Hyperglycemia Enhances Angiotensin II-induced Janus-activated Kinase/STAT Signaling in Vascular Smooth Muscle Cells*

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We have shown previously that angiotensin II (Ang II) activates the janus-activated kinase (JAK)/signal transducers and activators of transcription (STAT) pathway in vascular smooth muscle cells (VSMCs) and that activation of the JAK/STAT pathway is required for Ang II induction of VSMC proliferation. In the present study, we examined the effects of hyperglycemia (HG) on Ang II-induced JAK/STAT signaling events in cultured VSMCs. HG increases Ang II-induced JAK2 tyrosine phosphorylation and promotes a partial tyrosine phosphorylation of the enzyme under basal conditions. In addition, HG increases both basal and Ang II-induced complex formation of JAK2 with the Ang II AT1 receptor. The extent of STAT1 and STAT3 tyrosine and serine phosphorylation are also increased under HG conditions. Furthermore, the tyrosine phosphorylation and activities of the SHP-1 and SHP-2 tyrosine phosphatases, enzymes that regulate Ang II-induced JAK2 tyrosine phosphorylation, are altered by HG, SHP-1, which is responsible for JAK2 tyrosine dephosphorylation in VSMC, is completely deactivated in HG, resulting in a prolonged duration of JAK2 phosphorylation under HG conditions. HG also enhances Ang II induction of VSMC proliferation. Taken together, these data suggest that HG augments Ang II induction of VSMC proliferation by increasing signal transduction through the JAK/STAT pathway.

A major pathologic complication of diabetes is atherosclerosis (1). One of the basic underlying causes of diabetic atherosclerosis appears to be hyperglycemia-induced vascular smooth muscle cell (VSMC) proliferation. VSMCs cultured under hyperglycemic (HG) conditions, for example, proliferate at a significantly faster rate than those cultured under normal glucose (NG) conditions (2). HG increases the de novo synthesis of the protein kinase C-activator, diacylglycerol. Thus, one hypothesized mechanism by which HG induces VSMC proliferation is through the chronic activation of one or more isoforms of protein kinase C (3). Other mechanisms by which HG has been suggested to stimulate VSMC proliferation are through nonenzymatic modification of macromolecules to form advanced glycation end products, changes in sorbitol and myoinositol metabolism, increased oxidant formation, and increased production of extracellular matrix molecules (1, 4).

VSMC proliferation is also stimulated by a number of growth factors and hormones including angiotensin II (Ang II) (5–8). Furthermore, Ang II stimulation of VSMC proliferation is very likely enhanced by HG because Ang II activation of mitogen-activated protein kinases (MAPKs) is increased in VSMCs cultured under HG conditions (9), and we have shown previously that activation of the MAPK pathway is essential to Ang II induction of VSMC proliferation (7, 8). In addition to the MAPK pathway, a second pathway involved in Ang II induction of VSMC proliferation is the janus-activated kinase (JAK)/signal transducers and activators of transcription (STAT) pathway (8). The JAK/STAT pathway involves the tyrosine phosphorylation and consequent activation of JAK tyrosine kinases. Activated JAK kinases phosphorylate latent transcription factors termed STATs, resulting in STAT homo- and heterodimerization and translocation of STAT dimers to the nucleus. In the nucleus, STAT dimers bind to specific response elements in gene promoters and direct transcription (10). Cross-talk also appears to exist between the MAPK and the JAK/STAT pathways because serine phosphorylation of STAT1 and STAT3 by MAPK appears to be essential to Ang II induction of VSMC proliferation (11).

We have shown previously that Ang II stimulation of VSMCs results in the tyrosine phosphorylation of JAK2 and complex formation of JAK2 with the Ang II AT1 receptor (12). We have also demonstrated that Ang II-induced JAK2 tyrosine phosphorylation is regulated by the protein-tyrosine phosphatases known as SHP-1 and SHP-2 (13) and that phosphorylation of JAK2 is accompanied by the tyrosine and/or serine phosphorylation of STAT1 and STAT3 and translocation of the two proteins to the nucleus (12, 14, 15). This signaling cascade appears to be essential to Ang II induction of VSMC proliferation, because treatment of VSMCs with the JAK2 inhibitor, AG-490, or electroporation of anti-STAT1 or anti-STAT3-neutralizing antibodies completely blocks Ang II induction of cell proliferation (8).

