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Role of GPER in Cadmium-Induced Phosphorylation of ERK1/2 in Ovarian Adenocarcinoma

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ABSTRACT

Cadmium, a carcinogenic heavy metal, is an environmental contaminant found in air, water, and soil. It also exhibits endocrine disruptive properties by mimicking the proliferative effects of the hormone estrogen and is classified as a metalloestrogen. At low concentration levels in some cancer cells, cadmium induces cell proliferation and phosphorylation of ERK1/2, a key protein in the estrogen signaling pathway. While the signaling pathways for cadmium- induced phosphorylation of ERK1/2 have been discovered in breast and lung cancer cells, it has not yet been fully determined in ovarian cancer cells. The fairly recent discovery of a transmembrane receptor found in estrogen responsive tissues, G protein-coupled estrogen receptor (GPER), presents a possible pathway for studying cadmium's effects in ovarian cancer cells. To determine the role of GPER in cadmium-induced phosphorylation of ERK1/2, two human ovarian adenocarcinoma cell lines, OVCAR3 and SKOV3, were treated for 30 minutes with 10 μM G15, a GPER inhibitor, followed by treatment with 100 nM $CdCl_2$ for 10 minutes. Immunoblot analysis was performed to measure cadmium-induced phosphorylation of ERK1/2. The results indicate that in both cell lines, G-15 decreased cadmium-induced phosphorylation of ERK1/2 suggesting that GPER may be play an important role in cadmium's proliferative effect in ovarian adenocarcinomas.

INTRODUCTION

Ovarian Cancer

Ovarian cancer is the most fatal gynecologic cancer and the fifth most common cancer-related cause of death in women.[1] Epidemiologically, diagnosis rates are highest in non-Hispanic, post-menopausal, white women.[1] Worldwide, there are approximately 300,000 new cases, with high mortality rates of 150,000 deaths every year.[2] According to the American Cancer Society, 19,710 women in the United States will be diagnosed with ovarian cancer in 2023. Out of those women, about 13,270 are estimated to die from the disease.[3] Unfortunately, many women diagnosed with ovarian cancer face a five year survival rate below 50% which could have been avoided with the availability of effective screening procedures for early-stage diagnosis of the disease.[1] Often known as a silent killer, over 70% of ovarian cancer is usually not diagnosed until it progresses to stage III and IV.[1] The lack of proper screening methods and presentation of vague signs and symptoms are key factors resulting in the late detection of ovarian cancer. The main symptoms include bloating, abdominal pain, upset stomach, frequent and urgent urination, back pain, and fatigue.[3] Unfortunately, these symptoms are also present in patients affected by cancer from other organs and those affected with non-cancerous diseases which creates barriers for early diagnosis and treatment.[3] Some current screening options include transvaginal ultrasound (TVU) and measurement of protein biomarker cancer antigen (CA 125).[4] TVU is the the most popular imaging method used to detect ovarian cancer. It involves the imaging of the fallopian tube, uterus, and ovaries, and the detection of irregularities in size and shape of ovarian tissues.[3,4] Clinicians check for the presence of ascites, papillary projections, and/or internal blood

flow to predict the stage of malignant masses.[4] CA125 is a widely used ovarian cancer biomarker that has been found to promote cancer cell growth and metastasis.[4] Both of these screening methods, however, have major drawbacks. CA125 lacks specificity and sensitivity, and it was recently discovered not to be elevated in about 50% of patients during their earlier stages of the disease.[1,4] It has also been shown that epidemiological components such as ethnicity, obesity, age, and race influence serum levels of CA125, regardless of whether ovarian cancer is present or not.[4] As a sonographic imaging, TVU has poor abilities in determining if masses are malignant or benign. Once a mass is found, a core-needle biopsy is required to confirm the presence of ovarian cancer.[1] This procedure is also not free from risks as it can lead to the development of abdominal wall metastasis. The study of new protein biomarkers such as the human epididymis protein 4 (HE4), and the discovery of more accurate imaging modalities are two major areas of research needed in order to produce more effective screening and diagnostic of ovarian cancer.[1,4]

The threage andypes of ovarian cancer are epithelial ovarian cancer (EOC), germ cell, and sex-cord-stromal.[1] EOCs make up about 95% of all ovarian cancer types, while the latter two account for only 5% of ovarian cancers.[1,5] EOC has four subtypes: mucinous, clear cell, endometrioid, and serous which is further divided into high-grade serous carcinomas (HGSC) and low-grade serous carcinomas (LGSC).[1,6] HGSC accounts for 90% of all EOCs and has a more fatal prognosis with a 10 year mortality rate of 70%.[1] LGSC is usually diagnosed at a younger age and has a longer expected survival time.[1]

EOCs were first thought to originate only in the outer epithelium of ovaries, but recent evidence suggests that there are three main origin sites.[1] EOCs can originate in the epithelium of ovaries, fallopian tubes, and other epithelial sites in the pelvis.[1] These malignancies can stay

benign or metastasize to other parts of the body where they pose bigger problems as treatments options become limited.

