Published in final edited form as:

Apoptosis. 2009 May ; 14(5): 665–673. doi:10.1007/s10495-009-0331-0.

Insulin Receptor Substrate (IRS)-2, Not IRS-1, Protects Human Neuroblastoma Cells Against Apoptosis

Bhumsoo Kim, Ph.D. and Eva L. Feldman, M.D., Ph.D.

University of Michigan, Department of Neurology, Ann Arbor, Michigan 48109 USA

Abstract

Insulin receptor substrates (IRS)-1 and -2 are major substrates of insulin and type I insulin-like growth factor (IGF-I) receptor (IGF-IR) signaling. In this study, SH-EP human neuroblastoma cells are used as a model system to examine the differential roles of IRS-1 and IRS-2 on glucose-mediated apoptosis. In the presence of high glucose, IRS-1 underwent caspase-mediated degradation, followed by focal adhesion kinase (FAK) and Akt degradation and apoptosis. IRS-2 expression blocked all these changes whereas IRS-1 overexpression had no effect. In parallel, IRS-2, but not IRS-1, overexpression enhanced IGF-I-mediated Akt activation without affecting extracellular regulated kinase signaling. While IRS-1 was readily degraded by caspases, hyperglycemia-mediated IRS-2 degradation was unaffected by caspase inhibitors but blocked by proteasome and calpain inhibitors. Our data suggest that the differential degradation of IRS-1 and IRS-2 contributes to their distinct modes of action and the increased neuroprotective effects of IRS-2 in this report are due, in part, to its resistance to caspase-mediated degradation.

Keywords

IRS; apoptosis; caspase; neuroblastoma

INTRODUCTION

Insulin-like growth factor-I (IGF-I) plays crucial roles in the regulation of growth, survival, and normal metabolism of many cell types (1). IGF signaling plays important roles in the malignant transformation of many tumors, including neuroblastoma (2). Most of the physiological effects of IGF-I are mediated by the type I IGF receptor (IGF-IR) (3). Ligand binding of IGF-IR causes IGF-IR autophosphorylation, which subsequently initiates the binding of specific docking molecules to the phosphorylated receptor. One such docking molecule is insulin receptor substrate (IRS), which binds to phosphotyrosine residues on IGF-IR β -subunits. Receptor-IRS binding is required for IGF-I-mediated downstream signal transduction (4).

The most thoroughly characterized members of the IRS protein family are IRS-1 and IRS-2. Even though they display similar structural and functional characteristics, IRS-1 and IRS-2

mediate distinctly different cellular responses. IRS-2 knockout mice, but not IRS-1 deficient mice, develop diabetes (5, 6), and disruption of the IRS-2 gene results in reduced neuronal proliferation and impaired brain growth (7). In hepatocellular carcinoma, IRS-1 mediates the mitogenic effects of IGF-I (8), whereas IRS-2 is responsible for the metabolic and anti-apoptotic effects of insulin in adult and neonatal hepatocytes (6, 9). IRS-1 and IRS-2 also have differential roles during serum deprivation-induced apoptosis in pre-adipocytes (10) and activation-induced cell death in T cell hybridomas (11). Collectively, these reports suggest that IRS-1 and IRS-2 may have unique roles for cell survival and proliferation.

Our laboratory studies the regulation of apoptosis in primary and transformed neuronal cells. High concentrations of glucose or mannitol induce apoptotic cell death in primary dorsal root ganglia (DRG) neurons and glia, and in SH-SY5Y and SH-EP human neuroblastoma (NBL) cells (12–14). Apoptosis in SH-EP cells is accompanied by dephosphorylation and degradation of focal adhesion kinase (FAK) and Akt, disruption of actin stress fibers, loss of focal adhesion sites, and cell detachment, all of which are prevented by IGF-I treatment, overexpression of IGF-IR (13, 15) or caspase inhibition (16). We previously reported differential expression of IGF-IR and IRS in NBL cells. SH-EP cells express low levels of IGF-IR and IRS-2 and high levels of IRS-1. In contrast, SH-SY5Y cells express high levels of IGF-IR and IRS-2 and virtually no IRS-1. By transfecting SH-SY5Y cells with IRS-1 and SH-EP cells with IRS-2, we demonstrated that the differential regulation of IRS proteins by IGF-I is a key pathway modulating IGF-IR signaling in NBL cells (17).

Neuroblastoma is the most common solid tumor found in children and accounts for 7–10% of pediatric malignancies (18), with most cases detected in children younger than 10 years of age (19). Unfortunately, less than 20% of high-grade neuroblastoma patients survive despite the currently available chemotherapeutic and radiation therapies (20). While most solid tumors respond to chemotherapeutic agents by undergoing apoptosis (21), neuroblastoma is frequently chemoresistant. To further define the development of apoptosis, we examined the differential roles of IRS-1 and IRS-2 in neuroblastoma, using SH-EP NBL cells as a model system. In this report we show evidence suggesting that differential resistance to degradation by proteases may contribute to the unique protective role of IRS-2 in neuronal cells.

EXPERIMENTAL PROCEDURES

Materials

Anti-IRS-1, anti-IRS-2, anti-FAK, anti-HA, anti-p85, anti-Shc, and anti-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against total and phosphorylated forms of Akt and extracellular regulated kinase 1 and 2 (ERK1/2), and caspase-3 were purchased from Cell Signaling Technology (Beverly, MA). MG132 and calpain inhibitor III were purchased from Calbiochem (La Jolla, CA). General caspase inhibitor, ZVAD, was purchased from Biomol (Plymouth Meeting, PA). Recombinant human IGF-I was kindly provided by Cephalon, Inc. (Westchester, PA). HA-tagged IRS-1 and IRS-2 containing vectors were kindly provided by Dr. D. Yee of the University of Minnesota (22).

