Insulin-Like Growth Factor I Prevents Mannitol-Induced Degradation of Focal Adhesion Kinase and Akt

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Running Title: Mannitol-induced FAK and Akt degradation

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SUMMARY

In our laboratory, we are interested in hyperosmolarity-induced apoptosis in neuronal cells. We have shown that high concentrations of glucose or mannitol induce apoptotic cell death in dorsal root ganglia in culture and in SH-SY5Y and SH-EP human neuroblastoma cells. Focal adhesion kinase (FAK) is a cytoplasmic tyrosine kinase that has a critical role for transmitting integrin-mediated-signals. In this study, we report that hyperosmolar treatment mediates FAK dephosphorylation and cleavage, which is prevented by IGF-I treatment. Mannitol treatment of SH-EP cells transfected with vector (SH-EP/pSFFV) results in concentration- and time-dependent dephosphorylation and degradation of FAK. Dephosphorylation and degradation of FAK is tightly correlated with apoptotic morphological changes, including the disruption of actin stress fibers, the loss of focal adhesion sites, membrane blebbing and cell detachment. Treatment of SH-EP/pSFFV cells with IGF-I or transfection of IGF-IR prevents these changes. Treatment of cells with pharmacologic inhibitors of the MAP kinase or PI 3-kinase pathways does not affect mannitol-induced FAK dephosphorylation and degradation. However, PI 3-kinase is necessary for IGF-I-mediated protection against FAK alteration. Mannitol treatment also results in the degradation of Akt. Mannitol induces the activation of caspases -3 and -9 in a time course similar to the dephosphorylation and degradation of FAK. Treatment of the cells with ZVAD, a general caspase inhibitor, blocks the mannitol-induced FAK and Akt degradation as well as cell detachment and apoptosis. These results suggest that 1) one of the pathways of mannitol-mediated apoptosis is through the degradation of FAK and Akt and 2) IGF-I protects the cells from apoptosis by blocking the activation of caspases, which may be responsible for the loss of FAK and Akt.
FOOTNOTE

1 The abbreviations used are: DMEM, Dulbecco’s modified Eagle’s medium; ECM, extracellular matrix; FAK, focal adhesion kinase; pFAK, phosphorylated FAK; IGF-I, insulin-like growth factor-I; IGF-IR, type I IGF receptor; MAP kinase, mitogen-activated protein kinase; PI 3-kinase, phosphatidylinositol 3-kinase
INTRODUCTION

Insulin-like growth factor-I (IGF-I) is a potent mitogen and survival factor (1). The actions of IGF-I are mediated via the type 1 IGF receptor (IGF-IR). Cell viability correlates with IGF-IR expression and intact IGF-I/IGF-IR signaling pathways, including activation of mitogen-activated protein (MAP) kinase/phosphatidylinositol (PI)-3 kinase (2). *In vitro*, the first morphological effect of IGF-I/IGF-IR involves redistribution of filamentous (F)-actin with ruffling of actin bundles, followed by lamellipodia formation (3, 4). IGF-I/IGF-IR mediated actin redistribution and signaling is dependent on integrin ligand binding by cells to the extracellular matrix (ECM) (5, 6). Integrin:ECM binding results in enhanced association of integrin β subunits with the actin cytoskeleton via adhesion sites (7). These sites contain many focal adhesion proteins, including focal adhesion kinase (FAK) and paxillin (8) which, upon tyrosine phosphorylation, stimulate focal adhesion assembly. Like integrin-ligand binding, IGF-I stimulates the downstream phosphorylation of focal adhesion proteins with F-actin redistribution and subsequent cytoskeletal changes (3, 9, 10).

