



Insulin attenuates apoptosis in neuronal cells by an integrin-linked kinase-dependent mechanism

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ARTICLE INFO

Keywords:

Neuroscience
Cell biology
Nervous system
Diabetes
Developmental biology
Nervous system
Metabolic disorder
IGF-1
Serum and depolarization withdrawal
Integrin-linked kinase
Integrin
Insulin
Inhibitor of apoptosis

ABSTRACT

Insulin promotes neuronal survival by activating a phosphatidylinositol 3-kinase (PI 3-kinase)/AKT-dependent signaling pathway and reducing caspase activation. We investigated a role for integrin-linked kinase (ILK) in insulin-mediated cell survival in cultured neurons and differentiated R28 cells. We used a serum and depolarization withdrawal model to induce apoptosis in cerebellar granule neurons and a serum withdrawal model to induce apoptosis in differentiated R28 cells. ILK knock-out decreased insulin-mediated protection as did the addition of pharmacological inhibitors of ILK, KP-392 or QLT-0267. Prosurvival effects of insulin were rescued by Boc-Asp (O-methyl)-CH₂F (BAF), a pancaspase inhibitor, in the presence of KP-392. Insulin and IGF-1 decreased caspase-3 activation, an effect that was inhibited by KP-392 and QLT-0267. Western blot analysis indicates that insulin-induced stimulation of AKT Ser-473 phosphorylation was decreased after the ILK gene was conditionally knocked-out, following overexpression of AKT-DN or in the presence of QLT-0267. Insulin and IGF-1 stimulated ILK kinase activity in primary neurons and this was inhibited following ILK-DN overexpression. Western blot analysis indicates that insulin exposure upregulated the expression of the cellular inhibitor of apoptosis protein c-IAP2 in an extracellular matrix-dependent manner, an effect blocked by KP-392. These results indicate that ILK is an important effector in insulin-mediated neuroprotection.

1. Introduction

Insulin is an important survival factor for neurons. Impaired insulin signaling has been linked to abnormal brain development, neurodegenerative diseases and retinal neurodegeneration [1, 2, 3]. *In vitro* insulin provides trophic support for a wide variety of neuronal cells including cerebellar granule neurons, sensory neurons, cortical neurons, spinal motor neurons, retinal neurons and R28 cells [4, 5, 6, 7, 8]. The pro-survival effects of insulin and insulin-like growth factor (IGF-1) can be largely attributed to a signaling cascade involving phosphatidylinositol 3-kinase (PI 3-kinase) and the serine threonine kinase AKT (also known as protein kinase B) [9, 10, 11]. Activated AKT inhibits apoptosis by phosphorylating and inactivating a growing list of apoptotic factors including caspases-9, glycogen synthase kinase-3 β (GSK-3 β), BCL-2-associated death promoter, transcription factors of the forkhead family, and IKK, a kinase that regulates the NF- κ B transcription factor [12, 13, 14].

ILK regulates the phosphorylation of AKT at Ser 473 and glycogen synthase kinase-3 β (GSK-3 β) in various cell types including neuronal cells. ILK is an ankyrin repeat containing serine/threonine protein kinase that interacts with integrin β 1 and β 3 cytoplasmic domains [15]. ILK activity can be stimulated by both matrix attachment and growth factor stimulation in a PI 3-kinase-dependent manner [16, 17, 18]. In response to growth factor treatment, ILK phosphorylates AKT at Ser-473, one of two phosphorylation sites required for AKT activation [19, 20, 21, 22, 23, 24]. ILK has been shown to stimulate AKT activity *in vitro* and *ex vivo* [19, 23, 24, 25, 26]. In PC12 cells, nerve growth factor (NGF) stimulates AKT via ILK while in dorsal root ganglion neurons ILK regulates GSK-3 β in an NGF-induced, PI 3-kinase dependent pathway [27, 28]. Activation of AKT may, in turn, phosphorylate and thereby negatively regulate GSK-3 β [29]. Alternatively, phosphoinhibition of GSK-3 β by ILK may be direct as ILK has been shown to phosphorylate GSK-3 β *in vitro* [30, 31]. Given that ILK regulates AKT and other kinases in this pathway, it is not surprising that ILK has also been shown to suppress apoptosis in a variety of models

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<https://doi.org/10.1016/j.heliyon.2019.e02294>

Received 8 November 2018; Received in revised form 4 June 2019; Accepted 8 August 2019

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[22, 32, 33, 34]. By promoting AKT phosphorylation, ILK stimulates signalling pathways that regulate survival, including those that inhibit caspase activity (reviewed in [35, 36]).

A role for ILK in the prosurvival effects of trophic factors such as insulin remains largely unstudied. In nonneuronal cells, both insulin and IGF-1 stimulate ILK activity [19, 37] and stimulation of AKT by insulin requires integrin-linked kinase (ILK) [19]. Although a role for ILK in the neuroprotective effects of insulin has not been studied, ILK has been shown to be involved in neuroprotection via other AKT-dependent signalling pathways. In hippocampal neurons, ILK regulates integrin survival signalling via AKT [38]. Indirect evidence suggests a role for ILK in insulin signalling in neurons as the expression of ILK pathway components in neuronal cells are altered in long-term studies of rat diabetic models [39, 40]. As ILK regulates insulin-stimulated kinases in neurons [27, 38] and is an important effector of insulin and IGF-1 in nonneuronal cells, our aim was to investigate a role for ILK in insulin- and IGF-1-mediated neuronal cell survival signalling. We chose the serum and depolarization withdrawal model to induce apoptosis in cerebellar granule neurons and a serum withdrawal model to induce apoptosis in differentiated R28 cells as these neuronal models have already been used for studying insulin-mediated and AKT-dependent survival pathways.

2. Results

2.1. ILK knock-down and ILK inhibition decrease insulin-mediated neuroprotection

First, we studied insulin-induced neuroprotection in cerebellar granule neurons using the serum and depolarization withdrawal model as this has been a preferred model for studying AKT-dependent survival signaling. In keeping with past studies for this model, we used insulin at pharmacological concentrations (10 μ g/ml or 1.72×10^3 nM) [9]. To test whether ILK is required for insulin-induced neuronal survival we knocked down the expression of ILK in cerebellar cultures using the Cre-Lox system or we inhibited ILK pharmacologically with KP-392 (also called KP-SD1) as previously described [21, 23, 24, 27, 41, 42, 43, 44] (Fig. 1A, B). By inhibiting ILK, KP-392 has also been shown to inhibit downstream kinases such as PKB and GSK-3 in a variety of cell types, including neurons [21, 27, 45, 46].

To excise the ILK gene and eliminate its expression, ILK floxed/floxed (fl/fl) cerebellar cultures prepared from the pLox-ILK mice [25] were infected with Cre recombinase-expressing adenovirus (AdCre) particles. ILK fl/fl cultures were then subject to serum and depolarization withdrawal with or without insulin for 24 h (Fig. 1A). Apoptosis was measured by terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick (TUNEL) labeling. Insulin-mediated neuroprotection was compromised in ILK excised cultures as compared to control ILK fl/fl cultures (Fig. 1A). In control cultures subject to serum and depolarization withdrawal, insulin exposure resulted in a significant reduction in apoptotic cells, while in ILK excised cultures insulin had no effect. In contrast, basal levels of apoptosis in ILK fl/fl cultures exposed to AdCre and maintained in serum were not significantly different from control cultures maintained in serum (Fig. 1A). Data are from 3 separate experiments (3 separate platings from different animals); error bars indicate SEM. Statistical comparison was performed using a paired Student's t-test, * $P < 0.05$.

