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The Role of Estrogens in Insulin Secretion. Implications for Aromatase Inhibitor Treatment

Ioannis Vlachodimitris[1*], Christos Markopoulos[2], Ifigenia Kostoglou-Athanassiou[3], Eustathia Papageorgiou[3], Helen Gogas[4], Michael Koutsilieris[5]

[1]*Department of Gynecology, Hospital Bethesda Duisburg, Germany*

[2]*Department of Surgery, Medical School, National and Kapodistrian University of Athens, Athens, Greece*

[3]*Department of Endocrinology, Asclepeion Hospital, Voula, Athens, Greece*

[4]*Department of Internal Medicine, Medical School, National and Kapodistrian University of Athens, Athens, Greece*

[5]*Department of Physiology, Medical School, National and Kapodistrian University of Athens, Athens, Greece*

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***Corresponding author :** Ioannis Vlachodimitris, Department of Gynecology, Hospital Bethesda Duisburg, Germany. Address: M. Asias 76, 11527 Goudi, Athens-Greece; E-mail: gvlachodimitris@gmail.com*

Abstract

Introduction

The main hormonal treatment of estrogen sensitive breast cancer includes the use of selective estrogen receptor modulators, aromatase inhibitors and GnRH-Analogs. It has been observed that administering aromatase inhibitor to breast cancer patients not only impairs their glucose metabolism but it can even cause frank diabetes mellitus. Moreover, it has been hypothesized that aromatase inhibitors may have an impact on glucose metabolism by their effect on estrogen levels. Therefore, we designed an experiment in order to assess the effect of estrogens on insulin secretion from rat beta pancreatic insulinoma INS-1 cells.

Methods

Experiments were conducted on an INS-1 cell line, a rodent beta cell line derived from a rat insulinoma induced by X-ray irradiation, which displays high insulin content, production of both proinsulin I and II and responsiveness to glucose and hormones. INS-1 cells were routinely cultured in 75cm^2 flasks (T75) containing 10ml of appropriate culture media and then were transferred into 6-well plates and treated with 17β-E2. Proliferation, as well as insulin expression in the 17β-E2 treated cells in mRNA and protein level was then investigated. Cell cultures were treated with 12.5mM, 25mM, 50mM, 100mM and 200mM estradiol. After 24 hours, RNA as well as protein extraction was carried out. Gene expression was examined in mRNA and protein level, by real time quantitative reverse transcriptase PCR and by western blotting, respectively. Extraction of total RNA was achieved with the use of NucleoZOL (Macherey-Nagel, Duren, Germany). Protein concentration of samples was measured by using the bicinchoninic acid (BCA) assay and bovine serum albumin (BSA) as the standard (Thermo Scientific TM Pierce TM BCA Protein Assay Kit, USA).

Results

Real time PCR showed a dose-dependent up regulation of insulin gene expression by estradiol. The findings were consistent with the results from western blot, although the estradiol dose-dependent increase in gene expression was not so clear. Finally, both protein and mRNA expression of insulin increased in a dose dependent manner, with mRNA reaching a plateau at 100nM estradiol treatment.

Conclusion

The experimental data suggest that there might be a direct effect of estrogen on beta cells and insulin secretion.

Introduction

Breast cancer is the most common cancer in women all over the world [1]. The disease is currently characterized by increased survival [2,3]. Therefore, the effects of the medications used for breast cancer treatment are of paramount importance [4]. A class of drugs used for breast cancer treatment is aromatase inhibitors [5-7]. It has been observed that aromatase inhibitors may impair glucose metabolism or even cause frank diabetes in patients with breast cancer [8]. Diabetes mellitus (DM) is currently a health problem with the size of a pandemic [9-12]. It is characterized by high blood glucose levels for a prolonged period of time [13]. Therefore, the tested hypothesis was that estrogens may affect glucose metabolism. Estrogens may affect both, insulin sensitivity and insulin secretion while altered estrogen levels may affect insulin secretion or promote insulin resistance [14-17]. In the paper here in the association between estrogen levels and insulin secretion was tested in an insulinoma rat cell line. The level of both protein and mRNA insulin expression were measured after treatment of cells with different doses of estradiol.

