Insulin Promotes Neuronal Survival via the Alternatively Spliced Protein Kinase C\(\alpha\)II Isoform*

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**Background:** Alternatively spliced PKC\(\alpha\)II is a pro-survival protein.

**Results:** Insulin regulates alternative splicing of PKC\(\alpha\)II pre-mRNA, which promotes Bcl2 and bcl-xL expression.

**Conclusion:** PKC\(\alpha\)II is a key regulator of insulin-mediated neuronal survival.

**Significance:** Elucidation of the molecular mechanisms by which insulin promotes survival in neuronal cells is necessary to understand how intranasal insulin improves cognition.

Insulin signaling pathways in the brain regulate food uptake and memory and learning. Insulin and protein kinase C (PKC) pathways are integrated and function closely together. PKC activation in the brain is essential for learning and neuronal repair. Intranasal delivery of insulin to the central nervous system (CNS) has been shown to improve memory, reduce cerebral atrophy, and reverse neurodegeneration. However, the neuronal molecular mechanisms of these effects have not been studied in depth. PKC\(\alpha\) plays a central role in cell survival. Its splice variants, PKC\(\alpha\)I and PKC\(\alpha\)II, are switches that determine cell survival and fate. PKC\(\alpha\)I promotes apoptosis, whereas PKC\(\alpha\)II promotes survival. Here, we demonstrate that insulin promotes alternative splicing of PKC\(\alpha\)II isoform in HT22 cells. The expression of PKC\(\alpha\)II splice variant remains unchanged. Insulin increases PKC\(\alpha\)II alternative splicing via the PI3K pathway. We further demonstrate that Akt kinase mediates phosphorylation of the splicing factor SC35 to promote PKC\(\alpha\)II alternative splicing. Using overexpression and knockdown assays, we demonstrate that insulin increases expression of Bcl2 and bcl-xL via PKC\(\alpha\)II. We demonstrated increased cell proliferation and increased BrdU incorporation in insulin-treated cells as well as in HT22 cells overexpressing PKC\(\alpha\)II. Finally, we demonstrate in vivo that intranasal insulin promotes cognitive function in mice with concomitant increases in PKC\(\alpha\)II expression in the hippocampus. This is the first report of insulin, generally considered a growth or metabolic hormone, regulating the alternative isoform expression of a key signaling kinase in neuronal cells such that it results in increased neuronal survival.

Strong links between insulin and cognitive function are supported by epidemiological data, studies of laboratory animals, and in vitro research (1–3). Intranasal delivery of insulin to the central nervous system (CNS) has been shown to improve memory, reduce cerebral atrophy, and reverse neurodegeneration caused by apoptosis (4–7). However, the neuronal molecular mechanisms of these effects have not been studied in depth. Insulin signaling in the CNS is important in learning, memory, and synaptogenesis and activates survival pathways in neurons. Protein kinase C (PKC) isoforms are serine/threonine kinases, which are involved in the regulation of cellular differentiation, growth, and apoptosis. The insulin and PKC pathways are integrated and function closely together. PKC activation in the brain is essential for learning, synaptogenesis, and neuronal repair (8–10). Insulin signaling pathways activate PKC and its substrates, many of which are essential components of learning, memory, and cognition. In particular, PKC\(\delta\) (PRKCD) has been implicated in memory, neuronal survival, and proliferation (11–13). PKC\(\delta\) improves learning and memory by preventing neuronal loss and maintaining the synapses (14). PKC\(\delta\) plays a central role in apoptosis and has dual effects: as a mediator of apoptosis and as a pro-survival effector (15–19). Our previous work demonstrated that the mouse splice variants, PKC\(\alpha\)I and PKC\(\alpha\)II, are a switch that determines cell survival and fate. PKC\(\alpha\)I promotes apoptosis, whereas PKC\(\alpha\)II promotes survival. PKC\(\alpha\)II is the mouse homolog of human PKC\(\alpha\)II; both are generated by alternative 5′ splice site usage, and their transcripts share >94% sequence homology. We have shown that PKC\(\alpha\)I and PKC\(\alpha\)II function as pro-survival proteins (20, 21); the functions of the other PKC\(\delta\) splice variants (PKC\(\delta\)III, -IV, -V, -VI, or -VII) are not yet established. A recently reported mouse splice variant, PKC\(\delta\)IX, is described as a dominant-negative inhibitor of the apoptotic property of PKC\(\delta\) in vivo (22). PKC\(\delta\)II is generated by the insertion of 78 bp (26 amino acids) via utilization of an alternative downstream 5′ splice site of PKC\(\delta\) pre-mRNA exon 9 in its caspase-3 recognition sequence (DILD). Previously, we showed that overexpression of PKC\(\delta\)II decreased apoptosis and promoted survival (20). It is established that insulin improves cognition and that neuronal survival is critical for promoting cognition and memory formation. However, the molecular determinants of insulin-mediated neuronal survival remain unknown. Here, we established the link between intranasal alternative splicing of

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a key signaling kinase, PKC\(\delta\), and increased neuronal survival, which ultimately improves cognitive function. We also demonstrate that PKC\(\text{II}\) is upstream of the Bcl2-mediated survival cascade.

