S100A11 Mediates Hypoxia-induced Mitogenic Factor (HIMF)-induced Smooth Muscle Cell Migration, Vesicular Exocytosis, and Nuclear Activation*§

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Hypoxia-induced mitogenic factor (HIMF) is a newly discovered protein that is up-regulated in murine models of pulmonary arterial hypertension and asthma. Our previous study shows that HIMF is a potent mitogenic, angiogenic, and vasoconstrictive chemokine associated with pulmonary arterial hypertension. Two-dimensional gel electrophoresis was used to investigate downstream molecules in HIMF-induced cell signaling, demonstrating that S100A11, an EF-hand calcium-binding protein, was exclusively altered and was decreased (2.7 ± 0.2-fold, p < 0.05) in pulmonary artery smooth muscle cells (SMCs) treated with HIMF for 5 min compared with untreated cells (n = 4). Immunofluorescence showed that in control cells S100A11 is a cytosolic protein, which then aggregates and translocates both to the plasma membrane with subsequent exocytosis and to the nucleus upon HIMF stimulation. Annexin A2, a known S100A11 binding partner, also colocalized with S100A11 during HIMF-induced membrane trafficking. To investigate the intracellular function of S100A11, siRNA was used to knock down S100A11 expression in SMCs. The S100A11 knockdown significantly reduced HIMF-induced SMC migration but did not affect the SMC mitogenic action of HIMF. Our data show that S100A11 mediates HIMF-induced smooth muscle cell migration, vesicular exocytosis, and nuclear activation. Molecular & Cellular Proteomics 10: 10.1074/mcp.M110.000901, 1–7, 2011.

The family of “resistin-like molecule (RELM)” proteins comprises a group of proteins with a conserved motif of 10 equally spaced cysteine residues within the C terminus. Hypoxia-induced mitogenic factor (HIMF), one member of the murine RELM/FIZZ family, is also known as RELMα because of its similarity to resistin (1) and FIZZ1 for its prominent presence in inflammatory lung of a murine model of allergic asthma (2). We have previously reported that HIMF is up-regulated in a mouse chronic hypoxia-induced model of pulmonary arterial hypertension (PAH) (3); that it has mitogenic, angiogenic, vasoconstrictive, antiapoptotic, and chemokine-like properties (3, 4); and that its overexpression can induce the vascular and hemodynamic changes of PAH (5). HIMF can stimulate multiple cell signaling pathways, but understanding of its molecular actions in the cell remains limited (3–5). For example, in bone marrow-derived mesenchymal cells, HIMF can bind Bruton’s tyrosine kinase (BTK), induce BTK autophosphorylation, and cause redistribution of BTK to the leading edge of the cells (6). HIMF has chemotactic actions on myeloid cells via BTK (6). As well, HIMF can activate Akt phosphorylation via the phosphatidylinositol 3-kinase (PI3K)-Akt pathway in pulmonary smooth muscle cells (SMCs) (3). It also activates the phosphorylation of ERK 1/2 via the mitogen-activated protein kinase (MAPK) pathway. More recently, we found that HIMF can stimulate the mobilization of intracellular calcium via the phospholipase C-inositol trisphosphate pathway in pulmonary SMCs. This calcium mobilization is independent of the Akt and ERK pathways but is dependent on tyrosine kinase phosphorylation (7).

The goal of the current study was to investigate cell protein changes and downstream pathways activated in association with HIMF-induced phospholipase C-inositol trisphosphate-Ca2+ pathway activation. Using two-dimensional gel electrophoresis and mass spectrometry (MS), we were able to identify proteins regulated by HIMF in human pulmonary artery SMCs (HPASMCs). The calcium-binding protein S100A11 was most dramatically decreased upon HIMF application. S100 proteins belong to the EF-hand calcium-binding protein SMC; FIZZ, found in inflammatory zone; BTK, Bruton’s tyrosine kinase; SBM, serum-free basal medium; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.
family, regulating a variety of cellular processes via interaction with different targeting proteins. Investigating the biological role of S100A11 in HIMF-induced cell signaling, we found that S100A11 translocates from the cytosol to the plasma membrane and to the nucleus upon HIMF stimulation and is involved in HIMF-induced cell migration.

