High glucose and insulin inhibit VSMC MKP-1 expression by blocking iNOS via p38 MAPK activation

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Begum, Najma, and Louis Ragolia. High glucose and insulin inhibit VSMC MKP-1 expression by blocking iNOS via p38 MAPK activation. Am. J. Physiol. Cell Physiol. 278: C81–C91, 2000.—Our laboratory has recently demonstrated a role for the phosphatidylinositol 3-kinase-mediated inducible NO synthase (iNOS) signaling pathway in acute regulation of insulin-induced mitogen-activated protein phosphatase-1 (MKP-1) expression in primary cultures of rat aortic vascular smooth muscle cells (VSMCs) (N. Begum, L. Ragolia, M. McCarthy, and N. Duddy. 2000 the American Physiological Society C81http://www.ajpcell.org). We now show that prolonged treatment of VSMCs with 100 nM insulin and high glucose (25 mM) for 12–24 h, to mimic hyperinsulinemia and hyperglycemia, completely blocked MKP-1 mRNA and protein expression in response to subsequent acute insulin treatment. To understand the mechanism of insulin resistance induced by high glucose and insulin, we studied the regulation of iNOS protein induction in these cells. Both high glucose and chronic insulin treatment caused a marked impairment of iNOS induction in response to acute insulin. Blocking of signaling via the p38 mitogen-activated protein kinase (MAPK) pathway by prior treatment for 1 h with SB-203580, a synthetic p38 MAPK inhibitor, completely prevented the inhibition of iNOS induced by high glucose and insulin and restored MKP-1 induction to levels observed with acute insulin treatment. In contrast, PD-98059, a MEK inhibitor, had no effect. Furthermore, high glucose and chronic insulin treatment caused sustained p38 MAPK activation. We conclude 1) that chronic insulin and high glucose-induced insulin resistance is accompanied by marked reductions in both iNOS and MKP-1 inductions due to p38 MAPK activation that leads to excessive cell growth and 2) that p38 MAPK/extracellular signal-regulated kinase pathways regulate iNOS induction, thereby controlling MKP-1 expression, which in turn inactivates MAPKs as a feedback mechanism and inhibits cell growth.

hyperglycemia; insulin resistance; cell growth; extracellular signal-regulated kinase signaling; inducible nitric oxide synthase; mitogen-activated protein phosphatase-1; mitogen-activated protein kinase; vascular smooth muscle cells

INSULIN RESISTANCE, hyperinsulinemia, and diabetes are closely associated with cardiovascular complications such as atherosclerosis and hypertension (8, 25, 33). The mechanisms linking hyperinsulinemia and hyperglycemia with these cardiovascular complications are poorly understood (8, 25, 33). Vascular smooth muscle cells (VSMCs) are a major constituent of blood vessel walls responsible for the maintenance of vascular tone (26). Accelerated VSMC growth, hypertrophy, and abnormal vascular tone play a central role in the development of atherosclerosis (30). Although alterations in insulin action of the vasculature due to hyperglycemia and hyperinsulinemia have been proposed to contribute to atherosclerosis and the regulation of vascular tone, little is known about the specific cellular signaling pathways that mediate the detrimental hyperinsulinemic and hyperglycemic effects in VSMCs.

Increasing evidence suggests that mitogen-activated protein kinase (MAPK) family members play a major role in the regulation of cell growth and differentiation in VSMCs (7, 22, 24, 27, 36). MAPKs are activated in response to growth factors and stress signals and have been implicated in VSMC proliferation, hypertrophy, and migration, all key processes in the pathology of vascular diseases such as atherosclerosis and hypertension. Four groups of MAPKs have been identified in mammalian cells: the extracellular signal-regulated kinases 1 and 2 (ERK1/ERK2, also known as p42/44 MAPK), the c-j un NH2-terminal kinases (JNKs, also known as stress-activated protein kinase or SAPK), p38 MAPK, and Big MAPK (ERK5) (24). Although MAPK family members are structurally related, they are generally activated via multistep phosphorylation cascades by distinct extracellular stimuli and phosphorylate different molecular substrates (27). The classic ERKs, ERK1 and ERK2, are activated through Ras-dependent signal transduction pathways by hormones and growth factors, whereas JNKs and p38 MAPKs are activated by environmental stress, oxidants, lipopolysaccharides, osmotic stress, heat shock, and cytokines (i.e., tumor necrosis factor-α and interleukin-1), leading to alterations in cell growth, prostanoid production, and other cellular dysfunctions (35).

The activities of all four members of MAPK family are regulated by the reversible phosphorylation of tyrosine and threonine residues, indicating that protein phosphatases play a critical role in regulating the activation status of these enzymes. Inactivation of MAPK signaling is mediated by a class of dual-specificity protein phosphatases (17, 31). These include mitogen-activated protein phosphatase-1 (MKP-1; also termed CL100, Erp, and hVH-1), which is encoded by the murine gene 3ch134 (17), MKP-2, MKP-3, PAC-1,
and B23 (17, 31). MKP-1, the most ubiquitously expressed and best studied of these phosphatases, has dual catalytic activity toward phosphotyrosine and phosphothreonine and is known to inactivate ERKs, JNK, and high-osmolality glycerol p38 (p38HOG) in vivo as well as in vitro (36). MKP-1 and the other family members are principally regulated at the transcriptional level, as evidenced by very low to undetectable mRNA expression in quiescent cells and a rapid mRNA induction after treatment of cells with growth factors or with agents that cause oxidative stress and heat shock (36). MKP-1 has been implicated in a feedback loop serving to inactivate MAPKs after stimulation by mitogens as well as during the cellular response to stress (36).