FAK is an important member of focal adhesion complexes that functions as a key mediator to transmit the signals from the extracellular matrix to cytoplasm (11). FAK plays important roles in many fundamental cellular functions including cell adhesion (12), migration (13), and survival (14, 15). Upon integrin ligation (11) or growth factor stimulation (10, 16), FAK undergoes autophosphorylation and forms complexes with other signaling molecules such as PI 3-kinase, Grb2, Shc, Src and paxillin (11). Cells denied anchorage undergo a form of apoptosis called anoikis (17). Growing evidences suggest that there is an association between apoptosis, cellular adhesion and the cytoskeleton (17, 18) and implicate FAK in this process. Disruption of FAK signaling results in apoptosis (15, 19) and overexpression of FAK can prevent apoptosis, including anoikis (19-21).
In our laboratory, we investigate mannitol-induced apoptosis in primary and transformed neuronal cells. We have shown that high concentrations of glucose or mannitol induce an apoptotic cell death in dorsal root ganglia and glial cells, as well as in SH-SY5Y and SH-EP human neuroblastoma cells (22-25). In addition, IGF-I treatment or overexpression of IGF-IR prevents glucose- or mannitol-induced cell death (24, 26, 27). Overexpression of anti-apoptotic members of the Bcl family (Bcl-2 or Bcl-xL) (25, 27) or inhibition of caspases (27, 28) also prevents apoptosis in neuronal cells. The PI 3-kinase pathway is critical for the anti-apoptotic effect of IGF-I (29-31). The current study extends our previous reports by investigating the role of FAK in mannitol-induced apoptosis in SH-EP neuroblastoma cells. Our results indicate that the initial effects of mannitol treatment are the disruption of the actin cytoskeleton and loss of focal adhesion sites, followed by cell detachment and death. This process is preceded by dephosphorylation and degradation of FAK, and degradation of the downstream signaling molecule Akt. These events are prevented by IGF-IR overexpression or activation. Treatment of the cells with ZVAD, a general caspase inhibitor, prevents the loss of FAK and Akt. Our study suggests that one mechanism for IGF-I/IGF-IR mediated survival is through prevention of alterations in FAK, which is essential in maintaining an intact cellular cytoskeleton.
EXPERIMENTAL PROCEDURES

Materials

Antibodies against phosphorylated FAK (pFAK; pY397 and pS722) were purchased from Biosource International (Camarillo, CA). Anti-FAK, anti-integrin β-1, and PY20 anti-phosphotyrosine antibodies were purchased from Transduction Laboratories (Lexington, KY), and antibodies against phosphorylated and unphosphorylated Akt and MAP kinase were purchased from Cell Signaling Technology (Beverly, MA). Anti-caspase 3 and 9 antibodies were from Pharmingen (San Diego, CA). Secondary antibodies for Western immunoblotting and Protein A/G-agarose bead were from Santa Cruz Biotechnology (Santa Cruz, CA). Texas Red-conjugated phalloidin, biotin-labeled secondary antibodies and FITC-avidin were from Vector Laboratories (Burlingame, CA). PD98059 and LY294002 were purchased from Calbiochem (San Diego, CA). ZVAD was purchased from Enzyme System Product (Livermore, CA). Recombinant human IGF-I was kindly provided by Cephalon, Inc (Westchester, PA).

Cell Culture

Transfection of SH-EP cells with pSFFV and the vector containing human IGF-IR cDNA has been described previously (26). Transfected SH-EP cells were maintained in DMEM containing 10% calf serum and 0.25 mg/ml G418. The cells were serum starved for 6 h before each experiment. When inhibitors were used, cells were preincubated with inhibitors for 30 min before mannitol and/or IGF-I treatment.

Immunoblotting, Immunoprecipitation and Immunocytochemistry

Immunoblotting and immunoprecipitation were performed as previously described (32). Typical representative results of at least three repeated experiments are shown in the figures. Immunocytochemistry was performed as described previously (3). For simultaneous staining of
pFAK and F-actin, Texas Red-phalloidin was added with FITC-avidin at the final incubation.

**Flow Cytometry**

Percent sub G₀ DNA was measured as the percent of apoptotic cells using flow cytometry as described previously (27). All results are expressed as the mean percent apoptotic cells of at least three experiments ± the standard error of the mean (S.E.M.).
RESULTS

Mannitol-induced Morphological Changes in SH-EP Cells

We began the current study by investigating the morphological changes induced by mannitol exposure in SH-EP neuroblastoma cells. When vector-transfected SH-EP (SH-EP/pSFFV) cells are serum-starved for a total of 10 h, most of the cells remain attached to the culture dishes (Fig. 1A). However, about 30% of the cells start to round up and display morphological features of cells undergoing detachment. Less than 15% of IGF-IR transfected SH-EP (SH-EP/IGF-IR) cells detach from the dishes in serum-free conditions (Fig. 1A).

