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The effects of insulin and insulin-like growth factor I on amyloid precursor protein phosphorylation in in vitro and in vivo models of Alzheimer's disease

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Abstract

Alzheimer's disease (AD) is a growing problem worldwide, and there are currently no effective treatments for this devastating disease. The neurotrophic growth factors insulin and insulin-like growth factor-I (IGF-I) are currently being investigated as potential therapeutic approaches for AD in preclinical and clinical studies. However, given that the metabolic syndrome (MetS) and diabetes are risk factors for AD, it is unknown how associated insulin resistance (IR) in the brain may impact the effectiveness of these therapies for AD. In this report, we therefore investigated the mechanisms underlying the effects of insulin and IGF-I on AD-associated pathology in the context of IR, with particular emphasis on phosphorylation of amyloid precursor protein (APP), a key step in promoting amyloid plaque formation in AD. Both insulin and IGF-I decreased APP phosphorylation in cultured primary cortical neurons, supporting their therapeutic use in AD. Induction of IR blocked the beneficial effect of insulin and reduced the effect of IGF-I on APP dephosphorylation. These effects were mediated by the phosphatidylinositol 3- kinase (PI3-K)/ protein kinase B (Akt) pathway, as inhibition of this pathway during IR restored the effect of IGF-I on APP dephosphorylation. Finally, we explored the translational relevance of these results *in vivo* by demonstrating that high fat diet fed mice, a robust model of IR and MetS, exhibited the expected increased brain APP phosphorylation. Overall, these data suggest that the beneficial therapeutic effect of insulin and IGF-I on APP phosphorylation is negatively impacted by IR, and suggest that insulin and IGF-I alone may not be appropriate therapies for AD patients with IR, MetS, or diabetes.

Keywords

Alzheimer's disease; IGF-I; insulin; amyloid precursor protein; insulin resistance

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Introduction

Alzheimer's disease (AD) affects millions of people worldwide (Brookmeyer et al., 2018; Fiest et al., 2016); however, there are currently no effective disease modifying treatments for patients. The two pathological hallmarks of AD are toxic protein aggregates of amyloid beta (A β) peptides, and neurofibrillary tangles composed of hyperphosphorylated tau (Masters CL, 2015; Selkoe et al., 2012). A β peptides are generated via amyloidogenic processing of amyloid precursor protein (APP) by β - and γ -secretases (Kim and Feldman, 2012; Masters CL, 2015). In addition to proteolytic cleavage, the phosphorylation state of APP contributes to amyloid pathology. APP phosphorylation at the threonine 668 residue increases A β generation (Lee et al., 2003) and produces neurotoxic cytoplasmic fragments that promote tau hyperphosphorylation (Shin et al., 2007). Pathological changes appear in the brains of patients long before the onset of clinical symptoms of cognitive decline (Ballard et al.; Villemagne et al., 2013), and many therapies aimed at combating cognitive symptoms and AD pathology have failed clinical trials (Anderson et al., 2017; Gauthier et al., 2016). Why these therapies have failed is a matter of debate; however, it is theorized that starting treatment following the onset of cognitive symptoms may be too late to alter disease progression (Aisen et al., 2013; Anderson et al., 2017; Mehta et al., 2017).

Insulin and insulin-like growth factor-I (IGF-I) are neurotrophic factors currently being investigated as therapies for AD and cognitive decline (Ashpole et al., 2015; Avgerinos et al., 2018; Benedict et al., 2004; Claxton et al., 2015; Rajasekaret al., 2017; Reger et al., 2008; Trejo et al., 2007). In the brain, insulin binds its cognate receptor to activate downstream pathways (Gubbi et al., 2018; Kim and Feldman, 2012; van der Heide et al., 2006), resulting in pleiotropic neuroprotective effects (Kim and Feldman, 2015; van der Heide et al., 2006). Insulin signaling via activation of phosphatidylinositol 3-kinase (PI3-K)/protein kinase B (Akt) and extracellular signal regulated kinase (ERK) pathways improves learning and memory (Kim and Feldman, 2015; van der Heide et al., 2006), promotes neuronal growth, and increases neuronal survival (Kim and Feldman, 2012; Renault et al., 2009). IGF-I primarily signals through the IGF-I receptor (IGF-IR) to activate PI3-K/Akt and ERK pathways, promoting neuronal differentiation, survival, and synapse formation (Hodge et al., 2007; McGinley et al., 2016; Zaka et al., 2005). IGF-I regulates neurogenesis (Mir et al., 2017; Nieto-Estevez et al., 2016; Ziegler et al., 2015), and like insulin, mediates neuroprotection (Fernandez and Torres-Aleman, 2012). IGF-I is produced locally within the central nervous system (CNS), and both insulin and IGF-I can enter from the systemic circulation by crossing the blood brain barrier (Carro et al., 2000; Csajbók and Tamás, 2016; Fernandez et al., 1999; Fernandez and Torres-Aleman, 2012). The receptors and downstream signaling molecules for both factors are expressed throughout the periphery and CNS (Ashpole et al., 2015; Avgerinos et al., 2018; Claxton et al., 2015; Kim and Feldman, 2012; Rajasekaret al., 2017; Trejo et al., 2007). Pre-clinical and clinical studies of ischemic injury (Lioutas et al., 2015) and amyotrophic lateral sclerosis (Dodge et al., 2008) further show that the neurotrophic effects of insulin and IGF-I contribute to neuroprotection and mitigate pathology in the CNS.

