Angiotensin II Signaling in Vascular Smooth Muscle Cells Under High Glucose Conditions

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Abstract—The mechanisms responsible for the accelerated cardiovascular disease in diabetes, as well as the increased hypertrophic effects of angiotensin II (Ang II) under hyperglycemic conditions, are not very clear. We examined whether the culture of vascular smooth muscle cells (VSMC) under hyperglycemic conditions to simulate the diabetic state can lead to increased activation of key growth- and stress-related kinases, such as the mitogen-activated protein kinases (MAPKs), in the basal state and in response to Ang II. Treatment of porcine VSMC for short time periods (0.5 to 3 hours) with high glucose (HG; 25 mmol/L) markedly increased the activation of the extracellular signal–regulated kinase (ERK1/2) and c-Jun/N-terminal kinase (JNK) relative to cells cultured in normal glucose (NG; 5.5 mmol/L). p38 MAPK also was activated by HG, and this effect remained sustained for several hours. Ang II treatment increased the activity of all 3 families of MAPKs. Ang II–induced ERK activation was potentiated nearly 2-fold in cells treated with HG for 0.5 hour. However, Ang II–induced JNK was not altered. In VSMC cultured for 24 hours with HG, Ang II and HG displayed an additive response on p38 MAPK activity. MAPKs can lead to activation of transcription factors such as activator protein-1 (AP-1). HG alone significantly increased AP-1 DNA-binding activity. Furthermore, Ang II and HG combined had additive effects on AP-1 activity. These results suggest that increased activation of specific MAPKs and downstream transcription factors, such as AP-1, may be key mechanisms for the increased VSMC growth potential of HG alone and of Ang II under HG conditions. (Hypertension. 1999;33[part II]:378-384.)

Key Words: angiotensin II ▬ hyperglycemia ▬ diabetes mellitus ▬ mitogen-activated protein kinases ▬ activator protein-1 ▬ muscle, smooth, vascular

Diabetes is associated with significantly increased rates of cardiovascular complications, including hypertension and atherosclerosis; however, the mechanisms responsible are not very clear. We have recently shown that vascular smooth muscle cells (VSMC) cultured under high glucose (HG; 25 mmol/L) conditions to simulate the diabetic state proliferate significantly faster than those cultured under normal glucose (NG; 5.5 mmol/L) conditions, suggesting 1 mechanism for the accelerated vascular disease seen in diabetes. However, the specific cellular signals activated in VSMC under hyperglycemic conditions are not clear. Because increasing evidence suggests that cellular effects in response to growth and stress signals are mediated by the specific activation of kinase cascades, including key mitogen-activated protein kinases (MAPKs), in the present study, we examined the hypothesis that HG-induced increases in VSMC growth is mediated by the activation of certain key growth-, stress-, and oxidant-related kinases, such as the MAPKs. Furthermore, because our earlier studies have shown that the hypertrophic effects of angiotensin II (Ang II) are significantly increased in VSMC cultured under HG conditions, we also evaluated whether Ang II–induced activation of the MAPKs are altered under HG conditions.

The MAPKs have been implicated in VSMC proliferation, hypertrophy, and differentiation, key responses in the pathology of vascular diseases such as hypertension and atherosclerosis. They are serine/threonine kinases that transduce signals in response to a wide variety of agonists acting through growth factor receptors with intrinsic tyrosine kinases, G protein–coupled receptors via nonreceptor tyrosine kinases, and cellular stress. There are at least 4 major MAPK cascades in mammalian cells: the extracellular signal–regulated kinases 1 and 2 (ERK1/2 or p42/44 MAPK), the c-Jun N-terminal kinases (JNK), p38 MAPK, and big MAPK1 (BMK1 or ERK5). The ERKs have been implicated in mitogenic as well as other cellular responses, whereas JNK and p38 activation is usually associated with inflammatory cytokine action, cellular stress, and apoptosis. Recent studies have associated JNK and p38 MAPK activation with both cellular growth and apoptosis. Activated ERKs can translocate to the nucleus and lead to the phosphorylation and activation of transcriptional factors such as Elk-1, Sap-1, c-fos, and CREB. JNK can phosphorylate and activate transcription factors such c-Jun and ATF-2, whereas p38 MAPK can activate transcriptional factors such as ATF-2 and CREB. The ERK, JNK, and p38 signaling cas-
cades run in parallel with distinct upstream activators. However, there can be cross-talk and cross-activation. Ang II can activate ERKs in VSMC, and very recent studies have shown that Ang II can also activate JNK and p38 MAPKs. We examined whether HG itself alters the activation of these key MAPKs and whether Ang II–induced activation of these kinases is altered by HG culture.

