

ERK5: A Novel IKK α -Kinase in Rat Hippocampal Neurons

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ABSTRACT: Background: Alzheimer's Disease (AD) is characterized in part by the increased presence of neurofibrillary tangles and amyloid beta (A β) plaques. Alzheimer's Disease is considered an inflammatory disease and, as such, nuclear factor-kappaB (NF κ B) plays an important role in the pathophysiology of AD. Insulin acts as a neurotrophic factor. Yet, in the context of insulin resistance, concomitant hyperinsulinemia may contribute to the pathogenesis of AD. **Methods:** Rat Primary Hippocampal Neurons (RPHN) were treated with insulin in the absence and presence of Wortmannin and ERK5 small inhibitory RNA and assayed for downstream effectors of activated ERK5. **Results:** Here we demonstrate that genetic inhibition of ERK5 blocks insulin stimulated (1) activation and translocation of ERK5 and NF κ B, (2) phosphorylation of IKK α via association with ERK5, (3) increases in A β 1-40 and A β 1-42 soluble proteins 3-fold and 2.2-fold, respectively, and (4) increases in tau phosphorylation in RPHN. **Conclusions:** ERK5 plays an active role in insulin signaling in neurons and may be a potential therapeutic target for neurodegenerative diseases.

RÉSUMÉ: ERK5 : une nouvelle kinase IKK α dans les neurones de l'hippocampe chez le rat. Contexte : La maladie d'Alzheimer (MA) se caractérise entre autres par la présence accrue d'amas neurofibrillaires et de plaques de bêta amyloïde (A β). La MA est considérée comme étant une maladie inflammatoire et donc le facteur nucléaire KappaB (NF κ B) joue un rôle important dans la physiopathologie de la MA. Étant donné que l'insuline agit comme facteur neurotrope, l'hyperinsulinémie présente dans le syndrome de résistance à l'insuline pourrait contribuer à la pathogenèse de la MA. **Méthode :** Des neurones primaires de l'hippocampe de rat (NPHR) ont été traités par de l'insuline en l'absence et en la présence de Wortmannin et de petits ARN inhibiteurs de ERK5 et nous avons recherché les effecteurs en aval de ERK5 activé. **Résultats :** Nous avons démontré que l'inhibition génétique de ERK5 bloque (1) l'activation stimulée par l'insuline et la translocation d'ERK5 et de NF κ B, (2) la phosphorylation de IKK α via son association avec ERK5, (3) augmente les protéines solubles A β 1-40 et A β 1-42 de 3 fois et de 2.2 fois respectivement et (4) augmente la phosphorylation de tau dans les NPHR. **Conclusions :** ERK5 joue un rôle actif dans la signalisation de l'insuline dans les neurones et pourrait être une cible thérapeutique dans les maladies neurodégénératives.

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Alzheimer's Disease (AD) is one of the leading forms of dementia in the elderly in the United States¹. Alzheimer's disease is not only a cognitive disorder, but also presents itself as continual loss of neuronal mass over time as seen in sequential advanced medical imaging scans. In particular, the most prevalent area of affliction is the hippocampus and adjacent areas of the brain. It appears that neurological connections that allow daily and conscious thought are hampered to the point of cessation by the presence of neurofibrillary tangles and neuronal plaques due in part to hyperphosphorylation of tau and increased accumulation of insoluble amyloid-beta (A β) protein, respectively^{2,3}.

Interestingly, computed tomography (CT) scans of brains of patients with long-term insulin resistance resemble those of newly diagnosed AD⁴. These observations suggest either a positive association or a correlation of insulin resistance and AD. It is difficult to reconcile whether there is a cause and effect or just a coincidental presence of the two disorders. Thus, exploration of similarities may be beneficial in order to determine if a common dysregulation occurs in these two seemingly distantly related diseases. One such commonality is that both AD and diabetes have inflammatory components. In

particular is the central role player of inflammation: nuclear factor-kappaB (NF κ B)⁵.

It has long been understood that insulin signals to NF κ B in part via the phosphatidylinositol-3 kinase/Akt (PI3K/Akt) pathway. The pathophysiology of insulin resistance is characterized in part by events at the cellular level that are post-receptor disturbances. In particular, many have determined that perturbation of the PI3K/Akt pathway contributes to insulin resistance^{6,7}. It has been theorized that disturbances of the PI3K/Akt pathway would decrease insulin's actions on NF κ B. A molecular model of a PI3K/Akt pathway interruption occurs in the presence of the PI3K inhibitor Wortmannin⁸. The question remains though: if down regulation of the PI3K/Akt pathway

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exists in the context of insulin resistance, then would not activation of the inhibitor of kappa-B kinases (IKK) be down regulated as well? It appears that even in the context of insulin resistance (i.e., dysregulation of the PI3K/Akt pathway) activation of the inflammatory NF κ B pathway still occurs, and may, to a greater extent, be mediated via the insulin-stimulated Extracellular Signal-regulated Kinase-5 / Inhibitor of kappa-B Kinase-alpha (ERK5-IKK α) molecular interaction.

Hyperinsulinemia occurs in the presence of insulin resistance. It is this condition of higher than physiologic levels of insulin that cells become primed to be more responsive to other growth factors and cytokines and hence increased NF κ B activity⁹⁻¹². This “priming” mechanism may be one explanation as to why some have proposed a correlation between diabetes and AD^{13,14}.

Insulin is important to neurons because it is a neurotrophic factor and in normoinsulinemia it is anti-atherogenic¹⁵⁻¹⁷. Yet, insulin signaling is associated with NF κ B activity¹⁸. Previous reports have demonstrated that the PI3K/Akt pathway mediates insulin's transduced signal and regulates NF κ B activity¹⁹. Yet, others have found that NF κ B regulation occurs via PI3K/Akt-independent pathways^{20,21}.

In the presence of insulin resistance and concomitant hyperinsulinemia, neurons respond differently than those seen in the normal physiologic state. Although insulin resistance is associated with perturbed and even complete down-regulation of the PI3K/Akt pathway, it appears that NF κ B activation can still be attained. Thus, we wanted to determine which PI3k/Akt-independent pathway is associated with increased NF κ B activation and its related down-stream effects of hyperphosphorylated tau and increased amyloid-beta (A β) content.

Recently, it has been shown that the mitogen-activated protein kinase (MAPK) Extracellular Signal-regulated Kinase-5 (ERK5) activates NF κ B in NIH3T3 cells and vascular smooth muscle cells^{18,20,22}. To continue this line of investigation and elucidation of insulin signaling in neuronal tissue, we were interested in determining whether insulin-treated hippocampal neurons, in the context of neuronal insulin-resistance, would show an association between ERK5 activation and NF κ B activity. Here we show for the first time that NF κ B is regulated by insulin-stimulated ERK5 via a novel ERK5/IKK α interaction, leading to NF κ B activation. Furthermore, we show that that inhibition of ERK5 decreases insulin-stimulated: (1) inhibitor of kappa-B alpha (I κ B α) dissociation from NF κ B, (2) nuclear import of ERK5 and NF κ B, (3) A β production and (4) hyperphosphorylation of tau. These results suggest that activation of ERK5, in the context of insulin resistance, may lead to events associated with AD and diabetes and that ERK5 may be a potential therapeutic target for anti-inflammatory strategies in neuronal tissue.

