Insulin-like Growth Factor-I Inhibits the Stress-activated Protein Kinase/c-Jun N-terminal Kinase*

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The pathways involved in the cellular responses to the insulin-like growth factors (IGFs) are numerous and vary according to cell type. Following activation of the IGF-I receptor, the mitogen-activated protein kinase and phosphatidylinositol 3'-kinase (PI3K) pathways are activated and result in cellular proliferation and inhibition of apoptosis. In this study, we analyzed the IGF-I effect on the stress-activated protein kinase/c-Jun N-terminal kinase (JNK) activity using human embryonic kidney 293 cells, 293 cells transiently expressing hemagglutinin-JNK, and 293 cells stably expressing a hemagglutinin-JNK transgene. In all cell types, endogenous or transfected JNK activity was strongly stimulated by anisomycin or tumor necrosis factor-α, and 10 nM IGF-I pretreatment suppressed the induced JNK activity. To determine whether the effect of IGF-I on JNK activity involves the mitogen-activated protein kinase or PI3K pathway, we used the specific MEK1 inhibitor PD098059 and the PI3K inhibitor LY 294002. PD098059 did not alter the IGF-I suppressive effect on stressor-induced JNK activity, but LY 294002 suppressed the IGF-I effect. Moreover, in transiently transfected parental 293 cells expressing dominant-negative Akt, anisomycin-increased JNK activity was not suppressed by pretreatment with IGF-I. Our results demonstrate that the action of IGF-I on JNK in these cells is via PI3K and Akt.

Insulin-like growth factors (IGFs) and the insulin-like growth factor-I receptor (IGF-IR) have multiple pleiotropic effects. During early embryogenesis, these factors play an essential role in growth and development. Null mutations of IGF-I and IGF-II are associated with growth retardation, whereas mice with a null mutation of the IGF-IR die at birth due to respiratory failure as a consequence of poorly developed dia phragmatic muscle (1, 2). The cellular events following IGF-I activation have also been extensively studied. Following ligand binding to the receptor, the receptor undergoes autophosphorylation and increased receptor tyrosine kinase activity. This is followed by tyrosine phosphorylation of a number of endoge nous substrates including the insulin receptor substrate (IRS) family of substrates, Src homology collagen (SHC), Syp, Crk, and Grb2 (3–5). These early signaling events result in activation of the Ras/Raf/mitogen-activated protein (MAP) kinase and phosphatidylinositol 3'-kinase (PI3K) pathways, which in turn culminate in the biological effects of the IGFs (6–9).

The IGFs, like other growth factors, are mitogens and play an essential role in the cell cycle and cellular proliferation (10, 11). Recently, studies have demonstrated the prominent role of the IGF-IR signaling in inhibiting programmed cell death or apoptosis (6, 11–16). Although the most studied signaling pathway involved in this effect is the PI3K and Akt pathway (12, 17, 18), other studies have shown that the MAP kinase pathway (19–22) and the stress-activated p38 MAP kinase pathways may also play a role (23). Thus, the IGF-IR signaling may utilize multiple different cascades to effect the final biological event in a cell type-specific manner. The stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) pathway has not as yet been demonstrated to play a role in IGF anti-apoptotic effects. To study the potential effects of IGFs on the JNK pathway, we activated JNK using anisomycin (an environmental stress) and tumor necrosis factor-α (TNF-α; a physiological stress) in human embryonic kidney 293 cells, 293 cells transiently expressing hemagglutinin (HA)-JNK, and 293 cells stably expressing an HA-JNK transgene. In each case, we demonstrate that IGF-I exerts a potent inhibitory effect on JNK activation by stress-inducing signals. To determine whether the effect of IGF-I on JNK activity involves the MAP kinase pathway or the PI3K pathway, we used the specific MEK1 inhibitor PD098059, the PI3K inhibitor LY 294002, and transiently transfected parental 293 cells with dominant-negative Akt. Our results demonstrate that the action of IGF-I on JNK in these cells is via PI3K and Akt.

