S100B-RAGE–Mediated Augmentation of Angiotensin II–Induced Activation of JAK2 in Vascular Smooth Muscle Cells Is Dependent on PLD2

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Angiotensin II (Ang II), a vasoactive peptide that is also considered a growth factor, has been implicated in both normal and diabetic cellular proliferation. We recently found that activation of janus kinase 2 (JAK2) is essential for the Ang II–induced proliferation of vascular smooth muscle cells (VSMCs) and that high glucose augments Ang II–induced proliferation of VSMCs by increasing signal transduction through activation of JAK2. Here, we demonstrate that S100B, a ligand for the receptor of advanced glycation end products (RAGEs), augmented both Ang II–induced tyrosine phosphorylation of JAK2 and cell proliferation in VSMCs in a receptor-dependent manner. We also found that S100B-RAGE interaction triggered intracellular generation of reactive oxygen species (ROS), VSMC proliferation, and JAK2 tyrosine phosphorylation via activation of phospholipase D (PLD)2. These results provide direct evidence for linkages between PLD2, ROS production, and S100B-RAGE–induced enhancement of Ang II–induced cell proliferation and activation of JAK2 in VSMCs. *Diabetes* 52:2381–2388, 2003

Nonenzymatic glycoxidation, which ultimately leads to the formation of advanced glycation end products (AGEs), occurs when free amino groups are exposed to aldoses, such as high levels of glucose, and under conditions of oxidant stress, both of which occur in diabetes (1). AGE-modified macromolecules are found in plasma, cells, and tissues and accumulate in vessel walls and the kidney.

The receptor for AGE (RAGE) is a member of the immunoglobulin superfamily of cell-surface molecules (1). As the biology of RAGE has evolved, several common themes have emerged. First, the multiligand character of the receptor is quite remarkable. Ligands of the receptor include AGEs, crossed-sheet fibrils characteristic of amyloid, amphoterin, and S100/calgranulins (1). Even within a particular ligand family, RAGE recognizes more than one species. For example, the S100/calgranulins comprise a family of >15 polypeptides. RAGE interacts with an S100a species and S100B (two rather divergent family members), raising the likely possibility that this receptor may well interact with multiple, and maybe even all, S100/calgranulins. A second salient feature of RAGE biology is the presence of more than one ligand in tissues for prolonged times (1). For example, in diabetic tissues, both AGEs and S100/calgranulins are present at increased levels in many cases. Another unusual feature of the receptor is its apparent colocalization to sites where its ligands tend to accumulate (1). Thus, where AGEs and S100/calgranulins accumulate at sites of vascular lesions, higher levels of RAGE are also identified in cellular elements. This observation raises the possibility that the presence of ligands upregulates expression of the receptor, potentially resulting in exaggerated RAGE-mediated cellular activation. This has been demonstrated directly with S100B and appears to be true with AGEs, amphoterin, and amyloids.

The broad consequences of RAGE-ligand interaction for cellular properties are emphasized by the spectrum of signaling mechanisms, which the receptor triggers following ligand occupancy. One such consequence of AGE-RAGE interaction is the generation of reactive oxygen species (ROS) such as H_2O_2 and O_2^- (2,3). The latter are well-known triggers of mitogenic pathways, such as the JAK/signal transducers and activators of transcription (STAT) signaling cascade (4). Several recent data suggested a link between the cellular milieu in diabetes, RAGE-mediated activation of JAK2 and NADPH, and elevated diacylglycerol (DAG)/activation of protein kinase C (PKC). RAGE-mediated activation of NADPH oxidase has been demonstrated previously (3), and another study has shown that binding of AGEs to RAGE activates JAK2 (5). In addition, a very recent study (6) has demonstrated that PKC is essential for activation of NADPH oxidase. The activation of PKC by elevated glucose levels and diabetes has been shown to be related to increases in DAG levels (7). DAG can be generated from multiple pathways, but in diabetes most of the de novo synthesis of DAG is done via the polyol pathway (7). However, it has recently been found that DAG can also be derived from the metabolism

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Received for publication 30 January 2003 and accepted in revised form 9 June 2003.

AGE, advanced glycation end product; Ang II, angiotensin II; AT_1, Ang II type 1; DAG, diacylglycerol; DCF, 2,7-dichlorofluorescein; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal–related kinase; JAK2, janus kinase 2; pJAK2, phosphotyrosine-specific JAK2; PKC, protein kinase C; PLD, phospholipase D; RAGE, receptor of AGE; ROS, reactive oxygen species; STAT, signal transducers and activators of transcription; TTBS, Tris buffered solution with 0.05% Tween-20; VSMC, vascular smooth muscle cell.

of phosphatidylcholine due to the activation of phospholipase D (PLD) (7).

In this study we show that incubating VSMCs with the RAGE receptor ligand S100B (3) induces generation of H_2O_2 and tyrosine phosphorylation of JAK2. Furthermore, we also found that preincubation of cultures with S100B augments the Ang II–induced tyrosine phosphorylation of JAK2 and this augmentation is dependent on PLD2 and NADPH oxidase. Therefore, we hypothesize that AGEs, generated by high glucose during diabetes, augment the Ang II–induced activation of JAK2 via the action of H_2O_2 generated by NADPH oxidase, the latter of which is activated by PLD2.

RESEARCH DESIGN AND METHODS

Materials. Molecular weight standards, acrylamide, SDS, *N*-*N*′-methylenebisacrylamide, *N*,*N*,*N*′,*N*′-tetramethylenediamine, protein assay reagents, and nitrocellulose membranes were purchased from Bio-Rad Laboratories. Bovine catalase was obtained from Boehringer-Mannheim, and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) was from Molecular Probes. Protein A/G-Agarose was obtained from Santa Cruz Biotechnology, whereas Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, trypsin, and all medium additives were obtained from Mediatech. Anti-JAK2 and anti-p47phox antibodies were procured from Transduction Laboratories. Anti-phosphotyrosine JAK2 and anti-PLD2 antibodies were obtained from Biosource. Polyclonal monospecific anti-RAGE IgG was prepared as described (1,4). The Pierce Supersignal substrate chemiluminescence detection kit was obtained from Pierce. Goat anti-mouse IgG and anti-rabbit IgG were acquired from Amersham, and Tween-20 and all other chemicals were purchased from Sigma Chemicals.

