J. Biochem. 130, 351–358 (2001)

Osmotic Stress Induces HB-EGF Gene Expression *via* Ca^{2+}/Pyk2/JNK Signal Cascades in Rat Aortic Smooth Muscle Cells[1]

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Received February 10, 2001; accepted June 5, 2001

The present study was undertaken in an attempt to clarify the pathway by which hyperosmotic stress induces HB-EGF gene expression in rat aortic smooth muscle cells (RASMC). Hyperosmotic stress induced by a high concentration of glucose or mannitol resulted in an increase in HB-EGF mRNA level in a dose- and time-dependent manner. HB-EGF induction was blocked by curcumin, a *c-jun/fos* antisense oligonucleotide and a dominant-negative mutant of JNK1. Electrophoretic mobility shift assay also showed the involvement of AP-1 in HB-EGF gene expression by glucose. In addition, hyperosmotic stress induced rapid phosphorylation of Pyk2 in RASMC. TPA and calcium chelating agents (BAPTA-AM and EGTA) blocked Pyk2 phosphorylation and HB-EGF gene expression. Furthermore, HB-EGF gene expression and JNK activation by hyperosmotic stress were sensitive to PP2, an Src kinase–specific inhibitor. These findings indicate that hyperosmotic stress activates JNK *via* calcium-Pyk2 signaling cascades, which in turn induce HB-EGF gene expression.

Key words: HB-EGF, hyperosmotic stress, JNK, Src.

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Osmotic changes in the extracellular environment can influence a variety of vitally important cell functions *via* the regulation of several ion transporters (*1–4*), the activity of key metabolic enzymes (*5*) and the transcription of certain genes (*6, 7*). Hyperosmotic stress stimulates a certain extracellular signal which is involved in the regulation of protein kinases (ERKs or mitogen-activated protein kinases) as well as stress-activated protein kinases (JNK or p38) (*8–10*). While the potential link between these serine/threonine kinases and the effector functions including gene transcription has remained elusive, pharmacological data indicate that protein tyrosine phosphorylations may also play a pivotal role in the mediation of some of the hypo- or hyperosmotic stress–induced functional responses.

Expression of heparin-binding epidermal growth factor–like growth factor (HB-EGF), a potent mitogen for smooth muscle cells (SMC), is regulated by a variety of stimuli such as thrombin, angiotensin, TPA, TNF-α, shear stress, lysophosphatidylcholine, hydrogen peroxide, and reactive dicarbonyl metabolites (*11–16*). Recently, Pascall *et al.* reported that the rat HB-EGF gene promoter contains two AP-1 sites (*17*), suggesting the possible role of the JNK pathway and AP-1 in HB-EGF gene expression.

The Ca^{2+} signal acts in part by controling serine and threonine protein phosphorylation through multiple mechanisms, for example, by modulating Ca^{2+}/calmodulin-dependent protein kinase (*18*) and protein kinase C (*19*) activities. Hormones, hyperglycemia and other agonists that lead to an increase in intracellular Ca^{2+} levels (*e.g.*, thapsigargin) increase tyrosine phosphorylation, in part by activating a soluble, Ca^{2+}-dependent tyrosine kinase (CADTK/Pyk2). Pyk2 may be responsible for linking Src kinase signaling to downstream signaling events such as the activation of JNK (*20*). The accumulation of glucose in the extracellular fluid increases the osmolality of the extracellular fluid (*21*).

The goal of this study was to examine the potential role of the Src family and JNK in hyperosmotic stress-induced HB-EGF. The issue of whether Pyk2 plays an essential role in the hyperosmotic regulation of HB-EGF was examined. Our results show that high glucose, through its osmotic stress, activates JNK through Pyk2 and Src, which then induce HB-EGF in RASMC.

MATERIALS AND METHODS

Materials—BAPTA-AM was obtained from Sigma (St. Louis, MO). PP2 and piceatannol were purchased from the Calbiochem (La Jolla, CA). Glucose and 12-*O*-tetra-decanoylphorbol-13-acetate (TPA) were purchased from the Wako Pure Chemical Industry (Osaka). Mannitol was from Nacalai Tesque (Kyoto). Other chemicals were of the highest grade available.

Cell Cultures—Rat aortic smooth muscle cells (RASMC) were isolated from the thoracic aorta of a Wistar rat (body

[1] This study was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan.