Cell Culture

SH-EP and SH-SY5Y human NBL cells were maintained in DMEM containing 10% calf serum. HA-tagged IRS-1 and IRS-2 containing vectors or empty pcDNA vectors were transfected into NBL cells using Lipofectamine 2000 reagent (Invitrogen Life Technology, Carlsbad, CA) according to the manufacturer's protocol. Transfected cells were maintained in DMEM containing 10% calf serum and 0.25 mg/ml G418 (Invitrogen Life Technology). The cells were serum starved 4–6 h before treatment. The inhibitors were added with glucose treatment. IRS-2 siRNA was purchased from Dharmacon (Chicago, IL). SH-SY5Y cells were transfected with 150nM siRNA using Lipofectamine 2000 reagent for 96 h before treatment.

Flow Cytometry

Hypodiploid (sub-G₀) apoptotic analyses of SH-EP transfectants were performed in triplicate using propidium iodide as previously described (13). Briefly, cells were serum starved overnight, treated with or without 300 mM glucose for 2, 4, or 8 h and collected. Cells were fixed using 70% ice cold ethanol and stained with 18 µg/mL propidium iodide containing 40 µg/mL RNase A. The percent apoptotic cells representing the sub-G₀ fraction was determined using an Epics flow cytometry system (Coulter Cytometry, Hialeah, FL).

Western Immunoblotting

Western immunoblotting and immunoprecipitation were performed as described previously (23). After treatment, the cells were lysed by incubation on ice for 5 min with modified RIPA buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 1% Triton X-100) with 1 µg/ml of aprotinin and leupeptin and 1 mg/ml of NaOV and PMSF. The cell lysates were centrifuged at 13,000 rpm for 20 min at 4°C, and the supernatants were collected. The protein concentration was measured by DC protein assay reagent (Bio-Rad, Hercules, CA). Equal amounts of protein were separated by SDS-PAGE and transferred to nitrocellulose (NC) membrane. After blocking with 5% skim-milk in TBS with 0.1% Tween-20, the NC membrane was incubated with appropriate primary antibodies followed by secondary antibodies conjugated with horseradish peroxidase. The signal was visualized using enhanced chemiluminescence reagents (Amersham Bioscience, Piscataway, NJ). In some experiments, the NC membranes were incubated at 70°C for 30 min in stripping solution (2% SDS, 100 mM dithiothreitol and 100 mM Tris, pH 6.8) whereupon they were utilized for immunoblotting with another antibody. All experiments were repeated at least 3 times, and representative results are presented in the figures.

RESULTS

IRS-2, but not IRS-1, transfection protects SH-EP cells from glucose-induced apoptosis

We previously described IGF-I:IGF-IR signaling in N and S type NBL cells (24) including differential expression of IGF-IR, IRS-1, and IRS-2. We also reported the transfection of IRS-1 and IRS-2 into S and N cells, respectively, and their differential regulation by IGF-I treatment (25). In this report, the role of IRS proteins in mediating IGF-IR protection of neural cells against glucose-induced apoptosis was investigated using SH-EP cells

(expressing high levels of endogenous IRS-1 but low levels of IGF-IR and IRS-2) transfected with HA-tagged IRS-2. First, vector (pcDNA3.1) or HA-IRS-2 transfected SH-EP cells are serum starved, treated with 300 mM glucose for 0–8 h, and stained with propidium iodide to measure sub G₀ DNA, indicative of apoptosis. In agreement with our previous reports (12, 13, 15, 16), glucose treatment results in time-dependent apoptosis in vector transfected cells (Fig. 1A). IRS-2 transfection significantly ($p < 0.02$) protects SH-EP cells from glucose-induced apoptosis at all time points (Fig. 1A). SH-SY5Y cells express high levels of endogenous IGF-IR and IRS-2 but virtually no IRS-1 (24). We demonstrated the different expression and specific roles of IGF-IR and its downstream signaling pathways. SH-SY5Y and SH-EP cells also demonstrated different expression of Shc, FAK, paxillin, Akt, and ERK (24). Our laboratory demonstrated that these signaling pathways play important roles in cell survival (12, 24, 26, 27), making SH-SY5Y cells intrinsically more resistant to apoptosis. Glucose treatment of vector-transfected SH-SY5Y cells induces a 34% increase in apoptosis over DMEM treatment (compared to a 431% increase in SH-EP cells). IRS-1 transfection into SH-SY5Y cells slightly decreases apoptosis, but the data do not reach statistical significance (Fig. 1B).

Caspases are important mediators of apoptosis (28); they are synthesized as inactive precursors and proteolytically cleaved to generate active enzymes. When SH-EP/vector cells are treated with 300 mM glucose, there is a time-dependent cleavage of caspase-3 (Fig. 1C), which precedes the detection of apoptosis. Increased expression of cleaved caspase-3 was accompanied by the simultaneous decrease in total caspase-3 bands. IRS-2 transfection prevents caspase-3 cleavage by glucose treatment (Fig. 1C).