We have recently shown that physiological concentrations of insulin rapidly induce MKP-1 expression in primary cultures of VSMCs (2, 4). Blocking of NO synthase (NOS) and cGMP (a downstream effector of NOS) signaling with N-[monomethyl-L-arginine (L-NMMA) and Rp-8-(4-chlorophenylthio)-guanosine 3′,5′-cyclic monophosphate (Rp-cGMP), two specific inhibitors of NOS and cGMP, respectively, as well as with wortmannin, an inhibitor of phosphatidylinositol 3-kinase (PI 3-kinase), completely abolished insulin-mediated induction of MKP-1. Moreover, VSMCs isolated from spontaneously hypertensive rats exhibited resistance to insulin with respect to MKP-1 expression because of defective signaling via the NOS signaling pathway, leading to sustained MAPK activation and excessive cell growth. These observations, together with the fact that the induction of inducible NOS (iNOS) by insulin precedes MKP-1 expression and the fact that induction of MKP-1 could be mimicked by sodium nitroprusside (an NO generator) and dibutyryl guanosine 3′,5′-cyclic monophosphate (a cGMP agonist), suggested that insulin regulates the induction of MKP-1 via the PI 3-kinase-NO-cGMP signaling pathway (2).

In this study, we tested the hypothesis that high glucose (hyperglycemia) and chronic insulin treatment inhibit vasorelaxation and promote excessive cell growth by blocking the induction of iNOS and MKP-1. In addition, we characterized the signaling mechanism by which sustained insulin level and elevated glucose level exert their growth-stimulatory effects in VSMCs. Because stress-related signals mediate hypertrophy in VSMCs and because many stress factors [such as hyperosmolarity, glycation end products, oxidant formation, and diacylglycerol protein kinase C (PKC) activation] have been shown to be present in diabetes and insulin-resistant states (6, 10, 18, 34), we examined the contribution of the stress signaling pathway in the regulation of iNOS and MKP-1 induction under conditions of high glucose and insulin.

The results of the present study indicate that prolonged treatment of VSMCs with insulin and high glucose to simulate hyperinsulinemia and hyperglycemia completely blocked the induction of iNOS protein and inhibited MKP-1 mRNA and protein expression due to elevations in p38 MAPK activity. Blocking of the signaling via p38 MAPK with SB-203580, a p38 MAPK inhibitor, restored cellular responsiveness of iNOS expression and MKP-1 induction.

**METHODS**

Materials. Fetal bovine serum, antibiotics, trypsin, l-glutamine, freezing medium, α-MEM, and DMEM containing high glucose were obtained from Life Technologies (Grand Island, NY). [α-32P]dCTP (sp act 3,000 Ci/mmol), and [γ-32P]ATP were purchased from DuPont NEN (Boston, MA). Type I collagenase was from Worthington Biochemical (Freehold, NJ). The antibodies against MKP-1 and iNOS and the activating transcription factor-2 (ATF-2) substrate (1–96) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphospecific antibodies against p38 MAPK and ERKs were obtained from New England Biolabs. Protein A/G-agarose was from Oncogene Science (Cambridge, MA). PD-98059 and SB-203580 were from Biomol (Plymouth Meeting, PA). SDS-PAGE supplies and reagents for Western blot analyses were from Bio-Rad (Hercules, CA). Rat MKP-1 cDNA was a kind gift of Dr. J. Jotirmoy Kusari (Tulane University, New Orleans, LA). FITC-conjugated α-actin antibody, mannnit, and all other chemicals and reagents were purchased from Sigma Chemical (St. Louis, MO).