A variety of risk factors have been associated with the development of ovarian cancer and it is most likely a combination of those factors that lead to the disease being manifested. One of the biggest risk factors associated with the disease is genetic predisposition through family history of ovarian or breast cancer, but this also occurs in women with no family history of the disease.[1,2] Although several genes have been identified to increase the likelihood of ovarian cancer, it is mostly due to the mutation of tumor suppressor genes BRCA1 and 2.[1,2] A BRCA1 mutation leads to a 40-50% risk of developing ovarian cancer at the age of 70 while BRCA2 is associated with 10-20% risk increase.[2] When a woman with a BRCA mutation develops EOC, it is referred to as a type II tumor, which is usually associated with fatal outcomes.[1,6] Type I tumors are not linked to genetic mutations but are thought to be caused by increased inflammation, endometriosis, and continuous ovulation cycles which are all risk factors associated with the disease.[1,6] Processes which halt ovulation such as pregnancies, breastfeeding, and use of oral contraceptives have been associated with decreasing the risk of acquiring the disease due to reduced stress on the reproductive system as a result of menstruation.[1,2] It is important, however, to note that these factors cannot be promoted as preventive measures since these are major lifestyle choices not every woman would wish to choose.[2]

The conventional treatment of ovarian cancer is a combination of both debulking surgery and chemotherapy.[6] Before surgery, neoadjuvant therapy is performed to reduce tumor mass. During debulking surgery, surgeons remove the majority of the cancerous masses to increase the effectiveness of chemotherapy.[1] Carboplatin and paclitaxel are given as primary adjuvant therapy after surgery has been performed.[1] Despite all these treatments, remission is not guaranteed,

especially in patients diagnosed with advanced stages of the disease who tend to experience greater risk of recurrence.[1] Having an in depth understanding of the pathophysiology of this deadly disease is required to develop more effective treatments for patients.

Estrogen

Estrogens are a group of hormones including estradiol, estriol, estetrol, and estrone belonging to the organic family of steroid compounds.[7,8] Estrogen is primarily a female hormone, but plays vital roles in both male and female reproductive and brain functions, glucose and lipid homeostasis, and bone metabolism.[7,9] The biosynthesis of estrogen begins with the translocation of cholesterol to the inner mitochondrial membrane where it is converted to pregnenolone.[7] Pregnenolone gets converted to androstenedione which can be converted to other androgens or transported to the granulosa cells of the ovaries.[7] At the granulosa cells, androstenedione becomes estrone through the enzymatic activities of aromatase.[7] Estrone is then converted to estrogen's most potent form, estradiol, which travels through the bloodstream to induce effects on varying tissues.[7,10] Endogenous estradiol production is highest in pre-menopausal women, but elevated levels in postmenopausal women have been associated with the development of endometrial, breast, and ovarian cancer.[7,10] It is therefore a normal and beneficial process for estrogen production to decrease as a woman ages, especially after menopause.[7]

Estrogen's biological actions are mediated through its interactions with estrogen receptors (ERs) in tissues.[7,9] In 1958, Elwood Jensen discovered ERs and later showed their ability to migrate to the nucleus and stimulate transcriptional changes.[8] About twenty years later, ERα, the first human ER was cloned using RNA from human MCF-7 breast cancer cell lines. Ten years after the discovery of ERα, the second human receptor, ERβ, was identified in ovary

granulosa cells and prostate epithelial cells.[8] Both ERα and ERβ belong to a nuclear receptor superfamily of ligand regulated transcription factors which play a role in breast, ovarian, and uterine cancer.[7,11] Previously, cellular changes mediated by estrogen was thought to occur only through nuclear ERα and ERβ through direct genomic signaling.[27] With direct genomic signaling, ERα or ERβ function as ligand activated transcription factors located in the cytoplasm.[27] As estradiol binds to these receptors, they translocate to the nucleus and form dimers through conformational changes (Figure 1).[7,8] This complex then binds to estrogen response element (ERE) sequences in the promoter regions of estrogen responsive genes and induces transcriptional modifications.[7,8] In cells lacking EREs, estrogen receptor complexes activate or suppress target genes through protein-protein interactions with other response elements or transcription factors.

