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Interaction Between Serine Phosphorylated IRS-1 and β 1-Integrin Affects the Stability of Neuronal Processes

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Abstract

Tumor necrosis factor- α (TNF α) released in the brain by HIV-activated macrophages/microglia is suspected to compromise neuronal survival. Previously, we have demonstrated that activated receptor for insulin-like growth factor I (IGF-IR) protects neurons from TNF α -induced neuronal damage (Wang et al. [2006] *J. Neurosci. Res.* 83:7–18). Because TNF α triggers phosphorylation of insulin receptor substrate 1 (IRS-1) on serine residues (pS-IRS-1; Rui et al. [2001] *J. Clin. Invest.* 107:181–189), and pS-IRS-1 binds integrins (Reiss et al. [2001] *Oncogene* 20:490–500), we asked how these events affect neuronal processes. We show that β 1-integrin and pS-IRS-1 colocalize in PC12 cells and in primary cortical neurons. TNF α treatment elevated membrane-associated pS-IRS-1, enhanced pSIRS-1 interaction with β 1-integrin, and attenuated cell attachment to collagen IV. In contrast, IGF-I inhibited pS-IRS-1– β 1-integrin complexes and improved cell attachment. The domain of IRS-1 involved in β 1-integrin binding mapped between amino acids 426 and 740, and the expression of 426–740/IRS-1 mutant attenuated neuronal outgrowth. Our results indicate that TNF α facilitates the interaction of pS-IRS-1 and β 1-integrin and destabilizes neuronal processes. IGF-I counteracts TNF α -mediated accumulation of pS-IRS-1– β 1-integrin complexes supporting the stability of neuronal processes.

Keywords

IRS-1; integrins; TNF α ; IGF-I; neuronal damage

Although the molecular mechanisms involved in insulin-like growth factor I receptor (IGF-IR)-mediated neuroprotection are associated with a strong antiapoptotic potential of this tyrosine kinase receptor, several reports suggest that neuronal damage may happen in the absence of apoptosis (Lin et al., 2004) and that IGF-I can be still protective (Ying Wang et al., 2003; Wang et al., 2006). This includes neuronal damage often observed in the presence of TNF α (Saha and Pahan, 2003; Takeuchi et al., 2006) and, possibly, TNF α -mediated serine phosphorylation of insulin receptor substrate 1 (IRS-1; Venters et al., 1999; Rui et al., 2001). Actually, the process of IRS-1 phosphorylation on serine residues has been associated with the inactivation of IRS-1 function as a signaling molecule, which when

phosphorylated on multiple tyrosine residues amplifies and diversifies the signal from IGF-IR (Rui et al., 2001; White, 1998). Several serine kinases have been implicated in the process of IRS-1 inactivation. For instance, JNK has been identified to mediate IRS-1 phosphorylation at Ser³⁰² and Ser³⁰⁷ (Rui et al., 2001; Werner et al., 2004). Other studies demonstrated that PKC ζ (Ravichandran et al., 2001), MAP kinases p42/p44 and p38 (Fujishiro et al. 2003), PI3-kinase, Akt, GSK3 β , and mTOR (Eldar-Finkelman and Krebs, 1997; Paz et al., 1999) could be also involved, indicating the importance of IRS-1 serine phosphorylation/inactivation during cell growth and differentiation.

Although TNF α -mediated neuronal damage is well documented, only a few reports have implicated serine phosphorylated IRS-1 (pS-IRS-1) in this process (Venters et al., 1999; Steen et al., 2005). One such study demonstrated that cerebellar granule neurons lost the ability of responding to IGF-I survival signal when treated with low doses of TNF α (Venters et al., 1999). IRS-2 serine phosphorylation, and a loss of signaling connection between IGF-IR and PI3-kinase were suspected for this TNF α -mediated IGF-I resistance. Previously, we have reported that differentiated PC12/IGF-IR neurons, which ectopically express IGF-IR, are able to maintain neuronal processes in the presence of TNF α (Ying Wang et al., 2003). Although we did not observe any significant changes in the phosphorylation status of cytosolic IRS-1 and IRS-2, our results suggested that TNF α -mediated degeneration of neuronal processes could be linked to the interaction between IRS-1 and α 5 β 1-integrin (Wang et al., 2006). Such an interaction has already been proposed by Vuori and Ruoslahti (1994), who first demonstrated the binding between IRS-1 and α v β 3 integrin in cells cultured on vitronectin. In addition, reciprocal effects of the interaction between insulin receptor–IRS-1 signaling axis and α 5 β 1-integrin showed both the improvement of insulin-mediated attachment to fibronectin and increased IRS-1 phosphorylation (Guilherme et al., 1998). Extensive studies on cell adhesion and motility of LNCaP prostate cancer cells demonstrated the formation of IRS-1– α 5 β 1-integrin immunocomplex in the condition in which IRS-1 is predominantly phosphorylated on serine residues (Reiss et al., 2001). The association between β 1-integrins and IGF-I has been further demonstrated in myeloma cells (Tai et al., 2003), and several other reports confirmed the presence of a functional cross-talk between integrins and the IGF-IR signaling system (Hermanto et al., 2002; Maile and Clemmons, 2002; Lynch et al., 2005).