MATERIALS AND METHODS

Cell Culture—HPASMCs were obtained from Lonza (Walkersville, MD) and cultured in SGM-2 growth medium supplemented with 5% fetal calf serum (FCS), 0.5 ng/ml human epithelial growth factor, 2 ng/ml human fibroblast growth factor, and 5 μg/ml insulin. The cells were maintained under the conditions of 5% CO2 and 21% O2 in a humidified incubator at 37 °C. Cells from passages 5 through 8 were used for experiments.

Sample Preparation for Two-dimensional Gel Electrophoresis—When cells reached 70–80% confluence, growth was arrested by maintaining cells in serum-free basal medium (SBM) for 48 h. For the treated cells, 50 nM HIMF was applied for 5 min, whereas untreated cells were used as controls. Each experiment was carried out four times. After HIMF treatment for 5 min, cells were immediately washed twice with cold phosphate-buffered saline (PBS) and then lysed with an isoelectric focusing (IEF) lysis buffer comprising 8 M urea, 2 M thiourea, 4% CHAPS, and 50 mM dithiothreitol (DTT) and centrifuged at 16,000 × g for 15 min at 4 °C (9). The supernatant was snap frozen and stored at −80 °C. Protein concentrations were determined by a modified Bradford method (9) using bovine serum albumin (BSA) as a standard.

Two-dimensional Difference Gel Electrophoresis—DIGE analysis was carried out as we have reported previously (9). Briefly, equal amounts of protein (50 or 400 μg, the latter for preparative gels) of control and HIMF-treated samples were labeled with two different CyDyes (CyDye™ DIGE Flours (GE Healthcare)) according to the manufacturer’s recommended protocol (10). Samples were resolved on 18-cm Immobiline DryStrips (pH 4–7; GE Healthcare), activated, and rehydrated in IEF buffer at 50 V for 11 h followed by 150 V for 1 h, 300 V for 1 h, 600 V for 1 h, 1000 V for 1 h, 2000 V for 1 h, 4000 V for 1 h, a 2-h rapid ramp up to 8000 V, and finally 10,000 V for 10 h. After equilibration in 1% DTT and 2.5% iodoacetamide (Sigma) for 15 min at room temperature, the strips were subjected to 10% Bis-Tris polyacrylamide gels for the second dimension SDS-PAGE (20 × 20 cm) (9). To avoid any dye-specific effects that might result from preferential labeling or different fluorescence characteristics of the gel or glass, repetitive gels that swap the dyes used to label samples were run. Gels were visualized using a Typhoon™ 9410 imager (GE Healthcare) and analyzed by using the DeCyder™ 2D 6.5 software (GE Healthcare). The protein spots were detected first using the differential in-gel analysis (DIA) mode and then analyzed using the biological variation analysis (BVA) mode of DeCyder according to the manufacturer’s user manual. The protein spots with ≥1.5-fold change (p < 0.05) are reported as changed.

To prepare gel spots for protein identification, 400 μg of protein were subjected to two-dimensional gel electrophoresis as indicated above without CyDye labeling. At the end of the run, the gels were fixed in methanol/water solutions, stained with silver nitrate (11), and visualized by using an Epson 10000XL Fastsilver Imager (Agilent Technologies, Wilmington, DE). After gel image acquisition, the spots of interest were excised and stored at −80 °C.