Preparation of rat aorta VSMCs. Rat aortic smooth muscle cells were harvested and maintained in DMEM supplemented with 10% (vol/vol) fetal bovine serum, 100 μg/ml streptomycin, and 100 U/ml penicillin at 37°C in a 5% CO_2-enriched, humidified atmosphere as previously described (8,9). Cells from passages 5 and 6 were routinely subcultured 1:5 at 7-day intervals, and medium was changed at 2- to 3-day intervals.

Immunoprecipitation and Western blotting. Cells were stimulated with Ang II for the time periods assigned. The immunoprecipitation and Western blotting were performed as previously described (10–12). We used anti-PLD2 (4 μg/ml) to immunoprecipitate PLD2, and the recovered immunoprecipitated proteins were transferred to nitrocellulose membrane and blotted with anti-PLD2 (4 μg/ml) antibodies. To ascertain the tyrosine phosphorylation of JAK2, serum-starved VSMCs were stimulated with 100 nmol/l Ang II for various times ranging from 0 to 10 min. At the end of stimulation, cells were washed twice with ice-cold PBS-V (PBS with 1 mmol/l Na_3VO_4). Each dish was then treated for 60 min with ice-cold lysis buffer (20 mmol/l Tris-HCl, pH 7.4, 2.5 mmol/l EDTA, 1% Triton X-100, 10% glycerol, 1% deoxycholate, 0.1% SDS, 10 mmol/l $Na_4P_2O_7$, 50 mmol/l NaF, 1 mmol/l Na_3VO_4, and 1 mmol/l phenylmethylsulfonyl fluoride), and the supernatant fraction was obtained as cell lysate by centrifugation at 58,000*g* for 25 min at 4°C. Protein concentration of the cell lysate was measured with the Bio-Rad detergent-compatible assay kit and bovine serum albumin as standard.

Samples were resolved by 10% SDS-PAGE gel electrophoresis, transferred to a nitrocellulose membrane, and blocked by 60-min incubation at room temperature (22°C) in TTBS (Tris buffered solution with 0.05% Tween-20, pH 7.4) plus 5% skim milk powder. The nitrocellulose membrane was incubated overnight at 4°C with affinity-purified anti-phosphospecific JAK2 antibodies or nonphospho anti-JAK2 antibodies. Then, the nitrocellulose membranes were washed twice for 10 min each with TTBS and incubated for various times with goat anti-rabbit IgG horseradish peroxidase conjugate. After extensive washing, the bound antibody was visualized on Kodak Biomax film with a Pierce Supersignal substrate chemiluminescence detection kit. Molecular weight markers assessed specificity of the bands.

Electroporation procedure. Cells were plated in 100-mm cell plates and growth arrested in serum-deprived DMEM for 24 h before experiments. VSMCs were electroporated using a multi-coaxial electrode (Model P/N 747; BTX, San Diego, CA), which was performed in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution containing either anti-p47 phox antibodies at a final concentration of 10 μg/ml or catalase (8 mg/ml), as previously described (8,10,13). Following electroporation, cells were incubated for an additional 30 min at 37°C, washed once with serum-free DMEM, and left in serum-free DMEM before experiments.

PLD2 assay. The assay for PLD2 activity in intact VSMCs was performed as described by Ushio-Fukai et al. (14). Briefly, the PLD2 sense or antisense oligonucleotide-transfected VSMCs were grown in 100-mm dishes and labeled for 24 h with 1 μCi of [^{3}H]choline chloride in 5 ml culture medium. Afterward, the VSMCs were washed and incubated at 37°C for 20 min in the following buffer: 5.5 mmol/l glucose, 130 mmol/l NaCl, 5 mmol/l KCl, 1 mmol/l $MgCl_2$, 1.5 mmol/l $CaCl_2$, and 20 mmol/l HEPES (buffered to pH 7.4 with Tris base). The incubation buffer was replaced with 5 ml buffer with or without 5 μg/ml S100B for the times indicated. The buffer was then removed and combined with a chloroform:methanol (1:2) cellular extract for determination of total phosphatidylcholine metabolite accumulation. The aqueous phase was further processed for separation of choline and phosphocholine using tetraphenylboron in heptanone. Radioactivity was then quantified by liquid scintillation spectroscopy.

Assay of intracellular ROS. Intracellular ROS production was measured by the method of Ushio-Fukai et al. (15) with some modifications. Briefly, dishes of confluent cells at various times after stimulation with S100B, Ang II, or both were washed with modified Eagle's medium without phenol red and incubated in the dark for 5 min in Krebs-Ringer solution containing 5 mmol/l DCFH-DA. DCFH-DA is a nonpolar compound that readily diffuses into cells, where it is hydrolyzed to the nonfluorescent polar derivative DCFH and thereby trapped within the cells (15). In the presence of a proper oxidant, DCFH is oxidized to the highly fluorescent adduct 2,7-dichlorofluorescein (DCF). Culture dishes were transferred to a Zeiss inverted microscope and equipped with an X20 Neofluor objective and Zeiss LSM 410 confocal attachment, and ROS generation was detected as a result of the oxidation of DCFH (excitation, 488 nm; emission, 515–540 nm). The effects of DCFH photo-oxidation were minimized by collecting fluorescent images with a single rapid scan (line average, 4; total scan time, 4.33 s) and identical parameters, such as contrast and brightness, for all samples. The cells were then imaged by differential-interference contrast microscopy. Five groups of 20–30 cells each were randomly selected from the image in the digital interference contrast channel for each sample, the fluorescence intensity was then measured for each group from the fluorescence image, and the relative fluorescence intensity was taken as the average of the five values. Therefore, the relative fluorescence intensity (given in arbitrary units) reflects measurements performed on a minimum of 100 cells for each sample. All experiments were repeated at least six times.