To clarify the role of IRS-1 and its anticipated interaction with integrins in the paradigm of TNF α -induced neurodegeneration, we have evaluated subcellular localization of β 1-integrin–IRS-1 complexes, the phosphorylation status of IRS-1 in these complexes, and its stability following IGF-I and TNF α stimulation. We have also asked, which domain of IRS-1 is responsible for β 1-integrin binding and attempted to determine whether a truncation mutant of IRS-1, which contains a putative integrin binding domain, can affect the stability of neuronal processes in our in vitro model of TNF α -mediated neuronal injury.

MATERIALS AND METHODS

Cell Culture

Culture conditions for PC12 rat pheochromocytoma cells (ATCC No. CRL-1721) were previously reported (Ying Wang et al., 2003). To induce neuronal differentiation, PC12 cells were plated on collagen IV (Sigma, St. Louis, MO)-coated dishes and treated with 20 ng/ml nerve growth factor (NGF; Invitrogen, Carlsbad, CA) in serum-free medium (SFM). Neuronal processes began to form within first 24 hr following the treatment and could be preserved as fully differentiated neuronal processes for at least 2 weeks. PC12/IGF-IR cells were generated by collecting a mixed population of PC12 cells after retroviral transduction with neomycin-resistant pGR15 expression vector in which IGF-IR cDNA was cloned into the MSCV-based retroviral expression vector (Romano et al., 2001). PC12 cells transduced

with the empty retroviral vector (PC12/EV) and pGR15 vector (PC12/IGF-IR) were allowed to differentiate in the presence of NGF for 5 days. Subsequently, the medium was changed to fresh SFM, and the differentiated cells were treated with 100 ng/ml TNF α (BD Biosciences, Palo Alto, CA) or 50 ng/ml IGF-I (Invitrogen). The measurements of neurite-like processes were taken from series of photographs from selected microscopic fields and reflect an average length of neurite-like processes in a particular microscopic field, as previously described (Ying Wang et al., 2003).

Rat Neuronal Primary Culture

Rat cortical neurons were obtained by enzymatic and mechanical treatment from E17 Sprague Dawley rats. Cortices were dissected out in dissecting medium (1.6 mM sucrose, 2.2 mM glucose, 1 mM Hepes, 16 mM NaCl, 0.5 mM KCl, 0.1 mM Na₂HPO₄, and 0.022 mM KH₂PO₄) and placed in Hibernate E medium (BrainBits, Springfield, IL). After careful removal of the meninges, the intact tissue was incubated with a TripleExpress enzyme (Gibco Invitrogen Corporation, Carlsbad, CA) at 37°C for 10 min, followed by three washes with Hibernate E medium (Apréa et al., 2006). Tissue trituration was performed in culture medium (see below) using a fire-polished glass Pasteur pipette, and single-cell suspension was diluted with culture medium. Finally, cells were plated on poly-D-lysine-coated dishes at a density of $4.5 \times 10^4/\text{cm}^2$ and cultured in Neurobasal medium containing B27 supplement, 0.25 mM Glutamax, and 0.25 mM L-glutamine (all from Gibco, Invitrogen Corporation).

Western Blotting and Immunoprecipitation

To determine the phosphorylation status of IRS-1, differentiated cultures of PC12/IGF-IR or cortical neurons were first starved in SFM for 24 hr and were then stimulated with IGF-I or TNF α . The membrane rafts fraction and detergent-soluble fractions were separated according to the methodology previously described (Tai et al., 2003). The protein concentrations of isolated fractions were determined by a Bio-Rad Protein Assay (Bio-Rad, Hercules, CA), and 50- μg protein aliquots were separated on a 4–15% gradient SDS-PAGE (Bio-Rad) and transferred onto nitrocellulose membranes according to standard procedure used repeatedly in our laboratory (Wang et al., 2006). The resulting blots were probed with the following primary antibodies: rabbit polyclonal antibodies against total IRS-1 (Upstate Biotechnology, Lake Placid, NY), tyrosine phosphorylated IRS-1 (pY612/pY608; human/rodents; BioSource, Camarillo, CA), serine phosphorylated IRS-1 (pS616/pS612; human/rodents; BioSource), and (pS312/307; human/rodents; Upstate Biotechnology). To detect the truncated form of IRS-1 between aa 426 and aa 740, we have utilized anti-IRS-1 antibody developed against the central portion of the IRS-1 molecule (PA1-1058; Affinity Bioreagents). Endogenous levels of β 1-integrin were determined by anti- β 1-integrin mouse monoclonal antibody (MAB1987; Chemicon, Temecula, CA). Anti-c-Src antibody (Santa Cruz, Santa Cruz, CA) and anti-IRS-2 antibody (Upstate Biotechnology) were utilized as markers for the membrane rafts fraction and detergent-soluble fraction, respectively. In some experiments, reciprocal IP/Western analyses were performed to detect immunocomplexes between IRS-1 and β 1-integrin in membrane rafts fraction of differentiated PC12/IGF-IR and cortical neurons. We have followed standard IP/Western protocol described in our previous work (Trojanek et al., 2003). Briefly, immunoprecipitations were performed either with anti- β 1-integrin (CD29; Immunotech, France) or with anti-IRS-1 (Upstate Biotechnology) antibodies, and corresponding Western blots were developed with anti-IRS-1 (Santa Cruz), anti- β 1-integrin (AB1952; Chemicon), and anti-pS⁶¹²IRS-1 (Cell Signaling, Beverly, MA) antibodies. The final figures were prepared in Adobe Illustrator.

