High Glucose Augments the Angiotensin II-induced Activation of JAK2 in Vascular Smooth Muscle Cells via the Polyol Pathway*

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Angiotensin II (Ang II), protein kinase C (PKC), reactive oxygen species (ROS) generated by NADPH oxidase, the activation of Janus kinase 2 (JAK2), and the polyol pathway play important parts in the hyperproliferation of vascular smooth muscle cells (VSMC), a characteristic feature of diabetic macroangiopathy. The precise mechanism, however, remains unclear. This study investigated the relation between the polyol pathway, PKC-β, ROS, JAK2, and Ang II in the development of diabetic macroangiopathy. VSMC cultured in high glucose (HG; 25 mM) showed significant increases in the tyrosine phosphorylation of JAK2, production of ROS, and proliferation activities when compared with VSMC cultured in normal glucose (5.5 mM NG). Both the aldose reductase specific inhibitor (zopolrestat) or transfection with aldose reductase antisense oligonucleotide blocked the phosphorylation of JAK2, the production of ROS, and proliferation of VSMC induced by HG, but it had no effect on the Ang II-induced activation of these parameters in both NG and HG. However, transfection with PKC-β antisense oligonucleotide, preincubation with a PKC-β-specific inhibitor (LY379196) or apocynin (NADPH oxidase-specific inhibitor), or electroporation of NADPH oxidase antibodies blocked the Ang II-induced JAK2 phosphorylation, production of ROS, and proliferation of VSMC in both NG and HG. These observations suggest that the polyol pathway hyperactivity induced by HG contributes to the development of diabetic macroangiopathy through a PKC-β/ROS activation of JAK2.

We have recently found that activation of Janus kinase 2 (JAK2)§ was essential for the angiotensin II (Ang II)-induced proliferation of vascular smooth muscle cells (VSMC) and that high glucose (HG) augmented the Ang II induction of VSMC proliferation by increasing signal transduction through the activation of JAK2 (1, 2). Current studies suggest that HG, via the polyol pathway, induces a rapid increase in intracellular reactive oxygen species (ROS) such as H₂O₂, which stimulates intracellular signal events similar to those activated by Ang II including stimulation of growth-promoting kinases such as JAK2 and extracellular signal-regulated kinase 1/2 (3–5). The polyol pathway generates ROS (H₂O₂ and O₂⁻) (6, 7), which can then act as signal mediators in the activation of mitogenic pathways, such as the JAK/STAT signaling cascade (8). For instance, in VSMC H₂O₂ has been shown to play an important role in regulating cell growth (5). It has also recently been reported that Ang II induces a rapid increase in intracellular H₂O₂ via NADPH oxidase, which subsequently activates growth-related responses plus the activation of JAK2 (5, 9). Similar results have also been found for PDGF-induced cell proliferation, which was shown to be dependent on H₂O₂ (8). Furthermore, PDGF uses H₂O₂ as a second messenger to regulate the activation of JAK2 in rat fibroblasts (8).

The HG-induced activation of protein kinase C (PKC) has also been recently shown to increase the production of ROS and to enhance VSMC proliferation. In addition, the synthesis and characterization of a specific inhibitor for PKC-β isoforms has confirmed the role of PKC activation in mediating HG effects on VSMC, and it provides in vivo evidence that the activation of the PKC-β isoform could be responsible for the abnormal ROS production and vascular growth in diabetic animals (10). For example, a recent study has concluded that VSMC can produce ROS through NADPH oxidase via activation of PKC. The study found that exposure of cultured VSMC to HG significantly increased ROS production and that treatment of the cells with phorbol myristic acid, a PKC activator, also increased ROS production. Furthermore, it was also found that the HG-induced ROS production was completely inhibited by GF109203X, a PKC-specific inhibitor. These results suggest that HG stimulates ROS production through PKC-dependent activation of NADPH oxidase in VSMC (11). In addition, a very recent study has also shown that the PKC-β2 isoform was essential for the activation of NADPH oxidase (12).