PLD2 antisense oligonucleotide treatment. PLD2 antisense oligonucleotide synthesis and treatments were carried out as previously described (16,17). After 12 h, the medium was removed, calf serum (0.1%)/DMEM in normal glucose was added, and the cells were allowed to recover for 30 min. Afterward, the VSMCs were washed once with serum-free DMEM and growth arrested in serum-free DMEM for 24 h in either S100B (5 μg/ml) or vehicle.

Cell proliferation assay and Coulter counting. VSMC proliferation was measured using the Cell Titer 96 AQ_{ueous} nonradioactive cell proliferation assay (Promega, Madison, WI) (18). This assay is based on the cellular conversion of the colorimetric reagent, MTS 3,4-[5-demethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium salt, into soluble fomazan by dehydrogenase enzymes found only in metabolically active proliferating cells. MTS in Dulbecco's PBS (pH 6.0) is mixed with the electron-coupling reagent, phenazine methosulfate. The absorbance of fomazan, measured at 490 nm using a 96-well enzyme-linked immunosorbent assay plate reader interfaced with a personal computer, is directly proportional to the number of living cells in culture. To confirm the accuracy of our MTS proliferation assay, the actual increase in cell number was also directly assessed with a Coulter counter (Model ZM; Coulter, Hialeah, FL). The cells were grown in a 75-mm^2 flask to confluence and detached with trypsin-EDTA (0.05% trypsin and 0.53 mol/l EDTA). Twenty thousand cells were plated in 96-well plates and allowed to settle for 4 h in DMEM supplemented with 10% fetal bovine serum. Before the experiments, cells were growth arrested in serum-deprived DMEM for 24 h and then stimulated with the various ligands. After timed ligand exposure, the PMS/MTS mix was added to each well (final volume of 20 μl/100 μl medium) and then incubated for an additional 60 min. An SDS solution (10%) was then added to stop the reaction, and the absorbance of formazan was measured at 490 nm.

Data analysis. The data were evaluated using ANOVA with Fisher's projected least significant difference (PLSD) post hoc test to compare significant differences between individual means. Densitometry was performed using a digital imaging system (Alpha Innotech, Staffordshire, U.K.) and analyzed with ANOVA. Data are the means ± SE for the number indicated, and $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Effects of S100B on Ang II–induced activation of the JAK/STAT pathway and VSMC proliferation. We have

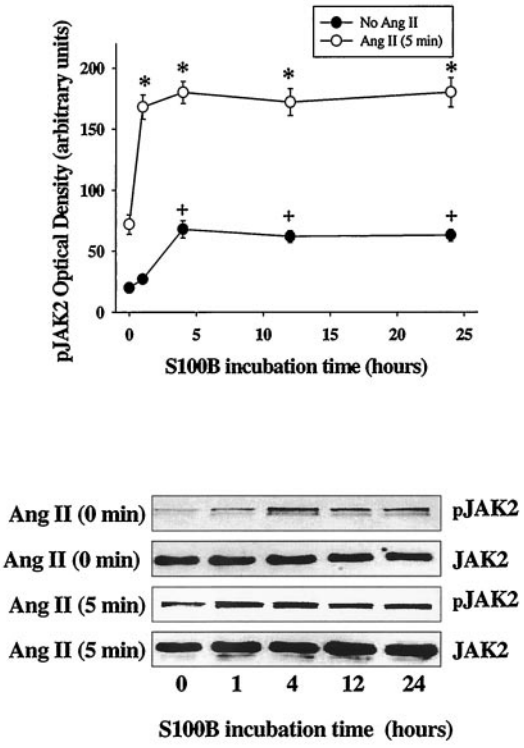

FIG. 1. Activation of JAK2 by Ang II in VSMC incubated for various periods of time (hours) with S100B. Quiescent VSMCs were treated with 100 nmol/l Ang II for 5 min in serum-free medium exposed for various periods of time to 5 μg/ml of S100B; 0 min indicates cells not exposed to Ang II. In each case, cells were lysed, and lysates were immunoblotted with either phosphotyrosine-specific or non–phospho-specific anti-JAK2 antibodies. Exposure of VSMCs to S100B induced a significant ($+P < 0.05$) tyrosine phosphorylation of JAK2 within 4 h, which was still maintained at 24 h. In addition, preincubating the VSMCs with 5 μg/ml of S100B from 4 to 24 h significantly ($*P < 0.05$) augmented the Ang II–induced JAK2 tyrosine phosphorylation. Representative immunoblots and densitometric analysis of three immunoblots probed with either the JAK2 phosphotyrosine-specific antibody (pJAK2) or JAK2 antibody (JAK2) are shown.

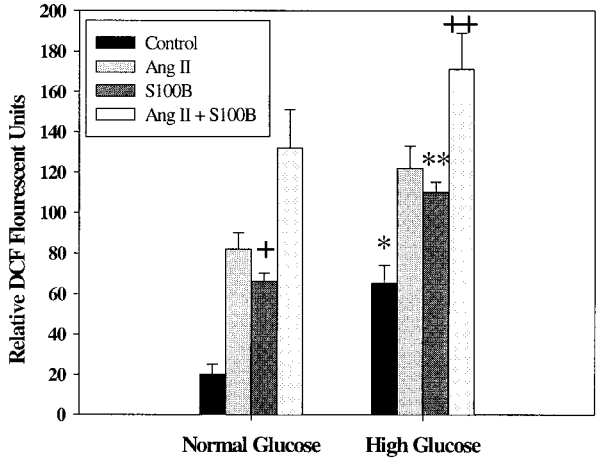

FIG. 2. Effects of high glucose and S100B on Ang II–induced H_2O_2 production. VSMCs were preincubated for 24 h with or without 5 μg/ml of S100B in normal (5.5 mmol/l) or high (25 mmol/l) glucose and then treated with Ang II (100 nmol/l, 4 h) in cells incubated with DCF-DA (5 μmol/l), an H_2O_2-sensitive dye that is incorporated into the cell. We found that both high glucose ($*P < 0.01$) alone or S100B ($+P < 0.01$) in normal glucose caused a significant increase in H_2O_2 production when compared with the normal glucose control cells. In VSMCs exposed to high glucose and S100B, significant differences ($**P < 0.01$) in H_2O_2 production were observed compared with cells treated with S100B or high glucose alone. Moreover, Ang II in normal glucose caused a significant ($++P < 0.01$) two- to threefold increase in H_2O_2 production, and this increase in H_2O_2 production was augmented when the VSMCs were preincubated with either high glucose or S100B. Data are means ± SE of six independent cultures.