Culture of VSMCs and treatment with high glucose and insulin. VSMCs in primary culture were obtained by enzymatic digestion of the aortic media of male normotensive Wistar Kyoto (WKY) rats (body wt 200–220 g), as described in our recent publications (2, 4). Subcultures of VSMCs at passages 3–5 were used in all the experiments. VSMCs prepared from these rats were not contaminated with fibroblasts or endothelial cells as evidenced by a >95% positive immunostaining of smooth muscle α-actin with FITC-conjugated α-actin antibody (data not shown). All experiments on MKP-1 induction, iNOS, p38 MAPK, and DNA synthesis were performed on highly confluent cells (9–11 days in culture) at passage 5. Before each experiment, cells were serum starved for 24 h in α-MEM containing 5.5 mM glucose and antibiotics. The next day, cells were exposed to either normal glucose (5.5 mM) or high glucose (25 mM) in the presence and the absence of insulin (100 nM) for 12–24 h, followed by acute insulin treatment for 30 min. Before acute insulin treatment, cells that were exposed to chronic insulin for 12 and 24 h were rinsed exhaustively with serum-free α-MEM containing 5.5 mM glucose and antibiotics. The next day, cells were exposed to either normal glucose (5.5 mM) or high glucose (25 mM) in the presence and the absence of insulin (100 nM) for 12–24 h, followed by acute insulin treatment for 30 min. Before acute insulin treatment, cells that were exposed to chronic insulin for 12 and 24 h were rinsed exhaustively with serum-free α-MEM containing 5.5 mM glucose to completely remove insulin and were left in this medium for 1 h. In some experiments, VSMCs were pretreated with various inhibitors for 30 min, followed by chronic exposure to insulin or high glucose. To prevent glucose and insulin depletion, the medium was changed to fresh medium containing high glucose or insulin every 7 h. The cells were used 24 h after exposure to high glucose or insulin. In some experiments, mannnit (19.5 mM) was used to control variations due to osmotic pressure.

Northern blot analysis of MKP-1 mRNA expression. Serum-starved VSMCs exposed to normal glucose, high glucose, and insulin for 24 h were incubated in the presence or absence of insulin (0–100 nM) for 30 min. Total RNA was extracted with guanidinium isothiocyanate using a Qiagen RNAeasy kit as per the manufacturer’s instructions and quantitated by measurement of the ratio of absorbance at wavelengths of 260 and 280 nm. Equal amounts of RNA (5 µg/lane) were separated on a 1.2% agarose-formaldehyde denaturing gel, transferred by capillary action overnight to a nitrocellulose membrane, hybridized with 32P-labeled MKP-1 cDNA, and detected by autoradiography with standard protocols (2, 4, 16). The
membrane was stripped by boiling for 5 min in 1% SDS and re-probed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The MKP-1 mRNA and GAPDH expressions were quantitated by densitometric analyses of the autoradiograms. The MKP-1 mRNA was normalized with respect to GAPDH.

Immunoblot analysis of MKP-1 and iNOS protein expression. Immunodetection of MKP-1 and iNOS proteins in control VSMCs and in VSMCs treated with normal glucose, high glucose, and insulin were performed by Western blot analyses as described in our recent publication (2). Briefly, 50–100 µg cell lysate proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were probed with anti-MKP-1 antibody and anti-iNOS antibody according to the manufacturer’s protocols. Visualization of the primary antibody was with horseradish peroxidase (HRP)-conjugated secondary antibodies, followed by enhanced chemiluminescence (ECL). Autoradiograms with linear signal were quantitated by densitometric scanning. In the initial studies, linearity of the ECL signal was established by blotting various dilutions of the second antibody conjugated to HRP.

Detection of p38 MAPK and ERK1/ERK2 phosphorylation by Western blot analyses. Serum-starved VSMCs were stimulated with insulin (100 nM) for 30 min (acute treatment) or 12–24 h in the presence and the absence of high glucose. The dishes were thawed on ice, and the cells were lysed with 12–24 h in the presence and the absence of high glucose. The membranes were probed with anti-MKP-1 antibody and anti-iNOS antibody antibody according to the manufacturer’s protocols. Visualization of the primary antibody was with horseradish peroxidase (HRP)-conjugated secondary antibodies, followed by enhanced chemiluminescence (ECL). Autoradiograms with linear signal were quantitated by densitometric scanning. In the initial studies, linearity of the ECL signal was established by blotting various dilutions of the second antibody conjugated to HRP.

Immunoprecipitation and assay of IRS-1-associated PI 3-kinase activity. Immunoprecipitation of cell lysates normal-ized to 100 µg protein were separated on 10% SDS-polyacrylamide gels and transferred to PVDF membrane (2–4). The membranes were probed with phosphospecific p38 MAPK antibodies and phosphospecific ERK1/ERK2 antibodies, followed by detection with HRP-conjugated secondary antibody using an ECL detection kit supplied by Amersham. Immunoprecipitation and assay of p38 MAPK activity. p38 MAPK activity was measured by immune complex kinase assay using p38 MAPK antibody with ATF-2 as a substrate. Briefly, equal amounts of cell lysate proteins (500 µg) from above were immunoprecipitated overnight at 4°C with 2 µg of anti-p38 MAPK antibody. The next day, the immunoprecipitates were captured by incubation with 100 µl (50% vol/vol) of protein A-Sepharose at 4°C. Cell lysates normalized to 100 µg protein were separated on 10% SDS-polyacrylamide gels and transferred to PVDF membrane (2–4). The membranes were probed with phosphospecific p38 MAPK antibodies and phosphospecific ERK1/ERK2 antibodies, followed by detection with HRP-conjugated secondary antibody using an ECL detection kit supplied by Amersham. Immunoprecipitation and assay of p38 MAPK activity. p38 MAPK activity was measured by immune complex kinase assay using p38 MAPK antibody with ATF-2 as a substrate. Briefly, equal amounts of cell lysate proteins (500 µg) from above were immunoprecipitated overnight at 4°C with 2 µg of anti-p38 MAPK antibody. The next day, the immunoprecipitates were captured by incubation with 100 µl (50% vol/vol) of protein A-Sepharose at 4°C with gentle shaking. The beads were washed four times with lysis buffer and twice with kinase buffer containing 25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate, 10 mM MgCl₂, and a cocktail of protease inhibitors. The beads were resuspended in 50 µl of kinase buffer containing 0.1 mM MgCl₂ and a cocktail of protease inhibitors. The reaction was initiated by the addition of 10 µl of a mixture of Mg²⁺-ATP containing 10 µCi of [γ-³²P]ATP. After 10 min of incubation at 30°C, the reaction was terminated by spotting 25 µl of reaction mixture on 2 × 2-cm phosphocellulose Whatman P-81 discs. The discs were washed four times with 0.75% phosphoric acid. The radioactivity bound to the filter paper was quantitated by liquid scintillation counting as described in our earlier publication (3).