To examine whether the above results are specific to IRS-2 or a general transfection of IRS proteins, we overexpressed HA-tagged IRS-1 in SH-EP cells. Anti-HA immunoblotting demonstrates that transfection of IRS-1 or IRS-2 does not affect the endogenous levels of IRS-1 or other molecules involved in IGF-IR signaling such as Shc or p85 (Fig. 2A). Even though IRS-1 transfection results in statistically significant protection against glucose-induced apoptosis, the effect is much less efficient than IRS-2 transfection (27% protection for IRS-1 transfected cells vs 76% protection for IRS-2 transfected cells) (Fig. 2B). IRS-1 transfection does not prevent the cleavage of caspase-3 (Fig. 2C). Both vector and IRS-1 transfected cells demonstrate caspase-3 cleavage bands as early as 2 h after glucose treatment, which lasts up to 8 h. In contrast, IRS-2 transfected cells induce minimal caspase-3 cleavage (Fig. 2C). To further confirm the relative roles of IRS-1 and IRS-2 during apoptosis, we transfected IRS-2 siRNA into IRS-1 transfected SH-SY5Y cells. IRS-2 siRNA significantly decreased IRS-2 expression in both vector (pcDNA) and IRS-1 transfected SH-SY5Y cells without affecting the IRS-1 expression level (Fig. 3A). Glucose treatment of these cells induces caspase-3 cleavage, and IRS-1 overexpression does not prevent glucose-induced caspase-3 cleavage (Fig. 3B) or apoptosis (Fig. 3C).

FAK and Akt degradation contributes to neuronal apoptosis (16). FAK is involved in anoikis (apoptosis due to cell detachment from the substrate) (29, 30), and the PI 3-kinase/Akt pathway is critical for survival of many cell types (31). Vector or HA-IRS-1 transfected SH-EP cells show time-dependent degradation of both FAK and Akt following glucose treatment (Fig. 4). In agreement with the apoptosis and caspase-3 cleavage data, HA-IRS-2

transfection inhibits glucose-induced FAK and Akt degradation. This effect is specific to FAK and Akt, since glucose treatment does not affect levels of ERK1/2 or actin (Fig. 4). Unlike IRS-1 degradation we were not able to detect any fragments of FAK or Akt degradation. It is possible that the fragments are too small to be detected or do not have the antigenic sites the antibodies can recognize. These results clearly show that IRS-2, but not IRS-1, specifically protects SH-EP cells from glucose-induced apoptosis and the degradation of FAK and Akt.

IRS-1 and IRS-2 degradation are mediated by different mechanisms

We speculate that the differential roles of IRS-1 and IRS-2 in neural cells could be due, in part, to distinct mechanisms of degradation. When serum starved SH-EP cells are treated with 300 mM glucose, both IRS-1 and IRS-2 exhibit time-dependent degradation. However, IRS-1 is degraded much earlier than IRS-2 (Fig. 5A). IRS-1 shows 48% degradation by 2 h and almost 80% by 8 h, compared to 18% by 2 h and 48% by 8 h for IRS-2. When analyzed using a higher percentage SDS-PAGE, IRS-1 degradation is accompanied by the appearance of a smaller fragment (Fig. 5A, lower panel). IRS-1 levels decrease between 1 and 2 h after glucose treatment (Fig. 5B) and a smaller fragment of IRS-1 appears at 1 h after glucose treatment (Fig. 5B, arrowhead), which coincides with caspase-3 cleavage. The cleavage of IRS-1 and caspase-3 is followed by FAK and Akt degradation, which precedes apoptosis (Fig. 1A).

Since IRS-1 and IRS-2 display different patterns of degradation (as indicated by the mobility shift in IRS-2 but not in IRS-1), possible mechanisms involved in IRS degradation were examined. This experiment employed inhibitors of caspases (ZVAD), proteasome (MG132), and calpain (calpain inhibitor III). Based on the results from Fig. 5, we chose 2 and 4 h time points for IRS-1 transfected cells and 8 h for IRS-2 transfected cells to examine the degradation. IRS-1 degradation is blocked by ZVAD and a caspase-3 specific inhibitor (data not shown) at 2 and 4 h after glucose treatment (Fig. 6, left panel). MG132 delays but does not prevent degradation and calpain inhibition has little effect on IRS-1 degradation. As expected the results are similar for FAK and Akt degradation; ZVAD inhibits FAK and Akt degradation, while MG132 delays and calpain inhibition has little effect on degradation (Fig. 6, left panel). IRS-2 degradation was examined at 8 h after glucose treatment (Fig. 6, right panel). In contrast to IRS-1, IRS-2 degradation is blocked by MG132 and the calpain inhibitor (Fig. 6, right panel). Interestingly, ZVAD is ineffective in preventing IRS-2 degradation. FAK and Akt levels are not changed by glucose treatment in IRS-2 transfected cells (Fig. 6 and Fig. 1C). These results demonstrate that during glucose-induced apoptosis, IRS-1 and IRS-2 degradation occurs through different mechanisms.

IRS-2 transfection increases IGF-I-stimulated Akt activation

IGF-I treatment activates the PI 3-K/Akt and ERK1/2 signaling pathways in NBL cells (23, 24, 32). Therefore, we next examined the effect of IRS transfection on IGF-I-stimulated Akt and ERK phosphorylation. In agreement with the previous reports, IGF-I treatment stimulates the phosphorylation of Akt, at both Ser⁴⁷³ and Thr³⁰⁸, and ERK1 and ERK2 (Fig. 7). IRS-1 transfection produces no detectable changes in IGF-I-induced phosphorylation of Akt and ERK (Fig. 7). In contrast, IGF-I-stimulated Akt activation significantly increases in

IRS-2 transfected cells (Fig. 7). IGF-I-stimulated ERK phosphorylation is not affected by IRS-2 transfection (Fig. 7).

