Short communication

Interactive Effects of Insulin and β-Estradiol on Protein Kinase B Phosphorylation in Adult Rat Cardiomyocytes

Emina Sudar,[1] Branislava Dobutovic,[1] Tijana Milosavljevic,[1] Biljana Putnikovic,[2] Zoran Gluvic[2] and Esma R. Isenovic[1]

[1] Institute Vinca, Laboratory of Radiobiology and Molecular Genetics, P.O. Box 522, 11001 Belgrade, Serbia

[2] Clinical Hospital Center Zemun, Department of Endocrinology and Cardiology, Vukova 8, 11080 Zemun, Serbia

** Corresponding author: E-mail: sudare @vinca.rs;*
Phon/Fax: +381-11-80-66-868

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Abstract

Insulin (INS) and β-estradiol (E_2) have vascular effects partially through stimulation of protein kinase B (Akt) phosphorylation. Employing adult rat cardiomyocytes (ARC), the effects of acute (15 min) stimulation with INS (10 mIU/ml) and prolonged (2 hours and 24 hours) stimulation with E_2 alone or in combination, were assessed with respect to protein levels of Akt. Exposure to INS for 15 minutes enhanced Akt Ser^{473} phosphorylation. The combined treatment had a greater effect on Akt phosphorylation then the effect of INS alone. The results suggest that INS and E_2 may interact through Akt in regulating metabolic processes.

Keywords: Adult rat cardiomyocytes, insulin, estradiol, signaling pathways, protein kinase B

1. Introduction

Insulin (INS), a hormone of the endocrine pancreas, and a sex steroid, β-estradiol (E_2), have multiple protective effects on the cardiovascular system (CVS)[1,2] and an important role in lowering risk factors of cardiovascular diseases. INS and E_2 interact with cognate receptors and initiate a signaling cascade that involves phosphorylation-dependent kinases, such as serin (Ser)/threonin (Thr) kinase-protein kinase B (Akt).

INS action is initiated trough the binding to, and activation of, its cell surface receptor-INS receptor (IR).[3] INS plays a major role in regulating the balance of metabolic fuels received by the myocardium[4] and has a number of acute metabolic actions on the heart: it promotes glycogen synthesis, as well as glycolysis and glucose oxidation, it inhibits free fatty acid (FFA) oxidation and it is one of the factors responsible for glucose uptake stimulation in adult rat cardiomyocytes (ARC).[5,6] In addition to its metabolic effects, the actions of INS on cellular gene expression may also contribute to cardiac myocyte growth in the normal heart or in hypertrophy.[6,7] Impaired INS signaling is a characteristic of the heart in INS-resistant states, and may lead to the loss of important cytoprotective mechanisms which increase susceptibility of the diabetic myocardium to injury in myocardial ischemia and cardiac hypertrophy.[8] The heart is an INS-responsive organ and disorders of INS action, such as diabetes and obesity, can have profound effects on cardiac function.[6]

Numerous studies have documented the sequence of events in INS signal transduction cascades emanating from cell surface receptors, and great progress in understanding these cascades in ARC have been accomplished in recent years.[3,5] Activation of the IR includes autophosphorylation on numerous tyrosine (Tyr) residues which in turn results in the phosphorylated Tyr providing docking sites for intracellular proteins like IR substrates (IRS). IRS-1 and IRS-2 are both expressed in the heart.[9,10] Phosphorylated IRS binds to intracellular signaling proteins containing src-homology 2 (SH2) domains. One such protein is the lipid kinase -phosphoinositol 3 kinase (PI3-K).[3] Activated PI3-K recruits and facilitate the activation of downstream target Ser/Thr kinase Akt.[11]