EXPERIMENTAL PROCEDURES

Cell Culture—The studies were carried out using an immortalized clonal mouse hippocampal cell line (HT22) obtained from Dr. D. R. Schubert (Salk Institute). HT22 cells were cultured in 75-cm\(^2\) flasks in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin (50 units/ml), and 2 mM glutamine. Cells were maintained at 37 °C in a humidified incubator containing 5% CO\(_2\). HT22 cells were sub-cultured into either 25-cm\(^2\) flasks or 100-mm\(^2\) dishes and used for experiments at 60—80% confluence.

Western Blot Analysis—Cell lysates (60 mg) were separated on 10% PAGE-SDS. Proteins were electrophoretically transferred to nitrocellulose membranes, blocked with Tris-buffered saline/0.1% Tween 20 containing 5% nonfat dried milk, washed, and incubated with a polyclonal antibody against either anti-PKC\(\delta\) (BioSource) or PKC\(\text{II}\)-specific polyclonal antibody (Patel laboratory). PKC\(\text{II}\)-specific antibody was raised against the amino acids of the extended hinge region of mouse PKC\(\text{II}\). This region is absent in PKC\(\delta\). PKC\(\delta\)-specific polyclonal antibody was raised in rabbits by Bio-Synthesis, Inc. (Lewisville, TX) to the synthetic peptide NH\(_2\)-HISLKSFSRAREKDDSET-COOH (corresponding to the V3-hinge domain of PKC\(\text{II}\)). The antibody specificity was confirmed by ELISA with the above epitope region peptide and was characterized alongside unreactive pre-immune antisera and shown to recognize PKC\(\text{II}\) in mouse HT22 cells. A peptide/antigen assay was also performed to confirm its specificity. Other antibodies, anti-Bcl2, anti-Bcl-xl, anti-pBAD, anti-PARP, anti-XIAP, and anti-GAPDH, were purchased from Cell Signaling. Following incubation with anti-rabbit IgG-HRP, enhanced chemiluminescence (Pierce) was used for detection.

Transient Transfection of Plasmid DNA—2 \(\mu\)g of PKC\(\text{II}\)-pTracer/CMV and PKC\(\text{II}\)-pTracer/CMV plasmids (from Dr. Harutoshi Kizaki, Tokyo Dental College, Japan) were transfected into HT22 cells using a Nucleofector kit from Amaxa. 10\(^6\) HT22 cells were combined with 100 \(\mu\)l of containing the insulin (or saline) was administered as described by previous studies (4,23), which established pharmacokinetic measurements, optimum concentrations, and uptake and demonstrated that intranasal insulin directly improves learning and memory and has no effect on circulating blood glucose levels. Briefly, mice are administered intranasal insulin (Humulin R, Eli Lilly, 100 units/ml) at final concentrations of 1.0 units/ml or 1% intranasal saline (control). A total of 24 \(\mu\)l of containing the insulin (or saline) was administered as four 6-\(\mu\)l drops in alternating nostrils every minute while each mouse was held in supine position with its neck in extension. Mice were administered intranasal insulin once daily for 4 weeks. Mice were tested in a radial arm water maze to assess their cognitive functions. At the end of the trials, the hippocampi of the mice were collected for RNA and protein analysis.