EXPERIMENTAL PROCEDURES

Materials—Recombinant IGF-I was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY); recombinant TNF-α was purchased from Calbiochem; LY 294002 was from BIOMOL Research Laboratories Inc. (Plymouth, ME); and anisomycin and poly-l-lysine were purchased from Sigma. Cell culture media and reagents were purchased from Biofluids, Inc. (Rockville, MD). G418 (Geneticin) was obtained from Life Technologies, Inc. The calcium phosphate transfection kit was purchased from 5 Prime 3 Prime, Inc. (Boulder, CO). Monoclonal anti-HA-II antibody was purchased from BAHCO (Richmond, CA). Polyclonal anti-JNK1 antibody (C-17), polyclonal anti-Akt-1 antibody (C-20), and glutathione S-transferase (GST)-c-Jun (79) fusion protein were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). GammaBind G-Sepharose was purchased from Pharmacia Biotech (Uppsala, Sweden). The specific MEK1 inhibitor PD098059 and the SAPK/JNK kinase assay kit were purchased from New England Biolabs Inc. (Beverly, MA). The radionucleotide (γ-32P)ATP (6000 Ci/mmol) was purchased from NEN Life Science Products. The enhanced chemiluminescence detection kit and donkey anti-rabbit IGFs coupled to horseradish peroxidase were purchased from Amersham Pharmacia Biotech.

Cell Culture and Stimulation—Human embryonic kidney 293 cells

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‡ The abbreviations used are: IGFs, insulin-like growth factors; IGF-IR, IGF-I receptor; IRS, insulin receptor substrate; SHC, Src homology collagen; MAP, mitogen-activated protein; PI3K, phosphatidylinositol 3'-kinase; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated protein kinase; MEK, MAP kinase/ERK kinase; MEKK, MAP kinase/ERK kinase kinase; TNF-α, tumor necrosis factor-α; HA, hemagglutinin; GST, glutathione S-transferase; MOPS, 4-morpholinoethanesulfonic acid.
were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Upstate Biotechnology, Inc.). Prior to growth factor stimulation, subconfluent cultures of cells in 100-mm poly-D-lysine-coated dishes were switched to serum-free Dulbecco's modified Eagle's medium supplemented with 0.1% insulin-free bovine serum and antibiotics. The cells were then grown in 25-cm diameter tissue culture flasks for 5 days in 20% HEPES (pH 7.5) for 2 h. In some experiments, the MEK1 inhibitor PD098059 (10 μM) was added to cells 30 min before growth factor stimulation, and the PI3K inhibitor LY 294002 (10 μM) was added 2 h before stimulation. Cells were treated with 10 nM IGF-I for 5 min, 10 μg/ml anisomycin for 20 min, or 50 ng/ml TNF-α for 15 min, all diluted in serum-free Dulbecco's modified Eagle's medium at 37 °C.

**JNK Kinase Assay**—JNK activity was determined as reported by Coso et al. (24) using GST-c-Jun (79) fusion protein as a substrate. Cells were lysed in a buffer containing 25 mM HEPES (pH 7.5), 0.3 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1% Triton X-100, 0.5 mM dithiothreitol, 20 mM β-mercaptoethanol, 0.1 mM sodium orthovanadate, 2 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 0.1% SDS, 0.5% deoxycholic acid-sodium salt, and 20 μg/ml aprotinin. Samples were sonicated four times for 5 s on ice. Endogenous JNK activity in parental 293 cells was determined following immunoprecipitation with 3 μg of polyclonal anti-JNK1 antibody and 35 μl of GammaBind G-Sepharose beads for 1 μg of total cellular protein. JNK activity in either transiently or stably transfected cells was determined following immunoprecipitation with 3 μg of monoclonal anti-HA-II antibody and 25 μl of GammaBind G-Sepharose beads for 500 μg of total cellular protein. Immunoprecipitates were washed twice in washing buffer (1% Nonidet P-40 and 2 mM sodium vanadate in phosphate-buffered saline), once in a second washing buffer (0.1 M Tris (pH 7.5) and 0.5 mM LiCl), and once in kinase reaction buffer (12.5 mM MOPS, 12.5 mM β-mercaptoethanol, 7.5 mM MgCl₂, 0.5 mM EGTA, 0.5 mM NaF, and 0.5 mM sodium orthovanadate). Immunoprecipitates were separated into two tubes for each sample; one was tested for JNK assay, and the other was used for Western immunoblotting. In vitro JNK assay was performed by resuspending the beads in 30 μl of kinase reaction buffer containing 1 μCi/reaction [γ-32P]ATP, 20 mM MgATP, 3.3 mM dithiothreitol, and 4 or 2 μg of GST-c-Jun (79) protein. After 30 min at 30 °C, reactions were terminated by adding 10 μl of 5× Laemmli buffer. Immunoprecipitates were fractionated by SDSPAGE on 10% polyacrylamide gels, and autoradiography was performed with the aid of an intensifying screen. The spots were quantified with a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). A second JNK assay using a commercial kit was performed without any radioactive materials. JNK was precipitated by c-Jun-conjugated beads, and JNK activity was determined by Western immunoblotting with a polyclonal anti-phosphorylated c-Jun antibody.