These MAPKs and associated transcription factors can lead to the induction of several genes, including c-Fos and c-Jun, which interact as the activator protein-1 (AP-1) complex. AP-1, a sequence-specific transcription factor, regulates the expression of several genes, including those mediating growth, inflammation, and differentiation. Ang II has been shown to induce AP-1 activation in VSMC. We examined for the first time whether HG can alter AP-1 activation and, further, whether Ang II effects on AP-1 are altered in VSMC under HG conditions. Our results reveal new mechanisms for increased VSMC growth under HG conditions.

**Methods**

**Cell Culture**

Primary cultures of porcine VSMC were obtained as described earlier. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing normal glucose (NG; 5.5 mmol/L) and 10% FCS. For studies under hyperglycemic conditions, cells were grown in DMEM with high glucose (HG; 25 mmol/L) and 10% FCS. Mannitol (19.5 mmol/L) was used in selected experiments as a control for osmolality.

**Incubation of Cells With HG or Ang II**

From 80% to 90% confluent VSMC growing in 100-mm dishes in NG or HG growth medium were serum starved for 24 hours in DMEM containing 0.2% BSA and 0.4% FCS. Washed cells in NG or HG were preincubated for 1 hour in medium containing 0.2% BSA only. HG, Ang II, or both then were added and incubated for various time periods. Cells maintained in NG for these same time periods did not show any increase in ERK activity (results not shown).

**Preparation of Nuclear and Cytosolic Extracts**

Nuclear extracts were prepared according to Marui et al. Briefly, after the incubations, cells from 100-mm dishes were scraped into 1 ml PBS and spun down at 3500 rpm for 4 minutes at 4°C. The cell pellets were lysed as described, and the lysates were centrifuged at 3500 rpm for 4 minutes. The supernates were stored at 70°C until use. HG treatment was significantly greater than the effect after 1 hour of HG addition. Figure 1A shows that HG treatment for 1 hour increased JNK activity as early as 30 minutes. This effect remained elevated up to 6 hours. Results from multiple experiments indicated that the stimulatory effect after 1 hour of HG treatment was significantly greater than the effect after 1 hour in NG (3.2±0.4-fold over NG; P<0.01; see bar graph in Figure 1E). The HG-induced increase in ERK activity decreased after 6 hours, and by 24 hours, values had returned to basal levels. Cells maintained in NG for these same time periods did not show any increase in ERK activity (results not shown).

**Electrophoretic Mobility Shift Assay**

Oligonucleotides corresponding to AP-1 consensus binding site for AP-1 c-Jun homodimer and Jun/Fos heterodimeric complexes were synthesized in the City of Hope Beckman Research Center DNA synthesis facility. Oligonucleotides with binding sites for Sp1 (controls) were obtained from Promega. After annealing, double-stranded DNA was labeled with γ-32P-ATP using T4 kinase (Stratagene) and purified on a Sephadex G-25 column. Then, μg of nuclear protein was incubated with 30P-labeled oligonucleotide (20 000 cpm) at room temperature for 20 minutes in binding buffer containing 12 mmol/L HEPEs (pH 7.9), 4 mmol/L Tris · HCl (pH 7.9), 60 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L DTT, 1 mmol/L PMSF, 12% glycerol, 5 μg of BSA, and 2 μg of poly(dI-dC). Protein/DNA complexes were resolved on 4% native polyacrylamide gels using 1X Tris-glycine buffer (pH 8.5). For the supershift analyses, 2 μg each of the antibodies to the AP-1 subunits, c-Fos and c-Jun (Santa Cruz Biotechnology), were preincubated with the nuclear extracts and the 32P-labeled probe for 1 hour at 4°C before the gel shift analyses. Gels were dried, and visualization and quantification of radioactive bands were carried out on a PhosphorImager (Molecular Dynamics) using ImageQuant software (National Institutes of Health).