Contrary to previous ideas, the presence of estrogen receptors have also been recognized in the plasma membrane. In 1977, Pietras and Szego reported rapid estrogen signaling in the cell membrane of uterine endometrium.[28] Membrane bound ERα or ERβ works through non-genomic rapid activation of intracellular signaling pathways. [27] In the non-genomic pathway, endogenous estrogen binds to membrane bound ER which interacts with signaling molecules such as protein kinase Src, and other adaptor proteins (Figure 1). This leads to the activation of multiple intracellular protein kinase cascades that causes indirect changes in gene expression.[7,8] One well known non-genomic pathway that has been associated with the development of many cancers is the Ras/Raf/MAPK pathway, which results in the phosphorylation and activation of protein kinase ERK1/2. This activation leads to the phosphorylation of numerous signaling proteins that induce cell proliferation.[26]

Although ERα and ERβ have some commonalities, they are also functionally distinct with each regulating unique sets of genes. It was discovered that both receptors diverged early on during evolution and differ at three domains in the N-terminal.[12] The differential functions of these ER receptors also play a crucial role in estrogen-sensitive cancers including ovarian cancer. ERα is thought to be a pro-tumorigenic gene whereas ERβ is reported to have anti-proliferative effects.[13] When comparing normal ovarian tissues with EOCs, a decrease in ERβ/ERα ratio as well as a loss in ERβ expression was observed.[14] Some studies have shown a positive correlation of ERβ and survival in ovarian cancer patients, and some speculate that ERβ may be used in treatment of estrogen-sensitive cancers.[12,14]

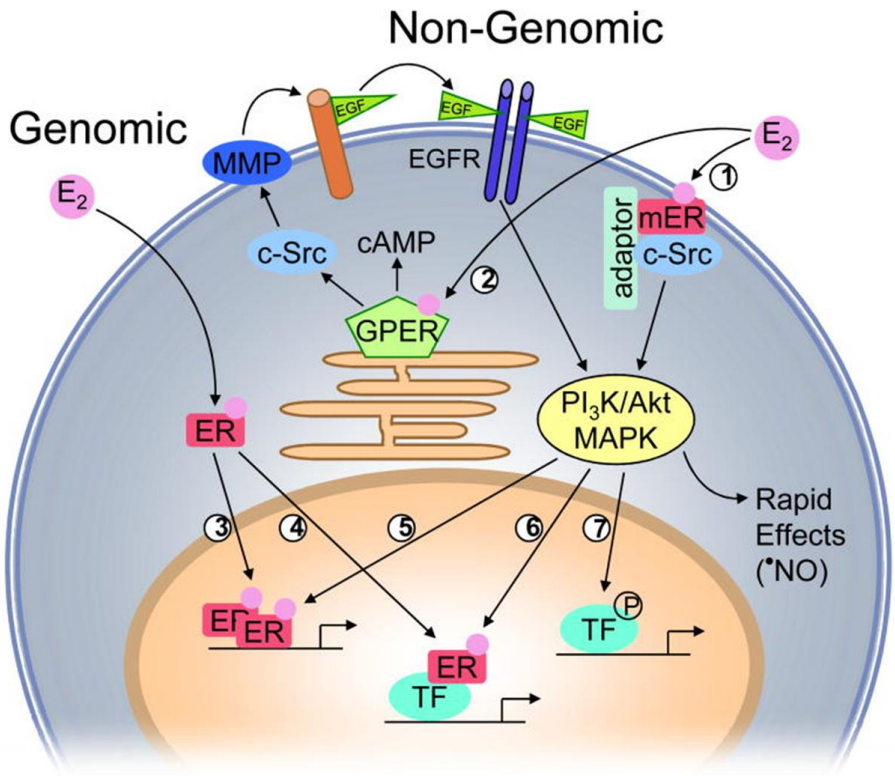

Figure 1. Proposed pathway of genomic and non-genomic estrogen receptor (ER) signaling. The genomic pathway shows endogenous estrogen (E2) binding to ER located in the cytosol. The non-genomic pathway shows E2 binding to either a plasma membrane-bound ER or G-protein coupled receptor (GPER) located on the endoplasmic reticulum. Figure adapted from *Meyer et al.* (2009).[27]