The role of insulin and IGF-I in the development and progression of cognitive aging and AD, however, is controversial (Deak and Sonntag, 2012; Sonntag et al., 2005; Van Fleemst,

2010). There is evidence for altered insulin receptor, IGF-IR, and IGF-I levels in AD patients (Gasparini and Xu, 2003; Gubbi et al., 2018; Moloney et al., 2010; Westwood et al., 2014; Zemva and Schubert, 2011; Zheng and Tong, 2017), suggesting that abnormalities in insulin/IGF-I signaling may be relevant to disease pathology, but implications of these abnormalities are yet to be elucidated. Preclinical evidence from murine models of AD has also revealed the complexity and inconsistency of the role of insulin and IGF-I signaling in AD pathology (Frater et al., 2018; Guo et al., 2017; Pardo et al., 2016). Insulin decreases intracellular A β and promotes A β delivery to the plasma membrane and extracellular space *in vitro* (Gasparini et al., 2001). Peripheral administration of IGF-I likewise increases A β clearance from the brains of aging rats in one study (Carro et al., 2002), but this finding is not replicated in other AD models (Lanz et al., 2008). Adding to the inconsistencies regarding the role of insulin and IGF-I on A β clearance, findings on IGF-IR activation in the context of neuronal toxicity also remain controversial. IGF-I protects hippocampal neurons against A β toxicity (Dore et al., 1997), yet several studies using neuronal knockout models demonstrate that reduced IGF-I signaling can mitigate disease pathology (George et al., 2017; Gontier et al., 2015; Gubbi et al., 2018). Specific to APP metabolism, insulin and IGF-I decrease APP phosphorylation in neuronal cell lines (Pandini et al., 2013) and promote non-amyloidogenic processing (Song et al., 2018). Collectively, these studies provide rationale for investigating the potential of insulin and IGF-I as treatments for AD (Avgerinos et al., 2018; Hu et al., 2016).

Insulin resistance (IR) is a common pathological characteristic of metabolic dysfunction (Frisardi et al., 2010; Grundy, 1999; Kim and Feldman, 2015; Tabák et al., 2009; Weyer et al., 1999) and there is growing literature to suggest the presence of IR in the CNS of AD patients (Arnold et al., 2018; Kim and Feldman, 2015). IR is a central feature of metabolic syndrome (MetS) and type 2 diabetes (Grundy, 1999; Tabák et al., 2009; Weyer et al., 1999), which are considered risk factors for AD (Frisardi et al., 2010; Kim et al., 2015; Li et al., 2015). IR develops primarily in response to lifestyle factors, particularly consumption of a high fat or western diet (Czech, 2017; Meikle and Summers, 2017), and has detrimental effects on many tissues and systems, including the CNS (Arnold et al., 2018; Kim and Feldman, 2015; Moloney et al., 2010; Talbot et al., 2012). Similar to pathological AD changes in the brain, IR can occur years prior to the onset of clinical symptoms (Talbot et al., 2012). Interestingly, some studies report that improving IR by metformin, the most widely prescribed anti-diabetic medicine, alleviates AD symptoms (Chen et al., 2016; Koenig et al., 2017; Li et al., 2012), although the efficacy and mechanisms of metformin in AD are still controversial (Barini et al., 2016; Chiang et al., 2016; Chung et al., 2017; Son et al., 2016). Hence, while clinical studies link IR and AD, currently little is known about how IR may influence the effectiveness of either insulin or IGF-I in the treatment of AD, and how the downstream signaling of insulin or IGF-I intersects with IR to affect APP processing in the brain. In the current study, we used our established *in vitro* (Kim et al., 2015; Kim et al., 2011b) model of AD pathology to investigate how insulin and IGF-I signaling impact APP phosphorylation alone and in the presence of IR, and we further utilized an *in vivo* model of IR and MetS (Kim et al., 2009; Kim et al., 2015) to examine the presence of APP phosphorylation in the brain under IR conditions.

Materials and methods

Antibodies and chemicals

All antibodies were purchased from Cell Signaling (Beverly, MA), except the polyclonal antibody against actin (Abcam, Cambridge, MA) and monoclonal antibody against total APP (Millipore, Billerica, MA). Inhibitors (LY294002 and U0126) were purchased from Calbiochem (La Jolla, CA). All other chemicals were purchased from either Sigma (St. Louis, MO) or Fisher Scientific (Fair Lawn, NJ).

Rat embryonic cortical neuron (eCN) preparation

Primary eCN were harvested from E15 embryos of Sprague Dawley rats according to published protocols (Kim et al., 2015; Kim et al., 2011b). Briefly, the cortex was dissected and dissociated using trypsin and plated in 12-well tissue culture plates coated with poly-L-lysine. Cells were maintained in feed media [(Neurobasal media (Invitrogen, Grand Island, NY), supplemented with 1X B27 (Invitrogen), antibiotics (penicillin, streptomycin, and neomycin; Sigma), 2.5 µg/ml albumin, 10 µg/ml apo-transferrin, 0.1 µg/ml biotin, 15 µg/ml D-galactose, 7 ng/ml progesterone, 16 µg/ml putrescine, 4 ng/ml selenium, 3 ng/ml µ-estradiol, 4 ng/ml hydrocortisone, 3 µg/ml catalase, and 2.5 µg/ml SOD]. Half of the media was replaced with fresh feed media every other day. On day 7, to exclude any effect of insulin contained in B27, culture media was changed to treatment media (feed media without B27) 2-4 h before treatment.

Western immunoblotting

eCN were lysed in RIPA buffer (Pierce, Rockford, IL) containing a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Lysates were collected, briefly sonicated, and centrifuged at 13,000 rpm for 20 min at 4°C. Western immunoblotting was performed as described previously (Kim et al., 2015). TBS with 0.01% Tween-20 and 3% BSA were used for blocking and primary antibody dilutions. Primary antibodies were incubated at 4°C overnight. Secondary antibodies were diluted in 5% fat-free milk and incubated for 2 h at room temperature. Signal was visualized using enhanced chemiluminescence reagents (ECL, Amersham Bioscience, Piscataway, NJ) or SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL). Images were captured using the Chemidoc XRS system and analyzed by Quantity One software (Bio-Rad Laboratory, Hercules, CA).