Immunoprecipitation and assay of IRS-1-associated PI 3-kinase activity. Immunoprecipitation of cell lysates normal-ized to 200 µg protein was performed overnight at 4°C with 2 µg of anti-rabbit IRS-1 antibody directed against the plek-strin homology domain (United Biotecnology). For negative control, 200 µg of lysate protein were immunoprecipitated with 2 µg of anti-rabbit IgG. The immunocomplexes were precipitated the next day by incubation with 50 µl of protein G plus/protein A-agarose beads (50% vol/vol; Calbiochem) for 2 h at 4°C with constant shaking. The immunoprecipitates were washed exhaustively with buffers, and PI 3-kinase activity was assayed in the immunoprecipitates as described previously (2). The reaction products were separated by TLC on oxalate-treated silica gel 60 plates in a solvent of chloroform-methanol-water-ammonia (60:47:12:5:2). Unlabeled phosphatidylinositol 3-phosphate was used as a standard and visualized by iodine vapor. The ³²P-labeled phosphatidylinositol 3-phosphate was identified by autoradiography and quantitated by the cut-and-count technique.

Protein assay. Proteins in the cellular extracts and lysates were quantitated by the bicinchoninic acid method (29) or by the Bradford technique (5).

RESULTS

Effect of chronic insulin and high glucose on MKP-1 mRNA induction in VSMCs. To understand the exact mechanism whereby high glucose and prolonged insulin treatment cause sustained MAPK activation and excessive growth of VSMCs, we examined the effect of high glucose and chronic insulin treatment on MKP-1 mRNA induction. MKP-1, a dual-specificity tyrosine/threonine protein phosphatase, dephosphorylates MAPKs and inactivates the MAPK signaling pathway. Acute treatment of serum-starved VSMCs with insulin or sodium nitroprusside (SNP; a nitric oxide donor) for 30 min caused a three- to fourfold increase in MKP-1 mRNA expression over basal levels (Fig. 1, top, compare lanes 2 and 3 with lane 1; quantitation in bottom). Prolonged exposure to insulin (100 nM for 24 h, to mimic hyperinsulinemia) completely blocked MKP-1 mRNA expression in response to subsequent acute insulin treatment (Fig. 1, top, compare lane 4 with lane 2) and decreased the MKP-1 mRNA expression to below basal levels. Furthermore, chronic exposure of serum-starved VSMCs to high glucose (25 mM) for 24 h also blocked the subsequent acute effects of insulin and SNP on MKP-1 mRNA induction (Fig. 1, top, compare lanes 6 and 7 with lanes 2 and 3). High glucose alone caused a 30% decrease in basal MKP-1 mRNA expression compared with cells exposed to normal glucose (Fig. 1, top, compare lane 5 with lane 1).

We next examined the effects of high glucose and prolonged insulin treatment on MKP-1 protein induction. As reported earlier (2, 4), acute insulin treatment resulted in a threefold increase in MKP-1 protein accumulation in VSMCs exposed to normal glucose (Fig. 2, top, compare lane 2 with lane 1; quantitation in bottom). A 12-h exposure to insulin resulted in a significant decrease in MKP-1 protein levels (Fig. 2, top, compare lane 3 with lane 2). A subsequent acute insulin treatment at the end of 12 or 24 h did not
To examine whether the inhibitory effects of high glucose on MKP-1 expression are due to an increase in glucose-induced osmolarity, we exposed VSMCs to 19.5 mM mannitol and 5.5 mM glucose for 24 h and examined the effect of acute and chronic insulin treatment on MKP-1 induction. Acute insulin treatment resulted in a more than twofold increase in MKP-1 protein expression over basal levels in mannitol-treated cells (Fig. 3, top, compare lane 2 with lane 1; quantitation in bottom). The extent of MKP-1 induction by acute insulin in mannitol-treated cells was more or less comparable to induction in cells exposed to normal glucose (Fig. 2). Chronic treatment with insulin for 24 h decreased MKP-1 protein expression to below basal values in response to a subsequent acute dose (Fig. 3, top, compare lane 3 with lane 2). Furthermore, the presence of 25 mM glucose in addition to 19.5 mM mannitol for 12 and 24 h also inhibited MKP-1 protein induction in response to a subsequent acute insulin dose (Fig. 3, top, compare lanes 4 and 5 with lane 2).