Akt plays an important role in the heart through regulation of cardiomyocytes growth, survival, function, and metabolism.[12] Acute activation of Akt has beneficial cardioprotective effects both *in vitro* and *in vivo*.[13] Activation of Akt also plays an important role in promoting cardiomyocytes survival and function in models of cardiac injury.[14] Akt is subsequently phosphorylated at both Thr^{308} and Ser^{473} and after activation, temporal changes in Akt localization occur. From the cytoplasm, Akt moves to a membrane proximal position and ultimately accumulates in the nucleus.[15] Multiple binding proteins and intracellular substrates for Akt have been identified.[15] The Ser/Thr kinase Akt represents the principal downstream effector of PI3-K, triggering several of its cellular effects.[15–17]

E_2 action is mediated through estrogen receptors (ERs). The cellular actions of E_2 are, in turn, usually mediated through the transcriptional regulation of target genes.[18] These effects mainly occur when E_2 binds to the nuclear ER, with the resulting complex then either binding to response elements expressed on various genes, [19] or modifying transcription through protein-protein interactions.[20] Classical nuclear-initiated signaling responses depend on ER dimerization, and nuclear translocation, and require hours to days to produce effects on gene expression. However, E_2 can also rapidly modulate cell functions through nongenomic actions, by activating signaling pathways outside the nucleus[21] mediated through plasma membrane proteins.[22] There are also an increasing number of studies characterizing cell signaling downstream of ER in various cell systems that are too rapid to be compatible with transcriptional mechanisms.

In CVS, E_2 activates several kinase cascades, including an Akt signaling pathway. It has been reported that in response to E_2, direct binding of ERα, to the p85 regulatory subunit of PI3-K, as well as increased PI3-K activity, results in the activation of Ser/Thr kinase Akt which is blocked by ER antagonists.[23] Numerous cellular and molecular studies have apparently reported favorable effects of E_2 on vascular structure, function, and cell signaling.[24] In the heart these non-nuclear signaling pathways mediate rapid vasodilatation, the inhibition of response to vessel injury reduction in myocardial injury after infarction, and attenuation of cardiac hypertrophy.[25]

Treatments with INS (like E_2 treatment) result in increased Akt phosphorylation in myocardial tissue.[26] Thus, E_2 alters the INS signaling cascade, and some cardiac effects of E_2 and INS are mediated by the same signaling pathways. Long-term estrogen replacement treatment might protect the CVS by decreasing blood pressure and inducing the expression of IRS-1 in the myocardium.[27] Based upon (i) our recently published observation that E_2 treatment decreases the FFA plasma level,[28] which is important for cardiac INS sensitivity and heart fuel utilization,[29] (ii) data indicating that INS and E_2 regulate the same processes in the heart,[30] (iii) reports that E_2 exerts nongenomic effects through the Akt pathway, [24,31] and (iv) data indicating that E_2 regulates genes of some INS signaling molecules,[32–34] we initiated the present study based on the hypothesis that E_2 is influeantial in heart insulin signaling.

Thus, we examined possible interactions of INS and E_2 in stimulating phosphorylation of Akt using primary cultures of ARC. The aim of the present study was to examine the involvement of Akt signaling in individual/interactive effects of INS and E_2 in ARC, specifically addressing the hypothesis that in ARC, the interaction of INS and E_2 is mediated *via* activation of phosphorylation of Akt (Figure 1).

