Recruitment of Nox4 to a Plasma Membrane Scaffold Is Required for Localized Reactive Oxygen Species Generation and Sustained Src Activation in Response to Insulin-like Growth Factor–I* [S]

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Background: Nox4-derived ROS is required for Src oxidation and activation.

Results: Grb2 recruits Nox4 to the scaffold protein SHPS-1, and Nox4 colocalization with Src on SHPS-1 allows Nox4 to activate Src.

Conclusion: Nox4 recruitment to SHPS-1 is essential for sustained Src activation and cell proliferation.

Significance: This is the first report that localized ROS generation mediates growth factor signaling in vitro and in vivo.

Nox4-derived ROS is increased in response to hyperglycemia and is required for IGF-I-stimulated Src activation. This study was undertaken to determine the mechanism by which Nox4 mediates sustained Src activation. IGF-I stimulated sustained Src activation, which occurred primarily on the SHPS-1 scaffold protein. In vitro oxidation experiments indicated that Nox4-derived ROS was able to oxidize Src when they are in close proximity, and Src oxidation leads to its activation. Therefore, we hypothesized that Nox4 recruitment to the plasma membrane scaffold SHPS-1 allowed localized ROS generation to mediate sustained Src oxidation and activation. To determine the mechanism of Nox4 recruitment, we analyzed the role of Grb2, a component of the SHPS-1 signaling complex. We determined that Nox4 Tyr-491 was phosphorylated after IGF-I stimulation and was responsible for Nox4 binding to the SH2 domain of Grb2. Overexpression of a Nox4 mutant, Y491F, prevented Nox4/Grb2 association. Importantly, it also prevented Nox4 recruitment to SHPS-1. The role of Grb2 was confirmed using a Pyk2 Y881F mutant, which blocked Grb2 recruitment to SHPS-1. Cells expressing this mutant had impaired Nox4 recruitment to SHPS-1. IGF-1-stimulated downstream signaling and biological actions were also significantly impaired in Nox4 Y491F-overexpressing cells. Disruption of Nox4 recruitment to SHPS-1 in aorta from diabetic mice inhibited IGF-I-stimulated Src oxidation and activation as well as cell proliferation. These findings provide insight into the mechanism by which localized Nox4-derived ROS regulates the sustained activity of a tyrosine kinase that is critical for mediating signal transduction and biological actions.

The generation of cellular reactive oxygen species (ROS) has been shown to influence cell proliferation, migration, apoptosis, and the progression of various diseases (1). However, previous clinical trials have failed to show antioxidants that suppress the generalized increase of cellular ROS reduce the risk and/or progression of atherosclerosis (2) or metabolic syndrome (3). Two recent studies have suggested that localization of ROS generation in a specific subcellular compartment can regulate cellular signaling and biological functions. Inactivation of membrane-associated peroxiredoxin I led to local accumulation of H2O2, which was required for healing cutaneous wounds (4). In another study, NADPH oxidase 2 (Nox2) was found to localize in the leading edge of lamellipodial focal complex, and this was shown to play a role in endothelial cell migration (5).

In vascular cells, Nox4 are the major sources of cellular ROS (6), and in vascular smooth muscle cells (VSMC), Nox1 and Nox4 are the major isoforms (7). Nox4-derived ROS has been shown to be required for angiotensin II– (8) and IGF-I-stimulated Src activation (9). Differential subcellular localization of Nox4 has been correlated with alteration in specific cellular functions. For instance, endoplasmic reticulum localization of Nox4 has been shown to regulate protein tyrosine phosphatase 1B-mediated alterations in EGF signaling (10). Mitochondrial localized Nox4 has been shown to mediate high glucose-induced ROS generation (11), and vinculin-colocalized Nox4 was shown to play an important role in mediating focal adhesion formation (12). However, only one of these studies (10) showed an alteration in a signal pathway that was dependent upon Nox4 oxidation of a specific target protein, and that study did not determine the significance of the change in vivo.

Our previous studies have shown that SHPS-1, a plasma membrane protein, functions as a localized scaffold for the formation of a signaling complex, which is required for mediating IGF-I signaling and biological functions in VSMC in response to IGF-I stimulation (13). The significance of localized ROS generation required for IGF-I signaling and biological functions in VSMC has been demonstrated (14). Overexpression of a Nox4 mutant, Y491F, prevented Nox4/Grb2 association and impaired Nox4 recruitment to SHPS-1. These findings indicate that localized ROS generation is essential for IGF-I-stimulated Src activation and cell proliferation.
Function of SHPS-1-localized Nox4

to hyperglycemia (13, 14). This complex includes Src, and SHPS-1-localized Src plays an essential role in mediating IGF-I actions (15). Using unbiased mRNA display, we showed that Nox4 associated with SHPS-1 in response to IGF-I stimulation (14). However, the biological consequences and underlying mechanism of Nox4 recruitment to SHPS-1 have not been investigated. Because Nox4-derived ROS mediates IGF-I-stimulated Src oxidation and activation (9), we hypothesized that recruitment of Nox4 and Src to SHPS-1 results in localized ROS generation, which is required for oxidation of Src and its sustained activation. Furthermore, we determined how Nox4 is recruited to SHPS-1 in response to IGF-I stimulation in VSMC and the consequences of disruption Nox4 recruitment for IGF-I signaling and biological actions in vivo.