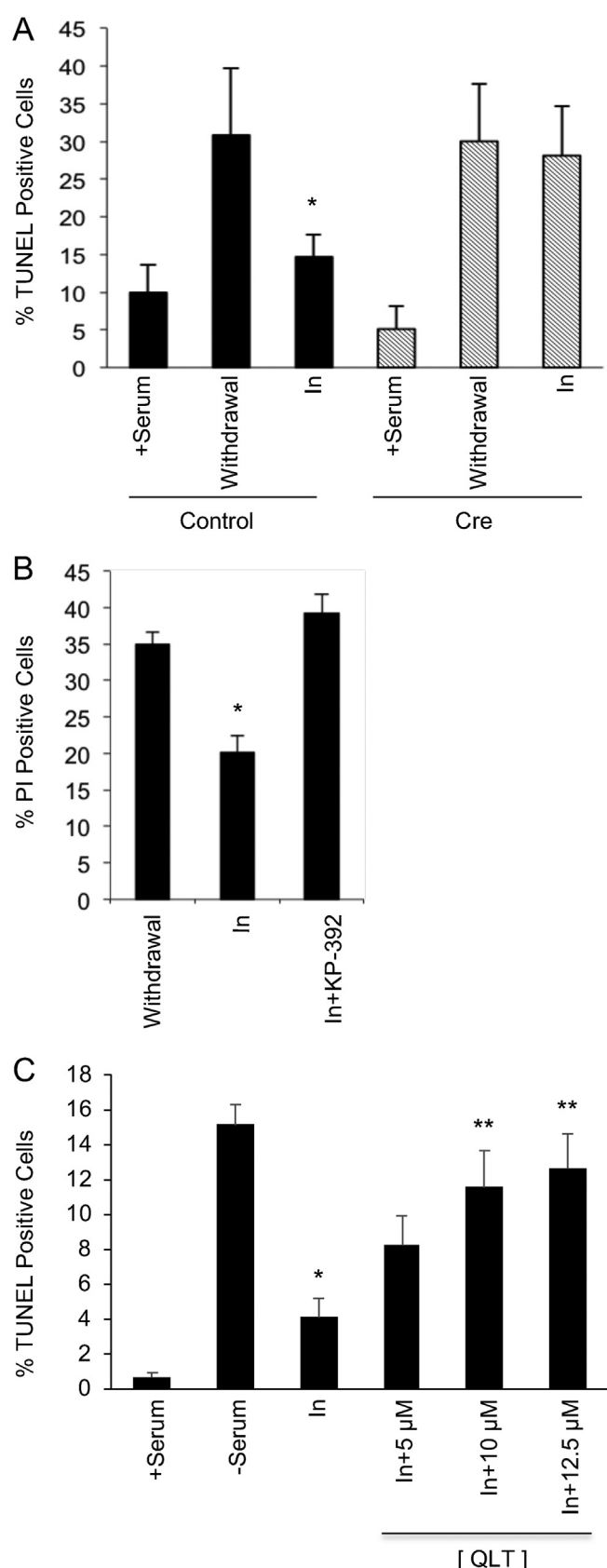
To determine whether or not ILK activity is required for insulin-induced neuroprotection we measured apoptosis in the presence of insulin alone or together with the ILK inhibitor KP-392. We assessed the number of apoptotic cells by propidium iodide staining and this was quantified as percentage of total cells. Mouse cerebellar granule neurons that had been plated onto laminin were subject to serum and depolarization withdrawal for 48 h. Under these conditions the percentage of apoptotic cells was 34.9 ± 1.68 . Insulin (10 μ g/ml) reduced the number of apoptotic cells and insulin-mediated protection was significantly inhibited in the presence of 100 μ M KP-392 (Fig. 1B). Data are from 3

separate experiments; error bars indicate SEM. * $P < 0.05$, different from cells subject to serum and depolarization withdrawal alone. Statistical comparison was performed using a one-way analysis of variance (ANOVA) and Tukey's multiple comparison test.

Next, we studied insulin-induced neuroprotection following serum withdrawal in another neuronal cell model, R28 cells. These are retinal neuro-glial progenitor cells that acquire a neuronal phenotype when exposed to cAMP [8, 47, 48, 49, 50]. In this retinal neuronal model, physiological levels of insulin have been shown to protect R28 cells from apoptosis induced by serum withdrawal. To inhibit ILK pharmacologically we used QLT-0267 a selective, small molecule inhibitor of ILK that has been characterized previously [23, 51, 52]. We found that the pro-survival effect of physiological levels of insulin (10 nM), was blocked by the ILK inhibitor QLT-0267 in a concentration-dependent manner (Fig. 1C). A significant increase in apoptosis was observed at 10 and 12.5 μ M concentrations of QLT-0267 relative to insulin alone. Data represent 3–4 separate experiments; error bars indicate SEM. * $P < 0.05$, different from cells subject to serum withdrawal alone. ** $P < 0.05$, different from cells treated with insulin alone. Statistical comparison was performed by a one-way ANOVA followed by Tukey's multiple comparison test.

To determine whether or not ILK's role in insulin-induced neuroprotection involves caspases, we compared the effects of ILK inhibition on insulin-induced survival in the presence or absence of a pancaspase inhibitor. Apoptosis in cerebellar cultures plated on laminin and exposed to serum and depolarization withdrawal was measured by TUNEL labeling (TUNEL-positive cells were expressed as a percentage of total cell number). After 24 h, insulin (10 μ g/ml) significantly reduced neuronal cell death while KP-392 (100 μ M) blocked this effect (Fig. 2A, B). The effect of insulin on cell death was rescued by 100 μ M of Boc-Asp (O-methyl)-CH₂F (BAF), a pancaspase inhibitor that prevents neuronal apoptosis [53] and this occurred in the presence of KP-392 (Fig. 2A, B). Representative fluorescence photomicrographs of cells labeled for TUNEL (right panel) and DAPI (left panel) for each treatment group are shown (Fig. 2A). A bar graph (Fig. 2B) indicates the percentage of cells that were TUNEL positive for each treatment group ($n = 3$ –4 separate culture platings). * $P < 0.05$, different from cells subject to serum and depolarization withdrawal alone and different from insulin with KP-392. Statistical comparisons were performed by a one-way ANOVA and Tukey's multiple comparison test.

The fact that an ILK inhibitor decreased insulin-induced survival effects and that this was rescued by BAF, indicates that insulin stimulates an ILK-mediated survival pathway that ultimately regulates caspases. Indeed, insulin has been shown to decrease caspase-3 activity, a common effector of most apoptotic pathways and this effect is blocked by ILK inhibition [22]. To determine whether caspase-3 is a target of an ILK-dependent survival pathway in cerebellar neuronal cells, we used the serum and depolarization model to measure caspase-3 activity in the presence of insulin or IGF-1. IGF-1 was chosen as a related anti-apoptotic hormone that also promotes neuronal cell survival via AKT and other signaling pathway proteins shared by ILK [11, 54]. Activated caspase-3 was detected by immunofluorescent cytochemistry with the polyclonal antibody CM-1, an antibody that detects activated caspase-3 p20. Bar graph (Fig. 2C) represents CM-1 immunoreactive neuronal cells subject to serum and depolarization and normalized to control (neuronal cells maintained in serum and 25 mM KCl). In the control group there was little immunoreactivity for active caspase-3. In contrast, cerebellar cells exposed to serum and depolarization withdrawal were highly immunoreactive for active caspase-3. Insulin (10 μ g/ml) and IGF-1 (25 ng/ml) reduced the number of immunoreactive cells, an effect that was antagonized in the presence of KP-392 (Fig. 2C). Data are from 3 separate culture platings; error bars indicate SEM. * $P < 0.05$, different from cells treated with the same first messenger together with KP-392 as determined by a Student's t-test. Likewise, we measured caspase-3 activity in our other neuronal cell model, differentiated R28 cells. In these cells, apoptosis was induced by serum withdrawal alone. Western blot analysis was used to measure caspase-3 activity indirectly by measuring a