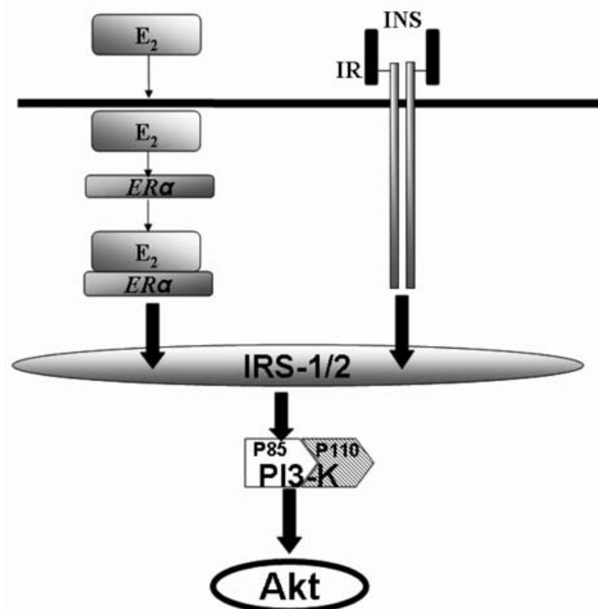

Figure 1: Schematic diagram of insulin (INS) and β-estradiol (E_2) interaction *via* Akt in adult rat cardiomyocytes (ARC): Abbreviations: **INS**-insulin; **IR**-INS receptor; **IRS-1/2**-IR supstrate 1 and 2; **PI3-K**-phosphatidylinositol 3 kinase; **Akt**-protein kinase B; E_2 -β-estradiol; **ERα-E_2 receptor α; ERα/E_2 -complex.**

2. Experimental

2. 1. Reagents

Cell culture dishes were obtained from Corning (Int, Corning, NY, USA), and the cell culture materials, media and all other chemicals, including β-Estradiol, were obtained from Sigma (St. Louis, MO, USA). Insulin-Actrapid was obtained from Novo Nordisk (Bagsvaerd, Denmark). Penicillin and streptomycin were obtained from In vitrogen (Paisley, UK)

2. 2. Antibodies

Anti-phospho-Akt Ser^{473} and the total Akt monoclonal antibody were purchased from Cell Signaling (Be-

verly, MA,USA), and the anti-actin antibody was purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Anti-rabbit antibodies conjugated to horseradish peroxidase (HRP) were purchased from New England Biolabs (Beverly, MA, USA). Briefly, the antibodies used against phospho-Akt detects the Ser/Thr kinase when it is phosphorylated on Ser^{473} as required for its activation.

2. 3. Cell Culture

ARC were isolated from Wistar rats (wt. 300–400 g) by collagen digestion using Langendorf's system as described previously.[35] The experimentation and housing of rats was completed in accordance with the laws and regulations controlling experiments and procedures of live animals in Norway. Isolated cells were maintained in Medium 199 with 2 mg/ml BSA, 2 mM dl-carnitine, 5 mM creatine, 5 mM taurine, 0.1 µM INS, 0.1 nM triiodothyroine, 100 U/ml penicillin and 100 µg/ml streptomycin. This procedure yields approximately 75% rod shaped cardiomyocytes after 24 hours in culture as previously reported.[36] It has previously been shown that ARC maintained in a culture in a serum-free medium remains INS responsive.[37] Cells were stimulated with INS (10 mIU/l; 15 minutes) or 1 µM E_2 (alone, or in combination with INS) for 2 and 24 hours.

2. 4. Western Blotting Analysis

Quiescent ARC were pretreated and stimulated as indicated by the figures. After protein extraction, aliquots with 15 µg of cell proteins were prepared with Leammli buffer, heated at 95 °C for 5 minutes, in order to completely dissociate proteins, and centrifuged at 22 000 *g* for 15 seconds and SDS-PAGE was carried out essentially as described.[38–40] The transfer of proteins from the gel onto the polyvinylidene difluoride (PVDF) membrane was performed for 1 hour at 200V in a Mini-trans-Blot cell with Bio-Ice cooling unit (Bio-Rad Laboratories, Hercules, CA, USA). The transfer buffer contained 25 mM Tris, 192 mM Glycin and 10% methanol. Membranes were washed (3x10min) in PBS-T (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, 0.1% Tween 20). To avoid nonspecific binding of antibodies to the PVDF, membranes were blocked in PBS-T containing 5% nonfat milk for 2 hours at room temperature. After blocking, membranes were washed in PBS-T (2 x 30 seconds).

