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The Effects of the Environmental Estrogens Cadmium and Arsenite on Phosphorylation of ERK1/2 via GPR30 in Human Lung Adenocarcinoma Cells

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A Senior Honors Thesis Presented in
Partial Fulfillment of the Requirements of the
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ABSTRACT

Nanomolar concentrations of both cadmium and arsenite, two environmental estrogens present in cigarette smoke, have been documented in rapidly phosphorylating ERK1/2, a type of MAPK, in the human non-small cell lung cancer (NSCLC) line NHI-1793 in a manner similar to that of estrogen. Pretreatment of cells with a general, nonspecific estrogen receptor antagonist reduced the levels of phosphorylated MAPK, indicating that this phosphorylation event is achieved through use of an estrogen signaling pathway. The specific estrogen receptor involved in this process, however, is currently unknown. To determine whether GPR30, one of the three types of estrogen receptors, is necessary for the phosphorylation of MAPK, NHI-1793 cells were treated with 17 β -estradiol, cadmium chloride, and sodium arsenite both in the presence and absence of G15 – a GPR30-specific antagonist – and MAPK levels were measured using immunoblot analysis. As expected, the data revealed these nanomolar concentrations of endogenous and environmental estrogens were sufficient in phosphorylating ERK1/2 in under ten minutes and pretreatment with G15 reduced phosphorylation in cells treated with 17 β -estradiol and cadmium chloride, but not in those treated with arsenite. Therefore, cadmium and estradiol likely utilize GPR30 to phosphorylate ERK1/2, while further research is needed to elucidate the mechanism of arsenite-induced ERK1/2 activation. These results are important in understanding the polarity of lung cancer diagnosis and progression between genders, as well as in the development of pharmaceuticals.

INTRODUCTION

Lung Cancer

Lung cancer kills more men and women in the United States than any other cancer, according to the Centers for Disease Control and Prevention (2016). Out of the 210,828 people diagnosed with lung cancer in 2012, 157,423 died as a result of this disease (CDC, 2016). This corresponds to nearly a 75 percent fatality rate. Approximately 85 to 90 percent of diagnosed lung cancers are non-small cell lung cancers (NSCLC). There are three types of NSCLC: squamous cell carcinoma, adenocarcinoma, and large cell carcinoma. At a 40 percent occurrence, adenocarcinoma is the most common of these three categories of non-small cell lung cancers (American Cancer Society, 2015).

Adenocarcinomas are cancers which form in the glandular cells responsible for secreting mucus in the lung. As with all lung cancers, there is a strong correlation between smoking and the development of lung adenocarcinoma, but this type of cancer is also the most common lung cancer found in non-smokers. Among non-smokers, adenocarcinomas are often found in younger individuals and significantly more common in women than men (American Cancer Society, 2015).

Lung Cancer in Women

While lung cancer is concerning in both men and women, several gender differences associated with the disease make it clear that further research should focus on women. As of 1987, lung cancer – not breast cancer – has been the number one cause of cancer-related deaths among women. Though men have a higher age-adjusted death rate from lung cancer than women and seem to suffer from the disease more often than their female counterparts, women are being diagnosed at a rapidly increasing rate. In the last 37 years, the number of diagnoses for men have

dropped 28 percent while having increased 98 percent for women. It seems as though the peak of diagnoses for women was in 1998, 14 years after the peak for men, and though the rates are now beginning to decline, survival rates are still shockingly low. The five-year survival rate for lung cancer patients is a mere 17.8 percent and over half of the individuals who are diagnosed with the disease pass away within a year of diagnosis (American Lung Association, 2016). These numbers show not only how serious a lung cancer diagnosis is, but the gender differences between men and women who are diagnosed.

The differential rates at which women have been diagnosed with lung cancers in recent years is only one of the differences seen between genders suffering from lung cancer. In fact, the later peak in diagnoses among women may have little to do with biological differences between genders and a great deal to do with social trends of the times. Women started smoking much later than men in society, and the rates at which women were being diagnosed with lung cancer may be correlated to these social behaviors (Baik & Eaton, 2012). Smoking habits between men and women, however, are not the only differences which may explain the variances in gender-related lung cancer rates. About 15 percent of women who have never smoked are diagnosed with some form of lung cancer as compared to only 5 to 10 percent of non-smoking men in the North American continent. Looking beyond North America, 80 percent of the lung cancer diagnoses in men are related to smoking, as compared to 50 percent in women. Additionally, a mutation in the epidermal growth factor receptor (EGFR), which is present in certain types of lung cancers, is found more often in women and individuals who have never smoked (Baik & Eaton, 2012). These biological differences in lung cancer between men and women have suggested that estrogen could have a role in the development and/or progression of lung cancer.

The Role of Estrogen in Lung Cancer

Estrogen has a variety of roles in the human body beyond its well-known role in the female reproductive system. In addition to its functions in reproduction, estrogen can also bind to receptors elsewhere in the body to allow for proliferation and growth, especially in NSCLC. These estrogen receptors could be activated either by direct binding of the steroid hormone to the receptor, or independently of the hormone through phosphorylation of the receptor (Siegfried et al, 2009). In this manner, estrogen could be responsible for increasing the growth, metastasis, and likelihood that a NSCLC adenocarcinoma will form within the female lung.