Animals

Animal protocols were approved by the University of Michigan Institutional Animal Care and Use Committee. Mice were housed in a pathogen-free environment and cared for in accordance with University of Michigan guidelines. C57BL6 mice, a strain commonly used to study diet-induced obesity (Hinder et al., 2017; O'Brien et al., 2018; O'Brien et al., 2014), were purchased from Jackson Laboratory (C57BL/6J #000664). Obesity was induced by placing mice on a high fat diet (HFD) consisting of 54% kcal from fat (Research Diets Inc., New Brunswick, NJ #D05090701) at 4 wk of age until 24 wk of age, while age-matched controls were fed a standard diet consisting of 10% kcal from fat (Research Diets Inc. #D12450B). Our laboratory has previously published a detailed phenotypical analysis of

these HFD mice (Hinder et al., 2017; Kim et al., 2015; O'Brien et al., 2018), which exhibit a MetS phenotype, including obesity, impaired glucose tolerance, and hyperinsulinemia, as well as cognitive deficits.

Mice were euthanized per our published protocols (O'Brien et al., 2018; Oh et al., 2010) with an overdose of sodium pentobarbital (Fatal-plus, Vortech Pharmaceutical, Dearborn, MI). For Western immunoblotting analyses, brains were cut in half and the cortex halves were separated, snap frozen in liquid nitrogen, and stored at -80°C until use. At least 6 animals were analyzed for each treatment, and representative Western immunoblotting results are presented in the corresponding figure.

Statistical analysis

All experiments were repeated at least 4 times and results are presented as the mean \pm standard error of the mean (SEM). Statistical analysis was performed by Student's *t*-test using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA). Statistical significance was defined as $p < 0.05$.

Results

Insulin and IGF-I reduce T668-APP phosphorylation via Akt

APP phosphorylation at threonine 668 (T668) is one of the key posttranslational modifications that yields the production of A β (Matsushima et al., 2012; Pandini et al., 2013; Ramelot and Nicholson, 2001). Therefore, we first examined the effect of insulin and IGF-I on the phosphorylation of T668-APP in rat eCN. Both insulin and IGF-I decreased APP phosphorylation for up to 6 h in a time-dependent manner, with phosphorylation levels returning to baseline after 24 h (Fig 1A). This effect was more significant with IGF-I (68% of the control, $p < 0.0001$) compared to insulin (85% of the control, $p < 0.05$; Fig 1C). We also confirmed that insulin and IGF-I increased Akt Ser473 and ERK Thr202/Thr204 phosphorylation (Fig. 1A). We found that the effect of IGF-I on APP phosphorylation was blocked by inhibiting Akt activation using the PI3-K/Akt inhibitor LY294002; however, the ERK inhibitor, U0126, had no effect on IGF-I-mediated APP dephosphorylation (Fig 1D & E). LY294002 and U016 had a similar effect on insulin-induced decreases in APP phosphorylation (data not shown). These results demonstrate that insulin and IGF-I decrease APP phosphorylation, and that this decrease in phosphorylation is mediated through the PI3-K/Akt pathway.

IR reduces the effects of insulin and IGF-I on T668-APP phosphorylation

We next employed our previously established *in vitro* model of hyperinsulinemia induced IR in eCN (Kim et al., 2011a; Kim et al., 2011b), which consists of chronic 50 nM insulin pretreatment for 24 h. We then treated the cells acutely with 20 nM insulin or IGF-I. In agreement with our previous report (Kim et al., 2011b), acute insulin treatment of IR eCN failed to further increase Akt phosphorylation ($p > 0.05$; Fig 2A). However, IGF-I retained the ability to significantly increase Akt phosphorylation in IR eCN, although this increase was significantly lower (60% lower, Fig 2B bars b3 vs b6, $p < 0.005$) than in non-IR cells. Insulin- and IGF-I-induced ERK phosphorylation was unaffected by chronic insulin

pretreatment ($p>0.05$; Fig 2A). Induction of IR increased T668-APP phosphorylation (12% greater than the control, Fig 2E compare bars e1 to e4; $p<0.05$). In addition, both insulin (80% of the controls, Fig 2E bar e2; $p<0.05$) and IGF-I (70% of the controls, Fig 2E bar e3; $p<0.005$) reduced APP phosphorylation under normal non-IR conditions. However, in IR eCN, insulin failed to reduce APP phosphorylation (Fig 2E bar e5; $p>0.05$). Although IGF-I maintained the ability to reduce APP phosphorylation in IR cells (Fig 2E bar e6; $p<0.05$), its effects were diminished, with a significant difference in APP dephosphorylation response between IR and normal non-IR conditions (15% difference, Fig 2E bars e3 vs e6, $p<0.05$). These results suggest that IR induced by chronic insulin treatment prevents the effect of insulin and reduces the effect of IGF-I on T668-APP phosphorylation.

Akt mediates IR effects on APP phosphorylation

We and others previously hypothesized that over-activation of Akt signaling due to hyperinsulinemia contributes to decreased insulin sensitivity and signaling, resulting in IR (Kim et al., 2011a; Kim et al., 2011b; Liu et al., 2009). We therefore confirmed the role of PI3-K/Akt signaling on T668-APP phosphorylation. We blocked this pathway using specific inhibitors for PI3-K (LY294002) and ERK (U0126) in our *in vitro* model of IR. During chronic insulin pretreatment for 24 h (IR), eCN were incubated without or with the inhibitors LY294002 or U0126, followed by acute insulin or IGF-I treatment without inhibitors. In line with the above results (Fig 2B), the ability of IGF-I to decrease APP phosphorylation was again diminished in IR (Fig 3B). However, LY294002 restored the effect of IGF-I on APP phosphorylation in the presence of IR (Fig 3C compare bars c2, c4 & c5). The ERK inhibitor U0126 had no effect (bars 4 vs 6, $p>0.05$), indicating a biological regulation specific to the Akt pathway. Densitometric analysis (Fig 3C) confirmed that LY294002 treatment restored APP dephosphorylation (compare bars c2 and c5, $p>0.05$), which was significantly lower than in cells without inhibitors (bar c4, $p<0.005$) or in cells treated with U0126 (bar c6, $p<0.05$). Additionally, there was no significant difference between LY294002 (Fig 3C bar c5) and non-IR neurons stimulated acutely with insulin (bar c2, $p>0.05$). These results suggest that IR induces chronic hyperactivation of Akt and subsequent desensitization of PI3-K/Akt pathway, which prevents an insulin and IGF-I-mediated decrease in T668-APP phosphorylation.