Radial Arm Water Maze Testing—An aluminum insert was extended 5 cm above the surface of the water, allowing the mice to easily view surrounding visual cues, which were generously placed outside of the pool. Visual/spatial cues consisted of large, brightly colored objects, including a beach ball, poster, and inflatable pool toys. In one arm, a transparent 9-cm escape platform was placed 1.5 cm below the surface of the water near the wall end. Each mouse was given thirteen 1-min trials per day. The last of 12 consecutive acquisition trials (Trial 12) and a
60-min delayed retention trial (Trial 13) are indices of working memory. The next day (24 h later), the mice were given the trial once again to measure the long term memory retention. The escape platform location was placed at the end of one of the six arms and was moved to a different arm in a semi-random fashion for each day of testing. Moving the escape platform forces the mouse to learn a new platform location daily and therefore evaluates working memory. On each day, different start arms for each of the daily trials were selected from the remaining five swim arms in a semi-random sequence that involved all five arms. For any given trial, the mouse was placed into the start arm, facing the center swim area, and given 60 s to find the platform. When the mouse made an incorrect choice, it was gently pulled back to the start arm, and an error was recorded. An error was also recorded if the mouse failed to make a choice in 20 s (in which case it was returned to the start arm) or entered the platform-containing arm but failed to locate the platform. After finding the platform, the mouse was allowed to stay on it for 30 s. If the mouse did not find the platform within 60 s, it was guided to the platform, allowed to stay for 30 s, and assigned a latency of 60 s. Errors (incorrect arm choices) and escape latency were recorded for each daily trial.

Cell Viability and Proliferation Assays—HT22 cells were assessed for cell viability and proliferation. HT22 cells were either treated with insulin or transfected with PKC\textsuperscript{II} plasmid or PKC\textsuperscript{II} siRNA as described above. The treatments were performed in triplicate in a 48-well plate. The 5-bromo-2′-deoxyuridine (BrdU) cell proliferation assay kit was purchased from Millipore, catalogue no. 2750, and used as per manufacturer’s instructions to quantitatively evaluate the number of actively proliferating cells. Briefly, 100 \( \mu L \) of BrdU was added per well of the 48-well plate and incubated overnight. BrdU incorporation was detected using peroxidase conjugate. The plate was read using a spectrophotometer microplate reader set at a dual wavelength of 450 nm/550 nm. The results were normalized against the blank and background readings. The cell proliferation kit was purchased from Chemicon International, catalogue no. 2210, and uses WST-1/ECS as per the manufacturer’s instructions. The formazan dye produced by viable cells is quantified using a spectrophotometer set at a wavelength of 440 nm.

Statistical Analysis—The significance for the animal studies was assessed by the matched Student’s \( t \) test in which the mice cohort was \( n = 6 \) per treatment for every time trial. A level of \( p < 0.05 \) was considered statistically significant. The gels were densitometrically analyzed using UN-SCAN-IT\textsuperscript{TM} software (Silk Scientific, Inc.). PRISM\textsuperscript{TM} software was used for statistical analysis. The results are expressed as mean \( \pm \) S.E. of densitometric units or as a percentage of exon inclusion.

RESULTS

Insulin Increases PKC\textsuperscript{II} Expression—Because the hippocampus is critical for memory and cognition, we used the immortalized mouse hippocampal cell line HT22 for our studies. These cells have been used to study neuronal survival and are established as an \textit{in vitro} model for mechanistic studies for neurodegenerative diseases such as Alzheimer disease (24–26). PKC\textsuperscript{I} and PKC\textsuperscript{II} are alternatively spliced variants of PKC\textsuperscript{I} pre-mRNA; PKC\textsuperscript{I} is pro-apoptotic, while PKC\textsuperscript{II} promotes survival. Insulin was added in increasing doses (10 to 100 nM) to HT22 cells for 24 h. Total protein lysates were harvested, and Western blot analysis was performed with a PKC\textsuperscript{II}-specific antibody raised by our laboratory (see “Experimental Procedures”) or a COOH-terminal PKC\textsuperscript{II} antibody, which recognizes both variants. In separate experiments, total RNA was extracted and analyzed by RT-PCR with primers that detect PKC\textsuperscript{I} and PKC\textsuperscript{II} simultaneously. Insulin increased PKC\textsuperscript{II} expression, whereas PKC\textsuperscript{I} levels remained constant (Fig. 1, \( a \) and \( b \)). In subsequent experiments, the lowest dose (10 nM) of insulin was used. This is the first report demonstrating that insulin regulates PKC\textsuperscript{II} pre-mRNA alternative splicing in neuronal cells.

Insulin Mediates Its Effect via a PI3K Pathway—Tyrosine phosphorylation of the insulin receptor and its downstream substrates initiates a series of events through tyrosine and serine/threonine kinases that mediate the effects of insulin. Insulin may mediate its effects by activating kinase pathways such as the mitogen-activated protein kinase pathway, the Janus-activated kinase/signal transducers and activators of transcription pathway, or the phosphatidylinositol 3-kinase (PI3K) pathway. To determine which pathway is involved in insulin-mediated PKC\textsuperscript{II} alternative splicing in neuronal cells, we used inhibitors to these pathways. Inhibitors were added 30 min before insulin (10 nM) treatment. The PI3K inhibitor LY294002 (1 \( \mu M \)) blocked the insulin-mediated increase in PKC\textsuperscript{II}, whereas the mitogen-activated protein kinase inhibitor PD98059 (10 \( \mu M \)) did not (Fig. 2a). Rapamycin, a p70/S6 kinase inhibitor, had no effect (data not shown), and the Janus-activated kinase inhibitor AG490 decreased PKC\textsuperscript{II} by 5%.