When both SH-EP/pSFFV and SH-EP/IGF-IR cells are stained with Texas Red phalloidin, well-organized actin stress fibers are seen in attached cells. In contrast, when SH-EP/pSFFV cells are serum-starved for 6 h then incubated with 300 mM mannitol for 4 h, most (94.5 ± 5.8%) of the cells are rounded and detached from the dishes and subsequently undergo apoptosis (Fig. 1A-B). Actin stress fibers in mannitol-treated SH-EP/pSFFV cells are completely disrupted, even in the attached cells (Fig. 1A). Although the cells start to detach, significant numbers of SH-EP/IGF-IR cells still adhere to the dishes after 4 h mannitol treatment (less than 40% detached cells) (Fig. 1B). However, the adherent cells have less organized actin stress fibers than cells in DMEM alone, and mannitol treated SH-EP/IGF-IR cells show dense actin staining at the periphery of the cells, which represents active membrane ruffling (arrowheads, Fig. 1A). After mannitol treatment, the cells were stained with propidium iodide to measure sub G0 DNA, indicating apoptosis. Mannitol treatment results in apoptosis in both SH-EP/pSFFV and SH-EP/IGF-IR cells; however, significantly less SH-EP/IGF-IR cells undergo apoptosis (Fig. 1C). These results demonstrate that the initial cellular events after mannitol treatment are
the loss of actin stress fibers and detachment of the cells from substrate, leading to an anoikis-like cell death. Consistent with our previous results (26, 27), IGF-IR overexpression significantly prevents or delays these early apoptotic events.

**Mannitol Induced Concentration- and Time-dependent Degradation and Dephosphorylation of FAK that Correlates with the Morphological Changes**

FAK is involved in anoikis in many cell types (14, 19, 21). Since our results suggest an anoikis-like cell death induced by mannitol treatment, we investigated changes in FAK in SH-EP/pSFFV and SH-EP/IGF-IR cells. Treatment with mannitol results in a concentration-dependent decrease in the phosphorylation of FAK at Tyr 397 in both SH-EP/pSFFV and SH-EP/IGF-IR cells (Fig. 2A). However, FAK phosphorylation is maintained in SH-EP/IGF-IR cells treated with 150 and 300 mM mannitol. FAK is cleaved during apoptosis, resulting in smaller fragments (33-35).

Mannitol treatment also induces the degradation of FAK, resulting in two major cleavage fragments between 97 and 66 kD (Fig. 2A). However, mannitol treatment has no effect on the protein levels of actin (Fig. 4E), IGF-IR β subunit (Fig. 4F) or MAP kinase (Fig 7), suggesting that the changes in FAK are specific. Mannitol-induced FAK dephosphorylation and degradation are also time-dependent (Fig. 2B). FAK phosphorylation starts to decrease after 45 min of mannitol treatment, and FAK is completely dephosphorylated by 90 min (Fig. 2B). This dephosphorylation occurs simultaneously with the disappearance of 125 kD bands, indicating degradation. FAK degradation also begins at 45 min and continues up to 6 h. The time course of FAK dephosphorylation and degradation in SH-EP/IGF-IR cells is delayed compared to SH-EP/pSFFV cells, indicating an important role of IGF-IR signaling in the prevention of mannitol-induced FAK alterations.

FAK dephosphorylation and degradation results in distinct morphological changes.
These morphological changes, which include membrane blebbing, loss of contacts with neighboring cells, rounding up, and detachment, are noticeable 45 min after mannitol treatment (Fig. 3). Morphological changes continue through the time course, and most of the cells lose their contact by 90 min. These results suggest that mannitol-induced apoptosis involves alterations of phosphorylation and protein levels of FAK, resulting in cell detachment.

**IGF-I Signaling Can Prevent FAK Dephosphorylation, Degradation and Cytoskeletal Changes**

Our results suggest that IGF-I signaling through the IGF-IR prevents FAK dephosphorylation and degradation. We therefore investigated the effect of IGF-IR activation on mannitol-induced FAK alteration. Serum-starved SH-EP/pSFFV cells were treated with IGF-I and immunoblotted with anti-pFAK antibodies raised against pTyr397 and pSer722 sites (Fig. 4A and B). In the absence of mannitol, IGF-I treatment slightly increases the phosphorylation of FAK (Fig. 4A). Mannitol treatment results in almost complete dephosphorylation on both sites and, like IGF-IR transfection, IGF-I treatment blocks this effect of mannitol (Fig. 4B). Similar results are obtained by immunoprecipitation with anti-FAK followed by immunoblotting with anti-phosphotyrosine antibody (Fig. 4C). IGF-I treatment also blocks the degradation of FAK by mannitol (Fig. 4D). Mannitol treatment has little effect on the protein levels of actin (Fig. 4E) or IGF-IR β subunit (Fig. 4F).

