Inhibition of p38 Mitogen-activated Protein Kinase by Insulin in Cultured Fetal Neurons*

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Kim A. Heidenreich† and Jennifer L. Kummer
From the Department of Pharmacology, University of Colorado Health Sciences Center, Denver, Colorado 80262 and the Denver Veteran Affairs Medical Center, Denver, Colorado 80220

Insulin supports the survival and differentiation of many types of fetal neurons. To determine if mitogen-activated protein (MAP) kinases play a role in mediating the neurotrophic actions of insulin, we identified the MAP kinases present in fetal chick forebrain neurons and examined their regulation by insulin. Cell extracts were fractionated on Mono Q columns, and phosphotransferase activity was measured using myelin basic protein as the substrate. In control neurons, four peaks of MAP kinase activity were resolved. Peaks I, II, and IV were identified by immunoblotting as c-jun N-terminal kinase (JNK), extracellular signal-related kinase (ERK), and p38 MAP kinase, respectively. Neurons treated with insulin showed a dramatic decrease, 80-90%, in p38 MAP kinase activity without significant changes in the other MAP kinase activities. Insulin decreased the phosphoryrosine content of p38 MAP kinase with maximal effects observed within 5 min. Pretreatment of neurons with sodium orthovanadate blocked the ability of insulin to inhibit the tyrosine phosphorylation and activity of p38 MAP kinase, suggesting that activation of a tyrosine or dual specific phosphatase is necessary for the inhibition of p38 MAP kinase by insulin. Since p38 MAP kinase has been recently implicated in neuronal cell apoptosis, negative regulation of this kinase by insulin may be critical for the neurotrophic actions of insulin.

MAP1 kinase cascades are key signaling systems by which cells transduce extracellular stimuli into intracellular responses (for recent reviews see Refs. 1 and 2). Many steps of these signaling cascades are conserved in Caenorhabditis elegans, Drosophila melanogaster, and mammalian cells. In mammalian cells, the extracellular signal-regulated kinases, ERK1, and ERK2, are the prototypic members of the MAP kinase family. ERK1 and ERK2 were originally found to be activated by a number of growth factors that interact with cell surface tyrosine kinase receptors. The main features of the ERK cascade involve p21ras-mediated translocation of an upstream serine kinase, c-Raf, which phosphorylates and activates its downstream protein kinase, MAP kinase (also referred to as MEK or MKK1). MAP kinase, a dual specific protein kinase, activates ERK1 or ERK2, which, in turn, regulates the activity of a variety of cytosolic enzymes and nuclear transcription factors (3). Thus, a signal generated at the cell surface is translated to the nucleus where gene expression is regulated. Recently, other MAP kinases have been identified including c-jun N-terminal kinase (JNK), which is also referred to as stress-activated protein kinase (4, 5) and p38 MAP kinase, the mammalian homologue of the yeast HOG1 protein kinase (6, 7). A characteristic feature of all the MAP kinases is their activation by phosphorylation on Tyr and Thr residues within a TXY phosphorylation motif, where X can be Glu, Pro, or Gly (1, 2). The newly discovered MAP kinases, JNK and p38, appear to have equivalent but distinct upstream kinases that are activated by a wide variety of extracellular stimuli including growth factors, differentiating factors, UV irradiation, heat shock, and changes in osmolarity (4–7). In some cases, a given extracellular signal appears to activate multiple MAP kinase pathways, whereas other signals appear to activate a single MAP kinase pathway. In yeast, six distinct MAP kinase pathways have been identified (8), and it is likely that many more exist in mammalian cells.