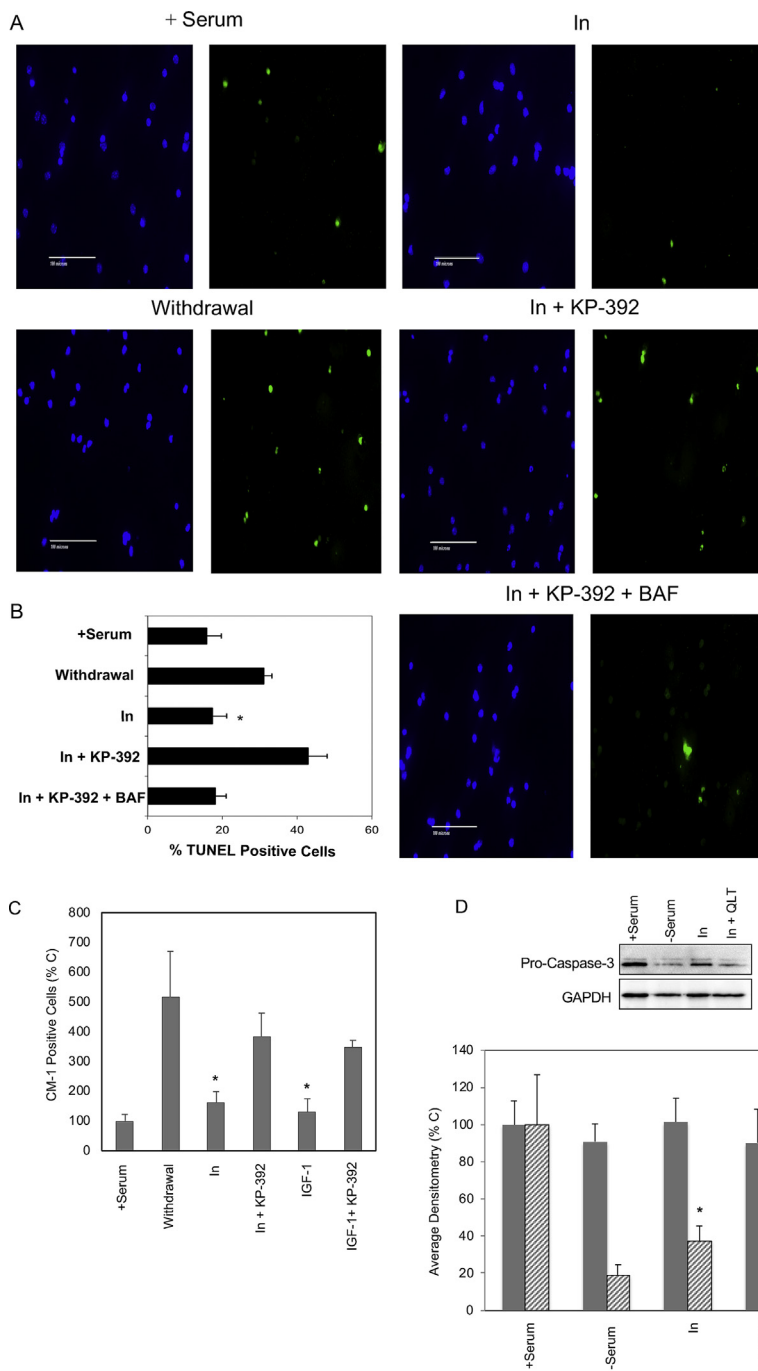
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Fig. 1. Insulin-Promoted Neuroprotection is Mediated by ILK A, Excision of ILK inhibits insulin induced neuroprotection. A TUNEL assay was performed in ILK fl/fl cerebellar cultures plated onto laminin and exposed to insulin (10 μ g/ml) following AdCre (Cre) or Control infection. In control cultures subject to serum and depolarization withdrawal, insulin exposure resulted in a significant reduction in apoptotic cells, while in ILK excised cultures insulin had no effect. The percentage of TUNEL positive cells maintained in serum and 25 mM KCl did not differ between control and ILK excised cultures. $n = 3$ (3 separate platings from different animals); error bars indicate SEM. * $P < 0.05$ as determined by a Student's t-test. B, The ILK inhibitor KP-392 inhibited insulin-promoted survival in cerebellar cultures plated onto laminin. Cells were plated onto laminin and subject to serum and depolarization withdrawal (Withdrawal), in the presence of 10 μ g/ml insulin (In) alone or with 100 μ M of the ILK inhibitor KP-392 (In+KP-392). After 2 days cells were fixed and the number of apoptotic cells determined by staining with the DNA dye propidium iodide. Apoptotic cells have been expressed as a percentage of the total number of cells. $n = 3$ (3 separate experiments); error bars indicate SEM. * $P < 0.05$, different from cells subject to serum and depolarization withdrawal alone as determined using a one-way ANOVA and Tukey's multiple comparison test. C, The ILK inhibitor QLT-0267 inhibited insulin-promoted survival in R28 cells. A TUNEL assay was performed in differentiated R28 cells subject to serum withdrawal in the absence or presence of 10 nM insulin (In) alone or with increasing concentrations of QLT-0267 (In + 5, 10 and 12.5 μ M QLT). $n = 3-4$ separate experiments; error bars indicate SEM. * $P < 0.05$, different from cells subject to serum withdrawal alone. ** $P < 0.05$, different from cells treated with insulin alone. Statistical comparison was performed by a one-way ANOVA followed by Tukey's multiple comparison test.

decrease in the levels of the pro-form of caspase-3 (pro-caspase-3) which would occur following cleavage (Fig. 2D). A representative Western blot of R28 cell lysates probed with the pro-form of caspase-3 antibody (Fig. 2D Top) indicate that there was a decrease in the pro-form of the caspase-3 following serum starvation (-Serum), an effect that was partially rescued by 10 nM insulin (In). The ILK inhibitor QLT-0267 (QLT) significantly decreased insulin-induced pro-caspase-3 levels indicating that ILK inhibition increased caspase-3 activity. GAPDH served as a loading control. Bar graph (Fig. 2D Bottom) represents densitometric analysis of blots probed for pro-caspase-3 or GAPDH and expressed normalized to control (cells maintained in serum). Data are mean $\pm$ SEM of 6-7 independent trials. * $P < 0.05$, different from the serum starved group and cells treated with insulin together with QLT-0267 as determined by a Student's t-test.

2.2. Insulin stimulates AKT via ILK in primary neurons

Insulin and ILK have both been shown to regulate AKT activity in a wide variety of cell types including neurons [19, 23, 24, 25, 26, 27, 38, 55]. Whether or not insulin activates AKT via ILK is unknown. Using Western blot analysis, we characterized insulin-stimulation of AKT phosphorylation in cerebellar cultures plated on laminin. We found that insulin stimulated AKT Ser 473 phosphorylation in a time-dependent manner over a 120 min exposure period (Fig. 3A). The increased phosphorylation observed at 120 min (relative to 0 min) was inhibited in the presence of adenovirus-mediated overexpression of dominant negative AKT (AKT-DN) (compare lanes 2 and lanes 6; Fig. 3A). Actin loading controls indicate that the overexpression of AKT-DN did not affect protein expression overall. A recombinant AKT protein (rAKT) was loaded as a positive control for AKT phosphorylation using Western blotting. In order to determine the importance of ILK in insulin-induced AKT phosphorylation, ILK was excised from ILKfl/fl cerebellar cultures by AdCre prior to insulin exposure. Four days post-infection cells were serum starved for 12 h and then exposed to serum depolarization withdrawal with or without insulin for 15 min. Lysates were run and Western blots probed with an antibody to phospho-AKT Ser-473, AKT or ILK. Western blots probed with an antibody to ILK indicate that ILK protein levels were substantially reduced following AdCre infection (Fig. 3B). Western blots probed with an antibody to phospho-AKT Ser-473 indicate that



insulin-induced stimulation of AKT Ser-473 phosphorylation was reduced in cells concomitant with a reduction of ILK expression (Fig. 3B). AKT loading controls indicate that AdCre infection reduced ILK expression levels selectively as the AdCre virus did not alter AKT levels. Bar graph represents densitometric analysis of blots from AdCre excised cultures normalized to the nonexcised cultures treated in the same manner. Data are mean $\pm$ SEM. * $P < 0.05$, different from serum withdrawal treatment group as determined by a Student's t-test. In R28 cells, insulin induced a marked increase in AKT phosphorylation in 15 min and this was significantly inhibited by the ILK inhibitor QLT-0267 (Fig. 3C). Insulin resulted in a prominent increase in AKT Ser-473 phosphorylation above serum withdrawal, an effect that was significantly inhibited by 10 μ M QLT-0267. AKT served as a loading control. Bar graph represents densitometric analysis of blots probed for AKT Ser-473 or AKT. Protein