Immunocytofluorescence

PC12/IGF-IR cells were cultured on collagen IV-coated chamber slides, and rat cortical neurons on poly-D-lysine-coated chamber slides (Nalge Nunc International, Rochester, NY). For immunolabeling, cells were fixed and permeabilized with buffer containing 0.02% Triton X-100 and 4% formaldehyde in phosphate-buffered saline (PBS). Fixed cells were washed three times in PBS and blocked in 1% bovine serum albumin (BSA) for 30 min at 37°C. α 1 β 1-Integrin complex was detected by anti- α 1 β 1-integrin mouse monoclonal antibody (MAB2513; Chemicon), followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody (Molecular Probes, Eugene, OR). Total IRS-1 was detected with rabbit polyclonal antibody (Upstate Biotechnology), and serine phosphorylated IRS-1 was detected with anti-pS⁶¹²IRS-1 rabbit polyclonal antibody (Cell Signaling). The images were visualized with an inverted Nikon Eclipse TE300 microscope equipped with a Retiga 1300 camera, motorized Z-axis, and deconvolution software (SlideBook4). Three-dimensional images of each individual picture were deconvoluted to one two-dimensional picture and resolved by adjusting the signal cutoff to near maximal intensity to increase resolution. Nikon Plan Fluor 100 $\times$ /1.3 oil, 40 $\times$ /1.3 oil, and 20 $\times$ /0.45 objectives were used to generate images in Figure 4A, Figure 6C, and Figure 6A, respectively. The final figures were prepared in Adobe Illustrator.

GST Pull-Down Assay

The protocol for GST pull-down assay described in our previous work (Lassak et al., 2002; Trojanek et al., 2003) was followed. Five overlapping IRS-1 truncation mutants and membrane rafts protein extracts from differentiated PC12/IGF-IR neurons were utilized. Fusion proteins were generated on the basis of the pGEX-5x-1 vector expressed in IPTG-induced bacteria cultures and purified on glutathione-agarose beads. Hela membrane extract was used as a positive control for β 1-integrin.

Cell Adhesion Assay

The procedure described in our previous work was followed (Wang et al., 2006). Briefly, after TNF α , IGF-I, or obtustatin treatment, 5-(chloromethyl) fluoresceine diacetate-labeled PC12/IGF-IR cells or PC12/IGF-IR expressing the 426–740/IRS-1 mutant were placed on collagen IV-coated 96-well plates in 0.2 ml HBSS. After incubation for 30 min at 37°C, unbound cells were washed out, and bound cells were lysed with 0.5% Triton X-100. The number of adhered cells was calculated from a standard curve as previously described (Marcinkiewicz et al., 1996, 2003). Cells treated with a specific α 1 β 1-integrin inhibitor, obtustatin (kindly provided by Dr. Cezary Marcinkiewicz, Temple University, Philadelphia, PA), were used as a control for the inhibition of cell attachment.

RESULTS

Effects of IGF-I and TNF α on IRS-1 Phosphorylation and Its Association With Membrane Rafts Fraction

We have previously demonstrated the involvement of IGF-I and integrins in neuroprotection against TNF α -induced degeneration of neuronal processes (Ying Wang et al., 2003; Wang et al., 2006). To investigate mechanisms of IGF-I-mediated neuroprotection, we have utilized stable clones of PC12 cells, which express human IGF-IR (Ying Wang et al., 2003; Wang et al., 2006) and primary cultures of rat cortical neurons (Bergonzini et al., 2004; Aprea et al., 2006). We have prepared detergent-insoluble fraction (membrane rafts fraction) and detergent-soluble fraction from differentiated PC12/IGF-IR neurons kept in SFM or stimulated with IGF-I (Fig. 1A) and TNF α (Fig. 1B). The results in Figure 1A demonstrate that β 1-integrin is localized almost exclusively in the membrane rafts fraction. In contrast,

the majority of IRS-1 was found in the detergent-soluble fraction, and only a small amount of IRS-1 was associated with the membrane rafts fraction. This membrane-associated IRS-1 was strongly phosphorylated on serine residue pS⁶¹². Interestingly, IGF-I stimulation affected only minimally the levels of membrane-associated pS⁶¹²IRS-1, and tyrosine phosphorylated IRS-1 (pY⁶⁰⁸) was not detected in this fraction.

