The receptor for advanced glycation end products (RAGE) and its ligands have been implicated in the activation of oxidant stress and inflammatory pathways in vascular smooth muscle cells (VSMCs) leading to the initiation and augmentation of atherosclerosis. Here we report that non-receptor Src tyrosine kinase and the membrane protein caveolin-1 (Cav-1) play a key role in the activation of RAGE by S100B in VSMCs. S100B increased the activation of Src kinase and tyrosine phosphorylation of caveolin-1 in VSMCs. A RAGE-specific antibody blocked both these effects. An inhibitor of Src kinase, PP2, significantly blocked S100B-induced activation of Src kinase, mitogen-activated protein kinases, transcription factors NF-κB and STAT3, superoxide production, tyrosine phosphorylation of Cav-1, VSMC migration, and expression of the pro-inflammatory genes monocyte chemotactic protein-1 and interleukin-6. Cholesterol depletion also inhibited S100B-induced effects indicating the requirement for intact caveolae in RAGE-specific signaling. Nucleofection of either a Src dominant negative mutant, or Cav-1 short hairpin RNA significantly reduced S100B-induced inflammatory gene expression in VSMCs. Furthermore, VSMCs derived from insulin-resistant and diabetic db/db mice displayed increased RAGE expression, Src activation, and migration compared with those from control db/+ mice. The RAGE antibody blocked enhanced migration in db/db cells. These studies demonstrate for the first time that, in VSMCs, Src kinase and Cav-1 play important roles in RAGE-mediated inflammatory gene expression and migration, key events associated with diabetic vascular complications.

Vascular smooth muscle cell (VSMC) proliferation, migration, and inflammatory gene expression play important roles in the development of atherosclerotic lesions. Diabetic conditions have been shown to enhance these processes, which lead to accelerated atherosclerosis (1–4). Evidence shows that the accumulation of advanced glycation end products (AGEs) and activation of the receptor for AGEs (RAGE) are key factors mediating these events (5–8). RAGE is a member of the immunoglobulin superfamily of cell-surface molecules and is expressed in many cell types, including VSMCs. It can be activated by multiple ligands such as amphoterin, β-amylloid peptide, and several short peptides belonging to the S100/calgranulin family, which includes S100B (9, 10). Recent studies using animal models showed that diabetes-induced accelerated atherosclerosis in apoE null mice is associated with enhanced accumulation of RAGE ligands and increased expression of RAGE itself (11, 12). The expression of RAGE as well as its ligands, including S100B, was increased in neointimal and medial cells. Administration of sRAGE could reduce neointimal thickening as well as VSMC proliferation in these animal models. Furthermore, RAGE null mice showed reduced arterial injury responses, whereas transgenic mice expressing DN-RAGE specifically in smooth muscle cells displayed significantly less neointimal thickening (13–16). These results clearly demonstrate the in vivo role of RAGE signaling in VSMC dysfunction.

Some of the in vitro effects mediated by RAGE and its ligands in VSMCs include increased oxidant stress, cell migration, proliferation, inflammatory gene expression, and extracellular matrix production (17–20). RAGE-mediated signaling can activate multiple signaling pathways, including Ras-mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase, protein kinase C (PKC), and the Janus tyrosine kinases (JAKs), and transcription factors, including STAT3, AP1, and NF-κB (21–26). However, the role of key upstream tyrosine kinases in signaling mechanisms triggered by the ligation of RAGE in VSMCs is not known.

Src tyrosine kinases are utilized by several receptors lacking intrinsic tyrosine kinase activity to transduce intracellular signals involved in diverse biological processes, including gene expression, proliferation, and migration (27). In VSMCs, Src kinases play an important role in platelet-derived growth factor-induced migration (28), angiotensin II-induced activation of STAT transcription factors, and transactivation of the epidermal growth factor receptor (29, 30). In this study we tested the hypothesis that Src kinases play a key role in signaling associated with VSMC migration and inflammatory gene expression induced by the RAGE ligand S100B. We also examined the downstream effectors of Src in these events, including caveolin-1 (Cav-1), which is tyrosine-phosphorylated by Src (31). Cav-1 is an integral protein of caveolae that are specialized cholesterol-rich membrane microdomains existing as vesicular invaginations in several cells, including VSMCs. Cav-1 plays an important role in the assembly and integration of signaling complexes within caveolae (32, 33). Evidence shows that RAGE and Src are co-localized in caveolae (34), and Cav-1 function has been implicated in diabetes and cardiovascular abnormalities (35). Our results demonstrate for the first time that S100B activates Src kinase in a RAGE-de-
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Dependent manner and that Src kinase is required for the S100B-induced RAGE signaling leading to migration and inflammatory gene expression in VSMCs. Furthermore, our results also show the involvement of Cav-1 in S100B-induced inflammatory gene expression in VSMCs and provide evidence for the involvement of RAGE signaling in enhanced atherogenic responses in VSMCs derived from diabetic mice.