In the present study we have inhibited by either pharmacological or molecular methods the polyol pathway or PKC-β to examine their effects on the Ang II, HG, and Ang II plus HG-induced tyrosine phosphorylation of JAK2, ROS production, and VSMC proliferation. We hypothesize that HG augments the Ang II-induced activation of JAK2 and growth responses in VSMC through ROS generated via the polyol pathway activation of PKC-β.

**EXPERIMENTAL PROCEDURES**

**Materials**—Molecular weight standards, acrylamide, SDS, N,N'-methylenebisacrylamide, N,N',N’'-tetramethylethylenediamine, protein assay reagents, and nitrocellulose membranes were purchased from Bio-Rad. Bovine catalase was obtained from Roche Applied Science, and 2,7-dichlorofluorescin (DCFH) diacetate was from Molecular Probes.

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Protein AG/A-galactosidase was obtained from Santa Cruz Biotechnology, and Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, and all medium additives were obtained from Mediatech Inc. Antibodies to phosphotyrosine (PY20), anti-SHIP-1, anti-SHIP-2, and the PKC-β isomers were procured from Transduction Laboratories. Anti-phosphotyro sine JAK2 and anti-JAK2 antibodies were obtained from BIOSOURCE International. The aldose reductase inhibitor opizorestat and the PKC-β inhibitor LY379196 were gifts from Pfizer and Eli Lilly, respectively. The Supersignal substrate chemiluminescence detection kit was obtained from Pierce. Goat anti-mouse IgG and anti-rabbit IgG were acquired from Amersham Biosciences, and Tween 20 and all other chemicals were purchased from Sigma.

Preparation of Rat Aorta VSMC—Rat aorta smooth muscle cells were harvested in DMEM supplemented with 10% (v/v) fetal bovine serum, 5.5 mM glucose, 10 mg/ml streptomycin, and 100 units/ml penicillin at 37 °C in a 5% CO₂ enriched, humidified atmosphere as previously described (13, 14). Cells from passages 5–6 were routinely subcultured 1:5 at 7-day intervals, and medium was changed every 24 h.

Aldose Reductase and PKC-β Antisense Oligonucleotide Treatment—Aldose reductase (15) and PKC-β (16) antisense oligonucleotides synthesis and treatment were carried out as previously described (17, 18). After 12 h, medium was removed, calf serum (0.1%) in Dulbecco’s modified Eagle’s medium in NG (normal glucose) was added, and the cells were allowed to recover for 30 min. The VSMC were washed once with serum-free DMEM and growth-arrested in serum-free DMEM in NG for 24 h. Afterward, the VSMC were placed in either NG or HG media for 24 more hours.

Western Blotting of JAK2—To ascertain the tyrosine phosphorylation of JAK2, growth-arrested VSMC were placed in either NG or HG for 24 h and stimulated with 0.1 μg Ang II or 0.33 mM PDFO for various times ranging from 0 to 10 min. At the end of stimulation, cells were washed twice with ice-cold phosphate-buffered saline with 1 mM/liter Na₃VO₄, and 1 mmol/liter 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 lysate was measured with the Bio-Rad detergent-compatible assay kit and bovine serum albumin as the standard. Subsequently, samples were resolved by 10% SDS-PAGE gel electrophoresis, transferred to a nitrocellulose membrane, and blocked by a 60-min incubation at room temperature (22 °C) in Tris-buffered saline with 0.05% Tween 20, pH 7.4, plus 5% skimmed milk powder. The nitrocellulose membrane was incubated overnight at 4 °C with affinity-purified anti-phospho-specific JAK2 antibodies or non-phospho anti-JAK2 antibodies. Subsequently, the nitrocellulose membranes were washed twice with 10 min each with Tris-buffered saline with 0.05% Tween 20, pH 7.4, and incubated for various times with goat anti-rabbit IgG horseradish peroxidase conjugate. After extensive washing, bound antibody was visualized on Kodak Biomax film with a Pierce Supersignal substrate chemiluminescence detection kit. Molecular weight markers assessed specificity of the bands.