The MAP kinase cascade involving ERK1 and ERK2 has been implicated in signal transduction by the insulin receptor. Insulin activates p21ras, Raf-1, MEK, and ERK1/ERK2 in a number of cell types (9). Activation of this cascade is mediated by GRB2, a 23-kDa SH2-containing protein, which couples tyrosine-phosphorylated IRS-1 or SHC (both substrates for the insulin receptor kinase) to a p21ras-specific GDP/GTP exchange factor (10). Microinjection of Xenopus oocytes with a neutralizing p21ras antibody or a dominant negative p21ras mutant inhibits the meiotic maturation induced by insulin (11, 12). Moreover, overexpression of p21ras oncogenes mimics the effects of insulin on 3T3 cell differentiation, and dominant negative mutants of p21ras block the differentiation process triggered by insulin (13, 14). Taken together, these data suggest that p21ras-mediated activation of ERK1/ERK2 is critical for certain actions of insulin. However, there is also accumulating evidence that ERK1/ERK2 activation is cell-specific and may not be required or sufficient for all of the cellular effects of insulin. In this study, we examined the regulation of MAP kinases by insulin in cultured fetal neurons from chick forebrain. Insulin acts as a potent neurotrophic factor for these neurons (15) and other neurons from cerebellum, mesencephalon, cerebral cortex, spinal cord, and hippocampus. Insulin’s neurotrophic actions include supporting the growth of neurons in serum-free medium (16, 17), stimulating neuronal protein synthesis (18), inducing neurite outgrowth (16, 17), and regulating the expression of certain neurofilament and early intermediate genes (19, 20). We report here that insulin has no significant effect on ERK or JNK activities in chick forebrain neurons but markedly inhibits a high basal level of p38 MAP kinase in these cells. These results are the first to demonstrate an inhibitory regulation of a MAP kinase by a growth factor.
The inhibition of p38 MAP kinase, a kinase that has recently been linked to neuronal programmed cell death (21), may represent the mechanism by which insulin supports neuronal survival.

**EXPERIMENTAL PROCEDURES**

**Materials—**Porcine monocomponent insulin and recombinant human insulin-like growth factor I were generously supplied by Dr. Ronald Chance and Margaret Niedenthal, respectively, of Lilly. Fertilized White Leghorn chicken eggs were obtained from Lower Poultry Farm (Fort Collins, CO). Fetal calf serum was obtained from Irvine Scientific (Irvine, CA). Tissue culture media, balanced salt solutions, and laminin were obtained from Life Technologies, Inc. All other chemicals were obtained from Sigma. Mono Q HR 5/5 columns were purchased from Pharmacia Biotech Inc. Monoclonal anti-ERK1/ERK2 antibodies were purchased from Zymed (San Francisco, CA). PY20 monoclonal anti-Tyr(P) antibodies were obtained from Transduction Laboratories (Lexington, KY). Polyclonal anti-jNK antibodies were a kind gift from Dr. Lynn Heasley (University of Colorado Health Sciences Center). Polyclonal anti-p38 antibodies were generously provided by Drs. J. Han and R. Ulevitch (Scripps Research Clinic).

**Cell Culture—**Neurons were cultured from day 8 chick forebrain as described previously (22).

**Treatment of Neurons with Growth Factors—**After removing the growth medium, the neurons were rinsed with 2 ml of phosphate-buffered saline (PBS, pH 7.4) and incubated in 2 ml of Dulbecco's modified Eagle's medium (pH 7.4) containing 20 mM HEPES and 1% bovine serum albumin at 37°C in the absence or presence of growth factors. The times of incubation are indicated in the text. Experiments were terminated by aspirating the buffer and rinsing twice with PBS. After washing in PBS, the neurons were scraped from the dishes, pelleted, and solubilized in lysis buffer (pH 7.2) containing 50 mM β-glycerophosphate, 5 mM EDTA, 1 mM dithiothreitol, 2 mM sodium orthovanadate, 10 mM leupeptin, 5 μg/ml aproptin, and 1 mM phenylmethylsulfonfyl fluoride.

**Mono Q Column Chromatography—**Cellex extracts (3–5 mg of protein) were loaded at a flow rate of 0.8 ml/min to a Mono Q column (Pharmacia fast protein liquid chromatograph HR5/5) equilibrated with Buffer A (50 mM β-glycerophosphate, 5 mM EDTA, 1 mM dithiothreitol, 2 mM sodium orthovanadate, pH 7.2). The column was developed at the same rate with a linear NaCl gradient (0–800 mM) in Buffer A. Fractions (200 μl) were collected and assayed for protein kinase activity.