T668-APP phosphorylation is increased in the cortex of MetS mice

Our *in vitro* results suggest that neuronal IR negatively affects the therapeutic impact of insulin and IGF-I on AD pathology. Therefore, we confirmed the translational relevance of our *in vitro* results using a HFD mouse model of MetS that presents with obesity, IR, and cognitive decline (Kim et al., 2015; Sims-Robinson et al., 2016). We observed a significant increase in T668-APP phosphorylation in the cortex of HFD mice compared to age-matched controls fed a standard diet (20% higher than control, $p<0.005$; Fig 4B). Our results provide a promising animal model to study the effects of AD therapies, including insulin and IGF-I, on tau and APP pathology in the presence of IR.

Discussion

Although AD is the most common neurodegenerative disease worldwide (Fiest et al., 2016), there are no viable treatments for this morbid disorder (Anderson et al., 2017; Liao et al., 2015). In humans, metabolic dysfunction, specifically MetS and diabetes, increases the risk of AD, with IR playing a key role in this process (Ferreira et al., 2018; Kim and Feldman, 2012; Kim and Feldman, 2015). Therefore, the current study addressed the impact of IR on the therapeutic potential of insulin and IGF-I in AD. We show that in the absence of IR, acute insulin and IGF-I treatment reduce T668-APP phosphorylation in eCN. These results suggest that in addition to their neuroprotective effects (Hamabe et al., 2005; Hodge et al., 2007), insulin and IGF-I could improve AD pathology by reducing APP phosphorylation, which may then lead to a decrease in pathogenic A β plaques (Shin et al., 2007). In contrast, IR increased T668-APP phosphorylation in eCN and blocked the ability of insulin to reduce APP phosphorylation. IGF-I retained the ability to reduce APP phosphorylation, but to a lesser degree than under non-IR conditions, secondary to continued IGF-I activation of the PI3-K/Akt pathway. Further, we confirmed the translational relevance of our *in vitro* results when we observed an increase in T668-APP phosphorylation in the cortex of a mouse model of MetS, IR, and cognitive decline (Sims-Robinson et al., 2016), providing a murine model for testing the effects of evolving therapies on cognition and APP biology. Not only do the novel data presented here further support an important role for IR in AD pathology, but they also indicate that insulin and IGF-I alone may not be effective treatments for AD in patients with IR.

Insulin and IGF-I are currently being investigated as novel therapeutics for AD and cognitive decline; however, effects on cognitive deterioration and AD pathology have been mixed. Clinical studies demonstrate that intranasal insulin administration in humans (for >3 weeks) with mild cognitive impairment (MCI) or AD positively impacts cognitive performance (Claxton et al., 2015; Craft et al., 2017). It appears that IGF-I administration has not been investigated in humans with AD or MCI, but treatment of both healthy and MCI patients with growth hormone releasing hormone (GHRH), which increases serum IGF-I levels via the somatotrophic axis, results in improved executive function and a trend towards improved memory (Baker et al., 2012; Sevigny et al., 2008). In contrast, use of a GH secretagogue in patients with AD did not alter disease progression (Sevigny et al., 2008). While these studies evaluated the clinical outcome using cognitive function measures, the role of insulin and IGF-I in regulating amyloid and tau pathology in humans is unclear.

The published literature on the effects of insulin and IGF-I, including endogenous protein and exogenous treatment, on AD pathology to date suggest a complex and often contradictory role for these growth factors in regulation of APP processing, A β , or tau pathology. Insulin administration protects against A β induced synapse loss in neurons (De Felice et al., 2009; Lourenco et al., 2013), and IGF-I protects against A β induced toxicity in hippocampal neurons (Dore et al., 1997). Based on existing studies, it is unclear whether IGF-I promotes A β clearance from the brain (Dore et al., 1997; Lanz et al., 2008). In contrast to findings suggesting that IGF-I reduces A β levels and toxicity, IGF-IR knockout studies suggest that reducing IGF-IR activation may be beneficial (George et al., 2017; Gontier et al., 2015; Gubbi et al., 2018). Similar to the anti-amyloidogenic effects presented

here, others have also observed decreased T668-APP phosphorylation (Pandini et al., 2013), decreased A β production (Song et al., 2018), and decreased APP processing (Wang et al., 2014) in transformed human neuroblastoma cell lines and/or primary rat eCN upon insulin or IGF-I treatment. Furthermore, we and others have shown *in vitro* effects of insulin and IGF-I treatment on tau pathology in primary eCN, including decreased tau phosphorylation (Flong and Lee, 1997; Kim et al., 2015). Collectively, our new data in a primary nontransformed cell, eCN, along with our previously published findings on tau biology (Kim et al., 2015), support the idea that insulin and IGF-I may mitigate AD-associated pathology.

