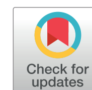

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Insulin as a Potent Stimulator of Akt, ERK and Inhibin-βE Signaling in Osteoblast-Like UMR-106 Cells

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Abstract

Insulin is a peptide hormone of the endocrine pancreas and exerts a wide variety of physiological actions in insulin sensitive tissues, such as regulation of glucose homeostasis, cell growth, differentiation, learning and memory. However, the role of insulin in osteoblast cells remains to be fully characterized. In this study, we demonstrated that the insulin (100 nM) has the ability to stimulate the phosphorylation of protein kinase B (Akt/PKB) and extracellular signal-regulated kinase (ERK) and the levels of inhibin-βE in the osteoblast-like UMR-106 cells. This insulin-stimulated activities were abolished by the PI3K and MEK1 inhibitors LY294002 and PD98059, respectively. This is the first report proving that insulin is a potential candidate that enables the actions of inhibin-βE subunit of the TGF-β family. The current investigation provides a foundation for the realization of insulin as a potential stimulator in survival signaling pathways in osteoblast-like UMR-106 cells.

Key Words: Akt, ERK, Inhibin-βE, Insulin, UMR-106

INTRODUCTION

Insulin is a peptide hormone secreted from beta cells of the endocrine pancreas (Steiner and Oyer, 1967). It exerts a wide variety of physiological actions in insulin sensitive tissues (adipocytes, liver, muscle and brain), such as regulation of glucose homeostasis, cell growth, differentiation, learning and memory (Cheatham and Kahn, 1995). In addition to these, a variety of insulin actions have accumulated to suggest its important roles in osteoblast cells, such as regulating integrin-linked kinase (ILK) (Yoon *et al*., 2015) and thereby collagen synthesis, alkaline phosphatase and gene expression (Han *et al*., 2010). However, the role of insulin in osteoblast cells remains to be fully characterized. Insulin receptors (IRs) and insulin receptor substrates (IRSs) have been identified in osteoblast cells, suggesting insulin's role in the cells (Ju Ha and Kim, 2013).

The IRSs are required for normal insulin signaling (Ramalingam and Kim, 2016a). These signaling networks employs kinase-linked cascades including phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt/PKB) and mitogen-activated protein (MAP) kinase or extracellular signal-regulated kinase (ERK) in UMR-106 cells (Ju Ha and Kim, 2013; Yoon *et al*., 2015). Akt is a key anti-apoptotic effector in the growth factor signaling pathway. Activation of PI3K/Akt pathway by various growth factors produces an anti-apoptotic signal in various cell types. Recently, we discovered that insulin treatment inhibited the degradation of IRs and IRSs protein levels and activates the down-regulation effectors of insulin signaling such as PI3K/Akt/GSK-3 in retinoic acid (RA)-differentiated SH-SY5Y neuroblastoma cells (Ramalingam and Kim, 2016a). Moreover, ERK exists as isozymes including ERK I (44 kDa) and ERK II (42 kDa). Insulin induces a rapid translocation of the MEK from the cytoplasm to the nucleus and activates resident nuclear ERK I/II in UMR-106 cells (Kim and Kahn, 1997).

In addition, transforming growth factor-β (TGF-β), a family of secreted polypeptide growth factors utilizes a multitude of intracellular signaling pathways in addition to Smads to regulate a wide array of cellular functions (Zhang, 2009). These non-Smad pathways include various branches of PI3K/Akt, MAPK and Rho-like GTPase signaling pathways (Zhang, 2009). Inhibins are secreted polypeptides, representing a subgroup of the TGF-β superfamily or growth and differentiation factors. The novel inhibin-βE subunit was predominantly observed in normal and malignant human cervical tissue and cervical cancer cell lines and its physiological functions are still quite unclear (Bergauer *et al*., 2009). The above mentioned pathways

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are activated after the activation of intrinsic tyrosine kinase associated with IRs, but how signaling progresses remains poorly understood (Cheatham and Kahn, 1995).