EXPERIMENTAL PROCEDURES

Reagents—Bovine brain S100B peptide and the kinase inhibitors used in this study (PP2, GFX109203, AG490, and SB202190) were purchased from Calbiochem. S100B stock solutions were assayed for endotoxin contamination using E-TOXATE kit (Sigma-Aldrich) and were found to have less than 1.6 enzyme units/mg of protein. Phospho-(Tyr-14)-Cav-1 antibody specific for Tyr-14 and monoclonal Cav-1 antibody was purchased from BD Biosciences. Polyclonal Cav-1 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA), Src antibody (both soluble and agarose-conjugated) was from Oncogene Research Products (La Jolla, CA), and p65 (NF-κB) antibody was from Rockland Immunochemicals (Gilbertsville, PA). All other antibodies used in the Western blot analyses were purchased from Cell Signaling (Beverly, MA). RNA-STAT60, Quantum RNA 18 S primers, and RT-PCR kits were purchased from Tel-Test (Friendswood, TX), Ambion (Austin, TX), and Applied Biosystems (Foster City, CA), respectively. Nucleofection kits were obtained from Amaza (Gaithersburg, MD). All other reagents were obtained from Sigma-Aldrich. The expression vectors for SrcRF and Cav-1 scaffolding domain mutants were from Dr. Joan Brugge (Harvard University, Boston) and Dr. R. G. Anderson (University of Texas Southwestern Medical Center, Dallas), respectively. RAGE antibody was provided by Dr. A. M. Schmidt (Columbia University, NY).

Cell Culture and Treatments—Porcine VSMCs (PVSMCs) were isolated using explant culture method and maintained in culture as described earlier (36). Rat VSMCs (RVSVMCs) were isolated from aortas by enzymatic digestion as described earlier. The db/db mice (type 2 diabetic animals) and their genetic control db/+ mice were obtained from Jackson Laboratory (Bar Harbor, ME). They were sacrificed at 9–10 weeks of age to isolate MVSMCs by enzymatic digestion as described earlier (38). Both RVSVMCs and RVSVMCs were cultured in Dulbecco’s modified Eagle’s medium/F-12 containing 10% fetal bovine serum, 2 mM glutamine, and streptomycin/penicillin. Human aortic smooth muscle cell (HSMCs) were purchased from Cambrex (East Rutherford, NJ) and cultured according to the manufacturer’s specifications. In all the experiments, cells were grown to 80% confluence and serum-starved for 48 h in Dulbecco’s modified Eagle’s medium containing 0.2% bovine serum albumin before subjecting them to experimental conditions unless mentioned otherwise. VSMCs were stimulated with S100B at a concentration of 10 μg/ml, unless indicated otherwise. In some experiments cells were pretreated with inhibitors PP2, N-acetylcyesteine, bisindolylmaleimide (GFX109203), β-cyclodextrin, or SB202190 at the indicated concentrations for 30 min to 1 h prior to stimulation with S100B.

Determination of Superoxide Formation—S100B-induced superoxide production was detected using dihydroethidium staining as described earlier (37). Briefly, VSMCs were seeded on coverslips placed in 12-well cluster dishes at 50,000 cells per well. Cells at 80% confluence were serum-starved for 48 h and then treated with S100B for 30 min and stained with dihydroethidium (20 μM) for 10 min at 37 °C. The coverslips were then rinsed with phosphate-buffered saline and sealed in mounting medium. Fluorescence was detected using a confocal laser-scanning microscope (wavelength: 510 nm/595 nm). Fluorescence intensity was quantified using Image Pro software (Media Cybernetics, Inc., Silver Spring, MD).

Preparation of Cell Lysates, Immunoprecipitation, and Western Blotting—After stimulation with S100B, total cell lysates were prepared in SDS sample buffer for Western blotting. To perform immunoprecipitations, cells were lysed in radioimmune precipitation assay buffer and immunoprecipitated with the indicated antibodies as described previously (38), except that, when Cav-1 was immunoprecipitated, cells were lysed in radioimmune precipitation assay buffer containing β-octylglucoside instead of deoxycholate. Protein samples were fractionated on 12.5% SDS-polyacrylamide gels to detect Cav-1 or on 10% SDS-polyacrylamide gels for all other proteins. Fractionated proteins were transferred to nitrocellulose membranes and immunoblotted with the indicated antibodies, and blots were developed using chemiluminescence reagents as described earlier (38).