previously shown that activation of JAK2 is required for Ang II–mediated induction of VSMC proliferation and that high glucose augments both Ang II–induced activation of JAK2 and VSMC proliferation (10,19). Since formation of AGEs is accelerated by hyperglycemia and oxidant stress, as in diabetes (20,21), we examined the effects of RAGE activation on JAK2 signaling by using the well-characterized ligand S100B. Exposure of VSMCs to S100B induced a significant tyrosine phosphorylation of JAK2 within 4 h, which was still maintained at 24 h (Fig. 1). Furthermore, we also found that preincubating VSMCs with S100B from 4 to 24 h significantly augmented the Ang II–induced JAK2 tyrosine phosphorylation (Fig. 1). These studies suggest that activation of RAGE, in a fashion similar to high glucose, augments the Ang II–induced activation of JAK2. VSMC lysates were also immunoblotted with anti-JAK2 antibodies that recognize both phosphorylated and non-phosphorylated forms of JAK2 (Fig. 1). Equal amounts of JAK2 were detected in all conditions tested, indicating that differences detected with the phosphotyrosine-specific antibodies (pJAK2) were not due to differences in the amount of total JAK2 loaded in each lane.

Effects of S100B on basal and Ang II–induced production of ROS in VSMC. S100B, high glucose, and Ang II induce the formation of intracellular ROS, such as H_2O_2, (3,15,22), and ROS stimulate intracellular signaling events similar to those triggered by Ang II, including activation of growth-promoting kinases, such as JAK2, pp60c-src, and extracellular signal–related kinase (ERK)1/2 (23,24). Therefore, to determine whether S100B augments Ang II–induced intracellular H_2O_2 production, VSMCs were preincubated for 24 h with or without S100B in normal (5.5 mmol/l, control) and high (25 mmol/l) glucose and then treated with Ang II (0.1 μmol/l for 4 h). Cultures were also incubated with DCFH-DA, an H_2O_2-sensitive dye incorporated into cells. Either high glucose or S100B in normal glucose caused a significant increase in H_2O_2 production when compared with control cultures in normal glucose (Fig. 2). VSMCs exposed to both high glucose and S100B show a significant potentiation in H_2O_2 production when compared with VSMCs treated with S100B or high glucose alone (Fig. 2). Moreover, Ang II in normal glucose caused a two- to threefold increase in H_2O_2 production, and this increase in H_2O_2 production was further augmented when VSMCs were preincubated with high glucose and S100B (Fig. 2). To confirm the nature of the ROS under study, catalase was introduced intracellularly by electroporation. In the presence of catalase, there was no increase in the fluorescence of DCF in the presence of S100B, high glucose, or Ang II (data not shown).

Effects of electroporation of the NADPH oxidase neutralizing antibody, anti-p47phox, on basal and Ang II–induced tyrosine phosphorylation of JAK2 in VSMCs preincubated in S100B. A number of recent studies (15,25) have suggested that most of the H_2O_2 produced by Ang II in cells comes from the NADPH oxidase system, and anti-p47 phox antibody has been shown to neutralize the NADPH oxidase system (25). In this study we found that electroporation of VSMCs with

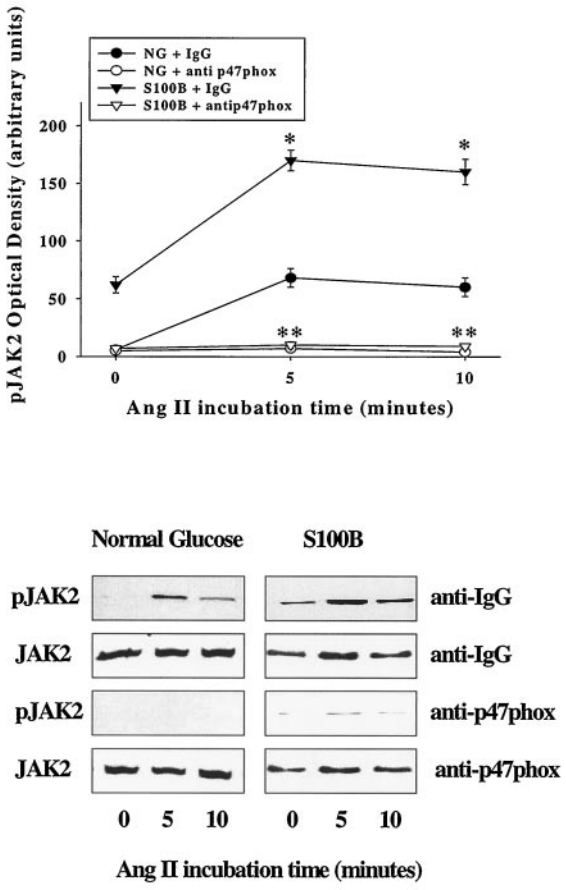

FIG. 3. Effects of electroporating rabbit IgG or anti-p47phox antibodies on S100B-induced augmentation of Ang II–induced JAK2 tyrosine phosphorylation. VSMCs were electroporated as described in RESEARCH DESIGN AND METHODS. Quiescent VSMCs in serum-free medium were then exposed for 24 h to S100B (5 μg/ml) or vehicle (control) and treated with Ang II (100 nmol/l) for 5 and 10 min. Cells were lysed, and lysates were immunoblotted with either phosphotyrosine-specific or non–phospho-specific anti-JAK2 antibodies. Electroporation of VSMCs with anti-p47 phox antibodies significantly ($P < 0.05$) suppressed JAK2 tyrosine phosphorylation in all groups tested, whereas electroporation of a control rabbit IgG had no significant (*$P < 0.05$) effect. Representative immunoblots and densitometric analysis of three immunoblots probed with either the JAK2 phosphotyrosine-specific antibody (pJAK2) or JAK2 antibody (JAK2) are shown.**