Since estrogen elicits a response by binding to estrogen receptors, it is not surprising that previous studies have confirmed that estrogen receptors are found within cancerous tumors extracted from patients and in established lung cancer cell lines. Several preclinical estrogen studies showed that these receptors are responsive to estrogen and might contribute to cancerous growth (Baik & Eaton, 2012). One study in particular treated immunocompromised xenografts of lung tissue *in vivo* with estrogen, fulvestrant – an ER inhibitor – or a mixture of estrogen and fulvestrant. This study showed that tissue treated with estrogen alone led to significant tumor growth while the fulvestrant treatment inhibited the growth of the tumor by 40 percent (Baik & Eaton, 2012). It seems apparent, therefore, that estrogen and estrogen receptors do have some impact on the growth and proliferation of lung cancer cells.

Types of Estrogen

Estrogen is a general term used to refer to a family of steroidal hormones produced in the ovaries, testes, and adrenal cortex, all of which are responsible for the development of secondary sexual characteristics, among other functions. There are three types of endogenous estrogens present in the human body: estrone (E₁), estradiol (E₂), and estriol (E₃). The predominant and

most potent form of estrogen circulating in the blood stream of women during their reproductive years is 17β -estradiol (Rettberg et al., 2013). Estrone sulfate, however, becomes the most common circulating estrogen in women following menopause. This compound is also detected in the human placenta and the urine of pregnant women (Estrogens, Steroidal, 2002). Estrone is not thought to play a large role in cancer biology at this point in time.

Estriol is another type of estrogen which is weaker in its impact than 17β -estradiol. This form of estrogen is the most common type detected in pregnant women because it is synthesized in the placenta. Previous studies have provided evidence that estriol is capable of binding to estrogen receptors with reduced binding affinity when compared to 17β -estradiol (Melamed et al., 1997). This is considered a weaker form of estrogen due to its ability to act similarly to 17β -estradiol, but bind to receptors with less affinity.

17β -estradiol is the most active and powerful form of estrogen in the body. This estrogen is the most studied in terms of its potential to impact cancer, possibly because of its roles in controlling the function and growth of the reproductive system, especially in females (Kim et al., 2015). It has been shown that 17β -estradiol can bind to the three different types of estrogen receptors and result in varying functions in the body.

Environmental Estrogens

The interaction between endogenous estrogen and lung cancer has been established, making it equally important to consider whether environmental estrogens may also be involved in the formation, growth, or proliferation of lung cancer. Environmental estrogens are considered endocrine disruptors – a category of pollutants which have been studied for the past fifty years due to the effects they can have on human beings and natural wildlife. These chemicals are a unique type of pollutant because they are not a toxin which adversely impacts the body, but

rather a compound capable of mimicking natural hormones, resulting in improper hormonal signaling and potential changes in gene expression (McLachlan & Arnold, 1996). One of the first identified environmental estrogens was DDT – an insecticide widely utilized to combat mosquitos from the 1930s through the 1960s. In the late 1960s, following wide use of this chemical, Louis Guillette and his team at the University of Florida began reporting “feminized” animals, such as male alligators with penises smaller than standard males. The presence of excess DDT acting as an environmental estrogen also has the potential to lower sperm counts in males and promote the growth of breast cancer and other hormone-related cancers. Research on the mechanism of DDT action elucidated that this compound was able to enter the body and behave in the same manner as natural estrogen, leading to these feminized characteristics (McLachlan & Arnold, 1996). To date, a large number of these environmental estrogens have been identified to possess the ability to enter the body in a variety of ways. *In vivo*, these compounds can act in manners which mimic endogenous estrogen or block androgens. Two of these environmental estrogens are cadmium and arsenite – heavy metals found in cigarette smoke, among other sources.

Cadmium

Cadmium is an environmental estrogen specifically known as a metalloestrogen which has the potential to activate estrogen receptors within the body. Previous research has shown that this heavy metal acts similarly to estrogen in estrogen receptor positive breast cancer cells. Studies suggest that cadmium is able to bind to a specific type of estrogen receptor by complexing with the domain which normally binds estrogen through an interaction with a number of amino acids in the active site of the receptor (Stoica et al, 2013). In this manner,

cadmium is capable of activating one type of estrogen receptor, justifying the classification of cadmium as an environmental estrogen.

Further studies analyzing the effect of cadmium binding to an estrogen receptor have shown that this blocks estrogen from binding to the receptor and that cadmium can function in the same way as the endogenous hormone. Exposure to cadmium has allowed for the activation of estrogen-dependent genes, including those which lead to increased growth. In breast cancer tissue, research has shown that an increased cadmium level has resulted in double the risk of breast cancer compared to individuals with normal levels of cadmium (Strumylaiate et al., 2010).

Cadmium, while being both a metalloestrogen and an environmental estrogen, is also categorized as a group 1 carcinogen due to its effects on cancerous and healthy tissue. This classification means that this chemical can cause cancer in some manner, which it does by inhibiting tumor suppressor genes, damaging repair mechanisms, and disabling enzymes involved in oxidative damage metabolism. Cadmium is toxic and found in low levels in the environment, so the main sources of exposure to this compound is through pollution of crops, waterways, seafood, battery dumping, or workplace exposure (Kim et al, 2015). Most commonly, this chemical is found in contaminated food products or cigarette smoke (Satarug & Moore, 2004). The toxic effects of cadmium on the body are seen primarily through unwanted apoptosis, oxidative stress, and the response to DNA damage. Of particular interest to this study, cadmium poisoning has been shown to elicit the expression of mitogen-activated protein kinase 1 (MAPK-1), an indicator of cellular growth and proliferation. (Kim et al, 2015).

