Phosphatidylinositol 3-kinase and Akt effectors mediate insulin-like growth factor-I neuroprotection in dorsal root ganglia neurons

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SPECIFIC AIMS

The present study investigates *1)* the signaling pathways underlying insulin-like growth factor-I (IGF-I) neuroprotection in embryonic rat dorsal root ganglia (DRG) neurons and *2)* how these pathways interrupt apoptosis of DRG neurons induced by high glucose.

PRINCIPAL FINDINGS

1. IGF-I activates the PI3K/Akt but not the ERK/MAPK pathway in DRG neurons

We investigated IGF-I induced effects on two known survival signaling pathways: the MEK/ERK pathway and the phosphatidylinositol 3-kinase (PI3K) pathway (which activates Akt). We demonstrate that 10 nM IGF-I causes phosphorylation of Akt in cultured embryonic rat DRG neurons. In contrast, IGF-I does not increase phosphorylation of ERK in DRG neurons. These data suggest that IGF-I primarily signals through the PI3K/Akt pathway.

2. IGF-I activates cytosolic and nuclear proteins important for survival of DRG neurons

DRG treated with 10 nM IGF-I for 15 min exhibit phosphorylation of Akt and of three known Akt effectors: glycogen synthase kinase-3β (GSK-3β), the apoptotic transcription factor forkhead (FKHR), and the survival transcription factor cyclic AMP response element binding protein (CREB), which binds to DNA sequences containing the cyclic AMP response element (CRE) (**Fig. 1*A*, *B***). IGF-I causes phosphorylation of these proteins even in high glucose media (45 mM). Inhibition of the PI3K/Akt pathway with LY294002 blocks phosphorylation of Akt, GSK-3β, FKHR, and CREB. IGF-I increases DNA binding to CRE-containing sequences, suggesting that IGF-I increases CREB-mediated DNA binding and transcription (Fig. 1*C*).

3. IGF-I causes Akt translocation

We demonstrate that IGF-I causes phosphorylated Akt to translocate from the cytosol to the nuclei of DRG neurons within 15 min in control and high-glucose media (**Fig. 2**). Nuclear localization is retained for at least 60 min. Inhibition of the PI3K/Akt pathway blocks redistribution of phosphorylated Akt from the cytosol to nucleus.

4. IGF-I causes nuclear FKHR phosphorylation

IGF-I causes increased phosphorylation of FKHR in the nuclei of DRG neurons within 5 min in control and 45 mM glucose media. By 15 min of IGF-I treatment, most neurons exhibit phospho-FKHR primarily in the cytosol; by 30 min, phospho-FKHR is found exclusively in the cytosol. Treatment with IGF-I and LY294002 results in nuclear phospho-FKHR expression similar to control levels. These data suggest that IGF-I induces export of phospho-FKHR from the nuclei of DRG neurons.

5. IGF-I inhibits glucose-induced apoptosis in DRG neurons via the PI3K/Akt pathway

We previously demonstrated that rat DRG neurons survive optimally in media containing 25–30 mM glucose, but higher glucose concentrations induce apoptosis. Our current work demonstrates that treatment with 45 mM glucose for 24 h induces apoptosis in 55.3% of embryonic rat DRG neurons. Addition of IGF-I inhibits apoptosis to control levels. However, rescue is blocked