To examine IGF-I-induced morphological changes, cells were immunostained with anti-FAK and phalloidin (Fig. 5). Serum-starved SH-EP/pSFFV cells show well-organized actin stress fibers in most of the cells (Fig. 5A). FAK staining displays a typical punctate pattern at the focal adhesion sites. When the two images are overlayed, most FAK staining is localized at the termini of actin stress fibers. IGF-I addition to the control serum-starved cells results in little change in both actin and FAK immunostaining (Fig. 5B). As in Fig.1, mannitol treatment
results in the disruption of actin stress fibers, and FAK staining cannot detect the focal adhesion sites (Fig. 5C). IGF-I treatment prevents mannitol-induced detachment of the cells, the disruption of actin stress fibers, and the loss of focal adhesion sites, although the focal adhesions are not as well maintained as in control or control + IGF-I cells (Fig. 5D). Most of the focal adhesion sites in the mannitol + IGF-I treated cells are smaller than the control or control + IGF-I cells and are localized at the periphery of the cells. Immunostaining with anti-pFAK showed similar pattern of staining (data not shown).

**PI 3-kinase Pathway Plays an Important Role for the Protective Effect of IGF-I Against Mannitol**

IGF-I mediates its biological effect through two major signaling pathways, the MAP kinase and PI 3-kinase pathways (2, 36). We have previously reported that the MAP kinase pathway is important for neurite outgrowth (4, 32) and the PI 3-kinase pathway is responsible for membrane ruffling, motility, and survival (9, 28, 29, 37) in neuronal cells. To test which pathway is important for IGF-I prevention of FAK dephosphorylation and degradation, we treated the cells with pharmacologic inhibitors of each pathway. Treatment of the cells with PD98059, an inhibitor of MAP kinase pathway, has no effect on either mannitol-induced FAK alterations or the preventive effect of IGF-I (Fig. 6). The PI 3-kinase inhibitor, LY294002, also has no effect on mannitol-induced FAK dephosphorylation or degradation, although it made the cells detach earlier than mannitol-alone treatment (data not shown). However, LY294002 completely inhibits the protective effect of IGF-I, which is in agreement with our previous reports (28, 29). PD98059 and LY294002 block basal and IGF-I induced MAP kinase and Akt activation, respectively.

One of the important downstream signaling molecules in the PI 3-kinase pathway is Akt (38). PI 3-kinase/Akt pathway is critical for the survival of many cell types (39). We have shown that
transfection of dominant negative Akt prevents IGF-I mediated survival in Schwann cells (31). Incubation of SH-EP/pSFFV cells with mannitol results in a time-dependent degradation of Akt (Fig. 7A), which occurs later than FAK degradation. Akt degradation begins 1.5 h after mannitol treatment, and Akt expression is undetectable by 4 h. Activation of IGF-I signaling prevents the degradation of Akt (Fig. 7B). 4 h treatment with IGF-I in the absence of mannitol has no effect on the protein levels of Akt in both SH-EP/pSFFV and SH-EP/IGF-IR cells. However, mannitol treatment in SH-EP/pSFFV cells results in the degradation of Akt without affecting MAP kinase. IGF-I treatment or IGF-IR overexpression prevents mannitol-induced loss of Akt. Interestingly, MAP kinase levels are unaffected by mannitol or IGF-I treatment in both cell types. These results suggest that the modulation of Akt protein levels plays a role in mannitol-induced apoptosis.

**ZVAD Prevented FAK Dephosphorylation and Degradation**

Caspases are important mediators of apoptosis (40) and are responsible for the cleavage of FAK during apoptosis (33-35). Caspases -3 and -9 are synthesized as inactive precursors that are proteolytically cleaved to generate active enzymes (41). We have shown that caspases are involved in neuronal apoptosis (25, 27, 30). When SH-EP/pSFFV cells are treated with 300 mM mannitol, there is a time-dependent cleavage of caspases -3 and -9 (Fig. 7A). This time course is similar to the time course of FAK dephosphorylation and degradation, suggesting the involvement of caspases in FAK alterations. To study the role of caspases in FAK cleavage, we used ZVAD, a general caspase inhibitor. ZVAD treatment blocks the activation of caspase-3 and also inhibits caspase-9 cleavage to some degree (Fig. 7B). 50 µM ZVAD partially blocks mannitol-induced dephosphorylation and degradation of FAK (Fig. 7C). ZVAD treatment also prevents mannitol-induced degradation of Akt (Fig. 7D). As in Fig. 6, mannitol treatment results in Akt...
degradation, and treatment with either IGF-I or ZVAD blocks this effect. Unlike the effect on FAK, ZVAD almost completely blocks the degradation of Akt. 4 h of incubation with mannitol results in cell detachment and apoptosis, and IGF-I treatment significantly prevents these events. Consistent with the effect on FAK and Akt alterations, ZVAD also inhibits mannitol-induced cell detachment and apoptosis in SH-EP/pSFFV cells (Fig. 7E).
DISCUSSION