In-gel Digestions and MS—Excised spots were subjected to in-gel tryptic digestion (9). The resulting peptides were dissolved in 10 μl of 0.1% trifluoroacetic acid (TFA) in water and desalted with ZipTip C18 pipette tips (Millipore, Billerica, MA). Peptides were analyzed using an LTQ-Orbitrap LC/MS/MS instrument (ThermoFisher). The mass spectrometer was equipped with an on-line nano-HPLC system (1200 Series, Agilent Technologies). The peptides were separated on a reverse-phase analytical column packed with 10 cm of C18 beads (Biobasic C18 PicoFrit column, New Objective, Woburn, MA). A linear AB gradient comprising 5–60% B for 25 min was used where solvent A was 0.1% formic acid and solvent B was 90% acetonitrile in 0.1% formic acid followed by 100% B for 2 min. The flow rate was 300 nl/min. The LTQ-Orbitrap LC/MS/MS instrument was operated in a data-dependent mode in which a full scan was followed by MS/MS scans of the five most intensive ions. The ions were automatically selected for collision-induced dissociation (CID). Peak lists were generated by BioWorks 3.2 software. The raw data were searched against the International Protein Index human database (Version IPI3.19, which includes 60,397 sequence entries in total) using Mascot 2.205 (Matrix Science, London, UK). Search parameters allowed one miss cleavage site, cysteine residues to be modified by carbamidomethylation, and variable oxidation of methionine residues and phosphorylation of serine, threonine, and tyrosine residues. Precursor and fragment ion mass tolerance were set to 0.8 and 0.8 Da, respectively. A peptide was considered as identified with probability greater than 95% (Mowse scores were above 38). A protein was considered as identified with at least two peptides. Common contaminant keratins were excluded.

Immunofluorescence—HPASMCs were grown on glass coverslips and were starved in SBM for 48 h before HIMF treatment (7). Cells were fixed with cold methanol for 10 min at the indicated time of HIMF stimulation and washed twice with PBS for 5 min each time. Then, the cells on coverslips were permeabilized with 0.2% Triton X-100 in PBS followed by blocking with 2% BSA in PBS before being incubated overnight at 4 °C with the primary antibodies (rabbit anti-human S100A11 and mouse anti-annexin A2 (BD Biosciences)). FITC-conjugated donkey anti-rabbit IgG and rhodamine-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.) were used as secondary antibodies. Nuclei were stained with 50 ng/ml 4′,6′-diamidino-2-phenylindole dilactate (DAPI) for 5 min at room temperature. A Zeiss 510 Meta confocal microscope (Carl Zeiss International) was used for the imaging. Each experiment was done twice.

siRNA-mediated Knockdown of S100A11 Expression—A double-stranded 23-mer siRNA specific for S100A11 (sequence, 5′-CAG CTA GAT TTC TCA GAA TTT CT-3′) was designed in our laboratory and synthesized and annealed by Integrated DNA Technologies, Inc. (Corvallis, IA). Blast analysis confirmed that it does not overlap in recognition with any known S100 protein other than S100A11 or any other proteins. We confirmed the efficiency for S100A11 by screening an siRNA clone set targeted against human S100A11 from Open Biosystems (see supplemental Fig. S3). Control siRNA that has proven non-cross-reactive with any known genes was obtained from Qiagen (Valencia, CA). The transfection of siRNA was carried out by using the Amaxa Basic Nucleofector kit for primary SMCs (Amaxa Inc., Gaithersburg, MD) according to the manufacturer’s guidelines. Briefly, once HPASMCs had grown to 70–80% confluence, the cells were trypsinized and resuspended in the Basic Nucleofector solution. 20 pmol of siRNA were added to 20 μl of cell suspension and mixed by gentle pipetting. HPASMCs were transiently transfected by electroporation with the Amaxa Nucleofector II device and program CM137 (Amaxa Inc.) and were immediately transferred into wells containing...
37 °C prewarmed culture medium. Culture medium was changed once the cells attached to the bottom of the wells. Negative controls included untreated cells, cells in Nucleofector solution exposed to electroporation without siRNA, and cells transfected with control siRNA. 48 h after the electroporation, the cells were lysed for immunoblot analysis to confirm the down-regulation of S100A11 expression or were used to examine HIMF-induced proliferation and migration. Experiments were done in triplicate. ImageJ (National Institutes of Health, Bethesda, MD) was used to quantify the protein levels in Western blotting.