Cadmium is a particularly dangerous environmental toxin because humans do not have a mechanism by which to eliminate the metal – therefore, cadmium accumulates in the liver, kidney, and blood vessels (Schroeder, 1972). This metal is found in the food human beings

consume because it is easily brought up from the soil into plants. Even at low levels of less than 0.5 µg cadmium/g urinary creatine, cadmium has been linked to adverse effects on the kidney (Satarug et al., 2010). The more cadmium that accumulates in the body due to environmental exposure, the higher the likelihood of developing cancer.

This metal enters the body through a variety of sources, these largely being food-based, as previously mentioned. Mollusks and crustaceans, being filter feeders, sequester a number of pollutants from water which has been contaminated with cadmium. Other sources of cadmium include oilseeds, such as sunflower seeds, flaxseed, and peanuts. These seeds obtain cadmium from contaminated soil, and the compound is then stored in various portions of the plant, including the seed. Offal, which is often fed to livestock and other animals as a food source, has been shown to have high levels of cadmium (Satarug et al., 2010). When a human consumes cadmium from any one or more of these sources, the heavy metal is stored in the body and can have toxic effects on the individual. A potential mechanism by which cadmium adversely impacts the human being is through its mimicking of estrogen and resulting in increased cellular proliferation of cancer cells.

Arsenite

Arsenite, like cadmium is an environmental estrogen, endocrine disruptor, and group 1 carcinogen with the ability to cause oxidative stress which can upset cell signaling, damage cellular DNA, and ultimately result in cell death (Kim et al, 2013). The activity of arsenite on lung cancer tissue could exacerbate cancerous proliferation by mimicking endogenous estrogen and binding to estrogen receptors. When a breast cancer cell line was treated with arsenite, there was an increase in growth and proliferation as compared to the control cells grown in the absence of estrogen (Stoica et al, 2013). In another study, mice exposed to arsenic resulted in cancers in

the liver, ovary, uterus, and other organs. Long term exposure to arsenic results in cancerous tumors with increased transcription of genes under estrogen control and an increase in some types of estrogen receptors on cells (Xu et al., 2014). The fact that arsenite appears to interact with estrogen receptors to increase the growth of cancerous cells in mouse models and cell line work, justifies its categorization as an environmental estrogen.

Arsenic enters the human body through a variety of different sources. It is present in many pesticides, which has increased the amount of this toxin found in the environment. The pollutant is also released into the air through the burning of coal and some woods which have been treated with preservatives containing arsenic. Industrialization has led to an increase in arsenic concentration found in drinking water, which has resulted in some fish and crustaceans containing high levels of arsenic if they reside in these contaminated waterways (Pershagen, 1981). Arsenic exposure, therefore, is not uncommon, especially in developed, industrialized areas, and can have a number of negative impacts on the body.

Estrogen Receptors

Just as there are multiple types of estrogen, both endogenous and environmental, there are three different receptors capable of reacting with estrogen to elicit cellular responses – estrogen receptor alpha ($ER\alpha$), estrogen receptor beta ($ER\beta$), and the G Protein-Coupled Estrogen Receptor (GPCR/GPR30). Though it is known, to an extent, which estrogens these receptors preferentially bind in some tissues, not all of the actions performed by these receptors have been characterized. Further, it is currently unknown as to which receptors that estrogens, endogenous or environmental, may utilize to induce increased growth in lung cancer tissue. $ER\alpha$ and $ER\beta$ are both known to regulate growth at the transcriptional, or genomic level, though $ER\alpha$ appears to be involved in the proliferative actions of cells and $ER\beta$ seems to repress these

proliferative effects (Williams et al., 2008). The role of GPR30 is not as well characterized as these other two receptors, but it currently seems to be the more likely candidate for nongenomic, or nontranscriptional, impacts. For these reasons, further study as to the roles of the three estrogen receptors is needed to further elucidate their functions *in vivo*.

Estrogen Receptor Alpha

Estrogen receptor alpha (ER α), which was first characterized in 1986, functionally binds all three endogenous forms of estrogen. The gene for this receptor is located on chromosome 6 and is known as ESR1. This estrogen receptor appears to be most common in the ovaries, breasts, and endometrium (Baik & Eaton, 2012). ER α does not currently have a clearly defined role in lung cancer though it is detected primarily as a cytoplasmic protein in lung cancer tumors (Siegfried et al, 2009). ER α contains two functional domains, AF1 and AF2. The AF1 domain is dependent on ligand binding and is responsible for transcription while the AF2 domain is also ligand-dependent and functions in activating the receptor. When an estrogen ligand binds the AF2 domain, the receptor can form homo- or heterodimers and proceed to bind the estrogen responsive element which is located in the upstream region of an estrogen-responsive gene (Baik & Eaton, 2012). This allows for the ER α to control transcription within the cell.

Estrogen Receptor Beta

Estrogen receptor β (ER β) was initially discovered in 1996 – ten years after the discovery of ER α – and acts similarly to ER α in many ways. The gene for this receptor is known as ESR2 and is located on chromosome 14. ER β binds all three estrogens similarly to ER α , though ER β will bind estriol more tightly than estradiol or estrone. This receptor is located in many more tissues than ER α , including the brain, colon, lung, bone, kidney, prostate, ovaries, testes, and endothelial cells. Just like ER α , ER β is made up of the same functional domains – AF1 and AF2

– though AF1 is not as active as the corresponding domain in ER α , meaning ER β is more reliant on the activity of AF2 (Baik & Eaton, 2012). Unlike its ER α counterpart, however, ER β can be localized both in the cytoplasm and in the nucleus of the cell in 45 to 69 percent of lung cancer cells. This implies the possibility that ER β may be more important than ER α in regulating increases in growth of both cancer and normal tissue (Siegfried et al, 2009).