anti-p47 phox antibodies significantly suppressed the tyrosine phosphorylation of JAK2 in all groups tested, whereas electroporation of a control rabbit IgG had no effect (Fig. 3). These results suggest that NADPH oxidase is the ROS-generating system responsible for most of the H_2O_2 produced after experimental treatments with Ang II in VSMCs cultured in the presence or absence of S100B. Again lysates were immunoblotted with an anti-JAK2 antibody that recognizes both phosphorylated and nonphosphorylated forms of JAK2. Equal amounts of JAK2 were detected for all the conditions tested (Fig. 3), indicating that the differences detected with the phosphotyrosine-specific antibodies (pJAK2) were not due to differences in the amount of total JAK2 loaded in each lane. Finally, both the Ang II–stimulated increases in H_2O_2 and Ang II–induced JAK2 tyrosine phosphorylation in all the experiments tested were inhibited when the cells were preincubated with candesartan, a specific Ang II type 1 (AT_1)-receptor blocker (26). This indicates that both the induction of H_2O_2 and the tyrosine phosphorylation of JAK2 by Ang II were specific for AT_1-receptor activation (data not shown).

Effects of anti-RAGE IgG on JAK2 activation in VSMCs. AGE formation occurs during normal aging and at an accelerated rate in diabetes, in which accumulation of AGEs in the plasma and vessel wall has been speculated to contribute to the pathogenesis of secondary complications, especially those in the vasculature (2,21,27,28). One of the principal ways in which AGEs impact cellular elements is through interaction with cellular binding proteins. Although there are several possible cell-associated polypeptides to which AGEs might bind, recent studies (29,30) have demonstrated that the RAGE is a key site for the interaction of such nonenzymatically glycated adducts, especially in terms of triggering subsequent changes in cellular properties. For example, AGE-stimulated mononuclear phagocyte migration and activation, endothelial expression of vascular cell adhesion molecule-1, and increased monolayer permeability were prevented by blocking the interaction of AGE-modified proteins with RAGE (29,30). Consistent with the latter data in cell culture, in vivo studies have also shown RAGE to mediate the early and rapid removal of AGEs from the intravascular space, AGE induction of vascular oxidant stress, and vascular hyperpermeability in diabetic animals and rodents infused with AGE (29–31). These results highlight a potential role for RAGE activation in pathologic states such as diabetes and have led us to assess the effect of RAGE blockade in activation of signal transduction mechanisms in VSMCs. For these studies, we employed the RAGE ligand S100B because it is well characterized compared with the inevitably more heterogeneous mixture of biologically active AGEs (1). Cultured VSMCs express RAGE (29). Preincubation of aortic VSMCs with neutralizing anti-RAGE IgG (70 μg/ml) (3,29) blocked S100B-induced enhancement of JAK2 tyrosine phosphorylation subsequent to exposure to Ang II (Fig. 4). By contrast, preincubation of cultures with nonimmune IgG (100 μg/ml) had no effect (Fig. 4).

In this study we also wanted to examine the effects of high glucose on the S100B augmentation of Ang II–induced tyrosine phosphorylation of JAK2. We recently found (10,19) that high glucose augmented the Ang II induction of VSMC proliferation by increasing signal transduction through the activation of JAK2, and current studies (15,23,24) have also shown that high glucose stimulates intracellular signal events similar to those activated by Ang II, including stimulation of growth-promoting kinases such as JAK2 and ERK1/2. In addition, the formation of AGEs is considered a potential link between high glucose and chronic diabetes complications, including disturbances in cell signaling. Furthermore, a recent study (32) has also suggested that high glucose is capable of modulating RAGE-mediated effects, such as increasing the expression of several AGE-binding proteins. In this study we found that the combination of both high glucose and S100B caused a significant increase in the tyrosine phosphorylation of JAK2 (Fig. 4). On the other hand, preincubation with the anti-RAGE IgG had no effect on the high glucose augmentation (Fig. 4). These results suggest that the high glucose augmentation of the Ang II–induced JAK2 activation is not dependent on RAGE, but rather that both high glucose and the RAGE augment the Ang II–induced