Immunoprecipitation Studies of SHP-1 and SHP-2—To determine the protein-tyrosine phosphatase SHP-1 and SHP-2 tyrosine phosphorylation, serum-starved VSMC grown in HG for 24 h were stimulated with 0.1 μg Ang II for various times ranging from 0 to 10 min. At the end of stimulation, cells were washed twice with ice-cold phosphate-buffered saline with 1 mM/liter Na₃VO₄. Each dish was then treated for 60 min with ice-cold lysis buffer (20 mM/liter Tris-HCl, pH 7.4, 2.5 mM/liter EDTA, 1% Triton X-100, 10% glycerol, 1% deoxycholate, 0.1% SDS, 10 mM/liter Na₃PO₄, 50 mM/liter NaF, 1 mM/liter phenylmethylsulfonyl fluoride, and 1 mM/liter phenylmethylsulfonyl fluoride), and the supernatant fraction was obtained as cell lysate by centrifugation at 58,000 × g for 20 min at 4 °C. Protein concentration of the lysate was measured with the Bio-Rad detergent-compatible assay kit and bovine serum albumin as the standard. Subsequently, samples were resolved by 10% SDS-PAGE gel electrophoresis, transferred to a nitrocellulose membrane, and blocked by a 60-min incubation at room temperature (22 °C) in Tris-buffered saline with 0.05% Tween 20, pH 7.4, plus 5% skimmed milk powder. The nitrocellulose membrane was incubated overnight at 4 °C with 10 μg/ml of affinity-purified anti-phosphotyrosine antibodies, and the bound antibodies were visualized using a Pierce Supersignal chemiluminescence detection kit.

PKC Assay—PKC activation was determined by the method of Tardif et al. (19). Briefly, serum-starved VSMC grown in either NG or HG for 24 h were treated with Ang II (0.1 μM) for 1 min. Cells were washed, resuspended in buffer, and sonicated, and homogenates were ultracentrifuged to isolate the plasma membrane fractions. Equal amounts of plasma membrane protein in each sample were mixed and separated by SDS-PAGE under reducing conditions, and the plasma membrane distribution of PKC-β1 and PKC-β2 was visualized by Western blot using specific monoclonal antibodies against each isoform of PKC-β.

Electroporation Procedure—Cells were plated in 100-mm cell plates and growth-arrested in serum-deprived DMEM for 24 h before experiments. As previously described (2, 13, 20), VSMC were electroporated using a Multi-Coaxial electrode (Model P/N 747, BTX Inc., San Diego, CA) that performed in Ca²⁺− and Mg²⁺-free Hanks’ balanced salt solution containing anti-p47phox antibodies at a final concentration of 10 μg/ml. After 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 the experiments.

Assay of Intracellular ROS—Intracellular ROS production was measured by the method of Ushio-Fukai et al. (5) with some modifications. Briefly, dishes of confluent cells after stimulation with Ang II were washed with modified Eagle’s medium without phenol red and cultured in Krebs-Ringer bicarbonate solution containing 5 mM DCFH diacetate. DCFH diacetate is a nonpolar compound that readily diffuses into cells, where it is hydrolyzed to the non-fluorescent polar derivative DCFH and thereby trapped within the cells (5). In the presence of a proper oxidant, DCFH is oxidized to the highly fluorescent 2,7-dichlorofluorescein. Culture dishes were transferred to a Zeiss inverted microscope equipped with a 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 effect of DCFH photo-oxidation was minimized by collecting the fluorescent image with a single rapid scan (time average, 4; total scan time, 4.33 s) and identical parameters, such as contrast and brightness, were maintained 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.