EXPERIMENTAL PROCEDURES

Human IGF-I was a gift from Genentech (South San Francisco, CA). Immobilon-P membranes, anti-2,4-dinitrophenyl, and Src antibodies were purchased from Millipore Corp. (Billerica, MA). DMEM containing 4500 and 1000 mg of glucose/liter, streptomycin, and penicillin were purchased from Invitrogen. Grb2 and caveolin antibodies were purchased from BD Bioscience (San Diego, CA). Antibodies against phospho-AKT (Ser-473), total AKT, phospho-Src (Tyr-419), phospho-Erk1/2 (Thr-202/Tyr-204), total Erk1/2, and HA were from Cell Signaling Technology Inc. (Beverly, MA). Anti-phosphotyrosine (Tyr(P)-99), p22phox, and β-actin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-Nox4 and prohibitin antibodies were purchased from Abcam Inc. (Cambridge, MA). SHPS-1 polyclonal antiserum was prepared as described previously (16). The horseradish peroxidase-conjugated mouse anti-rabbit, goat anti-mouse, and mouse anti-rabbit light chain-specific antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). All other reagents were obtained from Sigma unless otherwise stated. A synthetic peptide was prepared that contained the TAT sequence that confers cell permeability followed by a YYVI-containing sequence (underlining indicates residues 486–496 in Nox4 except that Tyr was substituted with Asp) YARAARQARAENRPDDVNIQL. A peptide containing a scrambled sequence (YARAARQARAEDNPRFONQLV) was synthesized to serve as a control peptide. In this peptide, the Tyr was substituted with Phe. The peptides were synthesized by the Protein Chemistry Core Facility at the University of North Carolina at Chapel Hill. The sequences were verified by mass spectrometry.

Cell Culture—VSMC were isolated from porcine aortas using a method described previously (17). The cells were maintained in DMEM containing high glucose (25 mM glucose) and supplemented with 10% fetal bovine serum (HyClone, Logan, UT), streptomycin (100 μg/ml), and penicillin (100 units/ml). The cells were used between passages 5 and 15.

Construction of cDNAs and Establishment of VSMC Expressing Wild Type Nox4, Nox4 Y491F, Wild Type Pyk2, Pyk2 Y881F, Wild Type SHPS-1, SHPS-1 Cytoplasmic Domain-deleted, and Mitochondria-localized Catalase Cells—Wild type human Nox4 cDNA contained in the pCMV-SPORT6 vector was purchased from Thermo Fisher Scientific (Huntsville, AL). PCR with the forward and reverse primers 5’-caccatggctgtgctctgtggaggagctg-3’ and 5’-taAGCCTAATCTGGAACATCGTATGGTACTggaaactttattagg-3’ was used to prepare the full-length cDNA. The PCR product contained a Kozak sequence (CACC) at the 5’ end and a HA sequence (capitalized) at the 3’ end. Wild type Nox4 cDNA was used as a template to generate a cDNA encoding Nox4 Y491F. The forward and reverse primers that were used were: 5’-gaacagactgctTtgctacactcactctg-3’ and 5’-cagctggatgtagcAaaTtagctgttgtc-3’. The underlined bases indicate the substitutions that changed tyrosine 491 to phenylalanine. Point mutations were introduced using a standard protocol (QuickChange; Stratagene, La Jolla, CA). Mitochondria-localized catalase cDNA was obtained from pCAGGS (a gift from Dr. Robinovitch, University of Washington). PCR was used to add the HA tag and a Kozak sequence with the following forward and reverse primers: 5’-caccatgctgggattagcagcatctg-3’ and 5’-taAGCCTAATCTGGAACATCGTATGGTACTgAatcgccgctacaaaggtg-3’. That the constructs contained the correct sequences was verified by DNA sequencing. 293FT cells (Invitrogen) were prepared for generation of virus stocks, and VSMC expressing Nox4, Nox4 Y491F, and mitochondria-localized catalase were established using procedures that were described previously (18). VSMC expressing wild type Pyk2, Pyk2 Y881F, wild type SHPS-1, and cytoplasmic domain-deleted SHPS-1, were prepared as described previously (14, 19).

Isolation Cytoplasmic and Membrane Fraction Proteins—The cells were grown to confluence and were serum-starved 16 h before IGF-I treatment. After IGF-I stimulation for the indicated times, membrane and cytoplasmic fractions proteins were isolated following a procedure described previously (18).

Immunoprecipitation, Double Immunoprecipitation, and Immunoblotting—The cell monolayers were lysed in a modified radioimmunoprecipitation assay buffer as described previously (18). Immunoprecipitation was performed by incubating 0.5 mg of cell lysate protein with 1 μg of each of the following antibodies: anti-Src, SHPS-1, Nox4, and HA at 4 °C overnight. Double immunoprecipitation was performed using a procedure described previously (19). Briefly, the first round immunoprecipitation was performed with an anti-Nox4 antibody or a non-immune IgG (control). The beads were resuspended in 30 μl of SDS buffer (20 mM Tris, pH 7.5, 50 mM NaCl, 2% SDS) containing 1 mM DTT. The resuspended supernatant was added to cold radioimmunoprecipitation assay buffer (final concentration, 0.1% SDS), incubated overnight, and then used for a second immunoprecipitation with an anti-Src antibody (1: 500 dilution). The second immunoprecipitation was followed by immunoblotting that was performed as described previously (18) using a dilution 1:1000 for anti-pAKT (Ser-473), AKT, 2,4-dinitrophenyl, pSrc (Tyr-419), Src, SHPS-1, pErk1/2 (Thr-202/Tyr-204), Erk, and β-actin antibodies, a dilution of 1:500 for an anti-Grb2 antibody, and a dilution of 1:5000 for an anti-Nox4 antibody, and a dilution of 1:5000 for anti-Grb2 antibody. The proteins were visualized using enhanced chemiluminescence (Thermo Fisher Scientific). Total cellular protein in the lysates was determined using BCA (Thermo Fisher Scientific).