 ***Corresponding author email:** gvlachodimitris@gmail.com*

Materials and methods

Experiments were performed on an INS-1 cell line (AddexBio, USA), which is a rodent beta cell line, derived from a rat insulinoma induced by X-ray irradiation, that displays many important functional features of pancreatic beta cells such as high insulin content, production of both proinsulin I and II and responsiveness to glucose and hormones. INS-1 cells were cultured in 75cm^2 flasks (T75) containing 10ml of culture media. The media was composed of RPMI 1640 supplemented with 10% (v/v) FBS, 10mM HEPES, 2mM L-glutamine, 1x sodium pyruvate, 0.05mM 2-mercaptoethanol, 100 U/ml penicillin and 0.1g/l streptomycin antibiotics (Gibco, USA). Cultured cells were maintained in a humid incubator containing 5% (v/v) CO2 at 37oC, and they were checked every two to three days for any possible contamination or deterioration, before replacement of the old culture medium with new. After incubation, the cells were observed under the microscope, to check if they were detached and if they appeared sufficiently rounded.

Cell proliferation assays

(a) The rate of proliferation/metabolism of cells was measured using the 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt (XTT) (Applichem). After treatment with estradiol, 15μl of XTT was added to each well in a humidified atmosphere (37°C, 5%CO2) for up to 4 hr. Then XTT was aspirated and 150 ml of DMSO was added to each well. The optical density (OD) was measured at 450nm using a microplate reader (VersaMax; Molecular Devices, Sunnyvale, CA, USA).

(b) The actual number of alive INS-1 cells in cultures was assessed using the trypan blue exclusion assays. Therein, cells were plated (at a cell density of 8x104 cells/well) in 12-well plates and exposed to various doses of the synthetic peptides under investigation. After 48 hours, the cells were harvested and counted by trypan blue exclusion method.

Treatment of cells with estradiol solution

Using 17β-estradiol or 17β-E2 ≥98% (molecular weight 272,38kg/mol) (Sigma Aldrich, USA) a 7.400nM 17β-E2 solution was prepared and used as treatment for the INS-1 cell cultures. Cells from the flask were transferred into 6-well plates in order to perform subculturing of cells under treatment with different concentrations of 17β-E2.

Isolation of total RNA

Extraction of total RNA was achieved with the use of NucleoZOL (Macherey-Nagel, Duren, Germany) and the estimation of extracted RNA concentration with a spectrophotometric assay. Gene expression in cells is achieved through the transcription of genes into messenger RNA (mRNA). So, the amount of mRNA copies is representative of the amount of gene expression[18]. This relationship between gene expression and mRNA, was utilized in the experimental work herein, in order to investigate the regulation of insulin expression by estrogen. Real time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) is a method that allows the detection and quantification of the desired mRNA. The principle for real time qRT-PCR requires the reverse transcription (RT) of RNA into complementary DNA (cDNA). The cDNA is then used as a template for the real time quantitative PCR. Real time PCR requires the use of fluorescent molecules, which report the presence of amplification products during each cycle of PCR, a machinery to detect the fluorescent dye and hence monitor amplification in real-time, and a computer software for quantitative analysis [19].

Isolation of total cellular protein

For protein extraction to be achieved, cell culture medium from the 6-well plate cultures was aspirated using a pipette, and cells were washed with ice-cold Tris-buffered saline (TBS) (Sigma Aldrich, USA) (about 1ml on each well was enough to cover the cells). Protein concentration of samples was measured using the bicinchoninic acid (BCA) assay and bovine serum albumin (BSA) as the standard (Thermo ScientificTM PierceTM BCA Protein Assay Kit, USA). Gel electrophoresis was performed to separate the proteins of the sample lysates. Separation was achieved with SDS-PAGE, which allows protein separation by size. SDS-PAGE gel electrophoresis employs polyacrylamide gels (PAGE) and buffers loaded with sodium dodecyl sulphate (SDS).

Western Blot

Proteins were transferred to a membrane and then antibody incubation to detect the levels of protein.