DISCUSSION

Many of the biological effects of IGF-I are mediated by IGF-IR-dependent phosphorylation of IRS proteins. Despite structural and biochemical similarities, IRS-1 and IRS-2 regulate distinctly different cellular responses (5, 6). The current results indicate a major role of IRS-2 over IRS-1 in the prevention of glucose-mediated apoptosis in human NBL cells. These data also suggest that different pathways involved in IRS degradation are responsible for the distinct ability of IRS-2 to support cell survival. We previously demonstrated that both glucose and mannitol induce apoptosis in SH-SY5Y and SH-EP NBL cells (13, 16). However, high concentrations of mannitol are needed to induce apoptosis (13), and even at high concentrations, cell death is considerably higher with equiosmolar glucose than with mannitol, indicating that glucose induces cell death independent of an osmolar effect (12). In agreement with apoptosis prevention, IRS-2, not IRS-1, transfection enhanced IGF-I-mediated Akt activation.

The current report demonstrates that IRS-2, but not IRS-1, plays a major role in the survival of SH-EP NBL cells following glucotoxicity. IRS-2 transfection into SH-EP cells prevents glucose-mediated apoptosis and caspase cleavage, while IRS-1 transfection has little effect. Several reports suggest distinct anti-apoptotic roles of IRS proteins in numerous cell types despite their structural similarities. IRS-2, but not IRS-1, plays a critical role in the maturation and survival of photoreceptor cells in murine retina (33). In brown adipocytes, IRS-1, IRS-2, and IRS-3 mediate the anti-apoptotic effect of IGF-I, with IRS-1 being most effective (10). Insulin fails to rescue serum-starvation-induced apoptosis in neonatal hepatocytes lacking IRS-2 (33). Adenovirus-mediated expression of IRS-2 protects a pancreatic β -cell line, INS-1, against fatty acid-induced apoptosis (34). In addition, reducing endogenous IRS-2 using siRNA in INS-1 cells increased basal and fatty acid-induced apoptosis threefold. In our current report, reducing IRS-2 expression by siRNA did not affect the basal apoptosis in SH-SY5Y cells, and IRS-1 overexpression could not rescue glucose-induced caspase-3 cleavage and apoptosis. We speculate that the lack of the effect of IRS-2 siRNA on basal apoptosis is due to the high expression of IGF-IR in SH-SY5Y cells (24), which can activate survival signals through other mechanisms (35, 36). The unique anti-apoptotic role of IRS-2 may be a characteristic of tumor cells, as IRS-2 expression appears critical for tumorigenicity (37, 38). IRS-2 is overexpressed in two different animal models of hepatocarcinogenesis and also in human carcinomas (8). We also reported that only tumorigenic N cells express IRS-2, whereas non-tumorigenic S cells express IRS-1 (24). These reports and ours suggest that different isoforms of IRS have distinct anti-apoptotic effects depending on the specific cell type used.

Protein degradation is a common feature of apoptosis. We previously reported IRS-1, FAK, and Akt, which all play important roles in cell survival (1, 39, 40), are degraded during SH-EP cell apoptosis (16, 25, 41). In the current report, we show that IRS-1 and IRS-2 are also degraded during glucose-induced apoptosis. Interestingly, the degradation patterns of IRS-1 and IRS-2 are different during apoptosis. IRS-1 degradation occurs early during glucose

treatment and is accompanied by the appearance of a smaller cleavage fragment. The time course of IRS-1 fragment formation coincides with caspase-3 cleavage, and the pan-caspase inhibitor, ZVAD, completely blocks IRS-1 degradation. A proteasome inhibitor, MG132 (Fig. 4), and lactacystin (data not shown) delay, but do not prevent, IRS-1 degradation. In contrast, IRS-2 degradation involves a mobility shift typical of serine/threonine phosphorylation. MG132 and the inhibitor of calcium-dependent cysteine protease calpain (42), but not ZVAD, inhibit IRS-2 degradation. ZVAD is also less effective in preventing mannitol and glucose-induced IRS-2 degradation in SH-SY5Y cells (B. Kim, unpublished data), which express only IRS-2 (24).

Studies show that various proteases are implicated in IRS degradation. IRS-1 and IRS-2 are degraded by caspase-10 during mammary epithelial cell apoptosis, regulated by the MAPK pathway (43). IRS-1 cleavage during this process produces a small fragment, even though it has a lower molecular weight than our study (43). Calpain inhibitor, but not caspase or proteasome inhibitors, prevents IRS-1 degradation during thapsigargin-induced apoptosis in human prostate epithelial cells (44). We previously demonstrated that caspase inhibition prevents IRS-1 degradation during mannitol, but not okadaic acid, induced apoptosis in SH-EP cells (25). Chronic exposure of INS-1 cells to glucose or IGF-I decreases IRS-2 expression, which is accompanied by a mobility shift and Ser/Thr phosphorylation (44). Ser/Thr phosphorylation and subsequent degradation of IRS-2 are prevented by the mTOR inhibitor rapamycin and the proteasome inhibitor lactacystin (45). Though there are many reports concerning the differential degradation of either IRS-1 or IRS-2 during apoptotic stimuli in specific cell types, few studies directly compare IRS-1 and IRS-2 degradation in the same cellular environment. Our results clearly demonstrate that IRS-1 undergoes rapid degradation primarily through caspase activation, while IRS-2 degradation involves proteasome and calpain activation during NBL apoptosis. We speculate reduced susceptibility to caspases renders IRS-2 more resistant to degradation and provides better anti-apoptotic capability by protecting the degradation of downstream signaling proteins such as FAK and Akt (see below). Our results may explain the different effects of IRS isoforms during apoptosis of different cell types (10, 33, 34, 46).