It is known that ER β is present in both bronchial epithelial cells and pneumocytes in the lung and plays an important role in preserving the extracellular matrix. When organisms are lacking ER β , as shown in a knockout mouse model, the lung is more likely to develop uncharacteristic arrangements and dysfunctional lung structures which could lead to oxygen deficiency. These knockout models also displayed fewer alveoli and less surfactant, among other lung abnormalities (Baik & Eaton, 2012). This data makes it apparent that the ER β receptor is playing some role in normal lung structure and function and irregularity in its expression can lead to lung abnormalities.

GPR30 Estrogen Receptor

GPR30 is a more recently discovered estrogen receptor, and therefore there is less information regarding its function. This receptor was discovered in the late 1990s, but it was not until many years later that it was discovered that estrogen was the ligand for GPR30 and that estrogen agonists could also have an effect on this receptor (Wang et al., 2014). Since ER α and ER β are both nuclear hormone receptors, and are largely responsible for the genomic, or transcriptional, effects of estrogen, the discovery of GPR30 provided further understanding in how estrogen could activate the ERK1/2 pathway and lead to cellular growth and proliferation (Prossnitz & Hathaway, 2015).

The GPR30 receptor is a member of the G protein-coupled receptor (GPCR) superfamily composed of seven transmembrane proteins (Ferguson, 2001). GPR30 is important for a variety of estrogen binding effects including immune, nervous, vascular, renal, and reproductive responses (Kim et al, 2015, pg. 79). Along with these normal cellular responses to estrogen, GPR30 has been associated with cancerous growth, especially in ovarian, testicular, breast, and endometrial cancers (Wang et al., 2014). Current research is attempting to determine whether this receptor also plays such a role in lung cancer, and increasing evidence is suggesting that it may be.

GPR30 functions in the same way as other known GPCRs to allow for signal transduction as a result of the binding of a ligand. Ferguson (2001) describes this process in detail, explaining how the binding of a ligand elicits a conformational change in the receptor, leading to a GTP exchange from a GDP to a GTP attached to the α -subunit of the associated G protein. This phosphorylation causes the G protein to dissociate into the $G\alpha$ -subunit and the $G\beta\gamma$ -subunit. These subunits are then free to move around the cell and regulate specific cellular targets.

Previous studies support the idea that GPR30 is primarily involved in many of these non-genomic, or non-transcriptional, estrogen effects throughout the body. Prossnitz and Hathaway (2015) studied GPERs – a generic name for all G protein-coupled estrogen receptors – in breast tissue to determine whether these receptors played a role in tumor proliferation. To achieve this, the researchers developed GPER-knockout (KO) mice and treated them with estrogen along with a wild type mouse population. The KO mice had smaller, less aggressive tumors than the control population supporting role of GPERs in cancerous growth and metastasis. By the end of the study, the researchers noted a strong correlation between a poor prognosis in endometrial and breast cancers and the amount of GPER being expressed. This study suggests that GPERs are, in

at least some capacity, playing a role in cancer development and aggression in breast cancer tissue.

Estrogen Action

Natural estrogen is synthesized from cholesterol in the ovaries, testes, and potentially the adrenal cortex. When the appropriate signal is received from the brain or some other organ, estrogen is released from its area of synthesis into the blood stream, where it is carried to target cells – often the reproductive system or the breasts. Estrogen, unlike many other biochemical signals in the body, can diffuse easily through the cellular membrane due to its solubility in fats, including the phospholipids making up the plasma membrane of cells. For the same reason, estrogen can pass through the nuclear membrane quite easily (McLachlan & Arnold, 1996). The hormone can bind to estrogen receptors localized in the nucleus or cytoplasm, and through binding, can lead to activation or repression of estrogen-regulated genes – including a number of growth factors.

The binding of estrogen to a nuclear estrogen receptor elicits a conformational change on the receptor, allowing the associated proteins to interact with transcription machinery and regulate transcription in this manner. Another function of estrogen in the cell is to increase the levels of calcium and cyclic AMP (cAMP) in a manner similar to that seen in the presence of growth factors. This similar mechanism of secondary messenger activation implies that both epidermal growth factors (EGFs) and estrogens are both able to increase cell growth and proliferation. EGF has been shown to mimic the effects of estrogen binding to estrogen receptors by also phosphorylating MAPK in an overlapping manner. It has been shown that 17 β -estradiol is also able to activate MAPK, which can aid in increasing the transcriptional impact of the EGFs (Improtera-Brears et al., 1999). This pathway can help explain how estrogen is able to impact

cellular growth and proliferation in a manner similar to epidermal growth factors through the phosphorylation of ERK1/2 or other MAPKs.

Through these studies, it is now believed that estrogen signaling has two important mechanisms of action. First, estrogen acts by binding to a receptor and causing the activation or repression of transcription factors, thus impacting transcription. This pathway, however, can take several hours if not days to elicit a change in the cell. The second mechanism involves estrogen's ability to phosphorylate, and thus activate, MAPKs through a nongenomic pathway which results in an exponentially quicker response. Though estrogen receptors are often localized at the nucleus of target cells, estrogen has also been shown to elicit a response at the cellular membrane. In this event, estrogen interacts with ligand-gated ion channels and G protein-coupled receptors to elicit the non-genomic responses associated with MAPK phosphorylation and ERK activation (Improtera-Brears et al, 1999). Estrogens, therefore, have a variety of roles in target cells, including both genomic and non-genomic pathway activation which can result in increased growth and proliferation of the cells.