Cell Proliferation Assay—VSMC proliferation was measured using the Cell Titer 96™AQсенс microplate proliferation assay kit (Promega, Inc., Madison, WI) (21). This assay is based on the cellular conversion of the colorimetric dye 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) into soluble formazan by dehydrogenase enzymes found only in metabolically active, proliferating cells. MTS in Dulbecco’s phosphate-buffered saline, pH 6.0, was mixed with the electron-coupling reagent phenazine methyl sul fate. The absorbance of formazan was 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 Corp., Hialeah, FL). The cells were grown in a 75-mm² flask to confluence and dispersed with trypsin/EDTA (0.05% Trypsin X-100, 1% trypsin-EDTA, 1% FBS, and 0.02% EDTA). A 0.1% SDS, 10 mM/liter Na₃PO₄, 50 mM/liter NaF, 1 mM/liter NaVF₄, and 1 mM/liter phenylmethylsulfonyl fluoride), and the supernatant fraction was obtained as cell lysate by centrifugation at 58,000 × g for 20 min at 4 °C. The cell lysate was incubated with 10 μg/ml either anti-SHIP-1 or anti-SHIP-2 monoclonal antibodies at 4 °C for 2 h on ice and precipitated by the addition of protein A/G-agarose at 4 °C overnight. The immunoprecipitates were then recovered by centrifugation and washed 3 times with ice-cold wash buffer (Tris-buffered saline, 0.1% Triton X-100, 1 mM/liter phenylmethylsulfonyl fluoride, and 1 mM/liter NaVF₄). Immunoprecipitated proteins were dissolved in 100 μl of Laemml sample buffer, and 80 μl of each sample was subsequently electrophoresed in a 10% SDS-PAGE gel electrophoresed to a nitrocellulose membrane and blocked by 60-min incubation at 22 °C in Tris-buffered saline with 0.05% Tween 20, pH 7.4, plus 5% skimmed milk powder. The nitrocellulose membrane was incubated overnight at 4 °C with 10 μg/ml of affinity-purified anti-phosphotyrosine antibodies, and the bound antibodies were visualized using a Pierce Supersignal chemiluminescence detection kit.
High Glucose, JAK2, and the Polyol Pathway

![Diagram](http://www.jbc.org/Downloaded from)
MKP-1, JAK2, and the Src kinases Src and Fyn in studying the regulation of STAT1 and STAT3 tyrosine phosphorylation and activation in VSMC (17, 18). In addition, two recent studies show that both aldose reductase (15) and PKC-ζ/H9252 (16) antisense oligonucleotides inhibit the synthesis of these two proteins. VSMC were treated with aldose reductase and PKC-ζ sense and antisense oligonucleotides for the times indicated, the cells were lysed, and aldose reductase and PKC-ζ were immunoprecipitated from the lysates with the specific anti-aldose reductase or anti-PKC-ζ antibody. Precipitated proteins were then immunoblotted with the specific anti-aldose reductase or anti-PKC-ζ antibody.

![Graph](image1.png)

**Fig. 4.** Effects of zopolrestat on the high glucose-induced activation of PKC-β1 and PKC-β2. PKC isoforms were characterized by using monospecific antibodies against PKC-β1 or PKC-β2 isoforms. The expression of PKC-β2 isoform protein in the plasma membrane fraction of VSMC cultured in HG or exposed to Ang II were significant (p < 0.01) when compared with those VSMC cultured in NG. Zopolrestat blocked the HG-induced activation of PKC-β2, but it had no effect on the Ang II activation.

![Graph](image2.png)

**Fig. 5.** Effect of aldose reductase and PKC-ζ antisense oligonucleotides on aldose reductase and PKC-β2 expression in vascular smooth muscle cells. VSMC were treated with aldose reductase and PKC-ζ sense and antisense oligonucleotides for the times indicated, the cells were lysed, and aldose reductase and PKC-ζ were immunoprecipitated from the lysates with the specific anti-aldose reductase or anti-PKC-ζ antibody. Precipitated proteins were then immunoblotted with the specific anti-aldose reductase or anti-PKC-ζ antibody.