In recent years, another non-genomic pathway was also discovered in estrogen responsive tissues. The G Protein-Coupled Estrogen Receptor (GPER) was identified through molecular cloning methods in 1996.[8] GPER can be localized at the plasma membrane or the endoplasmic reticulum.[8,27] As shown in Figure 1, estrogen's non-genomic action through GPER begins with the activation of Src, which activates matrix metalloproteinases (MMP). MMP causes the transactivation of epidermal growth factor receptors (EGFR), which results in the activation of signaling cascade via PI3K/Akt and Ras/ Raf/MAPK pathways before inducing transcriptional changes within the nucleus.[27] Some studies indicate that Src and EGFR are also involved in cancerous cell growth, which could highlight their key roles in estrogen signaling and cell proliferation in estrogen sensitive cancers including ovarian cancer.[23,29]

The use of anti-estrogen therapy has become a common method in treating estrogen sensitive cancers.[9,14] In hormone dependent breast cancer, an estrogen antagonist, tamoxifen, is the main treatment option used to regulate transcriptional activities of ERs.[9] Tamoxifen proves effective in about 50% of ER^+ breast cancer patients, but when applied to ER^+ ovarian cancer patients, only 15-18% responded positively with the anti-estrogen treatment.[14] One theory suggests that tamoxifen's effectiveness in blocking ERα and ERβ inhibits the antiproliferative properties of ERβ in ovarian cancer cells.[14] Estrogen signaling pathways also crosstalk with other oncogenic pathways making it possible for the potential benefits of tamoxifen to be masked during treatment.[13] The complexities of hormone signaling and the challenges they pose highlight the importance of future research studies on combinatorial anti-estrogen treatments of ovarian cancer.

Endocrine Disruptors and Metalloestrogens

Along with endogenous hormones produced in the body, there are large numbers of substances with natural and synthetic origins that can alter the normal functioning of the endocrine system. These are known as endocrine disruptors (EDs).[15,16] These exogenous compounds interfere with homeostatic mechanisms of normal steroid hormones in the body by disrupting synthesis, secretion, transportation, binding, or elimination of those hormones.[15] EDs may act as agonists or antagonists by mimicking or blocking the effects of hormones, especially those relating to reproduction and sexual development.[17] It is therefore suggested that EDs may have vital roles in the formation of reproductive health issues in both humans and wildlife.[17]

Because of their industrial significance, a wide variety of EDs are used in our day to day lives without awareness of their hazardous effects.[15] One ED known by many, DDT, was commonly used as a pesticide in agricultural, public, and household settings before being banned due to its dangerous nature on humans and the environment.[15,18] It was classified as an ED because of its ability to interfere with multiple hormonal functions, including thyroid, estrogen, and androgen action.[15] Since DDT discovery, several other EDs have been found and classified.

DDT and some other EDs are also capable of impairing female reproduction and disrupting fertility in numerous species, including humans, when exposure occurs early in development.[18] Another popularly known ED, Bis-phenol-a (BPA), is a stabilizing agent commonly used in plastic containers and linings of canned food products.[15] BPA began raising concerns in recent years due to its estrogenic effects on the body, which makes it capable of directly interacting with ERs and their regulated pathways.[17] A study done by *Levy et al.* (2004) showed that BPA is capable of interacting with ER as an exogenous estrogen and inducing feminizing effects on *X. laevis* tadpoles. The expected sex ratio of male to female tadpoles under

normal conditions is 1:1, but with exposure to 10^{-7} M doses of BPA, the percentage of females rose significantly to 70%.[17] This demonstrates that BPA's estrogenic effects could lead to sex determination of tadpoles at larval stages, which in turn ruins the balance of female to male frogs in natural habitats.[17]

Another study on mice done by *Nicolas et al.* (2009) showed that BPA exposure on perinatal subjects resulted in decreased fertility and fecundity.[19] Female reproductive functions are greatly dependent on the balance of hormones, especially estrogen. The addition of exogenous hormones would likely cause unwanted effects including increased risk of developing ovarian cancer.[20] Being able to identify and limit the exposure of endocrine disruptors becomes very important in maintaining not just female health but that of the entire environment.

Recent research studies have shown that heavy metals can also possess estrogenic properties and are referred to as metalloestrogens. While these heavy metals do not chemically resemble estrogens, it is believed that the large ligand-binding cavity of the ERs causes low specificity which enables many EDs to easily bind and can mimic or antagonize the actions of estrogen in the body.[21,22,23] Many heavy metals are also carcinogenic, and may increase cancer cell growth in estrogen-responsive tissues by stimulating increased cell proliferation through different ER pathways.[22] The specific pathways and full mechanism of action metalloestrogens use is still being researched in many carcinomas including ovarian cancer.