Detergent-soluble fraction from PC12/IGF-IR neurons was characterized by an abundant presence of total as well as tyrosine phosphorylated IRS-1, which was surprisingly high in SFM. A weak labeling with anti-pS⁶¹²IRS-1 and anti- β 1-integrin antibodies was detected in this fraction as well. Anti-c-Src and anti-IRS-2 antibodies were used as markers for the membrane rafts fraction and the detergent-soluble fraction, respectively. Densitometric data in Figure 1A were calculated by normalizing intensities of the bands by corresponding bands from c-Src blot for the membrane rafts fraction and by IRS-2 blot for the detergent-soluble fraction.

Next, we evaluated levels of pS⁶¹²IRS-1 and pS³⁰⁷IRS-1 in membrane rafts following cell stimulation with TNF α . Results in Figure 1B demonstrate the presence of pS⁶¹²IRS-1, pS³⁰⁷IRS-1, and total IRS-1 in the membrane fraction following 48-hr incubation of differentiated PC12/IGF-IR neurons in SFM. TNF α stimulation increased the intensity of pS³⁰⁷IRS-1 and pS⁶¹²IRS-1 at 1 hr after stimulation, and the bands remained elevated 24 hr after stimulation. Total IRS-1 was not affected by TNF α at the indicated time points and was considered a loading control.

Effects of TNF α and IGF-I on the Interaction Between IRS-1 and β 1-Integrin

By applying immunoprecipitation/Western blot analysis (IP/Western), we have detected the presence of IRS-1– β 1-integrin immunocomplexes in the membrane rafts fraction of differentiated PC12/IGF-IR cells (Fig. 2). Immunoprecipitation with anti- β 1-integrin antibody resulted in the detection of total IRS-1 as well as pS⁶¹²IRS-1. This protein–protein interaction was significantly enhanced by TNF α at 1 hr and 6 hr and for pS⁶¹²IRS-1 remained elevated 24 hr after the treatment. In contrast, IGF-I strongly attenuated the binding between IRS-1 and β 1-integrin (Fig. 2B). Reciprocal IP/Western analysis demonstrated β 1-integrin–IRS-1 immunocomplex in SFM. After IGF-I stimulation, the amount of IRS-1 that coprecipitated β 1-integrin decreased as early as 5 min after IGF-I stimulation and remained low at 60 min.

Blots depicted in Figure 2C illustrate results of control IP/Westerns in which IP reactions were carried out with anti- β 1-integrin antibody (β 1), anti-BrdU irrelevant antibody (BrdU), anti-IRS-1 antibody (IRS), and agarose beads only (beads). In the last lane, 50 μ g of membrane rafts proteins were loaded without immunoprecipitation (W), and the resulting blots were probed with anti- β 1-integrin or anti-IRS-1 antibody.

To determine whether the interaction between IRS-1 and β 1-integrin is restricted to PC12/IGF-IR neurons, we have analyzed this protein–protein interaction in the membrane rafts from primary cultures of rat cortical neurons (Fig. 3). Reciprocal IP/Westerns depicted in Figure 3A demonstrate the presence of IRS-1– β 1-integrin complex in membrane rafts of cortical neurons cultured for 24 hr in SFM. After 30 min of IGF-I stimulation, the intensity of IRS-1– β 1-integrin complex declined about twofold (Fig. 3A), and TNF α stimulation significantly up-regulated the complex (Fig. 3B).

Detection of IRS-1– β 1-Integrin Colocalization Foci in Neuronal Processes

Double immunolabeling of differentiated PC12/IGF-IR with anti-IRS-1 (green fluorescence) and anti- β 1-integrin (red fluorescence) antibodies revealed a significant number of colocalization foci between these two proteins in neuronal processes (yellow fluorescence;

Fig. 4A). Quantitatively, $22.3\% \pm 0.5\%$ of total IRS-1 colocalized with $\beta 1$ -integrin in differentiated PC12/IGF-IR neurons cultured in SFM. After TNF α stimulation, the amount of IRS-1 detected in the colocalization foci with $\beta 1$ -integrin increased to $41.6\% \pm 3.5\%$, and the cells treated with IGF-I showed only $14.5\% \pm 4.3\%$ of IRS-1 bound to $\beta 1$ -integrin. We have demonstrated a significant number of IRS-1– $\beta 1$ -integrin colocalization foci by anti-pS⁶¹²IRS-1 antibody (35.1%) and have determined that membrane-associated IRS-1 is serine phosphorylated (Fig. 1); these observations strongly suggest that the fraction of IRS-1 that binds $\beta 1$ -integrin is indeed phosphorylated on serine residues. Note that anti-IRS-1 antibody from Upstate Biotechnology recognizes both phosphorylated and nonphosphorylated IRS-1.