In Vitro Binding Assay—The in vitro [35S]methionine (MP Biomedical, Solon, OH) labeled wild type Nox4 was generated.
by a transcription/translation (TnT) reaction (Promega, Madison, WI). An aliquot of the TnT mixture (0.4 μM total protein) was incubated with 200 ng of purified active Src (Millipore, Billerica, MA) in binding buffer (HEPES, pH 7.6, 50 mM KCl, 1 mM DTT, 0.5 mM PMSF, 0.2% Triton X-100, and 10% glycerol), using a 100-μl final volume for 2 h at 4°C. 0.5 μg of anti-Src antibody and 30 μl (50% slurry) of protein A/G-agarose beads were added and incubated for 2 h at 4°C. After extensive washing with the binding buffer, the precipitated proteins were eluted in 30 μl of 2× Laemmli sample buffer, boiled for 5 min, and separated using 10% SDS-PAGE. The images were developed and analyzed using a Storm860 phosphor imager (GE Healthcare). For Nox4/Grb2 or SHP-2 binding assays, TnT expressed Nox4 WT or Nox4 Y491F (100 ng each) was enriched by immunoprecipitation with an anti-Nox4 antibody and phosphorylated by active Src (150 unit) in a buffer containing 50 mM HEPES-NaOH (pH 7.6), 3 mM MnCl2, 10 mM MgCl2, 0.1 mM EGTA, 1 mM dithiothreitol, 0.1 mM Na2O4, and 0.2 mM ATP and incubating at 30°C for 30 min. After extensive washing with the above buffer, purified SHP2 (Millipore, Billerica, MA; 500 ng) or Grb2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; 500 ng) was added and incubated for 2 h at 4°C in the binding buffer (500 μl). After extensive washing with the binding buffer, the precipitated proteins were separated using 10% SDS-PAGE and immunoblotted with an anti-Grb2 or SHP2 antibody, respectively.

In Vitro Src Protein Oxidation Assay and H2O2 Measurement—Nonoxidized Src was prepared from quiescent VSMC cultured in DMEM containing normal glucose (5 mM) by immunoprecipitating with an anti-Src antibody (1:500 dilution). The activated Nox4/p22phox enzymatic complex was prepared from VSMC cultured in DMEM containing high glucose (25 mM) followed by IGF-I (100 ng/ml) stimulation for 10 min. This has been shown to activate Nox4 enzymatic activity (9). The complexes were immunoprecipitated from cell lysates using an anti-Nox4 antibody (1:500 dilution). Control immune complexes were precipitated using lysates prepared from the cells cultured using the same conditions and precipitated with a nonimmune IgG. The immune complex was released using 50 μl of SDS buffer (20 mM Tris, pH 7.5, 50 mM NaCl, 2% SDS) and a 3-h incubation at room temperature. To detect Src oxidation, nonoxidized Src (10 μg of IP) was incubated with Nox4 complex (10 μg of IP) in 50 μl of 50 mM phosphate buffer (pH 7.0, 1 mM EGTA, 150 mM sucrose, and 100 μM NADPH) for 1 h at 30°C. After extensive washing with radioimmunoprecipitation assay buffer, Src was released from the immune complexes using 12% SDS. The above procedures were completed using anaerobic conditions. Src oxidation was measured using a modified OxyBlot™ protein detection kit (Millipore, Billerica, MA) as described previously (9).

After immunoprecipitating with an anti-SHPS-1 antibody, the immune complex was released using 50 μl of SDS buffer and a 3-h incubation at room temperature. 500 μl of the above-mentioned phosphate buffer was added, and hydrogen peroxide was measured using an Amplex red hydrogen peroxide assay kit (Invitrogen) by following the manufacturer’s instructions.

Animal Preparations and Treatments—Animal maintenance conformed to the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. Hyperglycemia was induced in C57B6 mice (Taconic, Hudson, NY) using the low dose streptozotocin protocol previously described (20). Briefly, after withdrawal of food for 4 h, the mice were injected intraperitoneally with streptozotocin (50 mg/kg) (n = 72) daily for 5 days. Blood glucose levels were checked, and mice that had serum glucose concentrations >250 mg/dl were considered diabetic. They were maintained for 2 weeks before any treatment. During that time, they maintained normal activity and food intake, and no insulin injections were required. The DVNI-containing peptide (n = 24) (10 mg/kg) or the control peptide (n = 24) (10 mg/kg) was administered intraperitoneally daily for 2 days (24 h and 30 min before sacrifice). IGF-I (1 mg/kg) was administered intraperitoneally 15 min (n = 24) before sacrifice when signal transduction was being assessed and 24 h (n = 12) before sacrifice when measuring Ki67 staining.

Preparation of Aortas for Analysis—The mice were euthanized by injecting Nembutal (100 mg/kg intraperitoneally). The thoracic aortas were harvested and placed into ice-cold PBS. Connective tissue and endothelium were removed prior to protein extraction. The aortas were homogenized in ice-cold buffer (20 mM Tris, 150 mM sodium chloride, 2 mM EDTA, and 0.1 mM dithiothreitol, 0.1 mM Na3O4, and 0.2 mM ATP) and incubated at 30°C for 30 min. After extensive washing with the above buffer, purified SHP2 (Millipore, Billerica, MA; 500 ng) or Grb2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; 500 ng) was added and incubated for 2 h at 4°C in the binding buffer (500 μl). After extensive washing with the binding buffer, the precipitated proteins were separated using 10% SDS-PAGE and immunoblotted with an anti-Grb2 or SHP2 antibody, respectively.

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Function of SHPS-1-localized Nox4

Immunohistochemistry—The aortas from mice were fixed with 4% paraformaldehyde overnight, and paraffin-embedded sections were prepared by the University of North Carolina histology core facility. An immunohistochemistry paraffin protocol described previously (9) was followed to stain the Ki67 positive nuclei. A DAPI-containing mounting medium (Vector Laboratories, Burlingame, CA) was used to stain the total nuclei. The Ki67-positive nuclei and the total nuclei were counted in the aortic ring and expressed as the percentage of positive nuclei.

Cell Proliferation Assay—Assessment of VSMC proliferation was performed as described previously (21). Briefly, the cells were incubated with or without IGF-I (50 ng/ml) in serum-free DMEM containing high glucose (25 mM) and 0.2% platelet-poor plasma for 48 h, and the cell number in each well was counted.