In response to excess glucose, FAK and Akt are degraded in parallel with IRS-1 in IRS-1 transfected SH-EP cells but remain intact in IRS-2 transfected cells. We previously demonstrated the importance of FAK and Akt during mannitol and okadaic acid-induced apoptosis in SH-EP cells (16, 41). FAK is an important member of focal adhesion complexes, which transmit signals from the extracellular matrix to the cytoplasm (39). IRS-1 binds and activates FAK (47) and disruption of FAK signaling results in apoptosis (48), while overexpression of FAK prevents apoptosis, especially anoikis (29, 30). The PI 3-kinase/Akt pathway is critical for the survival of many cell types (40, 49). Dominant negative Akt prevents IGF-I mediated survival in Schwann cells (26), and PTEN overexpression, which decreases Akt phosphorylation (50), enhances mannitol-induced NBL cell apoptosis (51). In agreement with our previous reports (16, 41), FAK and Akt are degraded during glucose-induced apoptosis. IRS-1 degradation and caspase-3 cleavage are preceded by FAK and Akt degradation prior to the detection of apoptosis. Similar to our results, a recent report showed FAK cleavage during oxidative stress-induced apoptosis in HeLa cells (52). In contrast, FAK and Akt remain intact up to 8 h following glucose

treatment even when IRS-2 levels are decreased to 50% of control level. These results suggest that caspase-mediated rapid degradation of IRS-1 destabilizes FAK and Akt, resulting in their degradation and facilitation of cell detachment and apoptosis.

IRS-2, but not IRS-1, transfection enhances IGF-I-stimulated PI 3-K/Akt signaling in SH-EP cells. Akt is a serine/threonine kinase, which plays a critical role in many cellular responses, including glucose metabolism, apoptosis, and cell migration (31). Integrin ligation and growth factor activation of Akt require the phosphorylation of both Ser⁴⁷³ and Thr³⁰⁸ through a PI 3-kinase-mediated pathway (53, 54). Activated Akt regulates proteins involved in cell survival/apoptosis including caspases and Bcl family proteins (40, 49). PI 3-K/Akt signaling is critical for IGF-I's anti-apoptotic effect *in vitro* and *in vivo* (26, 51), as well as for cell motility (26, 51) and neurite outgrowth (55). The preferential phosphorylation (i.e. activation) of Akt in IRS-2 transfected cells correlates well with our results suggesting a distinct anti-apoptotic role of IRS-2 following glucotoxicity in human NBL cells. Interestingly, IRS-2 expression minimally affects ERK1/2 activation. This agrees with our previous report demonstrating that a Shc-Grb2 complex activates ERK1/2, whereas IRS-2 activates PI 3-K/Akt signaling (32). Shc phosphorylation is very low in SH-EP cells, and IRS-1 or IRS-2 expression does not change basal or IGF-I-induced Shc phosphorylation (data not shown).

In summary, we demonstrate that IRS-2, but not IRS-1, prevents glucose-mediated apoptosis in human NBL cells. We speculate that distinct degradation pathways involving different proteases for IRS-1 and IRS-2 are responsible for the unique anti-apoptotic role of IRS-2. We also demonstrate that IRS-2 transfection increases IGF-I-mediated Akt activation. These results support the hypothesis that IRS-2, not IRS-1, is the major signaling molecule for survival signaling in NBL cells and provide a rationale for developing new therapeutic compounds targeting IRS-2 in NBL treatment.

Acknowledgments

This work was supported by a grant from the the Juvenile Diabetes Research Foundation Center for the Study of Complications in Diabetes, the Program for Neurology Research and Discovery and the A. Alfred Taubman Medical Research Institute.

References

1. Laviola L, Natalicchio A, Giorgino F. The IGF-I signaling pathway. *Curr Pharm Des.* 2007; 13:663–669. [PubMed: 17346182]
2. Zumkeller W, Schwab M. Insulin-like growth factor system in neuroblastoma tumorigenesis and apoptosis: potential diagnostic and therapeutic perspectives. *Horm Metab Res.* 1999; 31:138–141. [PubMed: 10226794]
3. Riedemann J, Macaulay VM. IGF1R signalling and its inhibition. *Endocr Relat Cancer.* 2006; 13(Suppl 1):S33–43. [PubMed: 17259557]
4. Lee YH, White MF. Insulin receptor substrate proteins and diabetes. *Arch Pharm Res.* 2004; 27:361–370. [PubMed: 15180298]
5. Withers DJ, Burks DJ, Towery HH, Altamuro SL, Flint CL, White MF. Irs-2 coordinates Igf-1 receptor-mediated beta-cell development and peripheral insulin signalling. *Nat Genet.* 1999; 23:32–40. [PubMed: 10471495]