Fig. 2. Role of Caspase in ILK-Mediated Survival Signaling A, In cerebellar granule neurons inhibition of insulin-promoted survival by KP-392 is rescued by a pan caspase inhibitor BAF. A TUNEL assay was performed in cerebellar neurons plated onto laminin and exposed to serum or serum and depolarization withdrawal alone (withdrawal) or in the presence of insulin (10 μ g/ml) with or without KP-392 (100 μ M). In addition, BAF (100 μ M) was added to cells exposed to insulin (10 μ g/ml) and KP-392 (100 μ M). Representative fluorescence photomicrographs of cells labeled for TUNEL (right panel) and DAPI (left panel) for each treatment group are shown. Bar represents 100 μ m. B, Bar graph represents apoptotic cells, expressed as a percentage of the total number of cells for each treatment group, $n = 3-4$ separate culture platings. * $P < 0.05$, different from cells subject to serum and depolarization withdrawal alone and different from insulin with KP-392 as determined by a one-way ANOVA and Tukey's multiple comparison test. C, Insulin and IGF-1 prevent activation of caspase-3 by an ILK-dependent pathway. Cerebellar granule neurons plated onto laminin were exposed to serum (+Serum) or subject to serum and depolarization withdrawal for 8 h (Withdrawal) in the presence of insulin (In) or IGF-1 alone or after the addition of 100 μ M KP-392 (KP-392). Activated caspase-3 was detected by immunofluorescent cytochemistry with the polyclonal antibody CM-1. $n = 3$ (separate culture platings); error bars indicate SEM. * $P < 0.05$, different from cells treated with the same first messenger together with KP-392 as determined by a Student's t-test. D, Insulin-prevented procaspase-3 cleavage an effect that was inhibited by QLT-0267. Differentiated R28 cells were serum starved overnight and exposed to media containing 10% serum (+Serum) or media lacking serum alone (-Serum), or with 10 nM insulin (In) with or without 10 μ M QLT-0267 (QLT) for one day. Top, Representative Western blot of R28 cell lysates probed with an antibody for the pro-form of caspase-3 (pro-caspase-3) indicate that there was a significant decrease in the pro-form of caspase-3 following serum starvation and this was partially rescued by insulin. The ILK inhibitor QLT-0267 significantly decreased insulin-induced pro-caspase-3 levels. GAPDH served as a loading control. Bottom, Bar graph represents densitometric analysis of blots probed for pro-caspase-3 or GAPDH and expressed as % C ($n = 6-7$ independent trials). Data are mean $\pm$ SEM. * $P < 0.05$, different from the serum starved group and different from cells treated with the same first messenger together with QLT-0267 as determined by a Student's t-test. Full, non-adjusted images are not available as supplementary material for Fig. 2.

expression for AKT Ser-473 or AKT was averaged and normalized to control ($n = 3$ independent platings). Data are mean $\pm$ SEM. * $P < 0.05$, different from all other treatment groups. Statistical comparison was performed using a one-way analysis of variance and Tukey's multiple comparison test.

In nonneuronal cells, exposure to insulin has already been shown to result in a transient stimulation of ILK activity [19]. To determine whether insulin stimulates ILK in neurons, cerebellar cultures were stimulated with insulin and ILK kinase activity was measured using either AKT or glycogen synthase kinase 3 (GSK-3) as a substrate. In cerebellar cultures plated on laminin, insulin stimulated ILK activity (as determined by phosphorylation of GSK-3) and this occurred within 15 min (Fig. 4A). Bar graph represents densitometric analysis of 3 independent trials, averaged and normalized to control. Data are mean $\pm$ SEM. * $P < 0.05$,