Moreover, UMR-106 cells derived from a rat osteogenic sarcoma, possess many of the enzymatic properties of normal osteoblasts including high alkaline phosphatase activity and parathyroid hormone (PTH)-stimulated adenylate cyclase activity (Duncan and Misler, 1989). They produce bone-specific collagens and have similar resting membrane potentials as the osteoblast. UMR-106 cell contains insulin receptor and glucose transporters but signaling mechanisms regulated by insulin are not fully understood in the cell (Kim and Kim, 1997). Therefore, in the present study, we have been investigating the mechanisms whether insulin treatment has regulatory effects associated with Akt and ERK thereby regulate inhibin-βE on osteoblast-like rat UMR-106 cells.

MATERIALS AND METHODS

Materials

Dulbecco's modified essential medium (DMEM), penicillin streptomycin (Pen Strep), trypsin-EDTA and fetal bovine serum (FBS) were purchased from Life Technologies (GIBCO, Grand Island, NY, USA). Insulin was purchased from Roche Diagnostics (Mannheim, Germany). The PI3K inhibitor LY294002 and MEK1 inhibitor PD98059 were purchased from Abcam Biochemicals (Cambridge, UK). Plastic materials were purchased from SPL Life Science (SPL, Seoul, Republic of Korea). All other reagents were from commercial suppliers and of the highest purity available.

Cell culture and treatment

The rat osteogenic sarcoma line UMR-106 (ATCC CRL-1661) cells were grown to confluence in DMEM media supplemented with 10% fetal bovine serum, penicillin/streptomycin (100 U/ml; 100 μg/ml), and 2 mM L-glutamine at 37°C in a humidified atmosphere containing 5% CO_2/95% air. Cells from passages 10 to 24 were used for the experiments. UMR-106 cells were plated at a density of $5×10^6$ cells/ml in serum-free medium. After an overnight culture, the medium were replaced with fresh serum-free medium. To study the effects of insulin, cells were incubated with insulin for 24 h with or without inhibitors. The groups were G-1: control medium, G-2: insulin (100 nM; 24 h), G-3: insulin (100 nM; 24 h) plus LY294002 (20 μM; 24 h) and G-4: insulin (100 nM; 24 h) plus PD98059 (20 μM; 24 h).

Western blotting

Cells were washed twice with 1× PBS, harvested and lysed in an appropriate volume of lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% NP-40, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 30 mM NaF, 2.5 mM NaPPi, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 mM PMSF). Lysates were then centrifuged at 12 000 rpm for 10 min at 4°C and the supernatants were used as the total cell lysates. The protein concentration of each sample was determined by the Bradford assay (Bio-Rad, Hercules, CA, USA). About 50 μg of protein were separated on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to PVDF membranes (Millipore, Bellerica, MA, USA). Each membrane was blocked and then incubated with primary antibody of the phospho- or total forms of ERK, Akt (Cell Signaling, Danvers, MA, USA), or that recognized for inhibin β-E/C (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and β-actin (Abcam, Cambridge, MA, USA). Each protein was detected by the luminol-based chemiluminescence (ECL) system (West-Q Pico Dura ECL solution; GenDEPOT, Barker, TX, USA) with a ChemiDoc XRS imaging system (Bio-Rad). Densitometric analysis was performed using ImageJ (National Institute of Health, Bethesda, MD, USA) software.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted using TRIzol reagent (Life Technologies) and estimated. Total RNA (2 μg) was reverse transcribed for 60 min at 42°C, followed at 94°C for 15 min with oligo (dT)18 primer (Thermo Scientific, Bremen, GA, USA) using AccuPower RT PreMix K-2041 (Bioneer, Daejeon, Republic of Korea). Resultant cDNA was amplified in the presence of 1 nM forward and reverse primers using AccuPower PCR PreMix K-2016 (Bioneer). The PCR primers were AKT1 (NM_033230.2) forward 5'-GCCACGGATACCATGAACGA-3', reverse 5'-TGATGAAGG-TGTTGGGCCTC-3' (size 235 bp); ERK2 (NM_053842.2) forward 5'-GAAGACACAGCACCTCAGCAA-3', reverse 5'-TGG-AAGGCTTGAGGTCACGGT-3' (size 113 bp); INHBE (NM_031815.2) forward 5'-AGCCGTCCCAGAATAACTCG-3', reverse 5'-AGATTCCTCACTCCGCAAGC-3' (size 375 bp) and ACTB (NM_031144.3) forward 5'-GGCTGTGTTGTCCC-TGTAT-3', reverse 5'-CCGCTCATTGCCGATAGTG-3' (size 352 bp). The PCR consisting of denaturation at 94°C for 30 s, primer annealing at 56.5 (AKT1), 58.0 (ERK2), 55.0 (INHBE) and 54.3 (ACTB)°C for 30 s, and extension at 72°C for 30 s for 35 cycles was carried out. The amplified samples were then loaded at equal volumes onto 1% agarose gels in Tris-Acetate-EDTA (TAE) buffer. The PCR products were visualized with ethidium bromide by a ChemiDoc XRS imaging system (Bio-Rad). The band intensity was quantified by ImageJ (National Institute of Health) densitometric analysis software.