The current study investigates the effects of hyperosmotic stress induced by mannitol on cytoskeletal changes and FAK and Akt preceding apoptosis. We have previously shown that glucose and mannitol induce an apoptotic death in human neuroblastoma cells and primary neuronal cells (22-25), which is prevented by IGF-IR overexpression or activation (24, 26, 27). SH-EP neuroblastoma cells incubated with mannitol show morphological changes typical of apoptosis, including membrane blebbing, rounding up, and cell detachment, (42) about 1 h after treatment. Mannitol-treated cells also lose focal adhesions and actin stress fibers, consistent with changes induced by other apoptotic signals (43, 44). IGF-IR overexpression or activation prevents cell detachment, but the actin stress fibers are less organized and show dense staining at the periphery of the cells, representing active membrane ruffling. Mannitol-treated cells incubated with IGF-I and immunostained with FAK display typical features of focal complexes (smaller dot-like staining mostly at the edge of lamellipodium of the cells, considered to be the precursor of focal adhesions), rather than focal adhesions (flat, elongated and larger than focal complexes, located mainly at the periphery of the cells) seen in control cells (8).

FAK, an important molecule in focal adhesion formation (11), plays a critical role in various types of apoptosis. Inhibition of FAK signaling by microinjection of an anti-FAK antibody or antisense oligonucleotide can induce apoptosis (15, 19). Conversely, overexpression of FAK prevents anoikis (14) and apoptosis induced by ultraviolet irradiation (20), hydrogen peroxide treatment, or etoposide (21). In this report, we show that FAK is dephosphorylated and degraded during mannitol-induced apoptosis. FAK is cleaved during apoptosis in other systems (34, 44-46). Some reports show the appearance of another cleavage product around 40 kD (34, 44); however we could not detect this band, possibly due to the different cell type or antibody used. Given the importance of FAK
in focal adhesion formation, mannitol-induced dephosphorylation and cleavage of FAK likely leads to cell detachment, promoting anoikis.

Caspases are responsible for the cleavage of FAK and other signaling molecules during apoptosis (33-35). Our current data suggest involvement of caspases in FAK degradation during mannitol-induced apoptosis as well. The time course of caspase-3 and -9 cleavage (i.e. activation) is similar to that of FAK degradation and dephosphorylation. The general caspase inhibitor, ZVAD, however, does not completely prevent mannitol-induced caspase activation and FAK degradation, suggesting that another pathway is involved in these processes. The proteasome/ubiquitin pathway is responsible for apoptosis in other systems, and proteasomes can activate caspases (47, 48). The proteasome/ubiquitin pathway is activated during mannitol-induced apoptosis, and proteasome inhibitors block FAK alterations and apoptosis (B. Kim, unpublished data). Therefore, multiple proteolytic pathways are likely involved in mannitol-induced FAK degradation and dephosphorylation in neuroblastoma cells.

Although ZVAD partially prevents FAK degradation and apoptosis, this inhibitor has less effect on FAK dephosphorylation and detachment, similar to previous reports (43). SH-EP cells treated for 4 h with the serine/threonine phosphatase inhibitor okadaic acid display complete dephosphorylation and cell detachment with no effect on FAK degradation and apoptosis (B Kim, unpublished observation). Therefore, we believe that phosphorylation of FAK is required for the cells to maintain their attachment, but degradation of FAK is a prerequisite to the commitment of apoptosis.

IGF-I is a strong survival factor in neuronal cells (36). Consistent with its anti-apoptotic effect, IGF-I treatment inhibits mannitol-induced dephosphorylation and degradation of FAK. However, unlike SH-SY5Y cells (9), treatment of SH-EP cells with IGF-I alone has little effect on
FAK phosphorylation or changes in cell morphology. This may be due to high basal FAK phosphorylation or low IGF-IR levels present in SH-EP cells. IGF-I treatment in SH-EP/IGF-IR cells results in increased membrane ruffling (data not shown) without detectable changes in FAK phosphorylation.