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Abbreviations: HB-EGF, heparin-binding epidermal growth factor–like growth factor; RASMC, rat aortic smooth muscle cell(s); MG, methylglyoxal; 3-DG, 3-deoxyglucosone; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetraacetoxymethyl ester; Pyk2, proline-rich tyrosine kinase; JNK, c-Jun NH_2-terminal kinase.

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weight 200 g) as described previously (*22*) and cultured in Dulbecco's modified Eagle's medium (DMEM; Nikken Bio Med Lab) which contained 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin sulfate (100 μg/ml) in a humidified atmosphere of 5% CO at 37°C. The cells were passaged every 4–7 days. RASMC were cultured to about 80% confluence and then further incubated with fresh medium containing the above reagents. Throughout these experiments, the cells were used within passages 6–10.

Plasmids—Human JNK1 cDNA was prepared from a Hep3B cDNA library by the polymerase chain reaction. The polymerase chain reaction products were then cloned into the T vector (Novagen, Madison, WI) and confirmed by DNA sequencing (Applied Biosystem). The dominant-negative mutant of JNK1 (T183A/Y185F) in pcDNA3 was kindly donated by Dr. Roger Davis, Howard Hughes Medical Institute, University of Massachusetts Medical Center.

Transient Expression of the Dominant-Negative Mutants of JNK1—RASMC were plated at 1.7×10^5 cells/60-mm dish 1 day prior to transfection. Ten micrograms each of the mutants of the JNK1 expression vector was introduced into the cells using the LipofectAMINE reagent (Takara, Shiga). After incubation for 48 h, the cells were used for further analysis.

RNA Isolation and Northern Blot Experiments—Total RNA was extracted using acid guanidium thiocyanate-phenol-chloroform, as reported previously (*23*). Twenty micrograms of the total RNA was run on a 1% agarose gel which contained 2.2 mol/liter formaldehyde. The size-fractionated RNAs were transferred to Zeta-Probe membranes (Bio-Rad) overnight by capillary action. Rat HB-EGF cDNA was labeled with [α-^{32}P]dCTP (DuPont-New England Nuclear) using random hexanucleotide primers (Multiprime DNA labeling system; Amersham Pharmacia). After hybridization with the labeled probes at 42°C in the presence of 50% formamide, the membrane was washed twice with 2× sodium chloride-sodium citrate (SSC; 1× SSC, 15 mM sodium citrate 150 mM NaCl, pH 7.5), which contained 0.1% sodium dodecyl sulfate (SDS), at 50°C for 60 min, followed by a washing with 0.2× SSC with 0.1% SDS at 50°C for 30 min. Kodak X-AR films were exposed for 1–2 days to an intensifying screen at –80°C. The intensities of the bands on the X-ray films were quantitated with a CS-9000 gel scanner (Shimadzu, Tokyo).

Immunoprecipitation and Western Blotting—For immunoprecipitation, RASMC were lysed in an ice-cold lysis buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% NP-40, 10% glycerol, 10 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 10 mM β-glycerophosphate]. The lysate was centrifuged at 15,000 rpm for 15 min at 4°C, and an anti-Pyk2 monoclonal antibody (Transduction Laboratories, Lexington, KY) was added to the supernatant. The immunoprecipitates were collected by incubation with Protein A–Sepharose beads for 4 h, and the beads bearing the immunoprecipitates were washed three times with cold lysis buffer and used for Western blotting