Sample preparation and microarray analysis

The total RNA pellet was dissolved in diethylpyrocarbonate (DEPC water, and its quality and quantity was assessed by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Gene expression was analyzed with GeneChip Rat Genome 230 2.0 Arrays (Affymetrix, Santa Clara, CA, USA), which is comprised of over 31,000 probe sets representing approximately 28,700 well-characterized rat genes. For each gene, eleven pairs of oligonucleotide probes are synthesized in situ on the arrays.

Biotinylated cRNA were prepared according to the standard Affymetrix protocol from 500 ng total RNA (Expression Analysis Technical Manual, 2001, Affymetrix). Following fragmentation, 15 μg of aRNA were hybridized for 16 h at 45°C on GeneChip Rat Genome Array. GeneChips were washed and stained in the Affymetrix Fluidics Station 450. GeneChips were scanned using the Affymetrix GeneChip Scanner 3000 7G. The data were analyzed with Robust Multichip Analysis (RMA) using Affymetrix default analysis settings and global scaling as normalization method. The trimmed mean target intensity of each array was arbitrarily set to 100. The normalized, and log transformed intensity values were then analyzed using GeneSpring GX12.5. Fold change filters included the requirement that the genes be present in at least 150% of controls for up-regulated genes and lower than 66% of controls

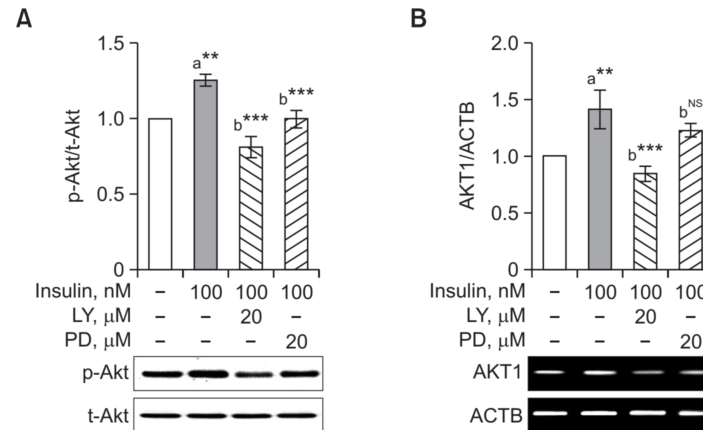

Fig. 1. Role of insulin on Akt expressions in UMR-106 cells. Cells were plated at a density of 5×10^6 cells/ml in serum-free medium and the experiment were carried out as explained in "Materials and Methods". After 24 h, the cells were collected, then the protein and RNA were prepared. The level of p-Akt/t-Akt (A) and AKT1/ACTB (B) were determined by Western blotting and RT-PCR analyses. All assays were performed at least three independent experiments (n=3). Statistical significance: [a]compared to control; [b]compared to insulin; ***p*<0.01, ****p*<0.001 and [NS]: non-significant.

for down-regulated genes. Hierarchical clustering data were clustered groups that behave similarly across experiments using GeneSpring GX12.5. Clustering algorithm was Euclidean distance, average linkage.

Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Life Technologies) and estimated. Total RNA (1.5 μg) was reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Resultant cDNA was amplified using 2X Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). The qPCR primers were INHBE (NM_031815) forward 5'-CCTGGCAACCGAGAGAAAGT-3', reverse 5'-CGGAGCGGTAGGTTGAAGTG-3' (size 70 bp) and GAPDH (NM_017008) forward 5'-ACAGTCAAGGCTGAGA-ATGG-3', reverse 5'-GATCTCGCTCCTGGAAGATG-3' (size 70 bp). The Real-Time PCR consisting of denaturation at 95°C for 15 s, primer annealing at 59°C for 30 s for 40 cycles was carried out in StepOnePlus real-time PCR system (Applied Biosystems).

Statistical analysis

All data analysis was completed using the Graphpad PRISM 5.0 software (Graphpad Software, Inc., La Jolla, CA, USA). Data are expressed as mean ± S.D. The significance level of treatment effects was determined using a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test between groups or paired *t*-test. Values of $p<0.05$ were considered to be statistically significant.

RESULTS

Insulin activity on Akt expression

Akt/PKB is a key anti-apoptotic effector in the growth factor signaling pathway. The Western blot assay was performed for phosphorylated Akt (p-Akt(Ser^{473})) and total Akt (t-Akt) to investigate the role of insulin in the activation of Akt. p-Akt(Ser^{473}) protein expression (Fig. 1A) and the Akt1 gene expression (Fig. 1B) were moderately increased ($p<0.05$) after treatment with 100 nM of insulin. Administration of 20 μM of PI3K and MEK1 inhibitors (LY294002 and PD98059, respectively) to insulin significantly inhibited ($p<0.001$) p-Akt (Ser^{473}) expression in UMR-106 cells. However, the increased expressions of Akt1 mRNA by insulin was abolished by the PI3K inhibitor LY294002 (20 μM) but not by the MEK1 inhibitor PD98059 (20 μM).

Insulin activity on ERK expression

To study the role of MAPK, the phosphorylation levels of ERK was measured against the total-ERK (Fig. 2A). Treatment with 100 nM of insulin resulted in a significant increase ($p<0.001$) in the p-ERK/t-ERK protein levels (Fig. 2A) when comparing normal control cells. In addition, PI3K and ERK inhibitors (20 μM of LY294002 and PD98059, respectively) prevented insulin action on p-ERK/t-ERK (Fig. 2A) and ERK2 mRNA (Fig. 2B) in UMR-106 cells. These results suggest that ERK pathway may play a role in the insulin-induced signaling pathway.

Insulin activity on inhibin-βE expression

To confirm the activation of Akt and ERK by insulin, inhibin-βE, other target of insulin signaling was determined. Present results shown that insulin (100 nM) treatment shows changes ($p>0.001$) on the inhibin-βE protein (39 kDa; Fig. 3A) and INHBE mRNA (Fig. 3B). However, the increased expressions of inhibin-βE protein and mRNA levels by insulin were abolished by 20 μM of the PI3K inhibitor LY294002 and MEK1 inhibitor PD98059 in UMR-106 cells.

The inhibin-βE expression profiling was performed by the microarray (Fig. 4A). The present study microarray analysis had revealed that insulin up-regulates inhibin-βE expression to 3.7 fold in osteoblast-like UMR-106 cells. The inhibin-βE gene was further analyzed by qRT-PCR (Fig. 4B). The results showed that the insulin significantly increased INHBE expression were in accordance with the microarray analysis.