One of the most toxic heavy metals and environmental pollutants, cadmium (Cd), has raised many concerns due to its harmful effects on the body by increasing oxidative stress, inducing DNA damage, and inhibiting DNA repair systems.[23,24] Cd exposure occurs through occupational means, use of cigarettes, and in contaminated air, water, food, and plants.[24] Several studies have shown that Cd can have impact on human health through exposure to chronic low-

levels.[22] Because Cd lacks an active biochemical pathway for elimination, it is excreted very slowly and can persists in soft tissues for 15-20 yrs, allowing enough time to cause irreversible damages in the body.[22,24] Classified as an important human carcinogen, Cd promotes cell proliferation in many cancer types including uterine, breast, prostate, and ovarian cancer.[24,25] In 2016, *Huff et al.* provided evidence that Cd also induced cell proliferation in lung adenocarcinoma cell lines. In these studies, proliferation was inhibited when ERs were blocked by the addition of ICI 182,780, an ER antagonist, in cell lines derived from female patients.[23] Along with the inhibition of cell proliferation, ICI 182,780 also blocked phosphorylation of ERK1/2, suggesting that Cd may be working through non-genomic estrogen signaling pathways.

In 2006, *Brama et al*. showed that a 10 μM $CdCl_2$ exposure in breast cancer cells induced cell proliferation and the activation of ERK1/2. Specifically, $CdCl_2$ treatment for 1,3,6, and 24 hrs showed increased phosphorylation of ERK1/2.[25] However, when cells were also treated with 1 μM ICI 182,780, an ER antagonist, cadmium-induced phosphorylation of ERK1/2 was inhibited, suggesting an ER-dependent mechanism of activation.[25] This study indicated that cadmium induced proliferation through non-genomic estrogen signaling pathway and could have an important effect in carcinogenesis.

Purpose of Study

In 2019, *Ataei et al.* showed that at low concentrations ranging from 0.00001- 0.1 μM, Cd significantly increased cell proliferation along with the activation of ERK1/2 in two ovarian cancer cell lines, SKOV3 and OVCAR3. Treatment with 10 μM ICI 182,780 significantly inhibited cadmium-induced cell proliferation and reduced ERK1/2 phosphorylation, indicating an ER-dependent mechanism of action.[24]

However, when Biochemistry and Molecular Biology student, Haley Todd, replicated similar experiments in SKOV3 and OVCAR3 cells, she found that exposure to low concentrations of Cd ranging from 0.001-1 μM only resulted in slight cellular proliferation. In fact, treatment with 10 μM ER antagonist, ICI 182,780, followed by a 10 minutes 0.1 μM Cd treatment did not inhibit Cd-induced ERK1/2 phosphorylation in OVCAR3, and only demonstrated a slight inhibition of phosphorylation in SKOV3. These contrasting results to the previously published study suggest that Cd might carry out its effects in an ER-α and ER-β independent manner in ovarian adenocarcinomas.

The discovery of the novel transmembrane receptor, GPER, shown to also be involved in cancer development provides a possible mechanism for an ER-α and ER-β independent pathway. In some breast cancer cells, estrogen can stimulate MAPK and ERK1/2 along with the transactivation of epidermal growth factor (EGFR) without interaction with ER-α and β.[7] This has already been established through inhibitor studies by *Huff et al*. (2016) where it was observed that cadmium-induced ERK1/2 phosphorylation involved GPER, Src, and EGFR in female lung adenocarcinoma.[23] Therefore, the purpose of this research is to determine if GPER plays an important role in Cd-induced phosphorylation of ERK1/2 in both OVCAR3 and SKOV3 ovarian cancer cell lines.