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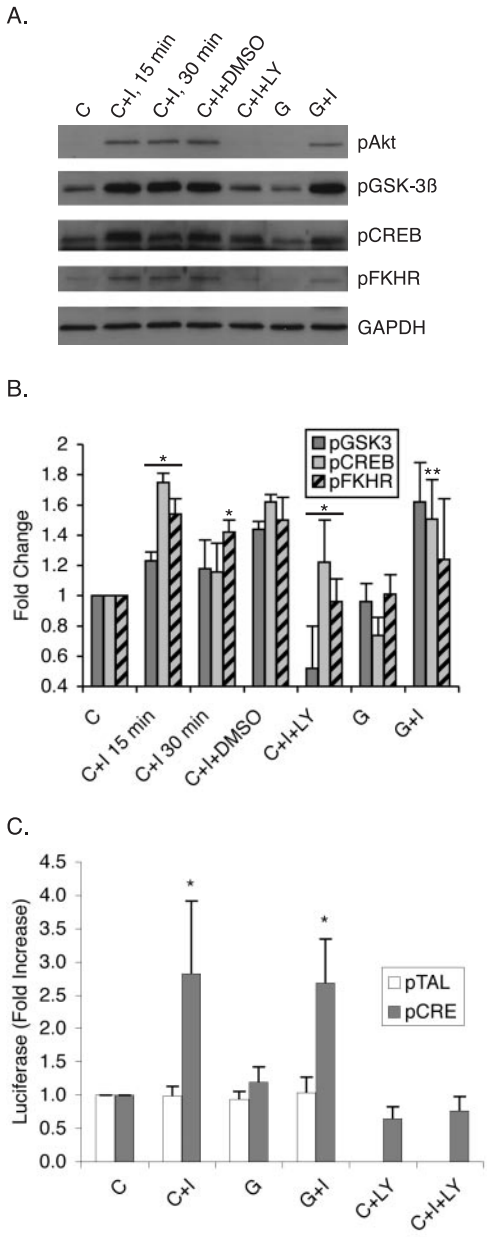

Figure 1. *A)* DRG neurons were treated with control media (C) or stimulated with 10 nM IGF-I for 15 min (C+I, 15 min) or 30 min (C+I, 30 min). DRG neurons were treated for 15 min with 10 nM IGF-I and 0.1% DMSO (C+I+DMSO) or LY294002 (C+I+LY), or separately with glucose (G) or glucose + 10 nM IGF-I (G+I). Immunoblots shown are representative of results from 3 separate experiments. *B)* Densitometry of pGSK-3β, pCREB, and pFKHR from immunoblots described in panel *A*. Expression is represented as the average fold change of samples compared with the controls (C), such that C = 1. Error bars represent standard error of the mean (SE). $*P < 0.05$ or less as determined by 1-way ANOVA. *C)* DRG neurons were transfected with plasmids containing control (pTAL) or CRE (pCRE) promoters directing expression of a firefly luciferase reporter gene. DRG neurons were transfected with a plasmid containing a constitutively active renilla luciferase reporter, used to normalize transfection levels between samples. Results are expressed as fold increase of luciferase fluorescence normalized to control treatment. DRG neurons were treated for 24 h with control media alone (C) or 10 nM IGF-I (C+I) or with high glucose (G) or high glucose + 10 nM IGF-I (G+I). Differences are significant (*) if $P < 0.005$ as determined by Students' *t* test, using a 2-tailed test and assuming equal variances.