Further increase MKP-1 protein expression (Fig. 2, top, compare lanes 4 and 5 with lane 2). Exposure to high glucose for 12 and 24 h caused a 75% decrease in basal MKP-1 protein levels compared with cells exposed to normal glucose (Fig. 2, top, compare lanes 6 and 8 with lane 1). In addition, high-glucose treatment for 12 and 24 h completely abolished the acute stimulatory effects of insulin on MKP-1 protein expression (Fig. 2, top, compare lanes 7 and 9 with lane 2). The inhibitory effects of high glucose on MKP-1 protein induction were more marked than the inhibition observed with chronic insulin treatment under normal glucose conditions (shown in Fig. 2, lanes 3–5). Combined addition of insulin and high glucose for 24 h did not further decrease MKP-1 induction (Fig. 2, top, lane 8).
High glucose and chronic insulin block iNOS induction. Results from our recent studies indicated that insulin rapidly induces the expression of iNOS protein in VSMCs (2). Blocking the signaling via the NOS/cGMP pathway with synthetic inhibitors, L-NMMA and Rp-cGMP, respectively, abolished the effects of insulin on MKP-1 induction (2). To further understand the molecular basis of the inhibition of MKP-1 induction observed with high glucose and chronic insulin treatment, we examined the induction of iNOS protein under high-glucose and hyperinsulinemic conditions.

As shown in Fig. 4, acute exposure of serum-starved VSMCs to insulin for 30 min results in a rapid threefold induction of iNOS protein (Fig. 4, top, compare lane 2 with lane 1; quantitation in bottom). Chronic exposure to insulin for 12 and 24 h, respectively, abolished the subsequent effects of acute insulin treatment on iNOS induction (Fig. 4, top, compare lanes 3 and 4 with lane 2). Chronic exposure to high glucose for 12 and 24 h, respectively, also abolished the effect of insulin on iNOS protein induction (Fig. 4, compare lanes 6 and 7 with lane 2). The inhibitory effects of high glucose and chronic insulin were observed only after 12 and 24 h. Shorter periods of 2–6 h caused a very small decrease in insulin-induced iNOS induction or MKP-1 expression (results not shown).

Effect of high glucose and chronic insulin treatment on insulin receptor content and PI 3-kinase activation. The observed inhibitory effects of high glucose and chronic insulin on iNOS and MKP-1 induction may be due to the downregulation of insulin receptor and/or desensitization of the downstream signaling molecules. Therefore, we examined the insulin receptor content and IRS-1-associated PI 3-kinase activity in these cells. Western blot analyses of equal amounts of cell lysate proteins with anti-insulin receptor antibodies detected a 95-kDa band corresponding to the β-subunit of the insulin receptor. High glucose and chronic insulin treatment did not alter insulin receptor content in VSMCs (Fig. 5). Furthermore, prolonged exposure to high glucose did not inhibit PI 3-kinase activation by insulin, as evidenced by comparable PI 3-kinase activity in IRS-1 immunoprecipitates (Fig. 6). However, chronic exposure to insulin for 24 h did result in a 40% decrease in insulin-stimulated PI 3-kinase activity in the IRS-1 immunoprecipitates (Fig. 6).
Inhibition of p38 MAPK signaling with SB-203580 prevents the inhibitory effects of high glucose and chronic insulin on iNOS and MKP-1 protein induction. Our previous studies suggested a potential cross talk between MAPKs and iNOS signaling pathways, since inhibition of ERKs with PD-98059 completely blocked insulin-mediated iNOS induction and MKP-1 protein expression (2). In addition, a number of recent studies indicated that oxidative stress leads to p38 MAPK and/or ERK1/ERK2 activation (12, 19, 23, 32). To further explore the possibility that inhibition of iNOS induction observed by chronic incubation with high glucose and insulin may be due to activation of ERKs and/or p38 MAPKs, we examined the effect of the inhibitors of these signaling pathways on iNOS and MKP-1 protein induction in cells exposed to high glucose and chronic insulin. As seen in Fig. 7, pretreatment of VSMCs with 0.3 µM SB-203580, a specific p38 MAPK inhibitor, for 30 min before chronic insulin exposure completely abolished the inhibitory effects of chronic insulin on iNOS induction (Fig. 7, top left, compare lane 5 with lane 3; quantitation in bottom) and restored insulin responsiveness to levels comparable to those seen with the acute insulin treatment of cells (Fig. 7, top left, compare lanes 5 with lane 2). SB-203580 by itself did not alter basal iNOS protein levels when present for 24 h (Fig. 7, top left, compare lane 4 with lane 2).
with lane 1). In our earlier studies, we demonstrated that SB-203580 did not affect the acute stimulatory effects of insulin on iNOS induction when added 30 min before acute insulin treatment (2). In contrast to inhibition by SB-203580, inhibition of MEK with PD-98059 did not prevent the inhibitory effects of high glucose and insulin on iNOS induction (Fig. 7, top left, lane 7) but decreased iNOS protein levels below the basal values (Fig. 7, top left, compare lanes 6 and 7 with lane 1). In separate experiments, we observed that SB-203580 also partially prevented the inhibitory effects of high glucose on insulin-mediated iNOS induction (Fig. 7, top right, compare lane 5 with lane 3), whereas PD-98059 was ineffective (Fig. 7, top right, compare lane 6 with lane 3). It should be noted that the presence of SB-203580 together with high glucose did decrease basal iNOS protein levels by 40% compared with normal glucose controls (Fig. 7, top right, compare lane 4 with lane 1).