The JAK/STAT pathway and HG, thus, both have important roles in promoting VSMC proliferation. It is not known, however, whether HG affects JAK/STAT signaling in VSMC. Therefore, in the present study we examined the effects of HG on various Ang II-induced JAK/STAT signaling events associated with Ang II stimulation of VSMC proliferation.

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† The abbreviations used are: VSMC, vascular smooth muscle cell; HG, high glucose or hyperglycemic; NG, normal glucose; Ang II, angiotensin II; JAK, janus-activated kinase; STAT, signal transducer and activator of transcription; MAPK, mitogen-activated protein kinase; SHP, SH2 domain-containing protein phosphatase.

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EXPERIMENTAL PROCEDURES

Materials—Anti-JAK2 phosphotyrosine-specific antibody was purchased from BIOSOURCE International. Anti-STAT1 antibody, anti-STAT3 antibody, anti-STAT1 phosphospecific antibody, and anti-STAT3 phosphospecific antibody were obtained from New England Biolabs. Anti-STAT1 phosphospecific antibody and anti-STAT3 phosphospecific antibody were procured from Upstate Biotechnology. Anti-AT1 receptor antibody, anti-JAK2 antibody, anti-SHP-1 antibody, and anti-SHP-2 antibody were purchased from Santa Cruz Biotechnology. Anti-phosphotyrosine antibody came from Transduction Laboratories. Cell Titer 96 AQueous nonradioactive cell proliferation assay kit was purchased from Promega. All other chemicals were purchased from Sigma.

Cell Culture—VSMCs were cultured from aortas of male Harlan Sprague-Dawley rats in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics as described previously (16). VSMC passages 8 to 15 were grown to 70 to 80% confluence, washed once in serum-free medium, and incubated for 24 h in either NG (5.5 mM), HG (25.0 mM), or NG (5.5 mM) plus mannitol (19.5 mM) before treatment with Ang II (100 nM).

Immunoprecipitation and Immunoblotting—Immunoprecipitation and immunoblotting were carried out as described previously (13–15).

Tyrosine Phosphatase Activity Assay—VSMC lysates (1 mg of protein) were incubated in 3 μl of anti-SHP-1 or anti-SHP-2 antibody at 4°C. After 3 h, protein A/G agarose was added, and incubation was continued for 3 h. Immunoprecipitates were recovered by centrifugation, then washed three times in ice-cold wash buffer containing 50 mM Tris-HCl, pH 7.4, 60 mM NaCl, 60 mM KCl, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Phosphatase activity was assayed as described previously (13, 17) by measuring the rate of formation of p-nitrophenol phosphate from p-nitrophenyl phosphate. The final immunoprecipitated pellet was suspended in a total of 100 μl of wash buffer containing 1 mg/ml bovine serum albumin, 5 mM EDTA, and 10 μM diethiothreitol. The reaction was initiated by the addition of p-nitrophenyl phosphate (10 mM final concentration) and was allowed to proceed for 30 min at room temperature. The reaction was terminated by the addition of 1 ml NaOH, and the absorbance of the sample at 410 nm was determined in a spectrophotometer.

Cell Proliferation Assay—VSMC proliferation was quantitated using the Cell Titer 96 AQueous nonradioactive cell proliferation assay kit (Promega). This assay is based on measuring the cellular conversion of the colorimetric reagent 3,4-(5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt into a soluble formazan by dehydrogenase enzymes found only in metabolically active, proliferating cells. The absorbance of formazan was measured at 490 nm using a 96-well enzyme-linked immunosorbent assay plate reader.

