Introduction

17-β Estradiol (E2) is a female sex hormone and is the most abundant and potent of the estrogens [1]. E2 causes females to sexually mature by promoting the further development of the mammary glands, the lining of the endometrium, vagina, the cervical glands, and the fallopian tubes, as well as increasing adhesion tissues [2]. Even though doses within the human body prevent cell lysis and proliferation, high dose E2 therapy can potentially be used as cancer therapy [3]. Studies suggest that high-dose E2 therapy is potent as some leading cancer therapies, with higher survival rates and without the side effects [5]. However, the mechanism of action is not fully understood in estrogen receptor negative cancers.

The two main proteins of interest with regard to E2 are protein disulfide isomerase (PDI) and mammalian target of rapamycin (mTOR). PDI are a family of multifunctional proteins which are involved in the formation, ligation, and rearrangement of disulfide bonds, as well as the folding of other proteins [4]. Recent studies suggest that E2 inhibits PDI [4]. When inhibited by E2, PDI loses its isomerase activity, which is mainly involved in protein folding, leading to the production of misfolded proteins in the endoplasmic reticulum (ER), thus causing ER stress [5]. With the onset of ER stress, the cell attempts to adapt to the stress. However, if adaptations fail, cells will induce apoptosis [5]. The ability of PDI to fold other proteins reportedly allows it to assemble and form mTOR. mTOR is a conserved serine/threonine kinase that plays a crucial role in various cellular functions such as regulation of cellular metabolism, growth and proliferation, as well as migration [2].

The goal of this study was to understand the mechanism of action of high dose E2 treatment in estrogen receptor negative cancers. It was hypothesized that with high dose E2 therapy, the concentration of active PDI will decrease in cells. With a decrease in PDI, there will also be a lower concentration of mTOR, which will decrease cellular proliferation. It is also hypothesized that apoptosis may be induced as a result of increased ER stress caused by the inhibition of PDI by E2.

Methods

Cell Culture

MDA-MB-231 (MDA) cells were cultured in DMEM/12 media (Gibco) supplemented with 10% FBS (Gibco) and 2% penicillin/streptomycin and incubated at 37°C and 5% CO2. Cells media was replenished and cells passed as needed.

E2 Treatment

MDA cells were treated with concentrations of E2 in DMEM, ranging from 0.01 µM to 100 µM. The time intervals for the E2 treatment varied based on assay protocol.

Cell Viability Assay for E2 MDA cells were seeded in a 96-well plate at 20,000 cells/well in 100 µl of cell culture medium. MDA cells were treated with E2 concentrations ranging from 0.01 µM to 100 µM for 48 hours. Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (MTS) was then performed on the cells and absorbance was read using a microplate reader at 490 nm.

ELISA of PDI and Phospho-mTOR
PDI and Phospho-mTOR were measured using immunoprecipitated PDI (EUSA Life Sciences) and Phospho-mTOR (Cell Signaling), respectively. Cell lysates of MDA were collected after 48 hr treatment of E2 and stored at -80°C. ELISAs were performed per manufacturer’s instructions.

Caspase 3/7 Assay
Caspase 3/7 assay (Invitrogen) (Promega) for Caspase 3/7 or Caspase 9 was used. In a flat-bottom 96-well cell culture plate, cells were treated with E2 for 48 hr. Assays were performed as per manufacturer’s instructions.

Sample Preparation for TEM Imaging
Cells were treated as described above and then fixed with 4% glutaraldehyde/0.1M formaldehyde in 0.1M sodium cacodylate buffer, pH 7.4, scraped from the wells with Tellyn and gently spun down to form a pellet. The pellet was post-fixed in 1% osmium tetroxide, dehydrated in a graded series of acetone, and infiltrated/used in EMBTA 812 epoxy resin. 100nm sections were collected onto 200 mesh copper grids and stained with 2% uranyl acetate and 0.5% lead citrate. Images were sampled using a JEOL, JEM 2100.

Results & Discussion

In this study, the effect of E2 on MDA was studied regarding cell viability, metastasis, and endogenous PDI concentrations. MDA cell viability was found to increase in a dose-responsive fashion (Figure 1). At low concentrations, such as 0.01 µM, the decrease in cell viability was not statistically significant. However, higher concentrations ranging from 0.1 µM to 100 µM, the decrease in cell viability was shown to be statistically significant (p<0.05 or p<0.01) (Figure 2). The effect of E2 on MDA metastasis was shown to increase significantly at high concentrations, such as 100 µM (p<0.01) (Figure 3). However, for certain intermediate concentrations, such as 0.01 µM, it was non-significant increased (p>0.10). It was also shown that as the E2 concentrations increased, the total PDI concentration increased in a dose-responsive fashion, and the increase was found to be statistically significant (p<0.05 or p<0.01) (Figure 4). The increase in PDI concentration may be an adaptive response against high dose E2; however, not all of the protein may be active.

Phosphorylated mTOR was also analyzed, as the concentration of phosphorylated mTOR is an indicator of mTOR activity. There was shown to be an overall decrease in mTOR activity as the concentrations of E2 increased (Figure 5). For 0.01 µM and 100 µM of E2, the decrease in mTOR activity was statistically significant (p<0.01). Even though E2 was causing cell death, as shown in Figure 1, it was crucial to understand whether death was occurring due to apoptosis or necrotic toxicity of E2. Therefore, levels of caspase 3/7 and caspase 9 were analyzed. Caspase 3/7 is the final effector apoptotic enzyme, which is involved in inducing apoptosis. Caspase 9 acts as marker for intrinsic apoptosis signaling. As E2 concentrations increased, caspase 9 showed a statistically significantly increased at the 1µM and 100µM concentrations of E2 (p<0.05 or p<0.01 respectively) (Figure 5). However, caspase 3/7 increased significantly only at 100µM E2 (p<0.01) (Figure 6). These findings suggest that E2 caused intrinsic apoptosis.

Light microscopy images revealed that at 100µM E2, cells had an abnormal shape and granules were present outside of the cells, suggesting that the cells were stressed and trying to remove the E2 that was taken up (Figure 7). This suggests that intrinsic apoptosis could be the result of cellular stress. TEM analysis of cells treated at 100µM E2 revealed that E2 was present as cells had abnormal dilations of the lumen of the ER, as compared to the control (Figure 8).

These findings suggest that increased ER stress may be one of the factors causing cellular stress. Additionally, these findings are contradictory to the analysis of the PDI concentrations present in the cells. If active PDI increased in quantity, PDI would increase protein folding, thus preventing ER stress. As a result, PDI requires further study. From this study, it can be seen that high-dose estrogen therapy has anti-cancer properties in estrogen receptor negative cancers as it was able to induce intrinsic apoptosis and increase cellular stress.

References


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E2 Concentration (µM)