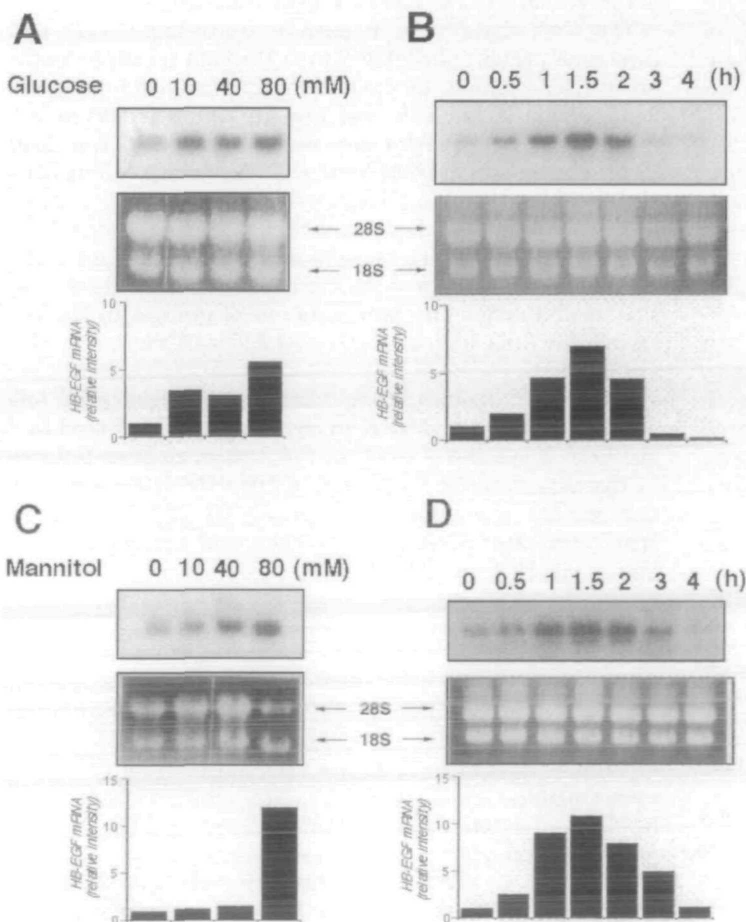

Fig. 1. **Induction of HB-EGF mRNA by glucose and mannitol.** Dose-dependence of induction by glucose (A) and mannitol (C) in 1.5 h. Time courses of induction by 80 mM glucose (B) and mannitol (D). After incubation of RASMC with glucose or mannitol, the total RNA was extracted, and a 20-μg aliquot was analyzed by Northern blotting with a ^{32}P-labeled rat HB-EGF probe. Densitometric analysis of HB-EGF mRNA levels was normalized to the level of 28S rRNA. Similar results were obtained in three separate experiments.

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with anti-Pyk2 antibody or anti-phosphotyrosine monoclonal antibody, 4G10.

JNK Assay—JNK activity was measured with a solid-phase (S-P) kinase assay (*24*) using GST-c-Jun (1–79 amino acid sequence). Following treatment with various reagents, RASMC were harvested and washed with PBS. The resulting cells were suspended in WCE buffer (20 mM HEPES, pH 7.7, 0.05% Triton X-100, 0.1 mM EDTA, 75 mM NaCl, 2.5 mM $MgCl_2$, 0.5 mM DTT, 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 100 μg/ml PMSF, 2 μg/ml leupeptin) and rotated at 4°C for 30 min. After centrifugation at 10,000 ×*g* for 10 min, the supernatant was collected and used as the whole-cell extract. The protein concentration in this extract was determined by means of a Bio-Rad protein assay kit. Ten microliters of glutathione-Sepharose and 1 μg GST-c-Jun (Santa Cruze Biotechnology) were added to the extract, which was then rotated at 4°C for 3 h. After washing, the pelleted glutathione beads, which contained the bound proteins, were incubated at 30°C for 30 min in the reaction buffer [25 mM HEPES (pH 7.5), 10 mM magnesium acetate, 50 μM ATP] containing 5 μCi [γ-^{32}P]ATP and 1 μg GST-c-Jun. After termination of the reaction by adding sample buffer, the proteins were separated by 10% SDS-PAGE, followed by drying and autoradiography.

Electrophoretic Mobility Shift Assays—23-mer double-stranded oligonucleotides containing the consensus sequence TGAGTCA of the AP-1 binding site were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Takara, Osaka). Binding reactions were performed for 30 min at room temperature with 5 μg of protein sample in 20 μl of binding buffer [30 mM Tris-HCl, pH 7.4, 30 mM KCl, 0.6 mM EDTA, 12% (v/v) glycerol, 5 mM DTT, and 0.5 mM PMSF] containing 1 μg of poly(dI-dC) and 20,000 cpm of ^{32}P-labeled oligonucleotide. DNA-protein complexes were electrophoresed on 6% native polyacrylamide gels in 1× TAE buffer (6.7 mM Tris-HCl, 3.3 mM NaOAc, and 1 mM EDTA, pH 7.5). The resulting gel was dried and then exposed to a Kodak X-ray film at –80°C.

Preparation of Oligonucleotides—Antisense *fos* (5′-TGC-GTT-GAA-GCC-CGA-GAA-3′) and *jun* (5′-CGT-TTC-CAT-CTT-TGC-AGT-3′) unsubstituted oligodeoxynucleotides were obtained as synthesized materials (Life Technologies). These nucleotide sequences were complementary to the first 18 bases after the AUG sequences of the *fos* (*25*) and *jun* (*26*) mRNAs, and the corresponding sense oligonucleotides were also synthesized and used as controls.