METHODS

Inhibitor Studies

Ovarian adenocarcinoma cell line, SKOV3, was obtained from ATCC (Manassas, VA) and grown according to manufacturer's instructions. For SKOV3, cells were plated at 400,000 cells in 3 mL of McCoys 5a media containing 10% fetal bovine serum and 1% penicillin/streptomycin

in a 60 mm petri dish for 48 hrs. For OVCAR3, cells were plated at 400,000 cells in 3 mL RPMI 1640 containing 10% fetal bovine serum, 1% penicillin/streptomycin in a 60 mm petri dish for 48 hrs. The media was then replaced with 3 mL plate media containing charcoal-stripped fetal bovine serum and no hormones for 48 hrs. To determine the role of G-protein coupled receptor (GPER) in cadmium-induced phosphorylation of ERK1/2, cells were treated with 10 µM G-15 (Tocris Bioscience, Bristol, U.K) dissolved in DMSO (Sigma Aldrich, St. Louis, MO) for 30 minutes followed by 100 nM $CdCl_2$ for 10 minutes. For controls, cells were either untreated or treated with DMSO alone. To prepare whole cell lysates, the media was removed, and cells were rinsed with ice cold phosphate buffered saline (PBS) two times. Cells were then treated with 60 µL cell lysate buffer prepared in RIPA buffer (Sigma) containing 1/1,000 volume Phosphatase Inhibitor Cocktail II (Sigma), 1/1,000 volume Phosphatase Inhibitor Cocktail III (Sigma), 1/1000 volume of Protease Inhibitor Cocktail I (Sigma), and 100 µM PMSF. Cells were scraped from the petri dish and collected in labeled microcentrifuge tubes on ice. Each lysate was sonicated two times for 1 sec at lowest strength using Microson™ ultrasonic cell disruptor (Barcelona, Spain). After sonication, lysates were centrifuged at high speed for 10 mins at 4°C. The supernatants were transferred to precooled labeled microcentrifuge tubes and stored at -80°C.

SDS Gel Electrophoresis and Western Blot Analysis

Protein concentrations were measured using Bradford Assay (Bio-Rad, Hercules CA), and then separated on a 4-20% polyacrylamide SDS gel at 300 volts for about 15-30 mins. Wells were loaded with 10 µL standards and 25 µg of protein. After separation, proteins were transferred for 7 minutes to a nitrocellulose membrane for western blot analysis using the Trans-Blot Turbo Transfer System (Bio-Rad) The membrane was blocked in 5% milk in TTBS (20 mM Tris pH

7.4, 140 mM NaCl, 0.1% Tween-20) for 1 hour at room temperature. The blot was then washed with TTBS 1X for 15 minutes and 3X for 5 minutes, and then incubated with a polyclonal antibody raised to P-p44/42 ERK1/2 as the primary antibody (Cell Signaling Technology, Danvers, MA) at a 1/1,000 dilution overnight with shaking at 4°C. Following another round of washes as described above, the blot was then incubated with a 1/5,000 dilution of goat anti-rabbit conjugated with horseradish peroxidase (GAR)-HRP secondary antibody (Bio-Rad) for 1 hour. After washing, the blot was developed using a 1:1 dilution of the ECL reagents (Bio-Rad) for five minutes before being imaged on a Chemidoc system (Bio-Rad).

To measure the total amount of ERK1/2, proteins were removed from the membrane using Stripping Buffer (Thermo Scientific, Waltham, MA) for 15 minutes, washed 3X with TTBS and incubated for 1 hour in 5% milk in TTBS. The blot was then incubated with a polyclonal antibody raised to p-44/42 ERK1/2 (Cell Signaling Technology) overnight at 4 C with shaking. After washing 3X in TTBS, the secondary antibody was added as described above. Following a final wash in TTBS, the blot was then exposed to ECL reagents as described above and imaged once more. To measure the density of the immunoblot bands, the images were analyzed by densitometry using Unscan-it software (Silk Scientific, Provo, UT). Images are reported as relative amounts of phospho-ERK1/2 to total ERK1/2.

RESULTS

Inhibitor Studies

Previous studies performed by Haley Todd in Huff's Lab showed that ERK1/2 was activated within 10 minutes of 0.1 μM Cd treatment in SKOV3 and OVCAR3 cell lines. However, treatment with the ER antagonist, ICI 182,780, did not inhibit ERK1/2

phosphorylation in OVCAR3 and demonstrated only slight inhibition in SKOV3, suggesting that Cd does not use an ER-α and ER-β receptor pathway. The recent discovery of the transmembrane receptor, GPER, provides another possible mechanism of action. To determine if Cd-induced phosphorylation of ERK1/2 involves GPER, SKOV3 and OVCAR3 cells were treated with the GPER inhibitor, G15, for thirty minutes before treating with Cd for ten minutes, and immunoblot analysis was used to measure the amount of ERK1/2 phosphorylation. As shown in Figure 2, Cd alone increased ERK1/2 phosphorylation in SKOV3 and OVCAR3. When cells were treated with the GPER antagonist, there was a 45.4% decrease in ERK1/2 phosphorylation in SKOV3 cells, while OVCAR3 had a 44.3% decrease. These results suggest that GPER plays a key role in Cd-induced ERK1/2 phosphorylation in OVCAR3 and SKOV3 cells, and that G15 can inhibit this process.