We have shown that the PI 3-kinase pathway, but not the MAP kinase pathway, is important for the survival effect of IGF-I (25, 29). Treatment of SH-EP cells with a PI 3-kinase inhibitor completely blocks the ability of IGF-I to inhibit mannitol-induced FAK alterations. This suggests that PI 3-kinase is an upstream regulator of FAK, in contrast with previously published reports (21). However, we, and others, have shown that cytochalasin D and LY294002 or wortmannin block IGF-I induced FAK phosphorylation in SH-SY5Y neuroblastoma (9) and Swiss 3T3 fibroblast cells (10). PI 3-kinase also plays an important role in maintaining the cytoskeletal integrity (49). Therefore, we propose that PI 3-kinase regulates cytoskeletal structures in our system, not FAK directly.

Akt is a serine/threonine kinase that plays a critical role in many cellular responses, including glucose metabolism, apoptosis, and cell migration (39). Integrin ligation or growth factor activation phosphorylates and activates Akt through PI 3-kinase (38, 50, 51). Activated Akt regulates cell survival by interacting with molecules involved in apoptosis including caspases (52), Bad (53) and Forkhead (54). In our laboratory, we have shown the PI 3-kinase/Akt pathway is important for IGF-I signaling in cell survival (25, 29), motility (31, 37) and neurite outgrowth (55). IGF-I treatment or IGF-IR transfection prevents mannitol-induced degradation of Akt. Unlike the effect on FAK, ZVAD almost completely inhibits the loss of Akt. Apoptotic signals also induce inactivation of Akt through caspase-mediated cleavage in U937 and Jurkat cell lines (35, 56). There are at least three caspase cleavage consensus sequences in Akt upon which caspase-3, -6, and -7 can operate (56, 57).
Furthermore, cell detachment induces the cleavage of Akt by a death receptor-dependent pathway that results in anoikis (58). We have shown that mannitol treatment reduces anti-apoptotic Bcl-2 protein level in neuroblastoma cells (59). Therefore Akt degradation, along with the decreased levels of Bcl-2, could accelerate mannitol-induced apoptosis.

In summary, we demonstrate in this study that mannitol treatment results in FAK dephosphorylation and degradation resulting in morphological changes. Mannitol treatment also results in the degradation of Akt. Based upon these data, we believe that mannitol induces caspase-mediated degradation of FAK, which results in cell detachment, Akt loss, and apoptosis. Activation of IGF-I signaling through the PI-3K pathway prevents caspase activation and the loss of FAK, resulting in cell attachment and survival.
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FIGURE LEGENDS

Figure 1. Mannitol-induced morphological changes in SH-EP cells. (A) Vector (pSFFV)-
or IGF-IR-transfected SH-EP cells were serum starved for 6 h and treated
without or with 300 mM mannitol for 4 h. For F-actin staining, the cells were
fixed and incubated with Texas Red conjugated phalloidin. Note that during the
staining procedure most of the detached cells were washed away, resulting in
fewer cells showing in the immunocytochemistry compared to the light
microscopy pictures. Arrowheads indicate peripheral actin staining, which
represent membrane ruffling. (B) The attached and detached cells were counted
from the light microscopy pictures. Cells from at least 10 different random
microscopy fields of 3 different experiments were counted. $p < 0.001$ (by t-test)
between control and mannitol-treated cells and also between SH-EP/pSFFV and
SH-EP/IGF-IR cells. (C) The cells were treated as in (B) and the apoptotic cells
were measured using flow cytometry. $p < 0.001$ between control and mannitol-
treated cells and $p < 0.01$ between mannitol-treated SH-EP/pSFFV and SH-
EP/IGF-IR cells.

Figure 2. Mannitol induces time- and concentration-dependent dephosphorylation and
degradation of FAK. Vector- or IGF-IR-transfected SH-EP cells were serum
starved for 6 h and then treated with increasing concentrations of mannitol (Man)
for 4 h (A) or with 300 mM mannitol for the indicated times (B). The cell lysates
were separated by SDS-PAGE, transferred to nitrocellulose paper and then
immunoblotted with antibodies against phosphoFAK (pFAK) or regular FAK
(FAK). pFAK was raised against pTyr 397.
Figure 3. Mannitol induces time-dependent detachment of the cells. Serum-starved vector-transfected SH-EP cells were incubated without or with 300 mM mannitol and the light microscopy pictures were taken at the indicated times. Arrowheads indicate membrane blebbing.