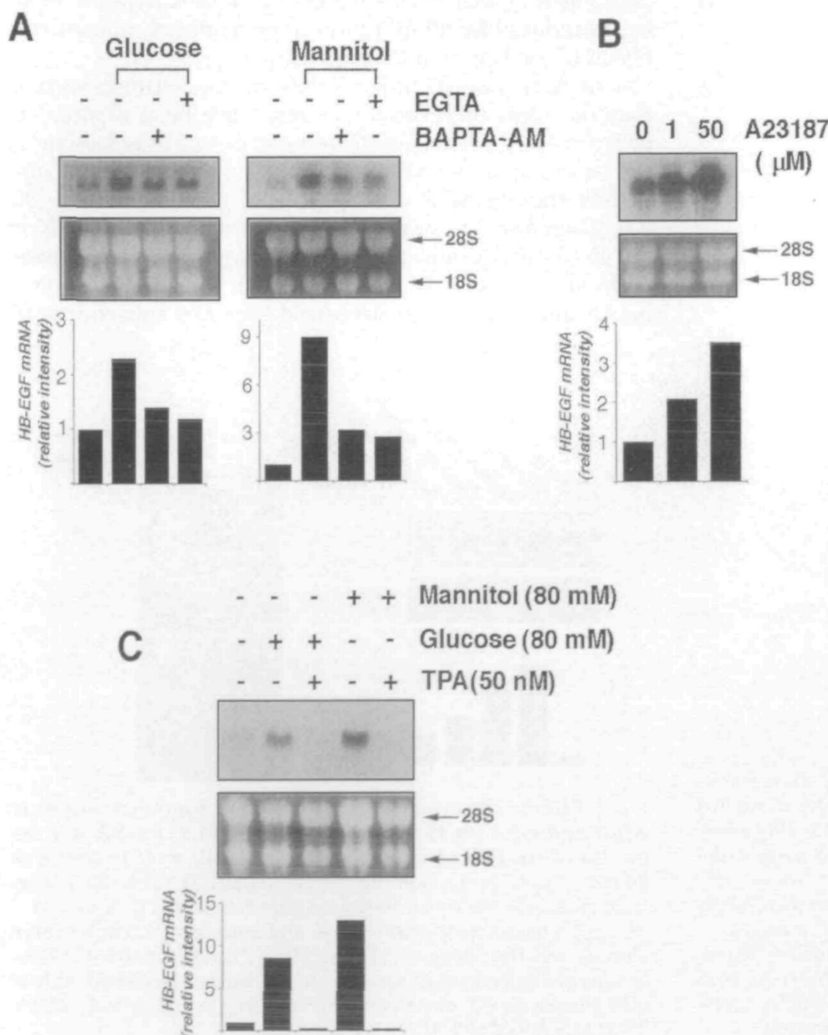

Fig. 2. **Effects of Ca^{2+} on the enhancement of HB-EGF mRNA levels by glucose or mannitol.** (A) Cells were incubated in the presence of 80 mM glucose or mannitol for 1.5 h after preincubation for 1 h in the presence of 3 mM EGTA or 10 μM BAPTA-AM. (B) Dose-dependence of induction by A23187 in 1 h. (C) After incubation of RASMC with 50 nM TPA for 24 h, the cells were treated with 80 mM glucose or mannitol. Northern blotting was then done using a rat HB-EGF cDNA probe. Similar results were obtained in two separate experiments.

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RESULTS

Induction of HB-EGF mRNA by Glucose and Its Analogues in RASMC—The effect of glucose on HB-EGF gene expression levels in RASMC was examined by means of Northern blot analyses. When RASMC were incubated with various levels (5.5–80 mM) of glucose for 2 h, a dose-dependent increase was observed in HB-EGF mRNA levels, compared to the controls (Fig. 1A). The levels of HB-EGF mRNA were increased 3–6-fold in response to treatment with 10–80 mM glucose for 2 h. Eighty millimolar glucose induced HB-EGF mRNA in a time-dependent manner. After the addition of glucose, an increase in the level of HB-EGF mRNA was observed at 30 min and reached a maximum level after 1.5 h. The elevated level of HB-EGF mRNA returned to near basal level after 3 h (Fig. 1B). Mannitol induced a similar increase in HB-EGF mRNA level to glucose (Fig. 1, C and D), indicating that hyperosmotic stress could be involved in the upregulation of HB-EGF gene expression in RASMC, as it is in vascular endothelial cells (*27, 28*).