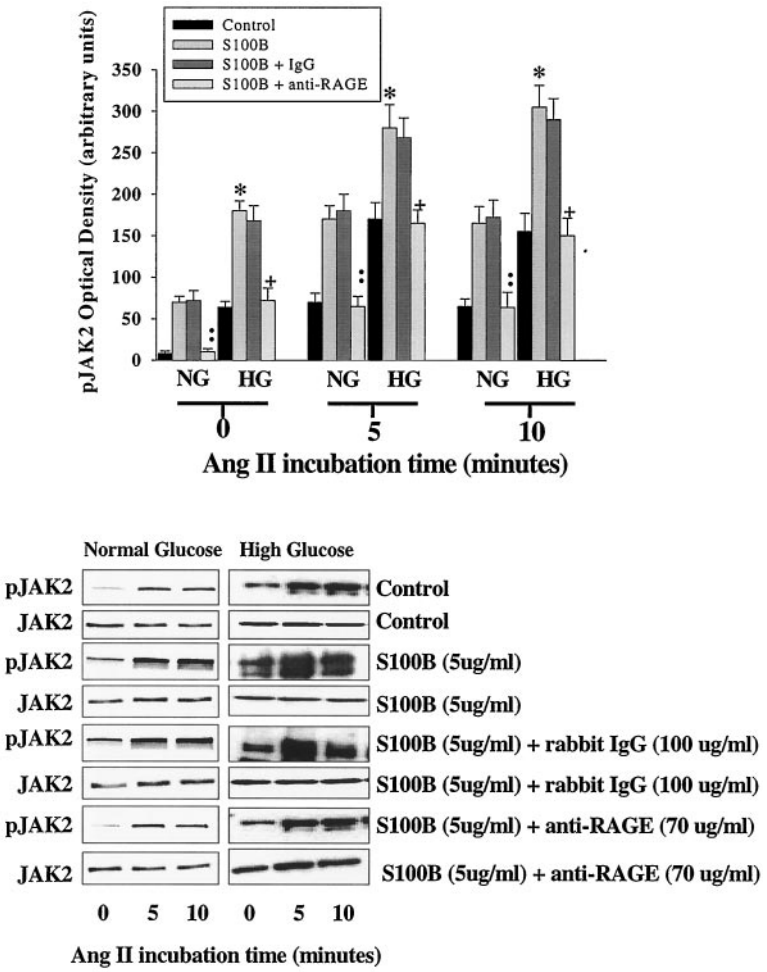

FIG. 4. Effects of anti-RAGE IgG on S100B augmentation of Ang II–induced tyrosine phosphorylation of JAK2. VSMCs in either normal or high glucose media were preincubated with the rabbit IgG anti-RAGE IgG (70 μg/ml) or nonimmune rabbit IgG (100 μg/ml) as previously described (3,29). Quiescent VSMCs in serum-free medium were then treated as described in Fig. 3. Preincubation of VSMCs with neutralizing anti-RAGE IgG (70 μg/ml [3,29]) significantly (:$P < 0.05$) blocked S100B-induced enhancement of JAK2 tyrosine phosphorylation subsequent to exposure to Ang II. By contrast, preincubation of cultures with nonimmune IgG (100 μg/ml) had no effect. S100B in high glucose caused a significant (*$P < 0.05$) increase in the tyrosine phosphorylation of JAK2 when compared with S100B or high glucose alone. Preincubation with the anti-RAGE IgG had no significant (+$P > 0.05$) effect on the high glucose augmentation. Representative immunoblots and densitometric analysis of three immunoblots probed with either the JAK2 phosphotyrosine-specific antibody (pJAK2) or JAK2 antibody (JAK2) are shown.

JAK2 tyrosine phosphorylation separately, perhaps via a common system.

As we have previously done, lysates were also immunoblotted with an anti-JAK2 antibody that recognizes both phosphorylated and nonphosphorylated forms of JAK2, and equal amounts of total JAK2 were noted in each case (Fig. 4).

Role of PLD in S100B-induced augmentation of Ang II–induced activation of JAK2 in VSMCs. The activation of PKC by elevated glucose levels and diabetes has been shown to be related to increases in DAG (7). Multiple pathways may generate DAG, though in diabetes most de novo synthesis of DAG occurs via the polyol pathway (7). However, it has recently been found that DAG can also be produced from the metabolism of phosphatidylcholine due to activation of PLD (7). This led us to evaluate the effects of PLD on S100B enhancement of Ang II–induced JAK2 phosphorylation. 1-Butanol has been used by many groups to evaluate PLD activity because it participates in a transphosphatidylation reaction that diverts PLD away from production of DAG (33). We found that 1-butanol (0.2% [vol/vol]) significantly prevented the S100B augmentation of the Ang II–induced JAK2 activation (Fig. 5). On the other hand, 0.2% [vol/vol] of 2-butanol, a butanol isomer that does not participate in the transphosphatidylation reaction (33), had no effect on the S100B augmentation (Fig. 5). The lysates were also immunoblotted with an anti-JAK2 antibody, and equal amounts of JAK2 were detected for all the conditions tested (Fig. 5). These results suggest that PLD is an integral part of the signaling pathway triggered by RAGE, leading to augmentation of Ang II–induced phosphorylation of JAK2.

Suppression of PLD2 expression prevents S100B enhancement of the Ang II–induced activation of JAK2. Thus far, our studies suggest that ligand engagement of RAGE (based on the inhibitory effect of anti-RAGE IgG) (Fig. 4) activates a signaling pathway including NADPH oxidase (based on the inhibitory effect of anti-p47 phox) (Fig. 3) and PLD (based on the inhibitory effect of 1-butanol) (Fig. 5), which ultimately enhances Ang II–induced phosphorylation of JAK2. To further confirm the involvement of PLD in these events, we used an antisense approach. PLD2 was targeted because it is mostly associated with the plasma membrane, has high intrinsic basal activity, and is activated by endothelin-1, platelet-derived growth factor, and Ang II in VSMCs (7). Therefore, we synthesized an oligonucleotide that targets the translational start site of the rat PLD2 coding sequence (5′-CAA CTGCTGTTCCGGCT-3′). We found that this antisense oligonucleotide blocked the expression of PLD2 (Fig. 6). That is, when we treated the cells with PLD2 sense or antisense oligonucleotides for various times, the PLD2 antisense but not the sense oligonucleotide was effective in completely inhibiting PLD2 expression within 12 h (Fig. 6). We then tested the effects of this PLD2 antisense on Ang II–, S100B-, and S100B plus Ang II–induced activation of