SB-203580 also prevented the inhibitory effects of chronic insulin treatment (Fig. 8, top left, compare lane 5 with lane 3; quantitation in bottom), as well as the effect of high glucose on MKP-1 induction (Fig. 8, top right, compare lane 5 with lane 3), whereas PD-98059 was ineffective (Fig. 8, top left, compare lane 6 with lane 3; Fig. 8, top right, compare lane 7 with lane 3). Thus inhibition of p38 MAPK signaling with SB-203580 abrogates the deleterious effects of chronic insulin and restores insulin sensitivity of VSMCs in terms of iNOS and MKP-1 induction.

High glucose and chronic insulin treatment activate p38 MAPK and ERK1/ERK2. To further confirm whether high glucose and chronic insulin treatment results in sustained activation of p38 MAPK and/or ERKs, we examined the phosphorylation status of p38 MAPK and ERK1/ERK2 using phosphospecific antibodies. Initial studies were performed to examine the dose-response and kinetics of the acute effects of insulin on p38 MAPK phosphorylation in cells maintained in normal glucose.

In unstimulated cells, a small amount of p38 MAPK was phosphorylated in the basal state (Fig. 9). Acute insulin treatment for 30 min caused a twofold increase in p38 MAPK phosphorylation compared with control cells (Fig. 9, compare lane 2 with lane 1; quantitation in bottom). The level of p38 MAPK phosphorylation 12 h after exposure to insulin was comparable to the increase observed with acute insulin treatment for 30 min (Fig. 9, compare lanes 3 and 4 with lane 2). More important, exposure to chronic insulin for 24 h further increased the phosphorylation in response to a subsequent acute insulin treatment (Fig. 9, compare lane 5 with lanes 2–4). Exposure to high glucose alone for 12 and 24 h, respectively, resulted in a time-dependent twofold increase in basal p38 MAPK phosphorylation compared with cells exposed to normal glucose (Fig. 9, compare lane 6 with lane 1). Subsequent acute insulin treatment of these cells (Fig. 9, lane 7 and lane 9), as well as combined addition of insulin and high glucose for 24 h (Fig. 9, lane 10), did not further increase p38 MAPK phosphorylation but rather caused a small reduction.

Quantitation of p38 MAPK activation by the in vitro kinase assay in the immunocomplexes confirmed the results shown in Fig. 9. With ATF-2 as a substrate, acute insulin treatment caused an approximately threefold increase in p38 MAPK activity compared with controls (Fig. 10). The stimulation persisted in cells exposed to chronic insulin for 24 h. Moreover, SB-203580 blocked p38 MAPK activity stimulated by insulin (Fig. 10). High-glucose exposure for 24 h also increased p38 MAPK activity by 200% compared with cells exposed to normal glucose. Insulin treatment of VSMCs exposed to high glucose did not further increase p38 MAPK activity in the immunoprecipitates (Fig. 10). The glucose effect was not entirely due to hyperosmolarity, since mannitol at 19.5 mM caused only a small increase in p38 MAPK activity compared with cells grown under normal glucose conditions (Fig. 10).

In contrast to its effect on p38 MAPK, acute insulin treatment caused only a small increase (45% over basal) in the levels of phospho-ERK1s (Fig. 11, compare lane 2 with lane 1; quantitation in bottom). However,
high glucose and chronic insulin treatment for 12 and 24 h, respectively, did result in a twofold increase in the phosphorylation status of ERKs (Fig. 11, compare lanes 3 and 4 and lanes 6 and 7 with lanes 1 and 2). Pretreatment with PD-203580 blocked chronic effects of insulin and high glucose, whereas SB-203580 was ineffective.

High glucose and chronic insulin treatment increase DNA synthesis in VSMCs via p38 MAPK activation. To examine the impact of sustained p38 MAPK activation on cell proliferation, we measured DNA synthesis in cells exposed to chronic insulin and high glucose. As seen in Fig. 12, chronic treatment of VSMCs with insulin for 24 h caused a 70% increase in \[^{3}H\]thymidine incorporation compared with basal levels. Pretreatment with SB-203580 partially abolished the stimulatory effects of insulin on DNA synthesis. SB-203580 by itself did not significantly alter basal \[^{3}H\]thymidine incorporation into DNA (Fig. 12). Exposure to high glucose resulted in a fourfold increase in \[^{3}H\]thymidine incorporation into DNA. The presence of insulin together with high glucose further increased \[^{3}H\]thymidine incorporation by 25% above high-glucose controls. Pretreatment with SB-203580 decreased the stimulatory effects of high glucose by 50%. Combined addition of PD-98059 completely inhibited the effect of insulin on DNA synthesis. PD-98059 alone blocks the effects of insulin on DNA synthesis (see Ref. 4).