Statistical Analysis—The results that are shown in all of the experiments are the representative of three separate experiments and expressed as the means ± S.E. The Student’s t test was used to compare differences between control and one treatment or control cells and one mutant for some in vitro experiments. One- or two-way analysis of variance was applied for all data obtained from in vivo studies or when multiple treatments or multiple cell types were compared using data from in vitro studies. p ≤ 0.05 was considered statistically significant.
RESULTS

Sustained Src Activation in Response to IGF-I Primarily Occurs on the SHPS-1 Complex—Although Src activation is required for mediating the IGF-I signaling response during hyperglycemia (15), it is unknown whether IGF-I stimulates sustained Src activation. This is important because sustained Src activation has been linked to sustained MAP kinase activation and cell proliferation (22). Analysis of whole cell lysates obtained at several time points showed that peak IGF-I stimulated Src activation was sustained for 3 h and had not declined to baseline after 6 h (Fig. 1A). Because our previous studies showed that hyperglycemia induced the formation of a signaling complex on the cytoplasmic domain (CD) of the scaffolding protein SHPS-1 and that IGF-I stimulated Src recruitment to that complex (13, 14), we determined whether Src recruitment to SHPS-1 resulted in sustained activation. The results showed that SHPS-1-associated Src activation was also sustained for at least 6 h (Fig. 1B). In contrast, non-SHPS-1-associated Src activation was transient and declined after 1 min of IGF-I stimulation (Fig. 1B). In addition, although IGF-I stimulated Src recruitment to SHPS-1 (supplemental Fig. S1A), the total amount of Src in whole cell lysate was not significantly different from SHPS-1 free cell lysate (supplemental Fig. S1B), indicating that only a small amount of Src was recruited to the SHPS-1 complex.
complex after IGF-I stimulation. To confirm the importance of SHPS-1 association, we compared Src oxidation and activation in cells expressing full-length SHPS-1 and a SHPS-1 mutant in which the CD had been deleted. The results showed that there was a much greater increase in oxidized Src and Tyr-419 phosphorylation in response to IGF-I in cells expressing SHPS-1 with an intact CD (Fig. 1C).

Nox4 and Src Coassociate on SHPS-1—Nox4-derived ROS is required for IGF-I-stimulated Src activation (9); however, it is unclear whether ROS that is generated by Nox4 acts in multiple cellular locations or whether it acts locally to activate Src. Because ROS is highly reactive and short lived, it is likely to be highly concentrated at cellular locations or whether it acts locally to activate Src. Because ROS is highly reactive and short lived, it is likely to be highly concentrated at the site of its generation.

To determine whether Nox4-generated ROS could directly oxidize Src, an in vitro oxidation assay was employed. Nonoxidized Src (inactive) was oxidized when it was incubated with the activated Nox4 enzymatic complex in vitro (Fig. 2A). Control immune complexes that did not contain Nox4 showed no Src oxidation. Our previous study showed that both Src and Nox4 are recruited to the SHPS-1 complex in response to IGF-I (14); therefore, we examined whether a Nox4/Src complex was formed on SHPS-1. A double immunoprecipitation (IP) analysis of the Nox4/Src complex after IGF-I stimulation demonstrated the formation of a ternary complex following IGF-I stimulation (Fig. 2B). Ternary complex formation could not be detected when control IgG was used in the initial IP. To determine whether this interaction was direct, we utilized an in vitro binding assay. The results showed that Nox4 did not directly bind to Src (Fig. 2C), suggesting that Nox4 associated with Src via an intermediary protein(s) and that Src did not directly recruit Nox4 to SHPS-1.

Nox4 Phosphotyrosine 491 Mediates Its Recruitment to SHPS-1 in Response to IGF-I—To determine how Nox4 is recruited to SHPS-1, we initially determined that Nox4 was tyrosine-phosphorylated in response to IGF-I stimulation (Fig. 3A). To determine whether this facilitated a protein-protein interaction that led to SHPS-1 recruitment, we analyzed the potential SH2 binding sites in Nox4 using Net-Phos 2.0, a web-based phosphorylation site prediction program. Six tyrosine phosphorylation sites with high potential were detected. Among them, Tyr-491 had the highest score (0.987), and its flanking sequences showed a typical SH2 domain-binding motif (YVNI) (supplemental Fig. S2A). We then utilized a cell-permeable synthetic peptide containing the DVNI motif and Nox4 flanking sequences to determine whether this altered Nox4 recruitment to SHPS-1. Cell exposure to the peptide for 2 h inhibited IGF-I-stimulated Nox4/SHPS-1 association, whereas a control peptide had no effect (Fig. 3B). To definitively determine the significance of Tyr-491 phosphorylation, we expressed a mutant form of Nox4 in which Tyr-491 was substituted with phenylalanine. WT Nox4 was used as a control. Expression of Nox4 was equivalent in both cell types (supplemental Fig. S2B). Analysis of VSMC expressing the Nox4 Y491F mutant showed significant inhibition of IGF-I-stimulated Nox4 tyrosine phosphorylation (Fig. 3C). Importantly, IGF-I–induced Nox4/SHPS-1 association was abolished in Nox4 Y491F-overexpressing cells, indicating that Tyr-491 phosphorylation mediates Nox4 recruitment to SHPS-1 after IGF-I stimulation (Fig. 3D). In addition, p22phox was also present in the SHPS-1 complex after IGF-I stimulation in Nox4 WT-overexpressing cells but absent in Nox4 Y491F-overexpressing cells (Fig. 3D).