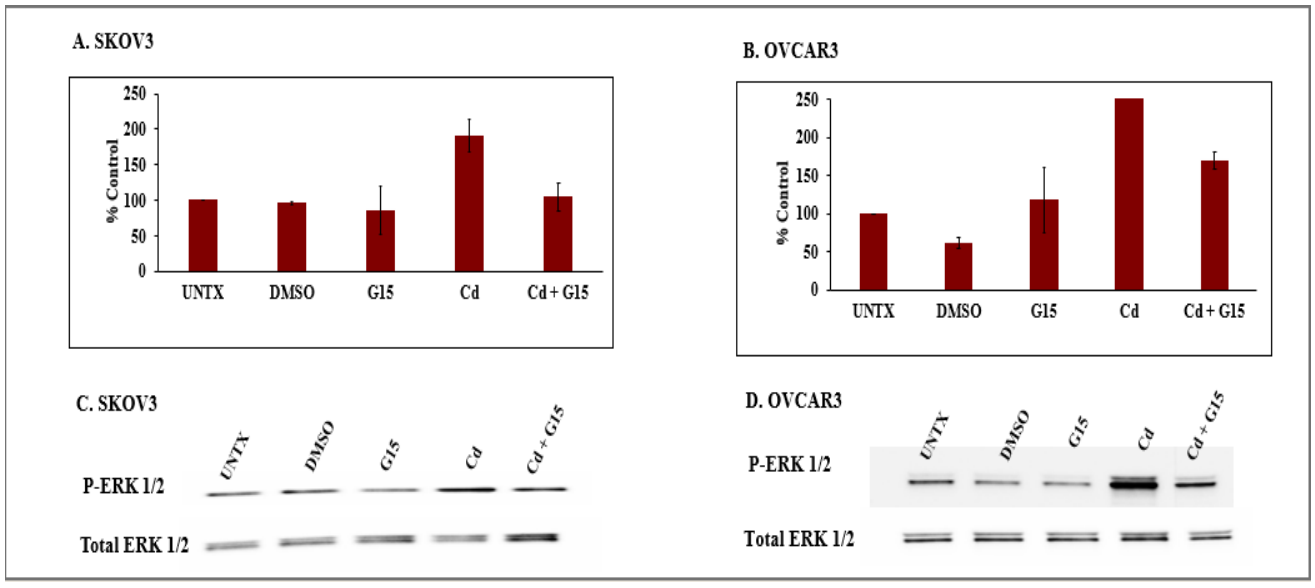

Figure 2: Inhibition of GPER with G15 inhibits Cd-induced ERK1/2 phosphorylation in OVCAR3 and SKOV3. (A&B) Quantitative analysis of relative P-ERK1/2 to Total ERK1/2 performed using Unscan-it software. (C&D) Results of immunoblots for SKOV3 and OVCAR3, respectively. Cells were treated with 10 µM G15 for 30 minutes followed by a ten-minute treatment with 100 nM CdCl2. Results are reported as % P-ERK/Total ERK relative to the

untreated control and averaged for three different experiments for OVCAR3 and two different experiments for SKOV3.

DISCUSSION

Exposure to chronic low levels of the environmental contaminant, Cd, has been associated with lung, breast, and ovarian cancer.[23,24,25] In breast and lung cancer cells, Cd has been shown to activate estrogen signaling, utilizing the non-genomic pathway by direct interaction with, ER-α and ER-β, resulting in phosphorylation of ERK1/2, and cellular proliferation. In contrast, previous studies in ovarian cancer cells have shown that Cd induces ERK1/2 phosphorylation and cellular proliferation independent of ER-α and ER-β. The results presented here suggest that phosphorylation of ERK1/2 occurs through GPER, a G-protein coupled estrogen receptor in both SKOV3 and OVCAR3 when cell lines are exposed to low levels of Cd.

Other studies have demonstrated the involvement of GPER in Cd-induced phosphorylation of ERK1/2. Huff *et al* (2016) found that GPER was involved in Cd-induced ERK1/2 phosphorylation in lung adenocarcinomas. Ali *et al* (2015) also reported that inhibition of GPER using G15, a specific GPER inhibitor, reduced Cd-induced ERK1/2 phosphorylation in human hepatocellular carcinoma and breast cancer cells, but not in endometrial carcinomas, indicating that Cd only mediates effects through GPER in some cell types. The observation that treatment with G15 inhibits ERK1/2 phosphorylation in OVCAR3 and SKOV3 cell lines align with the results found in lung, hepatocellular, and breast cancer cell lines.