**Transient and Stable Transfections**—Cells were transfected by the calcium phosphate method, adjusting the total amount of DNA to 5–10 μg/plasmid with vector alone. Plasmids expressing hemagglutinin epitope-tagged JNK (pcDNA3-3-4-HA-JNK) (24) and kinase-dead Akt (pCEFL-HA-Akt K179M) or wild-type Akt (pCEFL-HA-Akt wt) (25) were used. Selection of 0.5 mg/ml G418 began 48 h after transfection. After 2–3 weeks of selection in G418, individual colonies were isolated using 0.8-mm glass cloning cylinders (PGC Scientifics, Gaithersburg, MD). As a control, empty vector pGEM-7zf(+). (Promega, Madison, WI) was transfected.

**Western Immunoblots**—For analysis of JNK protein, immunoprecipitates using monoclonal anti-HA-II antibody were analyzed by Western blotting with monoclonal anti-HA II antibody. Immunoprecipitates were visualized by enhanced chemiluminescence detection using donkey anti-rabbit IgGs coupled to horseradish peroxidase as a secondary antibody. Immunoblots were scanned by FotoLook 2.08 and quantified by MacBAS 2.31.

**Data Analysis**—Each experiment was performed at least three times. Calculations were performed using Excel Version 6.0 (Microsoft).

**RESULTS**

**Effect of IGF-I and Anisomycin on JNK Activity**—HA-tagged-JNK was transiently transfected into 293 cells, and JNK activity was determined following stimulation with 10 nM IGF-I for 0, 5, 30, and 60 min. The maximum effect was seen following 5 min of stimulation with 10 nM IGF-I (data not shown). Basal JNK activity was increased slightly by 0.1 nM IGF-I, whereas 1 and 10 nM IGF-I inhibited JNK activity. Treatment with 10 nM IGF-I suppressed JNK activity by ~40% (Fig. 1). Anisomycin potently increased JNK activity, and pretreatment with 1 and 10 nM IGF-I inhibited the anisomycin-stimulated JNK activity in a dose-dependent manner (Fig. 1). In fact, 10 nM IGF-I abrogated the anisomycin-enhanced JNK activity (p < 0.005). Thus, at physiological concentrations (26), IGF-I is a potent inhibitor of the increased JNK activity caused by anisomycin, an inducer of oxidative stress in 293 cells.

**IGF-I Suppresses Activated Endogenous JNK Activity in Parental 293 Cells**—We then wished to determine if physiological concentrations of IGF-I inhibited endogenous JNK activity. JNK activity was analyzed by performing kinase reactions with immunoprecipitates obtained from 1 mg of total cell protein. Treatment with 10 nM IGF-I alone did not change basal JNK activity (Fig. 2). Anisomycin (10 μg/ml) stimulated endogenous JNK activity by 1120 ± 276% (mean ± S.D.). IGF-I treatment suppressed the anisomycin-induced JNK activity by 76 ± 17% (p < 0.001) (Fig. 2). To explore whether IGF-I could also reduce the enhancement of JNK activity in response to a physiological stress, we performed similar experiments with TNF-α without or with IGF-I pretreatment. TNF-α (50 ng/ml) stimulated endogenous JNK activity by 825 ± 371%; however, this effect was suppressed by 62 ± 23% (p < 0.05) by IGF-I pretreatment (Fig. 2). In contrast, the TNF-α effect was suppressed by only 16% when the IGF-I was applied after TNF-α (Fig. 2). Thus, IGF-I inhibition of stress-induced JNK activity requires that the IGF-I treatment be given before the stressor, not after. Thus, activation of JNK by both anisomycin and TNF-α could be reduced by a short pretreatment with 10 nM IGF-I.