**Data Analysis**

Results are expressed as mean±SEM. Student’s t tests or ANOVA with Dunnett’s or Tukey-Kramer post-tests were used to determine the statistical significance of the effects of glucose or Ang II at one time point using the Prism software (GraphPad). Blots from kinase assays were quantified in the linear range on a densitometer (SCISCAN 5000; US Biochemical). Electrophoretic mobility shift assay (EMSAs) were quantified on a PhosphorImager using the ImageQuant software.

**Results**

**Treatment of VSMC With HG Leads to Activation of ERK1/2, JNK, and p38 MAPKs**

Porcine VSMC were treated for various time periods with HG (25 mmol/L). Control cells were cultured in NG (5.5 mmol/L). Treatments of <24 hours were under serum-free conditions, whereas treatments of >24 hours were in the presence of serum. Figure 1A shows that HG treatment for 1 hour increased ERK1/2 activity as measured with the in-gel kinase assay. This effect remained elevated up to 6 hours. Results from multiple experiments indicated that the stimulatory effect after 1 hour of HG treatment was significantly greater than the effect after 1 hour in NG (3.2±0.4-fold over NG; P<0.01; see bar graph in Figure 1E). The HG-induced increase in ERK activity decreased after 6 hours, and by 24 hours, values had returned to basal levels. Cells maintained in NG for these same time periods did not show any increase in ERK activity (results not shown).

Figure 1B shows the effect of short-term HG addition on JNK activity in the porcine VSMC. Here, HG markedly increased JNK activity as early as 30 minutes. This effect peaked at 1 hour and remained elevated for 1.5 hours (2.9±0.2-fold over NG after 1 hour of HG, P<0.001; see Figure 1E). Cells in NG alone at these time periods showed no change in JNK activity relative to time zero (results not...
Effect of Ang II–Induced Activation of ERK, JNK, and p38 MAPK Activity Under NG Versus HG Conditions

We next compared the effect of Ang II (10^{-7} mol/L) on the activation of the key MAPKs under NG versus HG conditions. Figure 2A shows Ang II–induced ERK1/2 activity in cells treated with NG or with HG for time periods ranging from 0.5 to 6 hours. As seen earlier, HG (1 to 6 hours) alone increased basal ERK activity. Ang II (5 minutes) increased ERK activity under NG conditions, and this effect was markedly increased in cells treated with HG for 0.5 hour (Ang II in NG, 4.6±0.3-fold over NG control; Ang II after 0.5-hour HG, 8.8±0.7-fold over NG control, P<0.01; see also bar graph Figure 2F). At 1 and 3 hours, Ang II and HG displayed additive effects. Figure 2B shows that HG for longer periods of 72 hours to 5 days did not alter either basal or Ang II–induced ERK activity.

Figure 2C depicts the effect of HG treatment on Ang II–induced JNK activity. The first 4 lanes show the stimulatory effect of HG treatment for 0.5 to 1.5 hours on basal JNK activity (2.9±0.2-fold over NG at 1 hour, P<0.01). The last 4 lanes show that Ang II (10^{-7} mol/L, 30 minutes) was a potent inducer of JNK activity in these cells (4.9±0.4-fold over control, P<0.001). However, HG treatment for 0.5 to 1.5 hours did not have additive effects to that of Ang II (see also Figure 2F). Similarly, longer treatments with HG (24 and 72 hours) did not increase either basal or Ang II–induced JNK activity (Figure 2D).