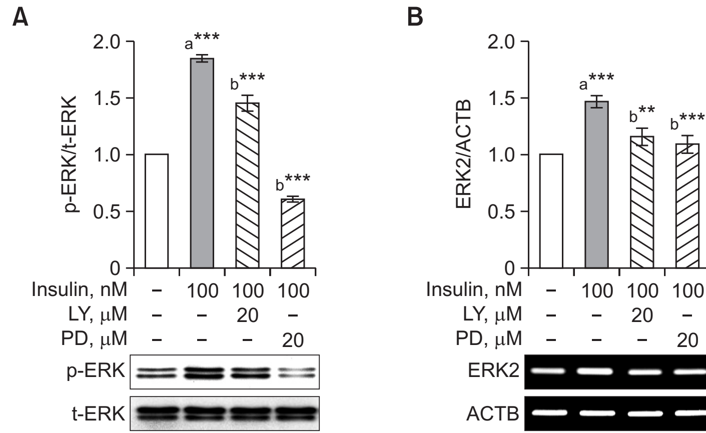

Fig. 2. Role of insulin on ERK expressions in UMR-106 cells. Cells were plated at a density of 5×10^{6} cells/ml in serum-free medium and the experiment were carried out as explained in "Materials and Methods". After 24 h, the cells were collected, then the protein and RNA were prepared. The level of p-ERK/t-ERK (A) and ERK2/ACTB (B) were determined by Western blotting and RT-PCR analyses. All assays were performed at least three independent experiments (n=3). Statistical significance: [a]compared to control; [b]compared to insulin; ***p*<0.01 and ****p*<0.001.

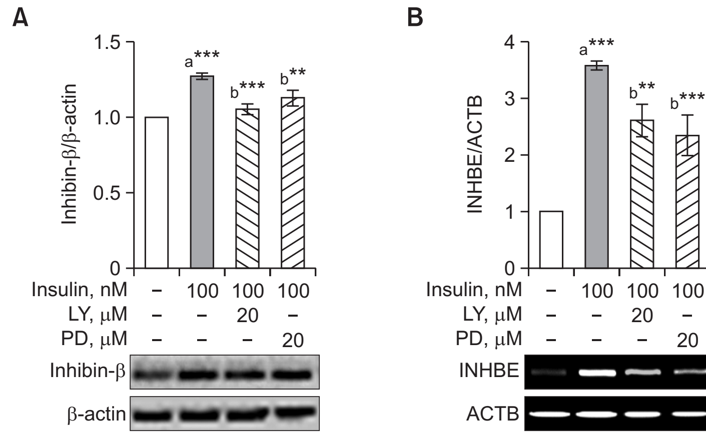

Fig. 3. Role of insulin on inhibin-βE expressions in UMR-106 cells determined by Western blotting and RT-PCR. Cells were plated at a density of 5×10^{6} cells/ml in serum-free medium and the experiment were carried out as explained in "Materials and Methods". After 24 h, the cells were collected, then the protein and RNA were prepared. The level of inhibin-βE/β-actin (A) and INHBE/ACTB (B) were determined. All assays were performed at least three independent experiments (n=3). Statistical significance: [a]compared to control; [b]compared to insulin; ***p*<0.01 and ****p*<0.001.

DISCUSSION

Insulin treatment activates the insulin receptor tyrosine kinase initiated by phosphorylation of insulin receptor substrate and followed by the association of SH2 containing proteins including PI3K. Its activation leads to translocation of glucose transporter to plasma membrane and take part in the regulation of gene expression, cell growth, and differentiation (Seol and Kim, 2003). In the present study, we present evidence that insulin stimulates Akt and ERK and regulates inhibin-βE in osteoblast-like UMR-106 cells.