Figure 4. IGF-I prevents mannitol-induced FAK dephosphorylation and degradation. Serum-starved vector-transfected SH-EP cells were incubated without or with 300 mM mannitol (Man) in the absence or presence of 10 nM IGF-I for 4 h. The cell lysates were immunoblotted with the anti-phosphospecific FAK antibodies raised against Tyr397 (A) or Ser722 (B) or with regular anti-FAK antibody (D). (C) The cell lysates were immunoprecipitated with anti-FAK antibody and immunoblotted with anti-phosphotyrosine antibody. To prove the specificity of mannitol treatment the cell lysates were also immunoblotted with anti-actin (E) or anti-IGF-IR β subunit antibodies (F).

Figure 5. Mannitol and IGF-I induced morphological changes in SH-EP cells. Vector-transfected SH-EP cells were serum starved for 6 h and treated without or with 300 mM mannitol ± 10 nM IGF-I for 4 h. The cells were fixed and stained with Texas red-phalloidin and anti-FAK antibody.

Figure 6. PI 3-kinase pathway plays an important role during the mannitol-induced dephosphorylation and degradation of FAK. Serum-starved vector-transfected SH-EP cells were preincubated with 50 µM PD98059 (PD) or 20 µM LY294002 (LY) for 30 min and then treated with 300 mM mannitol and/or 10 nM IGF-I for 4 h. The cell lysates were immunoblotted with anti-phosphoFAK (pFAK), anti-FAK or anti-phosphoAkt (pAkt) + anti-phosphoMAP kinase (pMAPK)
antibodies.

**Figure 7.** Mannitol induced degradation of Akt that can be prevented by IGF-I signaling.

(A) Vector- or IGF-IR-transfected SH-EP cells were treated without or with 300 mM mannitol for 4 h. The cell lysates were immunoblotted simultaneously with anti-Akt and MAP kinase antibodies.  

(B) Serum-starved SH-EP/pSFFV and SH-EP/IGF-IR cells were treated without or with 300 mM mannitol ± 10 nM IGF-I for 4 h and the cell lysates were immunoblotted simultaneously with anti-Akt and MAP kinase antibodies.

**Figure 8.** ZVAD can prevent mannitol-induced FAK and Akt dephosphorylation and degradation.  

(A) Serum-starved vector-transfected SH-EP cells were treated with 300 mM mannitol for the indicated times and the cell lysates were immunoblotted with anti-caspase 3 or 9 antibodies.  

(B-D) Vector-transfected SH-EP cells were treated without or with 300 mM mannitol (Man) in the absence or presence of 10 nM IGF-I or 50 µM ZVAD for 4 h. ZVAD was preincubated for 30 min before mannitol treatment. The cell lysates were immunoblotted with indicated antibodies.  

(E) The cells were treated as above and the cells were stained with Texas red phalloidin. Apoptotic cells were measured using flow cytometry and the values are indicated. $p < 0.005$ (by t-test) compared to mannitol treated cells.
Figure 1

A

SH-EP / pSFFV

Control

Mannitol

SH-EP / IGF-IR

Control

Mannitol

B

C

% Detached Cells

% Apoptotic Cells

SH-EP / pSFFV

SH-EP / IGF-IR

Control

Mannitol

Control

Mannitol
Figure 2

A

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pFAK

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Figure 4

A

IGF-I

| - | + | - | + |

IB: pFAK (pY397)

B

| - | + | - | + |

pFAK (pS722)

C

IGF-I

| - | + | - | + |

IP: FAK

IB: pTyr

D

| - | + | - | + |

IB: FAK

E

IGF-I

| - | + | - | + |

IB: Actin

F

| - | + | - | + |

IGF-IR β-subunit
Figure 5

F-Actin | FAK | Overlay
---|---|---
Control |  |  |
Control + IGF-I |  |  |
Mannitol |  |  |
Mannitol + IGF-I |  |  |
Figure 6

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- pFAK
- FAK
- pAkt
- pMAPK
Insulin-like growth factor I prevents mannitol-induced degradation of focal adhesion kinase and Akt
Bhumsoo Kim and Eva L. Feldman

J. Biol. Chem. published online May 14, 2002

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