We next examined the effect of Ang II on p38 MAPK activity in VSMC cultured for 24 hours under NG versus HG conditions (Figure 2E). Ang II (10^{-7} mol/L) treatment for 5 minutes led to a small but significant increase in p38 activity in cells cultured in NG (1.5±0.2-fold over NG control; see Figure 2E and bar graph in Figure 2F). Short-term HG treatment (0.5 to 3 hours) did not significantly alter Ang II–induced p38 MAPK activation (results not shown). In contrast, in cells treated with HG for 24 hours, Ang II and HG combined displayed an additive response on p38 MAPK activity (HG alone at 24 hours, 2.8±0.3-fold over NG at 24 hours; Ang II after 24 hours HG, 3.8±0.4-fold over NG control, P<0.01; see also bar graph in Figure 2F).

Effect of HG on AP-1 DNA-Binding Activity in VSMC

Because MAPK activation and subsequent c-Fos and c-Jun expression can lead to increased AP-1 activity, we first examined the effect of HG treatment on AP-1 DNA-binding activity in VSMC. EMSAs were performed on nuclear extracts from cells treated with HG for various time periods. Figure 3A shows that cells treated with 18 or 25 mmol/L HG for 6 days had markedly increased AP-1 activity in the basal state, with the 25 mmol/L concentration being more effective. Figure 3B depicts the time course of HG effects. Increased
AP-1 activity was observed as early as 3 days of HG treatment and remained elevated at 9 days. Our unpublished observations indicate that the AP-1 activity also remained sustained for >2 weeks of HG treatment.

Effect of Ang II on AP-1 Activity in Cells Cultured in NG Versus HG

We next examined whether Ang II treatment leads to differential activation of AP-1 DNA binding activity under HG conditions. VSMC cultured under NG or HG conditions for 6 to 10 days were treated with increasing concentrations of Ang II for 2 hours. Figure 4 shows that Ang II increased AP-1 DNA-binding activity in a dose-dependent fashion in cells cultured in NG as well as in HG. Basal AP-1 activity was greater in HG, and it is clearly seen that the effects of Ang II were additive to those of HG. Figure 4B is a bar graph quantification of the effects of HG and Ang II (10^{-7} mol/L) from multiple experiments and reveals a significant increase in AP-1 activity by HG alone (2.5±0.4-fold over NG control) and by Ang II, as well as the additive effect of Ang II and HG.

To determine the subunit composition in the Ang II–induced AP-1–binding complex, we performed supershift assays using antibodies specific to the most common AP-1 component proteins: c-Fos and c-Jun. As seen in Figure 5 (left), antibodies to both c-Fos and c-Jun induced supershifting of the DNA complex, with the former eliciting a stronger response. Thus, the Ang II–induced AP-1 complex contained heterodimers of c-Fos and c-Jun but may also contain other members of the Jun family because the c-Jun antibody elicited a lesser response than that expected for a 1:1 Fos/Jun complex. The specificity of the effect of Ang II is shown in Figure 5 (right). The addition of excess cold AP-1, but not
nuclear factor-κB oligonucleotide, to the reaction resulted in complete loss of DNA-binding activity induced by Ang II. In addition, we showed that the Ang II and HG effects were specific for AP-1 because Sp1 DNA binding was not affected by either Ang II or HG (results not shown).

**Discussion**

We have shown for the first time that the treatment of VSMC with HG to simulate the diabetic state could lead to increased activation of the growth- and stress-related kinases ERKs, JNK, and p38 MAPKs. Ang II also increased the activity of all three kinases. Furthermore, HG and Ang II had additive effects on ERK and p38 MAPK activation. There was a temporal difference in the effects of HG and that of Ang II plus HG on these key kinases. HG-induced increases in ERK and JNK activation were seen much earlier (0.5 to 3 hours), after which the effects were reduced almost to basal NG levels. However, stimulatory effects on p38 MAPK were evident after a few hours and remained elevated for several hours. In unpublished studies, we have also noted that p38 MAPK activity remains elevated even after several days in HG. This suggests that activation of these kinases may play specific roles in the cellular effects of glucose. ERK and JNK, as well as p38 MAPK, activation may lead to transcriptional activation of early response genes such as c-fos and c-jun, which can further lead to gene expression via AP-1 and other transcription factors. p38 MAPK may also be activated by oxidant stress induced by HG and Ang II, which usually manifests after a few hours. This initial p38 activation may then lead to gene expression via activation of other transcription factors that are targets of p38, such as CREB.