Immunofluorescent Staining of VSMCs—Immunofluorescent staining of VSMCs grown on coverslips was performed as described before (39). Briefly, serum-depleted VSMCs were treated with S100B as indicated. In some experiments cells were pretreated with the indicated inhibitors for 1 h prior to stimulation with S100B. At the end of the incubation period the cells were washed with phosphate-buffered saline and fixed in 3% formaldehyde for 20 min. The cells were then washed in phosphate-buffered saline and immunostained with the indicated antibodies followed by appropriate secondary antibodies conjugated to rhodamine or fluorescein isothiocyanate. Coverslips were mounted on slides and viewed under a confocal microscope LSM510 (Carl Zeiss, Inc., Thornwood, NY).

Cell Migration Assay—VSMC migration assays were performed in a modified 48-well Boyden’s microchemotaxis chamber as described earlier (40). In some experiments cells were pretreated with indicated inhibitors, RAGE antibody, or control IgG for 1 h.

Preparation of Human Cav-1-specific shRNA Expression Vectors—The plasmids vectors expressing Cav-1 shRNA were constructed using methods described earlier (41, 42). Briefly, shRNAs under the control of U6 promoter were cloned into a vector, which also expresses enhanced green fluorescent protein from a CMV promoter to monitor transfection efficiency. The shRNA vectors targeting several regions of human Cav-1 were co-transfected with a Cav-1 expression vector into HEK293 cells and inhibition of Cav-1 protein levels monitored by Western blottting of the cell lysates. The two most efficient shRNA vectors were selected to transfect HVSMCs as described below. Sequences used for the shRNAs in the experiments were: Cav1–100, 5′-gacagtctgacagagaac-3′; and Cav1–383, 5′-ggcagttgacctgatcata-3′ corresponding to 100–120 and 383–403 bp of human Cav-1.

RNA Preparation and Relative RT-PCR—Total RNA from VSMCs was isolated using RNA-STAT60 reagent, and the mRNA levels of interleukin-6 (IL-6) and monocyte chemotactic protein-1 (MCP-1) determined by previously described methods (43). Briefly, relative RT-PCRs were performed with 0.5 μg of RNA using gene-specific primers and Quantum RNA 18 S internal standards. The PCR primer sequences have been described earlier (41, 42). PCR products were fractionated on 2.0% agarose gels and photographed using an Alphalmager 2000 Documentation and Analysis system (Alpha Innotech, San Leandro, CA). The densities of amplified products corresponding to specific genes and 18 S RNA were determined with Quantity One software (Bio-Rad). Results are expressed as fold stimulation over control after normalizing with the levels of internal standard (18 S RNA).

Nucleofection of VSMCs with SrcRF, Cav-1 Mutants, Cav-1 shRNAs, and Control Scrambled shRNA—HVSMCs were trypsinized and resuspended at 1 × 10⁶ cells/ml and 1 × 10⁶ cells were nucleofected with the indicated plasmids (3 μg each) using a nucleofection equipment as described by the manufacturer (Amaza, Inc., Gaithersburg, MD). The
cells were allowed to recover overnight and serum-depleted for 48 h prior to stimulation with S100B. Transfection efficiency was monitored by examining enhanced green fluorescent protein expression, and we were able to get ~50% transfection efficiency.

RESULTS

Activation of Src Kinase by S100B—Tyrosine kinase activity plays an important role in VSMC function, including migration. Receptors lacking tyrosine kinase activity utilize Src, a non-receptor tyrosine kinase. Because RAGE does not have intrinsic tyrosine kinase activity, we hypothesized that Src kinases might play an important role in RAGE signaling. To test this we first examined whether the RAGE-specific ligand S100B can activate Src kinase in VSMCs. Serum-depleted PVSMS were stimulated with S100B for 0–60 min, and the cell lysates were immunoprecipitated with a Src monoclonal antibody. Src immunoprecipitates were analyzed by immunoblotting with a polyclonal phospho-Src antibody that recognizes both phosphorylated and non-phosphorylated Src (total Src). Numbers above the lane represent time period (min) of S100B stimulation. Results showed that S100B treatment quickly induced Src activation (5 min), and this was sustained up to 60 min (Fig. 1A, upper panel). When the blot was stripped and probed with an antibody that recognizes both phosphorylated and non-phosphorylated Src (total Src), the immunoprecipitates contained equal amounts of Src protein in all the samples (Fig. 1A, lower panel). Quantitation of Src activation at 5 min from multiple experiments is shown in Fig. 1B. These results demonstrate for the first time that RAGE ligands can activate Src kinase in VSMCs.