For immunoblotting, the levels of proteins were controlled using antibodies directly against Akt phosphorylation and a nonphosphorylated form of Akt. HRP-linked anti-rabbit IgG was used as a secondary antibody, and immunoreactive bands were detected by using an enhanced chemiluminescence -ECL kit from Amersham Pharmacia Biotech (Buckinghamshire, UK). The same membranes were striped at 65 °C for 45 minutes in a stripping buffer (pH = 6.8) containing 0.7% β-mercaptoethanol, SDS 2%, and 62.5 mM Tris and treated for immunoblotting with specific anti total Akt antibodies as well as anti actin antibodies as described above. Since we have observed that under the conditions used for these experiments, and in respect, to the agonists used total Akt did not vary, we choose to express the phosphorylation level of the pAkt/Ser^{473} versus total Akt. Furthermore, we also included the results of Western blot to other independent loading control as actin, to demonstrate the specificity of action of E_2 and INS. Thus, we did strip our membranes and re-probed with an actin antibody. No changes in the loading were detected. Densitometry analysis of immunoblots was carried out using National Institutes of Health (NIH) Image software. Since control bands of immunoblots have no signal, the background levels were not subtracted. In that way, the data were not normalized to the arbitrary level of the image background.

2. 5. Statistical Analysis

Results are presented as mean±SEM with N values representing the number of experiments. Each experiment involved one distinct cell culture. Statistical significance was evaluated by student's t-test or ANOVA, with the appropriate correction for multiple comparisons (Newman–Keults method). Arbitrary density units in the control sample were set at 1 and values for all treatments were normalized to 1 (-fold changes versus CTL) $P < 0.05$ was consider significant compared with control, unless otherwise specified.

3. Results

3. 1. Effects of INS on Akt Phosphorylation in ARC

ARC were treated with INS (10 mIU/ml) for 15 minutes and membranes were probed with anti-p-Akt/Ser^{473} antibody (Figure 2). Densitometry quantification of Western blot revealed that INS increased Akt phosphorylation at Ser^{473} after 15 minutes of hormone treatment.

3. 2. Interactive Effects of INS and E_2 on Akt Phosphorylation in ARC

We further examined whether Akt, an important downstream target for PI3-K[41–44] is involved in INS and E_2 interactions in ARC. Therefore we assessed the effects of these hormones on phosphorylation state of Akt at Ser^{473} by immunoblotting with a phosphospecific Akt antibody that recognizes Akt only when phosphorylated at Ser^{473} (Figure 3). Stimulation with E_2 (1 µM) for 2 or 24 hours and then with INS (10 mIU/ml) for an additional 15 minutes, enhanced Akt phosphorylation at Ser^{473}, with the effect of E_2/INS being much greater than INS alone (Figu-

re 3). However, the pre-treatment with E_2 (1 μM) for 2 or 24 hours before INS (10 mIU/ml) for an additional 15 minutes, produced an enhancement in the increase of Akt phosphorylation much greater than E_2 alone (Figure 3). This data indicate that Akt is involved not only in E_2/INS mediated Akt activation but also in the maintenance of basal activity of Akt in ARC.

4. Discussion

The principal new finding in the present study is that the Akt signaling pathway mediates, in part, the interactive effects of INS and E_2 in ARC. This data extends our previous findings, that Akt is involved in the molecular mechanisms of INS and E_2 interactions in the rat heart.[28] Exposure to INS increased Akt phosphorylation. In the latter two cases, combined E_2/INS treatment had a greater effect than either agonist alone. Current data indicates that INS can induce expression of other downstream E_2 signaling molecules, including the Akt. Furthermore, the results suggest that regulation of this molecule produces potentiation of INS on the E_2 stimulation of Akt and its activation and vice-versa.