Because Akt kinase is a key downstream mediator of the PI3K pathway, we designed experiments to assess its phosphorylation state. HT22 cells were treated with LY294002 before addition of insulin. Insulin increased the phosphorylation of Akt kinase (Ser-473) and simultaneously increased PKC\textsuperscript{II} levels; the latter increase was inhibited by LY294002, and total Akt levels remained the same (Fig. 2b). These findings indicate that insulin promotes alternative splicing of PKC\textsuperscript{II} via the PI3K-Akt pathway.

Insulin Increases Alternative Splicing of PKC\textsuperscript{II} Pre-mRNA via Phosphorylation of Splicing Factor SC35—In pre-mRNA, sequences exist as auxiliary cis-elements that recruit \textit{trans}-acting factors to promote alternative splicing. Exonic or intronic splicing enhancers often bind serine/arginine-rich nuclear factors (SR proteins)\textsuperscript{2} to promote the choice of splice sites in the pre-mRNA. The binding of SR proteins to exonic or intronic sites determines splice site choice. The mouse splice variant PKC\textsuperscript{II} is generated by use of an alternative downstream 5′ splice site in exon 9 of PKC\textsuperscript{I} pre-mRNA. Therefore, we sought to identify the \textit{trans}-factors that bind to the PKC\textsuperscript{II} pre-mRNA upon insulin treatment and that influence splice site use.

HT22 cells were treated with insulin (10 nM) for 24 h, and lysates were analyzed by Western blotting with mAb104. Used extensively by RNA biologists, this antibody recognizes the common epitope of all SR proteins and can detect them simul-

\textsuperscript{2}The abbreviations used are: SR, serine/arginine-rich; RS, arginine/serine-rich; CA, constitutively active; PARP, poly-(ADP-ribose)polymerase.
Insulin increased the expression of a protein of ~30 kDa, either SRp30a or SRp30b. Using specific antibodies to individual SR proteins, we observed an increase in SRp30b (SC35) expression concurrent with increased PKCδII levels in response to insulin; SRp30a (SF2/ASF) levels were unaffected (Fig. 3a). Hence, SC35 was the logical factor to pursue.
SC35 has an NH₂-terminal RNA recognition motif domain and a COOH-terminal arginine-serine-rich (RS) domain. The RNA recognition motif domain interacts and binds to the target pre-mRNA, whereas the RS domain is highly phosphorylated and is the protein interaction region. Phosphorylation of SR proteins may result in their differential distribution to alternate sites. Splicing factors are phosphorylated on their RS domain, and the phosphorylation state (hypo- or hyper-) also affects splice site selection. SC35 has several AKT motifs (RXX(S/T)) in its RS domain (Fig. 3b). In separate experiments, HT22 cells were treated with insulin and an AKT inhibitor (124005, Calbiochem), which inhibits all Akt isoforms. Inhibition of AKT prevented insulin-mediated increase of alternative splicing of PKCδII mRNA (Fig. 3c). A slight decrease of PKCδI was also observed, and the graph reflects the percent PKCδII exon inclusion for each lane and may be attributed to Akt being the central kinase mediating splicing events. Our previous publications and several other laboratories have shown that Akt2 kinase is central to insulin signaling and phosphorylation of SR proteins in insulin-mediated alternative splicing. Akt2 kinase

**FIGURE 3.**

a, insulin increases alternative splicing of PKCδII mRNA via SC35. HT22 cells were treated with increasing doses (10, 50, and 100 nM) of insulin for 24 h or left untreated (control). Whole protein lysates were separated and analyzed by Western blot analysis using either mAb104, PKCδII, SF2/ASF, or SC35 antibody as indicated. The gels represent three experiments performed separately with similar results. b, SC35 protein sequence showing Akt kinase consensus sequences RXRX(S/T) in its RS domain. c, HT22 cells were treated with the Akt inhibitor (124005, 1 μM) for 30 min prior to the addition of 10 nm insulin for 24 h. Whole cell lysates were analyzed by Western blot analysis using antibodies as indicated. The gels represent three experiments performed separately with similar results. The graph represents the percentage of exon inclusion calculated as SSI/(SSI + SSI) × 100 and is representative of four experiments performed separately. The results were analyzed with a two-tailed Student’s *t* test using Prism4 statistical analysis software (GraphPad). A level of *p* < 0.05 was considered statistically significant. ***, *p* < 0.0001. Significance was determined after three or more experiments. d, HT22 cells were treated with increasing doses (10, 50, and 100 nM) of insulin for 24 h or transiently transfected with CA-Akt2 or WT-Akt2. Whole protein lysates were separated and analyzed by Western blot analysis using either PKCδII, phospho-SC35, or GAPDH antibody as indicated. The gels represent three experiments performed separately with similar results.