Stimulation of Cav-1 Tyrosine Phosphorylation by S100B—We next examined the downstream effectors of Src kinase in S100B-stimulated VSMCs. Evidence shows that, in cells exposed to oxidant stress, Src phosphorylates Cav-1, the structural component of caveolae, on Tyr-14 (44). Because RAGE also induces oxidant stress, we tested whether the activation of Src kinase by S100B may lead to Tyr-14 phosphorylation of Cav-1. First, we examined whether RAGE co-localizes with Cav-1 in VSMCs by immunostaining with a monoclonal antibody and a polyclonal antibody. The slides were observed under a confocal microscope by taking several z-sections. As shown in Fig. 2A, both RAGE antibody as well as Cav-1 antibody staining could be seen on the cell surface, and a merge of the two images clearly showed that RAGE is co-localized with Cav-1 in VSMCs.

Having established the co-localization of RAGE and Cav-1, we next examined if Cav-1 is phosphorylated in S100B-stimulated VSMCs. Cell lysates prepared from PVSMS stimulated with S100B for 5–60 min were immunoprecipitated with a polyclonal Cav-1 antibody. Immunoprecipitates were subjected to immunoblotting with a monoclonal phospho-Cav-1 antibody that recognizes only Cav-1 protein phosphorylated at tyrosine phosphorylation site (Tyr-14). Results showed that S100B treatment rapidly induced Cav-1 phosphorylation (5 min), and this was sustained up to 60 min (Fig. 2B). The phosphorylation was apparent by 10 min, and this returned to basal levels by 60 min. Stripping and re-probing of this blot with Cav-1 antibody showed that total Cav-1 levels did not change under these conditions. Cell lysates from this experiment were also immunoprecipitated with Src antibody, and these Src immunoprecipitates were immunoblotted with a phospho-Src antibody. Results showed that the activation of Src parallels that of Cav-1 tyrosine phosphorylation (Fig. 2B). Fig. 2C shows the quantitation of Cav-1 tyrosine phosphorylation from multiple experiments. These results demonstrate for the first time that RAGE ligands can stimulate Cav-1 tyrosine phosphorylation in VSMCs.

RAGE Mediates S100B-induced Src Activation and Tyrosine Phosphorylation of Cav-1—Next we examined whether the S100B-induced activation of Src kinase and tyrosine phosphorylation of Cav-1 are dependent on RAGE signaling. Serum-depleted VSMCs were preincubated with either a polyclonal RAGE-specific antibody (70 µg/ml) or control normal rabbit IgG for 1 h. Immunoprecipitates of Src or Cav-1 antibodies from these cell lysates were immunoblotted with phospho-Src and phospho-Cav-1 antibodies, respectively. Results showed that the RAGE antibody could block S100B-stimulated Src activation (Fig.
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3A, upper panel) as well as tyrosine phosphorylation of Cav-1 (Fig. 3B, upper panel). The lower panels in both figures show that the immunoprecipitates contain equal amounts of Src or Cav-1. Fig. 3 (C and D) shows bar graph quantitation of the results expressed as fold-over control. Thus, these results clearly demonstrate that S100B acts via the RAGE receptor to induce Src activation and Cav-1 phosphorylation in VSMCs.

Involvement of Src Kinases in S100B-induced Oxidant Stress in VSMCs—Because activation of RAGE induces oxidant stress, we next examined whether Src kinases are involved in S100B-induced oxidant stress. PVSVMCs grown on coverslips were serum-depleted and left untreated or pretreated with a Src kinase inhibitor PP2 (10 μM) for 60 min. The cells were then stimulated with S100B for 30 min and stained with a cell-permeable fluorescent dye dihydroethidine to detect superoxide formation using a fluorescent microscope. The intensity of fluorescence in each field was quantitated using Image Pro software. As shown in Fig. 4, S100B was able to induce superoxide formation by 2-fold in 30 min (panel B) compared with control cells (panel A), and the Src kinase inhibitor PP2 blocked S100B-induced superoxide production (panels F versus B). The vehicle (MeSO) for PP2 did not have any effect (panels C and D). These results demonstrate the involvement of Src kinases in S100B-induced oxidant stress in VSMCs.