Next we determined the effect of inhibiting Nox4 recruitment to SHPS-1 on localized ROS generation. H$_2$O$_2$ generation from the SHPS-1 complex was detected using an Amplex red hydrogen peroxide assay kit in Nox4 WT-overexpressing cells in response to IGF-I but not in Nox4 Y491F-overexpressing cells, indicating that inhibition of Nox4 recruitment to SHPS-1 prevented localized ROS generation (Fig. 3E). To confirm this result, an in vitro oxidation assay was performed using the same conditions. The results show that SHPS-1-localized ROS is able to oxidize Src, and prevention of Nox4 recruitment to SHPS-1 prevents the increase in Src oxidation (Fig. 3F). Furthermore, we examined IGF-I-stimulated Src oxidation and activation in cells after inhibition of Nox4 recruitment. IGF-I-stimulated Src oxidation, which is a prerequisite for Src activation (9), was significantly impaired when Nox4 recruitment was prevented (Fig. 3G). Predictably, IGF-I-stimulated Src activation was also significantly impaired in Nox4 Y491F-overexpressing cells, compared with WT Nox4-overexpressing cells (e.g., a 29 ± 3% versus a 105 ± 14%, p < 0.05) (Fig. 3H).
**Function of SHPS-1-localized Nox4**

Grb2 Mediates Nox4 Recruitment to SHPS-1 in Response to IGF-I—To determine the component of the SHPS-1 signaling complex that mediated Nox4 recruitment, we focused on known components that had a high probability of binding to the Nox4 Y491 site. Because YNVI is a typical SH2 domain-binding motif, we analyzed the two SH2 domain-containing components in the SHPS-1 complex: SHP-2 and Grb2. Two forms of Nox4 (WT and Y491F) were expressed using the TNT system. This tyrosine-phosphorylated Nox4 (Fig. 4A) was prepared as described under “Experimental Procedures” and used to inves-

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**FIGURE 3. Phosphorylation of tyrosine 491 mediates Nox4 recruitment to SHPS-1 in response to IGF-I.** VSMC, VSMC expressing Nox4 wild type (Wt), Nox4 Y491F mutant, or LacZ were cultured in DMEM containing 25 mM glucose plus 10% FBS and serum-deprived for 16 h before IGF-I treatment for 10 min. A, cell lysates were immunoprecipitated with an anti-Nox4 antibody and immunoblotted with an anti-Tyr(P)-99 antibody. To control for loading, the blot was reprobed with an anti-Nox4 antibody. B, a peptide containing the DVN1 sequence (10 μg/ml) was incubated 2 h before IGF-I treatment. Cell lysates were immunoprecipitated with an anti-Nox4 antibody and immunoblotted with an anti-SHPS-1 antibody. To control for loading, the blot was reprobed with an anti-Nox4 antibody. The value of each bar is the ratio of the mean value of the scanning units for SHPS-1 divided by the scanning units of Nox4. *** (p < 0.001) indicates a significant difference between two treatments. NS indicates no significant difference between two treatments. C, cell lysates were immunoprecipitated with an anti-Nox4 antibody and immunoblotted with an anti-Tyr(P)-99 antibody. To control for loading, the blot was reprobed with an anti-Nox4 antibody. D, cell lysates were immunoprecipitated with an anti-HA or p22phox antibody, respectively, and immunoblotted with an anti-SHPS-1 antibody. To control for loading, the blots were reprobed with an anti-HA or anti-p22phox antibody, respectively. E, cell lysates were immunoprecipitated with an anti-SHPS-1 antibody. The immune complexes were used to measure H2O2 generation following a procedure described under “Experimental Procedures.” * (p < 0.05) indicates a significant difference between two treatments. F, cell lysates were immunoprecipitated with an anti-SHPS-1 antibody. The immune complexes were used to perform an in vitro Src oxidation assay following the procedure described under “Experimental Procedures.” To control for Src loading, the blot was probed with an anti-Src antibody. G, cell lysates were immunoprecipitated with an anti-Src antibody. Oxidized Src levels were measured following the procedure described under “Experimental Procedures.” To control for Src input, the blot was reprobed with an anti-Src antibody. H, cell lysates were immunoblotted with anti-p419Src and Src antibodies. The figures are representative of three independent experiments. Ctrl, control; DNP, 2,4-dinitrophenol.
tigate its potential interaction with Grb2 or SHP2. Grb2 but not SHP2 bound to WT Nox4 but not to the Y491F mutant (Fig. 4B). To determine whether Grb2 mediated Nox4 recruitment to SHPS-1 in the cells, we initially determined whether IGF-I stimulated Nox4/Grb2 binding. IGF-I stimulated Nox4/Grb2 binding, and this was disrupted using a DVNI-containing peptide (Fig. 4C). Consistently, IGF-I-stimulated Nox4/Grb2 binding was not detected in cells expressing the Nox4 Y491F mutant (Fig. 4D). In addition, the increase of SHPS-1-associated p419Src in the membrane faction was abolished in response to IGF-1 when Nox4 Y491F was overexpressed, whereas non-SHPS-1-associated Src activation was still detectable, although it was attenuated (Fig. 4E). To further confirm that Grb2 mediated Nox4 recruitment to SHPS-1, we utilized VSMC expressing a Pyk2 Y881F mutant that we had previously shown eliminated Grb2 recruitment to SHPS-1 (19). IGF-I-stimulated Nox4/SHPS-1 association was not detected in the Pyk2 Y881F mutant cells (Fig. 4F).