Results from the current study are in accordance with prior studies in rat hearts,[28,45] showing that E_2 enhance INS effects to increase Akt activity.[28] This data could be explained by the redistribution of IR as well as increa-

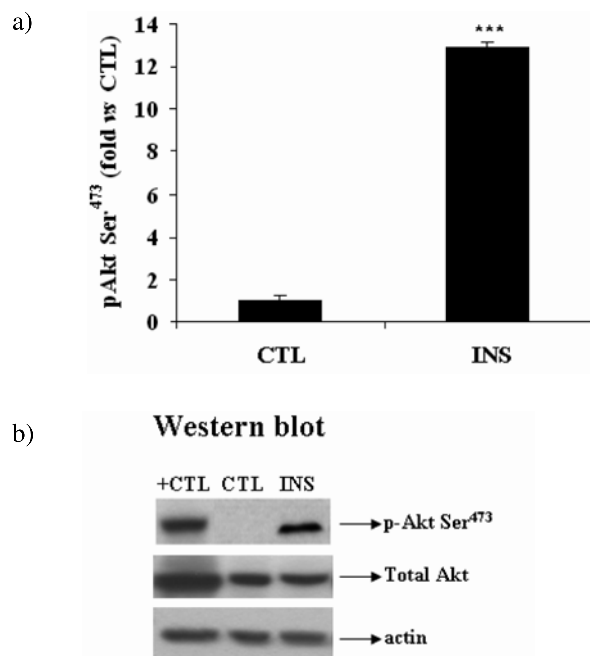

Figure 2: **Effects of Insulin (INS) on Akt Ser^{473} phosphorylation in adult rat cardiomyocytes (ARC): (a)** ARC were treated with INS (10 mIU/ml) for 15 minutes. The results are expressed as a -fold of control (CTL is arbitrary set at 1). **(b)** Representative immunoblots. Results are mean ± SEM, n = 3; *** p < 0.001 indicates INS *vs.* CTL; +CTL indicates positive control and CTL indicates control ARC.