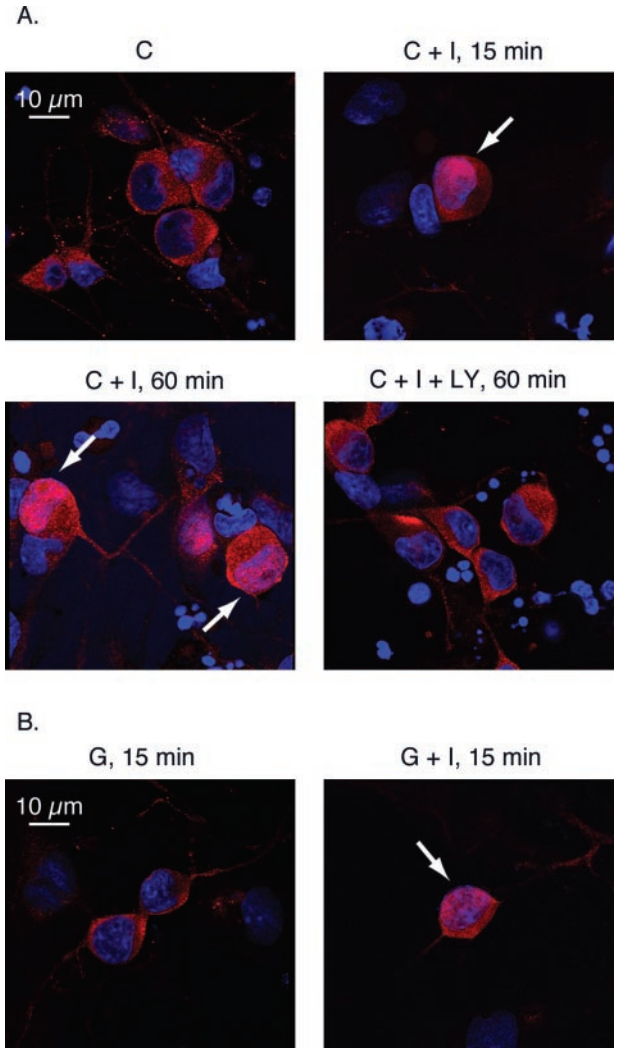

Figure 2. IGF-I induces accumulation of phospho-Akt in the nuclei of DRG neurons. *A)* DRG neurons were treated with control media (C) or 10 nM IGF-I for 15 (C+I, 15 min) or 60 min (C+I, 60 min) ± LY294002 (C+I+LY). Samples were probed with antibodies against phospho-Akt ser473 (red) and nuclei were labeled with Hoechst 33342 dye (blue). Samples were analyzed via confocal microscopy. Arrows show phospho-Akt accumulation in nuclei, represented by punctate pink nuclear staining (overlap of red phospho-Akt and blue nuclei). *B)* DRG neurons were treated for 15 min with 45 mM glucose (G, 15 min) or glucose + 10 nM IGF-I (G+I, 15 min) and assayed as in *A*. Images in photomicrographs are representative of 9 separate fields across 3 sample sets.

by addition of IGF-I with the PI3K (and thus, Akt) pathway inhibitor LY294002. In contrast, neurons are protected when treated with glucose, IGF-I, and the ERK/MAPK pathway inhibitor PD98059. These data suggest that IGF-I prevents high glucose-induced apoptosis of DRG neurons through the PI3K/Akt pathway.

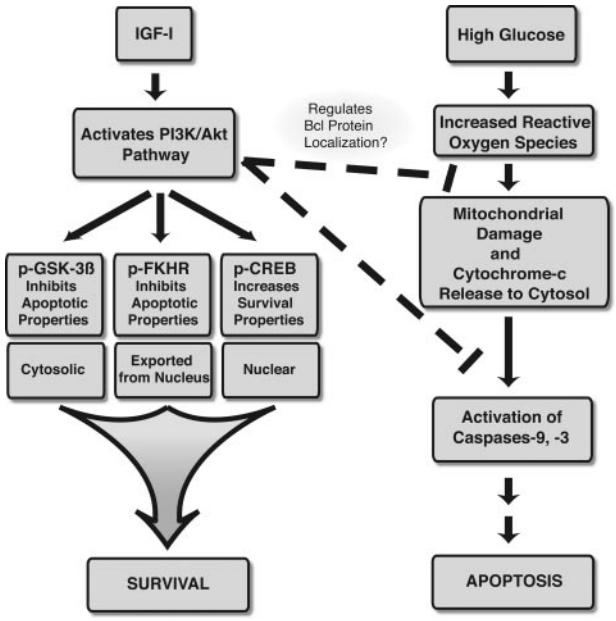

Figure 3. Schematic of IGF-I neuroprotective signaling in DRG neurons. IGF-I activates the PI3K/Akt pathway, which phosphorylates 3 known Akt effectors: GSK-3β, FKHR, and CREB. IGF-I-induced regulation of these Akt effectors collectively promotes survival. High glucose increases creation of reactive oxygen species, leading to mitochondrial damage, cytochrome *c* release, activation of the caspase-9 cascade, and apoptotic death. IGF-I inhibits processing of caspases-9 and -3, suggesting it protects DRG neurons at the mitochondrial level upstream of cytochrome *c* release. IGF-I may protect mitochondria via regulating localization of the Bcl family of proteins.