Effects of Ca^{2+} on the Enhancement of HB-EGF mRNA Levels by Glucose—The observed increase of intracellular calcium concentration appears to be related to hyperosmotic stress (*29, 30*). Recently Kayanoki *et al.* showed that HB-EGF gene expression was induced by Ca^{2+} influx in both vascular endothelial cells and smooth muscle cells (*31*). To investigate whether calcium-dependent pathways are involved in HB-EGF mRNA induction by glucose, the effects of EGTA and BAPTA-AM, chelators of intracellular Ca^{2+}, were examined. RASMC were treated with 10 μM BAPTA-AM for 1 h before stimulation with glucose and mannitol. As shown in Fig. 2A, 3 mM EGTA and 10 μM BAPTA-AM partially inhibited HB-EGF expression induced by glucose or mannitol in a similar manner. These data indicate that an increase in intracellular calcium concentrations is associated with the induction of HB-EGF in RASMC by glucose and mannitol. Furthermore, incubation of RASMC with various levels (1–50 μM) of A23187, a calcium ionophore, for 1 h increased the levels of HB-EGF mRNA in a dose-dependent manner (Fig. 2B). To examine whether HB-EGF mRNA induction by hyperosmotic stress was PKC-dependent, the effect of PKC depletion on HB-EGF mRNA expression was also investigated. Pretreatment with 50 nM TPA for 24 h abolished the stimulatory effect of 80 mM glucose or mannitol on HB-EGF gene expression (Fig. 2C). These results show that hyperosmolarity, as induced by 80 mM glucose or mannitol, upregulates HB-EGF *via* Ca^{2+} and PKC activation.

JNK Activation by Hyperosmotic Stress—Stimuli such as osmotic stress and oxidative stress have been reported to activate JNK, which phosphorylates c-Jun, thus leading to the stimulation of AP-1 transcription activity, and, ultimately, the control of AP-1–regulated gene expression (*32, 33*). Therefore, we were interested in the role of JNK in HB-EGF mRNA induction by hyperosmotic stress. Glucose-induced activation of JNK occurred rapidly, reaching a maximum at 1 h, then decreased over the subsequent 90

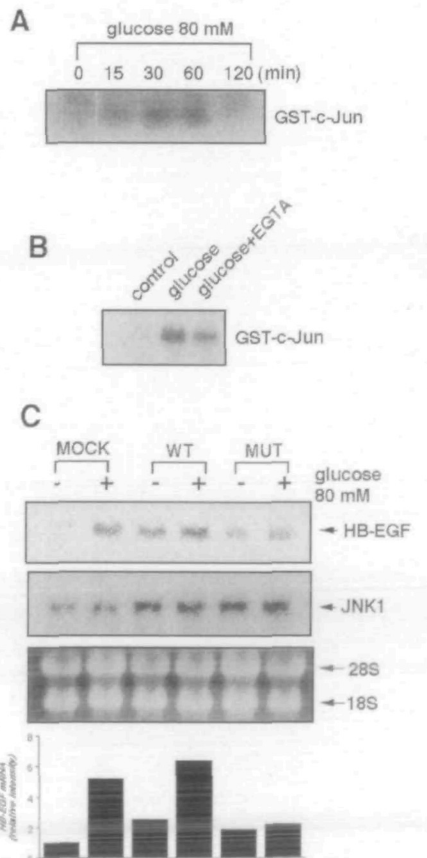

Fig. 3. **Stimulation of JNK activity by glucose.** (A) Time course of activation of JNK by glucose (80 mM). (B) Cells were incubated with 80 mM glucose with or without 3 mM EGTA for 1 h. Whole-cell extracts were used to determine JNK activity by the S-P assay as described under "MATERIALS AND METHODS." (C) Vector only (MOCK), JNK1 wild type (WT), or pcDNA3 JNK1 dominant-negative mutant (MUT) was transfected into RASMC, and a portion of the transfected cells was stimulated with 80 mM glucose. Stimulated and unstimulated cells were then subjected to Northern blotting using a rat HB-EGF cDNA probe and JNK1 cDNA probe. Similar results were obtained in three separate experiments.