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phosphorylates splicing factors such as SRp40 (27) and SRp75, SC35, and SRp55 (28). To determine whether Akt2 kinase phosphorylates SC35 thereby increasing PKC\(\alpha\)II expression, we transfected constitutively active (CA) or wild-type (WT) Akt2 kinase into HT22 cells compared with HT22 cells treated with increasing amounts of insulin. Akt1 or Akt3 did not have a significant effect on splicing (data not shown). Whole cell lysates were analyzed by Western blotting with antibodies against PKC\(\alpha\)II and phospho-SC35, a monoclonal antibody (S4045, Sigma) that recognizes the phospho-epitope of SC35. Phosphorylation of SC35 increased in cells transfected with CA-Akt2 kinase, thereby mimicking the effects of insulin (Fig. 3d).

**Insulin Increases Expression of Pro-survival Proteins Bcl2 and bcl-xL with a Concomitant Increase in PKC\(\alpha\)II—PKC\(\alpha\)II is a pro-survival protein. We showed that its overexpression decreases PARP cleavage and DNA fragmentation, both of which are indicators of apoptosis (20). Because our data showed an increase in PKC\(\alpha\)II expression with insulin treatment, we used an apoptosis micro-array (SuperArray, catalogue no. PAHS-012A) to determine the profiles of proteins associated with the apoptotic cascade. RNA was isolated from control and insulin-treated HT22 cells. Real-time RT-PCR was performed according to the manufacturers’ protocol, and data were analyzed by SuperArray software. Two genes, Bcl2 and bcl-xL, were increased 5-fold in response to insulin (data not shown), which were concurrent with an increase in PKC\(\alpha\)II levels with insulin treatment. We observed a moderate increase (0.25-fold) in Mcl-1. To confirm this finding, we treated HT22 cells with insulin (10 nM) for 24 h and analyzed whole cell lysates by Western blot. As shown (Fig. 4), expression of Bcl2 and bcl-xL increased along with PKC\(\alpha\)II expression following insulin treatment.

When Bad is in a complex with Bcl2, the mitochondria-mediated survival pathway is inhibited. Upon phosphorylation, p-BAD dissociates thereby allowing Bcl2-Bcl-xL complex to promote survival. p-BAD is then sequestered by 14-3-3 further inhibiting apoptosis. Other studies have shown that p-AKT phosphorylates BAD in several cell types (29, 30). Because our data (Fig. 4) indicated that insulin mediated its effects via AKT kinase and that p-AKT levels were increased with insulin treatment, we immunoblotted with p-BAD antibody. Our results (Fig. 4) show an increase in phosphorylation of BAD at Ser-136 and not Ser-122 or Ser-115, data not shown) with insulin treatment.

**PKC\(\alpha\)II Overexpression Mimics Insulin Treatment in HT22 Cells—**Next, we determined whether overexpression of PKC\(\alpha\)II directly affects the levels of these proteins. PKC\(\alpha\)II or PKC\(\alpha\)II pTracer/CMV expression plasmids were transiently transfected into HT22 cells. Overexpression of PKC\(\alpha\)II increased expression of Bcl2 and bcl-xL thereby mimicking insulin effects (Fig. 5a). Interestingly, overexpression of PKC\(\alpha\)II increased phosphorylation of BAD. This finding suggests that insulin promotes cell survival via the splice variant PKC\(\alpha\)II by increasing the expression of the pro-survival proteins Bcl2 and Bcl-xL. PKC\(\alpha\)II overexpression did not promote these effects of insulin.