Once we established the presence of pS-IRS-1– $\beta 1$ -integrin in differentiated neurons, we asked how these membrane colocalization foci affect cell attachment to collagen IV. The cell attachment assay presented in Figure 4B demonstrates a significant decrease in the ability of PC12/IGF-IR cells to adhere to the collagen IV-coated surface 1 hr following TNF α treatment. Interestingly, TNF α -mediated inhibition of cell attachment was partially reversed when the TNF α treatment was accompanied by simultaneous cell stimulation with IGF-I. As expected, cells cultured in SFM or stimulated with IGF-I alone were characterized by efficient attachment to collagen IV, and cells treated with a specific $\alpha 1\beta 1$ -integrin inhibitor, obtustatin (Wang et al., 2006), were used as a control for the inhibition of cell attachment.

Characterization of IRS-1– $\beta 1$ -Integrin Binding

To determine which domain of IRS-1 mediates the binding with $\beta 1$ -integrin, we have utilized GST pull-down assay and previously characterized overlapping truncation mutants of mouse IRS-1 cDNA cloned in frame with GST (Trojanek et al., 2003). The results in Figure 5 show that the fragment of IRS-1 capable of pulling down $\beta 1$ -integrin is mapped within the central portion of IRS-1 between amino acids 426 and 740. Other truncation mutants of IRS-1 and GST alone were negative. The diagram shown in Figure 5B demonstrates that the IRS-1 fragment that binds $\beta 1$ -integrin contains Ser⁶¹², the residue that is phosphorylated at the membrane rafts of differentiated PC12/IGF-IR (Figs. 1, 2).

Inhibition of Neuronal Outgrowth by 426–740/IRS-1 Mutant

Once we established the binding site for $\beta 1$ -integrin on the IRS-1 molecule, we asked whether overexpression of the IRS-1 truncation mutant (426–740/IRS-1) could interfere with the formation and stability of neuronal processes in the absence of TNF α . The IRS-1 fragment was cloned into the BamH1-EcoRI site of pcDNA3.1zeo(+) expression vector and was transfected into undifferentiated PC12/IGF-IR cells using the Amaxa nucleoporator. The results in Figure 5C show the level of 426–740/IRS-1 protein in two stable clones (clones 2 and 5) of PC12/IGF-IR cells in comparison with PC12/IGF-IR cells expressing empty vector (EV). Note that anti-IRS-1 antibody used in this assay recognizes the central portion of the IRS-1 molecule, which includes the 426–740 fragment (see Materials and Methods).

As shown in Figure 6A, PC12/IGF-IR cells expressing 426–740/IRS-1 (clone 2) are characterized by a dramatic reduction in the number and length of neuronal extensions in comparison with control cells expressing EV. Quantitatively, $76\% \pm 11\%$ ($n = 3$) decrease in a total length of neuronal processes per microscopic field was calculated at day 5 after neuronal differentiation on collagen IV. In a parallel study, we observed a significant decrease in cell attachment (cell adhesion assay) when PC12/IGF-IR cells expressing 426–740/IRS-1 mutant were compared with PC12/IGF-IR cells expressing EV. Cells treated with

the $\alpha 1\beta 1$ -integrin inhibitor obtustatin were used again as a control for cell attachment inhibition.

An apparent inhibition in the formation of neuronal processes was observed when the 426–740/IRS-1 mutant was delivered into cortical rat neurons by transient transfection (Amaxa nucleoporator; efficiency of transfection varied between 60% and 75% in three separate experiments; Fig. 6C). To further evaluate the negative effects of 426–740/IRS-1 mutant on neuronal outgrowth, we applied double immunolabeling with anti- β III-tubulin antibody (green) and anti-IRS-1 antibody (red). We found that all neurons expressing the 426–740/IRS-1 mutant did not develop neuronal extensions (cells with strong red fluorescence indicated by arrowheads). In the same microscopic field, we detected well-differentiated β III-tubulin-positive neurite-like cell. These well-differentiated cells were characterized by much less apparent red fluorescence, most likely associated with the detection of endogenous IRS-1. Detailed inspection of multiple microscopic fields from three separate experiments confirmed that all neuronal cells positive for the 426–740/IRS-1 mutant did not develop neuronal extensions. Note also that anti-IRS-1 antibody used in this experiment recognizes the central portion of IRS-1, so it binds endogenous IRS-1 (weak red fluorescence) as well as the 426–740/IRS-1 mutant (strong red fluorescence). Other IRS-1 truncation mutants (Fig. 5B) expressed in PC12/IGF-IR cells or in primary rat cortical neurons did not affect neuronal outgrowth (data not shown).