MATERIALS AND METHODS

1. Materials: All general lab reagents were purchased from Sigma-Aldrich (St. Louis, MO.). Primary rabbit polyclonal antibodies from Cell Signaling Technology (Boston, MA) were ERK5(#3372), inhibitor of kappa-B alpha (I κ B α)(#9242), inhibitor of kappa-B beta (I κ B β)(#9248), inhibitor of kappa-B kinase-alpha (IKK α)(#2682), inhibitor of kappa-B kinase-beta (IKK β)(#2370), NF κ B(#3034), A β 1-40(#2454), phospho-ERK5(#3371), phospho-IKK α (#2697), phospho-I κ B β (#9245),

and α -tubulin (#2125). Primary monoclonal antibodies from Cell Signaling Technology were tau (#4019) and phospho-I κ B α (#9246). Primary antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) were the monoclonal antibody TATA binding Protein (TBP) (#SC74596) and polyclonal antibody Ab1-42 (#SC9129). Western blot accessories and secondary horse-radish peroxidase (HRP)-conjugated antibodies were from GE Healthcare/Amersham (Piscataway, NJ). Rat Primary Hippocampal Neurons (RPHN) (# A1084101) and culture media Neurobasal (#21103), B27 (#17504) and 25 μ M glutamate for RPHN were from Gibco/Invitrogen (Carlsbad, CA). Small interfering RNA (siRNA), control (scrambled) and functional siERK5 were from Invitrogen (Carlsbad, CA) (kit #1320003). Promega Nuclear Extract NE-PER Kit (#78835) was from Thermo Fisher (Waltham, MA).

2. Cell Culture: Prior to plating RPHN, all culture surfaces were pre-coated with Poly-D-Lysine (Sigma-Aldrich, Cat# P7280). Briefly, Poly-D-Lysine (0.1 mg/mL) was applied to all surfaces necessary for cell cultures and allowed to dry at room temperature for two hours (h). Subsequently, RPHN were plated, cultured and treated with designated cytokines in complete neurobasal medium (CNM) [500 mL of neurobasal medium (Gibco; Cat# 21103), 200 mM GLUTAMAX™ -1 (Gibco; Cat# 35050) and 1X B27 Supplement (Gibco; Cat# 17504)]. For initial plating and growth purposes (i.e., first four days of growth) an additional supplement of 25 μ M L-Glutamate was used. For time-course studies, growth medium was aspirated, cells were washed twice with sterile 1X phosphate-buffered saline (PBS) and then incubated in serum-free medium (SFM) (Neurobasal medium, 200 mM GLUTAMAX™ -1, and B27 (minus insulin) for 24 h. Cells were then incubated in serum-free medium (SFM) without or with Wortmannin (1 μ M) for one h, and then treated without or with insulin (1 nM) for indicated times.

3. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Analysis and Protein Expression: Rat primary hippocampal neurons were cultured in serum-free medium (SFM) for 24 h before any treatments were performed. Thereafter, cell monolayers were treated without or with Wortmannin (1 μ M) for one hour and then treated without or with insulin (1 nM) for indicated times. Whole cell lysates were prepared using lysis buffer (50mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 150mM NaCl, 15mM MgCl₂, 1mM PIPES (piperazine-N,N'-bis(2-ethanesulfonic acid), 1mM NaHPO₄, 1mM DTT (dithiothreitol), 1mM Na Vanadate, 1% TX-100, 0.05% SDS (sodium dodecyl sulfate), 10 μ g/mol Aprotinin, and 10 μ g/mol Leupeptin). Lysates were cleared and protein concentrations were determined. Equal protein amounts were placed in 2X Laemmli Sample Buffer, frozen overnight and then boiled for five minutes (min) just before use. Forty microliters were loaded into each well of an 8-16% Pierce Precise Protein gel (Thermo-Fisher, Waltham, MA) and were run in 1X Tris/HEPES/SDS running buffer at 100V for 45 min. Proteins were then transferred to PVDF membranes (Millipore, Billerica, MA), using a standard wet transfer protocol. After completion of protein transfer, membranes were washed two times in 1X tris-buffered saline-tween-20 (TBS-T) solution for ten min.

Membranes were then incubated in 5% bovine serum albumin (BSA) in 1X TBS-T blocking solution for two h at room temperature, washed two times in 1X TBS-T for five min and then incubated with a designated primary antibody solution (1:1000 in 1% BSA/TBS-T) overnight at 4°C. Membranes were washed three times with TBS-T and then incubated with a designated secondary antibody (1:2000 in 1% BSA/TBS-T) conjugated with horseradish peroxidase at room temperature for two hours. Membranes were washed two times with TBS-T for ten min at room temperature and washed once with 1X TBS for ten min. One milliliter of enhanced chemiluminescence (ECL) (GE/Amersham) detection solution was added to each membrane and incubated for one min. Excess ECL was removed and membranes were exposed to HyperFilm (GE/Amersham) for visualization of proteins. Densitometry analysis was performed using the ImageQuant TL v2005 (GE/Amersham) software program.

4. Immunoprecipitation: 100 μ g of whole cell lysate was incubated with 0.2 μ g of designated antibody at 4°C with constant mixing for one h. Twenty microliters of Protein-G plus/Protein A agarose (EMD; Gibbstown, NJ) were then added to each sample and incubated at 4°C with constant mixing for 24 h. The mixture was then centrifuged at 2500 RPM for one min to pellet the agarose/protein bead complex. Supernatants were discarded and the pellets were washed four times each with 200 μ L PBS. After the final wash, the supernatant was discarded and the pellets were resuspended in 40 μ L of 2X Laemmli Sample Buffer and stored overnight at 20°C. Samples were boiled for five minutes and then thirty microliters of this mixture for each sample was loaded into a designated well and proteins were resolved by SDS-PAGE. Membrane proteins were immunoblotted for immunoprecipitated proteins or designated proteins potentially associated with immunoprecipitated proteins.

5. Nuclear and Cytoplasmic Determination of NF κ B and ERK5: Nuclear Factor kappa-B and ERK5 proteins were determined in nuclear and cytoplasmic fractions of RPHN using the Promega Nuclear Extract NE-PER Kit (Thermo Fisher, Waltham, MA). Briefly, RPHN were grown in 100 mm dishes or 6-well plates containing CNM. Complete Neurobasal Medium was replaced with SFM for 24 hours and subsequently incubated in SFM without or with Wortmannin (1 μ M) for one h and then treated without or with insulin (1 nM) for designated times. Cells were collected and plasma membranes were lysed, leaving the cytoplasmic remains and intact nuclei. The cytoplasmic fraction was isolated from the nuclear fraction and kept for biochemical assays. The nuclei were subsequently lysed and the supernatant (nuclear remains) were retrieved for biochemical assays. Cytoplasmic and nuclear extracts were normalized to designated protein concentrations in preparation for further analyses.