There are two splice variants of bcl-x (BCL2L1): the short form is Bcl-xS, which is apoptotic, and the long form is Bcl-xL, which is pro-survival. Bcl-x splicing is shown to be regulated by ceramide in lung adenocarcinoma cells where Bcl-xS is increased (31, 32). Another regulatory mechanism affecting the expression of Bcl-x splice variants is shown to be coupled with PKC signaling (33). Our data (Fig. 5a) demonstrated that PKC\(\alpha\)II increased the endogenous expression Bcl-xL isoform. We hypothesized that PKC\(\alpha\)II can regulate the alternative splicing of Bcl-x isoforms such that the expression is switched to the Bcl-xL splice variant. HT22 cells were transfected with PKC\(\alpha\)II-pTracer/CMV vector. Total RNA was isolated, and RT-PCR was carried out with primers that detect both Bcl-xL and Bcl-xS. PKC\(\alpha\)II increased expression of the pro-survival protein Bcl-xL and decreased expression of the apoptotic protein Bcl-xS (Fig. 5b).

** Knockdown of PKC\(\alpha\)II Expression Decreases Bcl2 and bcl-xL Expression—**Next, we determined whether knockdown of PKC\(\alpha\)II affects Bcl2 and bcl-xL levels after insulin treatment. These experiments provide insights into the extent to which PKC\(\alpha\)II affects the expression of these pro-survival proteins after insulin treatment, because there are other mechanisms by which Bcl2 and bcl-xL may be expressed in neuronal cells. siRNAs have been used to specifically knock down PKC\(\delta\) isoforms and have been used successfully by our laboratory previously (21). HT22 cells were transfected with specific siRNA for PKC\(\alpha\)II (increasing doses) or scrambled siRNA (control) in the presence or absence of insulin. Bcl2 and bcl-xL expression levels were determined in control and PKC\(\alpha\)II-siRNA-transfected HT22 cells by Western blot analysis with antibodies against...
COOH-terminal PKCα, which detects PKCαl and PKCαII simultaneously, anti-Bcl2, anti-pBAD, or anti-Bcl-xL. Our results (Fig. 6) show that knockdown of PKCαII decreased Bcl2 and bcl-xL expressions, whereas the expression of PKCαl and GAPDH remained unaffected. Further, insulin treatment of cells with PKCαII siRNA failed to increase Bcl2 and bcl-xL expression. This indicates that PKCαII is a crucial signaling kinase mediating neuronal survival cascade.

Insulin and PKCαII Decrease Neuronal Apoptosis—We have previously shown that PKCαII is a pro-survival protein (20). XIAP is bound to caspase-3 (p20/p12, intermediate caspase-3 form) in the cytosol and prevents the onset of apoptosis by activated caspase-3. We have shown that PKCαII overexpression increases the expression of XIAP (21). Here we sought to establish that insulin treatment decreases apoptosis via PKCαII. HT22 cells were transfected with PKCαII pTracer/CMV expression plasmid or PKCαII-specific siRNA as described above and then treated with insulin. Whole cell lysates were separated, and Western blot analysis was performed with antibodies against PKCαII or poly-(ADP-ribose)polymerase (PARP), an indicator of apoptosis. In apoptotic cells, PARP is cleaved by caspase-3 into an 85-kDa fragment, which is detected, along with the 116-kDa fragment, by Western blot analysis with anti-PARP antibody. PARP is differentially processed in apoptosis and necrosis, and hence its activity can be used to distinguish the two forms of cell death (35). Insulin increased PKCαII levels with concurrent decrease in PARP cleavage and increase in XIAP expression. Further, knockdown of PKCαII prevented insulin-mediated decrease in PARP cleavage and inhibited XIAP expression (Fig. 7). These results indicate that insulin decreases neuronal apoptosis via PKCαII.

PKCαII Overexpression Promotes Neurogenesis—Increased Bcl2 and bcl-xL expression is shown to enhance neurogenesis. Further, hippocampal neurogenesis is linked to the formation of memories and cognition. To test whether PKCαII-mediated increases in Bcl2 and Bcl-xL expression ultimately result in new cell birth, we performed a BrdU-coupled enzyme-linked immunosorbent assay in HT22 cells overexpressing PKCαII or PKCαII siRNA-treated cells in the presence of insulin. The incorporation of BrdU into replicating DNA was used to label proliferating cells. BrdU is incorporated into S-phase cells, serves as a proliferation marker, and can be quantitatively assayed to determine cell proliferation. BrdU is detected immunohistochemically allowing for the assessment of neuronal cells syn-
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FIGURE 7. Insulin decreases neuronal apoptosis via PKC\textit{II}. HT22 cells were transfected with either 2 \(\mu\)g of PKC\textit{II}-pTracer vector or 50 nM PKC\textit{II} siRNA for 48 h and then were treated with 10 nM insulin overnight as indicated. Whole cell lysates were analyzed by Western blot analysis using antibodies against PKC\textit{II}, XIAP, or PARP. PARP,\textit{F}, full-length PARP; PARP,\textit{C}, cleaved fragment of PARP. The gels represent three experiments performed separately with similar results. The graph shows the percentage of densitometric units normalized to GAPDH for each antibody and represents three separate experiments.