ERK1/2 and MAPK Phosphorylation

There are several signal transduction pathways known to respond to extracellular signals and, through a cascade of phosphorylation events, result in either the activation or inactivation of target proteins. One such pathway that has been identified is the extracellular-signal-related kinase 1/2 (ERK1/2) pathway. Binding of the appropriate ligand to a specific G protein-coupled receptor on the cell's surface results in the activation of the G protein, which can, in turn, recruit a guanine nucleotide exchange factor (SOS) protein to the cell surface. SOS is then able to activate Ras, which brings in and activates Raf through phosphorylation. Activated Raf, a mitogen-activated protein kinase kinase kinase (MAPKKK), can phosphorylate MEK1/2, a

MAPK kinase kinase (MAPKK). From here, MEK1/2 is capable of phosphorylating the MAPK, ERK1/2. Active ERK1/2 phosphorylates target proteins, such as Elk-1, in the nucleus which are necessary for transcription of proteins needed for cellular growth (Mebratu & Tesfaigzi, 2009). ERK1/2 has also been shown to induce the phosphorylation of nuclear estrogen receptor, increasing estrogen binding and facilitating estrogen action through transcription of growth proteins (Wortzel & Seger, 2011).

Previous Laboratory Research

Prior work in the lab has demonstrated that relatively low nanomolar concentrations of the heavy metals cadmium and arsenite are sufficient in activating MAPK phosphorylation in under 10 minutes and inducing the growth and proliferation of the lung adenocarcinoma cell line NCI-H1793. These effects have also been seen using similar concentrations of 17β -estradiol. Additionally, the use of a generic estrogen receptor antagonist capable of inhibiting all types of estrogen receptors prevents this increase in phosphorylated MAPK and thus inhibits the increase in growth observed in the uninhibited assays. As there are three types of estrogen receptors, it is important to study which estrogen receptor is responsible for allowing the binding of the heavy metals and phosphorylating MAPK. From the research presented in this introduction, it seems that the G Protein-Coupled Estrogen Receptor is likely responsible for these nongenomic effects. By inhibiting this receptor and treating cells with nanomolar concentrations of cadmium, arsenite, and estrogen, the the purpose of this study is to determine if cadmium and arsenite induce ERK1/2 phosphorylation through activation of GPR30.

MATERIALS AND METHODS

Cell Culture and Treatments

The cell line NCI-H1793 is a non-small cell lung adenocarcinoma that was cultured from a 52 year old, nonsmoking woman in December of 1987 and was purchased from ATCC (Manassas, VA). Within the laboratory, this cell line was grown and maintained according to manufacturer's directions in the suggested growth medium at 37°C with 5% CO₂. Cells were split every 5-7 days to sustain less than 90% confluence until it was time for experimentation.

Treatment of the NCI-H1793 cells with estrogen, sodium arsenate, cadmium chloride, and DMSO – used as a control because the treatments were suspended in DMSO solutions – both in the presence and absence of the G15 inhibitor required the cells to be grown to 80% confluency. Upon reaching this density, cells were trypsinized, counted using a hemocytometer and trypan blue, and plated at a concentration of approximately 400,000 cells/3 milliliters growth media in 60 cc³ petri plates. Plated cells were placed in the 37°C incubator and permitted to grow overnight.

Two or three days later, depending on cell growth, the growth media was removed from the plates and replaced with plate media – a media containing charcoal-stripped FBS and lacking any hormones to avoid any external influence on cellular growth. This media was changed every 24 hours for two days leading up to treatments.

To treat cells, they were split into two groups – one group received a treatment of an estrogen agonist in addition to treatment of the G15 inhibitor – an antagonist specific for the GPR30 receptor, and the second group received treatment of an estrogen agonist with no inhibitor present. Pretreatment of 3 μM G15 (GPR30 inhibitor) was applied to the appropriate cells for 20 minutes, while the other cell group received no pretreatment. Next, cells were treated

with either 100 nM concentration of 17 β -estradiol, cadmium chloride, or sodium arsenite for 10 minutes on both pretreated and non-pretreated cells for comparison. Controls for this experiment include those cells that received 0.1% DMSO both in the presence and absence of G15. A second control received no treatment in addition to no pretreatment.

Following the treatments, cells were washed twice with ice cold phosphate-buffered saline (PBS), then lysed using 60 μ L of a lysis buffer created with 1 mL RIPA buffer, a 1/1000 dilution of phosphatase inhibitor cocktail II purchased from Sigma Aldrich (St. Louis, MO), a 1/1000 dilution of phosphatase inhibitor cocktail III purchased from Sigma Aldrich (St. Louis, MO), a 1/1000 dilution of protease inhibitor, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Collection of cells was achieved using a cell scraper in 60 μ L lysis buffer on ice and transferred to an ice-cold microcentrifuge tube. Samples were sonicated using a Microson Ultrasonic Cell Disruptor Sonicator at 1 RMS, then centrifuged for 10 minutes at 4°C using a Beckman and Coulter Microfuge 22R Centrifuge. The supernatant was collected and stored at -80°C. This experiment was repeated three times.