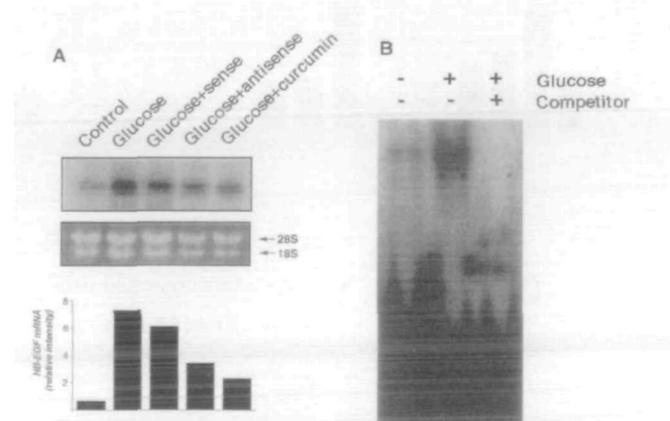

Fig. 4. **Effects of curcumin and antisense oligonucleotides to *c-jun* and *c-fos* on the induction of HB-EGF mRNA and increase of AP-1 binding activity.** (A) RASMC were treated with 80 mM glucose for 1.5 h in the presence of 20 μM curcumin or were treated 2.5 μM antisense and sense oligonucleotides to *c-jun* and *c-fos* for 3 h before treatment with 40 mM glucose for 1.5 h. Northern blotting was then done using a rat HB-EGF cDNA probe. (B) Electrophoretic mobility shift assay for AP-1. RASMC incubated with 40 mM glucose for 30 min. Detailed procedures are described in "MATERIALS AND METHODS."

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min (Fig. 3A). The glucose-induced activation of JNK was partially blocked by 3 mM EGTA (Fig. 3B). To test whether JNK activation modulates HB-EGF gene expression, plasmids containing the dominant-negative mutant of JNK1 (T183A/Y185F) were transfected into RASMC. The expression of HB-EGF induced by 80 mM glucose was decreased in comparison with a mock transfectant (Fig. 3C). These results suggest that JNK activation by hyperosmotic stress leads to the upregulation of HB-EGF gene expression.

Involvement of AP-1 in High Glucose–Induced HB-EGF Gene Expression—We further examined the effect of curcumin, an inhibitor of c-Jun/AP-1 (*34*), on high glucose–induced HB-EGF gene expression in RASMC. As shown in Fig. 4, the HB-EGF gene expression induced by 80 mM glucose was slightly inhibited by 20 μM curcumin. As curcumin is also known to have inhibitory effects on JNK or NF-κB, we examined the effect of antisense of *c-fos* and *c-jun*. The cells were pretreated for 3 h with 2.5 μM antisense or sense oligonucleotides, then treated with 80 mM glucose and analyzed by Northern blot assay. The simultaneous addition of *c-fos* and *c-jun* antisense oligonucleotides to the cells inhibited 80 mM glucose–induced HB-EGF mRNA expression, compared with the sense oligonucleotides (Fig. 4A). Further, we employed the electrophoretic mobility shift assay using double-stranded AP-1 oligonucleotides as a probe. We found that high glucose stimulated the binding of nuclear proteins to AP-1 in a time-dependent manner (data not shown). High glucose–stimulated binding was eliminated when an unlabeled AP-1 oligonucleotides were added as a competitor (Fig. 4B). These observations indicated that AP-1 plays an important role in HB-EGF expression in RASMC.

Pyk2 Tyrosine Phosphorylation by Hyperosmotic Stress—To assess the link between these hyperosmotic stress–induced signaling events, the effect of high concentrations of glucose on the proline-rich tyrosine kinase (Pyk2) was examined. Pyk2 was reported to be activated by a variety of stimuli that increase $[Ca^{2+}]_i$ or activate PKC. The rapid phosphorylation of Pyk2 was observed in cells which had been stimulated with 80 mM glucose (Fig. 5A). Further, phosphorylation of Pyk2 by 80 mM glucose was blocked by 3 mM EGTA, 10 μM BAPTA-AM, and pretreatment with 50 nM TPA for 24 h (Fig. 5B). These results are in agreement with the observation that hyperosmotic stress induces the rapid tyrosine phosphorylation of Pyk2 through PKC and Ca^{2+}.

Effects of PP2 on the Hyperosmotic Stress–Induced HB-EGF Gene Expression—The Ca^{2+}-dependent activation of Pyk2 induced MAP kinase *via* a direct interaction with Src (*35, 36*). Therefore, effect of the Src kinase–specific inhibitor PP2 on the hyperosmotic stress–induced HB-EGF gene expression was investigated. Because Syk protein–tyrosine kinase is rapidly activated by osmotic stress (*37*), the effect of a Syk-specific kinase inhibitor (piceatannol) was also investigated. HB-EGF gene expression by 80 mM glucose, 40 mM sorbitol, and 50 mM NaCl was blocked by treatment with 10 μM PP2, but not with 10 μM piceatannol (Fig. 6). Further, the effect of the Src kinase–specific inhibitor PP2 on the hyperosmotic stress–induced JNK activation was investigated. As shown in Fig. 7, hyperosmotic stress–induced JNK activation was blocked by PP2. These results suggest that the induction of Pyk2 phosphorylation by hyperosmotic stress involves activation of Src, which regulates HB-EGF gene expression. However, the induction of HB-EGF gene expression by hyperosmotic stress does