In conclusion, our data, summarized in Figure 7, suggest a mechanism by which TNF α compromises development and stability of neuronal processes. It involves inhibition of the interaction between neuronal processes and extracellular matrix. The inhibition is caused by serine phosphorylated IRS-1 (pS-IRS-1), which accumulates at the membrane of differentiated neurons after TNF α stimulation. In this scenario, pS-IRS-1 binds $\beta 1$ -integrin, and this colocalization complex is strongly suspected to decrease the binding between $\alpha 1\beta 1$ and extracellular matrix, possibly compromising stability of neuronal processes. IGF-I stimulation attenuates the binding between IRS-1 and $\beta 1$ -integrin in membrane rafts of differentiated neurons and improves their attachment to collagen IV. Further studies are required to evaluate the clinical relevance of the pS-IRS-1– $\beta 1$ -integrin complex in the paradigm of neurological disorders in which TNF α is elevated.

DISCUSSION

Activation of inflammatory cytokines, including TNF α , and their effects on the nervous system have been repeatedly demonstrated in vitro (Ying Wang et al., 2003; Dorr et al., 2005; Huang et al., 2005; Wang et al., 2006) and are strongly associated with neurodegenerative disorders such as Alzheimer's disease, HIV-associated dementia, and neuronal injuries that develop after ischemia and type 2 diabetes (Munoz-Fernandez and Fresno, 1998; Hermann et al., 2001; Tarkowski et al., 2003; Dorr et al., 2005). Reports from several laboratories indicate that TNF α -mediated effects on neuronal cells can be associated with the serine phosphorylation (inactivation) of the major IGF-I receptor signaling molecule, IRS-1 (Venters et al., 1999, 2000, 2001). To clarify the role of IRS-1 serine phosphorylation and its anticipated interaction with integrins in the paradigm of TNF α -induced neurodegeneration, we have employed PC12/IGF-IR neurons, which strongly respond to IGF-I stimulation and express only one type of integrin complex, $\alpha 1\beta 1$ -integrin (Wang et al., 2006). We have detected $\beta 1$ -integrin in the complex with serine-phosphorylated IRS-1 (pS-IRS-1) in the membrane rafts fraction isolated from differentiated PC12/IGF-IR neurons and from primary rat cortical neurons. We have also demonstrated that the fraction of IRS-1 associated with membrane rafts is serine phosphorylated, and that at least two serine residues, S⁶¹² and S³⁰⁷, are involved. In addition, the interaction between IRS-1 and $\beta 1$ -integrin was enhanced by TNF α and strongly inhibited by IGF-I. By utilizing

a GST pull-down assay, we found that the IRS-1 domain responsible for the interaction with β 1-integrin is located between aa 426 and aa 740, the fragment that contains Ser⁶¹². Finally, we have demonstrated that PC12/IGF-IR neurons and primary cortical neurons expressing the 426–740 mutant of IRS-1 lost the ability to develop and maintain neuronal processes.

An important aspect of IRS-1 serine phosphorylation, which also implicates TNF α , has been demonstrated in type 2 diabetes and insulin resistance (Gao et al., 2003; Kim et al., 2003; Hartman et al., 2004; Danielsson et al., 2005; Liberman and Eldar-Finkelman, 2005). The mechanism by which TNF α could induce insulin resistance implicates 55-kD TNF α receptor (TNF-R55) and its death domain. This cytosolic portion of TNF-R55 has been shown to activate/recruit sphingomyelidases, which catalyse ceramide production at the membrane and in the lysosomal compartment. Subsequent ceramide-mediated JNK and PKC ζ activation results in the direct phosphorylation of IRS-1 on serine residue 307/312 rodents/human, which has also been shown to repress IGF-I- and insulin-mediated tyrosine phosphorylation of IRS-1 (Peraldi et al., 1996; Gao et al., 2003; Csehi et al., 2005). Our results shown in Figure 1B demonstrate the presence of pS-IRS-1 (both pS307 and pS612) in the membrane rafts of serumstarved PC12/IGF-IR neurons and show that TNF α further enhances their accumulation. Because the binding between IRS-1 and β 1-integrin was mapped between aa 426 and aa 740, we conclude that serine residues within this region may play a role in the interaction between IRS-1 and β 1-integrin. Among several serine residues, S^{616/612} (human/rodents) has been shown to be phosphorylated via TNF α -mediated activation of ERK kinase (Engelman et al., 2000; Rui et al., 2001). Note, however, that, in our experimental setting, serine-phosphorylated IRS-1 colocalizes with the membrane rafts fraction, and activated ERKs usually translocate to the nucleus. Therefore, further experiments are required to clarify the mechanism(s) by which pS-IRS-1 accumulates in the membrane rafts of differentiated neurons following TNF α stimulation.