To determine the effect of disruption of Nox4 recruitment to SHPS-1 on downstream signaling, AKT and MAP kinase activation were analyzed. Significant attenuation of AKT (e.g., 3.7 ± 0.5-fold versus 7.8 ± 1.1-fold versus 10.8 ± 1.1-fold increase, respectively) and MAP kinase activation (e.g., 1.9 ± 0.2-fold versus 3.4 ± 0.5-fold versus 5.2 ± 0.2-fold increase, respectively) was detected in Nox4 Y491F mutant cells as compared with LacZ and WT Nox4 cells (Fig. 5A and B). Consistently, IGF-I-stimulated cell proliferation was significantly impaired in Nox4 Y491F mutant cells (e.g., a 1.26 ± 0.11-fold increase, p < 0.05), compared with cells expressing LacZ control vector (e.g., a 2.06 ± 0.16-fold increase) (Fig. 5C), whereas overexpression of WT Nox4 significantly increased IGF-I-stimulated cell proliferation (e.g., a 2.50 ± 0.12-fold increase, 

**FIGURE 4.** Grb2 mediates Nox4 recruitment to SHPS-1 in response to IGF-I. VSMC expressing LacZ, Nox4 wild type (WT), Nox4 Y491F mutant, Pyk2 wild type, or Pyk2 Y881F mutant were cultured in DMEM containing 25 mM glucose plus 10% FBS and serum-deprived for 16 h before IGF-I treatment for 1 or 10 min. A and B, the in vitro phosphorylation and binding assay were performed following the procedures described under "Experimental Procedures." C, the DVNI containing peptide (10 μg/ml) was incubated for 2 h before IGF-I stimulation. Cell lysates were immunoprecipitated with an anti-Nox4 antibody and immunoblotted with an anti-Grb2 antibody. To control for loading, the blot was reprobed with an anti-Nox4 antibody. D, cell lysates were immunoprecipitated with an anti-HA antibody and immunoblotted with an anti-Grb2 antibody. To control for loading, the blot was reprobed with an anti-HA antibody. E, membrane fractions were isolated following the procedures described under "Experimental Procedures." The fractions were immunoprecipitated with an anti-SHPS-1 antibody. The immune complex (Pellet) and supernatant (Super.) were immunoblotted with an anti-p419Src antibody, respectively. To control for loading, the blots were reprobed with anti-SHPS-1 and caveolin antibodies, respectively. F, cell lysates were immunoprecipitated with an anti-Nox4 antibody and immunoblotted with an anti-SHPS-1 antibody. To control for loading, the blot was reprobed with an anti-Nox4 antibody. The figures are representative of three independent experiments. Ctrl, control.
These results clearly demonstrate that the recruitment of Nox4 to SHPS-1 after IGF-I stimulation is a crucial step in mediating IGF-I signal transduction and biological actions. To understand why IGF-I-stimulated signaling and biological actions were impaired in Nox4 Y491F mutant-expressing cells compared with the LacZ cells, we examined the effect of overexpression of Nox4 or the Nox4 Y491F mutant on the expression of endogenous Nox4. The results clearly showed that endogenous Nox4 expression was significantly suppressed in both cell types that overexpressed Nox4 (Fig. 5D).

Mitochondria-derived ROS Does Not Contribute to IGF-I-stimulated Src Oxidation and Activation or to IGF-I Downstream Signaling—To exclude the possibility that mitochondrial derived ROS plays a role in Src oxidation and activation in response to IGF-I, a mitochondrial targeted catalase was overexpressed in VSMC (Fig. 6A). Overexpression of catalase specifically in mitochondria did not alter IGF-I stimulated Src, MAP kinase, or AKT activation (Fig. 6, B–D). Importantly, IGF-I-stimulated cell proliferation was also not changed by overexpression of catalase in mitochondria (Fig. 6E). As an alternative approach to prevent an increased mitochondria-derived hydrogen peroxide, DIDS was used. Consistently, the results showed that preincubation with DIDS did not affect IGF-I-stimulated Src oxidation (supplemental Fig. S3A) and activation (supplemental Fig. S3B). In addition, IGF-I-stimulated AKT activation and MAP kinase activation were also not affected when this inhibitor was used (supplemental Fig. S3, C and D).

SHPS-1/Grb2/Nox4 Association in Vivo and Disruption of This Association Alters IGF-I-stimulated Signaling and Cell Proliferation—Because our previous study showed that hyperglycemia enhanced IGF-I-stimulated Src oxidation and activation in mice (9), the importance of Nox4 recruitment to SHPS-1 for optimal Src activation after IGF-I stimulation in vivo was investigated in diabetic animals. Importantly, administration of a synthetic peptide containing the DVNI motif but not a control peptide disrupted IGF-I-induced association of Nox4/Grb2 and impaired the Nox4 recruitment to SHPS-1, thereby confirming that Grb2 mediates Nox4 recruitment to SHPS-1 in vivo (Fig. 7A). Consistent with the in vitro results, disruption of Nox4 recruitment to SHPS-1 using this peptide impaired IGF-
I-stimulated Src oxidation (Fig. 7B) and SHPS-1-associated Src activation (Fig. 7C), whereas the control peptide had no effect (Fig. 7, B and C). Similarly, a DVNI-containing peptide abolished the ability of IGF-I to stimulate MAP kinase activation during hyperglycemia, but the control peptide had an effect (Fig. 7D). To determine the biological significance of Nox4 recruitment, VSMC proliferation was determined using Ki67 staining of diabetic mouse aortas. The results showed that IGF-I stimulated cell proliferation from 27.1 ± 1.4 to 40.0 ± 0.8% (p < 0.01) in diabetic mice, and this stimulation was significantly impaired following exposure to the DVNI containing peptide (from 19.8 ± 1.3 to 21.5 ± 1.1%, p was not significant), whereas the control peptide had no effect on IGF-I-stimulated cell proliferation (29.8 ± 1.2 and 42.6 ± 2.2%, p < 0.01) (Fig. 7E).