SMC Proliferation Assay—HPASMCs were plated in 6-well plates at a density of 5000 cells/cm² in SGM-2 medium for 48 h. Cells were then starved in SBM-2 without serum and growth factors to synchronize for an additional 24 h. Cells were then treated with 10 nM HIMF or FLAG for 48 h in the presence of 2.5% FBS. At 48 h after treatment, the cells were washed, trypsinized, and counted with a hemocytometer. In some experiments, rabbit polyclonal anti-human S100A11 antibody was applied to neutralize S100A11. Each experiment was repeated in triplicate.

SMC Migration Assay—SMC migration was evaluated in a Transwell chamber according to the method by Goncharova et al. (12) with modification. Briefly, after HPASMCs had reached 70–80% confluency, they were maintained in serum-free medium for 48 h, then detached by trypsin, and washed with PBS. Cells were plated at a density of 2×10⁵ cells/well in 24-well Transwell plates (8-µm pore size; Corning, Corning, NY) in smooth muscle basal medium (Lonza) supplemented with 0.2% FBS. 50 nM HIMF was added to the lower chamber to induce cell migration; FLAG peptide was used as control. Cells were allowed to migrate for 6 h under 5% CO₂ and 21% O₂ in a humidified incubator at 37 °C. Then the chamber inserts were removed, and the cells were fixed for 10 min in cold methanol and stained for 15 min in Coomassie Brilliant Blue R-250 (Bio-Rad). The upper side of the insert membrane was wiped and cleaned with a cotton swab, and the remaining migrated cells on the lower side of the insert were viewed under a microscope. Images of three randomly selected fields were taken, and ImageJ software was used to measure the area covered by migrated cells for quantification. Each condition was repeated in triplicate.

S100A11 Antibody Neutralization—To neutralize extracellular S100A11 that is released into medium via exocytosis, a monoclonal S100A11 antibody (Proteintech Group Inc.), was applied to the media. In the SMC migration assay, 10 µg/ml antibody was added into the cell suspension 30 min before the cells were placed in the upper chamber for migration. Experiments were done in triplicate.

Statistical Analysis—All results are presented as means ± S.E. Statistical significance was tested with Student’s t test. A p value of <0.05 was considered statistically significant.

RESULTS

S100A11 Is Identified to Be Down-regulated by HIMF—A two-dimensional DIGE (pH 4–7) proteomics approach was used to identify HPASMC proteins regulated by HIMF within 5 min of treatment. Over 400 protein spots were resolved, and quantification showed that the abundance of only one protein significantly changed (see criteria under “Materials and Meth-
The protein was identified by mass spectrometry to be S100A11, also called S100C, which is a calcium-binding protein. It was down-regulated (2.7 ± 0.2-fold, p < 0.05) (Fig. 1A). To eliminate CyDye labeling preference, labeling of control and treated samples was switched, and results indicated consistent changes of S100A11 (supplemental Fig. S1). Two-dimensional immunoblotting for S100A11 was performed, and the decrease of S100A11 after HIMF treatment was further validated. -Actin was used as a loading control (Fig. 1B).

The identified proteins were annotated and are listed in supplemental Fig. S1 and supplemental Table 1. The MS raw data and search results have been uploaded to the Proteomics Identifications (PRIDE) database (accession numbers 12335–12346; http://www.ebi.ac.uk/pride/).

S100A11 Translocates upon HIMF Stimulation—Considering the short treatment time (5 min) and that only S100A11 was altered (decreased) with HIMF stimulation of HPASMCs, we speculated that the change in S100A11 might be caused by subcellular relocalization. Therefore, we used immunofluorescence of HIMF-stimulated (0–10 min) HPASMCs to track the distribution of S100A11 over time. As shown in Fig. 2A, S100A11 was diffusely distributed in the cytosol of untreated, resting cells. With continued HIMF stimulation, S100A11 aggregated and translocated to the plasma membrane (Fig. 2, B, C, and D, * and and D, arrows) and the nucleus (Fig. 2, C inset and D, arrows).