Metabolic dysfunction is a known risk factor for the development of AD (Frisardi et al., 2010; Kim et al., 2015; Li et al., 2015). In particular, IR is a common pathological characteristic linking metabolic dysfunction and AD in both animal models and humans (Friedlander et al., 2001; Frisardi et al., 2010; Kim and Feldman, 2015; Li et al., 2015; Sperling et al., 2019; Talbot et al., 2012). Studies in rodents have demonstrated that brain IR results in AD pathology and cognitive decline (de la Monte et al., 2017; Lester-Coll et al., 2006), and pathology is amplified when A β is infused under systemic diabetic conditions (Ma et al., 2013; Su et al., 2019). IR has also been identified in the CNS of AD patients postmortem (Moloney et al., 2010; Talbot et al., 2012). In the current study, using our *in vitro* model of IR induced by chronic insulin treatment (Kim et al., 2015; Kim et al., 2011b), we show for the first time that IR results in increased T668-APP phosphorylation. Phosphorylation at T668 leads to intracellular sorting and trafficking of APP (Pandini et al., 2013; Ramelot and Nicholson, 2001), which in turn impacts proteolytic cleavage and increases A β production (Matsushima et al., 2012). Phosphorylated T668-APP also promotes tau accumulation and A β plaques (Shin et al., 2007), promoting the spread of AD pathology (Aplin et al., 1996; Suzuki et al., 1994; Taru et al., 2002) and increasing cognitive impairment (Sperling et al., 2019). As data here and from others (Frisardi et al., 2010; Kim and Feldman, 2012; Moloney et al., 2010; Talbot et al., 2012) continue to emphasize that IR is not only present in AD, but contributes to AD pathology, it is essential to consider the clinical importance of IR when studying therapeutic approaches.

In the present study we also show that IR impacts the therapeutic effectiveness of insulin and IGF-I treatment. IR blocked the insulin-mediated decrease in T668-APP phosphorylation in eCN and partially prevented the ability of IGF-I to lower T668-APP phosphorylation in these same cells. A similar decrease in T668-APP phosphorylation has been reported in response to nerve growth factor treatment of mouse septal neurons that resulted in further anti-amyloidogenic effects (Triaca et al., 2016); however, studies assessing the treatment effects of insulin and IGF-I on AD pathology under IR conditions have not quantified functional changes in learning and memory *in vivo*, although they have demonstrated improvements in AD pathology (Pandini et al., 2013; Song et al., 2018; Wang et al., 2014). To our knowledge, only one other report demonstrates alterations in AD pathology in response to IR conditions, namely a change in A β generation and secretase activity in IR SH-SY5Y cells and in the brains of db/db mice (Son et al., 2012). However, in that study, positive results were only observed under IR conditions where supraphysiological insulin concentrations were used (Son et al., 2012). Insulin and IGF-I can both signal through the PI3-K/Akt pathway, which is typically protective in cells under metabolically normal, healthy conditions (Duarte et al., 2008; Sanderson et al., 2009). However, both insulin and

IGF-I signaling are impaired and the PI3-K/Akt pathway is disrupted as a consequence of IR in dorsal root ganglion neurons and HK-532 human cortical stem cell lines (Kim et al., 2015; Kim et al., 2011b), so the current data in eCN may be generalizable to other neuronal cell types as well. Our data support a mechanism whereby under normal metabolic conditions, treatment with insulin and IGF-I result in an PI3-K/Akt-mediated decrease in APP phosphorylation; however, under conditions of metabolic stress and IR, the beneficial effects of insulin and IGF-I are either blocked or reduced (Figure 5).

Another consideration that may also complicate the potential therapeutic effects of insulin and IGF-I is the presence of insulin/IGF-I hybrid receptors in the brain which have a higher affinity for IGF-I versus insulin and may therefore alter insulin signaling (Martinez-Rachadell et al., 2019; Slaaby, 2015). This may partially explain our results demonstrating that eCN are more sensitive to IGF-I treatment compared to insulin treatment. Further studies are thus warranted to better understand the potential contribution of the hybrid receptor to insulin- or IGF-I-mediated treatment effects in the presence of IR. Taken together, our data suggest that IR may be a confounding factor in the efficacy of therapeutic approaches for AD and emphasize the importance of efficacy testing in preclinical models that exhibit metabolic dysfunction as well as AD pathology.

We lastly established the translational relevance of our *in vitro* findings by demonstrating that a robust mouse model of MetS exhibited increased APP phosphorylation in brain cortex. Our laboratory has published a phenotypical analysis of this HFD model which presents with a MetS phenotype, including obesity and IR, as well as cognitive decline (Hinder et al., 2017; Kim et al., 2015; O'Brien et al., 2018; Sims-Robinson et al., 2016). Brain IR is present as early as 2 weeks after initiation of HFD (Sims-Robinson et al., 2016), and we have also previously shown that dysregulated IR and chronic overactivation of PI3-K/Akt signaling increases tau phosphorylation in the cortex of these mice (Kim et al., 2015). Here, we show that the other hallmark of AD pathology, APP phosphorylation (specifically T668-APP phosphorylation), is also increased in response to IR in this HFD mouse model of MetS. As it has been previously proposed that the development of brain IR is an early event in the progression of cognitive decline (Talbot et al., 2012), we propose a series of events where HFD feeding induces early IR, which then promotes T668-APP phosphorylation that drives subsequent generation of A β (Shin et al., 2007) and tau pathology (Kim et al., 2015). This supports our hypothesis that IR is an initial event contributing to pathological AD processing; however, more studies are needed to confirm our hypothesis and fully establish a time course of the disease.

IR-dependent hyperphosphorylation of T668-APP and the refractory response to insulin we observed *in vitro* are in line with other reports that brain IR in mouse models of diabetes results in increased AD pathology and cognitive decline (de la Monte et al., 2017; Lester-Coll et al., 2006; Ma et al., 2013; Su et al., 2019). Similarly, HFD or high sucrose diets in combination with altered insulin or IGF-I signaling promote AD pathology, memory deficits, and decreased A β uptake (Logan et al., 2018) (Busquets et al., 2017; Carvalho et al., 2012; Hiltunen et al., 2012; Ramos-Rodriguez et al., 2017). Most murine models of IR and AD are developed using genetically modified animals, and studies have shown mixed effects of insulin and IGF-I treatment on AD pathology and cognition (Lanz et al., 2008;

Yang et al., 2013). Our model provides a unique opportunity to investigate treatment effects on APP and tau pathology in a non-genetically modified animal under IR conditions. Additional preclinical studies using this model will allow us to elucidate these mechanisms and fully understand the therapeutic potential of insulin, IGF-I, and other AD therapies under IR conditions.