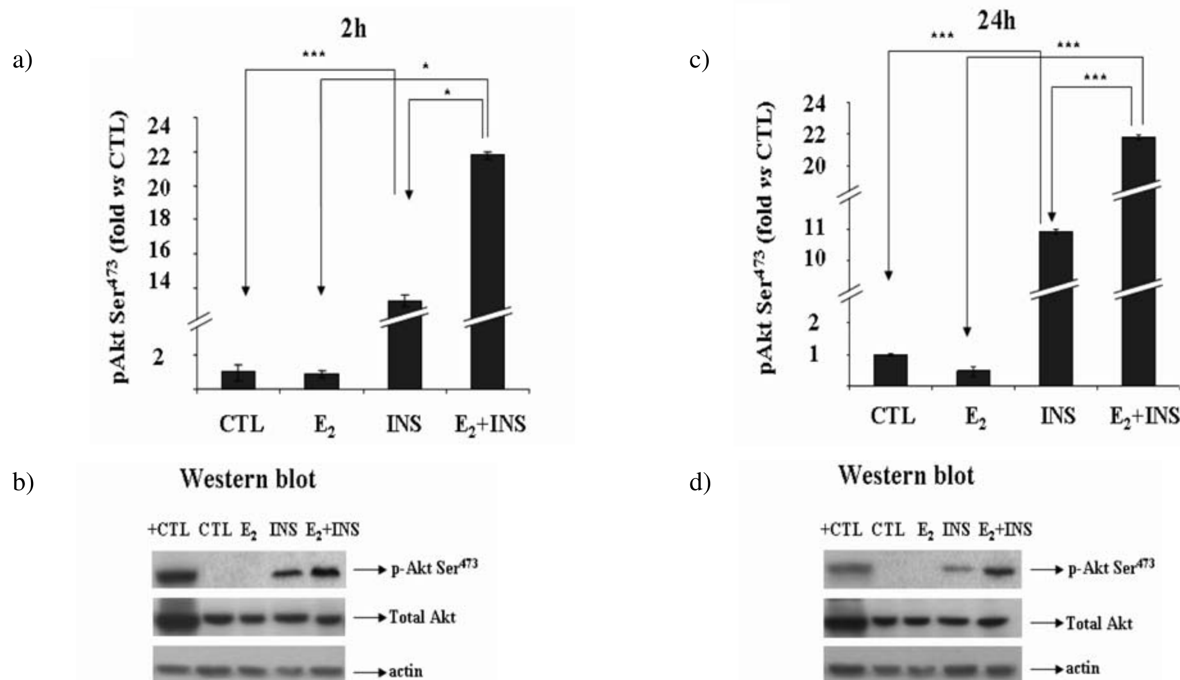

Figure 3: **Interactive effects of Insulin (INS) and β-Estradiol (E_2) on Akt Ser^{473} phosphorylation in adult rat cardiomyocytes (ARC):** ARC were treated with E_2 (1 μM) for 2 **(a)** and 24 hours **(c)** or with INS (10 mIU/ml) for 15 minutes **(a,c)**, and ARC were treated with E_2 (1 μM) for 2 **(a)** or 24 hours **(c)** and with INS (10 mIU/ml) for additional 15 minutes, **(a, c)**. The results are expressed as a -fold of **CTL** (CTL is arbitrary set at 1). **(b, d)** Representative immunoblots. Results are mean ± SEM, n = 3; *** p < 0.001 indicates INS *vs.* CTL (2 and 24 hours). ***p < 0.001 indicates E_2 + INS *vs.* INS (24 hours), *p < 0.05 indicates E_2 + INS *vs.* INS (2 hours). + CTL indicates positive control and CTL indicates control ARC.

sed IRS-1 expression following E_2 treatment of the cells. In addition, the combined treatment had greater effect on Akt Ser^{473} phosphorylation than INS alone, while E_2 has no effect alone.

In the current study, potentiation of Akt phosphorylation was the first observation of INS and E_2 interaction in ARC. There are several possibilities which explain the interactions of INS and E_2 in modulation of Akt in ARC. It is possible that E_2 maintains the proper local level of expression of INS and its receptor on ARC to allow for optimal INS regulation of Akt under normal conditions.[28,46] On the other hand, INS may also sensitize the ER on ARC into full function.[47] Together with our previously published data,[44,48] this indicates that E_2 enhanced INS induced Akt phosphorylation at Ser^{473} in ARC and other cell types.

5. Conclusions

In summary, the data suggests that the relative potency of INS versus E_2 in stimulation of Akt activation is related to the ability of these peptides to enhance Akt phosphorylation in ARC. Furthermore, the data suggests that both hormones, E_2 and INS, are necessary for optional Akt activation. In this study, we have elucidated some of the signaling mechanisms involved in the cross talk between the INS and E_2 in primary cultured ARC.

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Povzetek

Inzulin (INS) in β-estradiola (E_2) vplivata na ožilni sistem preko stimulacije protein kinaze B (Akt). Na podganjih kardiomiocitih (ARC) smo študirali vpliv akutne (15 min) stimulacije INS (10mIU/ml) in podaljšane (2 in 24 ur) stimulacije z E_2 brez in v kombinaciji z INS in opazovali raven proteina Akt. Stimulacija z INS za 15 min je zvišala stopnjo fosforilacije Akt Ser^{473} (CTL = 1-krat, INS = 11.83 ± 1.79-krat, $p < 0.001$). Vpliv kombinacije obeh stimulacij je bil močnejši kot vpliv posamezne stimulacije, pri čemer se je vpliv INS izkazal za mnogo močnejšega (CTL = 1-krat, INS = 11.83 ± 1.79-krat, INS/E_2 = 19.35 ± 2.34-krat pri 2 urah in INS/E_2 = 23.69 ± 1.84-krat pri 24 urah). Rezultati študije nakazujejo, da imata INS in E_2 interakcijski vpliv na uravnavanje metaboličnih procesov preko Akt.