**DISCUSSION**

The results of the present study clearly indicate that the simulation of hyperinsulinemia and hyperglycemia by chronic insulin and high-glucose treatment of VSMCs markedly inhibits the induction of MKP-1 mRNA and protein expression in response to a subsequent acute insulin stimulus. As expected, the high glucose- and chronic insulin-induced inhibition of MKP-1 induction was accompanied by a marked impairment in iNOS protein expression. The results of this study confirm our earlier observations that the iNOS/cGMP signaling pathway plays a major role in the acute stimulatory effects of insulin on the induction of MKP-1 expression (2). Our earlier studies also indicated a potential interaction between MAPK family members and iNOS (2). Thus blocking MAPKs by pretreatment with PD-98059 completely abolished the effect of insulin on iNOS induction (2). The inhibition of iNOS protein induction observed in this study in response to high glucose and high glucose in the presence of chronic insulin and HG treatment increase p38 MAPK phosphorylation in VSMCs. Serum-starved VSMCs were incubated with NG with or without insulin for 12 and 24 h or with HG for 12 and 24 h, followed by a subsequent acute treatment with insulin for 30 min. Equal amounts of cell lysate proteins were subjected to SDS-PAGE, followed by immunoblot analysis with phosphospecific p38 MAPK antibodies. Once phosphorylation status was known, blots were stripped and reprobed with p38 MAPK antibodies to normalize for variations in amount of p38 MAPK protein. Top: representative autoradiogram. Similar results were obtained in 3 or 4 experiments. Lanes 1, 6, and 8, NG and HG controls; lane 2, acute insulin treatment; lane 3, chronic insulin treatment for 12 h; lane 4, 12-h insulin treatment followed by acute insulin for 30 min; lane 5, 24-h insulin treatment followed by a subsequent acute insulin dose; lane 7, HG 12 h followed by 30-min insulin treatment; lane 9, HG 12 h followed by insulin 30 min; lane 10, HG 24 h plus insulin 24 h.

**Fig. 9.** Chronic insulin and HG treatment increase p38 MAPK phosphorylation in VSMCs. Serum-starved VSMCs were incubated with NG with or without insulin for 12 and 24 h or with HG for 12 and 24 h, followed by a subsequent acute treatment with insulin for 30 min. Equal amounts of cell lysate proteins were subjected to SDS-PAGE, followed by immunoblot analysis with phosphospecific p38 MAPK antibodies. Once phosphorylation status was known, blots were stripped and reprobed with p38 MAPK antibodies to normalize for variations in amount of p38 MAPK protein. Top: representative autoradiogram. Similar results were obtained in 3 or 4 experiments. Lanes 1, 6, and 8, NG and HG controls; lane 2, acute insulin treatment; lane 3, chronic insulin treatment for 12 h; lane 4, 12-h insulin treatment followed by acute insulin for 30 min; lane 5, 24-h insulin treatment followed by a subsequent acute insulin dose; lane 7, HG 12 h followed by 30-min insulin treatment; lane 9, HG 12 h followed by insulin 30 min; lane 10, HG 24 h plus insulin 24 h. Bottom: data from multiple experiments were quantitated by densitometric scanning and plotted as % of NG controls. *P < 0.05 vs. NG control.

**Fig. 10.** Assay of p38 MAPK activity in immunoprecipitates. VSMCs exposed to mannitol, NG, acute and chronic insulin, and HG in presence and absence of SB-203580 and PD-98059 were extracted in lysis buffer containing phosphatase and protease inhibitors. Equal amounts of cell lysates were immunoprecipitated with a polyclonal anti-p38 MAPK Ab at 4°C overnight. Immunocomplexes were sedimented with protein A-Sepharose, washed extensively, and resuspended in kinase assay buffer. Kinase activity was then measured using activating transcription factor-2 as a substrate. Details are given in METHODS. Results are means ± SE of 4 different experiments, expressed as % of normal control. *P < 0.05 vs. NG; **P < 0.05 vs. chronic insulin and HG.
chronic insulin treatment appears to be due mainly to sustained p38 MAPK activation. Thus it appears that a stress-related MAPK pathway such as p38 MAPK may represent the additional pathway necessary to link the high glucose- and chronic insulin-induced increase in intracellular oxidative stress to hypertrophy via inhibition of MKP-1, the phosphatase that turns off MAPK signaling by causing dephosphorylation and inactivation of MAPK family members. In support of our observations, recent studies by Igarashi et al. (13) showed PKC-dependent elevations in p38 MAPK activity in VSMCs isolated from diabetic rat aortae as well as those exposed to 16.5 mM glucose. The results of this study add a new dimension to the above observations by documenting that high glucose- and insulin-induced elevations in p38 MAPK result in inhibition of the stimulatory effects of insulin on MKP-1 protein expression. First, blocking p38 MAPK signaling by prior treatment with SB-203580, a selective p38 MAPK inhibitor, prevents the inhibitory effects of high glucose and chronic insulin on iNOS protein induction and restores the acute stimulatory effects of insulin on iNOS as well as MKP-1 protein induction. The effect is observed only with SB-203580; PD-98059, a MEK inhibitor that blocks MAPK signaling, does not prevent the inhibitory effects of high glucose and chronic insulin on iNOS and MKP-1 protein expression even though it blocks high glucose- and chronic insulin-induced ERK phosphorylation. Second, insulin rapidly and dose dependently increases p38 MAPK phosphorylation and its activity, and these elevations in the enzyme activity are maintained under conditions of high glucose and chronic insulin. Most important, p38 MAPK can be further stimulated in chronic insulin-treated cells by a subsequent acute insulin treatment. In contrast, ERK1/ERK2 are phosphorylated only after prolonged 24-h treatment with insulin and high glucose. Finally, a prolonged incubation period of 12–24 h with high glucose and insulin is needed to observe the inhibitory effects on iNOS induction and MKP-1 expression, suggesting that the p38 MAPK stress response pathway may downregulate the induction of the above proteins at the transcriptional level.