6. Withers DJ, Gutierrez JS, Towery H, et al. Disruption of IRS-2 causes type 2 diabetes in mice. *Nature*. 1998; 391:900–904. [PubMed: 9495343]
7. Schubert M, Brazil DP, Burks DJ, et al. Insulin receptor substrate-2 deficiency impairs brain growth and promotes tau phosphorylation. *J Neurosci*. 2003; 23:7084–7092. [PubMed: 12904469]
8. Boissan M, Beurel E, Wendum D, et al. Overexpression of insulin receptor substrate-2 in human and murine hepatocellular carcinoma. *Am J Pathol*. 2005; 167:869–877. [PubMed: 16127164]
9. Rother KI, Imai Y, Caruso M, Beguinot F, Formisano P, Accili D. Evidence that IRS-2 phosphorylation is required for insulin action in hepatocytes. *J Biol Chem*. 1998; 273:17491–17497. [PubMed: 9651339]
10. Tseng YH, Ueki K, Kriauciunas KM, Kahn CR. Differential roles of insulin receptor substrates in the anti-apoptotic function of insulin-like growth factor-1 and insulin. *J Biol Chem*. 2002; 277:31601–31611. [PubMed: 12082100]
11. Li L, Qi X, Williams M, Shi Y, Keegan AD. Overexpression of insulin receptor substrate-1, but not insulin receptor substrate-2, protects a T cell hybridoma from activation-induced cell death. *J Immunol*. 2002; 168:6215–6223. [PubMed: 12055235]
12. Leininger GM, Russell JW, van Golen CM, Berent A, Feldman EL. Insulin-like growth factor-I regulates glucose-induced mitochondrial depolarization and apoptosis in human neuroblastoma. *Cell Death Differ*. 2004; 11:885–896. [PubMed: 15105834]
13. Van Golen CM, Feldman EL. Insulin-like growth factor I is the key growth factor in serum that protects neuroblastoma cells from hyperosmotic-induced apoptosis. *J Cell Physiol*. 2000; 182:24–32. [PubMed: 10567913]
14. Russell JW, Feldman EL. Insulin-like growth factor-I prevents apoptosis in sympathetic neurons exposed to high glucose. *Horm Metab Res*. 1999; 31:90–96. [PubMed: 10226787]
15. van Golen CM, Castle VP, Feldman EL. IGF-I receptor activation and BCL-2 overexpression prevent early apoptotic events in human neuroblastoma. *Cell Death Differ*. 2000; 7:654–665. [PubMed: 10889510]
16. Kim B, Feldman EL. Insulin-like growth factor I prevents mannitol-induced degradation of focal adhesion kinase and *Akt*. *J Biol Chem*. 2002; 277:27393–27400. [PubMed: 12011046]
17. Kim B, van Golen CM, Feldman EL. Insulin-like growth factor I induces preferential degradation of insulin receptor substrate-2 through the phosphatidylinositol 3-kinase pathway in human neuroblastoma cells. *Endocrinology*. 2005; 146:5350–5357. [PubMed: 16150916]
18. Devary Y, Gottlieb RA, Smeal T, Karin M. The mammalian ultraviolet response is triggered by activation of Src tyrosine kinases. *Cell*. 1992; 71:1081–1091. [PubMed: 1473146]
19. Castleberry RP. Biology and treatment of neuroblastoma. *Pediatr Clin North Am*. 1997; 44:919–937. [PubMed: 9286292]
20. Schmidt ML, Lukens JN, Seeger RC, et al. Biologic factors determine prognosis in infants with stage IV neuroblastoma: A prospective Children's Cancer Group study. *J Clin Oncol*. 2000; 18:1260–1268. [PubMed: 10715296]
21. Kim PK, Mahidhara R, Seol DW. The role of caspase-8 in resistance to cancer chemotherapy. *Drug Resist Updat*. 2001; 4:293–296. [PubMed: 11991682]
22. Lee AV, Gooch JL, Oesterreich S, Guler RL, Yee D. Insulin-like growth factor I-induced degradation of insulin receptor substrate 1 is mediated by the 26S proteasome and blocked by phosphatidylinositol 3'-kinase inhibition. *Mol Cell Biol*. 2000; 20:1489–1496. [PubMed: 10669726]
23. Kim B, Leventhal PS, Saltiel AR, Feldman EL. Insulin-like growth factor-I-mediated neurite outgrowth in vitro requires mitogen-activated protein kinase activation. *J Biol Chem*. 1997; 272:21268–21273. [PubMed: 9261137]
24. Kim B, van Golen CM, Feldman EL. Insulin-like growth factor-I signaling in human neuroblastoma cells. *Oncogene*. 2004; 23:130–141. [PubMed: 14712218]
25. Kim B, Oh S, van Golen CM, Feldman EL. Differential regulation of insulin receptor substrate-1 degradation during mannitol and okadaic acid induced apoptosis in human neuroblastoma cells. *Cell Signal*. 2005; 17:769–775. [PubMed: 15722201]