Insulin decreases neuronal apoptosis via PKC\textit{II}. Our data (Fig. 8a) demonstrated that PKC\textit{II} overexpression increased the amount of BrdU concentration in HT22 cells thereby indicating that PKC\textit{II} overexpression promotes neurogenesis and proliferation thereby mimicking insulin action. PKC\textit{II} siRNA prevented the effects of insulin on neurogenesis and proliferation.

To further verify that PKC\textit{II} overexpression increases cell viability and proliferation, we performed a cell proliferation assay based on WST1 (a tetrazolium salt) cleavage to formazan by mitochondrial dehydrogenases. Increased viability of cells results in increased activity of the mitochondrial dehydrogenases in the sample, which can be measured quantitatively by increases in formazan dye production. Data from the assay (Fig. 8b) demonstrated that PKC\textit{II} overexpression increased HT22 cell viability and proliferation and knockdown of PKC\textit{II} inhibited the effect of insulin on HT22 cell viability and proliferation.

Intranasal Insulin Treatment in Mice Improved Cognitive Function with a Concomitant Increase in PKC\textit{II} Levels—Administration of intranasal insulin significantly improves cognitive performance in humans and rodent models (1–3). Insulin also prevents neuronal damage, thereby promoting healthy synapses, which are critical for cognition. It also improves memory, reduces cerebral atrophy, and reverses neurodegeneration due to apoptosis (4–7). Intranasal delivery is a noninvasive method that bypasses the blood-brain barrier and delivers insulin to the brain and the spinal cord, thereby avoiding systemic side effects (e.g. hypoglycemia and increased serum insulin levels) (36–38). Here we sought to measure the effects of insulin on cognitive function in mice and simultaneously evaluate PKC\textit{II} expression in the hippocampus. We tested six older mice (C57 BL/6, 18 months) treated with intranasal insulin (1 unit/ml, daily for 4 weeks) and matched controls in the radial arm water maze. The mice treated with intranasal insulin demonstrated improved cognition as shown by decrease in number of errors and decreased time to find the platform (Fig. 9a). At the end of the trials, the hippocampi of the mice were collected for RNA and protein analysis. Our data showed that intranasal insulin treatment in mice increased the expression of PKC\textit{II}, Bcl2, and bcl-xL in the hippocampus. These results taken together indicated that intranasal insulin improved performance in memory tests, and this was accompanied by increased expression of PKC\textit{II} in the hippocampus (Fig. 9, b and c). Hence, neuronal survival is a contributing factor in increasing cognition by intranasal insulin treatment.

DISCUSSION

Neuronal insulin signaling is critical in mediating learning and memory and functions as a neuroprotectant in the CNS. Insulin receptor signaling in the CNS is important in learning, memory, synaptogenesis, and regulation of neurodegeneration. Insulin also promotes the recovery of neurons after injury by activating survival pathways. Deficiencies in insulin and insulin receptor signaling are detrimental to learning and memory. Injected insulin lowers blood glucose, but the effects on cognition are not evident. Although it is not synthesized in the brain, insulin crosses the blood-brain barrier and is taken up by insulin receptors in the hippocampus. Reduced levels of insulin and insulin receptors in the brain are seen in the elderly and in patients with dementia and Alzheimer disease (39). Even in states of hyperinsulinemia, insulin levels are much lower in cerebrospinal fluid than in serum (40), and both type I and II diabetic patients often have larger declines in cognition at an earlier age. Human studies and epidemiological data suggest a strong link between insulin and cognition (41). Intranasal insulin treatment in humans and rodent models significantly improves cognition (7). Our in vivo data are in agreement with previous studies, and it further establishes that insulin affects cognition with concurrent increases in PKC\textit{II} expression in the hippocampus, the seat of memory and cognition. This knowledge will bridge the gap between insulin signaling, splicing, and cognition.

Insulin and PKC pathways are integrated and function closely together. PKCs, serine/threonine kinases, are involved in the regulation of cellular differentiation, growth, and apoptosis. PKC activation in the brain is essential for learning, synaptogenesis, and neuronal repair (8–10). In particular, PKC\textit{\(\delta\)} has been implicated in memory, neuronal survival, and proliferation and activates survival pathways in neurons (11, 43, 44). The two alternatively spliced variants of PKC\textit{\(\delta\)} expressed in mouse neuronal cells are PKC\textit{\(\alpha\)} and PKC\textit{\(\delta\)}. We have previously demonstrated that PKC\textit{\(\alpha\)} is apoptotic, whereas PKC\textit{\(\delta\)} promotes survival. Recent reports of clinical trials have emphasized the
role of intranasal insulin treatment for improving memory and cognition. However, the cellular target of insulin and its neuronal molecular mechanisms are less studied. Because insulin improves cognition and neuronal survival is critical for promoting cognitive function and memory formation, we sought to determine whether there is a link between insulin and PKCβ.