A previous student in Dr. Huff's lab, Haley Todd, showed that inhibition of ER with ICI 182,780 (ICI) resulted in a 50% increase in OVCAR3 cells, regardless of Cd treatment. While these studies support that Cd-induced phosphorylation of ERK1/2 does not work through an ER-

α and ER-β dependent manner in this cell line, it was surprising to see ERK1/2 phosphorylation increase significantly. However, it has been discovered that ICI, commonly known as fulvestrant, is a known agonist for GPER. This may explain why there was an increase in ERK1/2 phosphorylation that was even greater than treatment with Cd alone in the OVCAR3 cell line.[31] In this way, ICI in OVCAR3 cells may have activated GPER, resulting in Cd-induced ERK1/2 phosphorylation independently of ER-α and ER-β in OVCAR3 cell lines. On the contrary, SKOV3 cells showed a 23% decrease in phosphorylation in the presence of ICI, suggesting that Cd activation of ERK1/2 in SKOV3 cells might utilize ER-α and ER-β, to a small extent. Interestingly, GPER has been shown to be abundantly co-expressed along with ER-α and ER-β in SKOV3 cells unlike OVCAR3 which has 2-fold more GPER expression.[31,32] Perhaps, Cd is utilizing all three estrogen receptors to activate cellular changes in SKOV3 cells while GPER is the primary receptor in OVCAR3 cells. Further testing focused on the effects of both G15 and ICI on SKOV3 and OVCAR3 needs to be performed to fully understand how these two cell lines might be responding differently to Cd.

Studies that measured the expression of GPER in ovarian cell tumors indicated that GPER is overexpressed in a few ovarian cell tumors and is associated with lower 5 year survival rates.[31] Also, about 1/3 of malignant tumors overexpress GPER mRNA, and the co-expression of GPER with EGFR has been associated with reduced progression-free survival in patients diagnosed with ovarian cancer.[31] On the other hand, it has also been discovered that GPER can act as a tumor suppressor gene in ovarian cancer cell lines. When looking at differences in the 2-year survival rate, Ignatov *et al.* (2013) observed that patients with GPER positive cell lines had a 59.2% increase in survival as compared to patients with GPER negative cell lines who only had a 28.6% survival rate. In these studies, it appears that the presence of GPER promotes anti-

cancer effects and leads to favorable clinical outcomes. These contrasting studies make it unclear as to whether GPER is a proto-oncogenic or tumor suppressor gene. There is a possibility that the contradictory results of GPER's effect on cell proliferation may be due to different patient populations or cell types being studied, or other varying aspects that may have been reviewed in one research but omitted in another. However, future studies could be performed to determine if Cd-induced proliferation of SKOV3 and OVCAR3 cell lines is inhibited or activated in the presence of G15, a GPER antagonist.

Previous studies have shown the activation of multiple signaling proteins in the estrogen signaling pathway. Studies in lung and breast cancer cells have demonstrated that inhibition of EGFR with AG1478 also reduced Cd-induced ERK1/2 phosphorylation, suggesting that EGFR is also activated by low concentrations of Cd. Finding that Cd induced activation of ERK1/2 may be initiated through the activation of GPER, it is likely that other proteins in this signaling pathway including Src, MMP and EFGR may also be activated by Cd treatment. To determine if activation of EGFR may mediate Cd's effects in ovarian cancer cell lines, future inhibitor studies have to be carried out in both OVCAR3 and SKOV3 cells. This, in turn, will help delineate how Cd activates ERK1/2 through GPER.

The result of this research adds to the constantly increasing knowledge of Cd, estrogen receptors, and ovarian cancer. Because of its ability to persist in soft tissues for years, and its carcinogenic properties, Cd remains a harmful contaminant in the environment and the organisms that inhabit the land. With the likelihood that GPER may be associated with very low survival rates in ovarian cancer patients, the combination of GPER and Cd poses a serious threat on healthcare and a deadly force to be reckoned with. Therefore, it is of utmost importance to determine Cd's mechanism of action through GPER in ovarian cancer. Increased understanding

of this pathway can finally help tackle this “silent killer” and decrease the high mortality rates in female patients by leading to more effective treatment options, and the development of better diagnostic methods in the early stages of the disease.

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