6. Determination of Transactivation of NF κ B: Transactivation of NF κ B was determined using the TransAM™ Kit (#43298) from Active Motif (Carlsbad, CA). Briefly, consensus NF κ B response element oligonucleotides that have been immobilized to a 96-well plate format interact only with activated forms of NF κ B. Primary antibodies were used to detect activated NF κ B that were bound to the targeted DNA. The addition of a secondary HRP-conjugated antibody provided a sensitive colorimetric readout to quantify transactivation. Jurkat

nuclear extracts were used as positive controls for NF κ B activation. Wild-type consensus oligonucleotides acted as competitors of NF κ B binding in order to monitor the specificity of the assay. Mutated consensus oligonucleotide sequences acted as negative controls. Absorbance was read on a spectrophotometer at 450 nm with a reference wavelength of 655 nm. Absorbance readings of blanks were subtracted from sample readings.

7. Transfection of RPHN with siRNA: Rat primary hippocampal neurons were trypsinized and pelleted (ten minutes at 200X g) in a 50 ml conical tube. Using a hemocytometer, an aliquot of the cell suspension was taken for assessment of cell number. Cells were resuspended to a final concentration of 5 x 10⁵ cells per 100 μ l in pre-warmed in Nucleofector Solution HiFect® (#VBA-1001) (Lonza, Walkersville, MD). 1-5 μ g of control (scrambled) siRNA or functional siERK5 or siIKK α were added to each 100 μ l cell dilution. The cell/siRNA suspension was transferred to an AMAXA cuvette, and the suspension subjected to electroporation in the AMAXA device using program U-25. Once electroporation was completed, 500 μ l of growth media was added immediately to the cuvette and the cell/siRNA suspension was transferred to a 6-well plate. The 6-well plates were placed at 37°C in a 5% CO₂ incubator. Cell viability was checked two hours later using trypan blue. After 48 h of incubation, the CNM was replaced with SFM and RPHN were incubated for another 24 h. Cells were pre-treated with Wortmannin (1 μ M) and then treated with insulin (1 nM) for indicated times. Subsequently, RPHN were lysed and cellular contents were used for biochemical and other assay studies. Transfection efficiency was previously calculated at 90% using constitutively active GFP-expressing plasmids in RPHN in previous experiments, using the AMAXA system.

8. Amyloid-beta (A β) analysis: Briefly, RPHN were grown in 6-well dishes in CNM. After six days of culture, growth medium was replaced with one milliliter of serum-free medium for 24 h. Cells were pre-treated with Wortmannin (1 μ M) for one h, then treated with insulin (1 nM) for indicated times. Medium was collected and concentrated with Millipore Micron Centrifugal Filter Units (Cole Parmer; Vernon Hills, IL) from each well and A β was immunoprecipitated from solutions normalize for total protein content. Changes in total A β protein content were analyzed by SDS-PAGE and determined by Western blot analysis using antibodies (Cell Signaling, Cat# 2454) to A β protein.

9. Statistical Analysis: Data were analyzed by either unpaired Student's t test (two groups) or ANOVA with subsequent Tukey post test (several groups) as indicated. A "P" value of < 0.05 was considered significant. Results were expressed as the mean \pm SEM of three or more independent experiments. Prism 4 from GraphPad Software, Inc. (La Jolla, CA) was the statistical software used.

RESULTS

The major goal of this study was to determine the extent to which ERK5 regulated NF κ B activity in rat primary hippocampal neuronal cells. We first investigated whether insulin could stimulate the phosphorylation and activation of ERK5 (a possible upstream regulator of NF κ B activity) and

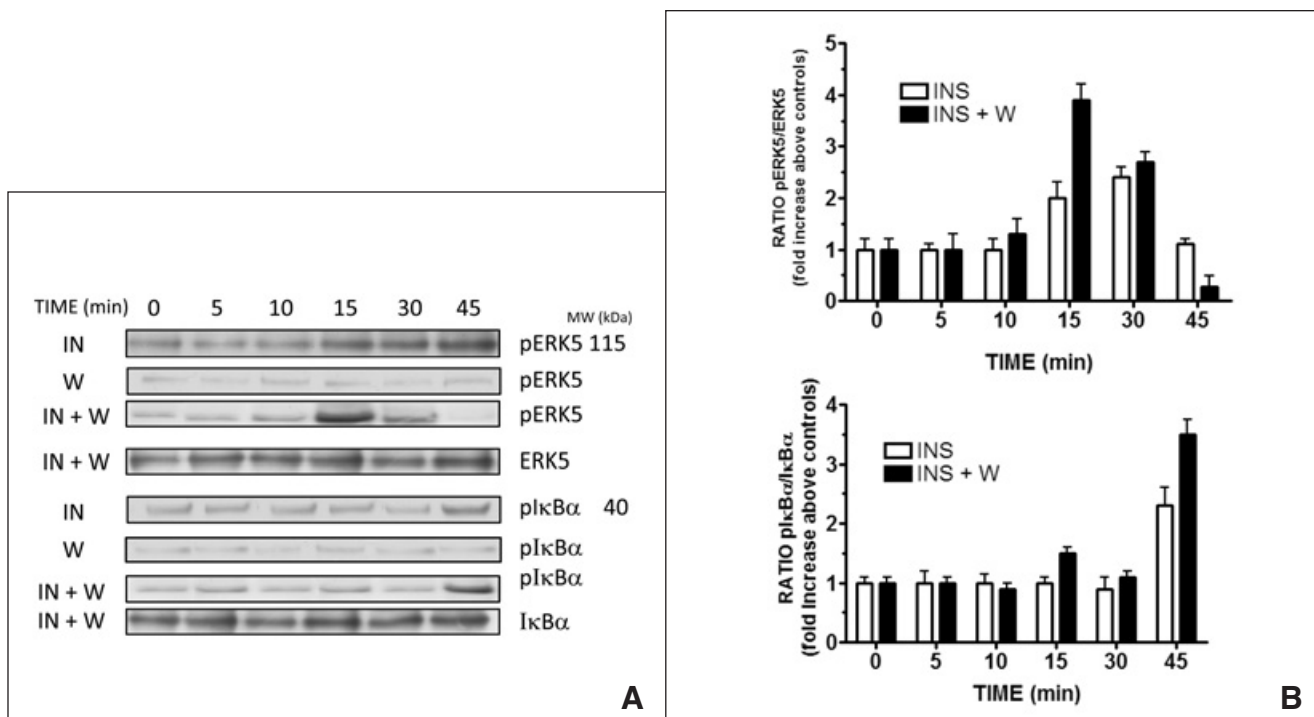


Figure 1: Insulin increases ERK5 and IκBα phosphorylation over time. Rat Primary Hippocampal Neurons (RPHN) were incubated in serum-free medium for 24 h and then cultured in the absence or presence of Wortmannin (W) (1 μM) for one hour and then with or without insulin (IN) (1 nM) for indicated times. Phosphorylated and total proteins were resolved by SDS-PAGE and determined by Western blot analysis. (A) Representative Western blot analyses of phosphorylated and total ERK5 and IκBα. (B) Changes in phosphorylated ERK5 and IκBα are expressed as ratios of the phosphorylated protein to its respective total protein and fold increase above controls (no insulin, no Wortmannin and zero time). The graphs represent the mean ± S.E. for each time point and condition. n=4.