Conclusion

In conclusion, our findings in eCN suggest that the therapeutic effectiveness of insulin and IGF-I on AD pathology is reduced under conditions of IR. Additional *in vitro* studies in other neuronal cell types as well as additional *in vivo* studies are needed to fully understand the use of insulin and IGF-I alone, or in combination, as therapeutic agents in preclinical models and in AD patients with IR. Further investigation of insulin and IGF-I in the context of IR will also help to clarify their complex role in AD. Overall, these data argue for a critical clinical impact of IR on AD therapies, and have important implications for the development and testing of future therapeutics.

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Abbreviations

(AD)	Alzheimer's disease
(Aβ)	Amyloid beta
(APP)	Amyloid Precursor Protein
(CNS)	Central nervous system
(eCN)	Embryonic cortical neurons
(ERK)	Extracellular signal regulated kinase
(HFD)	High fat diet
(IGF-I)	Insulin-like growth factor-I
(IGF-IR)	Insulin-like growth factor-I receptor
(IGFBP)	Insulin-like growth factor binding proteins
(IR)	Insulin resistance
(MCI)	Mild cognitive impairment
(PI3-K)	Phosphatidylinositol 3-kinase

(Akt)	Protein kinase B
(MetS)	The Metabolic Syndrome
(T668)	Threonine 668
(T2D)	Type 2 diabetes

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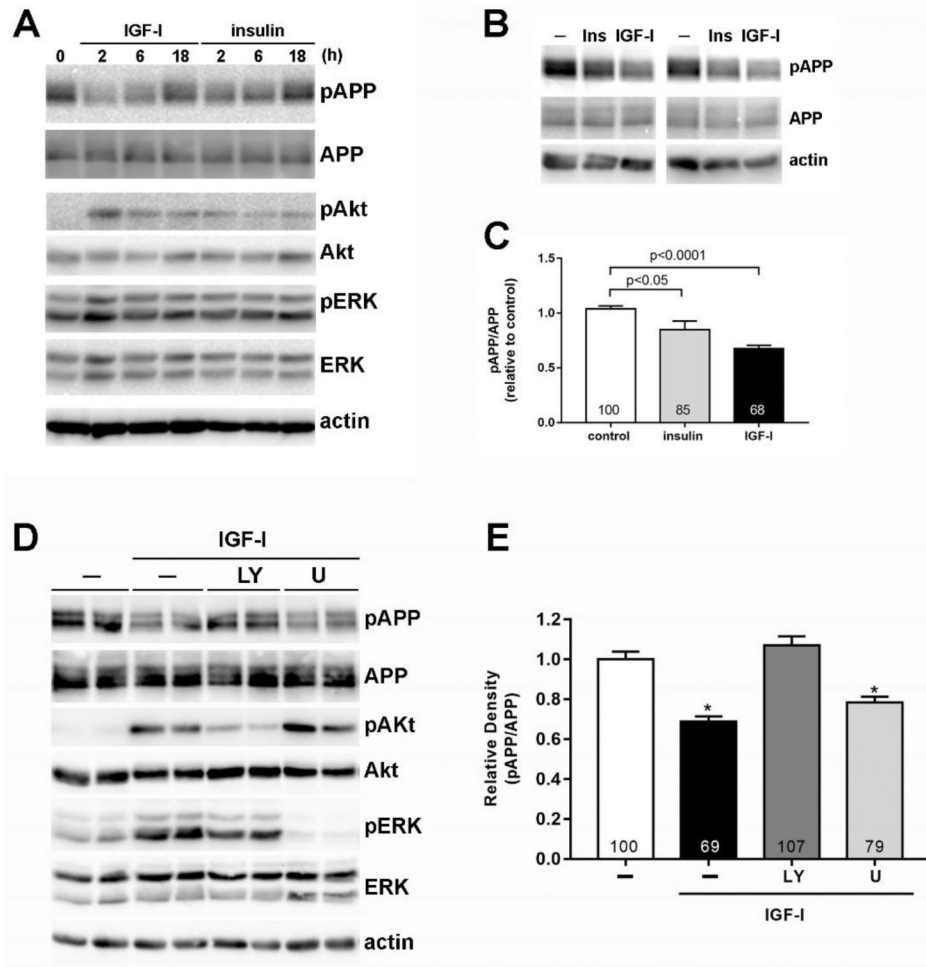


Figure 1. Insulin and IGF-I decrease APP phosphorylation through PI3-K/Akt-mediated pathways in embryonic cortical neurons (eCN).

(A) Rat primary eCN cells were treated with 20 nM insulin or IGF-I for the indicated times and cell lysates were analyzed by Western blotting with the indicated antibodies. (B) eCN were treated without or with 20 nM insulin or IGF-I for 2 h and (C) changes in APP phosphorylation were analyzed against total APP by densitometry. Fold changes compared to control are indicated in the bars. (D) eCN were treated with 20 μ M LY294002 (LY) or U0126 (U) for 1 h and then treated with 20 nM IGF-I for 2 h. (E) The effect of LY and U on APP phosphorylation was analyzed by densitometry. Results are shown mean \pm SEM by Student t-test. % changes compared to control are indicated in the bars. * p <0.05 compared to control or LY94002 treated cells. All experiments were repeated at least 4 times and representative images are shown.