Results

When the cultured cells (INS-1 rat insulinoma) reached about 80% confluence, they were treated with different concentrations of E2. Proliferation assays were performed as well and insulin expression in the E2 treated cells in mRNA and protein level was then investigated. Extraction of total cellular RNA was performed using NucleoZOL. The concentration of the extracted RNA samples was then measured by a Biospec-nano spectrophotometer. Besides, purity and integrity of total extracted RNA was assessed by calculating the ratio of optical density at 260nm and 280nm. A ratio close to 2.0 indicates RNA integrity and purity. The results are presented in table 1. Values of the ratio of optical density 260nm to 280nm ranged from 1.99 to 2.01, thus near 2, meaning that the extracted RNA was pure, and qualified to be used for qRT-PCR.

The expression of insulin was also assessed at protein level. Protein extracted from INS-1 cells was measured, and then analysed by Western Blot. The standard curve was plotted using Excel, Microsoft Office 2016. According to this curve the concentration of the protein samples was found to be as presented in Table 2 and Graph 2.

Insulin protein expression showed an increasing pattern, however the increase was not dose dependent. The change of the samples treated with 25nM and 50nM estradiol is the same, whereas in the sample treated with 200nM gene expression decreases, compared to the 100nM estradiol.

Table 1: Concentration and A260/A280 ratio of total RNA from INS-1 cells treated with different concentrations of E2.

Treatment	A_{260}	A_{280}	Ratio A_{260}/A_{280}	Concentration of total RNA(ng/μl)
Control	177.25	88.2	2.01	709
12.5nM	205	102.5	2.00	820
25nM	197.6	98.8	2.00	790.45
50nM	215.5	107.2	2.01	862
100nM	173.25	87.1	1.99	693
200nM	186.25	93.1	2.00	745

Table 2: Insulin protein concentration of the INS-1 cells treated with different E2 concentrations

Sample	Unknown Protein Concentration (μg/μl)
Control	1.23
Estradiol 12.5nM	0.97
Estradiol 25nM	1.33
Estradiol 50nM	1.12
Estradiol 100nM	1.35
Estradiol 200nM	1.01

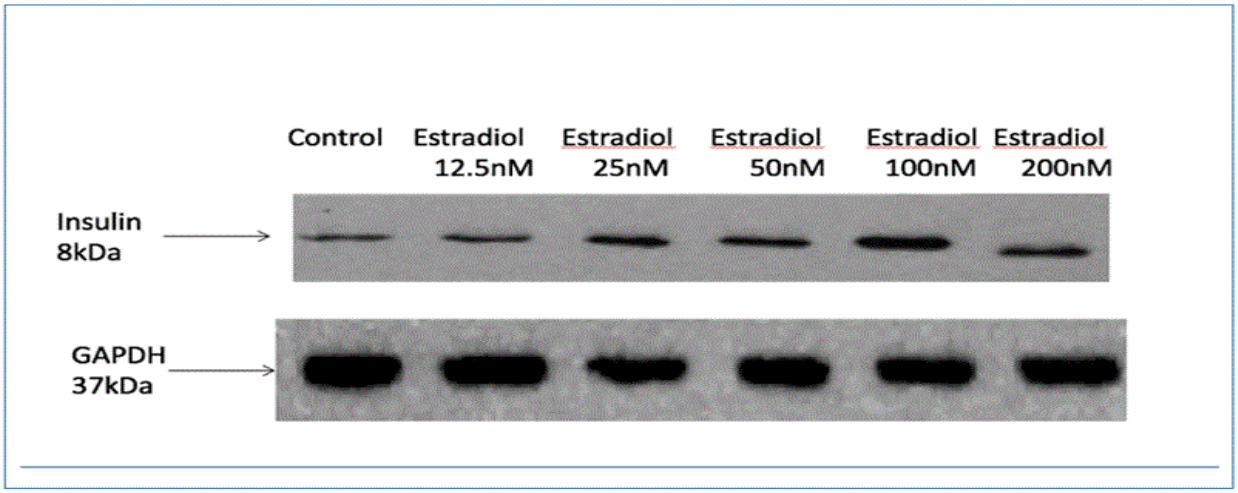


Figure 2: Western Blot results showing insulin as opposed to GAPDH (glyceraldehyde 3-phosphate dehydrogenase) as control.