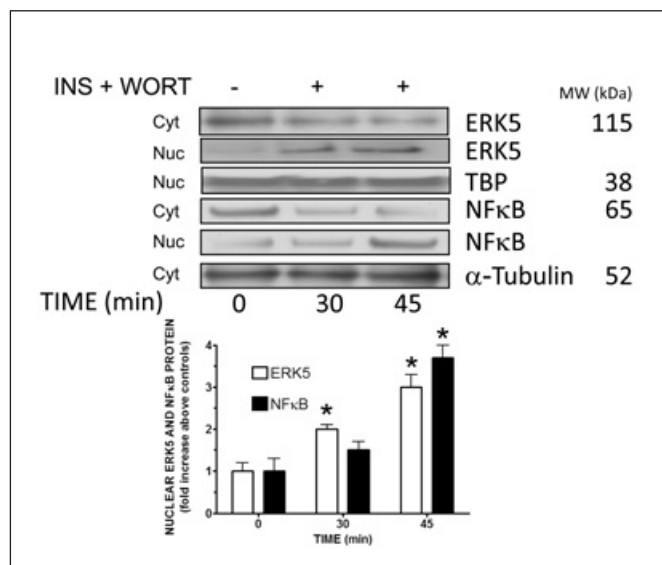


Figure 2: Insulin stimulates nuclear import of ERK5 and NFκB. Cells were incubated in serum free medium for 24h, cultured in the absence or presence of Wortmannin (WORT) (1 μM) for one h and then treated without or with insulin (INS) (1 nM) for indicated times. Upper panel depicts representative Western blot profiles. TATA Binding Protein (TBA) and α-tubulin were used as loading controls for nuclear (Nuc) and cytoplasmic (Cyt) fractions, respectively. Lower panel indicates changes in nuclear amounts of ERK5 and NFκB overtime that are expressed as fold increase above controls (zero time, no insulin, no Wortmannin) and represent the mean ± S.E. for each time point. n=5; *, P < 0.05 versus controls. ANOVA, Tukey.

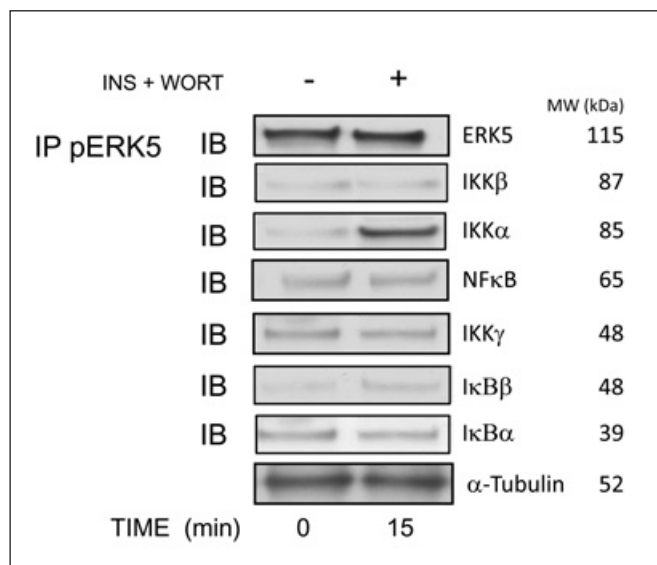


Figure 3: Insulin stimulates the interaction of ERK5 with IKKα. Neurons previously transfected with scERK5 or siERK5 were placed in serum-free medium for 24 h and then cultured in the absence or presence of Wortmannin (WORT) (1 μM) for one hour and incubated in medium with or without insulin (INS) (1 nM) for indicated times. Phospho-ERK5 was immunoprecipitated (IP) followed by SDS-PAGE and Western immunoblots (IB) of designated proteins. Representative Western blot analyses of ERK5, IKKβ, IKKα, NFκB, IKKγ, IκBβ and IκBα are shown with α-tubulin used as a loading control.

I κ B α (a definite regulator of NF κ B activity) in RPHN. Insulin (1 nM) induced a time-dependent increase in phosphorylation of both ERK5 and I κ B α , even in the presence of Wortmannin, a PI3K inhibitor (Figure 1).

Since it appeared that insulin stimulated the phosphorylation of ERK5 and I κ B α , we hypothesized that insulin would also stimulate the nuclear import of ERK5 and NF κ B. We observed that insulin-stimulated phosphorylation of ERK5 and I κ B α resulted in nuclear import of ERK5 and NF κ B with a concomitant decrease in their cytoplasmic concentrations, indicating activation of these two transcription factors (Figure 2). Similar to the relative time frames of ERK5 and I κ B α phosphorylation, nuclear import of ERK5 preceded that seen for NF κ B.

We next wanted to determine the role ERK5 played in regulating insulin-induced NF κ B phosphorylation and activation. Using the time frame for ERK5 activation (i.e., ERK5 phosphorylation) we immunoprecipitated activated ERK5 (i.e., phospho-ERK5), using antibodies towards phospho-ERK5 and immunoblotted for ERK5, NF κ B, I κ B α , I κ B β , IKK α , IKK β and IKK γ (i.e., NF κ B essential modulator (NEMO)). The only molecule that immunoprecipitated with phospho-ERK5 was IKK α (Figure 3).

In order to verify that ERK5 was in fact responsible for interacting with IKK α and regulating NF κ B activity, we employed the use of small inhibitory RNA (siRNA) for ERK5 (siERK5). First, we demonstrated that introduction of a

scrambled (sc) non-inhibitory siERK5 did not decrease ERK5 content in the cells. However, two different active small inhibitory siERK5, si(1)ERK5 and si(2)ERK5, decreased ERK5 content 80% and 70%, respectively, 24 hours after transfection (Figure 4A). In contrast, neither scERK5 nor the two active siERK5s affected ERK1/2 protein content, indicating that the presence of siERK5 significantly ($P < 0.05$) decreased the presence of endogenous and physiologic ERK5, but had no effect on ERK1/2 protein. Second, using si(1)ERK5 in cells pre-incubated with Wortmannin and treated with insulin, we immunoprecipitated total IKK α protein and immunoblotted for the phosphorylated IKK α and total IKK α . In the presence of si(1)ERK5 a significant ($P < 0.05$) decrease in phosphorylation of IKK α was observed as compared to that seen in the presence of scERK5 (Fig 4B).

Following this pathway downward, RPHN transfected with active si(1)ERK5 demonstrated significantly ($P < 0.05$) decreased insulin-stimulated NF κ B-I κ B α dissociation (Figure 5A) and I κ B α phosphorylation (Figure 5B). Furthermore, the presence of si(1)ERK5 inhibited translocation (Figure 6A) and transactivation (Figure 6B) of NF κ B. In a reciprocal set of experiments, we transfected RPHN with active siIKK α , treated the cells with Wortmannin and insulin as before and noted no phosphorylation or transactivation of NF κ B (data not shown).