Requirement of Caveolae, Src Kinase, and Oxidant Stress in S100B-induced Cav-1 Tyrosine Phosphorylation—Next we examined whether the integrity of caveolae, Src activation, and oxidant stress were required for Cav-1 tyrosine phosphorylation. VSMCs were pretreated for 1 h with the Src inhibitor PP2 (10 μM), or anti-oxidant N-acetylcysteine (NAC, 10 mM), or β-cyclodextrin (10 mM), which is known to deplete cholesterol and disrupt the integrity of caveolae. The cells were subsequently stimulated with S100B for 10 min, and the Cav-1 immunoprecipitates were immunooblotted with the phospho-Cav-1 antibody (Fig. 5A). The intensities of phospho-Cav-1 bands were quantified, and the results expressed as fold stimulation over control “no inhibitor” (NI) cells (Fig. 5E). Results in Fig. 5 (A and E) show that S100B-treated Cav-1 Tyr phosphorylation was significantly inhibited by both anti-oxidant (NAC) as well as Src inhibitor (PP2) compared with NI control, whereas MeSO (DM in Fig. 5), the vehicle for PP2, had no effect. Pre-treatment with βCD increased the basal tyrosine phosphorylation of Cav-1, but S100B had no further effect. Fig. 5B shows that total Cav-1 levels were not altered by these treatments. Src activation was also examined in these cell lysates after immunoprecipitation with Src antibody. As shown in Fig. 5C, the anti-oxidant and PP2 blocked Src activation, whereas cholesterol depletion and MeSO (DM) had no effect. Fig. 5D shows that total Src levels were similar in all the samples. Fig. 5F shows the bar graph quantitation of Src activation expressed as fold stimulation. These results show that S100B-induced Cav-1 tyrosine phosphorylation was dependent on Src activation and that oxidant stress is involved in both Src activation as well as Cav-1 tyrosine phosphorylation. Results with βCD indicate that disruption of caveolae leads to
nonspecific phosphorylation of Cav-1 and that the integrity of caveolae may be required for ligand-induced RAGE-specific phosphorylation.

To further confirm the role of Src in Cav-1 tyrosine phosphorylation, VSMCs were grown on coverslips, serum-depleted, and stimulated with S100B for 10 min in the absence or presence of PP2 (10 μM). Then the cells were fixed and stained with either a polyclonal phospho-Src antibody or a monoclonal phospho-Cav-1 antibody and observed under a confocal microscope. As shown in Fig. 6, compared with control cells, S100B treatment dramatically increased immunostaining of both phospho-Src (panel B versus A) and phospho-Cav-1 (panel F versus E). In contrast, S100B failed to stimulate activation of Src (panels C versus D) in cells pretreated with PP2 and tyrosine phosphorylation of Cav-1 (panels G versus H), further demonstrating the involvement of Src in RAGE-induced tyrosine phosphorylation of Cav-1 in VSMCs. DAPI nuclear staining is shown in the lowest panels (panels I–L).

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VSMCs were grown on coverslips were serum-depleted and pretreated with vehicle MeSO (control (Ctrl)) or Src inhibitor PP2 (10 μM). Then the cells were either left untreated (A, E, C, and G) or stimulated with S100B (B, F, D, and H) for 10 min, fixed and stained with polyclonal phospho-(Tyr-416)-Src antibody (pSrc, panels A–D) and monoclonal phospho-(Tyr-14)-Cav-1 antibody (pCav-1, panels E–H). The fluorescence was visualized using a confocal microscope. DAPI indicates nuclear staining with DAPI (panels I–L).

Role of Src kinase in MAPK activation by S100B in VSMCs.

We then examined the effects of S100B on MAPK activation to identify the signals downstream of Src kinases. Serum-depleted PVSMCs were stimulated with S100B for the indicated time periods, and cell lysates were immunoblotted with phospho-specific p38 MAPK or ERK1/2 MAPK antibody. As shown in Fig. 7A (upper panel), S100B potently activated p38 MAPK in 5 min with the activity returning to basal levels by 60 min. This blot was stripped and probed with total p38MAPK antibody, and the results showed that equal amounts of protein were loaded in all the lanes (middle panel). The lowest panel of Fig. 7A shows that S100B also activated ERK1/2 by 5 min, and this was sustained up to 60 min, returning to basal levels by 2 h. Thus S100B was able to activate both p38 MAPK and ERK1/2 in VSMCs.

Next we examined the role of Src in S100B-induced MAPK activation. PVSMCs were pretreated with specific inhibitors of Src (PP2, 10 μM), PKC (GFX109203, 10 μM), and p38MAPK (SB202190, 1 μM) for 30 min and stimulated with or without S100B for 5 min. The cell lysates were analyzed to detect phospho-p38 MAPK and phospho-ERK1/2 levels. As shown in Fig. 7B, the Src kinase inhibitor PP2 significantly blocked activation of both p38 MAPK (Fig. 7B, upper panel) and ERK1/2 (Fig. 7B, lower panel) compared with NI control. The PKC inhibitor (GFX) also inhibited both p38 MAPK and ERK activation as shown by others (27). As expected p38MAPK inhibitor (SB) blocked p38MAPK activation and did not have any effect on ERK activation. Immunoblotting with total p38MAPK antibody showed that the levels of total p38MAPK were the same in these lysates confirming that equal amounts of proteins were loaded. Fig. 7C shows the bar graph quantitation of p38 and ERK MAPK activation expressed as fold stimulation. These results demonstrate that Src kinase is involved in MAPK activation by S100B in VSMCs.