Akt/PKB, a serine/threonine kinase, has been demonstrated to be a downstream target of PI3K that mediates the anti-apoptotic actions by various growth factors (Ramalingam and Kim, 2014). Akt is regulated by the second messenger product of PI3-kinase action, PI(3,4,5)P_3, by direct binding to the pleckstrin homology domain of Akt and by the activation of upstream phosphoinositide PDKs, which phosphorylate threonine 308 and serine 473 residues required for its full activation. The phosphorylation of serine 473 of Akt is highly inducible by upstream signals (Persad *et al.*, 2001). In this study, p-Akt(Ser^{473}) protein expression and the Akt1 gene expression were increased with insulin treatment. Our previous study has shown that insulin treatment increased the ILK activity in UMR-106 cells (Yoon *et al.*, 2015). Other studies revealed that ILK fulfills many of the properties of an upstream regulator of phosphorylation of Akt(Ser^{473}). In addition, recombinant ILK can phosphorylate Akt(Ser^{473}) in vitro and this activity of ILK is stimulated in the presence of PI(3,4,5)P_3 (Delcommenne *et al.*, 1998). In this present study, LY294002 and PD98059 to insulin significantly inhibited p-Akt(Ser^{473}) expression in UMR-106 cells. However, the increased expressions of Akt1 mRNA by insulin was abolished by the LY294002 but not by the PD98059. Recently, we reported that p-Akt(Ser473) increased

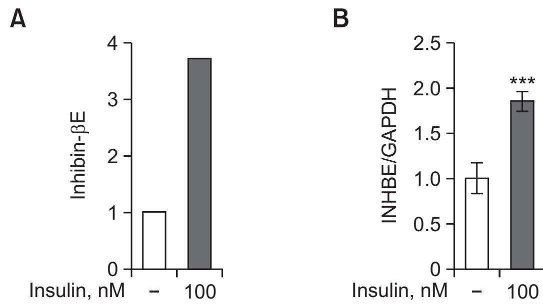

Fig. 4. Role of insulin on inhibin-βE expressions in UMR-106 cells determined by microarray and qRT-PCR assays. Cells were plated at a density of 5×10^6 cells/ml in serum-free medium and the experiment were carried out as explained in "Materials and Methods". After 24 h, the cells were collected, then the RNA was prepared. The level of inhibin-βE (A) and INHBE/GAPDH (B) were determined by microarray and qRT-PCR analyses. ***p<0.001.

in response to insulin in neuronal and glial cells (Ramalingam and Kim, 2015, 2016b). Taken together, the Akt phosphorylation in UMR-106 may be important components for the insulin signal transduction such as stimulation of many proteins (Seol and Kim, 2003).

The MAPK molecules are important upstream regulators of transcription factor activities, and their signaling is critical for the transduction of extracellular oxidative stress into intracellular events (Crossthwaite *et al*., 2002). The role of the MAPK pathway is still controversial that it can either enhance the cell survival or induce apoptotic cell death, depending on the specific cell types and insults (Wang *et al*., 2010). To study the role of insulin on MAPK, the phosphorylation levels of ERK was measured against the total-ERK. Insulin resulted in an increase in the p-ERK/t-ERK protein and ERK2 mRNA levels. In addition, LY294002 and PD98059 prevented insulin actions in UMR-106 cells. The increase in phosphorylation of ERK in response to insulin may be caused by translocation of the enzyme from cytoplasm to the membrane that requires tyrosine and threonine phosphorylation of the enzyme by MEK. Previously, significant increase in the amount of MEK2 in membranes with similar extent and kinetics of those of phosphorylation of membrane ERK2 by insulin was reported (Kim and Kim, 1997). In this present study, we report that the inhibition of Akt and ERK phosphorylation by their respective inhibitors suggests that the Akt and ERK pathways were upregulated in responsible to insulin treatment in UMR-106 cells. Regulation of ERK involves the activation of a series of signaling molecules that could interact with membrane receptors and regulating their functions. Since, insulin activate ERK in the UMR-106 cells, it is reasonable to propose that insulin stimulated ERK may phosphorylate osteoblast transcription factors (Kim *et al*., 2000).