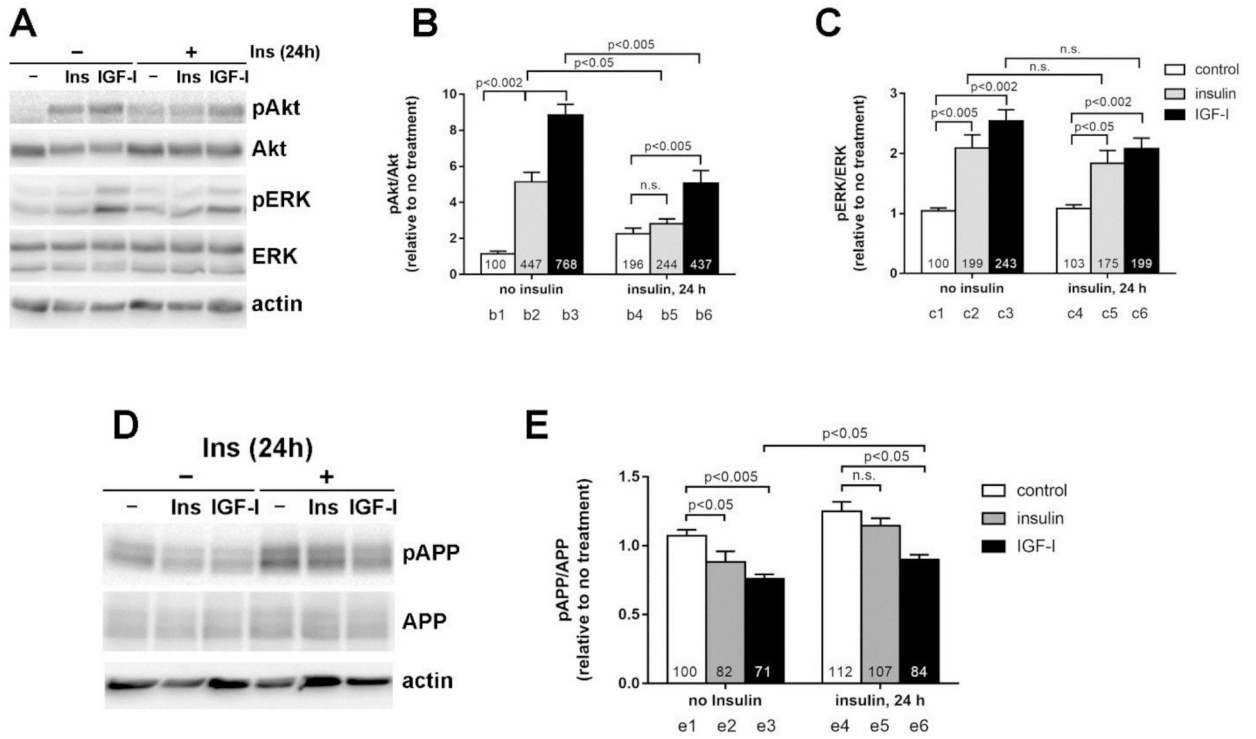


Figure 2. Chronic insulin treatment (IR) and reduced insulin- and IGF-I-mediated decrease in APP phosphorylation.

eCN were treated with 50 nM insulin for 24 h to model IR and then treated with 20 nM insulin or IGF-I for 2 h (A). Akt (B) and ERK (C) phosphorylation were analyzed against the total protein. (D) APP phosphorylation was analyzed by Western blotting and (E) measured against total APP level. The numbers inside the bars indicate the % changes compared to no treatment. Results are shown mean ± SEM by Student *t*-test.

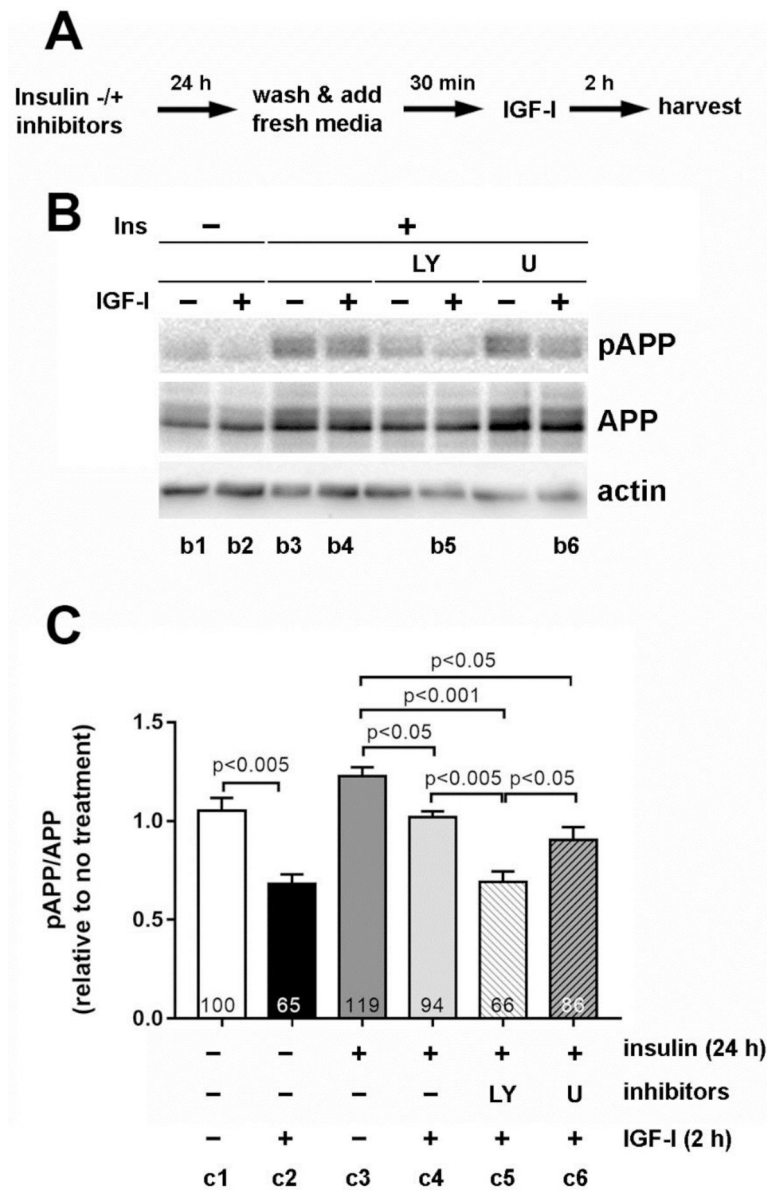


Figure 3. Preventing over-activation of PI3-K/Akt pathway restored IGF-I-mediated decrease in APP phosphorylation under IR conditions.