**IGF-I Pretreatment Suppresses Anisomycin-induced JNK Activity**—To confirm the above findings, we analyzed anisomycin-enhanced JNK activity and JNK protein levels in transiently transfected 293 cells. HA-tagged JNK was transiently transfected into 293 cells, and the JNK activity was normalized by the amount of p46 JNK protein, the predicted protein encoded by the transfected JNK vector (Fig. 3). The monoclonal anti-JNK antibody also detected a JNK-related protein migrating slightly slower than p46 JNK. This band is uncharacterized, but according to the supplier, is seen in other
IGF-I Suppresses JNK Activity via PI3K and Akt

Anisomycin increased JNK activity by ~530% in transfected cells, and pretreatment with 10 nM IGF-I suppressed the anisomycin-induced JNK activity by 102 ± 27% (p < 0.05) (Fig. 3). However, when 10 nM IGF-I was added after treatment with anisomycin, the IGF-I suppressive effect was not observed (data not shown). This was consistent with the importance of adding IGF-I before the stressor, as seen in Fig. 2 with TNF-α. TNF-α also induced JNK activity by ~270% in transfected cells, and pretreatment with 10 nM IGF-I suppressed the TNF-α-induced JNK activity by ~68% (data not shown). Thus, transient transfection of HA-JNK into 293 cells and stimulation with IGF-I affect JNK activity in a quantitatively similar manner compared with parental cells.

**PI3K/K Inhibitor Blocks IGF-I Effect on Anisomycin-induced Endogenous JNK Activity**—To determine whether the effect of IGF-I on suppression of anisomycin-induced endogenous JNK activity involved the MEK1/MAP kinase (ERK1 and ERK2) or the PI3K/Akt pathway, we used the specific MEK1 inhibitor PD098059 or the PI3K inhibitor LY 294002. Parental 293 cells were pretreated with the MEK1 inhibitor PD098059 (10 μM) for 30 min or with the PI3K inhibitor LY 294002 (10 μM) for 2 h before stimulation. Then the cells were treated with 10 nM IGF-I for 5 min without or with the addition of 10 μg/ml anisomycin and TNF-α in 293 cells. Parental 293 cells were treated with 10 nM IGF-I for 5 min followed by the addition of 10 μg/ml anisomycin for 20 min or 50 ng/ml TNF-α for 15 min. –, no addition of anisomycin or TNF-α; I, IGF-I treatment; A, anisomycin treatment; I-A, treatment with 10 nM IGF-I followed by treatment with 10 μg/ml anisomycin for 20 min; T, TNF-α treatment; I-T, IGF-I treatment followed by TNF-α treatment; T-I, TNF-α treatment followed by IGF-I treatment. 1 mg total cellular protein was immunoprecipitated with 3 μg of polyclonal anti-JNK1 antibody. JNK activity was analyzed by the addition of 4 μg of GST-c-Jun fusion protein, and detection of the phosphorylation of c-Jun was by autodigestion. The graph shows the results of six separate experiments (mean ± S.E.).
anisomycin for another 20 min. The MEK1 inhibitor (PD) did not significantly alter the IGF-I effect on anisomycin-induced endogenous JNK activity (Fig. 7). However, the PI3′K inhibitor (LY) blocked the IGF-I suppressive effect on anisomycin-induced endogenous JNK activity by 86 ± 39% (p < 0.05) (Fig. 7). The PI3′-kinase inhibitors similarly affected the IGF-I suppression of TNF-α-induced JNK activity by 90 ± 28% (p < 0.05) (Fig. 7). Thus, inhibition of the PI3′K pathway blocked the ability of IGF-I to suppress the stressor-induced activation of endogenous JNK.

Kinase-dead Akt Transfection Blocks IGF-I Effect on Anisomycin-induced Endogenous JNK Activity—To confirm the above findings, we analyzed anisomycin-increased JNK activity in parental 293 cells transiently expressing kinase-dead Akt or wild-type Akt. Endogenous JNK activity in mock-transfected cells was increased by anisomycin treatment by 1283 ± 337%, and pretreatment with 10 ng/ml IGF-I suppressed the anisomycin-induced JNK elevation by 56 ± 9% (Fig. 8). Compared with this result, IGF-I suppression of anisomycin-increased endogenous JNK activity in transiently transfected 293 cells with kinase-dead Akt was abrogated (Fig. 8). In 293 cells transiently expressing wild-type Akt, anisomycin-increased endogenous JNK activity was suppressed by IGF-I by 51 ± 10% (p < 0.0001) (Fig. 8). Thus, blockade of the Akt pathway abrogated the IGF-I suppression of stressor-induced JNK activation.