We then examined the possibility that inhibition of ERK5 activity resulted in decreased insulin-stimulated A β protein production. Paris et al²³ demonstrated that various inhibitors of

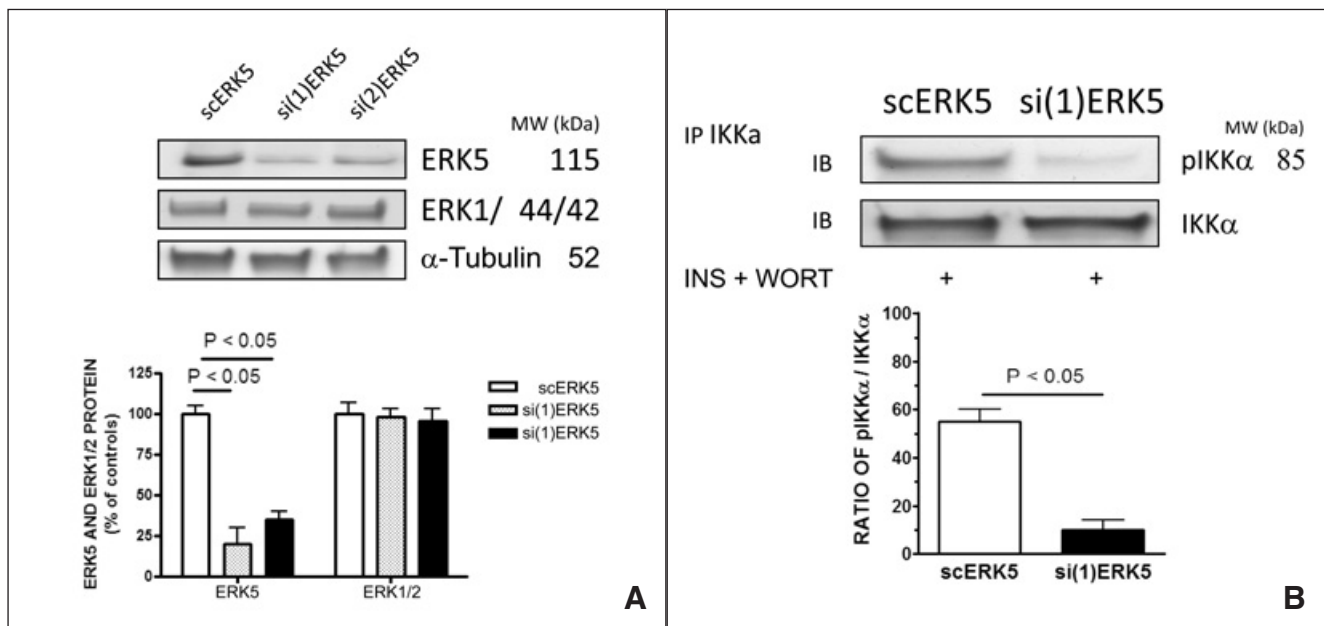


Figure 4: Small inhibitory ERK5 (siERK5) downregulates expression of endogenous ERK5 and inhibits insulin-induced phosphorylation of IKK α . Scrambled (scERK5) or active (siERK5) were transiently transfected into RPHN (see Methods section). Twenty-four hours later the amount of total ERK5, ERK1/2, pIKK α and IKK α content was determined. (A) Representative Western blot analyses of ERK5 and ERK1/2 in RPHN 24 h after transfection of either scERK5, si(1)ERK5 or si(2)ERK5. Alpha-tubulin was used for loading controls. Amounts of total ERK5 and ERK1/2 proteins are expressed as percent of controls (scrambled siERK5) and represent the mean \pm S.E. of four independent experiments. *, $P < 0.05$. (B) Representative Western blot profiles of phosphorylated and total IKK α protein in RPHN transfected with scERK5 or si(1)ERK5 and then pre-treated with Wortmannin (WORT) (1 μ M) for one h followed by 15 minutes of insulin (INS) (1 nM). Changes in phosphorylated IKK α protein are expressed as ratios of phosphorylated IKK α to total IKK α and represent the mean \pm S.E. for each condition. $n = 4$; *, $P < 0.05$ versus controls (scERK5).