26. Cheng HL, Steinway M, Delaney CL, Franke TF, Feldman EL. IGF-I promotes Schwann cell motility and survival via activation of Akt. *Mol Cell Endocrinol.* 2000; 170:211–215. [PubMed: 11162904]
27. Vincent AM, Mobley BC, Hiller A, Feldman EL. IGF-I prevents glutamate-induced motor neuron programmed cell death. *Neurobiol Dis.* 2004; 16:407–416. [PubMed: 15193297]
28. Chang HY, Yang X. Proteases for cell suicide: functions and regulation of caspases. *Microbiol Mol Biol Rev.* 2000; 64:821–846. [PubMed: 11104820]
29. Sonoda Y, Matsumoto Y, Funakoshi M, Yamamoto D, Hanks SK, Kasahara T. Anti-apoptotic role of focal adhesion kinase (FAK). Induction of inhibitor-of-apoptosis proteins and apoptosis suppression by the overexpression of FAK in a human leukemic cell line, HL-60. *J Biol Chem.* 2000; 275:16309–16315. [PubMed: 10821872]
30. Duxbury MS, Ito H, Zinner MJ, Ashley SW, Whang EE. Focal adhesion kinase gene silencing promotes anoikis and suppresses metastasis of human pancreatic adenocarcinoma cells. *Surgery.* 2004; 135:555–562. [PubMed: 15118593]
31. Frebel K, Wiese S. Signalling molecules essential for neuronal survival and differentiation. *Biochem Soc Trans.* 2006; 34:1287–1290. [PubMed: 17073803]
32. Kim B, Cheng HL, Margolis B, Feldman EL. Insulin receptor substrate 2 and Shc play different roles in insulin-like growth factor I signaling. *J Biol Chem.* 1998; 273:34543–34550. [PubMed: 9852124]
33. Valverde AM, Fabregat I, Burks DJ, White MF, Benito M. IRS-2 mediates the antiapoptotic effect of insulin in neonatal hepatocytes. *Hepatology.* 2004; 40:1285–1294. [PubMed: 15565601]
34. Lingohr MK, Dickson LM, Wrede CE, et al. Decreasing IRS-2 expression in pancreatic beta-cells (INS-1) promotes apoptosis, which can be compensated for by introduction of IRS-4 expression. *Mol Cell Endocrinol.* 2003; 209:17–31. [PubMed: 14604813]
35. Cosaceanu D, Carapancea M, Alexandru O, et al. Comparison of three approaches for inhibiting insulin-like growth factor I receptor and their effects on NSCLC cell lines in vitro. *Growth Factors.* 2007; 25:1–8. [PubMed: 17454144]
36. Edderkaoui M, Hong P, Lee JK, Pandol SJ, Gukovskaya AS. Insulin-like growth factor-I receptor mediates the prosurvival effect of fibronectin. *J Biol Chem.* 2007; 282:26646–26655. [PubMed: 17627944]
37. Neid M, Datta K, Stephan S, et al. Role of insulin receptor substrates and protein kinase C-zeta in vascular permeability factor/vascular endothelial growth factor expression in pancreatic cancer cells. *J Biol Chem.* 2004; 279:3941–3948. [PubMed: 14604996]
38. Nagle JA, Ma Z, Byrne MA, White MF, Shaw LM. Involvement of insulin receptor substrate 2 in mammary tumor metastasis. *Mol Cell Biol.* 2004; 24:9726–9735. [PubMed: 15509777]
39. Hanks SK, Ryzhova L, Shin NY, Brabek J. Focal adhesion kinase signaling activities and their implications in the control of cell survival and motility. *Front Biosci.* 2003; 8:d982–996. [PubMed: 12700132]
40. Toker A, Yoeli-Lerner M. Akt signaling and cancer: surviving but not moving on. *Cancer Res.* 2006; 66:3963–3966. [PubMed: 16618711]
41. Kim B, van Golen CM, Feldman EL. Degradation and dephosphorylation of focal adhesion kinase during okadaic acid-induced apoptosis in human neuroblastoma cells. *Neoplasia.* 2003; 5:405–416. [PubMed: 14670178]
42. Demarchi F, Schneider C. The calpain system as a modulator of stress/damage response. *Cell Cycle.* 2007; 6:136–138. [PubMed: 17264674]
43. Zhang H, Hoff H, Sell C. Downregulation of IRS-1 protein in thapsigargin-treated human prostate epithelial cells. *Exp Cell Res.* 2003; 289:352–358. [PubMed: 14499636]
44. Briaud I, Dickson LM, Lingohr MK, McCuaig JF, Lawrence JC, Rhodes CJ. Insulin receptor substrate-2 proteasomal degradation mediated by a mammalian target of rapamycin (mTOR)-induced negative feedback down-regulates protein kinase B-mediated signaling pathway in beta-cells. *J Biol Chem.* 2005; 280:2282–2293. [PubMed: 15537654]
45. Ando K, Fujita T. Role of lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) in the development of hypertensive organ damage. *Clin Exp Nephrol.* 2004; 8:178–182. [PubMed: 15480893]

46. Simmons JG, Ling Y, Wilkins H, et al. Cell-specific effects of insulin receptor substrate-1 deficiency on normal and IGF-I-mediated colon growth. *Am J Physiol Gastrointest Liver Physiol*. 2007; 293:G995–1003. [PubMed: 17823215]
47. Lebrun P, Mothe-Satney I, Delahaye L, Van Obberghen E, Baron V. Insulin receptor substrate-1 as a signaling molecule for focal adhesion kinase pp125(FAK) and pp60(src). *J Biol Chem*. 1998; 273:32244–32253. [PubMed: 9822703]
48. Bouchard V, Demers MJ, Thibodeau S, et al. Fak/Src signaling in human intestinal epithelial cell survival and anoikis: differentiation state-specific uncoupling with the PI3-K/Akt-1 and MEK/Erk pathways. *J Cell Physiol*. 2007; 212:717–728. [PubMed: 17443665]
49. Song G, Ouyang G, Bao S. The activation of Akt/PKB signaling pathway and cell survival. *J Cell Mol Med*. 2005; 9:59–71. [PubMed: 15784165]
50. Blanco-Aparicio C, Renner O, Leal JF, Carnero A. PTEN, more than the AKT pathway. *Carcinogenesis*. 2007; 28:1379–1386. [PubMed: 17341655]
51. van Golen CM, Schwab TS, Ignatoski KM, Ethier SP, Feldman EL. PTEN/MMAC1 overexpression decreases insulin-like growth factor-I-mediated protection from apoptosis in neuroblastoma cells. *Cell Growth Differ*. 2001; 12:371–378. [PubMed: 11457734]
52. Mian MF, Kang C, Lee S, et al. Cleavage of focal adhesion kinase is an early marker and modulator of oxidative stress-induced apoptosis. *Chem Biol Interact*. 2008; 171:57–66. [PubMed: 17888894]
53. Delcommenne M, Tan C, Gray V, Rue L, Woodgett J, Dedhar S. Phosphoinositide-3-OH kinase-dependent regulation of glycogen synthase kinase 3 and protein kinase B/AKT by the integrin-linked kinase. *Proc Natl Acad Sci U S A*. 1998; 95:11211–11216. [PubMed: 9736715]
54. Franke TF, Kaplan DR, Cantley LC. PI3K: downstream AKTion blocks apoptosis. *Cell*. 1997; 88:435–437. [PubMed: 9038334]
55. Kim B, Leventhal PS, White MF, Feldman EL. Differential regulation of insulin receptor substrate-2 and mitogen-activated protein kinase tyrosine phosphorylation by phosphatidylinositol 3-kinase inhibitors in SH-SY5Y human neuroblastoma cells. *Endocrinology*. 1998; 139:4881–4889. [PubMed: 9832424]