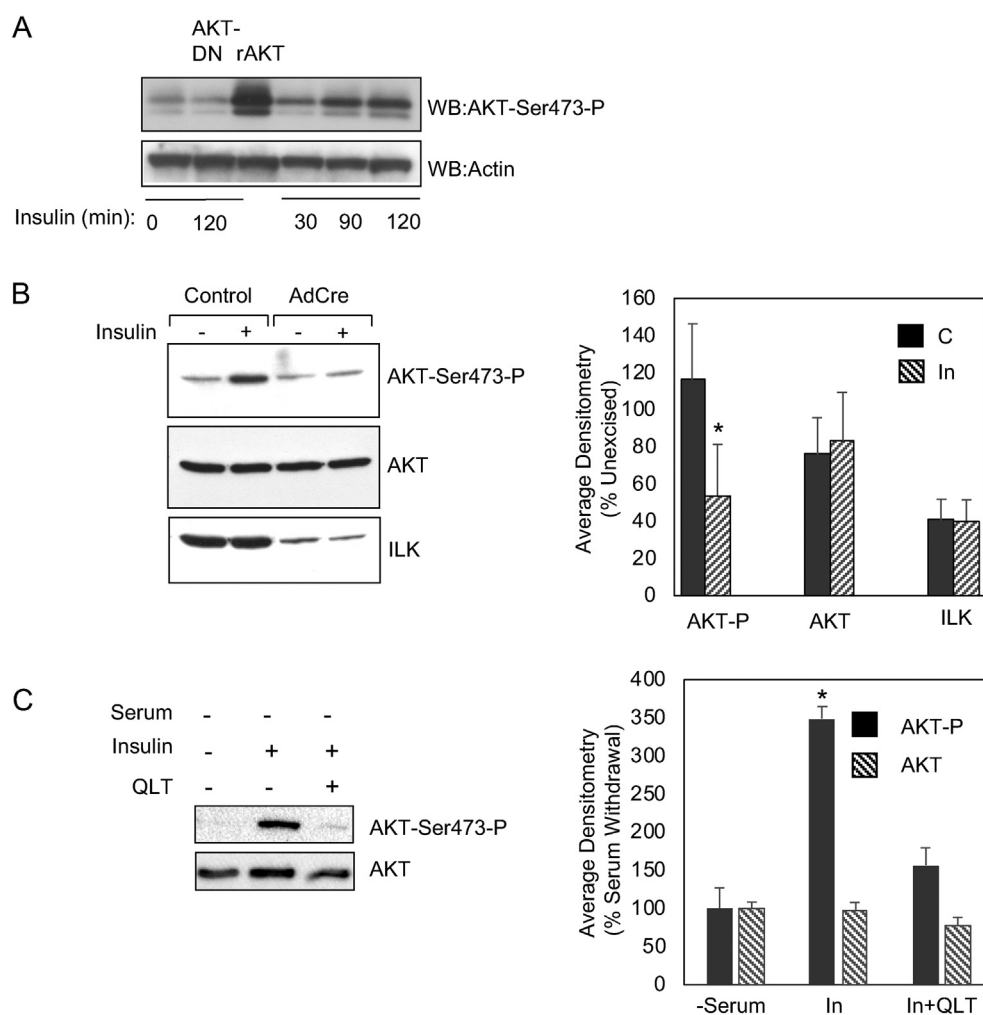


Fig. 3. Insulin-Induced Stimulation of AKT Occurs in an ILK-Dependent Manner. A, Insulin increases phosphorylation of AKT Ser-473: Effect of AKT-DN overexpression in cerebellar granule neurons. Western blots of cell lysates from cerebellar granule neurons grown on laminin and incubated with or without insulin for 30–120 min indicate that insulin markedly increased AKT Ser-473 phosphorylation during this time span. The increased phosphorylation observed at 120 min was significantly inhibited in the presence of adenovirus-mediated overexpression of dominant negative AKT (AKT-DN). A recombinant AKT protein (rAKT) was loaded as a positive control for AKT phosphorylation and actin served as a loading control. Phosphorylation of AKT1 (upper band) and AKT2 (lower band) was detected. B, Insulin increases phosphorylation of AKT Ser-473 in cerebellar granule cells: Effect of ILK excision. Cerebellar granule neurons generated from *ilK^{loxP/loxP}* mice were infected with an AdCre virus. Four days post-infection cells were serum starved for 12 h and then exposed to insulin for 15 min. Lysates were run and Western blots probed with an antibody to phospho-AKT Ser-473, AKT or ILK. Top, Representative Western blot of cell lysates probed with an antibody for phospho-AKT Ser-473 indicate that insulin stimulation of AKT phosphorylation was reduced in cells where ILK was excised. Middle, Membranes were also probed for AKT as a control for protein loading. Bottom, Western blots probed with an antibody for ILK confirm that AdCre infection resulted in a marked reduction in ILK expression levels. Bar graph represents densitometric analysis of lysates from ILK excised cultures expressed as a percentage nonexcised cultures. Data are mean $\pm$ SEM. * $P < 0.05$, different from serum withdrawal treatment group as determined by a Student's t-test. C, Insulin increased AKT Ser-473 phosphorylation in differentiated R28 cells, an effect that was significantly inhibited by the ILK inhibitor QLT-0267. R28 cells were differentiated for 24 h and then serum starved overnight before being exposed to serum free media alone or containing insulin with or without 10 μ M QLT-0267 (the percentage of DMSO drug vehicle was the same for all treatment groups). A 15 min exposure to insulin resulted in a prominent increase in AKT Ser-473 phosphorylation above serum withdrawal, an effect that was significantly inhibited by 10 μ M QLT-0267. Bar graph represents densitometric analysis of blots probed for AKT Ser-473 or AKT (AKT served as a loading control). Protein expression was averaged and normalized to control ($n = 3$ independent platings). Data are mean $\pm$ SEM. * $P < 0.05$, different from all other treatment groups. Statistical comparison was performed using a one-way ANOVA and Tukey's multiple comparison test. Full, non-adjusted images are available as supplementary material for Fig. 3C only (see Fig. 3C Supplementary).

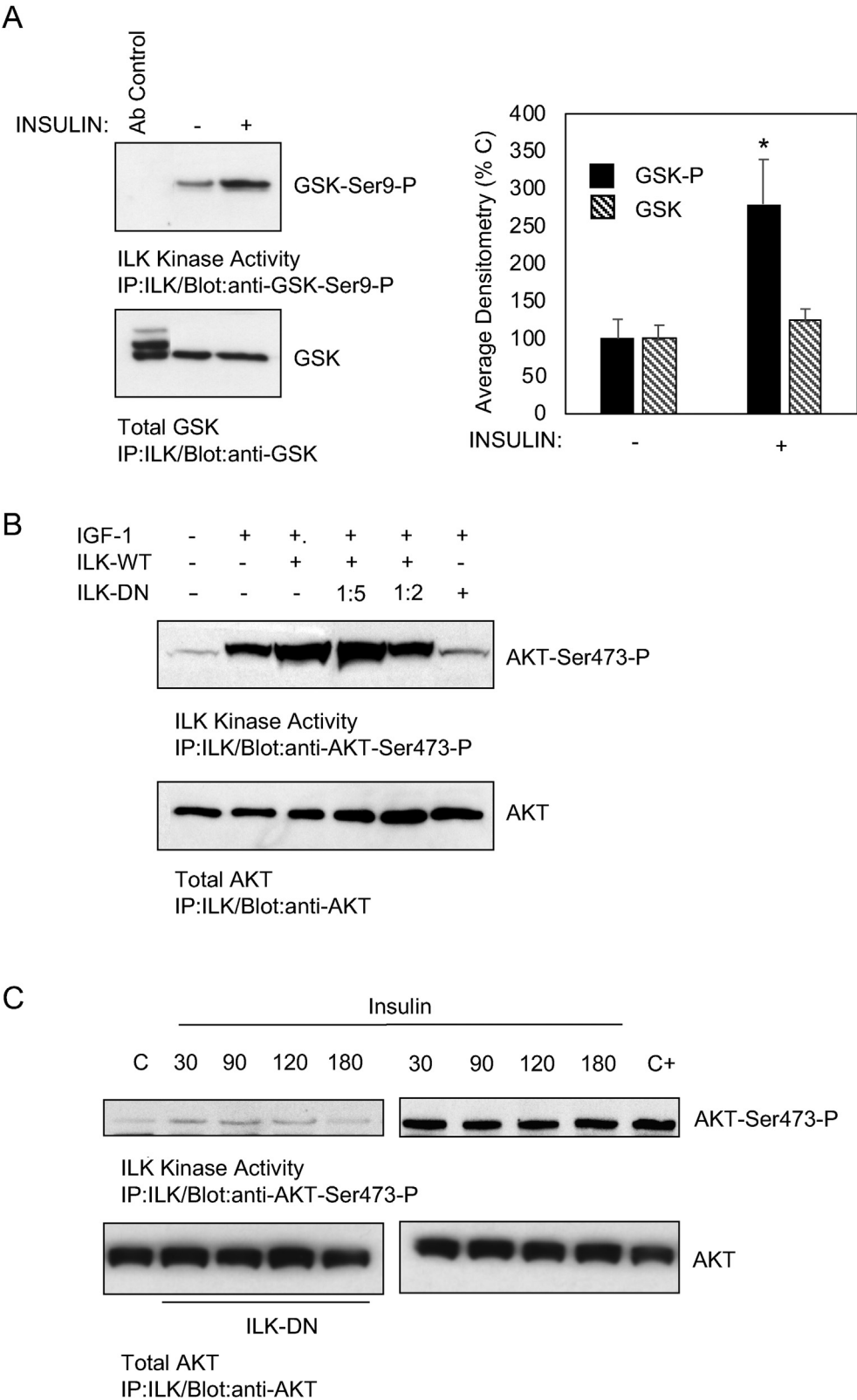


Fig. 4. Insulin and IGF-1 Stimulate ILK Kinase Activity. A, Insulin stimulates ILK kinase activity in cerebellar granule neurons. ILK kinase activity was measured in cells plated onto laminin after a 15 min exposure to insulin. The kinase assay was performed using a GSK-3 fusion protein (New England Biolabs) as a substrate. Phosphorylation of the substrate by ILK was detected using a phosphospecific anti-GSK-3 β (New England Biolabs) antibody. Bar graph represents densitometric analysis of 3 independent trials, averaged and normalized to control. Data are mean $\pm$ SEM. *P < 0.05, different from serum withdrawal treatment group as determined by a Student's t-test. B, IGF-1 stimulation of AKT Ser-473 phosphorylation: effect of ILK-DN and ILK-WT overexpression in primary cultures of rat cerebrocortical neurons. Cerebrocortical cultures were exposed to IGF-1 (100 ng/ml) for 20 min and subsequently harvested for kinase assays. Top, ILK *in vitro* kinase activity. IGF-1 increased ILK phosphorylation of AKT Ser-473. Growth factor stimulation of AKT phosphorylation was inhibited after overexpression of ILK-DN. Competition of ILK-WT was observed with increasing concentrations of ILK-DN (the ratio of ILK-WT:ILK-DN is indicated above). Bottom, Immunoblots were stripped and subsequently reprobed with an anti-AKT antibody as a control for His-tagged, AKT fusion protein loading. C, ILK-DN inhibits insulin stimulation of AKT Ser-473 phosphorylation. ILK *in vitro* kinase activity was measured in cerebellar cultures grown on laminin and transfected with ILK-DN prior to insulin exposure. Insulin stimulation of AKT phosphorylation was inhibited after overexpression of ILK-DN. An N-terminus, His-tagged, AKT fusion protein was used as a substrate. The addition of a recombinant AKT protein (+C) was loaded as a positive control for AKT phosphorylation. Full, non-adjusted images are not available as supplementary material for Fig. 4.

different from serum withdrawal treatment group as determined by a Student's t-test. Related first messengers such as IGF-1, that belong to the insulin/IGF-1 hormone family have been shown to inhibit apoptosis in primary neuronal models such as cerebrocortical cultures and this effect is mediated by PI 3-kinase and AKT [56]. To determine whether or not ILK is stimulated by IGF-1 in this neuronal model we examined ILK kinase

activity following a short-term exposure to IGF-1. In cerebrocortical cultures, a 20-minute IGF-1 (100 ng/ml) exposure resulted in a rapid stimulation of ILK kinase activity (Fig. 4B). IGF-1-induced stimulation was enhanced by ILK-WT overexpression and inhibited following overexpression of ILK-DN (Fig. 4B). Furthermore, competition of ILK-WT was observed with increasing concentrations of ILK-DN (the ratio of

ILK-WT:ILK-DN is indicated; see Fig. 4B). A time course measuring insulin-induced ILK kinase activity in cerebellar granule neurons was also performed using AKT as a substrate (Fig. 4C). Insulin-induced a prolonged stimulation of ILK activity that remained elevated between 30 and 180 min (Fig. 4C). Overexpression of ILK-DN significantly decreased insulin-mediated stimulation of AKT. A recombinant AKT protein (+C) was loaded as a positive control for AKT phosphorylation via Western blotting.