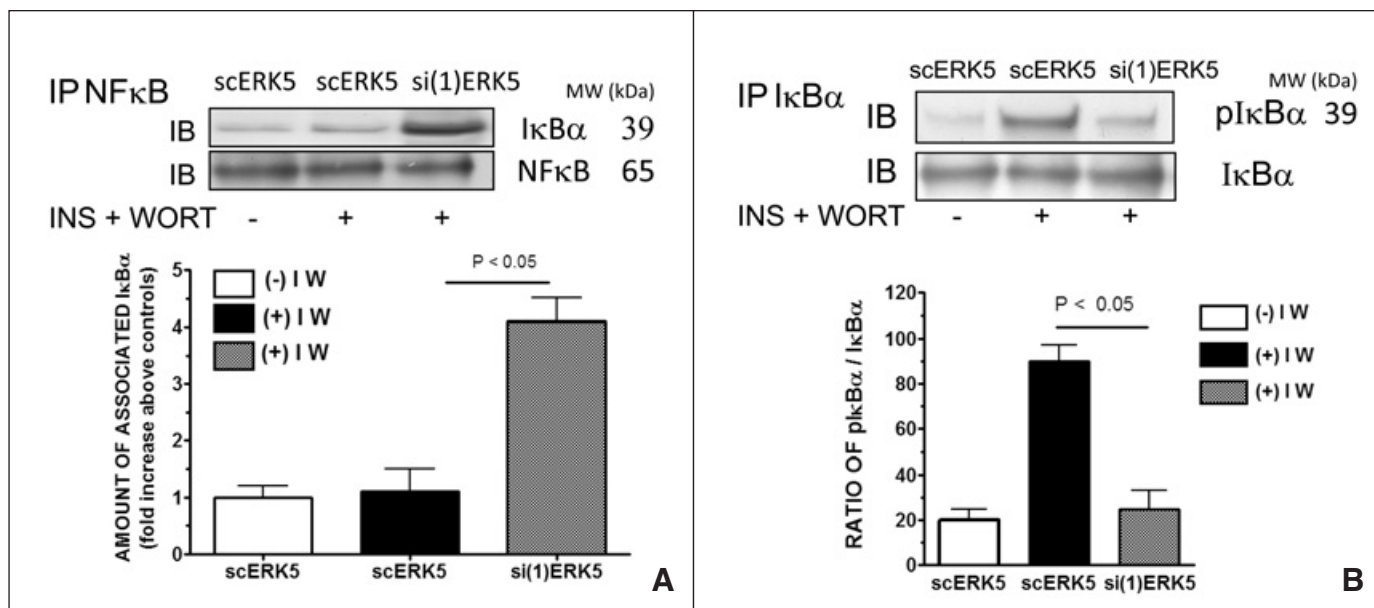


Figure 5: Small inhibitory ERK5 (siERK5) inhibits insulin-stimulated phosphorylation of IκBα and its dissociation from NFκB. Neurons transfected with either scERK5 or si(1)ERK5 were cultured in serum-free medium, pre-treated without or with Wortmannin (WORT)(1 μM) for one hour and then incubated for 45 min in serum-free medium containing insulin (INS)(1 nM) or no insulin. (A) Representative profiles of Western blots analyses of amounts of NFκB-associated IκBα protein in cells transfected with control, scERK5, or active, si(1)ERK5. Nuclear factor kappa-B was immunoprecipitated (IP) followed by immunoblotting (IB) of IκBα and NFκB. Changes in amount of IκBα protein associated with NFκB are expressed in fold increase above controls (no insulin or Wortmannin with scERK5) and represent the mean ± S.E. of five independent experiments. *, *P* < 0.05 vs controls. (B) Profiles are representative Western blot analyses of phosphorylated and total IκBα proteins in RPHN transfected with scrambled, scERK5, or active, si(1)ERK5 in the presence or absence of Wortmannin (WORT)(1 μM) and without or with insulin (INS)(1 nM). Changes in phosphorylated IκBα are expressed in ratios of phosphorylated IκBα to total IκBα and represent the mean ± S.E. for each condition. *n* = 3; *, *P* < 0.05 versus controls (no insulin, no Wortmannin and scERK5). Student's *t* test.

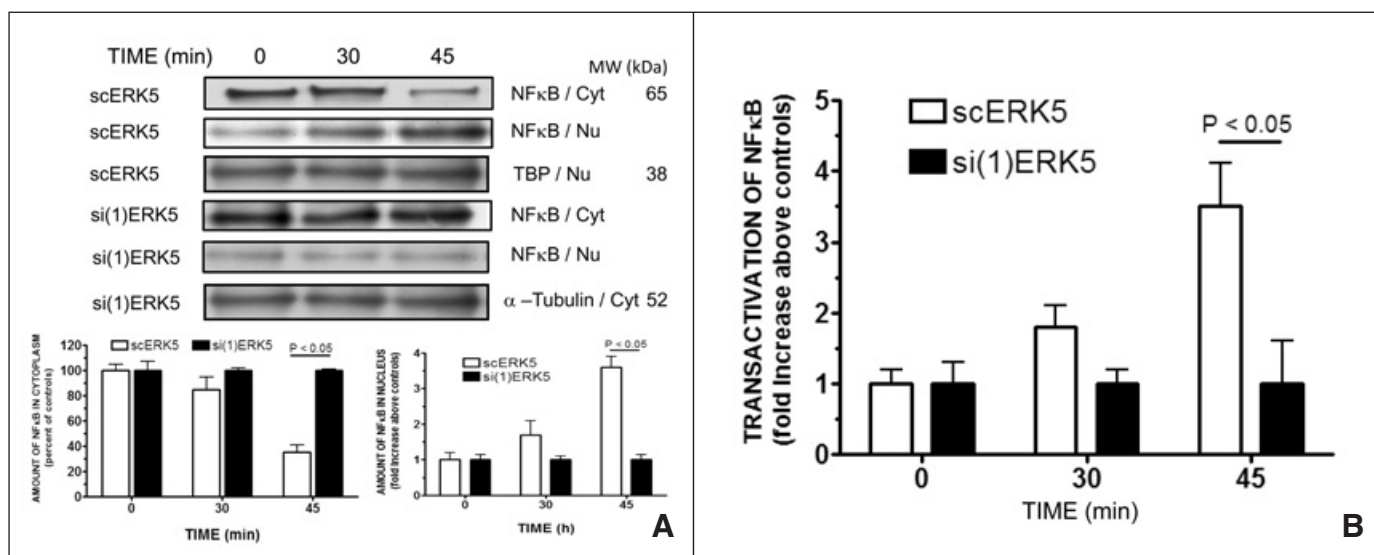


Figure 6: siERK5 inhibits insulin-stimulated NFκB nuclear import and transactivation. Neurons transfected with scERK5 or si(1)ERK5 were pre-treated without or with Wortmannin (1 μM) for one hour then treated without or with insulin (1 nM) for indicated times. Cytoplasmic (Cyt) and nuclear (Nu) amounts of total NFκB were determined over time. (A) Representative Western blot analyses of total NFκB protein in cytoplasmic (Cyt) and nuclear (Nu) fractions with respect to time are shown. TATA Binding Protein (TBP) and α-tubulin were also analyzed in order to demonstrate equal loading of lanes for nuclear and cytoplasmic fractions. Changes in amount of cytoplasmic and nuclear NFκB in cells transfected with scERK5 or si(1)ERK5 are expressed in percent of controls or fold increase above controls (zero time, no insulin, no Wortmannin) and represent the mean ± S.E. of six independent experiments. (B) Changes in amounts of transactivated NFκB protein were determined in transfected cells pretreated without or with Wortmannin (1 μM) for one h followed by treatment with insulin (1 nM) or serum-free medium alone for indicated times, using the Trans-AM™ methodology as described in the Methods section. Transactivated NFκB are expressed in fold increase above controls (zero time, no insulin, no Wortmannin) and represent the mean ± S.E. of each time point and condition. *n* = 3. Student's *t* test.

NF κ B activity blocked secreted A β 1-40 and A β 1-42 production in CHO cells. We wanted to examine the effect of si(1)ERK5 on A β production in RPHN treated with insulin and Wortmannin. RPHN transfected with si(1)ERK5 exhibited moderate, but statistically significant ($P < 0.05$) decreased secreted A β 1-40 and A β 1-42 production from cells treated with insulin and Wortmannin as compared to cells transfected with scERK5 (Figure 7).

Finally, we were interested in the role ERK5 played in insulin-stimulated hyperphosphorylation of tau. Insulin stimulated increased phosphorylation of tau as seen by its retarded profile, whereas in the presence of si(1)ERK5 insulin was without this effect (Figure 8).

DISCUSSION

The goal of this study was to determine the role of ERK5 regulation of NF κ B in rat primary hippocampal neurons (RPHN) in the context of insulin resistance. Since NF κ B plays an important role in inflammatory processes associated with AD and diabetes, and insulin plays an important role in neuronal physiology, the dysregulation of insulin signaling in neurons may be a key factor in cellular events that lead to increased NF κ B activity and the sequelae of events that may contribute to the pathologic characteristics of AD.

Nuclear factor kappa-B (NF κ B) plays a major role in the pathogenesis of AD and diabetes^{5,24}. Nuclear factor kappa-B

activity is strongly correlated with hyperphosphorylated tau and the presence of insoluble amyloid-beta (A β) protein in AD as well as the inflammatory aspects associated with atherosclerosis^{23,25}. The activity of NF κ B appears to be regulated in part by the activities of the serine/threonine kinase molecules, phosphatidylinositol 3-kinase (PI3K) and Akt¹⁵. Regulation of NF κ B by upstream activators is important to normal neuronal activity and the balance between normal and pathologic neuronal function lies, in part, within the tight regulation of NF κ B activity. Although regulation of NF κ B has been well studied, not all of the molecular mechanisms that regulate NF κ B activity have been elucidated.