Immunoblot Assay and Analysis

The protein concentrations of the cell lysates were determined using a Bio-Rad Protein Assay Dye Reagent (Hercules, CA) in comparison to a standard curve generated using bovine serum albumin (BSA) following manufacturer's directions. Generation of the curve was achieved by varying the BSA concentration from 0-12 μ g/mL in water with 200 μ L of the BioRad Microassay Protein Assay dye and determining absorbance at 595 nm after a 5 minute incubation at room temperature. To determine protein concentration, 2 μ L of each cell lysate were added to 798 μ L of water and 200 μ L of dye reagent and the absorbance was read after incubation. Both BSA and protein assays were performed in duplicate for accuracy. Protein

absorbance values were compared to the standard BSA curve and concentrations were determined.

Based on the concentrations of the protein solutions, 25 μg of each sample was separated on a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel. The gel was run in an SDS running buffer (1X Tris/Glycine/SDS Buffer purchased from Bio-Rad) at 200 volts for approximately one hour. Proteins were then transferred to a PVDF membrane through direct contact between the membrane and the gel in a cassette running at 30 volts overnight at 4°C with constant stirring in a transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol at pH 8.3).

The PVDF membrane was incubated in blocking buffer composed of 5% milk in TTBS (20 mM Tris at pH 7.5, 140 mM NaCl, 0.1% Tween 20) for one hour with gentle shaking following two quick washes in TTBS. Next, the membrane was washed once for 15 minutes and three times for 5 minutes each with TTBS. A 1/1000 dilution of primary antibody for phosphorylated MAPK purchased from Cell Signaling (Danvers, MA) was diluted in blocking buffer and incubated overnight at 4°C with shaking to allow the antibody to bind.

The following day, the membrane was washed once for 15 minutes and three times for 5 minutes each with TTBS. The secondary antibody of Goat antirabbit (GAR) conjugated to horseradish peroxidase purchased from Thermo Scientific (Waltham, MA) was applied in a 1/500 dilution in blocking buffer and incubated for 1 hour at room temperature with shaking. In preparation for development, the membrane was again washed once for 15 minutes and three times for 5 minutes each with TTBS. A Pierce ECL Western Blotting Substrate Kit purchased from Thermo Scientific (Waltham, MA) was utilized for visualization of the bands of phosphorylated MAPK and the blot was developed for a 30 second and 60 second exposure.

Images were captured on CL-X Posure Film 8 x 10 inches purchased from Thermo Scientific (Waltham, MA).

The PVDF membrane was stripped for 15 minutes with a Restore Western Blot Stripping Buffer purchased from Thermo Scientific (Waltham, MA) to remove the antibodies and then washed extensively with TTBS to be able to additionally test for total MAPK. Once stripped, the above methods for primary antibody attachment, secondary antibody binding, and development were repeated using a 1/1000 dilution of total MAPK primary antibody from the same manufacturer as stated previously and the same secondary antibody. Immunoblot analysis was performed for each treatment study.

Densitometry

Western blot data was quantified from the film using the UN-SCAN-IT gel 6.1 software. This program required the films to be scanned into a computer in grey scale. The bands could then be digitized to determine a pixel total of dark band compared to a background measurement. The number of pixels occupied by the dark bands can be translated to reflect the relative amount of phosphorylated MAPK. Data analysis performed in Microsoft Excel 2013 allowed for the determination of the increase in phosphorylation of MAPK in the presence and in the absence of the G15 inhibitor with the varying treatments. Results were reported as a ratio of relative phosphorylated MAPK to total MAPK.

RESULTS

In previous studies, it was observed that nanomolar concentrations of cadmium and arsenite were sufficient in stimulating ERK1/2 phosphorylation in a matter of minutes and that this activation was inhibited by the presence of a nonspecific estrogen receptor antagonist. Because there are three different estrogen receptors, the purpose of this study was to determine

whether cadmium and arsenite utilize GPR30 in the phosphorylation of MAPK in NCI-H1793 lung adenocarcinoma cells. In this study, cells were treated with 100 nM concentrations of cadmium, arsenite, or 17 β -estradiol in the presence or absence of G15, a GPR30 antagonist, as outlined in Table 1. The experiment was performed in triplicate to test for consistency and allow for comparison. Due to inactivation of the positive controls in the initial run of the experiment, only the second and third replicates are presented in this document for analysis.

Experimental Treatments				
Plate Number	Pretreatment	Stock for + 3 uL addition to 3 mL in 60 mm plate	Treatment (1:1000) Stock 100 uM (3 uL)	Time (min)
1	None	N/A	None	N/A
2	None	N/A	DMSO	10
3	None	N/A	100 nM estrogen	10
4	None	N/A	100 nM cadmium	10
5	None	N/A	100 nM arsenite	10
6	3 uM G15 (20 min)	3 uL of 3mM G15	DMSO	10
7	3 uM G15 (20 min)	3 uL of 3mM G15	100 nM estrogen	10
8	3 uM G15 (20 min)	3 uL of 3mM G15	100 nM cadmium	10
9	3 uM G15 (20 min)	3 uL of 3mM G15	100 nM arsenite	10

Table 1. Experimental treatment protocol to test the ability of E₂, CdCl₂, and NaAsO₂ to phosphorylate MAPK both in the presence and absence of a GPR30-specific inhibitor, G15. Nine experimental treatments were developed. Six of these involved E₂, CdCl₂, and NaAsO₂ with or without a pretreatment of G15 inhibitor. The remaining three treatments were negative controls in a completely untreated plate or plates treated with DMSO or DMSO with inhibitor for comparison.