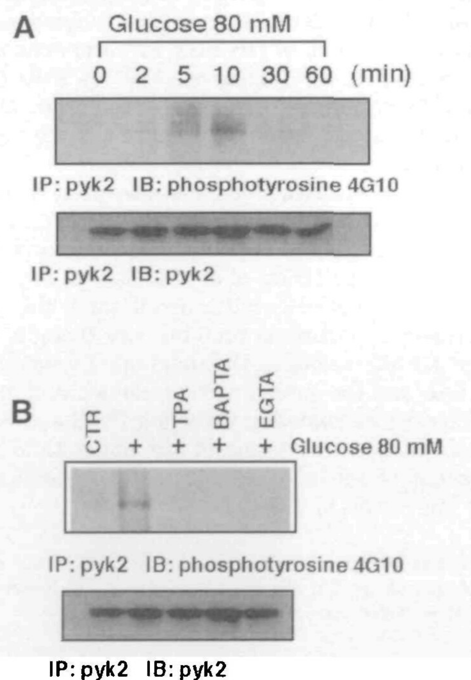

Fig. 5. **Phosphorylation of Pyk2 by glucose.** (A) RASMC were cultured with or without glucose (80 mM) for the indicated times. (B) RASMC were incubated with 80 mM glucose for 10 min with or without 3 mM EGTA, 50 nM TPA, 10 μM BAPTA-AM. Cell lysates were immunoprecipitated with anti-Pyk2 antibody, and the immunocomplexes were subjected to immunoblot assay with the anti-phosphotyrosine antibody (upper panel). The membrane was then stripped and restained with anti-Pyk2 antibody (lower panel). IP: immunoprecipitation; IB: immunoblotting.

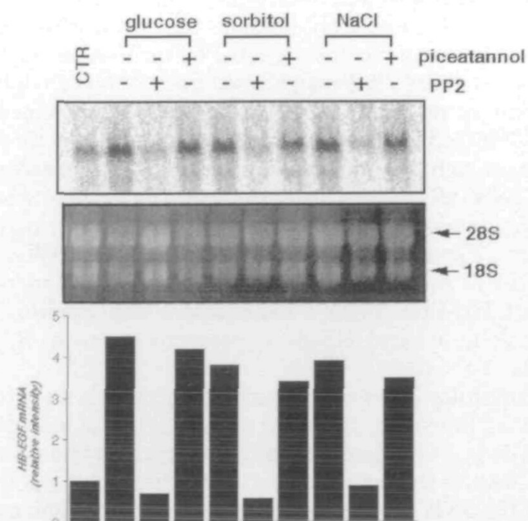

Fig. 6. **Effects of PP2 or piceatannol on the hyperosmotic stress–induced HB-EGF gene expression.** RASMC were treated with 80 mM glucose, 40 mM sorbitol or 50 mM NaCl for 1.5 h in the presence of 10 μM PP2 or piceatannol. Northern blotting was then done using a rat HB-EGF cDNA probe.

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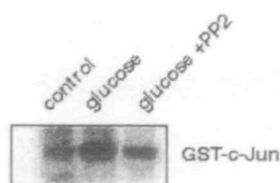

Fig. 7. **Effects of PP2 on the hyperosmotic stress–induced JNK activation.** RASMC were treated with 80 mM glucose for 1.5 h in the presence of 10 μM PP2. Whole-cell extracts were used to determine JNK activity by the S-P assay as described under "MATERIALS AND METHODS."

not need the Syk activation, because HB-EGF gene expression was not blocked by the Syk-specific kinase inhibitor piceatannol.

DISCUSSION

The present study was undertaken in an attempt to clarify the pathway by which hyperosmotic stress induces HB-EGF gene expression in RASMC. We have shown that osmotic stress induced by 80 mM glucose or mannitol rapidly induces Pyk2 phosphorylation and JNK activation *via* Ca^{2+}. The Src kinase inhibitor PP2 and the transfection of a dominant-negative mutant of JNK1 inhibited the induction of HB-EGF gene expression by hyperosmotic stress. From these studies, it could be concluded that osmotic stress induces HB-EGF *via* JNK activation by Pyk2 and Src signal cascade in RASMC.