Nuclear factor kappa-B is a family of dimeric transcription factors that consists of a combination of two of the subunits RelA/p65, p50, p52, c-Rel, and RelB (reviewed in²⁶). When inactive, NF κ B proteins are found largely in the cytoplasm and associated with one of three Inhibitory kappaB (I κ B) proteins: I κ B α , - β , or - ϵ ^{27,28}. Activation of NF κ B is caused by a variety of proinflammatory and noxious stimuli that stimulate the dissociation of the I κ B protein from NF κ B²⁹. Dissociation of the I κ B protein from NF κ B occurs as a result of phosphorylation of the I κ B protein, followed by ubiquitin-induced proteasome-mediated I κ B degradation³⁰. Phosphorylation and subsequent dissociation of the I κ B protein from NF κ B are, in turn, regulated by the upstream I κ B Kinase (IKK) proteins, IKK α - or IKK β -NEMO³¹. Inhibitor of kappa-B kinases (IKK) proteins are

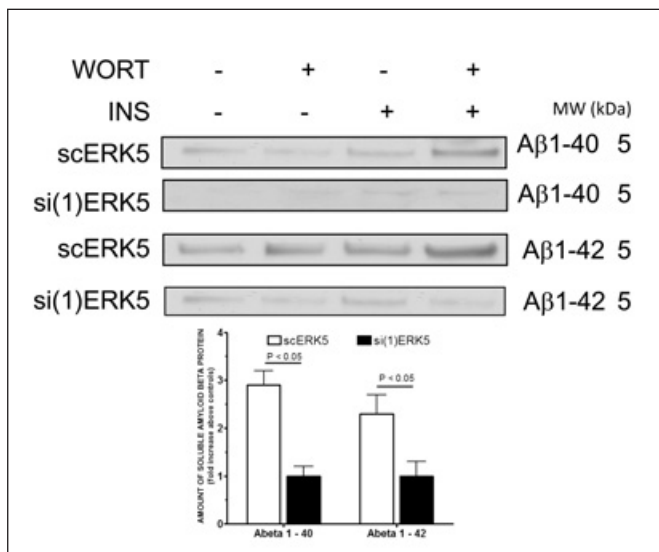


Figure 7: siERK5 inhibits insulin-induced secretion of amyloid beta protein from RPHN. Neurons transfected with scERK5 or si(1)ERK5 were incubated in serum-free medium without or with Wortmannin (W) (1 μ M) for one h and then insulin (INS) (1 nM) or serum-free medium for 24 h. Medium was collected, concentrated and A β 1-40 and A β 1-42 soluble amyloid proteins were immunoprecipitated as described in the Methods section. Representative Western blot analyses are of total soluble amyloid protein before and after Wortmannin and insulin treatment. Changes in the amounts soluble amyloid protein are expressed in fold increase above controls (zero time, no insulin, no Wortmannin) and represent the mean \pm S.E. of four separate experiments. Student's *t* test.

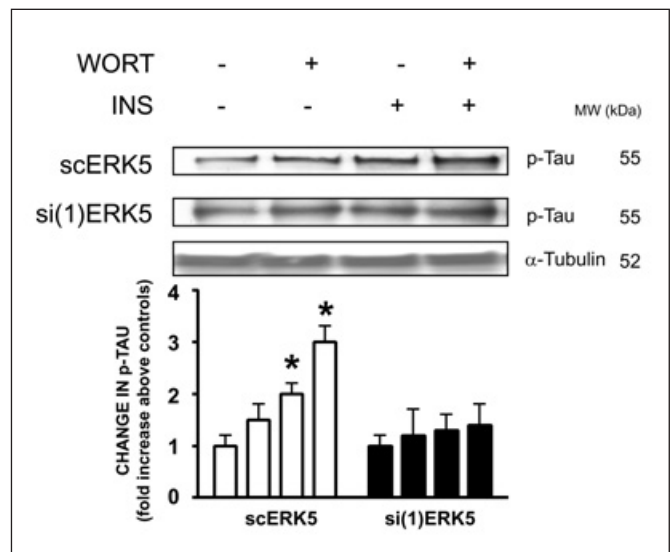


Figure 8: siERK5 inhibits insulin-induced hyperphosphorylation of tau in the context of insulin resistance. Neurons transfected with scERK5 or si(1)ERK5 were incubated in serum-free medium without or with Wortmannin (1 μ M) for one h and then insulin (1 nM) or serum-free medium for one h. (Upper Panel) Figure depicts a representative Western blot analysis of tau protein before and after Wortmannin and insulin treatment and α -tubulin as a loading control. (Lower Panel) Changes in the amounts of phosphorylated tau protein are expressed in fold increase above controls (zero time, no insulin, no Wortmannin) and represent the mean \pm S.E. of five separate experiments. *, $P < 0.05$. One way ANOVA with Tukey post test.

themselves activated by upstream kinases such as Akt and the well known Tumor Necrosis Factor Receptor Associated Factor (TRAF) family members^{32,33}.

We first determined that insulin stimulated both ERK5 and IκBα phosphorylation in RPHN and continued these effects even in the presence of the PI3K inhibitor Wortmannin; demonstrating that insulin could stimulate ERK5 and IκBα via a PI3K/AKT-independent process. Interestingly, insulin-stimulated phosphorylation of ERK5 increased at 15 minutes and then subsided at 45 minutes, whereas phosphorylation of IκBα did not occur until 30 minutes later. These two pieces of information suggest: (1) that insulin activates ERK5 for a short period of time and then there is a down regulation of ERK5 activation, possibly by ER5-specific phosphatases, and (2) that activation of ERK5 does not immediately activate IκBα, indicating intermediates between ERK5 and IκBα. The latter suggestion points to IKKα

as a potential mediator of ERK5-stimulated activation of IκBα. Yet, in reference to the former suggestion, it is not clear as to why ERK5 phosphorylation is transient even in the context of insulin resistance. Obviously further examination of this issue is warranted.

We then followed these experiments with translocation studies to determine that insulin-stimulated phosphorylation of ERK5 and IκBα resulted in nuclear import of both ERK5 and NFκB in RPHN; an indication of insulin's part in the activation and canonical nuclear import of these two transcription factors. Interestingly, the temporal sequence of nuclear import of ERK5 and NFκB matched that seen for insulin-stimulated ERK5 and IκBα phosphorylation; that is, ERK5 nuclear import preceded that of NFκB, indicating that activation of ERK5 occurred before that seen for NFκB.