	Fold Change
Control	1
Estradiol 12,5nM	1.1
Estradiol 25nM	1.5
Estradiol 50nM	1.5
Estradiol 100nM	2.3
Estradiol 200nM	2.1

Figure 3: Fold change in insulin-protein gene expression in INS-1 after treatment with different concentrations of 17β-E2 as compared to untreated INS-1 cells

Western blot

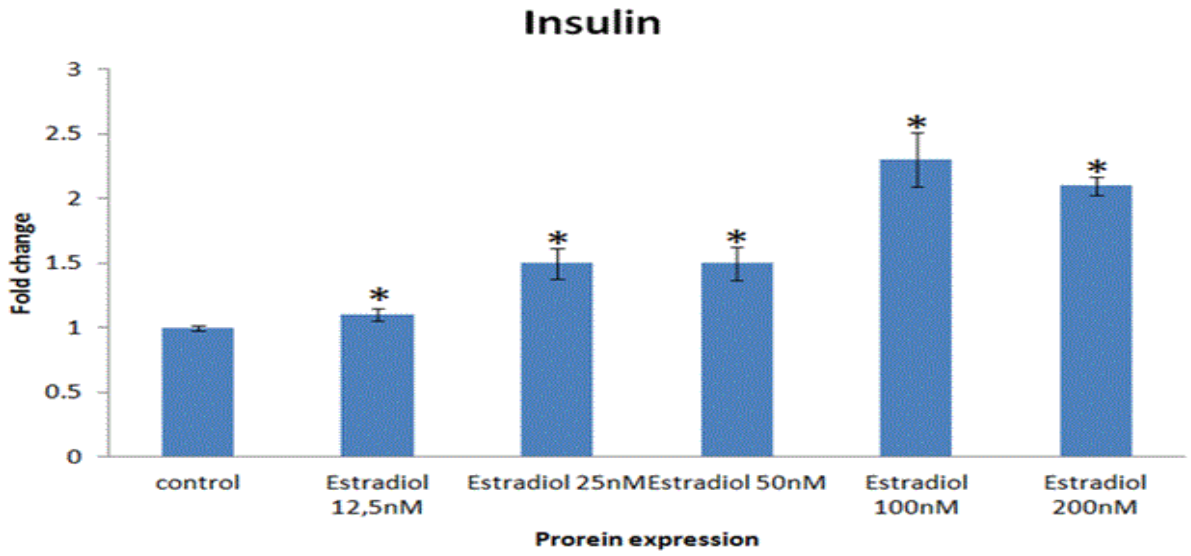
Protein separation and Western Blot

Protein separation was then done by gel electrophoresis. Equal amount of protein (20μg) from the 6 protein samples was loaded into each one of the wells of the formatted SDS-PAGE gel (BioRad, USA) as described in section 2.5. The gel was run at 50V for 5min and then at 130V for an hour. Then proteins were transferred on a PVDF membrane and placed on a cassette for overnight incubation at 10mA constant current as described in section 2.6.1.

The blot was then analysed by application of the Western Blot technique. The blot was incubated overnight with using primary Insulin (L6B10) Mouse (mAb #8138 by Cell Signalling, USA) and then incubated with anti-mouse IgG, HRP-conjugated secondary antibody (#7076 at 1:2000) and HRP-conjugated antibody (#7075 at 1:2000). GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as control in WB. Interpretation of results was achieved with X-ray exposure. The image obtained on western blotting film, for the proteins, insulin and GAPDH, is shown in figure 2.

This image was analyzed with band analysis tools of ImageLab software version 4.1 (Bio Rad). This software allowed the quantification of the band intensity, which is representative of the protein expression. Then the intensity of all samples was divided with the intensity of the control sample, and this is the fold change, shown in figure 3. Then, using SPSS software figure 4 was plotted to allow comparison of the change in gene expression.