RESULTS AND DISCUSSION

The JAK/STAT signaling pathway in VSMCs plays an essential role in Ang II induction of cell proliferation (8). VSMC proliferation is also stimulated by HG (2). Whether HG affects JAK/STAT signaling, however, has not been previously investigated. To determine whether HG alters either basal or Ang II-induced tyrosine phosphorylation of JAK2, cells were incubated for 24 h in serum-free medium containing either NG (5.5 mM glucose) or HG (25 mM glucose) before stimulation for 0, 1, 5, 10, and 30 min with Ang II (100 nM). Cells were then lysed, and equal amounts of lysate from each condition (50 μg of protein) were immunoblotted with a JAK2 phosphospecific antibody that recognizes the tyrosine-phosphorylated, but not the nonphosphorylated, form of JAK2. As shown in Fig. 1, under euglycemic conditions, Ang II induced a transient tyrosine phosphorylation of JAK2 (130-kDa band) that was maximal between 1 and 5 min. Under HG conditions, both the extent and the duration of JAK2 phosphorylation were increased, suggesting that HG increases the magnitude and prolongs the duration of Ang II-induced JAK2 activation in VSMC. In addition, in HG, JAK2 was partially phosphorylated even under basal conditions, suggesting that HG promotes a partial constitutive activation of JAK2 even in the absence of Ang II. Lysates were also immunoblotted with an anti-JAK2 antibody that recognizes both phosphorylated and nonphosphorylated forms of JAK2. Equal amounts of JAK2 were detected for all conditions by the nonphospho-specific antibody, indicating that the differences detected with the phosphotyrosine-specific antibody were due to differences in the amount of total JAK2 protein loaded in each lane. Similar results were also obtained in experiments in which JAK2 tyrosine phosphorylation was quantitated by immunoprecipitation with anti-phosphotyrosine antibody followed by immunoblotting of immunoprecipitates with anti-JAK2 antibody (data not shown). In the experiment shown in Fig. 1 and in all others described in the present study, controls were included to determine whether the effects of HG might be due to hyperosmolarity. Thus, in addition to the NG control (5.5 mM glucose), we also included a control of 5.5 mM glucose plus 19.5 mM mannitol. In this experiment, as well as in all other experiments described hereafter, no effects of hyperosmolarity (glucose plus mannitol) were seen on any of the JAK/STAT-signaling events examined.

Ang II-induced JAK2 tyrosine phosphorylation in VSMC is accompanied by complex formation of JAK2 with the Ang II AT1 receptor (12). Complex formation involves association of JAK2 with a YIPP motif in the C-terminal intracellular domain 4 of the AT1 receptor, an event that appears to be a prerequisite for JAK2 phosphorylation and, hence, activation (18). To determine whether HG alters either basal or Ang II-induced complex formation of JAK2 with the AT1 receptor, VSMC were incubated for 24 h in serum-free medium containing either NG or HG and then stimulated for 0 or 5 min with Ang II (100 nM). Cells were then lysed, and the AT1 receptor was immunoprecipitated from equal amounts of lysate (1 mg of protein) by the nonphosphospecific antibody, indicating that immunoprecipitated proteins were then immunoblotted with an anti-JAK2 antibody to determine whether the AT1 receptor and JAK2 coimmunoprecipitated as a complex. As shown in Fig. 2, under NG conditions, no AT1 receptor-JAK2 complex was detected in the absence of Ang II treatment. A complex between the two proteins was detected, however, following a 5-min exposure to Ang II. Under HG conditions, the extent of Ang II-stimulated complex formation was significantly increased, suggesting again that HG enhances Ang II-
induced JAK2 signaling in VSMC. Furthermore, in HG, a significant amount of JAK2 was associated with the AT1 receptor even under basal conditions, suggesting that HG may cause a non-Ang II-dependent constitutive activation of JAK2 in VSMC.

We have shown previously that STAT1 is tyrosine-phosphorylated and that STAT3 is both tyrosine- and serine-phosphorylated in VSMCs in response to Ang II stimulation (12, 14, 15). To determine whether HG alters either the basal or the Ang II-induced tyrosine or serine phosphorylation of STAT1 or STAT3, we carried out immunoblotting experiments with phosphospecific antibodies that recognize either the tyrosine- or the serine-phosphorylated, but not the nonphosphorylated, forms of STAT1 and STAT3. VSMCs were incubated for 24 h in serum-free medium containing either NG (5.5 mM) or HG (25 mM) and then stimulated with Ang II (100 nM) for 0, 1, 5, 10, 30, or 60 min. Cells were lysed, and lysates were immunoblotted with phosphotyrosine-specific and nonphosphospecific anti-STAT1 antibodies. Shown are representative immunoblots and densitometric analysis of three immunoblots (mean ± S.E.). Tyr-P, phosphotyrosine.