We have detected β 1-integrin–IRS-1 complex in quiescent neurons; the complex quickly destabilizes following IGF-I stimulation (Fig. 2B) and is significantly enhanced by TNF α (Fig. 2A). TNF α compromises the stability of neuronal processes, supports IRS-1 serine phosphorylation, and enhances the interaction between IRS-1 and β 1-integrin, so we hypothesized that its accumulation could interfere with the interaction between neurite-like processes and extracellular matrix proteins. In agreement with this hypothesis, we found that TNF α , in contrast to IGF-I, inhibits cell attachment (Fig. 4B), destabilizes neuronal processes (Fig. 6A), and facilitates the formation of IRS-1– β 1-integrin complex (Figs. 2A, 3B). However, we have also detected a significant amount of the β 1-integrin–IRS-1 complexes in quiescent neurons (SFM) in the absence of TNF α (Fig. 2), the condition in which neuronal processes are quite stable. This discrepancy could be explained by the presence of unbound β 1-integrin in SFM. Such an explanation is partially supported by our results shown in Figure 4A in which we have detected much more of free β 1-integrin (not associated with pS-IRS-1) in SFM (green fluorescence) in comparison with TNF α -treated samples (a significant increase of pS-IRS-1– β 1-integrin colocalization; yellow fluorescence).

In summary, our results demonstrate for the first time that the interaction between serine-phosphorylated IRS-1 and β 1-integrin may affect the stability of neuronal processes in the paradigm of TNF α -induced neuronal damage. We have also demonstrated that IGF-I inhibits the formation of IRS-1– β 1-integrin complex at the membrane rafts of differentiated neurons, and this could suggest a new mechanism of action for the IGF-I receptor, which in addition to its strong antiapoptotic signal could directly support integrin-mediated cell attachment, counteracting the detrimental effects of TNF α on the stability of neuronal processes.

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Fig. 1. Membrane-associated localization of IRS-1 and β 1-integrin in PC12/IGF-IR neurons. Western blot analysis showing protein levels for β 1-integrin and IRS-1 as well as IRS-1 serine (pS) and tyrosine (pY) phosphorylation. After serum starvation for 48 hr (SFM), differentiated neurons were stimulated with IGF-1 (50 ng/ml; **A**) or TNF α (100 ng/ml; **B**) at the indicated times. Proteins were extracted from the membrane rafts fraction and from the detergent-soluble fraction. The results indicate that β 1-integrin as well as IRS-1 phosphorylated on serine residues (pS⁶¹² and pS³⁰⁷) are located predominantly in the membrane rafts fraction. In contrast, total IRS-1 and IRS-1 phosphorylated on tyrosine

residue (pY⁶⁰⁸) are in the detergent-soluble fraction. Anti-Src and anti-IRS-2 antibodies were used as membrane rafts and detergent-soluble fraction markers, respectively. Densitometric analysis of the corresponding blots was prepared in Scion Image. Densitometric data in A were normalized to the levels of c-Src, for the membrane rafts fraction, and by IRS-2, for the detergent-soluble fraction. Densitometric data in B were normalized to total IRS-1. The results presented are averages from three independent blots ($n = 3$) with SDs. In B, an asterisk indicates values statistically different from SFM values ($P = 0.05$).

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Fig. 2.

Interaction between IRS-1 and β 1-integrin in the membrane fraction of PC12/IGF-IR neurons. IP/Westerns were performed with membrane proteins isolated from quiescent (SFM), TNF α -stimulated (**A**), and IGF-I-stimulated (**B**) PC12/IGF-IR neurons at the indicated times. In **B**, reciprocal PI/Western analyses were performed with mouse monoclonal anti- β 1-integrin and rabbit polyclonal anti-IRS-1 antibodies. Note the presence of an apparent interaction between IRS-1 and β 1-integrin in SFM, and a strong attenuation of the interaction following IGF-I stimulation. Histograms illustrate densitometric data normalized either to total β 1-integrin or to total IRS-1 from three independent experiments with SDs ($n = 3$). An asterisk indicates values statistically different ($P = 0.05$) from SFM.

Blots in **C** represent control IP/Westerns in which IP reactions were performed with anti- β 1-integrin antibody (β 1), anti-BrdU irrelevant antibody (BrdU), anti-IRS-1 antibody (IRS), and agarose beads only (beads). In the last lane, 50 μ g of membrane rafts proteins were loaded without immunoprecipitation (W), and the resulting blots were probed with anti- β 1-integrin or anti-IRS-1 antibody.

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Primary Rat Neurons (membrane rafts)

Fig. 3.

Interaction between IRS-1 and β 1-integrin in membrane fraction of rat cortical neurons. IP/ Western performed with membrane proteins isolated from the quiescent (SFM), IGF-I-stimulated (**A**), and TNF α -stimulated (**B**) primary cultures of rat cortical neurons. Reciprocal PI/Western analysis was performed with anti- β 1-integrin and anti-IRS-1 antibodies. Note the presence of an apparent interaction between IRS-1 and β 1-integrin in SFM, partial attenuation of the interaction after IGF-I stimulation, and increased interaction after TNF α stimulation. Histograms illustrate densitometric data normalized by total β 1-

integrin or total IRS-1 from three independent experiments ($n = 3$) with standard deviation. An asterisk indicates values statistically different from SFM values ($P = 0.05$).