Figure 4. Figure showing fold change of insulin protein gene expression of the 17β-E2 stimulated INS-1 cells. Untreated cells were used as the negative control with a normalized gene expression value of 1.00. The experiment was run in triplicate. Error bars in each bar chart represent the standard deviation. Student's test (two-sided) was used for statistical analysis of variance (SPSS v. 20 statistical package, SPSS Inc., Chicago, USA). The level of significance was set at $p<0.05$. * indicates $p<0.05$ which represents statistically significant change from untreated INS-1 cells as compared to E2 treated INS-1 cells.



Again, as in insulin mRNA, insulin protein expression showed an increasing pattern in expression, however, not dose dependent. The fold change of the samples treated with 25nM and 50nM estradiol is the same, whereas in the sample treated with 200nM gene expression decreases, as compared to the 100nM estradiol.

Discussion

The results of the present study provide evidence that protein and mRNA expression of insulin increased in a dose dependent manner after treatment of an insulin producing beta cell line with estradiol. Insulin gene expression increased proportionally to the concentration of estradiol. Gene expression reached a plateau, which may result from saturation of estrogen receptors.

In a study performed in female rats Kang et al[20] showed that ovariectomy reduces insulin expression as determined by stain intensity in beta cells as well as by reverse polymerase chain reaction. In the same experiments estradiol was shown to counteract the adverse effects induced by glycosamine on beta cells, including the attenuated by glycosamine insulin secretion. Soriano et al[21] have shown that 17β-estradiol, at physiological levels is implicated in normal beta cell function. They further showed that long term exposure to estradiol increases insulin content, insulin gene expression and insulin release via the estrogen receptor α (ERα), while rapid responses to 17β-estradiol regulate K(ATP) channels thereby increasing insulin release in beta cells. Ripoll et al[22] showed that in beta

cells 17β-estradiol regulates K(ATP) channel activity and glucose-induced calcium oscillations, modulating insulin release, while Alonso-Magdalena et al[23] proved that long-term exposure to physiological concentrations of 17β-estradiol increased beta cell insulin content, insulin gene expression as well as insulin release. The use of ER alpha and ER beta agonists in their experiments suggested that the estrogen receptor involved is ER alpha. Nadal et al [17] have shown that estrogen receptors ERalpha and ERbeta exist in beta-cells. They showed that ERalpha plays an important role in the regulation of insulin biosynthesis, insulin secretion and beta-cell survival. They proved that activation of ERalpha by 17β-estradiol promotes an increase of insulin biosynthesis. In addition, it has been shown that glucose induced insulin secretion was enhanced by estradiol in isolated cells[24]. ERbeta is also involved in modulating insulin release by beta cells. In accordance, an estrogen receptor beta agonist [25] was shown to be an insulinotropic agent, enhancing glucose stimulated insulin secretion from both mouse and human islets.

ERalpha antagonist tamoxifen was shown to induce diabetes in vitro and in vivo. Thus, tamoxifen may induce diabetes mellitus [26]. Aromatase inhibitors markedly decrease estrogen concentrations in postmenopausal women by inactivating the aromatase enzyme responsible for the synthesis of estrogen from androgenic substrates. Aromatase inhibitors have been shown to induce impaired glucose metabolism or even frank diabetes in breast cancer patients8, although not all reports are in agreement [27]. However, the relationship between aromatase inihibitor treatment and diabetes is in accordance with the fundamental role estrogens have been found to play in the control of energy metabolism [28,29] and the results of the present study, where estrogens were observed to increase insulin secretion. Furthermore, the mechanism underlying the effect of aromatase inhibitors on glucose metabolism has not been elucidated. These experiments provide some evidence for a possible effect of altered estrogen levels being responsible for altered glucose metabolism in patients on treatment with aromatase inhibitors. In the case of treatment with aromatase inhibitors the benefits outweigh the risks [8] and treatment should not be stopped. Patients should be offered guidance on lifestyle and diet in order to have the most benefit from breast cancer treatment.

In conclusion, we have shown that estrogens increase insulin gene expression and insulin secretion. These results are in accordance with the literature and provide a possible explanation for the effect of aromatase inhibitors on glucose metabolism.

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