Hyperglycemia, a recognized pathogenetic factor of long-term complications in diabetes mellitus, not only generates more reactive oxygen species but also attenuates antioxidative mechanisms *via* the glycation of the scavenging enzymes. Elevated glucose levels induce lipid peroxidation and oxidative stress, ultimately causing cell death and cellular insulin resistance, and enhancing the production of extracellular matrix components (*38–41*). Hyperosmolarity can be produced not only by glucose and sorbitol, but also by other sugar alcohols, neutral free amino acids and other metabolites. It is entirely possible that all of these might participate in the increase of HB-EGF mRNA levels. NaCl, fructose, sorbitol, 2-deoxyglucose, and 3-*O*-methylglucose all cause an increase in HB-EGF mRNA expression levels in RASMC (data not shown). Because hyperosmotic stress causes an increase of intracellular calcium concentration in many cells (*29, 30*), we also examined the issue of whether osmotic stress–induced HB-EGF is mediated by increased calcium. These experiments showed that EGTA and BAPTA-AM significantly suppressed glucose and mannitol-induced HB-EGF mRNA expressions, and calcium ionophore alone induced HB-EGF gene expression in RASMC (Fig. 2).

Intracellular calcium appears to function as an intermediate that links osmotic stress signals with the induction of HB-EGF. It is well known that osmotic stress and oxidative stress lead to the activation of JNK. Thereby, the potential role of the JNK pathway and AP-1 in HB-EGF gene expression was examined. HB-EGF gene expression induced by osmotic stress was blocked by the transfection of a dominant-negative JNK1 mutant. These findings suggest that HB-EGF gene expression is dependent on JNK activation.

Treatment of RASMC with TPA, which downregulates protein kinase C, had no effect on the MG-induced expression of the HB-EGF gene (*16*), but completely blocked high-glucose induction of HB-EGF mRNA (Fig. 2). We also found that MG induced JNK activation (data not shown). These data indicate that, although the signals of MG or high glucose are distinct, the two kinds of stress (MG and high glucose levels) are clearly dependent on the activation of JNK.

It has previously been shown that calcium-dependent activation of Pyk2, a novel focal adhesion kinase family protein–tyrosine kinase, occurs upstream of MAP kinase and the JNK pathway (*42, 43*). Pyk2 distribution was demonstrated in rat aortic tissue and in cultured VSMC by immunohistochemistry, revealing a cytosolic distribution which is distinct from smooth muscle α-actin, focal adhesion kinase, or paxillin (*44*). Recently, Yoshizumi *et al.* showed that Src and Cas mediate the activation of JNK, but not ERK and p38 kinases by H_2O_2 (*45*).

On the basis of these observations, we investigated the possibility that Pyk2 and Src signaling pathway is involved in hyperosmotic stress-induced HB-EGF gene expression. Treatment of RASMC with 80 mM glucose resulted in a time-dependent increase in Pyk2 tyrosine phosphorylation. Furthermore, HB-EGF gene expression and JNK activation by osmotic stress were blocked by PP2, an Src kinase inhibitor. These results suggest that Pyk2 and Src signaling cascades are involved in the induction of HB-EGF gene expression by osmotic stress.

Angiotensin II infusion dramatically promoted vascular pathology, including an increase in the extent of atherosclerosis, a change in the nature of the lesions and surrounding adventitial tissue, and formation of large abdominal aortic aneurysms. It was demonstrated that angiotensin II increased the expression of HB-EGF (*12*) and activated Pyk2 and the Src signaling cascade *via* Ca^{2+} in SMC (*44*). This and the observations herein suggest that the Ca^{2+}-dependent induction of HB-EGF may require a Pyk2 and Src signaling cascade.

In conclusion, this study demonstrates that hyperosmotic stress, by increasing intracellular calcium as an intermediate, induces HB-EGF in RASMC *via* JNK and Pyk2/Src pathways. The toxic effects of excess glucose, including protracted hyperosmotic activity, accelerated the nonenzymatic glycation of various proteins, are thought to be responsible for alterations in the function of vascular smooth muscle cells and the subsequent development of atherosclerosis. The calcium signaling involving Pyk2 and Src is also an important event in terms of our understanding of the development of atherosclerosis and the induction of HB-EGF by high levels of glucose.

We thank our laboratory colleagues for helpful advice during the course of this study. We are very grateful to Dr. Roger Davis for generously providing the dominant-negative JNK1.

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