Involvement of Src in S100B-induced NF-κB and STAT3 Activation—Transcription factors NF-κB and STAT3 play important roles in VSMCs function, including migration and inflammatory gene expression. Furthermore, they have been implicated in the actions of AGEs via RAGE. Hence, we examined the role of Src kinases and caveolar integrity in S100B-induced VSMC transcription factor activation. PVSMCs were pretreated with either PP2 (10 μM) for 30 min or with BCD (10 mM) for 1 h and stimulated with S100B for 10 min. Cell lysates were immunoblotted with phospho-specific antibodies that recognize IκB-α phosphorylated at Ser-32 and STAT3 phosphorylated at Tyr-705 to document NF-κB and STAT3 activation, respectively. Results showed that S100B potently stimulated phosphorylation of both IκB-α (Fig. 8A, upper panel) and STAT3 (Fig. 8A, lower panel) and the Src kinase inhibitor PP2 abolished activation of both the transcription factors. Cholesterol depletion by BCD increased the basal activation of both IκB and STAT3 and S100B had no further stimulatory effect indicating that integrity of caveolae is necessary for RAGE-specific activation. Fig. 8B shows bar graph quantitation of the data. These results demonstrate that both Src and intact caveolae are required for the RAGE-stimulated NF-κB and STAT3 activation.

Inhibition of p65 Nuclear Translocation by Src Inhibitor—NF-κB activation leads to nuclear translocation of its active subunit p65, which then binds to NF-κB elements on key gene promoters and mediates their trans-activation. To determine the role of Src in this process, VSMCs grown on coverslips were serum-depleted and stimulated with S100B for 30 min in the absence or presence of Src inhibitor PP2 (10 μM). Then the cells were fixed, immunostained with p65 antibody, and
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observed under a confocal microscope to detect nuclear translocation of p65. As shown in Fig. 8C, S100B treatment led to the nuclear translocation of p65 (panel S100B) compared with unstimulated cells (panel Control). In contrast, S100B failed to induce nuclear translocation of p65 in VSMCs pretreated with Src kinase inhibitor PP2 (panel PP2/S100B) further supporting the role of Src in S100B-induced downstream NF-κB activation. Merged images of p65 antibody and nuclear (DAPI) staining are shown in the lower panels.

Role of Src in S100B-induced Cytokine IL-6 and Chemokine MCP-1 Expression in VSMCs—To determine the functional significance of Src kinase-mediated NF-κB activation, we next examined whether Src kinase can modulate S100B-induced expression of two key inflammatory genes known to be regulated by NF-κB, namely IL-6 and MCP-1. These two genes play important roles in vascular inflammation and VSMC migration. RT-PCR analysis of RNA extracted from VSMCs stimulated with S100B for various time periods (0–24 h) showed that S100B could induce the expression of both MCP-1 as well as IL-6 mRNAs with a peak at 1 h and 6 h, respectively (data not shown). Next we examined the role of Src and p38MAPK in S100B-induced IL-6 and MCP-1 expression. As shown in Fig. 9, S100B-induced IL-6 (A) and MCP-1 (B) expressions in VSMCs were significantly inhibited by both 10 μM PP2 (PP2) and 1 μM SB202190 (SB) compared with cells without inhibitors (NI), indicating the involvement of Src kinase and its downstream effector p38MAPK. Fig. 9 (C and D) shows the bar graph quantitation of MCP-1 and IL-6 expression, respectively.

To verify the data obtained with the chemical inhibitor PP2, we tested the ability of a genetic inhibitor SrcRF to block S100B-induced gene expression. SrcRF is a dominant negative mutant form of Src kinase in which both the ATP binding site (Lys-295) and Csk (C-terminal Src kinase) phosphorylation site Tyr-527 are mutated to Arg (K295R) and Phe (Y527F), respectively. SrcRF has been shown to inhibit Src-induced VEGF expression by hypoxia (45). We introduced expression vector pCMVSrcRF into HVSMCs by nucleofection as described under “Experimental Procedures.” The nucleofected cells were allowed to recover overnight and serum-depleted for 48 h. They were then cultured with S100B, and IL-6 mRNA levels were determined by RT-PCR. As shown in Fig. 10 (A and B), SrcRF significantly blocked the S100B-induced IL-6 expression by 52% compared with cells nucleofected with empty vector (pCR). We also examined the expression of MCP-1 and noted that S100B-induced MCP-1 expression was also inhibited by 50% in cells nucleofected with SrcRF compared with cells nucleofected with empty vector (data not shown). These results demonstrate for the first
MCP-1 expression was also blocked by pCAV-scrambled shRNA (shScr) had no effect on IL-6 expression. S100B-induced inflammatory gene expression. Thus, these results demonstrate the role of Cav-1 in S100B-induced inflammatory gene expression in VSMCs.