Rat eCN were incubated with 50 nM insulin along with 20 μ M LY294002 (LY) or U0126 (U) for 24 h. The cells were washed and incubated in fresh treatment media for 30 min before 20 nM IGF-I treatment for 2 h. (A) The experimental scheme. (B) Immunoblotting was performed using antibodies against pAPP and APP. Equal protein loading was confirmed with actin immunoblotting. (C) Densitometric analysis of the immunoblot. The relative changes compared to control (c1) are indicated in % inside each bar. All experiments were performed at least 3 times in duplicate and representative images are shown. Results are shown mean \pm SEM and analyzed by Student *t*-test.

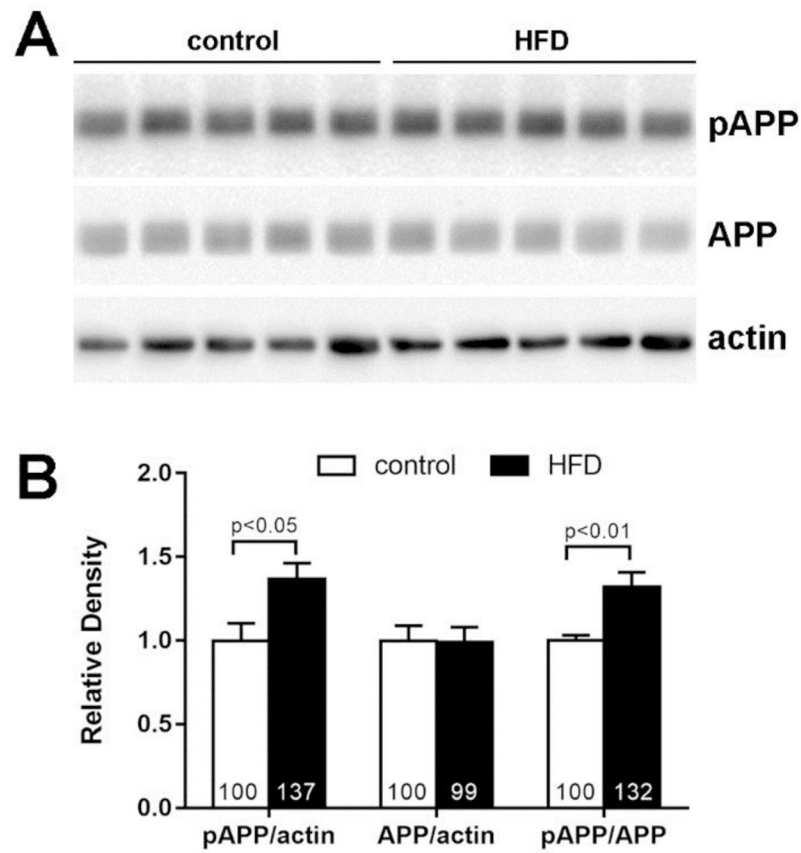


Figure 4. APP phosphorylation is increased in HFD mouse cortex.

(A) Cortex homogenates from 24 wk control (n=5) and high fat diet (HFD; n=5) mice were analyzed by the Western immunoblotting. Each lane represents a cortex from one animal.

(B) The relative density determined by densitometric analysis. The % change is indicated in the bars. Results are shown as mean \pm SEM and analyzed by Student *t*-test.

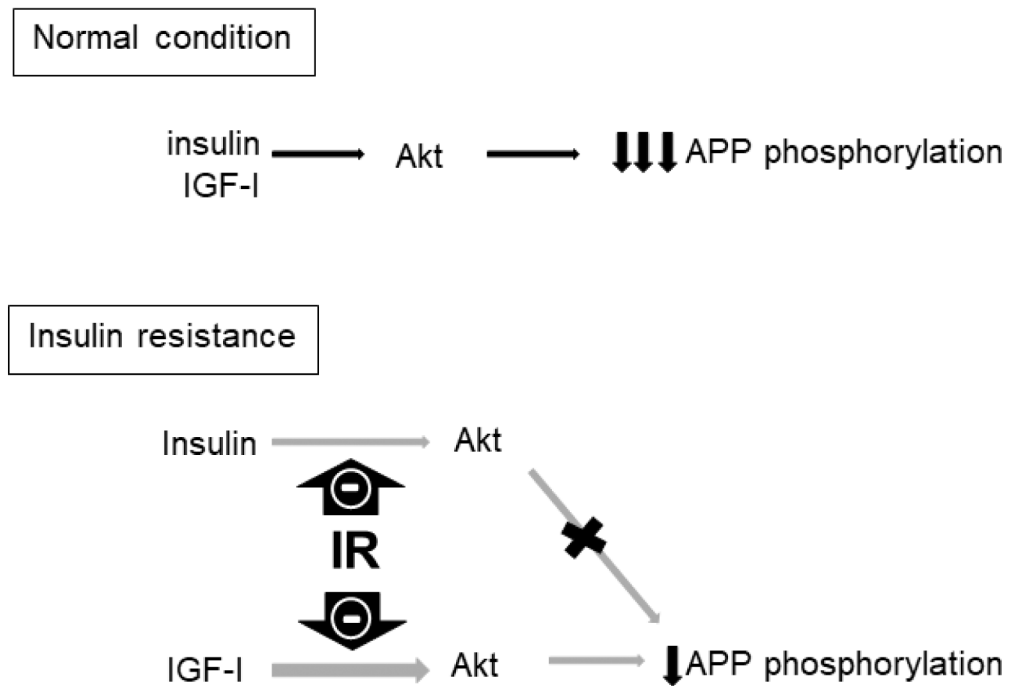


Figure 5.

Proposed model summarizing the findings of this study. Under normal conditions, both insulin and IGF-I reduce APP phosphorylation through an Akt-mediated pathway. In IR, the PI3-K/Akt pathway is compromised, leading to an almost complete inhibition of the insulin-mediated reduction in APP phosphorylation. IGF-I still is able to reduce APP phosphorylation under IR conditions, but its effect is greatly reduced.