**DISCUSSION**

Our prior study showed that Nox4 enzymatic activity was stimulated by hyperglycemia and that this increase was required for IGF-I-stimulated Src activation and cell proliferation. However, the underlying mechanism by which Nox4-activated Src leads to increased IGF-I-stimulated cell proliferation was not defined. In the present study, we demonstrate that IGF-I stimulates Nox4 recruitment to the plasma membrane scaffold protein, SHPS-1, which allows local ROS generation to activate Src. Because both proteins are recruited to SHPS-1 for a prolonged time period and they are in close proximity, this allows Nox4-derived ROS to oxidize Src. Because Src oxidation leads to increased enzymatic activity (9), their colocalization is an important step in the molecular events leading to sustained Src activation in response to IGF-I. Importantly, this sustained activation occurred almost exclusively on the SHPS-1 scaffold because abundant oxidized and activated Src could be coimmunoprecipitated with SHPS-1, but there was a minimal increase in Src activation that could not be sustained (e.g., 1 min) in the non-SHPS-1-associated subcellular fraction. Our present study also revealed that Grb2 is the intermediary protein that recruits Nox4 to SHPS-1. Disruption of Nox4 recruitment via either preincubation with a peptide that disrupted Nox4/Grb2 associated or overexpression of a Nox4 mutant that did not bind Grb2 significantly impaired IGF-I-stimulated Src oxidation and activation.

ROS, both superoxide and hydrogen peroxide, play vital roles in regulating growth factor signal transduction and biological functions. However, antioxidants that inhibit generalized increases in ROS do not result in any beneficial effect on reducing disease risk (2) (3). This suggests that localized ROS generation may be more important for activating specific signaling pathways and inducing pathophysiological changes. This conclusion is supported by Woo et al. (4), who showed that membrane-localized inactivation of peroxiredoxin 1 via tyrosine phosphorylation allowed the accumulation of Nox1-derived H₂O₂ in the membrane fraction, thereby resulting in enhanced tyrosine kinase receptor signaling. Because Nox1 and Nox4 are the major isoforms in vascular smooth muscle cells (7) and Nox4-derived ROS has been shown to be required for angiotensin II- (8) and IGF-I-stimulated Src activation (9), in this study we focused on Nox4. Although differential subcellular localization of Nox4 has been suggested to mediate different cellular functions (10, 12), only one study has identified a specific molecule, PTPB1, whose activity was altered in response to Nox4 colocalization. This change was linked to activation of EGF signaling (10), but these results were only studied in vitro.

Our previous study using unbiased proteomic screening showed that IGF-I stimulates Nox4 recruitment to phosphorylated SHPS-1, a transmembrane protein that serves as a scaffold for signaling complex formation in response to IGF-I (13, 14). IGF-induced Src activation occurs on this complex, and this is required for activation of downstream signaling (15). Because Nox4 derived ROS is responsible for Src oxidation (9), we hypothesized that Nox4 recruitment to SHPS-1 in response to IGF-I would allow localized ROS generation within the SHPS-1 complex. In support of this hypothesis, our in vitro oxidation experiments clearly show that Nox4 oxidizes Src when they are close proximity. In addition, our data demonstrate that deletion of SHPS-1 CD inhibits Src oxidation and activation, suggesting that Nox4 recruitment to the SHPS-1 CD is a prerequisite for Src activation in response to IGF-I.
Function of SHPS-1-localized Nox4

(A) Peptide

IB SHPS-1
IB Grb2
IB Nox4

IP Nox4

Peptide: —, DVNI, Ctrl

IB Src
IB DNP

IP Src

—, DVNI, Ctrl

IB p419Src
IB SHPS-1

IB pErk1/2
IB Erk1/2

IP SHPS-1

—, DVNI, Ctrl

IGF-I

—, +, +

—, +, +

55 KDa

100 KDa

25 KDa

70 KDa

(F) Percentage of Ki67 positive nuclei

No Pep
No Pep + I
DVNI Pep
DVNI Pep + I
Ctrl Pep
Ctrl Pep + I

p<0.01

p<0.01

p, NS

(p, NS)

Ratio of scan values

No Pep
DVNI
Ctrl Pep

p<0.01

p<0.05

p<0.01

No Peptide
DVNI Peptide
Ctrl Peptide

Ctrl

IGF-I
Previous studies have shown that sustained Src activation is essential for mediating optimal growth factor signal transduction and biological actions. For example, sustained Src activation is required for PDGF-stimulated human airway smooth muscle cell migration (23), and sustained Src activation is the essential for up-regulation of MAP kinase activity (24). Our previous studies have shown that Src activation is required for MAP kinase activation (15) and that sustained MAP kinase activation is required for optimal cell migration and proliferation in response to IGF-I (13). In addition, free radical-stimulated continuous activation of Src during ischemia/reperfusion has been reported in rat hippocampus (25), and oxidation of Src is a prerequisite for Src activation in response to IGF-I (9). However, free radicals diffuse quickly. Therefore, the mechanism by which they function to regulate signal pathways has not been defined. A recent study indicated that inactivation of peroxiredoxin I by its tyrosine phosphorylation reduced ROS elimination, which allowed localized H$_2$O$_2$ accumulation for cell signal transduction (4). Unfortunately, no specific signaling pathway or molecule was identified in their study. Based on our current results, which show sustained activity of Src when it is associated with SHPS-1, we postulate that to maintain sustained Src activation, the recruitment of Nox4 to SHPS-1 is required. Nox4/Src colocalization on SHPS-1 is an important step in the series of signaling events leading not only to sustained Src activation but also to increased VSMC proliferation. ER localization of Nox4 has been shown to regulate EGF signaling via oxidation and inactivation of PTP1B, which required colocalization of Nox4 and PTP1B in the ER (10). However, that study did not explore whether these two molecules directly bound to each other or whether their association was mediated by an intermediary protein(s). In contrast, our results identified a specific protein, Grb2, that mediated Nox4/Src colocalization. Importantly, our results were also confirmed in vivo in diabetic mice.