![Graph](image3.png)

**Fig. 6.** Effects of aldose reductase antisense on the high glucose augmentation on the angiotensin II-induced JAK2 tyrosine phosphorylation. Quiescent VSMC were transfected with either aldose reductase (AR) sense or antisense without for 12 h in serum-free medium in NG. Afterward the VSMC were further incubated for an additional 24 h in serum-free medium containing either NG (5.5 mM) or HG (25 mM) and treated with Ang II (0.1 μM) for 5 and 10 min. Cells were lysed, and lysates were immunoblotted with either phosphotyrosine-specific or nonphospho-specific anti-JAK2 antibodies. Representative immunoblots (of three experiments) probed with either the JAK2 phosphotyrosine-specific antibody (pJAK2) or JAK2 antibody (JAK2) are shown.

with HG alone or Ang II in either NG or HG. We found that preincubating the VSMC with the aldose reductase antisense oligonucleotide (but not the JAK2 sense oligonucleotide) significantly inhibited the HG stimulation of the Ang II-induced JAK2 tyrosine phosphorylation (Fig. 6). However, just like with zopolrestat, the aldose reductase antisense oligonucleotide had no effect on the Ang II-induced JAK2 tyrosine phosphorylation (Fig. 6). These results further support our previous findings, which showed that the Ang II-induced JAK2 activation is not dependent on the polyol pathway but, rather, that Ang II and...
crease in both the HG- and Ang II-induced H2O2 production that both the HG- or Ang II-induced production of H2O2 and observations further support our hypothesis, which suggests (Fig. 7) and JAK2 tyrosine phosphorylation (Fig. 8). These transfected with either PKC- 

Quiescent VSMC were transfected with either PKC-

ation.

tation on the angiotensin II-induced JAK2 tyrosine phosphoryl-

containing either NG (5.5 mM) or HG (25 mM) and treated with Ang II (0.1 μM, 4 h) in cells incubated with 2',7'-dichlorofluorescein diacetate (DCF, 5 μM), an H2O2-sensitive dye that is incorporated into the cell. We found that both HG (*, p < 0.01) or Ang II (**, p < 0.01) in NG caused a significant increase in H2O2 production when compared with the NG control cells. VSMC exposed to HG plus Ang II show a significant difference (#, p < 0.01) in H2O2 production when compared with cells treated with HG alone. PKC-β antisense blocked the HG-induced H2O2 production significantly (+, p < 0.01) and also the Ang II-induced H2O2 production in both NG (**, p < 0.01) and HG (#, p < 0.01). Data represent the mean ± S.E. of six independent cultures.

Fig. 7. Effects of PKC-β antisense oligonucleotide on the Ang II- and high glucose-induced H2O2 production. Quiescent VSMC were transfected with either PKC-β sense or antisense for 12 h in serum-free medium in NG. Afterward the VSMC were further incubated for an additional 24 h in serum-free medium containing either NG (5.5 mM) or HG (25 mM) and then treated with Ang II (0.1 μM, 4 h) in cells incubated with 2',7'-dichlorofluorescein diacetate (DCF; 5 μM), an H2O2-sensitive dye that is incorporated into the cell. We found that both HG (*, p < 0.01) or Ang II (**, p < 0.01) in NG caused a significant increase in H2O2 production when compared with the NG control cells. VSMC exposed to HG plus Ang II show a significant difference (#, p < 0.01) in H2O2 production when compared with cells treated with HG alone. PKC-β antisense blocked the HG-induced H2O2 production significantly (+, p < 0.01) and also the Ang II-induced H2O2 production in both NG (**, p < 0.01) and HG (#, p < 0.01). Data represent the mean ± S.E. of six independent cultures.

