figure 7. Cys-OH formation is increased in ischemic tissues in a mouse hindlimb ischemia model. **A**, Representative laser Doppler images of hindlimb ischemia model. Arrows indicate a low perfusion signal (dark blue) detected immediately after operation (day 0) and a high perfusion signal (yellow to red) detected on day 3 in the ischemic hindlimbs. **B**, DCP-Bio1 was injected into tibialis anterior (TA) muscles of both ischemic and non-ischemic legs 3 days after femoral artery ligation. The frozen sections of the middle part of TA muscles were incubated with avidinbBiotin complex (ABC) reagent and visualized with diaminobenzidine (DAB). Upper left: Representative images for DCP-Bio1 positive staining in small vessels (arrows) and skeletal myocytes in TA muscles. Upper right: Bar graph represents the numbers of DCP-Bio1-positive small vessels with different diameters, expressed as percentage of total vessel numbers in TA muscles of five randomly selected high power fields ($\times 400$, per mm^2). Lower left: The ischemic tissue sections without ABC reagent showing endogenous biotin used as a negative control. Lower right: DCP-Bio1 staining is not observed in TA muscles of the non-ischemic legs. Bars represent 20 μm. **C**, Lysates from ischemic tissue labelled with DCP-Bio1 were pulled down with streptavidin beads, followed by immunoblotting with anti-IQGAP1 antibody to detect biotin-bound Cys-OH-formed IQGAP1. In parallel, total lysates without biotin pull-down were blotted for IQGAP1 or ERK1/2 (loading control). Bar graph represents averaged data for Cys-OH-formed IQGAP1 proteins in response to hindlimb ischemia, expressed as fold change over day 0 ($n = 3$). $^{*}p < 0.05$.

formation is essential for VEGF-induced EC migration in this study. Identifying the Cys oxidation sites on IQGAP1 and determining the functional significance of their oxidation in VEGF-induced EC migration are currently under investigation.

Conclusions

Using a newly developed Cys-OH detecting probe as well as the parental reagent, dimedone, the present study demonstrates that VEGF stimulation increases Cys-OH formation in cultured ECs and in *vivo* model of angiogenesis. We show that Cys-OH-containing proteins including IQGAP1 accumulate at the leading edge, which may reflect the localized ROS production during VEGF-stimulated directional EC migration. Once ROS are formed, the ROS modulate the activity of proteins and regulate signalling pathways via oxidation of Cys residues. Thus, Cys-OH formation may be an important post-translational modification of proteins that may alter their function or influence protein-protein interactions, which regulate various cellular processes. Further identification of Cys-OH-containing proteins and their Cys oxidation sites involved in angiogenesis using redox proteomics approaches and addressing the functional significance of Cys-OH formation of individual proteins are necessary. Our findings should provide insight into the importance of detection and visualization of protein Cys-OH formation using specific probes to understand the mechanism of redox signalling involved in angiogenesis and other physiological and pathological responses. This could lead to the development of targeted therapy to *Cys oxidized proteins at specific compartments* for the treatment of various angiogenesis-dependent diseases.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper. This research was supported by National Institutes of Health (NIH) R01 Heart and Lung (HL)077524 and HL077524-S1 (to M.U.-F.), HL070187 (to T.F.), CA126659 (to L.B.P.), American Heart Association (AHA) Grant-In-Aid 0755805Z (to M.U.-F.) and AHA National Center Research Program (NCRP) Innovative Research Grant 0970336N (to M.U.-F), AHA Post-doctoral Fellowship 09POST2250151 (to N.U.), Ruth L. Kirschstein-National Service Research Award (Kirschstein-NRSA) T32 Training Grant (NK and

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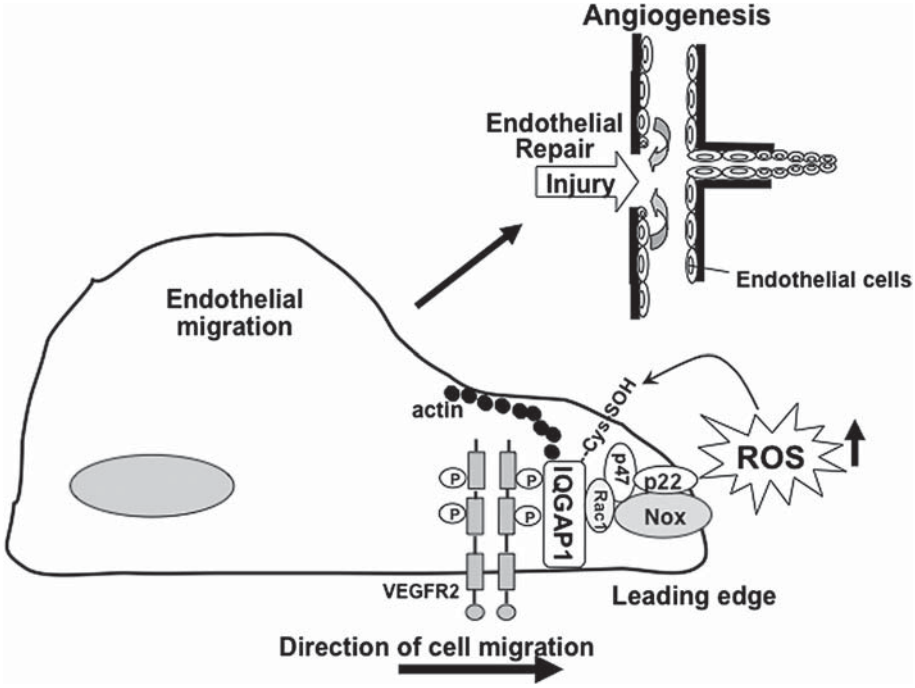

Figure 8. Proposed model for the role of protein Cys-OH-formation in VEGF signalling linked to EC migration and proliferation. Upon activation of VEGFR2 by VEGF, IQGAP1 translocates to the leading edge where it forms VEGFR2/IQGAP1/NADPH oxidase signalling complex in migrating EC. ROS derived from this complex leads to localized Cys-OH formation of IQGAP1/VEGFR2 at the leading edge, which may contribute to ROS-dependent directional EC migration. Thus, protein Cys-OH formation may play an important role in VEGF-induced redox signalling involved in endothelial repair in response to injury and post-natal angiogenesis.

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