In contrast to Src, the mechanism of Nox4 recruitment to SHPS-1 has not been reported previously. To determine the component of the SHPS-1 signaling complex that mediated Nox4 recruitment, we focused on three SH2 domain containing proteins in this complex, Src, SHP-2, and Grb2. In vitro binding assay results showed that Grb2 but not Src or SHP-2 could directly bind to Nox4. Our results show that binding occurred through an interaction between a phospho-YVNI motif in Nox4 and a SH2 domain in Grb2. That Tyr-491 was the specific phosphotyrosine that mediated Nox4 recruitment to SHPS-1 was shown by demonstrating that cells expressing a mutant form of Nox4 (Y491F) or a cell-permeable peptide containing a Tyr to Asp substitution at position 491, and the Nox4 flanking sequence prevented Nox4/Grb2 association as well as Nox4 recruitment to SHPS-1. To confirm this result, we utilized cells that expressing a Pyk2 Y881F mutant, because our previous study had shown that Pyk2 mediates Grb2 recruitment to SHPS-1 (19). As predicted, cells expressing this mutant had attenuated Nox4 recruitment. Therefore, we conclude that Grb2 mediates Nox4 recruitment to SHPS-1 in response to IGF-I, which allows Nox4-derived ROS to oxidize SHPS-1-associated Src leading to its activation. The importance of this interaction for IGF-I signaling and biological actions was shown by demonstrating that disruption of Nox4 recruitment to SHPS-1 not only inhibited IGF-I-stimulated Src oxidation and activation as well as cell proliferation in vitro, but also impaired IGF-I signal transduction and biological actions in the aorta of diabetic mice. Therefore, we conclude that Nox4 recruitment to SHPS-1 is an important step leading to IGF-I-stimulated VSMC proliferation. Although ROS has been proposed to mediate growth factor-stimulated signaling (26) and NADPH oxidases were involved in PDGF-induced ROS generation (27), this is the first study to demonstrate, in vitro and in vivo, how a growth factor, IGF-I, modulates ROS generation on a specific molecular scaffold to mediate increased downstream signaling and biological actions.

A previous study reported that mitochondrial localized Nox4 is responsible for high glucose-induced ROS generation (11). To specifically eliminate a mitochondrial source of H$_2$O$_2$, a mitochondrial localized catalase was introduced into the VSMC. Cells expressing this mutant showed no change in IGF-I-stimulated Src activation or in IGF-I-stimulated downstream signaling and biological actions. In addition, a mitochondrial superoxide inhibitor had minimal or no effect on the ability of IGF-I to enhance Src oxidation, and activation in VSMC exposed to high glucose, consequently, had no effect on IGF-I-stimulated downstream signaling. Similarly, overexpression of catalase targeted to mitochondria had no effect on Nox4-dependent EGFR signaling (10). In addition, a recent study demonstrated that, in skeletal muscle, mitochondria did not modulate cellular superoxide, but NADPH oxidases were the major contributors of ROS generation during skeletal muscle rest or contraction (28). Nox4 has been detected in different subcellular locations, such as perinuclear vesicles (29), focal adhesions (12), the nucleus (30), and plasma membrane (31). It is possible that expressing a mutant showed no change in IGF-I-stimulated Src activation and in IGF-I-stimulated downstream signaling and biological actions. Similarly, overexpression of catalase targeted to mitochondria had no effect on Nox4-dependent EGFR signaling (10). In addition, a recent study demonstrated that, in skeletal muscle, mitochondria did not modulate cellular superoxide, but NADPH oxidases were the major contributors of ROS generation during skeletal muscle rest or contraction (28). Nox4 has been detected in different subcellular locations, such as perinuclear vesicles (29), focal adhesions (12), the nucleus (30), and plasma membrane (31). It is possible that expressing a mutant showed no change in IGF-I-stimulated Src activation and in IGF-I-stimulated downstream signaling and biological actions. Similarly, overexpression of catalase targeted to mitochondria had no effect on Nox4-dependent EGFR signaling (10). In addition, a recent study demonstrated that, in skeletal muscle, mitochondria did not modulate cellular superoxide, but NADPH oxidases were the major contributors of ROS generation during skeletal muscle rest or contraction (28). Nox4 has been detected in different subcellular locations, such as perinuclear vesicles (29), focal adhesions (12), the nucleus (30), and plasma membrane (31). It is possible
that these examples represent cell type-specific responses and differential responses to distinct stimuli. Our previous study has shown that IGF-I stimulates Nox4 and p22 complex formation, leading to ROS generation and Src activation (9). The current results show that this occurs at a specific site in the plasma membrane (the SHPS-1 complex). This allows localized H₂O₂ generation within the plasma membrane, a site that is also a major focus of Src localization (32). However, we cannot exclude the possibility that superoxide is also produced in this complex. Based on our current results, we conclude that SHPS-1 localized Nox4-derived ROS, rather than mitochondrial or pancellular increases in Nox4-derived ROS, is critical for mediating IGF-I signaling and biological actions. Furthermore, as pointed out by Toledano et al. (33), localized H₂O₂ production may be important for protecting the rest of the cell from the unwanted effects of H₂O₂ production as well as providing an important mechanism for H₂O₂-dependent signal termination. Therefore, there may be multiple reasons why localized ROS generation is the predominant Nox4-dependent signaling mechanism.

This study presents a novel mechanism by which IGF-I stimulates and maintains Src activation during hyperglycemia. IGF-I-stimulated tyrosine phosphorylation of Nox4 creates a SH2-binding site, leading to Grb2 association and colocalization of Nox4 with Src on the SHPS-1 scaffold. SHPS-1-localized Nox4 oxidizes Src, leading to sustained Src activation and subsequent downstream signaling. Diabetic mice were used to confirm these occurrences in vivo. This study is the first report of an underlying mechanism by which localized Nox4-derived ROS mediates sustained Src oxidation and activation, IGF-I downstream signaling, and biological actions. The findings suggest several novel approaches that could be developed to prevent localized ROS generation in response to hyperglycemia and thereby prevent the progression of atherosclerosis in diabetic patients.

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