Fig. 8. Effects of PKC-β antisense on the high glucose augmentation on the angiotensin II-induced JAK2 tyrosine phosphorylation. Quiescent VSMC were transfected with either PKC-β sense or antisense for 12 h in serum-free medium in NG. Afterward the VSMC were further incubated for an additional 24 h in serum-free medium containing either NG (5.5 mM) or HG (25 mM) and treated with Ang II (0.1 μM) for 5 and 10 min. Cells were lysed, and lysates were immuno-blotted with either phosphotyrosine-specific or nonphospho-specific anti-JAK2 antibodies. Representative immunoblots (of three experiments) probed with either the JAK2 phosphotyrosine-specific antibody (pJAK2) or JAK2 antibody (JAK2) are shown.

The polyol pathway induce JAK2 tyrosine phosphorylation separately perhaps via a common system.

Last, our results with the PKC-β antisense oligonucleotide were almost identical to our studies with PKC-β specific inhibitor LY379196. For example, we found that incubation with the PKC-β antisense oligonucleotide completely suppressed the increase in both the HG- and Ang II-induced H2O2 production (Fig. 7) and JAK2 tyrosine phosphorylation (Fig. 8). These observations further support our hypothesis, which suggests that both the HG- or Ang II-induced production of H2O2 and JAK2 activation occurs through the PKC-β2 isoform.

Effects of Apocynin and Electroporation of the NADPH Oxidase-Neutralizing Antibody, anti-p47phox, on Both the Basal and Ang II-Induced Tyrosine Phosphorylation of JAK2 in VSMC Preincubated in HG—A number of recent studies have suggested that most of the H2O2 produced by Ang II stimula-

tion in cells comes from the NAD(P)H oxidase system (5, 9). Apocynin has been shown to be a specific inhibitor of the NAD(P)H oxidase system (5). In these studies, we found that incubation of VSMC with apocynin completely suppressed the increase in ROS production (Fig. 9) in VSMC treated with Ang II under both NG and HG conditions. Zopolrestat, on the other hand, only blocked the HG-induced production of H2O2. In addition, apocynin also completely blocked the JAK2 tyrosine
ROS-generating system responsible for most of the H$_2$O$_2$ production and the hypothesis, which suggest that NADPH oxidase might be the ROS-generating system responsible for most of the H$_2$O$_2$ produced after experimental treatments with Ang II in VSMC cultured in either NG or HG.

**Effect of Ang II and HG on Vascular Smooth Muscle Cell Growth and the Role of the Polyol Pathway and Reactive Oxygen Species**—As shown in Fig. 12a, VSMC exposed to Ang II for

As stated above, because most inhibitors are usually not very specific, we also used another additional approach to determine the influence of the NADPH oxidase system on both the HG- and the Ang II-induced JAK2 activation. The anti-p47phox antibody has been shown to neutralize the NADPH oxidase system in VSMC (9). In these studies, we found that electroporation of VSMC with anti-p47phox antibodies completely suppressed the JAK2 tyrosine phosphorylation in all groups tested, whereas electroporation of a control rabbit IgG had no effect (Fig. 11). These results again strongly support our hypothesis, which suggest that NADPH oxidase might be the ROS-generating system responsible for most of the H$_2$O$_2$ produced after experimental treatments with Ang II in VSMC cultured in either NG or HG.
activation of JAK2 via the polyol pathway. We found that transfection of the VSMC with the aldose reductase antisense oligonucleotide was able to prevent the HG-induced abrogation of SHP-1 tyrosine phosphorylation (Fig. 13). These results suggest that the JAK2-sustained tyrosine phosphorylation, which occurs under HG conditions in VSMC by examining their tyrosine phosphorylation states. We found that transfection of the VSMC with the aldose reductase antisense oligonucleotide was able to prevent the HG-induced abrogation of SHP-1 tyrosine phosphorylation (Fig. 13). On the other hand, the HG augmentation of the Ang II-induced tyrosine phosphorylation of SHP-2 was blocked by pretreatment with the aldose reductase antisense oligonucleotide (Fig. 13). These results suggest that the JAK2-sustained tyrosine phosphorylation, which occurs under HG concentration in VSMC, might be due to changes on SHP-1 and SHP-2 activation influenced by the polyol pathway.