DISCUSSION

The IGF-IR is an αβ2-heterotetrameric protein with ligand-stimulated tyrosine kinase activity (3). Binding of IGF-I to its receptor induces receptor autophosphorylation in the intracellular kinase domain of the β-subunit and results in activation of the intrinsic tyrosine kinase activity of the IGF-IR (27). The predominant substrate of the IGF-IR is IRS-1, a docking protein that has multiple tyrosines in YMXM or related motifs known to associate with proteins containing SH2 domains. Phosphorylated IRS-1 regulates the activity of certain SH2 domain-containing proteins such as PI3′ K (9). IRS-1 also associates with other SH2 domain-containing proteins involved in growth factor signaling pathways, including Grb2, Nck, and Syp (3). Other phosphotyrosine substrates of the IGF-I signaling pathway are phospholipase C-γ and SHC (3). Phosphorylated SHC associates with Grb2 and subsequently, through a Grb2/SOS (Son of Sevenless) complex, activates Ras and Raf-1, an intermediate in the Ras/ERK signaling pathway (8). The MAP kinases are a large family of serine/threonine protein kinases, divided into a number of major subfamilies. Activation of p42 and p44 extracellular signal-regulated kinases (ERK1 and ERK2) occurs via the Ras/Raf/MEK pathway (28, 29). p38 MAP kinase, the mammalian homologue of the yeast Hog1 protein kinase, is involved in a stress-activated pathway that is induced following environmental stresses such as changes in osmolarity, UV irradiation, and heat shock (30–32). In primary
fetal neurons, insulin was shown to inhibit p38 MAP kinase activity and to promote cell survival (23). The third major MAP kinase pathway involves SAPK/JNK, which has multiple isoforms derived from three related genes (JNK1, JNK2, and JNK3). Unlike the ERK and p38 MAP kinase pathways, the JNK pathway has not as yet been demonstrated to play a role in IGf anti-apoptotic effects, although activation of JNK by TNF-α or environmental stresses leads to phosphorylation of several transcription factors that regulate immediate-early gene expression (31, 33–36).

Thus, we set out to determine if IGf-I modulated the stress-induced pro-apoptotic JNK pathway in human embryonic kidney 293 cells. Multiple activators have been shown to increase JNK activity. Increased osmolality (e.g. sorbitol treatment) activates JNK via an as yet unidentified pathway. UV irradiation and anisomycin trigger activation of the JNK pathway via MAP kinase/ERK kinase-1 (MEKK1) and MAP kinase/ERK kinase-4 (MEK4) (also known as SEK-1, JNKK, or MKK4). Recently, it has been shown that TNF-α activates apoptosis signal-regulating kinase (ASK1, a JNK kinase kinase), which then activates MEK4, leading to JNK activation (37). Furthermore, a catalytically inactive form of ASK1 pre-kinase), which then activates MEK4, leading to JNK activation.

Interestingly, in these studies, no effect of the growth factors on stress-induced JNK was measured, however. In contrast to IGF-I, fibroblast growth factor blocked TNF-α-induced apoptosis, but did not affect JNK activation (20). Since JNK activity may produce pleiotropic effects, in some instances inducing apoptosis coincident with growth factor proliferation, we conclude that the different responses to these growth factors are likely cell type-specific and determined by variability in the cellular context, whether in the resting state or in the stress-activated state. Thus, other pathways may impact on these differential responses under varying conditions of cell stimulation.

Dominant-negative forms of MEK4 block Fas-induced JNK activation (45), providing evidence that Fas-induced apoptosis is mediated, at least in part, by the MEK4/JNK pathway (46). Daxx, a signaling protein that binds to the Fas death domain, stimulates both JNK activation and apoptosis. The Daxx apoptotic pathway is sensitive to Bel-2, which is itself regulated by IGf-I signaling (47). Furthermore, expressed Bel-2 inhibits ceramide and Fas activation of JNK (48, 49). Heat shock protein (hsp70) can prevent ceramide-induced activation of JNK and apoptosis (50). Presently, there is no evidence that IGf-I signaling converges with hsp70 upstream of JNK. Recently, two members of the mixed lineage kinases, MUK/DLK/ZPK and mammalian STE20-like protein kinase/MLK2, have been shown to activate MEK4 and JNK (51). These proteins appear to be JNK kinase kinases that are parallel to MEKK1. Therefore, any convergent effects on the JNK pathway that positively or negatively affect JNK activity could occur at the level of MEK4. At this point, there is no evidence that IGF-I signals through the mixed lineage kinases.

In summary, we have shown that IGf-I blocks the enhancement of JNK activity in response to anisomycin and TNF-α. Furthermore, we have shown results consistent with the model that IGf-I impacts on the JNK pathway at or upstream of JNK. It will be important to extend these studies of the IGf-I impact on the JNK pathway and provide identification of the specific pathways regulating the balance between cell proliferation and apoptosis when cells are exposed both to the growth factor IGf-I and stressors known to promote apoptosis.

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