6. IGF-I inhibits loss of pro-caspase-9 and cleavage of caspase-3 in DRG neurons via the PI3K pathway

We further explored glucose-mediated apoptosis in DRG neurons by examining the caspase cascade. Treatment for 3 h with 45 mM glucose causes loss of the proform initiator caspase-9 but does not alter cleavage levels of initiator caspase-8. IGF-I inhibits loss of proform caspase-9 in glucose-treated DRG neurons. High glucose induces cleavage of executioner caspase-3, which is inhibited by IGF-I. Addition of LY294002 blocks IGF-I inhibition of caspase-3 cleavage, but IGF-I can inhibit cleavage in samples treated with PD98059. Altogether, these data indicate that DRG respond to high glucose by activating initiator caspase-9 and executioner caspase-3, both of which can be inhibited by IGF-I signaling through the PI3K/Akt pathway.

7. High glucose up-regulates Bim but not Bcl-xL in DRG neurons

We demonstrate that protein expression of the pro-apoptotic Bcl family protein Bim, a transcriptional target of FKHR, is increased in DRG neurons treated with 45 mM glucose. However, addition of IGF-I ± LY294002 does not inhibit the glucose-induced expression of Bim. We further demonstrate that neither high glucose, IGF-I, or LY294002 alter expression of the anti-apoptotic Bcl family protein, Bcl-xL.

CONCLUSIONS AND SIGNIFICANCE

IGF-I is critical for the development, maintenance, and regeneration of neurons of the peripheral nervous system (PNS). However, little is known about the intracellular signaling systems underlying. We therefore used DRG neurons subjected to high glucose-induced apoptosis to investigate IGF-I survival signaling in primary PNS neurons.

Our results indicate that IGF-I potently activates the PI3K/Akt pathway in DRG neurons within 15 min and lasts at least 3 h. These data are in contrast to IGF-I activation of Akt in transformed neural SH-SY5Y and SHEP cell lines, where activation is transient and peaks within 15 min. However, our results are consistent with data showing IGF-I activation of Akt in primary adult sensory neurons at 6, 12, and 24 h. The rapid and prolonged responses of IGF-I-mediated Akt activation allow for regulation of anti-apoptotic Akt effectors (CREB) and inhibition of proapoptotic Akt effectors (GSK-3β, FKHR) via phosphorylation and cellular localization. The perseverance of these effects in the presence of high glucose confirms that an apoptotic environment does not interrupt IGF-I-mediated survival signaling in primary DRG neurons.

We previously demonstrated that IGF-I inhibits glucose-mediated apoptosis, but the intervention points remained unknown. Our current study shows that IGF-I suppresses processing of initiator caspase-9 and downstream executioner caspase-3. Previous data from our laboratory has shown that high glucose induces mitochondrial dysfunction and cytochrome *c* release, which is known to activate caspase-9. Therefore, our data collectively suggest that IGF-I inhibits mitochondrial distress upstream of cytochrome *c* release. The family of Bcl proteins is known to regulate mitochondrial integrity and may be targeted by IGF-I for mitochondrial protection. Our current work shows that IGF-I does not regulate protein expression of Bcl-xL or Bim. This contrasts with results from neural cell lines and neurons of the central nervous system in which IGF-I regulates expression of both anti-apoptotic and pro-apoptotic Bcl family proteins. However, as for Akt and FKHR, IGF-I may regulate localization of pro- and anti-apoptotic Bcl proteins, thus exerting mitochondrial protection.

In summation, IGF-I activates PI3K and Akt effectors in DRG neurons and inhibits glucose-induced apoptosis via maintenance of mitochondrial integrity. FJ