**Protein Kinase Assay—**The protein kinase assay was initiated by the addition of 20 μl of 2 x reaction buffer (50 mM β-glycerophosphate, 1 mM EGTA, 20 mM MgCl2, 100 μM sodium orthovanadate, 1 mg/ml MBP, or 0.1 mg/ml ATF2 (N-terminal half), 50 μM IP20, a peptide inhibitor of CaMP-dependent protein kinase, 200 μM ATP, and 0.9 M/cm3 [32P]ATP) to 20 μl of column fraction. The reaction was allowed to proceed for 10 min at 30°C and then stopped by the addition of trichloroacetic acid (15%) and placed on ice. A 40-μl aliquot was spotted onto a P-81 phosphocellulose paper square. After washing extensively with 75 mM phosphoric acid, the paper squares were dried, and radioactivity was determined by liquid scintillation spectroscopy. When reactions contained ATF2 as the substrate, the reactions were terminated by the addition of 2 x Laemmli sample buffer and analyzed by SDS-PAGE using 10% acrylamide gels. After electrophoresis, the gels were dried and subjected to phosphorimaging (GS-100, Bio-Rad).

**Immunoblotting—**Column fractions were concentrated 10-fold using Centricon 10 concentrators (Amicon), solubilized in 2 x Laemmli sample buffer, and analyzed by SDS-PAGE on 12% acrylamide gels. After electrophoresis, the proteins in the gel were transferred to PVDF membranes and probed with polyclonal anti-jNK antibodies (1:1,000), monoclonal anti-ERK1/ERK2 antibodies (1:1,000), and polyclonal anti-p38 antibodies (1:1,000) followed by secondary antibodies coupled to alkaline phosphatase. Bound alkaline phosphatase was visualized by the Western-Light (Tropix) chemiluminescent detection system.

**RESULTS AND DISCUSSION**

To investigate the MAP kinase activities present in fetal neurons, neurons from E8 chick forebrain were cultured for 5 days, and neuronal cell extracts were fractionated by anion exchange chromatography on Mono Q. Phosphotransferase activity was measured in each fraction using MBP as the substrate. Four peaks of MBP kinase activity were resolved (Fig. 1A), the first peak eluted at a NaCl concentration of about 30 mM. This peak contained jNK kinase identified by immunoblotting with anti-jNK antibodies (Fig. 1B) and had the greatest phosphotransferase activity against a GST-c-jun fusion protein relative to the other fractions (data not shown). Peak II eluted at about 170 mM NaCl and displayed immunoreactivity with an antibody that recognizes the C terminus of both ERK1 and ERK2 (Fig. 1, A and B). The identity of Peak III, which
Insulin inhibits neuronal p38 MAP kinase

When neurons were treated with insulin (50 ng/ml), a potent neurotrophin for these cells, for 15 min at 37 °C prior to fractionation of cell lysates on Mono Q columns, there was a dramatic and very consistent decrease (80–90%, n = 3) in the activity of Peak IV without major changes in the other MAP kinase activities (Fig. 1A). The inhibition of p38 MAP kinase activity by insulin was specific for this polypeptide since IGF-I at the same concentration had no effect on p38 MAP kinase activity, despite the 10-fold higher number of IGF-I receptors on these neurons. Exposure of the neurons to sorbitol (200 µM), a known activator of p38 MAP kinase (6), resulted in a 20% increase in Peak IV kinase activity (data not shown). The relatively low stimulation of p38 MAP kinase activity by sorbitol is most likely related to the high basal level of p38 MAP kinase activity in these neurons.

The inhibition of MAP kinase activity by insulin was specific for p38 MAP kinase since the other MAP kinases present were not significantly affected by insulin. This was demonstrated by examining the phosphotransferase activities of the various MAP kinases using the N-terminal half of ATF2 as the substrate (Fig. 2). Insulin inhibited the ATF2 phosphotransferase activity of p38 MAP kinase by 90% with no significant effect on the phosphotransferase activities of the other MAP kinases. ATF2 was observed to be the best substrate for p38 when compared with MBP and the epidermal growth factor receptor peptide (data not shown).