Fig. 11. HG and chronic insulin treatment increase phosphorylation status of extracellular signal-regulated kinase (ERKs). VSMCs exposed to NG, acute and chronic insulin, and HG in presence and absence of SB-203580 and PD-98059 were extracted in lysis buffer containing phosphatase and protease inhibitors. Equal amounts of cell lysates were subjected to SDS-PAGE followed by Western blot analyses with phosphospecific ERK Ab. Once linear signal had been obtained, blots were stripped and reprobed with anti-ERK antibodies to normalize results for variations in ERK protein levels. Top: representative autoradiogram. Bottom: data from multiple experiments were quantitated by densitometric scanning and plotted as % of NG controls. *P < 0.05 vs. NG control; **P < 0.05 vs. acute insulin; ***P < 0.05 vs. chronic insulin.

Fig. 12. Inhibition of p38 MAPK abolishes chronic insulin and HG-mediated DNA synthesis. Confluent serum-starved VSMCs were pretreated with SB-203580 (0.2 µM) for 30 min, followed by addition of NG or HG with or without insulin for an additional 24 h. [3H]Thymidine (1 µCi/ml) was added during last 3 h of incubation. Reaction was stopped by removing medium and rinsing cells four times with ice-cold PBS and once with 10% ice-cold TCA. Pellet was rinsed once with ether to remove TCA and solubilized in 1% SDS containing 0.1 N NaOH. An aliquot of cell lysate was counted in a scintillation counter. Results are means ± SE of 3 separate experiments performed in duplicate. DPM, disintegrations/min. *P < 0.05 vs. NG control; **P < 0.05 vs. insulin; ***P < 0.05 vs. NG controls; ****P < 0.05 vs. HG control; *****P < 0.05 vs. HG controls and HG + insulin.
level. Given that iNOS protein levels are regulated by transcription, mRNA stability, translation, and protein turnover, it is hard to determine exactly how elevations in p38 MAPK downregulate iNOS protein induction.

Further studies with constitutively active as well as dominant negative mutants of p38 MAPK will help in understanding the exact role of p38 MAPK in iNOS activation and MKP-1 induction. It should be noted that the NOS signaling pathway does not directly control MAPK activation in VSMCs, since inhibition of NOS with L-NMMA did not prevent ERK activation but increased its activation status, presumably due to inhibition of MKP-1 expression.

Our observations on the inhibitory effects of the p38 MAPK signaling pathway on iNOS induction coincide with the results of Guan et al. (11) reporting inhibition of NO synthesis by p38 MAPK pathway in renal mesangial cells stimulated by interleukin-1β. In contrast, studies by Da Silva et al. (7a) and LaPointe and Isonic (20) in mouse astrocytes and cardiac myocytes, respectively, indicate that blockade of p38 MAPK signaling results in inhibition of iNOS expression. The most likely explanation for these seemingly inconsistent results is that the regulation of iNOS induction is tissue specific and complex, involving both ERKs and various isoforms of p38 MAPK with different sensitivities to the inhibitor SB-203580.

The presence of detectable levels of p38 MAPK phosphorylation and activity in unstimulated VSMCs maintained under normal glucose conditions suggests that this enzyme or one of its isoforms may be needed to suppress MKP-1 expression in the basal state. When subjected to acute insulin treatment, VSMCs may use the ERK-mediated NOS signaling pathway to cause MKP-1 expression. Thus it appears that the acute stimulatory effects of insulin on NOS and MKP-1 protein induction are likely to be mediated via the MEK/ERK signaling pathway, whereas the chronic inhibitory effects of insulin and high glucose are mediated via the p38 MAPK stress-response pathway. Thus chronic activation of p38 MAPK provides a crucial signaling mechanism, which may negatively regulate iNOS induction, NO biosynthesis, and MKP-1 expression by a desensitizing mechanism, thereby resulting in the sustained MAPK activation and excessive cell growth commonly observed in VSMCs exposed to high glucose and chronic insulin (1, 9, 15).

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