Following treatment, western blot analysis was performed on the proteins extracted from the treated cells to determine the levels of phosphorylated MAPK. To control for protein loading, immunoblot analysis was repeated for each blot to measure for the levels of total MAPK since these levels are constant regardless of treatment. This data was then quantified using software to determine relative levels of phosphorylated MAPK over total MAPK for each treatment.

The results of the two studies are shown in Figures 1 and 2, corresponding to experimental replicates 2 and 3 respectively. In each experiment, it is clear that there is a

decrease in phosphorylated MAPK levels between cells treated with 17 β -estradiol and cadmium and those pretreated with the G15 inhibitor, suggesting that GPR30 is likely involved in the activation of MAPK by these two compounds. The results for arsenite are not as clear. In the second experimental replicate (Figure 1), the results suggest arsenite's involvement in MAPK phosphorylation while the third replicate (Figure 2) does not indicate an inhibition of MAPK phosphorylation following G15 treatment. Since western blot analysis is challenging to quantify and can often vary greatly from replicate to replicate, it is difficult to determine, from this data, whether GPR30 is involved in arsenite-induced activation of MAPK.

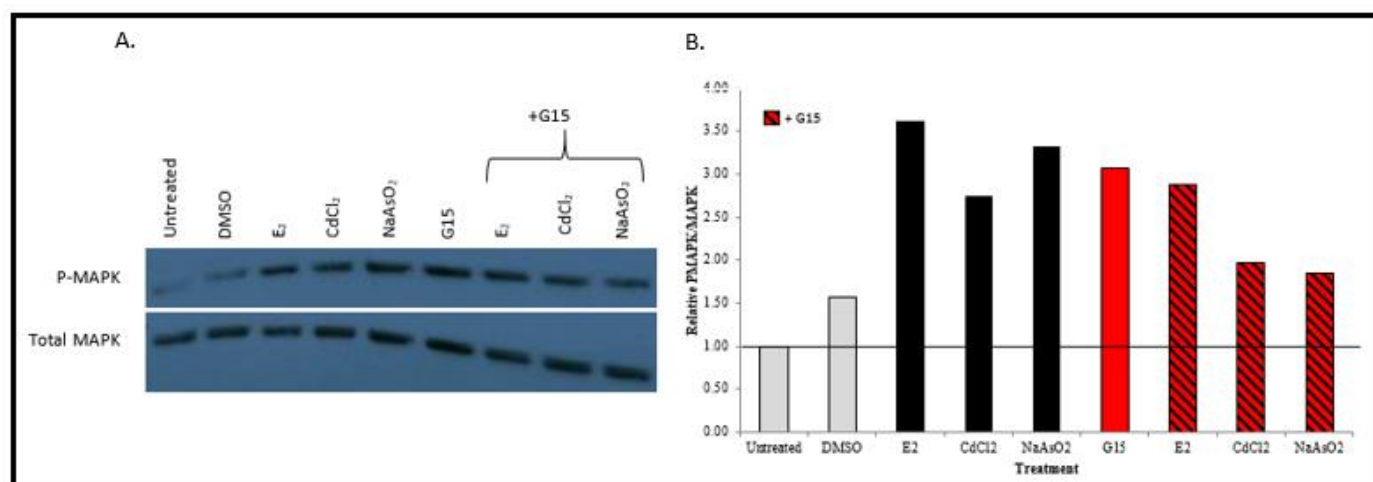


Figure 1. Second experimental replicate: MAPK phosphorylation induced by E₂, CdCl₂, and NaAsO₂ is reduced by the GPR30 inhibitor G15. A) Immunoblot analysis of E₂, CdCl₂, and NaAsO₂ in the presence and absence of G15. B) Quantification of the relative ratio of phosphorylated MAPK over total MAPK for E₂, CdCl₂, and NaAsO₂.