2.3. Insulin upregulated IAP expression via ILK

Members of the inhibitor of apoptosis family (IAP) are natural anti-apoptotic proteins have been shown to inhibit apoptosis induced by serum and depolarization withdrawal [55]. Extracellular matrices and integrins that regulate ILK have also been shown to increase IAP expression and survival of cells [38, 57, 58, 59]. To determine whether upregulation of IAP is a mechanism underlying insulin-induced neuroprotection, we examined insulin regulation of cellular IAP2 (c-IAP2) expression using Western blotting. c-IAP2 expression in cell lysates of cerebellar granule neurons plated on the extracellular matrix laminin or poly-D-lysine were subject to serum and depolarization withdrawal alone or in the presence of insulin (10 µg/ml). Western blots of cell lysates were immunoblotted with an antibody to c-IAP2 (also called human IAP1 and abbreviated HIAP1). Insulin increased c-IAP2 expression only in cells plated on laminin and KP-392 significantly blocked this effect (Fig. 5). Bar graph represents densitometric analysis of 3 independent trials,

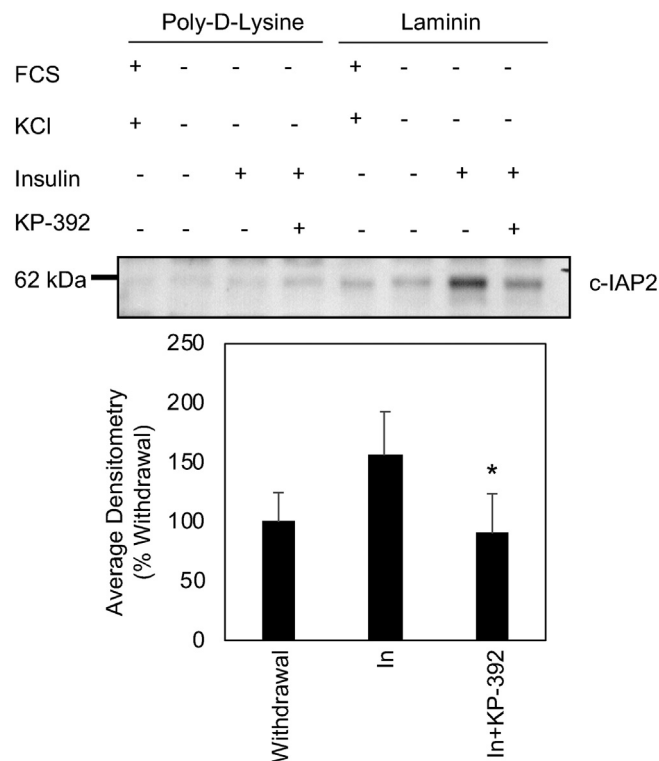


Fig. 5. Insulin increases IAP expression in an ECM-dependent manner in Cerebellar Granule Neurons: Inhibition by the ILK Inhibitor KP-392. Representative Western blots of lysates from cerebellar cultures grown on poly-D-lysine or laminin and exposed to serum and depolarization or serum and depolarization withdrawal alone or in the presence of insulin (10 µg/ml) with or without KP-392 (100 µM) for 24 h (n = 2–3). Immunoblots were probed with an antibody against c-IAP2. Bar graph represents densitometric analysis of 3 independent trials of cells grown on laminin, averaged and normalized to cultures subject to serum and depolarization withdrawal. Data are mean ± SEM. *P < 0.05, different from insulin treated group as determined by a Student's t-test. Full, non-adjusted images are not available as supplementary material for Fig. 5.

averaged and normalized to cultures grown on laminin and subject to serum and depolarization withdrawal. Data are mean ± SEM. *P < 0.05, different from insulin treated group as determined by a Student's t-test. Effects of other IAP family members such as endogenous X-linked IAP (XIAP) were examined however, insulin did not increase its expression (J. Tan and J. Mills, unpublished observations, n = 2).

3. Discussion

3.1. ILK and insulin survival signaling

In neuronal cells, anti-apoptotic effects of insulin have been shown to be mediated by a PI 3-kinase/AKT-dependent signaling pathway that lead to the inhibition of both GSK-3 [29,60] and caspase activity [55, 61, 62, 63, 64]. Although ILK is activated by insulin in nonneuronal cells [19] and regulates AKT, GSK-3 and caspases in other signaling pathways [22, 38, 65, 66], to date, a role for ILK in the neuroprotective effects of insulin has not been studied. We studied insulin-induced neuroprotection in cerebellar granule neurons and in R28 cells, a retinal cell line. We used the serum and depolarization withdrawal model for cerebellar neurons and serum withdrawal for differentiated R28 cells, as these models have been preferred for studying insulin survival signaling via an AKT-dependent mechanism. Insulin-mediated neuroprotection was compromised by the excision of ILK (Fig. 1) and by inhibiting its activity with the ILK inhibitors KP-392 or QLT-0267 [20,21,23,24,27,41,51,52] cells (Figs. 1 and 2). Collectively, these data indicate that ILK has an important role in the neuroprotective effects of insulin.

As ILK downregulation has been shown to activate caspase-dependent proapoptotic pathways in nonneuronal cells, we also determined whether or not caspase-3 activity was increased when ILK was down-regulated in insulin-induced neuroprotective pathways. The involvement of caspases was first suggested by rescue experiments using Boc-Asp (O-methyl)-CH₂F (BAF), a pancaspase inhibitor [53]. Specifically, in cerebellar neurons, the ILK inhibitor KP-392 antagonized insulin-induced neuroprotection while the protective effect of insulin was rescued by a Boc-Asp (O-methyl)-CH₂F (BAF), a pancaspase inhibitor [53] (Fig. 2A,B). Given that BAF, a pancaspase inhibitor, rescues neurons from death caused by ILK inhibition suggests that caspases may be a downstream target in this survival pathway. Caspase-3 was examined because it is a crucial effector of neuronal cell death induced by serum and depolarization withdrawal [55, 62, 64, 67] and is regulated by both ILK and insulin in other cell paradigms [8, 22, 32]. To determine whether caspase-3 is a target in this survival pathway we measured caspase-3 activity in the presence of insulin with or without the ILK inhibitors KP-392 or QLT-0267. Neuronal cells exposed to serum and depolarization withdrawal were highly immunoreactive for active caspase-3. Insulin (10 µg/ml) and IGF-1 (25 ng/ml) reduced the number immunoreactive cells, an effect that was antagonized in the presence of KP-392 (Fig. 2C). Likewise, in differentiated R28 cells insulin decreased pro-caspase-3 cleavage in serum free conditions and this effect was blocked by QLT-0267 (Fig. 2D). Together these experiments indicate that insulin-induced survival pathways involve ILK and ILK-dependent downregulation of caspase-3 activity.

ILK is an important regulator of AKT activity [19, 23, 24, 25, 26] a kinase well characterized for its role in insulin-induced neuroprotection [6]. AKT is activated through phosphorylation at two major sites, Thr-308 in the kinase domain and Ser-473 in the carboxy terminal tail. These AKT sites are phosphorylated by two different kinases: PDK-1 phosphorylates AKT at Thr-308 while ILK phosphorylates AKT at Ser-473 [12,14,17,19,21–24]. In neuronal cells, a role for ILK has been suggested in laminin- and NGF-induced AKT signaling [27, 38]. We found that in cerebellar cultures plated on laminin, stimulation of AKT phosphorylation increased following exposure to insulin for 30, 90 and 120 min relative to control (Fig. 3A). Stimulation of AKT-473 phosphorylation at 120 min was antagonized when dominant negative AKT (AKT-DN) was overexpressed in cells using an adenoviral construct [68].