Effects of Aldose Reductase Antisense on Both the Basal and PDGF-induced Tyrosine Phosphorylation of JAK2 in VSMC Preincubated in HG—Recently it has been shown that the growth factor PDGF, which is another growth factor just like Ang II, employs H2O2 as a second messenger to regulate the activation of JAK2 in rat fibroblasts (8). Therefore, we were interested in examining if HG also augments the PDGF-induced activation of JAK2 via the polyol pathway. We found that transfection of VSMC with the aldose reductase antisense oligonucleotide inhibited the HG stimulation of the PDGF-induced JAK2 tyrosine phosphorylation (Fig. 14). However, just

48 h under NG conditions resulted in a significant increase in cell proliferation when compared with control cells. Exposure of cells to HG alone also resulted in a significant increase in cell proliferation when compared with NG. In addition, the Ang II-induced cell proliferation was also significantly enhanced in cells incubated in HG when compared with cells incubated in NG. Finally, we also found, as we had previously shown that the phosphorylation state of JAK2 is tightly regulated by the two cytoplasmic protein-tyrosine phosphatases SHP-1 and SHP-2 (25). Furthermore, we have also shown that HG alters the tyrosine phosphorylation and activation of these two cytosolic protein-tyrosine phosphatases under HG conditions in VSMC by examining their tyrosine phosphorylation states. We found that transfection of the VSMC with the aldose reductase antisense oligonucleotide was able to prevent the HG-induced abrogation of SHP-1 tyrosine phosphorylation (Fig. 13). On the other hand, the HG augmentation of the Ang II-induced tyrosine phosphorylation of SHP-2 was blocked by pretreatment with the aldose reductase antisense oligonucleotide (Fig. 13). These results suggest that the JAK2-sustained tyrosine phosphorylation, which occurs under HG concentration in VSMC, might be due to changes on SHP-1 and SHP-2 activation influenced by the polyol pathway.

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as we previously showed with Ang II, the aldose reductase antisense had no effect on the PDGF-induced JAK2 tyrosine phosphorylation (26). This indicates that the induction by Ang II is not dependent on the polyclonal pathway but, rather, that both PDGF and the polyclonal pathway induce JAK2 tyrosine phosphorylation independently, perhaps via complementary pathways.

Lysates were all immunoblotted with an anti-JAK2 antibody that recognizes both phosphorylated and nonphosphorylated forms of JAK2. Equal amounts of JAK2 (Figs. 1, 3, 6, 8, 10, 11, 13, and 14) were detected for all the conditions tested, indicating that the differences detected with the phosphospecific 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 H2O2 and Ang II-induced JAK2 tyrosine phosphorylation in all the experiments tested was inhibited when cells were preincubated with candesartan, a specific AT1 receptor blocker (26), indicating that this induction by Ang II was AT1 receptor-specific (data not shown).

**DISCUSSION**

The specific cellular signals activated in VSMC under hyperglycemic conditions have not been completely elucidated. Recently Amiri and co-workers (1) found that hyperglycemia increased both the basal and Ang II-induced VSMC proliferation, tyrosine phosphorylation, and complex formation of JAK2 with the Ang II AT1 receptor and the extent of the tyrosine and serine phosphorylation of STAT1 and STAT3. They also found that hyperglycemia altered Ang II-induced tyrosine phosphorylation and the activities of SHP-1 and SHP-2 (1). These results suggest that increased and/or altered activation of tyrosine kinase (JAK2), tyrosine phosphatases (SHP-1 and SHP-2), and downstream transcription factors, such as STAT1 and STAT3, are important for abnormal ROS production and vascular growth in diabetes mellitus (Fig. 15).

In conclusion, the results from this study support our hypothesis, which states that the polyclonal pathway activation of PKC-β2 is an important mechanism by which HG augments the Ang II-induced ROS production and the activation of JAK2, which leads to VSMC proliferation that is associated with diabetes mellitus.
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