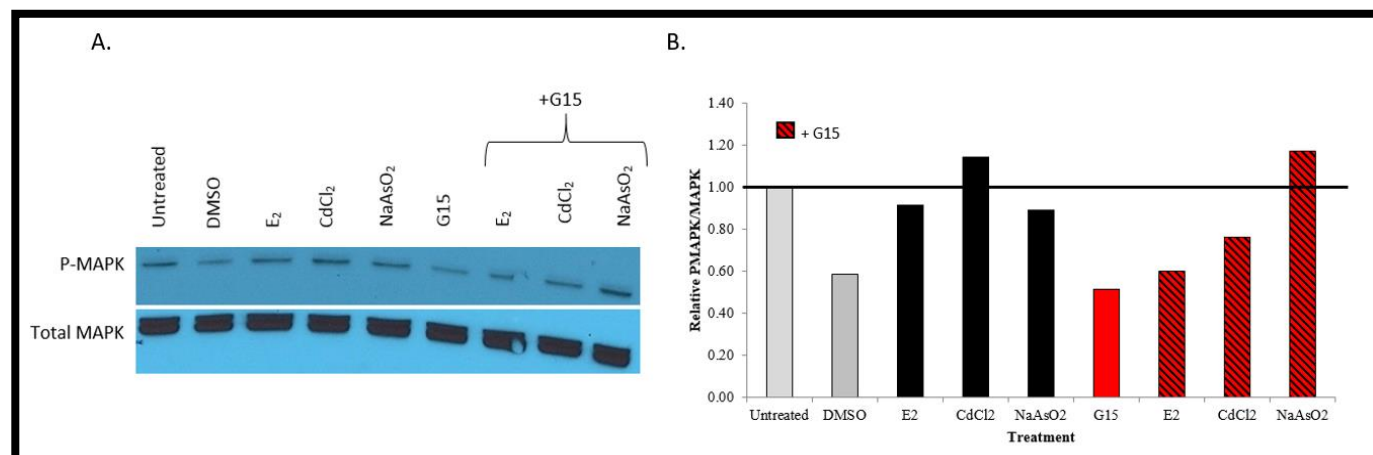


Figure 2. Third experimental replicate: MAPK phosphorylation induced by E₂ and CdCl₂, but not NaAsO₂, is reduced by the GPR30 inhibitor G15. A) Immunoblot analysis of E₂, CdCl₂, and NaAsO₂ in the presence and absence of G15. B) Quantification of the relative ratio of phosphorylated MAPK over total MAPK for E₂, CdCl₂, and NaAsO₂.

DISCUSSION

To determine if GPR30 is involved in the phosphorylation of MAPK by cadmium and arsenite, a number of treatments were performed on female lung adenocarcinoma cells to compare the impact of treatment with these heavy metals to 17 β -estradiol. Upon completion of western blot analysis of the cell lysates, relative ratios of phosphorylated MAPK over total MAPK revealed an increase in MAPK phosphorylation of cells treated with estrogen and cadmium, as well as a decrease in these levels of phosphorylation if pretreated with G15, a GPR30-specific antagonist. This data implies that cadmium may utilize GPR30 to phosphorylate MAPK in a manner similar to that of estrogen. Since other studies in the lab addressing the role of ER α and ER β have shown that these receptors are not involved, it is likely that GPR30 is the only estrogen receptor involved in cadmium activation of MAPK. Further experimental replicates will need to be performed to determine whether arsenite is also utilizing GPR30.

This novel data implies that cadmium is capable of binding to and activating the GPR30, leading to an increase in phosphorylated MAPK within the cell. This is further verified by the fact that inhibiting the receptor prior to treatment resulted in decreased levels of phosphorylated MAPK. Though Figure 2 shows an unexpectedly high untreated level of phosphorylated MAPK, the overall average results of both experimental replicates display increase in phosphorylated MAPK with treatment and a muted phosphorylation in the presence of inhibitor, at least for estrogen and cadmium. As phosphorylated MAPK is an indication of activation of the ERK1/2 pathway, leading to cellular growth and proliferation, it appears that exposure to some environmental or natural estrogens can exacerbate the growth of lung adenocarcinoma tumors within the body through GPR30. The biochemical impacts of these chemicals on tumors could explain, in part, the differences in adenocarcinoma tumors between men and women.

The data generated in this study supports the results of previous, published work on GPR30. Prossnitz and Hathaway (2015) displayed a positive correlation between number of tumors and the quantity of GPER in breast tissue cells, indicating a strong link between more aggressive forms of cancer and the presence of this estrogen receptor. If cadmium and arsenite are capable of activating GPR30 and phosphorylating MAPK in lung tissue, as the current data would suggest, this could provide a mechanism how estrogen stimulates lung cancer growth, at least in GPR30 positive adenocarcinoma cells.

The data revealed in this study has a number of potential implications and future directions for cancer biologists to explore. Medical professionals need to be aware of the different effects cadmium, arsenite, and estrogen can have on men and women. This data, in combination with some standing research, can provide new directions for potential therapeutics.

The next direction for this research should focus on determining whether cadmium and arsenite are capable of utilizing estrogen receptors other than GPR30, as well as repeating these arsenite assays to clarify whether arsenite utilizes GPR30. This research supports GPR30 activation by cadmium, which leads to a biochemical cascade which activates ERK1/2. Since previous studies have shown that cadmium and arsenite stimulate growth in these cells, it is likely that the activation of GPR30 is a key step leading to cellular proliferation, though further study would need to be performed to confirm this for arsenite. Perhaps these estrogen agonists are also capable of utilizing ER α , ER β , or some combination of the three estrogen receptors to result in MAPK phosphorylation. Further inhibition studies could test this possibility. It is possible that an increased understanding about how estrogens promote cellular proliferation in lung cancer could lead to more effective for this deadly disease.

While developing a pharmaceutical to block or inhibit GPR30 should be sufficient in slowing cancer development, further studies revealed that drug development and inhibition of the GPR30 is difficult to achieve. As explained by Ferguson (2001), GPCRs can become desensitized to their ligands upon continual stimulation. They achieve this by internalizing and sequestering their receptors so they cannot be activated. This process prevents over stimulation of the cellular response and can occur in a time period of between a few seconds to several hours.

This desensitization process cannot be permanent, however, or the cell would never be able to be stimulated again. Resensitization is the process by which the receptors are returned to the cell's surface. For this to occur, the ligand needs to be internalized into the endosome sequestering the GPCRs. Unlike the desensitization process however, resensitizing a cell is much less efficient, which would make it difficult to develop drugs and pharmaceuticals that would be effective for the long-term (Ferguson, 2001). Not only would this be a challenge for pharmaceutical companies, but it is also a fact which needs to be acknowledged when interpreting the data resulting from experimentation on the GPR30 receptor. Further research on developing therapeutics which can target GPR30 despite desensitization and resensitization should be prioritized, especially with this novel work proving the impact environmental estrogens can have on exacerbating the impacts of lung adenocarcinomas on patients.

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