Likewise, increased AKT-473 phosphorylation was observed in cerebellar granule neurons cultured from wild-type and *Ilk^{loxP/loxP}* mice following a 15 min exposure to insulin but was reduced after ILK was excised following an adenoCre virus infection (Fig. 3B). Bar graph represents densitometric analysis of lysates from ILK excised cultures (n = 3–5 separate platings) expressed as a percentage of nonexcised cultures. Data are mean $\pm$ SEM. *P < 0.05, different from serum withdrawal treatment group as determined by a Student's t-test. Insulin's effect on AKT-473 phosphorylation was also determined in a serum withdrawal model using differentiated R28 cells. R28 cells were differentiated for 24 h and then serum starved overnight before being exposed to serum free media alone or containing 10 nM insulin with or without 10 μ M QLT-0267. In differentiated R28 cells, insulin induced phosphorylation of AKT-473 following a 15 min exposure and this effect was significantly inhibited by the ILK inhibitor QLT-0267. AKT levels did not change significantly with the various treatments. Bar graph represents densitometric analysis of blots probed for AKT Ser-473 or AKT, taken from 3 independent trials that were averaged and normalized to control. Data are mean $\pm$ SEM. *P < 0.05, different from all other treatment groups. Statistical comparison was performed using a one-way ANOVA and Tukey's multiple comparison test.

This change in AKT activation by insulin is likely a direct effect of ILK, as insulin directly stimulates ILK activity. Indeed ILK stimulation by insulin and IGF-1 occurred within minutes in primary neurons (Fig. 4A,B). A time course measuring ILK kinase activity using AKT as a substrate revealed that insulin induced a prolonged stimulation of ILK activity and this effect was inhibited following overexpression of ILK-DN (Fig. 4C). The importance of ILK's kinase activity in insulin-induced neuronal cell signaling was also indicated using a small molecule pharmacological inhibitor of ILK, QLT-0267. In R28 cells, insulin-induced stimulation of AKT phosphorylation was inhibited by QLT-0267 (Fig. 4D). Together these studies indicate that an insulin-induced signaling pathway regulates AKT activation in an ILK-dependent manner.

Members of the inhibitor of apoptosis family (IAP) are natural anti-apoptotic proteins that inhibit caspases. Members of this family have been shown to inhibit apoptosis induced by serum and depolarization withdrawal [55]. In cerebellar neuronal cultures, insulin increased cellular IAP2 (c-IAP2 or HIAP1) expression only in cells plated on laminin (not poly-D-lysine) and KP-392 blocked this effect (Fig. 5). As c-IAP2 is known to inhibit caspase-3, upregulation of c-IAP2 may underlie a mechanism whereby insulin via ILK inhibits caspase-3 activity. Exactly how c-IAP2 expression is upregulated is beyond the scope of the present study but one worthy of further pursuit.

3.2. Multiple roles for ILK

ILK has been shown to be an upstream regulator of AKT following insulin- and NGF-mediated activation [19, 27]. Why then does inhibition of ILK inhibit insulin-induced survival of neuronal cells but does not inhibit NGF-induced survival of PC12 cells? Firstly, signaling pathways activated by NGF differ from those activated by insulin. In neuronal cells, although GSK-3 β has been shown to be critical for strictly PI 3-kinase/AKT-dependent survival signaling, GSK-3 β is not critical for NGF-induced survival [69]. Secondly, NGF-induced survival pathways may include redundant survival mechanisms not affected by ILK. For example, although the Ras-MAPK pathway is involved in NGF-induced survival, MAPK is not activated by insulin and therefore not involved in survival of cerebellar granule neurons [9]. In PC12 cells, inhibition of ILK did not decrease NGF stimulation of MAPK phosphorylation [27]. Therefore, the Ras-MAPK pathway may represent a mechanism whereby NGF promotes survival even when ILK signaling is compromised. Redundancy may also explain why ILK loss did not significantly decrease survival in the presence of serum (Fig. 1A). Thirdly, the downstream targets of these various survival pathways differ depending on the pro-survival factor. Therefore, the first messenger may ultimately determine the outcome of ILK inhibition. For example, in noneuronal cells,

GSK-3 β -mediated phosphorylation of insulin receptor substrate 1 (IRS-1) has been shown to inhibit insulin signaling [61]. Stimulation of GSK-3 β activity by ILK inhibition may lead to phosphorylation of insulin receptor substrate 1 (IRS-1) and inhibition of insulin signaling in cerebellar neurons. However, a role for IRS-1 in NGF signaling has not been reported [69]. Finally, in addition to AKT and GSK-3 β the list of ILK targets is still growing and include β -parvin, the 20 kDa regulatory light chains of myosin LC₂₀, the myosin targeting subunit of myosin light chain phosphatase, MYPT1 protein phosphatase inhibitors and α -NAC [70, 71, 72, 73, 74, 75, 76] making it difficult to predict the precise effects of ILK inhibition in different survival signaling pathways (for reviews see [35, 77]).

In conclusion, our study reveals an important role for ILK in the neuroprotective effects of insulin and other first messengers belonging to the insulin/IGF-1 hormone family. Insulin's stimulation of this ILK-mediated survival pathway was shown to regulate other survival kinases such as AKT and GSK-3 and to inhibit caspase-3 activity. The ILK-dependent effect of insulin on c-IAP2 expression indicate that this might be a mechanism whereby insulin via ILK inhibits caspase-3 activity. Insulin's ability to signal through ILK to protect neurons against death induced by trophic withdrawal extends our knowledge of the neuroprotective function of ILK and supports a role for ILK in the metabolic function of neurons.

4. Materials and methods

4.1. ILK inhibitors

KP-392 (also known as KP-SD-1; Valocor Therapeutics, Vancouver, BC, CA) was identified in a high-throughput kinase assay using highly purified recombinant ILK. KP-392 has been characterized previously and its specificity been determined by analyzing its effect on 150 protein kinases in vitro [21, 23, 24, 27, 41, 42, 43]. KP-392 was used in our earlier experiments that were performed when QLT-0267 (Valocor Therapeutics, Inc.) was not readily accessible. QLT-0267 is a second-generation ILK inhibitor, derived from KP-392. It has a similar selectivity profile to KP-392 but is more potent [45]. QLT-0267 has been characterized elsewhere and has also been shown to be highly selective when compared to 150 other kinases [23, 78].

4.2. Cell culture and drug exposure

Adult male and female C57 black 6 mice (Charles River) were bred in-house at the Jack Bell Research Centre, UBC. Mice were housed in a vivarium maintained on a standard 12 h light-dark cycle and given unrestricted access to food and water. Mice pups at 4–5 days were euthanized using 5% isoflurane/CO₂. The UBC Animal Care Committee approved this study (animal care certificate # A02-0295). Cultures of cerebellar granule cells were prepared from the cerebella of postnatal day 4–5 mice using a protocol that has previously been described for cultured rat granule neurons [79]. Granule neurons were plated onto either poly-D-lysine (100 μ g/ml) or laminin coated dishes (5 μ g/ml) dishes or onto coated coverslips in MEM containing 10% serum, 25 mM KCl, 0.5% glucose and 0.2 mM glutamine and allowed to adhere overnight as previously described [9, 27, 80]. Within 16 h of plating, serum containing media was replaced with a Neurobasal media (Thermo Fisher Scientific, CA, USA) supplemented with B-27 (Thermo Fisher Scientific, CA, USA) and 25 mM KCl. Cells were allowed to differentiate for 6–7 days *in vitro* as previously described [27, 80]. Cerebellar mouse cultures were used for the serum and depolarization withdrawal experiments in order to induce apoptosis. Media was removed prior to drug treatment and drug exposure was performed in MEM lacking serum and 25 mM KCl but containing 0.5% glucose and 0.2 mM glutamine (serum and depolarization controls contained serum and 25 mM KCl). Drug exposure media also contained 0.1 mg/ml bovine serum albumin as a carrier protein as previously described [9]. For drug exposures ($\leq$ 8 h) using cerebellar primary