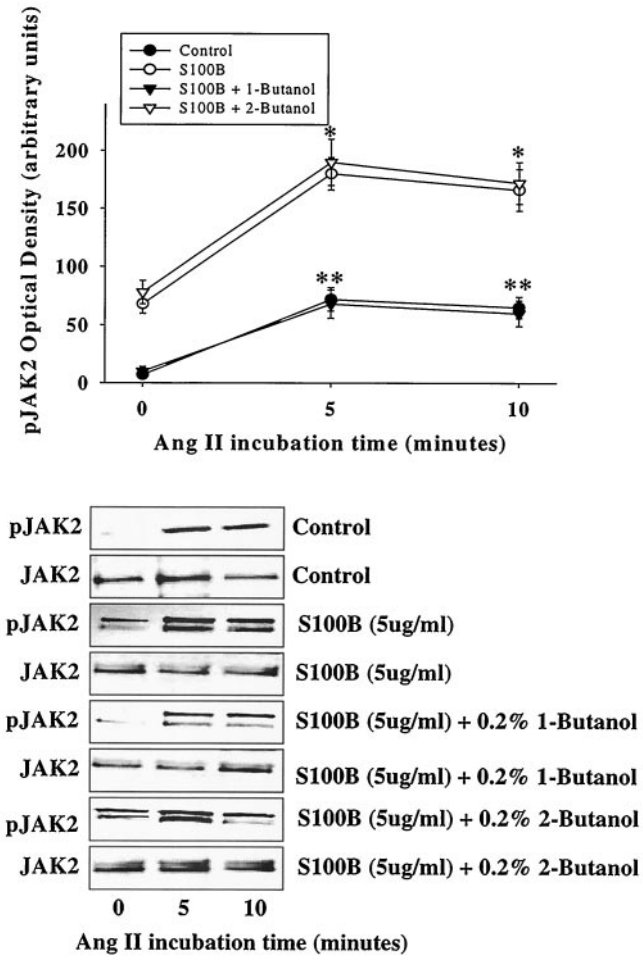

FIG. 5. Effects of 1- or 2-butanol on S100B augmentation of Ang II–induced tyrosine phosphorylation of JAK2. Quiescent VSMCs in serum-free medium were exposed for 24 h to S100B (5 μg/ml) or vehicle (control) in the presence or absence of 1-butanol (0.2% [vol/vol]) or 2-butanol (0.2% [vol/vol]) and treated with Ang II (100 nmol/l) for 5 and 10 min. VSMCs were lysed, and lysates were immunoblotted with either phosphotyrosine-specific or non–phospho-specific anti-JAK2 antibodies. 1-Butanol (0.2% [vol/vol]) significantly ($P < 0.05$) prevented the significant (*$P < 0.05$) S100B augmentation of the Ang II–induced JAK2 activation (Fig. 5). 2-Butanol at 0.2% [vol/vol], a butanol isomer, had no effect in the transphosphatidylation reaction. Representative immunoblots and densitometric analysis of three immunoblots probed with either the JAK2 phosphotyrosine-specific antibody (pJAK2) or JAK2 antibody (JAK2) are shown.**

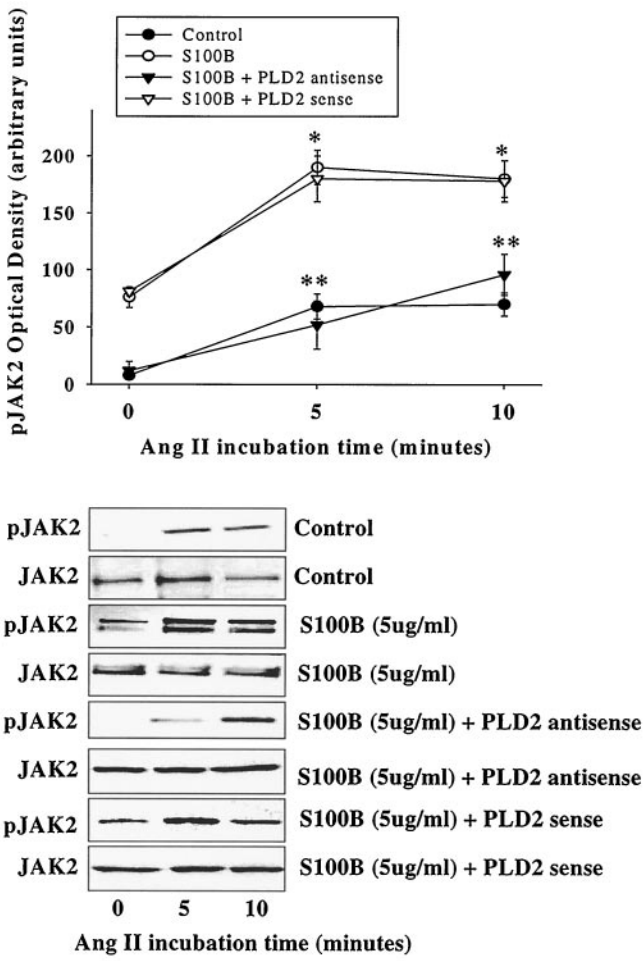

FIG. 7. Effects of PLD2 antisense on S100B enhancement of Ang II–induced tyrosine phosphorylation of JAK2. VSMCs were pretreated for 12 h with either PLD2 sense or antisense oligonucleotides and processed as described in RESEARCH DESIGN AND METHODS. Afterward, cells were washed once with serum-free DMEM, and incubated for a further 24 h in serum-free DMEM containing either S100B (5 μg/ml) or vehicle. VSMCs were then treated with Ang II (100 nmol/l) for 5 and 10 min and lysed. Lysates were immunoblotted with either phosphotyrosine-specific or non–phospho-specific anti-JAK2 antibodies. PLD2 antisense transfection blocked significantly ($P < 0.05$) the significant S100B (*$P < 0.05$) augmentation of the Ang II–induced tyrosine phosphorylation of JAK2. Representative immunoblots and densitometric analysis of three immunoblots probed with either the JAK2 phosphotyrosine-specific antibody (pJAK2) or JAK2 antibody (JAK2) are shown.**

JAK2. We found that PLD2 antisense transfection blocked the S100B augmentation of the Ang II–induced tyrosine phosphorylation of JAK2 (Fig. 7). The sense PLD2, on the other hand, had no effect (Fig. 7). These results support our hypothesis, suggesting that the PLD2 isozyme plays a critical role in the signaling pathway triggered by RAGE, which leads to the amplification of the Ang II–induced tyrosine phosphorylation of JAK2.

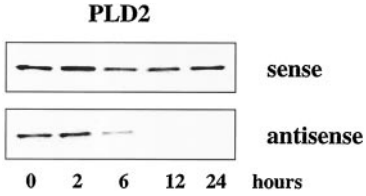

FIG. 6. Effects of antisense and sense oligonucleotides on PLD2 protein expression in VSMCs. VSMCs were treated with PLD2 sense or antisense oligonucleotides for the times indicated, the cells were lysed, and PLD2 was immunoprecipitated from the lysates with the anti-PLD2 antibody. Precipitated proteins were then immunoblotted with the anti-PLD2 antibody.