Involvement of Cav-1 in S100B-induced Gene Expression—Next we examined the involvement of Cav-1 in S100B-induced inflammatory gene expression using Cav-1 mutants and shRNAs. To inhibit the function of Cav-1, we used a vector pCavASF, which expresses a Cav-1 mutant in which the scaffolding domain (amino acids 80–100) required for interaction with Src has been deleted (46). We developed new vectors to express human Cav-1 shRNA (shCav) to inhibit the Cav-1 expression. Co-transfection of these shRNA vectors along with a target human Cav-1 expression vector effectively inhibited Cav-1 expression in HEK293 cells confirming the efficacy of the shRNAs. A control vector expressing scrambled shRNA (shScr) had no effect on Cav-1 expression (data not shown). Nucleofection of HVSMCs with a mixture of equal amounts of shRNAs Cav1–100 and Cav1–383 inhibited Cav-1 protein expression (by Western blotting) by −50% compared with cells nucleofected with shScr (data not shown). HVSMCs were nucleofected with either shScr or pCavASF or a mixture (1:1) of shCav1–100 and shCav1–383 vectors and serum-depleted for 48 h. Then nucleofected cells were stimulated with S100B and IL-6 mRNA expression was determined by RT-PCR. Interestingly, as shown in Fig. 10 (A and B), S100B-induced IL-6 mRNA levels were significantly reduced by 50 and 70% in cells transfected with pCavASF (CavASF) and Cav1 shRNA (shCav), respectively, compared with empty vector (pCR). In contrast the scrambled shRNA (shScr) had no effect on IL-6 expression. S100B-induced MCP-1 expression was also blocked by pCAVASF in HVSMCs (data not shown). Thus, these results demonstrate the role of Cav-1 in S100B-induced inflammatory gene expression.

Involvement of Src Kinase in VSMC Migration Induced by the RAGE Ligand S100B—Next, we examined the functional role of Src kinase activation in VSMC migration induced by S100B. Confluent VSMCs were serum-starved for 24 h, and migration assays were performed using a 48-well-modified Boyden’s microchemotaxis chamber apparatus. Cells were placed in the upper wells of the chamber, and S100B (10 μg/ml) was added to the lower wells of the chamber. The number of cells that migrated to the lower side of the filter was counted after 4 h of incubation, and results are expressed as -fold over control cells. In some experiments cells were pretreated with Src kinase inhibitor PP2 (10 μM). PP2 significantly inhibited the VSMC migration induced by S100B (, p < 0.001 versus control, n = 4; **, p < 0.0001 versus S100B, n = 4).

Enhanced RAGE Levels, Src Activation, and Migration in VSMCs from db/db Mice—To examine the in vivo relevance of Src kinase activation by RAGE ligands, we isolated VSMCs from db/db mice and their genetic control db/+ mice. The db/db mice with a mutation in the leptin receptor are obese and diabetic and have hallmarks of insulin resistance (47). Since diabetic animals are known to have elevated levels of AGEs and RAGE expression (16), we hypothesized that VSMCs in these animals may be in a pre-activated state. Recent studies showed that macrophages from db/db mice are hyper-responsive to lipopolysaccharide stimulation (48). We examined RAGE expression, Src activation, and migration in VSMCs derived from db/db mice and control db/+ mice. Results showed that RAGE expression was significantly increased in VSMCs from db/db mice compared with those from control db/+ mice (Fig. 12, A and B). Furthermore, immunoblotting of lysates from these cells showed that phospho-Src levels were also significantly greater in db/db VSMCs compared with db/+ VSMCs (Fig. 12, C and D). Finally, migration assay revealed that VSMCs from db/db mice displayed 3-fold increase in migration in the basal state compared with those from db/+ mice, and this was blocked by pretreatment with the RAGE antibody and not control IgG (Fig. 12E). Thus, these new results demonstrate that increased RAGE expression and elevated Src activity levels may contribute to VSMC dysfunction in diabetes.

DISCUSSION

In the present study, we showed for the first time that the treatment of VSMCs by S100B, a member of the calgranulin family of polypeptides and
also RAGE ligand, activates Src kinase in a RAGE-dependent manner, and this is associated with increased VSMC migration and inflammatory gene expression. Furthermore, we have also shown that caveolae and Cav-1 play important roles in these events. A RAGE antibody could block S100B-induced Src kinase activation and Cav-1 tyrosine phosphorylation.