Cells were lysed, and equal amounts of lysate (50 μg of protein) were immunoblotted with the phosphospecific antibodies. As shown in Fig. 3, both the extent and the duration of the Ang II-induced tyrosine phosphorylation of STAT1 (92-kDa band, which is sometimes resolved into a doublet on immunoblots (12)) was increased in HG. Lysates were also immunoblotted with an anti-STAT1 antibody that recognizes both phosphorylated and nonphosphorylated forms of STAT1. Equivalent amounts of STAT1 were detected for all time points by the nonphospho-specific antibody, demonstrating that the differences detected with the phosphotyrosine-specific antibody were not due to differences in the amounts of total STAT1 protein. It has not been previously determined whether Ang II induces serine phosphorylation of STAT1 in VSMC. Quantitation of serine phosphorylation of STAT1 in the present study, however, showed that Ang II does indeed induce the serine phosphorylation of STAT1 in VSMC and that the level of phosphorylated serine in STAT1 was increased by HG both in response to Ang II and in the basal state (Fig. 4). When lysates were
probed with the antibody that recognizes both phosphorylated and nonphosphorylated forms of STAT1, equal amounts of STAT1 protein were detected by the nonphospho-specific antibody in all conditions, indicating that the differences observed in STAT1 serine phosphorylation were not due to differences in the amounts of STAT1 protein.

We next examined the phosphorylation of STAT3. HG increased the extent of Ang II-induced tyrosine phosphorylation of the protein (89-kDa band, which is sometimes resolved into a doublet on immunoblots) (12) (Fig. 5) and increased both the extent and the duration of Ang II-induced serine phosphorylation (Fig. 6). HG also induced serine phosphorylation of STAT3 under basal conditions. Differences that were detected in the tyrosine and serine phosphorylation of STAT3 were not due to differences in the amounts of STAT3 protein as confirmed by immunoblotting with an anti-STAT3 antibody that recognizes both phosphorylated and nonphosphorylated forms of STAT3.

The results of these studies of tyrosine and serine phosphorylation of STAT1 and STAT3 demonstrate that HG not only...
enhances basal and Ang II-induced JAK/STAT signaling at the level of JAK2 phosphorylation and activation but also at the level of STAT1 and STAT3 phosphorylation and activation. We have shown previously that Ang II-induced serine phosphorylation of STAT3 is mediated by MAPK (15). To determine whether Ang II-induced serine phosphorylation of STAT1 is also mediated by MAPK, we investigated Ang II-induced serine phosphorylation of the factor following exposure of VSMC to the MAP kinase kinase (and hence MAPK) inhibitor, PD98059 (19). Preincubation of cells with the inhibitor (30 μM for 1 h) completely blocked Ang II-induced serine phosphorylation of STAT1 (data not shown), confirming that phosphorylation was catalyzed by MAPK.

In a previous study, we showed that the cytosolic SH2-domain-containing protein-tyrosine phosphatases SHP-1 and SHP-2 have important and opposite roles in Ang II-induced JAK2 tyrosine phosphorylation in VSMC (13). SHP-1 appears to be responsible for JAK2 dephosphorylation and undergoes tyrosine dephosphorylation itself (and consequently deactivation) during the time period when JAK2 is maximally phosphorylated. The protein-tyrosine phosphatase is subsequently tyrosine-phosphorylated and activated during the period of time when JAK2 is dephosphorylated. The time course of Ang II-induced SHP-2 tyrosine phosphorylation and activation, on the other hand, is very different. Phosphorylation and activation of SHP-2 is maximal during the period of time when JAK2 tyrosine phosphorylation is maximal. Furthermore, as we have also reported previously, SHP-2 functions as an adaptor protein for JAK2 association with the AT1 receptor (13). Because this association is required for JAK2 tyrosine phosphorylation (18), SHP-2, in its role as an adaptor, facilitates Ang II-induced JAK2 tyrosine phosphorylation. To determine whether HG affects the Ang II-induced tyrosine phosphorylation and activity of SHP-2 in VSMC, cells were incubated for 24 h in serum-free medium containing either NG or HG and then stimulated with Ang II (100 nM) for 0, 1, 5, 10, or 30 min. Cells were lysed, and equal amounts of lysate (1 mg of protein) were immunoprecipitated with anti-SHP-2 antibody. Immunoprecipitated proteins were immunoblotted with anti-phosphotyrosine antibody. As shown in Fig. 7A, in NG, Ang II induced a tyrosine phosphorylation of SHP-2 that was maximal at 5 to 10 min and was partially sustained for at least 30 min. In HG, the time course of Ang II-induced tyrosine phosphorylation of SHP-2 was similar to that in NG except that the extent of phosphorylation was approximately 2-fold higher at each time point. Immunoprecipitated SHP-2 proteins in these experiments were also assayed for tyrosine dephosphorylation activity by monitoring the rate of formation of p-nitrophenol from p-nitrophenyl phosphate as described previously (13, 17). As shown in Fig. 7B, SHP-2 activities for each time point and for each condition were altered in parallel with changes in SHP-2 tyrosine phosphorylation with approximately 2-fold higher activities in HG as compared with NG. HG thus appears to enhance the effects of Ang II on both SHP-2 tyrosine phosphorylation and SHP-2 activity.