Fig. 1. IRS-2 protects against glucose-induced apoptosis in SH-EP cells

SH-EP or SH-SY5Y cells transfected with vector, IRS-1, or IRS-2 are serum starved overnight and treated with 300 mM glucose for the indicated times. (A & B) Apoptosis is measured by flow cytometry. * $p < 0.02$ compared to vector control by t -test. The results are an average of at least 5 repetitions for each time point. (C) Whole cell lysates are separated by SDS-PAGE, transferred to NC membrane and immunoblotted with antibodies detecting both non-cleaved and cleaved caspase-3. The blots were stripped and reprobed with anti-

GAPDH antibody to verify equal loading of the protein. These experiments are repeated at least 3 times and a representative result is shown in duplicate. Con: control; Glu: glucose.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig 2. IRS-2, but not IRS-1, is responsible for the prevention of glucose-induced apoptosis in SH-EP cells

(A) SH-EP cells are transfected with vector (v), IRS-1 (1), or IRS-2 (2). Equal amounts of cell lysate are immunoblotted with the indicated antibodies. IRS-1 or IRS-2 transfection does not affect the endogenous level of IRS-1 or other signaling molecules (*upper panels*). IRS-1 or IRS-2 transfection does not affect the expression of Shc or p85. Anti-actin blot shows the equal loading of the proteins (*lower panels*). (B) The cells are serum starved overnight and treated with 300 mM glucose for 8 h. Apoptosis is measured by flow cytometry. The results are the average of at least five repeats. *a*, *p* < 0.01 and *b*, *p* < 0.001

compared to vector transfected cells. *c*, $p < 0.05$ and *d*, $p < 0.001$ compared to IRS-1 transfected cells by *t*-test. (C) Serum starved cells are treated with 300 mM glucose for 2–8 h. Equal amounts of cell lysate are immunoblotted with the antibody detecting both non-cleaved and cleaved caspase-3. The blots were stripped and reprobed with anti-GAPDH antibody to verify equal loading of the protein. The Western blots are repeated at least 3 times.

Fig. 3. IRS-1 overexpression does not rescue apoptosis in SH-SY5Y cells

SH-SY5Y cells expressing vector or IRS-1 are transfected with IRS-2 siRNA for 96 h. (A) Total IRS-1 and IRS-2 levels are measured by immunoblotting. IRS-2 siRNA reduced IRS-2 expression without affecting the IRS-1 level. (B) IRS-1 transfected SH-SY5Y cells are treated with 300 mM glucose for 2–8 h, and the cell lysates are immunoblotted with antibodies against non-cleaved and cleaved caspase-3. The blot is stripped and reprobed with the anti-GAPDH antibody to show the equal loading of the proteins. (C) Apoptosis is

measured by flow cytometry after 8 h glucose treatment. * $p < 0.05$ compared to no glucose treatment by t -test. The results are an average of at least 5 repetitions.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig 4. FAK and Akt degradation during glucose-induced apoptosis is blocked by IRS-2
SH-EP cells are transfected with vector, IRS-1, or IRS-2, serum starved, and treated with 300 mM glucose for 2–8 h. Equal amounts of cell lysate are immunoblotted with the indicated antibodies. The Western blots are repeated at least 3 times.

Fig. 5. Degradation of IRS-1 and IRS-2 by glucose treatment

(A) vector, IRS-1, or IRS-2 transfected SH-EP cells are treated with 300 mM glucose for the indicated times. Equal amounts of cell lysate are separated by 7.5% (upper panel) or 12.5% (lower panel) SDS-PAGE and immunoblotted with anti-HA antibody. Arrowheads indicate the IRS-1 fragment. (B) IRS-1 transfected SH-EP cells are treated with 300 mM glucose for 0.5–4 h and the cell lysates are immunoblotted with antibodies against indicated proteins. The representative results from at least three different experiments are shown. IB: immunoblotting.

Fig. 6. Different proteases are involved in the degradation of IRS-1 and IRS-2
 IRS-1 or IRS-2 transfected SH-EP cells are treated with 300 mM glucose for 2, 4, or 8 h, along with 20 μ M caspase (ZVAD, Z), proteasome (MG132, M), or calpain (Calpain inhibitor III, C) inhibitors. Equal amounts of cell lysate are immunoblotted with the indicated antibodies. The experiments are repeated at least three times with similar results.

Fig. 7. IRS-2 transfection increases Akt phosphorylation

Vector, IRS-1, or IRS-2 transfected SH-EP cells are treated with 10 nM IGF-I for 0–120 min. Equal amounts of cell lysate are immunoblotted with the indicated antibodies. The experiments are repeated at least 3 times and the representative result is shown.