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Fig. 4.
Colocalization between IRS-1 and β 1-integrin in differentiated PC12/IGF-IR neurons. **A:** Double immunolabeling of IRS-1 and β 1-integrin in differentiated PC12/IGF-IR neurons. Fluorescent images were collected from an inverted fluorescent microscope equipped with motorized Z-axis and deconvolution software (Slide-Book4). Anti- β 1-integrin mouse monoclonal antibody and antimouse rhodamine-conjugated secondary antibody (red fluorescence) as well as anti-IRS-1, anti-pS612IRS-1 rabbit polyclonal antibody, and anti-rabbit FITC-conjugated secondary antibody (green fluorescence) were utilized. Note the presence of a strong colocalization between IRS-1 and β 1-integrin detected in neuronal

processes after TNF α treatment (yellow fluorescence). **B:** The adhesion of PC12/IGF-IR cells to collagen IV was evaluated after 1 hr of exposure of the cells to IGF-I, TNF α , or the combination of both. The number of adhered cells was calculated from a standard curve, and the data obtained are presented as an average of three experiments ($n = 3$) with SD. Note an apparent loss of cell adhesion after TNF α treatment, which was significantly prevented by IGF-I. Cells treated with a specific $\alpha 1\beta 1$ -integrin inhibitor, obtustatin (kindly provided by Dr. Cezary Marcinkiewicz, Temple University, Philadelphia, PA), were used as a control for the inhibition of cell attachment. A single asterisk indicates values statistically different from SFM values ($P = 0.05$). Double asterisks indicate values statistically different from TNF α values ($P = 0.05$). Original magnification $\times 1,000$. Scale bar = 5 μm .

Fig. 5. Characterization of the interaction between $\beta 1$ -integrin and IRS-1 in PC12/IGF-IR neurons. **A:** Pull-down reactions between GST-IRS-1 fusion proteins (five overlapping IRS-1 truncation mutants) and $\beta 1$ -integrin were performed with protein extracts isolated from the membrane rafts fraction of differentiated PC12/IGF-IR neurons. After pull-down assay, one of the IRS-1 truncation mutants, GST-IRS-1 (426–740), binds $\beta 1$ -integrin. All other mutants and GST alone were negative. An aliquot of a protein extract from membrane rafts of HeLa cells was used as positive control. **B:** Schematic illustration of IRS-1 molecule with the indicated IRS-1 truncation mutants and their corresponding positions. Note that the IRS-1

fragment, which binds $\beta 1$ -integrin, contains serine residue at position 612, which is strongly phosphorylated following TNF α stimulation. **C:** Western blot analysis demonstrating protein levels for IRS-1 and 426–740/IRS-1 fragment in PC12/EV control cells and in two clones of PC12/IGF-IR cells stably expressing the 426–740/ IRS-1 mutant. Note that anti-IRS-1 antibody used in this experiment was developed against the central portion of IRS-1 and recognizes both total IRS-1 and the 426–740/IRS-1 truncation mutant.

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Fig. 6.

Inhibition of neuronal outgrowth and cell attachment by the IRS-1 truncation mutant that binds β 1-integrin. The ability of PC12/IGF-IR (**A**) and primary cortical neurons (**C**) to differentiate and to maintain neuronal processes was evaluated in stable clones of PC12/IGF-IR/426-740/IRS neurons, or in primary cortical neurons transfected with the 426-740/IRS expression vector. Cells transfected with empty vector (EV) were used as a reference sample. The efficiency of transient transfection evaluated by green fluorescent protein (GFP) expression vector ranged between 60% and 70%. Phase-contrast images illustrate a decrease in the formation and stability of neuronal processes in PC12/IGF-IR/426-740/IRS

cells in comparison with cells expressing the EV. Original magnification $\times 200$. **B:** The cell adhesion assay to collagen IV was performed with PC12/IGF-IR neurons transiently expressing the 426–760/IRS-1 mutant or control EV. The EV-expressing cells treated with the $\alpha 1\beta 1$ -integrin inhibitor obtustatin were used as a control. Data represent three independent experiments ($n = 3$) with SDs. An asterisk indicates values statistically different from SFM/EV values ($P = 0.05$). **C:** Double immunolabeling of primary cortical neurons with anti-IRS-1 and anti- β III-tubulin antibodies. Note that cortical neurons that express the IRS-1 mutant (strong red fluorescence, arrowheads) do not develop neuronal processes. In contrast, untransfected neurons (weak red fluorescence associated with endogenous IRS-1) retained the ability for neuronal outgrowth (arrow). Original magnification $\times 400$. Scale bars = 5 μ m.

Fig. 7.

Functional implications of the pS-IRS-1-β1-integrin interaction. The pS-IRS-1-β1-integrin complex formation in the membrane rafts of differentiated neuronal cells impairs the binding between integrins and collagen and is thought to compromise stability of neuronal processes. This protein-protein interaction is facilitated by TNFα, which triggers accumulation of pS-IRS-1 in membrane rafts and is attenuated by IGF-I, which facilitates tyrosine phosphorylation of IRS-1, impairs formation of the complex, and supports neuronal survival.