To determine whether HG influences the Ang II-induced tyrosine phosphorylation and activity of SHP-1 in VSMC, cells were incubated for 24 h under either NG or HG conditions before stimulation with Ang II (100 nM) for 0, 1, 5, 10, or 30 min. Cells were lysed, and SHP-1 was immunoprecipitated from lysates (1 mg of lysate protein from each condition) with equal amounts of lysate (1 mg of protein) were immunoprecipitated with anti-SHP-1 antibody. Immunoprecipitated proteins were cultured in NG, NG plus Ang II (100 nM), or HG plus Ang II (100 nM) for 0, 1, 5, 10, or 30 min. Immunoprecipitated proteins were immunoblotted with anti-SHP-1 antibody. SHP-1 tyrosine phosphorylation and activity were quantitated as described above for SHP-2. In NG, SHP-1 was transiently dephosphorylated in response to Ang II followed by a subsequent rephosphorylation to a level greater than that under basal conditions (Fig. 8A). SHP-1 catalytic activity in NG was also reduced in parallel followed by a subsequent increase (Fig. 8B). In HG, SHP-1 was completely dephosphorylated and completely devoid of catalytic activity both under basal conditions as well as after Ang II stimulation (Figs. 8, A and B). HG thus appears to cause a complete deactivation of SHP-1 in VSMC. Because SHP-1 is responsible for JAK2 dephosphorylation in VSMC (13), these results provide an explanation for the increased JAK2 phosphorylation observed under HG conditions (Fig. 2). Increased JAK2 tyrosine phosphorylation in HG both basally and in response to Ang II is likely due to a lack of SHP-1 activity in HG and, hence, a lack of dephosphorylation of JAK2.

Both hyperglycemia and Ang II are capable of stimulating VSMC proliferation (2, 7, 8). Furthermore, Ang II stimulation of VSMC proliferation may be augmented by HG. This hypothesis follows from the observation of Natarajan et al. (9) that Ang II activation of MAPKs in VSMCs is enhanced in HG. We have shown previously that MAPK activation is important in Ang II induction of cell proliferation (7, 8). Whether HG augments Ang II induction of VSMC proliferation, however, has not been previously demonstrated. To determine whether HG enhances the Ang II induction of VSMC proliferation, cells were cultured in NG, NG plus Ang II (100 nM), or HG plus Ang II (100 nM). Cell proliferation was then quantitated over a period of 48 h by a colorimetric assay that determines the level of activity of dehydrogenase enzymes found only in metabolically active, proliferating cells. As shown in Fig. 9, HG does indeed enhance Ang II induction of VSMC proliferation with the effect of Ang II in stimulating cell proliferation being approximately 2-fold higher in HG relative to that in NG.

In summary, the results of the present study demonstrate that HG has significant effects on multiple basal and Ang II-induced JAK/STAT signaling events in VSMC as well as on Ang II induction of VSMC proliferation. Thus, in addition to the previously known effects of HG on chronic activation of protein kinase C isoforms and on basal and Ang II-induced activation of MAPK isozymes, HG appears to also augment Ang II induction of VSMC proliferation by increasing signal transduction through the JAK/STAT pathway.

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