This is the first report showing that insulin regulates alternative splicing of PKCβ, a key signaling kinase in the neuronal cells. Alternative splicing events in the brain resulting in diverse proteins are now shown to promote specific functionality and to be the cause of several neurodegenerative diseases. Alternative splicing is a means by which multiple proteins can be produced from a single gene. The large, diverse proteome present at any given time in the human body is the direct evidence of the genome’s power to generate functional alternatively spliced variants of the gene. Alternative splicing in neurons is now considered to be a central phenomenon in development, evolution, and neuronal survival. We have demonstrated that insulin mediates PKCβ alternative splicing in neuronal cells. Insulin regulates alternative splicing of PKCβ pre-mRNA in other tissues where it regulates glucose uptake and cellular differentiation (45). However, its role in neuronal alternative splicing mediating the apoptotic pathway was not yet determined.

The human homolog PKCβVIII has a >94% sequence similarity with the mouse PKCβII. Both PKCβII and PKCβVIII splice variants are generated via utilization of an alternative 5’

![FIGURE 8. Overexpression of PKCβII increases HT22 proliferation and viability. HT22 cells were transfected with either 2 μg of PKCβII-pTracer vector or 50 nM PKCβII siRNA for 48 h and then were treated with 10 nM insulin overnight as indicated. The BrdU assay and cell viability assay were performed. The graphs represent BrdU incorporation in PKCβII-overexpressing cells as a percentage of control cells (a) and cell viability in PKCβII-overexpressing cells as a percentage of control cells (b). The measurements were made in triplicate in three separate experiments. The results were analyzed with a two-tailed Student’s t test using PRISM4 statistical analysis software (GraphPad). A level of p < 0.05 was considered statistically significant. ***, p < 0.0001. Experiments were performed in triplicate, and significance was determined after three experiments.](image-url)
splice site. Computational analysis showed a conserved SC35 binding site on the 5′ intron of PKCαII exon 9 pre-mRNA. However, the sequence of exon 9 and its flanking introns, which is the region of alternative splicing of PKCαII pre-mRNA, is different from the sequence comprising of PKCαⅧ exon 10. We are cloning a PKCαII splicing minigene to further study the molecular mechanisms of alternative splicing of PKCαII mRNA regulated by insulin, thereby evaluating the interplay of PKCαII cis-elements with their trans-factors.

Here we demonstrated that insulin acts through PKCαII to increase expression of the pro-survival proteins Bcl2 and bcl-xL, which increase neurogenesis and neuronal survival (34, 46–51). Our results with insulin treatment and in cells overexpressing PKCαII show an increase in phosphorylation of BAD at Ser-136, which indicates that this increase occurs via the AKT pathway and not the ERK or PKA pathways, because we did not see increases at Ser-122 or Ser-115. It may also be possible that PKCαII is an additional kinase phosphorylating BAD...
such that it dissipates from the Bcl2-BAD complex thereby initiating the survival pathway. We are currently expanding the study to evaluate this in our laboratory. Knockdown of PKCII further prevented insulin-mediated increases in Bcl2, Bcl-xL, and pBAD levels.

Currently, there is a lack of a good inhibitor for PKCδ. Rotterlin, the widely used PKC inhibitor, is shown to also inhibit other kinases such as PKA, calmodulin kinase, and other additional PKC isoforms (42). Hence we used siRNA in our studies, which also offers the additional benefit of selecting for a specific splice variant. Our data indicated that PKCII knockdown affected Bcl2 and bcl-xL levels, and the expression of PKCδ, which remained constant in the PKCII-siRNA cells, did not affect their levels. These results suggest that PKCδ is an important kinase in the Bcl2-mediated survival pathway.

In conclusion, our studies demonstrate that insulin regulates alternative splicing of a key signaling kinase, PKCδ, in neuronal cells. Further, we have shown that PKCII is a critical signaling component that modulates neuronal survival, which is essential to improve memory and learning. These findings contribute to the therapeutic potential of PKCII and elucidate molecular mechanisms underlying intranasal insulin treatment, which ultimately contribute to improved cognition and memory.

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