Suppression of PLD2 expression prevents S100B activation of PLD2 and the S100B enhancement of the Ang II–induced VSMC proliferation and H_2O_2 production. As previously shown in Fig. 2, both Ang II and S100B significantly induced H_2O_2 production. Furthermore, S100B augmented the Ang II–induced H_2O_2 production (Fig. 2). We have also found that transfection of VSMCs with PLD2 antisense significantly blocked both S100B-induced and S100B augmentation of Ang II–induced H_2O_2 production (Fig. 8*A*). Similar results were also found with the VSMC proliferation studies. Both ligands, Ang II and S100B, significantly induced the proliferation of VSMCs, and S100B significantly augmented the Ang II–induced VSMC proliferation (Fig. 8*B*). We also found that transfection of VSMCs with the PLD2 antisense blocked the S100B

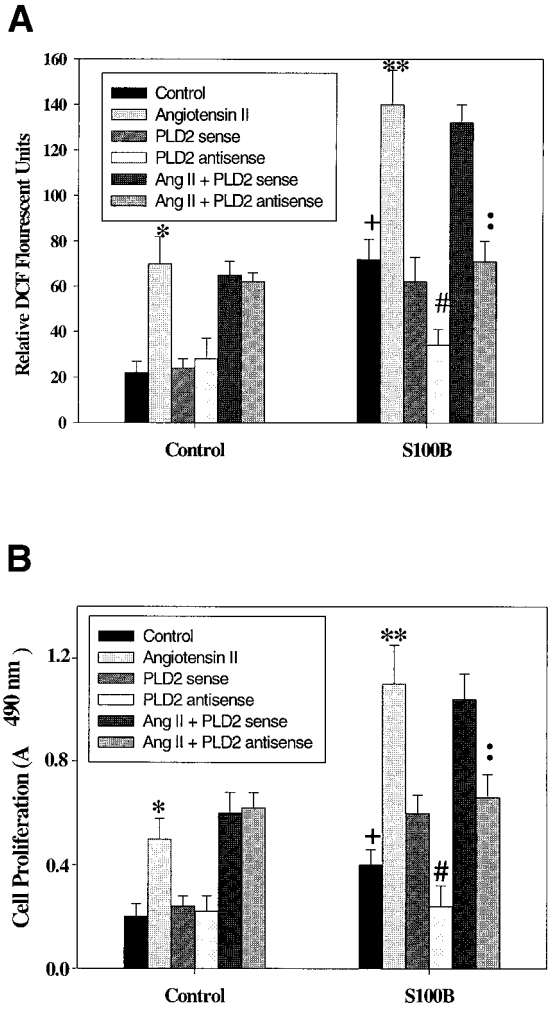

FIG. 8. *A*: Effects of PLD2 antisense on S100B enhancement of Ang II–induced H_2O_2 production. VSMCs were pretreated for 12 h with either PLD2 sense or antisense oligonucleotides. VSMCs were cultured as described in Fig. 7, and H_2O_2 production was assessed as described in Fig. 2. Data are means ± SE of six independent cultures. Both ligands, Ang II (P* < 0.05) and S100B (+*P* < 0.05), significantly induced H_2O_2 production. Furthermore, S100B significantly (***P* < 0.05) augmented the Ang II–induced H_2O_2 production. PLD2 antisense significantly blocked the S100B (#*P* < 0.05) and S100B augmentation (:*P* < 0.05) of the Ang II–induced H_2O_2 production. *B*: Effects of PLD2 antisense on S100B enhancement of Ang II–induced VSMC proliferation. PLD2 sense and antisense 12-h pretreated VSMCs were cultured for 48 h in DMEM containing either vehicle (control) or 5 μg/ml S100B with or without Ang II at 100 nmol/l. Cell proliferation was determined as described in RESEARCH DESIGN AND METHODS and is expressed as absorbance (*A*) of formazan at 490 nm. Data are means ± SE from six experiments. The Ang II induced a significant (**P* < 0.01) increase in cell proliferation, which was not significantly inhibited by PLD2 antisense oligonucleotide preincubation. S100B alone significantly induced (+*P* < 0.01) proliferation, which was significantly inhibited by preincubation with the PLD2 antisense oligonucleotide (#*P* < 0.01). PLD2 sense oligonucleotide pretreatment had no effect. PLD2 antisense also significantly (:*P* < 0.01) blocked the significant (***P* < 0.01) S100B augmentation of Ang II–induced H_2O_2 production.**

and S100B augmentation of the Ang II–induced VSMC proliferation (Fig. 8*B*).

Finally, we did direct measurement studies of S100B activation of PLD2. These studies were performed to identify whether the augmented response to Ang II caused by RAGE signal transduction is due to enhanced PLD2 activation since our previous data indicate that PLD2 expression is required. We found that S100B significantly increases the enzymatic activity of PLD2, since transfection of the VSMCs with the PLD2 antisense oligonucleotide blocked the S100B activation of PLD2 (Fig. 9).

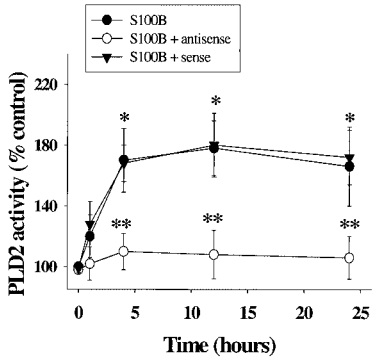

FIG. 9. S100B activation of PLD2. PLD2 activity was determined as described in RESEARCH DESIGN AND METHODS. S100B induced a significant (P* < 0.01) increase in PLD2 activity, which was significantly (***P* < 0.01) blocked by the transfection of the PLD2 antisense oligonucleotide. Data are means ± SE from six experiments.**

In summary, we have shown that in VSMC engagement of the RAGE with the ligand S100B (3) induces production of H_2O_2 (via the NADPH oxidase system), VSMC proliferation, and tyrosine phosphorylation of JAK2. Furthermore, S100B activation of VSMCs augments tyrosine phosphorylation of JAK2, VSMC proliferation, and H_2O_2 production in a manner dependent on PLD2 activation. Therefore, we hypothesize that AGEs, a class of RAGE ligands generated by high glucose and oxidant stress, both of which are conditions closely associated with diabetes, augment Ang II–induced activation JAK2 via the action of H_2O_2 generated by NADPH oxidase via a PLD2-dependent mechanisms in VSMCs. This signaling cascade then stimulates proliferation of VSMC, potentially contributing to vascular complications of diabetes.

ACKNOWLEDGMENTS

This study was supported by National Institutes of Health Grants HL58139 and DK50268 and an American Heart Association Established Investigator Award.

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