Our study identifies RAGE as another receptor without intrinsic tyrosine kinase activity that uses Src tyrosine kinase for downstream signaling. Upon activation, Src interacts with several cellular proteins through its Src homology domains, SH-2 and SH-3, to transmit downstream signals (49). Our results show that some of the downstream targets of Src in S100B and RAGE signaling in VSMCs include Cav-1, MAPKs (p38MAPK and ERK1/2), and transcription factors NF-κB and STAT3. On the other hand the JAK inhibitor AG-490 had no effect on the activation of p38 and p42/44 MAPKs (data not shown), suggesting Src may be the upstream mediator of Ras-MAPK pathway under these conditions. A PKC inhibitor could also block S100B-induced MAPK activation, suggesting that both Src and PKC are involved in these events.

Interestingly, the Src inhibitor also blocked S100B-induced STAT3 activation. STAT3 was shown to be activated by JAK kinases in VSMCs stimulated by growth factors, cytokines, and RAGE ligands (50). However, Src kinases can also directly activate STAT3 in various cell types stimulated by growth factors (51). In VSMCs, in addition to JAK, the Src family member Fyn was shown to be involved in angiostatin IL-stimulated STAT3 activation (52). Thus, Src kinase may directly activate STAT3 or via JAK2 in VSMCs treated with S100B. Our results suggest that Src activation may be another mechanism through which RAGE can activate STAT3.

Our data also indicate that Src is involved in S100B-induced oxidant stress and NF-κB activation. A general antioxidant NAC also blocked Src activation, suggesting that RAGE-induced oxidant stress is required for Src activation, which in turn can further induce oxidant stress. Recent studies have implicated a key role for Src in NF-κB activation by oxidant stress and inflammatory signals. Src activated NF-κB by both IκB kinase-dependent and -independent mechanisms (53–55). Further studies are required to verify the specific mechanisms by which Src regulates RAGE-induced NF-κB activation.

Inflammatory gene expression and inflammation play important roles in the development of atherosclerosis. Accumulation of RAGE ligands has been shown to amplify inflammation in diabetic animal models (16). We demonstrated the involvement of Src in S100B-induced inflammatory gene expression. Furthermore, a p38 MAPK inhibitor also blocked S100B-induced IL-6 and MCP-1 expression. Because p38 MAPK is downstream of Src kinase, Src may mediate inflammatory gene expression via p38 MAPK. Furthermore, because both IL-6 and MCP-1 are regulated by NF-κB, and the Src inhibitor blocked both NF-κB activation and downstream gene expression, our results suggest that Src mediates S100B-induced inflammatory gene expression via NF-κB.

VSMC migration is another key event in the development of atherosclerosis. Src kinase has been implicated in growth factor-stimulated VSMC migration, and our current results demonstrate for the first time that it is also required for the VSMC migration stimulated by RAGE ligands. In growth factor-stimulated cells Src activates focal adhesion kinase leading to cytoskeletal rearrangements required for cell migration (28). Further studies are required to determine if similar mechanisms are involved in VSMC migration induced by RAGE ligands.

We observed that the integrity of caveolae and its structural protein Cav-1 are required for RAGE signaling. Furthermore, Cav-1 was tyrosine-phosphorylated in a RAGE- and Src-dependent manner suggesting that tyrosine phosphorylation may play an important role in Cav-1 function under these conditions. Because Cav-1 also acts as a scaffold to hold signaling molecules in caveolae, it may serve to prevent inappropriate cell activation and also help in assembling the signaling complexes (56). Activation by specific ligands can then mobilize the signaling components, including Cav-1, into various subcellular locations (57). Thus, S100B- and RAGE-mediated tyrosine phosphorylation may be required for Cav-1 translocation to the cytoskeleton and also assist in translocating other signaling molecules involved in cytoskeleton rearrangements required for VSMC migration and gene expression.

Additionally, we have demonstrated that RAGE expression, Src activity levels, and migration are greater in VSMCs derived from db/db mice, a model of type 2 diabetes, obesity, and insulin resistance. Evidence shows that these animals are at a higher risk of developing cardiovascular disorders due to the hyperactivated state of their vascular cells (48, 58). Our studies now identify Src kinase as a potential mediator of VSMC dysfunction in these mice. Elevated levels of RAGE and its ligands may be responsible for these changes in the VSMCs, because a RAGE antibody could attenuate the enhanced migratory responses of the db/db VSMCs.

In conclusion, our results clearly demonstrate that Src kinase and caveolae play important roles in downstream signaling, inflammatory gene expression, and VSMC migration induced by RAGE ligands. These events can induce VSMC dysfunction and thereby enhance the risk of cardiovascular complications under diabetic conditions. Taken together, these results increase our understanding of the molecular mechanisms of RAGE signaling in vascular cells and could lead to the identification of novel therapeutic targets for diabetic vascular complications.

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