It is known that S100A11 is a calcium-binding protein, it exists as a non-covalent homodimer, and when Ca^{2+} binds to S100A11, it will undergo conformational changes that facilitate its binding and interaction with target proteins involved in its relocalization (13). Annexins, another calcium-binding protein family, bind to some S100 proteins and form a complex, participating in the processes of endocytosis and exocytosis (14–16). We found that annexin A2 colocalizes with S100A11 in HIMF-stimulated cells. In resting cells, annexin A2 localized in both a cytosolic and membrane distribution. Upon HIMF stimulation, the S100A11 formed conglomerates, annexin A2 colocalized with S100A11, and vesicles formed that enveloped the S100A11-annexin A2 complex. The vesicles were exocytosed at the brim of cell boundaries at 5 and 10 min, and dual staining extracellular vesicles for S100A11 and annexin A2 were found at 10 min (Fig. 3).

HIMF Induces SMC Migration via Ca^{2+}-S100A11 Pathway—To investigate the role of S100A11 in the HIMF-induced cell signaling pathway in SMCs, we used siRNA knockdown to inhibit S100A11 expression in HPASMCs. The introduction of siRNA significantly depressed the protein level as early as day 2, lasting at least through day 5 when 90% of S100A11 expression was still inhibited. Control siRNA did not affect the expression of S100A11 in SMCs. Western blotting for S100A11 was done to show the reduction of S100A11 protein level with siRNA knockdown (Fig. 4A).

HIMF can cause pulmonary hypertension with significant pulmonary vascular remodeling (5). SMC proliferation and migration contribute greatly to the process of vascular remod-
To test whether HIMF can induce SMC migration, using the Transwell migration assay, we found that 50 nM HIMF can stimulate profound migration of HPASMCs (Fig. 4B), which is similar to the effect of PDGF-BB at 10 ng/ml. When S100A11 expression was knocked down by siRNA, this migratory effect induced by HIMF was abolished (Fig. 4C), implying that S100A11 is pivotal to HIMF-induced SMC migration.

S100A11 Does Not Participate in HIMF-induced Cell Proliferation of HPASMCs—HIMF is known to be able to induce SMC proliferation via the PI3K-Akt pathway (3). We tested whether S100A11 reduction by siRNA knockdown would affect HIMF-induced SMC proliferation. siRNA to human S100A11 was designed and effectively down-regulated S100A11 expression (see supplemental Fig. S3). Fig. 4D shows that HIMF still was able to stimulate mitogenic activity in HPASMCs even when S100A11 expression was blocked. Quantification analysis revealed that cell proliferation induced by HIMF was not significantly different in siRNA-treated and control cells, suggesting that S100A11 is not a contributor to HIMF-induced SMC proliferation.

S100A11 Does Not Alter SMC Migration and Proliferation via Extracellular Exocytosis—As our data have shown that S100A11 is exocytosed in HIMF-induced SMC migration, we speculated that S100A11 might function via autosecretion. To
test this, we used an S100A11 antibody to neutralize extracellular S100A11 from exocytosis. The efficiency of antibody knockdown was confirmed by Western blot (see supplemental Fig. S4). We found that neither the proliferation nor migration of HPASMCs was affected (Fig. 5), suggesting that S100A11 does not function extracellularly during HIMF signaling.

DISCUSSION

In this study, S100A11 was shown for the first time to be involved in the downstream signaling and physiologic action of HIMF/FIZZ1/RELMα. This novel downstream protein S100A11 was down-regulated by HIMF in SMCs via translocation to the nucleus and the plasma membrane and exocytosis. We also demonstrated that HIMF can induce smooth muscle cell migration and that S100A11 is required during this process.