neurons, cultures were preincubated with the selective ILK inhibitor KP-392 [21,24,27,41,42] for 1 h; for long-term drug exposures, a preincubation with KP-392 was not performed. Stock KP-392 was suspended in DMSO and stored at -70 °C. An equivalent amount of drug vehicle was added to all controls during drug exposures. Cerebellar cultures assessed for caspase-3 immunoreactivity, were subject to serum and depolarization withdrawal for 8 h in the presence of insulin (10 µg/ml) or IGF-1 (25 ng/ml) after the addition of KP-392 (100 µM; cells were preincubated with KP-392 for 1 h). Cerebrocortical cultures were prepared from embryonic day 16 Sprague Dawley rats as previously described [81]. Cerebrocortical cultures were serum-starved 16 h before drug addition, exposed to IGF-1 (100 ng/ml) for 20 min in Earles balanced salt solution and subsequently harvested for kinase assays. R28 cells were grown in DMEM containing 10% FCS supplemented with 1 µg/ml puromycin (R28 cells were provided by Gail M. Seigel) [8]. R28 cells were plated onto coated coverslips and differentiated for one day in complete media containing 250 µM cell permeable pCPT-cyclic AMP using a previously described protocol with minor modifications [8]. Differentiated R28 cells were used for the serum withdrawal model to induce apoptosis. R28 cells were serum starved overnight in media containing 250 µM cell permeable pCPT-cyclic AMP and exposed to drug treatments for 24 h for TUNEL labelling or for 15 min for phospho-AKT Ser-473 analysis using Western blotting. For 15 min drug exposures, R28 cells were preexposed to the QLT-0267 for 4 h prior to drug exposure. Stock QLT-0267 was suspended in DMSO at a concentration of 25 mM and stored at -70 °C. Cells were exposed to QLT-0267 (or vehicle control; vehicle control did not exceed 0.04%).

4.3. Excision of the floxed ILK gene and adenovirus infection

Primary cerebellar neuronal cultures from *ilk^{loxP/loxP}* mice were infected with CRE recombinase adenovirus (AdCre) at 2 days after plating as previously described [26]. Conditioned media of cells plated on laminin was removed and cells were incubated with virus in OptiMEM for 4 h at 37 °C. The conditioned media was then added back and cells were incubated for 4 days. ILK protein expression was evaluated by Western blotting (as described below). ILK-DN [19] and AKT-DN was transiently introduced in cultures 24 h before experimentation via an adenoviral construct as previously described [68]. The ILK-DN was derived by site-directed mutagenesis containing a E359 → K mutation within the ILK kinase domain as described [82] and the AKT-DN (T308A, S473A) cannot be activated by phosphorylation and functions in a dominant negative manner as previously described [83].

4.4. Immunocytochemistry

Cultured mouse cerebellar neurons were assessed for caspase-3 immunoreactivity after serum and depolarization withdrawal. These cultured mouse cells were then fixed in 4 % paraformaldehyde and incubated with a rabbit polyclonal antibody against activated caspase-3 (CM-1, Idun Pharmaceuticals, CA, USA) at a dilution of 1:1000 [4]. CM-1 was detected with an anti-rabbit Texas Red-conjugated secondary antibody (Vector Laboratories).

4.5. Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end (TUNEL) labeling and propidium iodide staining

After a 24 h drug treatment, DNA fragmentation was measured in neuronal cells using the In Situ Cell Death Detection Kit (Fluorescein; Boehringer Mannheim Biochemicals, IN, USA) as previously described [84]. Cells were then washed in PBS and mounted in Vectashield mounting media containing DAPI (Vector Laboratories, Burlingame, CA, USA). For propidium iodide staining, cells were incubated in MEM containing propidium iodide (100 µg/ml) and Hoechst 33342 (50 µg/ml) for 15 min at room temperature, washed with phosphate buffered saline (PBS), and fixed with 4% paraformaldehyde. The number of

TUNEL-positive or propidium iodide-positive cells was calculated as a percentage of total cell number (DAPI or Hoechst-stained cells, respectively). For each trial, TUNEL-positive or propidium iodide-positive cells in each treatment group were manually counted from 4-5, randomly obtained fields of view (200x magnification). All data is representative of 3 or more independent culture platings.

4.6. Kinase assay and immunoprecipitations

For kinase assays using GSK-3 as a substrate, primary cerebellar cultures were serum starved for 16 h in exposure media and then insulin was added for 15 min. Cells were then washed in PBS and harvested in ice-cold lysis buffer as previously described [27]. Precleared lysates were immunoprecipitated for ILK and an ILK immunoprecipitation kinase assay was performed as previously described [20, 27]. In these cerebellar cultures ILK was immunoprecipitated with a monoclonal anti-ILK antibody (Upstate Biotechnology, Lake Placid, NY) and a GSK-3 fusion protein (New England Biolabs, Whitby, ON, CA) was used as a substrate [20, 27]. Alternatively, in cerebrocortical and cerebellar cultures using AKT as a substrate, ILK was immunoprecipitated with an anti-ILK antibody and an N terminus, His-tagged fusion protein corresponding to human AKT (Upstate Biotechnology, Lake Placid, NY) was performed using a previously published protocol [27, 68]. Basal substrate phosphorylation of the His-tagged fusion protein human AKT was reduced prior to kinase assays by incubating the fusion protein with shrimp alkaline phosphatase. This dephosphorylation step was omitted when the AKT fusion protein was used as a positive control for phospho-AKT. After the kinase assay, anti-ILK immune complexes were subject to Western blot analysis. Phosphorylation of the substrate was measured using either a rabbit anti-phospho AKT Ser-473 antibody (New England Biolabs, Whitby, ON, CA) or a rabbit anti-phospho GSK-3 Ser-21/9 antibody (New England Biolabs, Whitby, ON, CA).

4.7. Western blotting

Lysates from primary cultures were prepared in Tris-HCl buffer, pH 7.6 containing 1% NP-40, 150 mM NaCl, 1mM EDTA, 3.8 µg/ml aprotinin, 1 µg/ml leupeptin, 1 mM PMSF, 2 mM NaF, and 1 mM Na₃VO₄ as previously described [27]. c-IAP2 was detected using a rabbit anti-c-IAP2 antibody (a kind gift from Dr. Robert Korneluk, Aegera Therapeutics Inc., QC, CA), ILK was detected using a monoclonal anti-ILK antibody (BD Transduction Laboratories, San Jose, CA), caspase was detected using a monoclonal antibody caspase-3 p11 (Santa Cruz, ON, CA) and GAPDH was detected using a rabbit anti-GAPDH (Santa Cruz, ON, CA). Details regarding these and other antibodies have been previously described [27, 85].

4.8. Statistical analysis

Data depicted in the graphs represent the mean ± S.E.M. of results. Unless otherwise stated graphs or Western blots are representative of 3 or more independent trials (independent cell culture platings). The minimum level of statistical significance was set at $\alpha = 0.05$ unless otherwise stated. Inter-group comparisons were made with a paired *t*-test or analysis of variance and either the Tukey's multiple comparison test or the Newman-Keul post hoc test. Statistical tests were performed using XLSTAT, a statistical software package and data add-on for Excel.

Declarations

Author contribution statement

Jacqueline Tan, Stacy X.J. Wang: Performed the experiments; Analyzed and interpreted the data.

Murat Digicaylioglu: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed

reagents, materials, analysis tools or data.

Jonathan Dresselhuis: Conceived and designed the experiments; Performed the experiments.

Shoukat Dedhar: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Julia Mills: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Funding statement

This work was supported by a grant to Julia Mills from the TWU Diabetes Endowment Fund and to Shoukat Dedhar from the National Cancer Institute of Canada and Canadian Institutes of Health Research which made this work possible. Students in Mills' lab were supported by the M.J. Murdock Charitable Trust Science Research Initiative Grant. The molecular and cell biology laboratory in which these studies took place were made possible by a grant from Murdock Trust as a match to the Industry Canada Knowledge Infrastructure Program Award.

Competing interest statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at <https://doi.org/10.1016/j.heliyon.2019.e02294>.

Acknowledgements

We would like to thank Dr. Gail Seigel for providing the R28 cell line used in this study.

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