The ERKs have been implicated in Ang II–induced growth effects. Increasing evidence has now linked activation of p38 MAPK and associated transcription factors such as CREB with cellular growth and hypertrophy. A very recent study has shown that Ang II leads to the activation of p38 MAPK in VSMC by a redox-sensitive mechanism, whereas it activates ERK1/2 by a redox-insensitive mechanism. Furthermore, it was suggested that activation of both the ERKs and p38 may be necessary for the full hypertrophic response of Ang II in VSMC. However, the role of these kinases in the growth effects of HG in VSMC is not yet known. It is possible that augmented ERK and p38 activation in HG may be key mechanisms for the amplified growth effects under hyperglycemic conditions. We and other groups have demonstrated that VSMC cultured under HG conditions have increased protein kinase C (PKC) activity. Ang II also is a potent inducer of PKC. PKC has been shown to activate the MAPK pathway, presumably by the phosphorylation of raf-1. PKC may be the common upstream kinase whose activation under HG conditions leads to activation of the downstream MAPKs. This supports our observations with...
ERK and JNK in which HG increased activity only at early time periods. However, the more sustained pattern of activation of p38 by HG suggests the involvement of additional non–PKC-dependent mechanisms, such as oxidant stress or tyrosine kinase activation. Further studies will be needed to delineate the specific mechanisms, as well as functional roles, of HG-induced ERK, JNK, and p38 MAPKs in the augmented growth effects observed in HG.

In the present study, we also presented new evidence for the activation of JNK by short-term HG treatment. However, Ang II–induced increase in JNK activation was not altered by HG treatment for either short or long time periods. These observations suggest that there are subtle differences in the pathways leading to Ang II–mediated activation of JNK, p38, and ERK1/2. In general, ERKs are activated via a ras, raf, and MEK mechanism, whereas JNK and p38 are activated by a rac/Cdc42, PAK, MEKK, and MKK3/6 mechanism. Very recently, MKK7 was identified as the specific upstream activator of JNK; hence accumulating evidence indicates a high level of specificity in the ability of a cell to transmit signals from different agonists, and this is associated with the specific gene and cellular response that ensues.

Our studies have also shown that the effects of HG are not entirely a consequence of increased osmolality because the effects were only partially mimicked by mannitol. However, additional studies will be needed to determine whether HG effects on kinase activation are due to glucose transport and metabolism.

In this study, we also observed that HG alone increased AP-1 DNA-binding activity in the VSMC in a dose- and time-dependent fashion. Furthermore, Ang II increased AP-1 DNA-binding activity and had additive effects with HG. However, we noted a temporal dissociation between HG-induced ERK/JNK activation (~1 hour) and AP-1 activation (a few days), unlike with Ang II. This indicates that HG-induced AP-1 may be mediated by other mechanisms, such as PKC, which is usually activated by HG only after a few days, or oxidant stress or another, as-yet-unidentified, secondary signal activated by the initial induction of the MAPKs. Our earlier studies have also shown increased formation of 12-lipoxygenase products in VSMC cultured for several days in HG, and evidence shows that such lipoxygenase products can increase AP-1 activity in VSMC. Increased AP-1 activity under hyperglycemic conditions may account for increased expression of genes implicated in cellular growth and Ang II–induced VSMC hypertrophy under HG conditions. Studies are under way to confirm this hypothesis. It is likely that other transcription factors that are the targets of ERK, JNK, and p38 MAPKs, or other kinases, may also be activated by HG and Ang II. Examination of these will help to delineate key important signals mediating the aberrant behavior of VSMC under hyperglycemic/diabetic conditions and thereby lead to the potential development of novel therapeutic modalities for accelerated diabetic vascular disease.

Acknowledgments

This work was supported by Grant PO1-HL-55798, jointly funded by the National Institutes of Health and the Juvenile Diabetes Foundation (R.N. and J.N.) and a Grant-in-Aid from the American Heart Association (R.N.). The authors would like to thank Noe Gonzales for his technical help and expertise with the figures.

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Hypertension. 1999;33:378-384
doi: 10.1161/01.HYP.33.1.378

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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