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, 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. Control cells in NG were incubated for the same time periods. Cells then were processed for kinase assays or for nuclear and cytosolic protein extraction as described below.

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 as cytosolic fractions. Nuclear pellets were washed and resuspended in buffer containing 20 mmol/L HEPES, pH 7.9, 25% glycerol, 420 mmol/L NaCl, 1.5 mmol/L MgCl2, and 0.2 mmol/L EDTA with the protease inhibitors. This suspension was incubated at 34 minutes at 4°C, followed by centrifugation at 10 000g. The resulting supernatant containing nuclear proteins was stored at −70°C until use.

Electrophoretic Mobility Shift Assay

Oligonucleotides corresponding to AP-1 consensus binding site for c-Jun homodimer and Jun/Fos heterodimeric complexes5'CGCTTGATGACTCAGCGGAA-3' 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, 5 μg of nuclear protein was incubated with 32P-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 of 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).

Competition experiments were performed by 10 minutes preincubation of nuclear protein with 50 times excess unlabeled wild type AP-1 oligonucleotide or nonspecific oligonucleotide (NF-kB).

Activities of ERK1/2, JNK, and p38

At the end of the incubations, cells were rapidly lysed as described previously and processed for kinase assays. ERK activity was measured by the myelin basic protein (MBP) substrate-in-gel kinase assay as we described previously. Cell lysates were electrophoresed on SDS-gels containing MBP substrate for ERKs. The SDS was then washed, followed by denaturation, renaturation, and substrate phosphorylation on the gel with [γ32P]ATP. JNK activity was measured by a immune complex kinase assay wherein cell cytosolic extracts were first immunoprecipitated with a specific antibody to JNK-1 and protein A/G-Sepharose, and then activity was measured with a GST/c-Jun (1–79) substracte (Santa Cruz) as described previously. p38 MAPK activity was similarly evaluated by immune complex kinase assay using a p38 MAPK antibody and ATF-2 (1–505) protein as substrate (Santa Cruz).

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 assays (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 followed by a 24-hour serum-depletion period before the kinase assays. 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
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However, by 3 hours, there was a clear increase in p38 activity, and this was further increased by 24 hours, as seen by the increased phosphorylation of exogenous ATF2 substrate and a lower band that corresponded to p38 (Figure 1D) (3.7±0.6-fold over NG after 24-hour HG treatment, P<0.01 versus NG at 24 hours; see bar graph in Figure 1E). The last two lanes of Figure 1D show that mannitol treatment for 3 or 24 hours also had only a slight stimulatory effect on p38 MAPK activity.

Effect of Ang II–Induced Activation of ERK, JNK, and p38 Under NG Versus HG Conditions

We next compared the effect of Ang II (10⁻⁷ mol/L) on the activation of these 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

Figure 1. Effect of HG (25 mmol/L) on ERK, JNK, and p38 MAPK activation. A, Serum starved cells in NG medium were treated with HG for time periods ranging from 0.5 to 6 hours. Cells were then processed for ERK activity measurements. Shown is an autoradiogram of an MBP in gel kinase assay. B, Cells were treated similarly and processed for JNK activity determination. A representative autoradiogram of an immune complex kinase assay depicting phosphorylation of GST/c-Jun is seen. C, Cells in NG were treated with mannitol (19.5 mmol/L) for 30 minutes or 1 hour and then processed for ERK and JNK activity measurements. An autoradiogram showing phosphorylation of ATF-2 is seen. D, Serum-starved cells were treated for 3 or 24 hours with HG or with mannitol and processed for p38 MAPK activity measurements. A representative autoradiogram of an immune complex kinase assay depicting phosphorylation of GST/c-Jun is seen. E, Bar graph quantification of the results from 3 to 6 experiments expressed as mean±SEM. *P<0.01 vs effect in cells kept in NG for the same time period. Shown). The stimulatory effect of HG on JNK was lost at the 24- to 72-hour time period (see also Figure 2D), similar to ERK. Furthermore, no increase in JNK activity was seen in cells treated for time periods of HG treatment ranging from 5 days to several weeks (results not shown).

To determine whether the effect of HG was a consequence of increased osmolality, we examined the effect of mannitol (19.5 mmol/L) on ERK and JNK activity. Figure 1C shows that mannitol led to a slight increase in both ERK and JNK activity only at 30 minutes. Treatment with mannitol for longer time periods did not alter either ERK or JNK activities.

Figure 1D shows the effect of HG on p38 MAPK activity. Here, acute treatment with HG (0.5 to 1 hour) did not significantly alter p38 MAPK activity (results not shown). However, by 3 hours, there was a clear increase in p38 activity, and this was further increased by 24 hours, as seen by the increased phosphorylation of exogenous ATF2 sub-
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
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activator of JNK; hence accumulating evidence indicates a

Very recently, MKK7 was identified as the specific upstream

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pathways leading to Ang II–mediated activation of JNK, p38,

observations suggest that there are subtle differences in the

HG treatment for either short or long time periods. These

Ang II–induced increase in JNK activation was not altered by

the activation of JNK by short-term HG treatment. However,

mented growth effects observed in HG.

delineate the specific mechanisms, as well as functional roles,

of HG-induced ERK, JNK, and p38 MAPKs in the aug-

mented 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.3,4,18

Very recently, M KK7 was identified as the specific upstream

activator of JNK3; 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,1,23 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,25 and evidence shows that such lipoxy-

genase products can increase AP-1 activity in VSMC.26

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/dia-

betic conditions and thereby lead to the potential develop-

ment of novel therapeutic modalities for accelerated diabetic

vascular disease.

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