HIMF induces a rapid, dramatic, and sustained release of calcium from the internal stores in smooth muscle cells (7). To understand what relevant cell functions might be activated by this HIMF-induced calcium response, we carried out a two-dimensional gel proteomics investigation and found that the majority of the proteome remained constant and that only S100A11 was decreased significantly. Sakaguchi et al. (17, 18) reported that S100A11 translocated to the cell nucleus in keratinocytes after exposure to high calcium or TGF-β1. The nucleus translocation suggests that S100A11 regulates gene transcription that might lead to functional changes. In human fibroblasts and keratinocytes, the translocation of S100A11 to the nucleus led to cell growth inhibition (17, 19, 20). In addition, corticoids were shown to induce S100A11 shuttling that resulted in keratinocyte differentiation (21).

SMCs play a pivotal role in the development of PAH and hypertension. Their phenotype changes from an immobile, non-proliferative contractile state to one that is migratory and proliferative. The proliferation and accumulation of SMCs accelerate the thickening of the vessel medium and contributes to increases in blood pressure. In this study, we discovered that HIMF can induce SMC migration; this is a novel function of HIMF on SMCs. To investigate the role of S100A11 during this process, we used siRNA to knock down S100A11 expression and inhibit the subsequent translocation; we found that HIMF-induced HPASMC migration is completely blocked when S100A11 expression is inhibited by siRNA knockdown. As S100A11 lies downstream of HIMF in the pathway of calcium mobilization, we believe that HIMF-induced SMC migration is dependent on calcium and mediated by S100A11. When we tested the effects of S100A11 on SMC proliferation, however, this inhibition by siRNA knockdown did not affect cell proliferation in response to HIMF stimulation, implying that S100A11 is not a key mediator of SMC proliferation. In SMCs, the cell proliferation induced by HIMF is associated with the PI3K-Akt pathway, which is not dependent on calcium release. In addition, when we tested the effect of inhibitors of PI3K and Akt on HIMF-induced calcium release (our unpublished data), these inhibitors could not block HIMF-induced calcium mobilization. In sum, the evidence supports the idea that S100A11 and the calcium pathway do not participate in mediating HIMF-induced SMC proliferation. As our current observation of the HIMF-induced S100A11 translocation to the nucleus indicates that HIMF, via S100A11, may be involved in gene transcription, in future work we will focus on how S100A11 might regulate gene transcription in SMCs.

The confocal imaging showed that S100A11 also translocates to the plasma membrane. During this process, S100A11 is colocalized with annexin A2 and undergoes exocytosis. Annexins are a family of calcium-binding proteins that participate in membrane trafficking, endocytosis, and exocytosis. Some S100 family members, such as S100A8 and S100A9,
are inflammatory factors that are secreted extracellularly and induce cell migration; therefore, we hypothesized that S100A11 might affect SMC function in an autocrine manner after exocytosis. However, the application of S100A11 antibody did not affect HIMF-induced cell proliferation or migration, suggesting that S100A11 does not function extracellularly. Additional studies have revealed that annexin A2 is also involved in secretory processes. For example, in endothelial cells, it plays a role in the secretion of von Willebrand factor (22, 23), and it interacts with SNAP-23 to regulate the secretion of lung surfactant in alveolar type II cells (24). In the current study, the interaction of S100A11 with annexin A2 following HIMF stimulation and the resulting exocytosis indicate that HIMF may regulate the secretion process via these proteins. In conclusion, in this study, we demonstrated that HIMF stimulation of SMCs induces translocation of S100A11 from the cytosol to the plasma membrane and nucleus and leads to the formation of annexin A2–S100A11 complexes, indicating a role in regulating exocytosis/membrane trafficking. S100A11 is a mediator of HIMF-induced SMC migration.

* This work was supported, in whole or in part, by National Institutes of Health Specialized Centers of Clinically Oriented Research Grant P50084946 (to R. A. J.).

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In order to cite this article properly, please include all of the following information: Fan, C., Fu, Z., Su, Q., Angelini, D. J., Van Eyk, J., and Johns, R. A. (2011) S100A11 Mediates Hypoxia-induced Mitogenic Factor (HIMF)-induced Smooth Muscle Cell Migration, Vascular Exocytosis, and Nuclear Activation. Mol. Cell. Proteomics 10(3):M110.000901. DOI: 10.1074/mcp.M110.000901.