Taken together, PI3K/Akt and MAPK/ERK as two independent parallel pathways displays influence each other at different stages of signal propagation, both negatively and positively, resulting in dynamic and complex cross-talk (Aksamitiene *et al*., 2012). Moreover, TGF-β can rapidly activate PI3K, as indicated by the phosphorylation of its downstream effector Akt and this activation appears to be independent of Smad (Wilkes *et al*., 2005). Subsequently, rapid activation of ERK by TGF-β was also observed in rat cells (Yan *et al*., 1994;

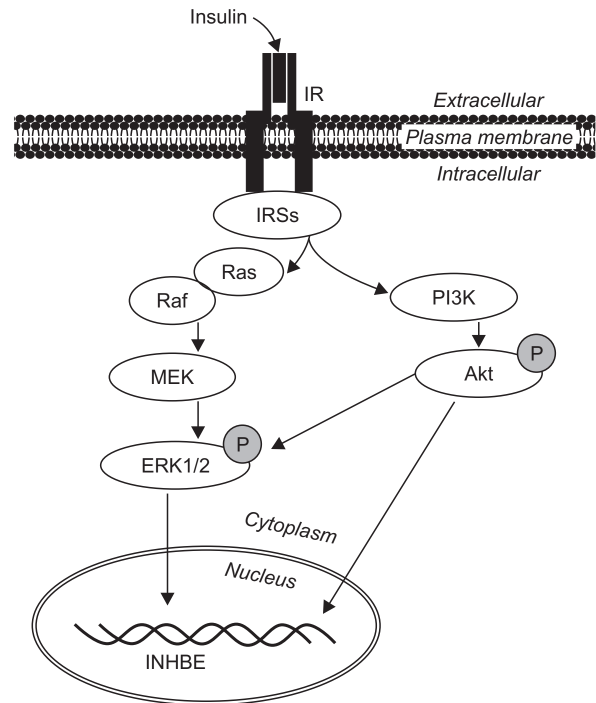

Fig. 5. Schematic diagram illustrating the insulin stimulation of INHBE gene expression by Akt and ERK pathways and their biological functions. ERK1/2, extracellular signal-regulated kinase 1/2; INHBE, inhibin-βE; IR, insulin receptor; IRS, insulin receptor substrate; PI3K, phosphoinositide 3 kinase.

Zhang, 2009). ERK activation is one of the non-Smad pathways necessary for TGF-β mediated roles in integrin-based cell-matrix interaction and promote cell motility (Zavadil *et al*., 2001). From above, the activation of Akt and MAPK influences on several downstream targets (Kim *et al*., 2015) which could result in attenuation of apoptosis mediated by pro- and anti-apoptotic proteins (Lee *et al*., 2015). Our recent study also shown that insulin mediated Akt-ERK interactions proved to be more efficient in activation of compensatory signaling, allowing to evade apoptosis in neuronal cells (Ramalingam and Kim, 2016a).

Bone cells synthesize a number of growth factors such as IGF-1, PDGF, TGF-β, IL and TNF suggesting growth factor-mediated signal transduction plays significant roles in bone cells (Kim *et al*., 2000). Inhibins are secreted polypeptides, represent a subgroup of the TGF-β superfamily or growth and differentiation factors. They are heterodimers composed of an α-subunit and one-two β-subunits namely inhibin-α and inhibin-β. They have been implicated in several important functions including tumor suppressor activity and apoptosis (Bergauer *et al*., 2009). Results from this study shown that insulin changes the inhibin-βE protein and gene levels and these increased expressions by insulin were abolished by PI3K and MEK1 inhibitors. Furthermore, the microarray analysis also showed that insulin up-regulated inhibin-βE gene to 3.7 fold increase in UMR-106 cells. From this, we suggest that the insulin may have a regulatory role on inhibins for protection and inhibin-βE is essential for normal growth and development, cell survival and function via PI3K/Akt and MAPK/ERK pathways (Fig. 5).

In this study, we demonstrated that the insulin has the abil-

ity to stimulate phosphorylation of Akt, ERK levels and inhibin-βE in the UMR-106 cells. Insulin stimulated activities were abolished by the PI3K and MEK1 inhibitors. In conclusion, this is the first report proving that insulin is potential candidates that enable inhibin-βE subunit of the TGF-β family. The current investigation provides a foundation for the realization of insulin as a potent stimulator in survival signaling pathways in insulin-sensitive cells including osteoblast-like UMR-106 cells.

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