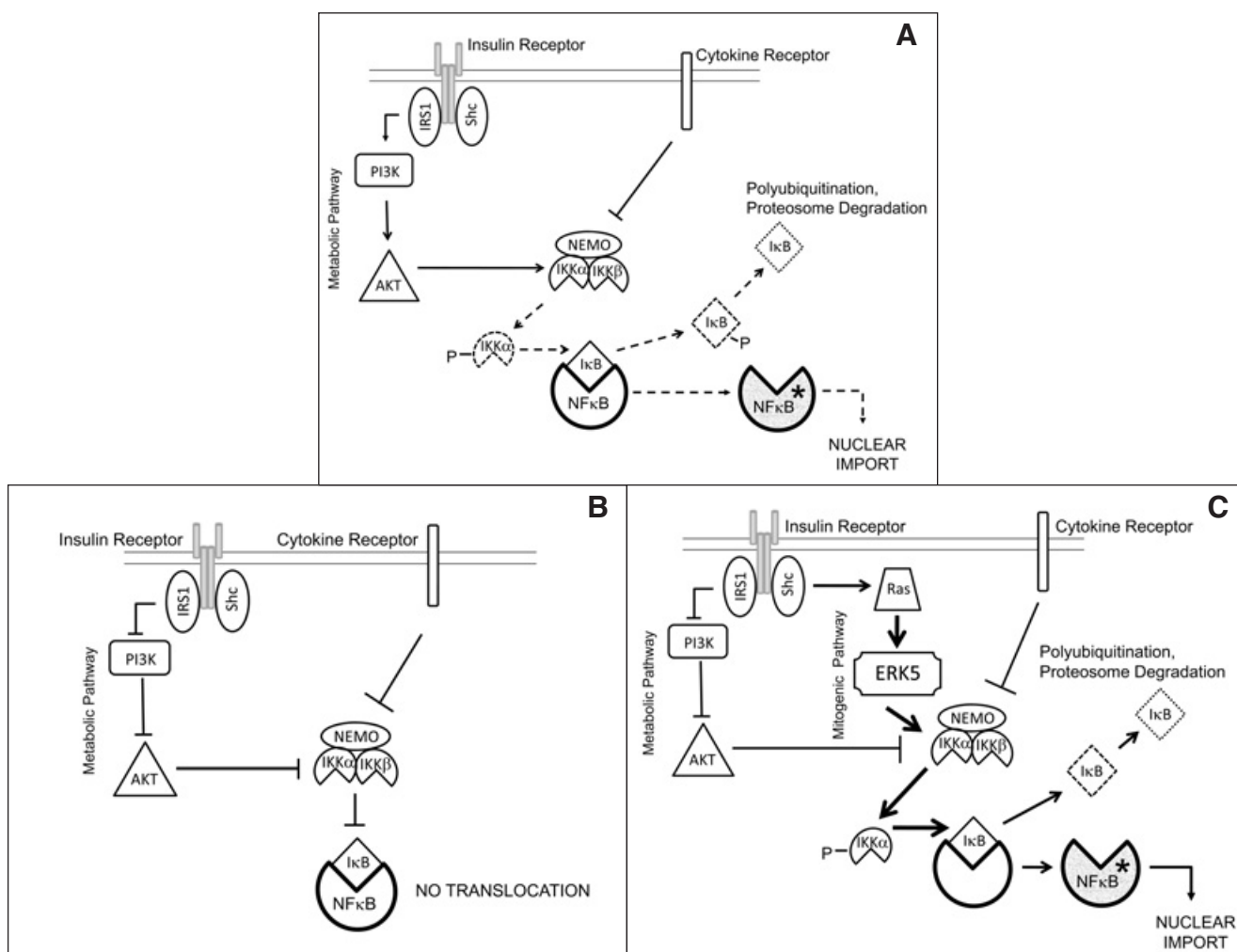


Figure 9: Model of insulin-stimulated NFκB activation via ERK5 in the context of insulin resistance. (A) In normal physiologic conditions the activation and nuclear import of NFκB is modulated by signals from insulin and other cytokines that interact with neurons. (B) In the presence of insulin resistance, insulin's metabolic pathway is perturbed and thus intracellular signaling to NFκB would appear to be decreased. (C) However, even in the context of insulin resistance, concomitant hyperinsulinemia signaling is still transduced within RPHN via a mitogenic pathway of insulin, mediated in part by ERK5, resulting in the phosphorylation and activation of IKKα, which in turn phosphorylates IκBα, leading to NFκB dissociation and nuclear import.

We next began to determine the mechanism by which ERK5 regulated NF κ B activity. It has been demonstrated in several studies that NF κ B is stimulated as a downstream event of both the activation of the IKK complex and subsequent phosphorylation and ubiquitination/degradation of I κ B α ³². Although it has been shown that the Toll-like Receptor (TLR) signaling family can activate the IKK complex via Tumor Necrosis Factor (TNF) receptor associated factor/Transforming Growth Factor-beta activated kinase (TRAF/TAK) pathway, others have indicated that non-TRAF-mediated kinases may be involved, especially in reference to other cytokine-stimulated pathways³³. We hypothesized that one of these other kinases is the MAP kinase ERK5, which is a newly characterized MAP kinase associated with insulin signaling.

Extracellular Signal-regulated Kinase-5 appears to play an important role in proliferation, differentiation and neuronal survival³⁴⁻³⁷. Although ERK5 has been classified as a mitogen-activated protein kinase (MAPK) its function within cells has not been fully elucidated. Similar to ERK1/2, translocation of ERK5 from the cytoplasm to the nucleus occurs as a result of extracellular cytokine stimulation. Yet, unlike ERK1/2, whose major transcription factor targets are Elk1 and c-fos; ERK5 targets Myocyte Enhancer Factor-2C (MEF2C)³⁷⁻³⁸. Here we show for the first time that ERK5 regulates NF κ B activity by targeting IKK α .

It has long been understood that insulin signals to NF κ B in part via the PI3K/Akt pathway (Figure 9A). Many have postulated that disturbances in the PI3K/Akt pathway would decrease insulin's actions on NF κ B (Figure 9B). Our findings set aside this theory since it appears that even in the context of insulin resistance (i.e., dysregulation of the PI3K/Akt pathway) activation of the inflammatory NF κ B pathway still occurs, and may, to a greater extent, be mediated via the insulin-stimulated ERK5-IKK α molecular interaction (Figure 9C).

Hyperinsulinemia occurs in the presence of insulin resistance. It is this condition of higher than physiologic levels of insulin that cells become primed to be more responsive to other growth factors and cytokines and hence increased NF κ B activity⁹⁻¹². This "priming" mechanism may be one explanation as to why some have proposed a correlation between diabetes and AD^{13,14}.

The association between diabetes and AD is a hotly debated topic. On one side many reviews and studies have stated that diabetes is a risk factor for AD and the incidence of hyperinsulinemia and insulin resistance plays a key role in the pathogenesis of AD^{13,39,40}. In contrast some have stated that diabetes is a risk factor for vascular dementia, but not for AD per se⁴¹. More research is needed in this field in order to better understand the pathogenesis of AD at the cellular and tissue level. The use of transgenic mouse models of AD and diabetes will not only help determine new pathways and interactions within neurons that contribute to the AD phenotype, but also assist in discovering molecular targets and therapeutic strategies to stave off this insidious disease.

Our future studies will focus on changes in (1) amyloid precursor protein levels, (2) amyloid precursor mRNA levels, and (3) beta and gamma secretase activity; all in the context of insulin resistance and hyperinsulinemia.

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