MAP kinases are activated by phosphorylation of critical tyrosine and threonine residues. To determine if the inhibition of p38 MAP kinase activity by insulin was due to changes in the tyrosine phosphorylation state of the enzyme, control and insulin-treated neuronal cell extracts were immunoprecipitated with anti-phosphotyrosine antibodies (PY20), and the phosphotyrosine-containing proteins in the precipitants were analyzed by Western blotting with anti-p38 antibodies. The results demonstrated that insulin, but not sorbitol or IGF-I, decreased the phosphotyrosine content of p38 (Fig. 3A). The effect of insulin was maximal by 5 min and sustained out to 15 min (Fig. 3, B and C). After 15 min, the phosphotyrosine content of p38 began to approach control levels. Similar results were obtained by immunoprecipitating neuronal cell extracts with p38 antibodies and then subsequently Western blotting with PY20.

To determine whether activation of a phosphatase might be involved in the inhibition of p38 MAP kinase by insulin, neurons were treated with sodium orthovanadate (1 mM), an inhibitor of tyrosine and dual specific phosphatases, 10 min prior to the addition of insulin. Sodium orthovanadate had no effect on the basal levels of phosphotyrosine content but totally blocked the ability of insulin to decrease the phosphotyrosine content of p38 MAP kinase (Fig. 4). The decrease in phosphotyrosine content was indicative of decreased kinase activity, suggesting that activation of a tyrosine phosphatase or a dual

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**Fig. 2. In vitro phosphorylation of ATF2 by Mono Q column fractions.** Column fractions (peaks I, II, III, and IV) from control (−) and insulin-treated (+) neurons were assayed for kinase activity as described in Fig. 1 except that 0.1 mg/ml ATF2 (N-terminal half) was added as the substrate. Reactions were terminated by the addition of 2 × Laemmli sample buffer and analyzed by SDS-PAGE using 10% acrylamide gels. After electrophoresis, the gels were dried and subjected to phosphorimaging (GS-100, Bio-Rad).

**Fig. 3. Insulin decreases tyrosine phosphorylation of p38 MAP kinase.** Panel A, neurons were incubated in the absence or presence of insulin (50 ng/ml), IGF-I (50 ng/ml), and sorbitol (200 µM) for 15 min at 37 °C. After washing 2 × in ice-cold PBS, the cells were solubilized, and cell lysates were analyzed by immunoprecipitation using PY20 monoclonal anti-Tyr(P) antibodies. The immunocomplexes were then analyzed by Western blotting using polyclonal anti-p38 antibodies. Bound horseradish peroxidase was visualized by the ECL chemiluminescent detection system. IP, immunoprecipitate. Panel B, neurons were incubated in the absence or presence of insulin (50 ng/ml) at 37 °C for the indicated times. The cells were solubilized and analyzed as described in A. Panel C, densitometric scans performed on experiments (n = 4) described in B.
specific phosphatase is necessary for the inhibition of p38 MAP kinase by insulin. This activated phosphatase may act directly on p38 MAP kinase or at steps upstream of p38 MAP kinase, which are regulated by tyrosine phosphorylation.

Although ERK, JNK, and p38 MAP kinase are structurally related kinases and are stimulated by similar kinase cascades, they are activated by different extracellular stimuli and have different substrate specificities (1,2). The p38 MAP kinase was originally described as the mammalian homologue of the Saccharomyces cerevisiae HOG1 kinase involved in osmoregulation in the yeast. In mammalian cells, p38 MAP kinase is activated by changes in osmolarity, bacterial endosomes, and heat shock (6). Very recently, p38 MAP kinase has been implicated in neuronal programmed cell death (21). In differentiated PC12 cells, NGF withdrawal results in apoptosis, which is preceded by an increase in p38 MAP kinase activity. Introduction of the dominant negative mutant of MKK3, the upstream regulator of p38 MAP kinase, blocks the apoptosis induced by NGF withdrawal, whereas introduction of the constitutively activated form of MKK3 induces apoptosis in PC12 cells. Thus, stimulation of p38 MAP kinase by NGF withdrawal appears to mediate neuronal programmed cell death. These data, taken together with the results from the present study, suggest that the mechanism by which insulin supports neuronal survival involves inhibition of p38 MAP kinase. This is the first evidence for negative regulation of p38 MAP kinase by a trophic factor and suggests that this MAP kinase may be an important target for other neurotrophic factors.

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